High-fat diet exposure induces IgG accumulation in hypothalamic microglia

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SUMMARY

The medio-basal hypothalamic arcuate nucleus (ARC), with its relatively “leaky” blood brain barrier that allows more circulating molecules to enter the brain, has emerged as a key sensor of blood-borne signals. In both the ARC and white adipose tissue (WAT), consumption of a high-fat diet (HFD) rapidly induces infiltration of microglia (ARC) or macrophages (WAT). Animals with HFD-induced obesity (DIO) and insulin resistance additionally accumulate B-lymphocytes in WAT, increasing local production of pathogenic antibodies. We therefore asked whether DIO mice or genetically obese \( \text{ob/ob} \) mice have increased IgG in the ARC, analogous to the recent observations in WAT. Following 16 weeks exposure to a HFD, WT mice had significantly increased IgG-ir signaling that was specific to the ARC and is exclusively concentrated in microglia. In contrast, IgG-ir of age-matched obese \( \text{ob/ob} \) mice fed standard chow had ARC IgG levels comparable to those in chow-fed WT control mice. However, following 2 weeks of HFD exposure, \( \text{ob/ob} \) mice also had a significant increase of IgG-ir in the ARC. In summary, our findings reveal a novel pathophysiological phenomenon specific for the hypothalamic ARC, which is induced by exposure to a HFD and can be enhanced, but not caused, by genetic obesity.

Running title: Arcuate nucleus IgG and obesity
INTRODUCTION
The brain is often considered an "immunoprivileged" organ, with immune signals having limited access under normal conditions. However, during infection and other brain neuropathologies (e.g., neurodegenerative diseases), the blood brain barrier (BBB) weakens and B cells can penetrate to infiltrate specific brain regions as part of a systemic immune response (Haire et al., 1973; McRae-Degueurce et al., 1988). In the hypothalamic arcuate nucleus (ARC) and median eminence complex, a differentially structured BBB allows more blood-borne signals to enter the brain (Gross, 1992), and it was recently reported that mice fed a high-fat diet (HFD, diet-induced obese (DIO) mice) have an increased presence of proinflammatory factors in these areas (Thaler et al., 2012). There is also an increase in microglia, the resident macrophages in the brain, in the ARC of DIO mice (Thaler et al., 2012). These increased indices of inflammation are reminiscent of what occurs in visceral white adipose tissue (WAT) of (DIO) mice, where both macrophages and B cells accumulate (Winer et al., 2011), leading to the production of pathogenic antibodies that may be involved in the complex process leading to insulin resistance in diet-induced obesity (Winer et al., 2011). Among the different types of immunoglobulin (Ig), IgG2c is predominantly found to be increased in visceral WAT in DIO mice (Winer et al., 2011), and the B cell infiltration and IgG deposition are also considered to be a pro-inflammatory marker. In hypothalamus, inflammatory factors resulting from calorie dense diet are substantially involved in developing central leptin and insulin resistance, this will increase food intake, reduce energy expenditure, increase hepatic glucose production and eventually cause obesity, diabetes and other metabolic syndromes such as cardiovascular disease (Obici et al., 2002; Munzberg et al., 2004; De Souza et al., 2005; Pocai et al., 2005; Posey et al., 2009; Thaler and Schwartz, 2010; Lumeng and Saltiel, 2011). Because of this similarity in inflammatory responses to a HFD in WAT and the ARC, we asked whether IgG accumulation is another parallel process that takes place in the ARC in response to exposure to a HFD, and we also asked whether the phenomenon could be triggered by genetically induced obesity, using ob/ob mice as a model.

RESULTS
HFD exposure, but not increased body weight alone, increases IgG accumulation in the ARC
In the hypothalamus of WT mice on standard chow (body weight (BW): 28.14±0.72 g),
modest IgG-immunoreactivity (ir) was observed in the ARC, but no signal was detected in other hypothalamic areas (Fig.1A and Fig.2). Following 16 weeks of exposure to a HFD, DIO mice weighed significantly more (50.81±1.68 g) than control mice fed chow (36.55±1.61 g, P<0.001). There was a significant increase of IgG-ir in the ARC of these DIO mice relative to the chow-fed controls (Fig.1B).

In chow-fed obese ob/ob mice with comparable BW as the DIO mice (48.39±1.45g, p=0.29 vs. DIO mice), ARC IgG-ir was comparable to that of control WT mice fed chow (Fig.1C). This implies that rather than increased body weight per se, increased exposure to dietary lipids is the predominant cause for IgG accumulation in the ARC. Consistent with this, only 2 weeks of exposure to a HFD led to greater IgG accumulation in the ARC of ob/ob mice than what had been observed in WT mice following 16 weeks of HFD exposure (Fig.1D).

**Hypothalamic IgG accumulation induced by exposure HFD occurs in microglia**

In the DIO mice, the strongest IgG-ir profile in the ARC shared a remarkable morphologic similarity with microglia, consistent with the possibility that the detected IgG is colocalized with microglia. We therefore co-stained brains of mice following 16 weeks of HFD exposure for IgG along with markers for microglia activity - ionized calcium binding adaptor molecule 1 (Iba1), and also co-stained with the astrocyte marker - glial fibrillary acidic protein (GFAP). Consistent with our earlier findings that in response to a HFD, activity of both Iba1-ir and GFAP-ir increased in the ARC (Thaler et al., 2011), we found that microglia transformed from cells with small somata and finely ramified processes to an activated phenotype associated with enlarged somata and highly ramified processes (Fig.3). In HFD mice, there was a clear co-localization of IgG-ir and Iba1-ir, indicating that substantial amounts of the HFD-induced IgG were located in the microglia. However, no IgG-ir was co-localized with GFAP, indicating that HFD-induced IgG accumulation did not occur in astrocytes (Fig.3). We conclude that HFD exposure leads to a strong and specific deposition of IgG in microglia.

