Accumulation of high-molecular-weight amylose in Alzheimer’s disease brains

Linjuan Huang², Rawle I. Hollingsworth³, Rudy Castellani⁴, and Birgit Zipser¹, ²
²Department of Physiology, 2195 Biomedical and Physical Sciences Bldg., Michigan State University, East Lansing, MI 48824; ³Department of Biochemistry, Michigan State University, East Lansing, MI 48824; and ⁴Department of Physiology, Division of Pathology, Michigan State University, East Lansing, MI 48824

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Although most of the glucose metabolized in the brain is taken up from the blood, glucose derived from glycogen stores is increasingly implicated in both normal brain function and injury repair. An impaired glucose metabolism is one of the features of Alzheimer’s disease (AD) entailing a reduction in glucose transporters and the uptake of glucose as well as alterations in the specific activity of glycolytic enzymes. Here we report that AD brains accumulate amylose, the unbranched α(1,4)-linked glucose polymer that is resistant to degradation by glycolytic enzymes. Neutral polysaccharides harvested from postmortem brains were purified with hydrazinolysis, ion exchange, and sizing chromatography and subjected to NMR spectroscopy, GC, GC-MS, and methylation analysis. Five percent of the polysaccharides (50 μg [0.3 μmol/g wet weight brain tissue] consisted of amylose with molecular weights exceeding 600,000 Da. There is no evidence for 1,6-branching, indicating that the polymer is not a form of high-molecular-weight glycogen. By GC analysis, the glucose content of the AD brains was almost three times greater than that of the age-matched control brains. A synthesis of amylose in AD brains at the expense of glycogen would compromise glucose metabolism and enhance neural degeneration.

Key words: corpora amylacea/glucose metabolism/glycogen stores/glycogen storage disease/glycopathobiochemistry

Introduction

Glucose metabolism during normal brain function and neurodegenerative disease is a field of active research. Although most of the consumed glucose is taken up from the blood, evidence is accumulating that brain glycogen is also an important energy source (Choi et al., 2003).

Glucose metabolism has commonly been measured using the uptake of a radiolabeled deoxyglucose, an inert glucose analog (Rapoport, 1999) and more recently of radiolabeled glucose (Hampel et al., 2002). Ultrastructural studies demonstrated that glycogen is primarily located in astrocytes, although neuronal, ependymal, and choroid plexus cells contain small amounts as well (Magistretti et al., 1993; Peters et al., 1970). It has been difficult to visualize or quantify brain glycogen because of its rapid postmortem degradation. However, glycogen levels as high as 12 μmol/g have been found using new protocols involving the extraction of glycogen and the handling of animals with minimal environmental stimuli (Cruz and Dienel, 2002). Furthermore, light microscopic analysis demonstrated that axonal tracts contain higher glycogen levels than neuropil regions (Kong et al., 2002).

Alterations in glucose metabolism are found in various disease states. During ischemia, both astrocytes and microglia exhibit an increase in glucose uptake and glycogen storage (Kajihara et al., 2001). In epileptic brains, astrocytes exhibit intense dehydrogenase activity, stopping at the amylose step in the synthesis of glycogen. This decreased dehydrogenase activity resulted in an accumulation of amylose, the unbranched α(1,4)-linked glucose polymer (Gilles-Pierlet, 1980). Amylose is also generated in type IV glycogen storage disease due to the lack in branching enzyme (as reviewed in Cavanagh, 1999). Amylose, in contrast to glycogen, is not a readily accessible source of energy because it is resistant to degradation by enzymes endogenous to the brain that break down glycogen. During normal aging, glucose polymers, considered to be amylose, accumulate in corpora amylacea (starch bodies) in the glial feltwork on brain surfaces, which so far have not been reported to contribute to cognitive decline.

In Alzheimer’s disease (AD), cognitive decline is paralleled by a reduction in cerebral blood flow and glucose metabolism. Other signs of impaired glucose metabolism reported for AD brains include the altered activity of glycolytic enzymes (Bigl et al., 1999) and alteration in insulin levels and insulin resistance (as reviewed in Watson and Craft, 2003).

In this study, we purified amylose, the unbranched α(1,4)-linked glucose polymer, from AD brains. Amylose occurs in two size populations, with molecular weights of about 600,000 Da and more than 1,000,000 Da. Thus by its high molecular weight, AD amylose differs from the glucose polymers (<4,000 Da) that are encapsulated in corpora amylacea during normal aging. The synthesis of amylose in AD brains at the expense of glycogen may partly explain metabolic complications leading to the neurodegeneration in AD.

Results

Glucose polymers were isolated from human brain material. Cerebral cortices were obtained at autopsy from three advanced AD cases (79–83 years; postmortem...
interval: 6–9 h) and three nondemented, age-matched control cases (67–90 years; postmortem interval: 4.5–15 h). Each cortical material was separately fractionated using three successive Bio-Gel size exclusion chromatographies: Bio-Gel P4, Bio-Gel P10, and Bio-Gel P60.

Gel filtration chromatography on a Bio-Gel P4 column of delipidated, hydrazinolyzed material from AD and control tissues gave polysaccharide peaks in the exclusion volume (Figure 1A, B, arrows) as judged by resonance between 3.0 ppm and 5.5 ppm (Figure 2) and by compositional analysis using gas chromatography (GC) (Table I). The average percentage of polysaccharide/wet weight tissue (or delipidated tissue) was 0.063% (or 1%) and 0.029% (or 0.5%) for AD and control cerebral cortices, respectively. The anomeric proton area of the proton spectra of the polysaccharides from AD brains contained a large resonance at 5.4 ppm, indicative of an anomeric proton that was lacking in the age-matched control brains (Figure 2A arrow, 2B).

The compositions of the polysaccharide peaks from the six different brain samples as determined by GC analysis is shown in Table I. The average glucose content of the polysaccharides purified from AD cerebral cortices was three times higher than that of the control cortices.

To investigate the origin of the resonance seen at 5.4 ppm in the NMR spectrum of the AD polysaccharides, the polysaccharides were subjected to further size fractionation. Bio-Gel P10 sizing chromatography yielded three peaks (Figure 3A). Examining each of these peaks by 1D ¹H nuclear magnetic resonance (NMR) spectroscopy demonstrated that the 5.4 ppm resonance was obtained only from peak I.

Table I. Compositional analysis by GC spectrometry of neutral polysaccharides from aged control and AD cerebral cortices after Bio-Gel P4 size chromatography

<table>
<thead>
<tr>
<th>Neutral polysaccharide</th>
<th>Fuc</th>
<th>Ara</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td>3.72</td>
<td>39.54</td>
<td>41.92</td>
<td>14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>10.43</td>
<td>2.65</td>
<td>1.4</td>
<td>33.05</td>
<td>27.66</td>
<td>24.82</td>
</tr>
<tr>
<td>control III</td>
<td>14.32</td>
<td>1.25</td>
<td>4.19</td>
<td>31.82</td>
<td>37.55</td>
<td>10.86</td>
</tr>
<tr>
<td>AD I</td>
<td>4.26</td>
<td>0.44</td>
<td>0.59</td>
<td>11.89</td>
<td>10.9</td>
<td>71.93</td>
</tr>
<tr>
<td>AD II</td>
<td>7.92</td>
<td>0.66</td>
<td>0.88</td>
<td>25.02</td>
<td>24.4</td>
<td>41.13</td>
</tr>
<tr>
<td>AD III</td>
<td>7.14</td>
<td>0.41</td>
<td>1.28</td>
<td>23.11</td>
<td>29.97</td>
<td>38.07</td>
</tr>
</tbody>
</table>

Information on GalNAc and GlcNAc is not included.

To separate peak I more fully from peaks II and III, it was further size-fractionated by Bio-Gel P60 chromatography (Figure 3B). This yielded two peaks. Examining each peak by 1D ¹H NMR spectroscopy demonstrated that the 5.4 ppm resonance was obtained only from peak Ia (Figure 3A). The peak Ia spectrum lacked the other anomeric protons with different resonances seen in Figure 2A. Peak Ia contains 5% of the neutral polysaccharides purified from the AD brains or 50 μg/g wet-weight brain tissue.
The polysaccharides in peak Ia are composed exclusively of glucose, as verified by GC. The 5.4 ppm chemical shift of the anomeric proton, together with a coupling constant of less than 4, indicates an α linkage. A spectrum of an authentic sample of amylose was acquired under the same conditions (Figure 4B) and was identical to the one obtained on the putative amylose sample from AD brain (Figure 4A).

The linkage of the glucose polymers was further investigated using methylation analysis (Figure 5). GC of the methylated alditol acetates showed one predominant peak and two trace peaks. The GC-mass spectrometry (MS) spectrum of the predominant peak shows a fragment 2,3,6-Me3-Glc, demonstrating that the linkage was 1,4-linkage. Some minor peaks corresponding to undermethylated products were also observed. There were no peaks corresponding to 1,6-branching or terminal residues (nonreducing residues).

To determine the molecular weight of amylose purified from AD brains, peak Ia was further fractionated using Sepharose 4B sizing chromatography (Figure 6). Two separate fractions were obtained. The molecular weight of fraction I was over 1,000,000 Da. The molecular weight of...
fraction II was about 600,000 Da. At the lower molecular weight limit, only 0.027% of the linkages should be terminal. This is consistent with no signal being observed for terminal residues in the methylation analysis.

We attempted to analyze the tissue distribution of the high-molecular-weight amylose we had purified in biochemical experiments. Paraffin sections of AD cortex were treated with potassium iodide/iodine followed by a rinse in sulfuric acid. As expected, corpora amylacea (not shown) stained bright purple (as reviewed in Cavanagh, 1999). In addition, senile plaques were lightly stained above background (Figure 7A, arrows). At higher magnification, plaques exhibited weak gray-purplish staining (Figure 7B). The light labeling of senile plaques using a stain for starch had not previously been reported.

Discussion

Glucose polymers in the form of glycogen are known to be present in a variety of tissues. However, the presence of unbranched glucose polymers had not previously been determined by structural analysis. Here we reported that the cerebral cortex of AD brains contains amylose, an unbranched glucose polymer. Glycogen is branched (1,6) every 8–12 residues and therefore methylation analysis should yield ~8–12% terminal (2,3,4,6-methylated) and branching (2,3-methylated) residues. The glucose polymers we isolated from AD brains did not show evidence for 1,6-branching, indicating that these polymers were not a form of high-molecular-weight glycogen. Amylose, unlike glycogen, does not serve as an accessible energy storage in the brain because it is only efficiently broken down by amylase, an enzyme present in the digestive tract. Therefore, the pathological synthesis of amylose may contribute to the impaired glucose metabolism documented in AD brains.

Although the brain depends on glucose uptake from the blood, brain glycogen is increasingly implicated in normal brain function and protective responses as a result of brain injury. Glycogen stores in the brain have been historically underestimated to be only 2–3 μmol/g because brain glycogen is rapidly metabolized and thus disappears within minutes postmortem. However, Cruz and Dienel (2003)
estimated that the concentration of glycogen is as much as 8–12 μmol/g by measuring glucose in ethanol-insoluble brain fractions after handling of animals with minimal environmental stimuli.

If amylose is synthesized at the expense of glycogen, one would expect an impaired glucose metabolism. The concentration of amylose that we purified from AD brains is 50 μg (0.3 μmol)/g wet weight of brain, which is only 3–10% of the glycogen concentration estimated for rat brain. However, if amylose were largely synthesized in particular brain regions, such as the AD-vulnerable regions displaying plaques and tangles, then the local concentration of amylose would be significantly higher.

Glycogen levels are dynamically regulated during normal brain activity. The turnover rate varies with synaptic stimulation (Swanson et al., 1992) and sleep deprivation (Kong et al., 2002) and is regulated by neurotransmitters, such as noradrenaline, and vasoactive intestinal peptide (Pellerin et al., 1997). Though glycogen granules are found in dendrites and synaptic terminals of neurons (Peters et al., 1970), astrocytes are generally thought to store most of the brain glycogen used as glucose source, especially during transient increases in metabolic demand (Magistretti and Pellerin, 1999).

During brain injury, for example, due to ischemia or epileptic activity, there is a dramatic increase in astrocytic glycogen (Swanson et al., 1989). In central white matter, as result of glucose deprivation, astrocytic glycogen is broken down to lactate, which is then transferred to axons for fuel (Choi et al., 2003), similar to the nourishing of neurons through astrocytic lactate, first described for synaptic areas (as reviewed in Meeks and Mennerick, 2003). Elevation in astrocytic glycogen is found in necrotic lesions resulting from ischemia (Kajihara et al., 2001) and therefore might also be used as an energy source during inflammatory responses.

AD brains are noted for their progressive reduction in glucose metabolism and blood flow, more so in association than in primary cortical regions (Hampel et al., 2002) as demonstrated by in vivo imaging of the uptake of the inert radiolabeled glucose analog deoxyglucose. This is compatible with the relative lack of amyloid plaques and neurofibrillary tangles in primary cortical areas.

One known indicator of impaired glucose metabolism in AD are the decreased levels of glucose transporters (Vanucci et al., 1998). Glucose uptake is impaired by lipid peroxidation that leads to the covalent modification of glucose transporters with aldehydes such as 4-hydroxynonenal (Mattson, 1998). Glucose transport is also impaired as vascular endothelial cells are exposed to amyloid-β peptide (Aβ) (Blanc et al., 1997), which presumably occurs during cerebral amyloid angiopathy. In contrast, the secreted form of neuroprotective amyloid precursor protein enhances glucose transport and protects against the Aβ effect (Mattson et al., 1999). The majority of the cerebral glucose uptake/metabolism was thought to be an insulin-independent process (Seaquist et al., 2001) because endothelial cells and astrocytes express the insulin-insensitive glucose transporters GLUT1 and 3. However, recent studies have reaffirmed that brain glycogen metabolism is highly insulin-sensitive (Choi et al., 2003), even though the insulin-sensitive glucose transporters GLUT4 and are only minor constituents in brain. Because GLUT4 and 8 are expressed by neurons, the observed insulin resistance in AD (as reviewed in Watson and Craft, 2003) presumably impairs neuronal glucose metabolism.

Insulin abnormalities and insulin resistance are increasingly implicated in the pathophysiology of AD (as reviewed in Watson and Craft, 2003). Patients with AD are hyperinsulinemic and hyperglycemic compared to healthy control individuals (Razay and Wilcock, 1994). Cell culture studies suggest that insulin accelerates trafficking of Aβ from the Golgi and trans-Golgi network to the membrane and thus accelerates the release of Aβ into the extracellular fluid (Gasparini et al., 2001). Furthermore, increased insulin levels may also interfere with the clearing of Aβ because both are proteolyzed by insulin degrading enzyme. The degradation of Aβ was shown to be competitively inhibited by insulin (Qiu et al., 1998).

Furthermore, glycosylation is affected in AD brains as demonstrated by the increased specific activities of phosphofructokinase, pyruvate kinase, and lactate dehydrogenase but the decreased activity in glucose-6-P dehydrogenase. The increase in glycolytic enzymes has been attributed to astrocytes that are activated by the inflammatory responses found in AD (Bigl et al., 1999). In AD brains, astrocytes show an association between glycogen granules and immunogold-labeled Aβ (Kurt et al., 1999). On the other hand, amylose, the unbranched glucose polymer, is relatively resistant to degradation and therefore easily detectable post-mortem. In type IV glucose storage disease, amylose accumulates instead of glycogen because of the lack of branching enzyme. The glucose polymer that accumulates during normal aging in the human brain in corpora amylacea is also considered to be amylose although its chemical structure was not determined. Its molecular weight has been reported to be less than 4,000 Da, which is consistent with the purple staining of corpora amylacea with potassium iodide/iodine followed by sulfuric acid (Sakai et al., 1969).

The glucose polymer that we purified from AD brains has significantly higher molecular weights than the glucose polymer found in corpora amylacea. It occurs as two size populations with molecular weights of about 610,000 Da and more than 1,000,000 Da, respectively. Though the single anemic proton of the glucose polymer (5.4 ppm) in the NMR spectrum is consistent with an α(1,4)-linkage, this linkage is conclusively demonstrated by the predominant fragment 2,3,6-Me3-Glc in the GC spectrum obtained by methylation analysis. There is no evidence for 1,6-branching indicating that the polymer is not a form of high-molecular-weight glycolgen. Branching at about every 7–10 glucose units is a characteristic feature of glycolgen that separates it from amylose.

Attempts to histochemically visualize the high-molecular-weight amylose that we purified in biochemical experiments only proved moderately successful. Using a histological stain for starch, senile plaques stained only weakly gray-purple in contrast to the intense staining of the lower-molecular-weight glucose polymer concentrated in corpora amylacea. This suggests that most of the high-molecular-weight amylose is soluble, and only traces are retained in paraffin-embedded sectioned material.
We undertook to purify amylose from AD brains because the 5.4 ppm resonance in NMR spectra from AD polysaccharides obtained after Bio-Gel P4 size fractionation suggested the presence of amylose. That high-molecular-weight amylose may also be present in aged control brains, albeit at a lower abundance, is suggested by the presence of unmetabolized glucose in its polysaccharides, as demonstrated by GC spectrometry. If amylose were synthesized in the AD brain at the expense of glycogen, the result would be an impairment of glucose metabolism leading to neural degeneration.

Materials and methods

Materials

Brain tissue was obtained from the Maryland Brain Bank at autopsy from three subjects with advanced AD (91 g, 23 g, 18 g; 79–83 years; postmortem interval: 6–9 h) and three nondemented, age-matched control subjects (113 g, 40 g, 25 g; 67–90 years; postmortem interval: 4.5–15 h). Bio-Gel P4, P10, and P60 separation media were purchased from Bio-Rad (Hercules, CA). Hydrazine, anhydrous, p-amino benzoic acid ethyl ester (ABEE), sodium cyanoborohydride, were purchased from Sigma (St. Louis, MO).

Delipidation of human brain tissue

As a first purification step, the human cortex was delipidated. After excision, the brain tissue was frozen and lyophilized. After pulverization, each gram of tissue was extracted with 40 ml of a chloroform-methanol-water mixture (4:8:3 by volume). The extract was filtered, and the residue was extracted again and filtered as before. The residue was then washed with ethanol, dried under vacuum, and made into a slurry with water. It was then exhaustively dialyzed against water at 4°C. The sample was lyophilized and then dried under vacuum for 1 day.

Liberation of glycans from glycoproteins by hydrazinolysis

Carbohydrate chains were released from glycoproteins by hydrazinolysis at 85°C for 12 h, and N-acetylated sugars were re-N-acylated following published procedures (Wing et al., 1992).

Purification of glycans by microcrystalline cellulose column chromatography

To remove peptides and amino acids, the preparation was applied to a column of microcrystalline cellulose equilibrated in 1-butanol-ethanol-water, 4:1:0.5 by volume. Peptides and amino acids were removed by elution with 10 column volumes of 1-butanol-ethanol-water, 4:1:0.5 by volume. The column was then washed with 1 column volume of ethanol, and glycans were recovered by elution with 5 volumes of water. The sample was lyophilized. The reducing termini of glycans were then quantitatively regenerated in distilled water containing 1 mM Cu (II) acetate and 1 mM acetic acid for 1 h at room temperature. Salts were removed with a Dowex AG 50W-X12 (H+ ) column and then lyophilized.

Separation of glycans into charged and neutral fractions by anion exchange chromatography

Glycans were fractionated into neutral and acidic glycans by anion exchange column chromatography (acetate form). Neutral glycans were recovered by elution with deionized water, and total acidic fractions were obtained by elution with 2 M ammonia formate.

Labeling with ABEE

Glycans were labeled with ABEE so that they could be monitored using a UV detector (314 nm): ABEE was dissolved in a freshly prepared mixture of glacial acetic acid/dimethyl sulfoxide (3:7 by volume). This was added to sodium cyanoborohydride and vortexed thoroughly until all the reductant had dissolved; 1 ml of this mixture was added to 50 mg dried glycans and incubated at 60°C for 4 h. The samples were spotted onto Whatman 3MM paper strips and allowed to dry at room temperature overnight. Excess reagents were removed by ascending paper chromatography in butanol-ethanol-water 4:1:1 by volume. After drying, labeled glycans from the origin were recovered by washing with water.

