Lipid Droplets Accumulate in the Hypothalamus of Mice and Humans with and without Metabolic Diseases

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Abstract

Background: In peripheral tissues, the lipid droplet (LD) organelle links lipid metabolism, inflammation, and insulin resistance. Little is known about the brain LDs. Objectives: We hypothesized that hypothalamic LDs would be altered in metabolic diseases. Methods: We used immunofluorescence labeling of the specific LD protein, PLIN2, as the approach to visualize and quantify LDs. Results: LDs were abundant in the hypothalamic third ventricle wall layer with similar heterogeneous distributions between control mice and humans. The LD content was enhanced by high-fat diet (HFD) in both wild-type and in low-density lipoprotein receptor deficient (\textit{Ldlr} \textasciitilde) HFD) mice. Strikingly, we observed a lower LD amount in type 2 diabetes mellitus (T2DM) patients when compared with non-T2DM patients. Conclusions: LDs accumulate in the normal hypothalamus, with similar distributions in human and mouse. Moreover, metabolic diseases differently modify LD content in mouse and human. Our results suggest that hypothalamic LD accumulation is an important target to the study of metabolism.

Introduction

The development of metabolic diseases, such as obesity and type 2 diabetes mellitus (T2DM), coincides with disturbed fatty acid metabolism and excess ectopic fat storage as lipid droplets (LDs) in many peripheral tissues \cite{1, 2}. While LDs are linked to insulin resistance in peripheral tissues, little is known about the role of LDs in

Keywords

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the brain and their putative role in the pathogenesis of metabolic disease and insulin resistance [2]. The impairment of glucose uptake induced by insulin in peripheral tissues is a core feature of T2DM, and insulin resistance also occurs in the central nervous system [3–5]. Insulin resistance, induced by high-fat diet (HFD) feeding, is accompanied by disruption of lipid metabolism with ectopic lipid deposition in intracellular LDs [6–9].

LDs are hydrophobic organelles formed in the majority of cells with a core of neutral lipids and a great variety of LD proteins that are related to lipid metabolism, cell signaling, and production of inflammatory mediators [10–12]. The perilipin (PLIN) family comprises specific LD structural proteins [13], and the most studied proteins are the adipocyte PLIN1 and the PLIN2 [14]. PLIN2 is present in all cells and tissues and is exclusively present in LDs, being considered the standard marker for this organelle [15].

In Drosophila, LDs were identified in microglia and shown important for the neuronal energy supply [16]. PLIN2 has also been described in LDs in isolated mammalian brain microglia under LPS stimuli [17, 18]. Microglia in the hypothalamus has been shown to be involved in the development of insulin resistance and lipid metabolism [19]; however, little is known about the role of other hypothalamic cell types, such as ependimocytes and tanyocytes, within the hypothalamic third ventricle (3V) region. These cells have barrier functions and are also important for the control of lipid and glucose metabolism/sensing [20–23], but whether LDs are present in the 3V wall cells remains unclear.

In the current study, we use the PLIN2 immunoreactivity as a marker for describing the physiological and pathological presence and distribution of LDs along the 3V in mouse and human with and without metabolic disorders.

Materials and Methods

Mice

C57BL/6 wild-type (WT) and low-density lipoprotein receptor deficient (Ldlr –/–) mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed in a pathogen-free environment, in conformity with institutional guidelines for animal experiments and approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. For the description of the normal LD distribution, we used C57BL/6 mice (age 8 weeks) fed with chow diet. The WT mice (age 8 weeks) were divided into 2 groups, one fed with chow diet, one with HFD containing high carbohydrates (D12079B Western diet, Research Diets, New Brunswick, NJ, USA, containing 17 kcal% protein, 42% carbohydrate, and 41 kcal% fat as well as a high-fructose corn syrup sweetened beverage) for 32 weeks. Ldlr –/– mice (age 8 weeks) were fed with the HFD for 26 weeks. All mice were housed on a 12-hour-light/12-hour-dark cycle at 22 ± 2°C with food and water ad libitum. At the end of the study, all mice were decapitated and brains were immerse-fixed in 4% paraformaldehyde 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4°C for 48 h. Brains were then equilibrated 48 h in 30% sucrose in 0.1 M Tris-buffered saline (TBS, pH 7.2), and coronally cut by a cryostat into 30-μm sections.