**Hypothalamic IgG accumulation on a HFD mainly consists of IgG1**

Specific immunofluorescent staining for IgG1, IgG2a, IgG2b and IgG3 revealed a clear increase only for IgG1 in the ARC of the HFD-fed mice (Fig.4A and D, Fig.5). IgG2a (B and D), IgG2b (immunoreactivity signal not detectable, data not shown) and IgG3 (C and
F) did not differ between chow and HFD mice (Fig.4B, C, E and F). In summary these data suggest that the increase of IgG in the ARC of HFD mice is mainly a consequence of accumulation of IgG1 (but not IgG2 or 3) in microglia in the ARC.

DISCUSSION

When challenged with a calorie-rich diet (HFD), numerous parallel molecular events occur in peripheral tissues and the central nervous system (CNS) that are involved in the pathogenesis of the metabolic syndrome. The development of resistance to key endocrine signals maintaining metabolic homeostasis, such as leptin and insulin, represents a hallmark of this process. Intrigued by recent reports suggesting that in visceral WAT of DIO mice, B cell-derived IgG may be involved in the pathogenesis of insulin resistance (Winer et al., 2011), we hypothesized that a similar phenomenon may play a relevant pathogenic role in the CNS. We therefore asked whether, when mice are exposed to a HFD, IgG would accumulate in key areas of the mouse brain known to control systemic metabolism and body weight. WT mice fed a standard chow and mice with morbid monogenetic obesity (leptin-deficient $ob/ob$ mice) were compared as controls. Sixteen weeks of HFD induced significant accumulation of IgG specifically in the hypothalamic ARC, but not in other regions of the CNS. Surprisingly, this increase was not observed in $ob/ob$ mice fed standard chow diet, but was rapidly, and even more markedly, induced when $ob/ob$ mice were exposed to HFD. These novel and unexpected observations suggested that induction of IgG in the ARC occurs in diet-induced obesity, and appears to be caused directly by diet exposure rather than by increased body weight per se, as it was absent in genetically induced obesity.

Subtype analysis of the IgG detected in the hypothalamus of mice on HFD revealed that among three IgG isotypes known to be present in the ARC, only IgG1 increased on the HFD. Neither IgG2 nor IgG3 were increased by HFD exposure. Co-staining of the microglia marker iba1 and the astrocyte marker GFAP identified clear co-localization of the hypothalamic IgG with microglia, with no IgG being found in astrocytes.

Microglia with IgG-like immunoreactivity have been observed in several strains of mice (including C57BL/6 used in this study) in several brain regions (Hazama et al., 2005). It is also the case that brain microglia can uptake serum IgG through Fc gamma receptors, which recognize IgG1/2b (Frei et al., 1987) and cause IgG accumulation inside the
microglia (Hazama et al., 2005). This raises the possibility that in DIO mice, microglia in the ARC, where the BBB has a specialized “leaking” structure for circulating molecules to enter brain tissue, can uptake IgG1 from the systemic circulation. However, we cannot exclude the possibility that a specific IgG1-producing B cell infiltrated into the ARC.

Our data indicate that the increased IgG1 in ARC microglia of mice on a HFD is different from what has been observed in visceral WAT, where IgG2c is the dominant isotype stimulated by a HFD (Winer et al., 2011). This indicates that different types of inflammatory response can take place in different tissues in response to a HFD challenge.

As the brain's innate immune cells, microglia are important for maintaining a homoeostatic balance in a normal CNS by clearance of cell debris with their highly dynamic ramified processes and constant phagocytosis (Neumann et al., 2009). Under certain pathological circumstances, antibodies produced by pathological elements can augment this clearance function. This is well exampled in Alzheimer disease (AD), in which antibodies against amyloid β-peptide can trigger microglial activation, and enhance phagocytosis and clearance of pre-existing amyloid through Fc receptors (Bard et al., 2000). Thus, there is the possibility that HFD-stimulated IgG in the circulation enters the microglia with the purpose of increasing microglial activity and boosts its scavenging function. This would be considered to be an immune-to-brain communication pathway.

On the other hand, highly activated microglia release cytokines to recruit or stimulate more microglia and even lymphocytes, such as B cells and T cells, into the ARC (Persidsky et al., 1999; Nelson et al., 2002). Recruited B cells could therefore produce antibodies locally in the ARC. Thus, the IgG accumulation could be the cause, but also the consequence, of microglial activation, and these two processes might eventually turn into a vicious cycle. Such a process would increasingly influence the microenvironment of the ARC and possibly damage other cell types in the ARC. Consistent with this, we observed in recent studies that POMC cells appear to decrease in number in the ARC after chronic exposure to a HFD, and this could be a consequence of neuronal damage triggered by HFD exposure (Thaler et al., 2011).
Our data suggest that activated microglia are associated with deposition of IgG during HFD exposure in mice. One remaining question is whether the newly discovered IgG deposition in the ARC would be helpful or harmful to the complex and delicately balanced neuronal-glial ARC circuits involved in metabolic control and body weight regulation. