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Dietary High Cholesterol and Trace Metals in the Drinking Water Increase Levels of ABCA1 in the Rabbit Hippocampus and Temporal Cortex

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Abstract

Background—Cholesterol-fed rabbits have been documented to show increased amyloid- β (A β) deposits in the brain that can be exacerbated by the quality of drinking water especially if rabbits drink tap water or distilled water containing copper. One mechanism of cholesterol and A β clearance may be through the ATP-binding cassette transporter A1 (ABCA1).

Objective and Methods—Using an ABCA1 antibody, we determined the number of ABCA1-immunopositive neurons in three areas of rabbit brain as a function of feeding 2% cholesterol and providing tap water, distilled water, or distilled water to which aluminum, copper, or zinc was added.

Results—The number of neurons with ABCA1 immunoreactivity was increased significantly as a result of dietary cholesterol in the rabbit hippocampus and inferior and superior temporal cortex. The number of neurons with ABCA1 immunoreactivity was further increased in all three areas as a result of cholesterol-fed rabbits drinking tap water or distilled water with copper. Finally, cholesterol-fed rabbits that drank distilled water with aluminum also showed an increased number of ABCA1-immunopositive neurons in inferior and superior temporal cortex.

Conclusions—These data suggest that ABCA1 levels increase in parallel with previously documented increases in A β levels as a result of high dietary cholesterol and copper in the drinking water. Addition of aluminum to distilled water may have a similar effect in the temporal cortex. ABCA1 has been proposed as a means of clearing A β from the brain and manipulations that increase A β also result in an increase of clearance machinery.

Keywords

Aluminum; ATP-binding cassette transporter A1; cholesterol-fed rabbit; copper; zinc

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INTRODUCTION

Debate about the role of trace metals in Alzheimer's disease (AD) has been long and sometimes contentious, with a number of meta-analyses weighing the merits of individual publications and providing some clarity as to the role of aluminum, iron, copper, and zinc [1–5]. We and others have found that elevated copper levels increase amyloid- β (A β) deposits [6–8], whereas some have argued that supplementing with copper may actually reduce A β pathology [9]. The case for the role of aluminum in AD is also unclear with some finding that it is a potential contributor to AD [10, 11], whereas others have found that it does not contribute to AD [12], and there have even been suggestions of political and economic factors influencing aluminum research [11, 12].

In contrast, the role of cholesterol in AD is more firmly established [13–15] although not without debate [16]. Even though cholesterol does not normally cross the blood-brain barrier (BBB) [17, 18], elevation of dietary cholesterol has been shown to induce A β accumulation in a number of species including rats, mice, guinea pigs, rabbits, and dogs [19–24]. Studies of the cholesterol-fed rabbit revealed at least a dozen features similar to the pathology observed in the AD brain including A β immunoreactivity, extracellular A β plaques, meningeal A β immunoreactivity, apolipoprotein E (ApoE) immunoreactivity, cathepsin D immunoreactivity, superoxide dismutase immunoreactivity, thioflavin-S immunoreactivity, microgliosis, apoptosis, vascular activation of superoxide dismutase, vascular inflammation indexed by mouse endothelial cell antigen immunoreactivity, breaches of the BBB, elevated brain cholesterol, and elevated A β concentration [25–30].

The report by three different groups in 1999 of a role for an ATP-binding cassette transporter in the HDL deficiency syndrome Tangier Disease opened new areas of research into the role these transporters in cholesterol efflux particularly ATP-binding transporter A1 (ABCA1) [31]. The role of ABCA1 in the regulation of ApoE and cholesterol transport as well as its localization in the brain and role in A β deposition and clearance has implicated it in AD [32–37]. In fact, ABCA1 has been shown to increase in both the hippocampus of AD patients and in A β PP/PS1 transgenic mice expressing familial AD genes [38]. Loss of function experiments in transgenic mice show that a reduction in ABCA1 results in an increase in A β [39, 40] whereas gain of function experiments show that an increase in ABCA1 results in a decrease in A β [41].