Human Brain Tissue

Postmortem hypothalamic tissues of 17 T2DM and 8 non-T2DM age-matched controls and 9 non-T2DM young subjects were obtained from the Netherlands Brain Bank, through autopsy approved by the Medical Ethics Committee of the VU Medical Center, The Netherlands. Subjects who had Braak stage V-VI or clinically diagnosed severe dementia were excluded. Subject details, including sex, age, postmortem delay, clinical diagnosis, and cause of death, are provided in Table 1 and online supplementary Table 1 (see www.karger.com/doi/10.1159/000508735 for all online suppl. material). The tissues were immersion fixed in 10% phosphate-buffered formalin, then paraffin embedded and coronally sectioned in 6-μm slices. Every 100th section was used for Nissl staining to predetermine the anatomical orientation of the infundibular nucleus, which was further analyzed by the range of NPY-positive neurons. For immunofluorescence, 2 sections of the infundibular nucleus areas were mounted on SuperFrost Plus slides and dried at 37°C. Sections were deparaffinized and hydrated, rinsed in 50 mM Tris TBS (pH 7.6), and were antigen retrieved for 10 min using microwave treatment at 700 W in 0.1 M sodium citrate (pH 6.0).

Immunofluorescence

Samples were washed (2×) with TBS before incubations. All antibodies and streptavidin conjugates were diluted in TBS buffered solution containing 0.25% gelatin and 0.5% Triton x-100, and all washes between incubations were done with TBS (4×; 5 min). In all experiments, control stainings were performed without the specific primary antibody. Samples were incubated with a guinea pig polyclonal anti-PLIN2 antibody (N-terminus aa 1–29, PROGEN, cat. GP40, 1:400 in mouse sections, 1:200 in human sections) and rabbit polyclonal anti-GFAP (DAKO, cat. Z0334, 1:100 in mouse sections, 1:500 in human sections) overnight at 4°C. Sections were then incubated for 1 h with a secondary anti-guinea pig conjugated with biotin antibody (Vector, cat. BA7000, 1:400). After that, sections were incubated for 3 h with Streptavidin-Alexa 488 (Thermo-Fisher, cat. S32354, 1:300) in the human slides, or Streptavidin-Alexa 647 (Thermo-Fisher, cat. S32357, 1:500) in the mouse free-floating slices. For the mouse sections, the Alexa-488 conjugated secondary anti-rabbit antibody (Thermo-Fisher, cat. A21206, 1:500) was added to the incubation, on the last hour. The sections were washed and incubated with DAPI (Thermo Fisher, cat. 62247, 1:2,000) and NeuroTrace Red (when indicated; Thermo Fisher, N21482) for 10 min. After final washing, all slides were then mounted with Vectashield Hard-Set mounting media (Vector).

Image Acquisition and Analysis

Images were acquired using a TCS SP8 Confocal in DMi8 inverted microscope (Leica Microsystems, Wetzlar, Germany). Identical hardware and software settings were used for each of the
mouse experiments and another fixed setting for the human samples. The quantification of the mouse LDs by the PLIN2 immunoreactivity (PLIN2-ir) was performed with the Imaris software (Oxford Instruments, Abingdon, UK). Images for quantification (z-stack, 20 μm) were taken with a 63× lens. The DAPI and the PLIN2-Alexa 647 images were used to generate surfaces corresponding to the total volume of nuclei and LDs, respectively, with fixed settings of threshold. The amount of LDs per nuclei was obtained by dividing the volume of PLIN2 labeling per volume of DAPI labeling. The GFAP labeling was used as a marker of astrocytes and specific α tanycytes.

The human PLIN2-ir LD quantification was done in images obtained with a 10× objective to cover half of the ventricle wall (left or right) on the slide, and 6–10 consecutive images were taken depending on the ventricle length. The analysis was made with the use of the ImageJ software and a macro developed by Ard Jonker (Cellular Imaging – Core Facility, Amsterdam UMC, location AMC). LD measurements were performed using an automatically generated stripe comprising the whole ependymal layer of the 3V half wall of each patient. The total length (from sulcus to infundibulum) was divided into 10 subsections to allow comparisons between patients. The region of interest to measure LD signal in the ependymal layer cells was determined by the nuclei (DAPI) staining, as detailed in the supplementary text file. The tissue fluorescence intensity outside the signal area was considered as the background signal and was subtracted from the signal fluorescence. The corrected signal of LD at the area of fluorescence was divided by the length of each subsection of the ventricle wall to give the LD fluorescence per µm of ventricle wall. The graphs and statistic calculations were performed with the GraphPad Prism software, and we used the Welch t test for the comparison between specific groups.

Results

Heterogeneous Distribution of LDs in Mouse 3V

The presence and distribution of LDs along the 3V wall in the naïve mouse hypothalamus was analyzed by the presence of PLIN2-ir in 3 regions determined by the distance to bregma along the rostral-caudal axis [24]. We observed that the majority of LDs were concentrated...
along the ependymal layer of the 3V in 3 different levels along the rostral-caudal axis with a heterogeneous distribution (Fig. 1). We compared the amount of LD at 3 different rostral-caudal levels, corresponding to specific surrounding hypothalamic nuclei, the suprachiasmatic (SCN; Fig. 1A), periventricular (PVN; Fig. 1B), and arcuate nuclei (ARC; Fig. 1C), and the 3 regions of the ventricle (i.e., top, middle, and bottom). Astrocytes (GFAP-ir) can be observed outside the ependymal layer (Fig. 1A2–7, B2–7, C2–7), but the majority of the astrocytes do not present LDs. At the level of bregma –1.8, the α tanycytes that project to the dorsal ARC and also express GFAP [25] coincide with an area with high amount of LDs (Fig. 1C4, C5). There is no significant variation between the different bregma levels when we compare the top and the middle regions. Interestingly, in the bottom region with GFAP-negative α2 tanycytes, known to project to the ventromedial ARC, we found negligible amounts of LD along the ventricle wall (Fig. 1C6, C7, E).

**LDs in the 3V Ventricle in Insulin-Resistant HFD-Fed Mice**

The ARC is a key brain region containing important neuron populations that are involved in the development of insulin resistance, and the tanycytes in this area of the 3V are involved in the control of systemic energy metabolism [26]. We investigated whether the LD heterogeneous distribution at the level of bregma –1.82 of the 3V was associated with the presence of insulin resistance. We studied the alterations induced by a HFD in the LD distribution in both WT mice and in the Ldlr –/– mice. The Ldlr –/– mice on a HFD present a complete phenotype for the study of metabolic diseases with enhanced adipose tissue, insulin resistance, and hypothalamic inflammation [7, 8]. The top area of the ventricle, with a majority of ependimocytes, has enhanced LDs in both the HFD-fed WT and the HFD-fed Ldlr –/– mice (Fig. 2A1, A2, B1, B2, C1, C2, E). The GFAP-ir α tanycytes region presents a high amount of LDs similarly to the top part of the ventricle in all the groups (Fig. 2A3, A4, B3, B4, C3, C4). The lower part of the ventricle wall with GFAP-negative α and β tanycytes (as shown in Fig. 1) has very low amounts of LDs in the WT on chow diet (Fig. 2A5, A6, E). Moreover, the HFD feeding in the WT mice did not induce LDs in this area (Fig. 2B5, B6, E). In the HFD-fed Ldlr –/– mice, with a more severe metabolic disruption, we can observe enhanced LDs at the ARC level (Fig. 2C5, C6, E).

**LDs in the 3V in Control (Non-Diabetic) and T2DM Subjects**

Insulin resistance induced by HFD in animal models does not fully mimic the human T2DM pathogenesis, and this is a major obstacle hampering translational studies on brain dysfunction in T2DM patients [19]. We therefore investigated the distribution of LDs in the human 3V, comparing control (non-diabetic patients) with T2DM patients. We used hypothalamic samples from the Netherlands Brain Bank selected with age matched between the groups (Table 1). We show here that the human 3V wall contains significant amounts of LDs as evidenced by the PLIN2 staining (Fig. 3A) and the LDs are mainly associated with the ependimocyte layer (Fig. 3B). All the patients with different ages, distinct causes of death, different body mass index (BMI, only part of the subjects had BMI data) and different postmortem delay present a significant amount of LD in the 3V wall. Moreover the LD content in this group of subjects did not correlate with age, BMI, or postmortem delay (online suppl. Fig. 1). It is important to point out that we also show the presence of LDs already in the 3V wall from a separate group of young brains (online suppl. Fig. 3; online suppl. Table 1). To compare the control patients with the T2DM patients, we performed the quantification of the LDs along the ventricle as described in the Methods section (Fig. 3C). In the control and T2DM subjects, we found the same pattern of distribution of LD as in mice, with higher amounts of LDs in the top and middle areas (PVN and DMH) and lower amounts in the bottom area (infundibular nucleus; Fig. 3A, C, D). Within the T2DM patients, this distribution is less pronounced with lower amounts of LDs in the ventricle wall cells (Fig. 3C, D, E).
Fig. 2. LD content is altered in the hypothalamus of insulin-resistant mice. Representative immunofluorescence images of the mouse hypothalamus (bregma –1.82) from WT (A1–A6), high-fat diet (HFD)-fed WT mice (WT HFD; B1–B6), and HFD-fed low-density lipoprotein receptor knockout mice (Ldlr KO HFD, C1–C6). Cell nuclei (DAPI; in grey), glial fibrillary acidic protein-immunoreactive (GFAP-ir) astrocytes and GFAP-ir α tanyocytes (in green), and LD (PLIN2-ir, in red; A, B, C). Quantification of the volume of LDs and nuclei are performed by generating the 3D surfaces with the IMARIS software (D). Representative image from the outlined area in A1 (original image; D1, D2; nuclei in grey; LD in red) (surface image; D3, D4; nuclei in cyan, LD in yellow). The volume of LDs was normalized by the volume of nuclei in order to compare the different areas (E). Scale bar, 40 μm in A1–C6; 10 μm in D1–D4. III, third ventricle. Data are presented as mean ± SEM of a minimum n = 4. * p < 0.05; ** p < 0.01 according to the Welch t test.
Fig. 3. Characterization of LD distribution and content in the third ventricle of non-diabetic controls and T2DM individuals. The anatomical orientation of different hypothalamic areas along the third cerebral ventricle and the distribution of perilipin 2 immunoreactive (PLIN2-ir, in green) LDs and cell nuclei (DAPI, in grey; A1). Higher-magnification images from 3 regions outlined in A1 are shown in A2–A4. The morphology of the LDs within the ependymocytes is demonstrated by images in B1 and B2 from the area selected in D1. The PLIN2-ir LD content was quantified as described in the Methods section from the images as in A1 and averaged for the 3 different regions of the ventricle wall according to the hypothalamic nuclei in the adjacent parenchyma (C). Representative images with higher magnification, showing the nuclei (DAPI, grey) and LD (PLIN2-ir, green), to evidence the variation of LDs between the control and the T2DM patients (D, E). III, third ventricle lumen; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; IFN, infundibular hypothalamic nucleus. Scale bar, 1 mm in A1; 200 μm in A2–A4; 10 μm in B1–B2; 40 μm in D1–D6, E1–E6. Data are presented as mean ± SEM of n = 8 (controls) and n = 17 (T2DM). *p < 0.05 according to the Welch t test.
Discussion

Here we provide evidence for the existence of significant amounts of LD in normal hypothalami in both mouse and human, concentrated in the wall of the 3V. We further demonstrate that with high fat feeding and genetic deletion of LDL receptor in mice, the LD content is augmented, depending on the degree of metabolic disturbances. Interestingly, in the human brain, the presence of T2DM disease was accompanied by lower amounts of LDs in the hypothalamus. Taken together, our data point to an important role for brain LDs in metabolism.