Starch is a typical sizing material used in the preparation of paper products. To confirm that this procedure did not lead to the contamination of ABEE-labeled polysaccharides with starch which could be present and eluted from Whatman 3MM paper, strips were incubated in 50 ml H2O while shaking for 8 h. The supernatant was filtered, lyophilized, and examined by NMR spectroscopy. No signals were observed corresponding to amylose.

Bio-Gel chromatography

Size chromatography was performed at room temperature on Bio-Gel columns P4 (120 × 1 cm), P10 (120 × 1 cm), and P60 (110 × 1 cm). Glycans dissolved in 0.5 ml redistilled water were applied to each column and eluted with redistilled water at a flow rate of 0.2 ml/min, collecting 1.6 ml/tube. The elution of glycans was monitored with a UV detector at 314 nm.

Sugar composition analysis by GC

Samples were incubated in 400 μl 2 M trifluoroacetic acid at 121°C for 2 h and then dried. Remaining traces of acid were removed by adding 250 μl 2-propanol. The dried hydrolyzed sample was dissolved and incubated in 250 μl 1 M ammonium hydroxide containing 10 mg/ml NaBH4 at room temperature for 3 h. Drops of acetic acid were added until effervescing ceased. Borate ions were removed from the dried sample by adding 0.5 ml methanol/acetic acid (9:1 by volume) and drying 7× at room temperature with the last time using only 0.5 ml methanol. The sample was acetylated with 0.2 ml pyridine and 0.2 ml acetic anhydride overnight at room temperature and then dried under an N2 stream. A mixture of chloroform and water was added to the sample, followed by vortexing. The organic phase was concentrated under a N2 stream and analyzed by GC. GC was performed on a DB-225 fused silica capillary column (0.25 mm × 30 m) using a Hewlett-Packard 5890 J gas chromatograph. The oven
temperature was programmed from 180 to 220°C at 2°C/min.

Methylation linkage analysis
Samples were permethylated (Ciucano and Kerek, 1984), hydrolyzed, reduced, and peracetylated according to the procedure of sugar composition analysis by GC. The partially methylated alditol acetates were analyzed on a JEOLAX-505 (JEOL USA, Peabody, MA) double-focusing mass spectrometer coupled to a Hewlett-Packard 5890 J gas chromatograph via a heated interface. GC separation employed a DB-225 fused-silica column (0.25 mm × 30 m). The oven temperature was programmed from 160 to 220°C at 5°C/min. The relative abundance of the partially methylated alditol acetates was determined by GC analysis on a Hewlett-Packard 5890 gas chromatography equipped with a DB-225 capillary column and flame ionization detector. The oven temperature was programmed from 180 to 220°C at 2°C/min. Helium was the carrier gas for both GC methods.

500 MHz proton NMR spectrometry
The glycan sample was exchanged 3× with D₂O (99.9 atom% D) by lyophilization and finally dissolved in D₂O (99.996 atom% D). The 500 MHz 1H NMR spectrum was obtained with a Varian VXR 500 spectrometer operating in the Fourier transform mode at a probe temperature of 50°C. Chemical shifts were expressed in ppm from acetone (2.225 ppm).

Histology
Paraffin sections of AD cortex were deparaffinized using xylene and then rehydrated through an ethanol series into water. The sections were then stained for 0.5 h in 2% potassium iodide/1% iodine dissolved in water. After a brief rinse with 0.1 N H₂SO₄, the sections were embedded in a mixture of glycerol, staining solution, and acid (5 cc glycerol/5 cc staining solution/1 cc 0.1 N H₂SO₄). The entire plaques were imaged on a Nikon DXM1200 digital camera using 10× and 100× objectives.

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Abbreviations
Aβ, amyloid-β peptide; ABEE, aminobenzoic acid ethyl ester; AD, Alzheimer’s disease; GC, gas chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance.

References