Although the normal physiological role of ABCA1 in the CNS remains less than clear [32, 42], it has been suggested that ABCA1 may be important in the removal of excess cholesterol [43, 44] although data from cell culture experiments suggest ABCA1 only increases cholesterol efflux from astrocytes and not for neurons [32, 45]. Nevertheless, ABCA1 is found to be highly expressed in both neurons and astrocytes [15, 32, 46]. Interestingly, efflux of cholesterol by ABCA1 in the form of the cholesterol metabolite 24S-hydroxycholesterol does occur in neuron-like cells cultures [42] and is hypothesized to be an important means of excretion of cholesterol from neurons to the vasculature [13, 46, 47].

In a series of studies, Sparks reported the effects of trace metals including aluminum, copper, and zinc on the accumulation of A β in the brain [8, 24, 48, 49] and on systemic

pathology [48]. It was suggested that elevated ABCA1 would increase ApoE-specific cholesterol efflux, decrease cellular cholesterol levels, and reduce A β production [50]. What is not known is the interplay between trace metals such as copper, aluminum, and zinc that have been implicated in AD [7, 51–57], cholesterol, and ABCA1. In the present experiment, we began to investigate the effects of elevated cholesterol and the presence of trace metals on ABCA1.

MATERIALS AND METHODS

Subjects

A total of 50 adolescent, male, New Zealand white rabbits (3–4 kg) were housed in the rabbit facility at the Banner Health Research Institute operating under USDA guidelines with a 12:12 light cycle, at $67 \pm 7^\circ\text{F}$, and 45–50% humidity. Animals were randomly assigned to one of ten groups in an Institutional Animal Care and Use Committee approved experimental protocol. Five groups of animals were fed a 2% cholesterol diet and given access to one of five different types of drinking water: tap water (Sun City, AZ, $n = 5$); distilled water (Arrowhead distilled drinking water, $n = 5$); distilled water with 0.12 PPM copper ($n = 5$); distilled water with 0.36 PPM zinc (as sulfate, $n = 5$); or distilled water with 0.36 PPM aluminum (as sulfate, $n = 5$). Another five groups of animals were fed normal chow and given access to one of five types of drinking water [tap water ($n = 5$); distilled water ($n = 5$); distilled water with 0.12 PPM copper ($n = 5$); distilled water with 0.36 PPM aluminum (as sulfate, $n = 5$); or distilled water with 0.36 PPM zinc (as sulfate, $n = 5$)]. The Sun City tap water used in this experiment has been independently analyzed across a number of studies and shown to routinely contain 0.2 PPM copper [49, 58]. Cholesterol and control chow diets were obtained from Purina Mills, Inc. (Laboratory Rabbit Diet with and without 2% cholesterol). Food intake was limited to one cup per day (225 grams) and *ad libitum* water consumption varied between 0.9 and 1.2 L/day.

Animals in each group were euthanized ten weeks after initiating the food and water protocol. On the day of euthanasia, animals were administered a cocktail of Ketamine and Xylazine (IM; 45–75 mg/kg and 5–10 mg/kg, respectively) and perfused under pressure with 120 ml of 4% paraformaldehyde at a constant rate of 5 ml/min using a constant pressure pump. The brain was removed and further fixed by immersion in 4% paraformaldehyde for 2 weeks before sectioning.

Histology

In order to determine the effects of the cholesterol diet and trace metals in the drinking water on the number of ABCA1-positive neurons, the hippocampus and temporal cortex—areas previously found to show A β immunoreactive neurons [29, 30, 59]—were examined. Fifty-micron vibratome sections of the hippocampus and surrounding cortex were immunostained with a commercial antibody to ABCA1 (Novus anti-ABCA1, NB400-105, 1:500 dilution) using published peroxidase immunohistochemical methods [30]. Briefly, after washing in 0.5 M Tris, sections were transferred to 3% H₂O₂ to block endogenous peroxidase. Sections were washed in 0.5 M Tris with 0.1% Triton X-100 (Tris-TX) and then placed in blocking solution (3% normal goat serum (NGS) in 0.5 M Tris-Triton X) for 1 h. Antigen retrieval

was achieved by placing tissue in 88% formic acid, washing, and then placing in undiluted pepsin reagent (Biomed, CA, M77). Tissue was placed in the ABCA1 antibody in Tris-Tx with 1% NGS overnight at 4°C. Tissue was then placed in a biotinylated secondary antibody (1:200, Chemicon) in Tris-Tx with 1% NGS. The antibody was visualized using Vectastain ABC reagents (Vector) for 1 h followed by DAB (Vector) for 4–5 min.

The cells positively stained for the ABCA1 antibody within a 0.5×0.5 mm square grid were counted and averaged across at least four randomly selected areas within the inferior and superior temporal cortex and at least four randomly selected areas within the hippocampus including CA1 and dentate gyrus within a randomly selected section using a 20 $\times$ objective as previously described [8, 24, 49, 60]. Counts were made by a histologist (GS) blind to the treatment conditions.

Statistical analyses

Data for three different brain regions (hippocampus, superior temporal cortex, inferior temporal cortex) were analyzed separately by between-subjects analysis of variance (ANOVA) using the SYSTAT statistical program. Significance was set at $p < 0.05$.

RESULTS

The three panels of Fig. 1 show the mean ($\pm$ SEM) number of ABCA1-immunoreactive neurons in the hippocampus (top), superior temporal cortex (middle), and inferior temporal cortex (bottom) of rabbits fed 2% cholesterol or normal chow and given access to tap water or distilled water with or without 0.12 PPM copper, 0.36 PPM aluminum, or 0.36 PPM zinc. Comparisons of interest included the effects of cholesterol (Cholesterol versus Chow), type of water (tap water versus distilled water), and the presence of trace metals in distilled water (none versus copper, aluminum, and zinc). Taken together, the panels of Fig. 1 show and statistical analyses confirmed that the presence of cholesterol in the food significantly elevated the number of ABCA1-positive neurons in the hippocampus [$F(1, 40) = 23.31, p < 0.001$], superior temporal cortex [$F(1, 40) = 50.53, p < 0.001$], and inferior temporal cortex [$F(1, 40) = 86.29, p < 0.001$], and that the type of water given to the rabbits made a significance difference to the number of ABCA1-positive neurons in the hippocampus [$F(4, 40) = 4.06, p < 0.01$], superior [$F(4, 40) = 11.01, p < 0.001$], and inferior temporal cortex [$F(4, 40) = 15.00, p < 0.001$]. This was particularly true for cholesterol-fed rabbits given tap water or distilled water with copper because the number of ABCA1-immunoreactive neurons was significantly higher than for cholesterol-fed rabbits given any other type of water in all three brain areas (*post hoc* comparisons all p 's < 0.01). However, the size and nature of the effects of the type of distilled water did differ among the three brain areas. In the hippocampus, the number of ABCA1-positive neurons was generally lower than in the temporal cortex and cholesterol in the food increased the number of ABCA1-positive neurons in rabbits given tap water, distilled water, and distilled water with copper (all p 's < 0.05). In the superior temporal cortex, cholesterol increased the number of ABCA1-positive neurons in rabbits given tap water, distilled water with copper (p 's < 0.01), and distilled water with aluminum ($p < 0.05$). In the inferior temporal cortex, the cholesterol diet increased the number of ABCA1-positive neurons in all cholesterol-fed rabbits regardless of

the type of water they were given. The differences in the number of ABCA1-positive neurons between the three brain areas were significant [$F(2, 88) = 114, 34, p < 0.001$] and these differences interacted with the presence of cholesterol in the food [$F(2, 88) = 17.66, p < 0.001$], and type of water given to the rabbits [$F(4, 88) = 3.41, p < 0.01$].

It is worth noting that the present results for ABCA1 differ only slightly from the levels of A β immunopositive neurons found in the temporal cortex of rabbits in a previous study [24]—rabbits that were treated in exactly the same manner as in the current experiment. Figure 2 is a plot of data from the Sparks et al. paper [24] and is included here to show that the mean number of A β immunopositive neurons in the superior temporal cortex were significantly higher in groups of rabbits fed cholesterol and given tap water, distilled water or distilled water with copper but not in groups given distilled water with aluminum or zinc.

DISCUSSION

The principal findings of the current experiment are: (1) the number of neurons that were ABCA1-immunopositive increased as a function of dietary cholesterol in the rabbit hippocampus, inferior and superior temporal cortex; (2) the number of neurons with ABCA1 immunoreactivity across all three areas were further increased as a result of cholesterol-fed rabbits drinking tap water that contained copper or distilled water with copper added; (3) drinking distilled water with aluminum also increased the number of ABCA1-positive neurons in inferior and superior temporal cortex of cholesterol-fed rabbits; and (4) the number of immunoreactive neurons in inferior temporal cortex was significantly increased by dietary cholesterol regardless of the type of drinking water although the effects were strongest for tap water and distilled water with copper.

It is clear from the present results that the effects of a high-cholesterol diet on ABCA1 were strong across all three brain regions with the number of ABCA1-positive neurons always higher in cholesterol-fed rabbits than chow-fed controls particularly in the inferior temporal cortex. This finding is consistent with a large number of reports from different laboratories on changes in a range of AD-related indices in the cholesterol-fed rabbit [28, 61–72]. Importantly, the increased number of ABCA1-immunoreactive neurons were significantly larger for cholesterol-fed rabbits given tap water or distilled water with copper added than those given distilled water with or without aluminum or zinc which is consistent with the previously reported significant increases in A β immunoreactivity resulting from feeding rabbits cholesterol and giving them tap water or adding copper to their distilled water [8, 24, 49, 73].

In addition to these effects, there were region-specific variations in the effects of the drinking water on the cholesterol-induced increases in the number of ABCA1 immunopositive neurons. First, the increased number of ABCA1-immunoreactive neurons in the hippocampus were also observed in cholesterol-fed rabbits given distilled water which is consistent with previous findings of the effects of dietary cholesterol *per se* on A β [8, 24, 48, 49] and tau pathology [58]. This increase in ABCA1-positive neurons in cholesterol-fed rabbits given distilled water was also observed in the inferior temporal cortex. Second, the addition of aluminum to distilled drinking water given to cholesterol-fed rabbits increased

the number of ABCA1-positive neurons in the inferior and superior temporal cortex suggesting that the temporal cortex is sensitive to aluminum in the water if cholesterol is present even if aluminum did not significantly elevate the level of A β in this region (Fig. 2) [24]. Finally, ABCA1 reactivity in the inferior temporal cortex seems particularly sensitive to a high-cholesterol diet because the number of ABCA1-positive neurons were elevated in all of the cholesterol-fed groups. It is worth noting, however, that here too the number of ABCA1-positive neurons were significantly higher for rabbits given tap water or distilled water with copper than for rabbits given distilled water with or without aluminum or zinc.

Regional differences in the number of ABCA1-positive neurons are consistent with previous research with cholesterol-fed rabbits in which there were similar regional differences in the level of A β as a function of cholesterol concentration [74] and cholesterol duration [75] where numbers of neurons in the cortex were double those found in the hippocampus. These differences are very consistent with many human studies in which A β deposition is much higher in the temporal cortex than the hippocampus [76]. If ABCA1 is involved in the removal of A β (see below), it would follow that the more A β that occurs in a region the more ABCA1 might accumulate in the brain's effort to remove it as evidenced by ABCA1 overexpression experiments [41].

We have previously reported the effects of cholesterol in the diet and aluminum, copper, or zinc in the drinking water on the accumulation of A β in the brain [8, 24, 48, 49] and on systemic pathology [48]. This earlier work showed that tap water and copper in distilled water but not aluminum or zinc increased A β in cholesterol-fed rabbits [24, 49]. The present data suggest strongly that these manipulations also elevated ABCA1 in the form of an increased number of immunopositive neurons in the hippocampus and inferior and superior temporal cortex. Indeed, an examination of the all three brain regions suggests a correspondence between previously reported A β levels from this laboratory and the current ABCA1 levels which is corroborated for the superior temporal cortex by a comparison of Figs. 1 and 2.

Data also of note were the differences in ABCA1 levels that occurred in both areas of the temporal cortex as a result of drinking distilled water with aluminum, a trace metal that has been long proposed to be involved in AD [10, 11]. The ubiquitous nature of aluminum both in manufactured products as well as in nature have prompted many to examine and discuss its toxic nature especially as it relates to AD [10, 11, 51, 55, 77, 78]. The present results taken together with those of our previous research suggest that the presence of aluminum elevates systemic pathology [48] and the number ABCA1 even though A β levels resulting from aluminum in distilled water are not significantly elevated by a high cholesterol diet [24]. Conversely, it is copper in the water—be it in tap water or distilled water—that prevents the clearance of A β and properties unique to copper account for the consistent elevation of A β and ABCA1 above levels for all other trace metal [23, 48, 50]. It has been argued elsewhere that the link between copper and ceruloplasmin is important in A β clearance and that both are involved in AD [23, 53, 79, 80].

There are two related aspects of ABCA1 function in the CNS that bear upon the present results: cholesterol efflux and clearance of A β . It has been suggested that ABCA1 may be

important in the removal of excess cholesterol [43, 44] although data from cell culture experiments suggest ABCA1 only increases cholesterol efflux from astrocytes and not for neurons [32, 45]. Nevertheless, ABCA1 is found to be highly expressed in both neurons and astrocytes [15, 32, 46] and the current results show the number of ABCA1-immunopositive neurons to be significantly increased by dietary cholesterol. Interestingly, efflux of cholesterol by ABCA1 in the form of the cholesterol metabolite 24S-hydroxycholesterol does occur in neuron-like cells cultures [42] and is hypothesized to be an important means of excretion of cholesterol from neurons to the vasculature [13, 46, 47]. Apart from disruption of the BBB that would permit direct entry of dietary cholesterol into the brain [25, 65], dietary cholesterol does not normally cross the BBB [18, 81, 82]. However, it has become clear that cholesterol may cross an intact BBB to the CNS in the form of the cholesterol metabolite 27-hydroxycholesterol [15, 22, 83–85]. Of particular interest to the current experiment is the finding that 27-hydroxycholesterol can increase ABCA1 expression [86]. Consequently, it is possible that the high levels of dietary cholesterol, which have been shown to elevate 27-hydroxycholesterol in the rabbit [22, 87, 88], would increase ABCA1 expression. However, this cannot be the entire story because there were significant differences in the number of ABCA1-immunopositive neurons among the rabbits that were all fed the same 2% cholesterol diet, a diet which has already been shown to increase ApoE and ApoE-specific cholesterol efflux [29].

As noted above, ABCA1 increases in the hippocampus of AD patients and in A β PP/PS1 transgenic mice expressing familial AD [38]. A series of loss of function experiments in transgenic mice show that a reduction in ABCA1 results in an increase in A β [39, 40]. More germane to the present experiment are gain of function experiments that show an increase in ABCA1 results in a decrease in A β [41]. Given, the relationship between trace metals and A β in the cholesterol-fed rabbit [8, 24, 49] as well as mice [89, 90], it seems reasonable to suggest that the significant increase in the number of ABCA1-immunoreactive neurons in hippocampus and temporal cortex resulting from the addition of copper to the drinking water and the presence of copper in the tap water was in response to the well-documented elevation in A β that results from the presence of cholesterol in the diet and copper in the water [8, 24, 48, 49]. If one were to assume from the present results that ABCA1 levels were increased in previous cholesterol-fed rabbits experiments where copper was added to the water [8, 24, 48, 49], the high levels of A β immunoreactivity in those experiments suggest that clearance of A β by ABCA1 in the presence of copper was not particularly successful. The number of different proposed mechanisms for such clearance are beyond the scope of this paper except perhaps to recapitulate speculation by Sparks [23] on the role of copper and the low-density lipoprotein receptor-related protein 1 (LRP1) shown by the Deane group to be critical for A β clearance [91]. Sparks suggested that if cholesterol is elevated, there is an overproduction of A β that is normally cleared to the blood but the presence of copper causes A β to accumulate in the brain because it cannot be cleared [23]. The Deane group showed that copper regulates LRP1-mediated clearance across the BBB to the blood [91]. Given this potential role of copper as well as other trace metals in AD, it is perhaps not surprising that recent dietary recommendations concerning AD have included a discussion of copper and aluminum [52].

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Fig. 1. Mean ($\pm$ SEM) number of ABCA1-immunoreactive neurons in the hippocampus (top), superior temporal cortex (middle) and inferior temporal cortex (bottom) of New Zealand white rabbits fed 2% cholesterol or normal chow and given access to tap water or distilled water with or without 0.12 PPM copper, 0.36 PPM aluminum, or 0.36 PPM zinc. * $p < 0.05$, ** $p < 0.01$.

Fig. 2.

Mean ($\pm$ SEM) number of A β -immunoreactive neurons in the superior temporal cortex of New Zealand white rabbits fed 2% cholesterol or normal chow and given access to tap water or distilled water with or without 0.12 PPM copper, 0.36 PPM aluminum, or 0.36 PPM zinc. ** $p < 0.01$. (Data plotted from Table 3 [24] and used with permission).