To our knowledge, we are the first to show a major LD accumulation within the ependymal layer of the 3V both in physiological and pathological conditions. Earlier studies have described the presence of LDs in the brain ventricle ependymal layer; however, they only identified significant LDs in the diseased brain with Alzheimer disease or ageing [27–29]. These authors propose that the presence of LDs in the brain is correlated directly to a pathological condition. We could demonstrate the age correlation when the group of younger subjects was analyzed together with the older non-T2DM control group (online suppl. Fig. 3). Our findings point to a broader physiological role for the accumulation of LDs along the ventricle, which is also modified in metabolic diseases and ageing. The heterogeneity of the distribution of LDs along the 3V is evident in the mouse and human brains and this pattern is specifically modified under pathogenic conditions. Interestingly, the changes in LDs in mouse brain with metabolic disruption either with HF feeding alone, or even more severe, through Ldlr deletion, was region specific. We found that the more metabolically disturbed, the more markedly the increase of LDs in the top of the ventricle, whereas there were discrete increases in LD in the ARC region of the 3V wall. Interestingly, in humans with T2DM, we observed a reduction of LDs throughout the ventricle regions. We can speculate that the complexity and chronic conditions of human T2DM may lead to a reduction of LDs when compared to the control, non-diabetic patients.

LD organelles present a hydrophobic core of neutral lipids that can be partially or completely extracted during fixation procedures. This characteristic makes it difficult to fully assess the presence of LDs in the brain by using hydrophobic probes solely. PLIN2 is the standard and very specific marker of LDs in all cells and the protein is readily degraded when not associated with the organelle [30]. LDs have distinct roles depending on the cell and stimuli. The most studied function is of lipid storage or-ganelles, but LDs are also known sites of inflammatory mediator production and intracellular signaling and vary in protein and lipid composition and size [31, 32]. We observed a great diversity of LD size (0.5–4 µm) in the ependymal layer. According to the literature, this suggests diverse roles for these LDs in lipid storage (larger) and in control of lipid metabolism, inflammation, and signaling (smaller) [32, 33].

A previous study proposed that only the tanycytes, and not the ependymal cells, would accumulate LDs only on HFD [20]. However, we show here that the majority of LDs in both mouse and in human brain is clearly associated with the ependimocytes, and there is a high content of LDs in areas with no tanycytes. These results show that ependimocytes and tanycytes accumulate LDs and may have a physiological role in brain lipid metabolism.

Recently, Geller et al. [34] showed that mouse tan- cytes can sense palmitate, in an oxidation-dependent manner, and modulate neurons to indirectly inhibit the lipolysis in the periphery. Interestingly, these palmitate-sensing tanycytes are exactly in the region of the GFAP-negative α tanycytes that we show here. Our suggestion is that this ARC region of the ventricle is a specialized lipid-sensing area and, therefore, does not physiologically accumulate LDs. Additionally, we suggest that this is an important feature since the same pattern was present in both mouse and human hypothalami.

Earlier reports showed LD accumulation in microglia and astrocytes being important in the control of neuronal metabolism [16, 35, 36]. In line, we also observed LDs in both microglia and astrocytes; however, less abundant when compared to the ependymal layer (as marked by Iba1-ir and GFAP-ir; online suppl. Fig. 2).

We conclude that the presence of hypothalamic LDs is region specific and is modified depending on the metabolic conditions in both mice and humans. The characterization of the distribution of the LDs opens possibilities for better understanding of lipid metabolism and lipid sensing in the brain. We propose that the evaluation of the LD organelle within the brain tissue should always be considered when studying the hypothalamic control of metabolism under physiological and pathogenic conditions.

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Statement of Ethics

Postmortem hypothalamic human tissues were obtained from the Netherlands Brain Bank, through autopsy approved by the Medical Ethics Committee of the VU Medical Center, The Netherlands.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

The conception of the study was done by C.M.M.-M., S.E.F., M.K.C.H., and C.-X.Y. The experimental design, execution, analysis, and interpretation of the data was done by C.M.M.-M., S.E.F., F.G.S., S.S.H., and C.-X.Y. The manuscript was written by C.M.M.-M. and C.-X.Y. and the final version was critically revised and approved by all the authors.

References


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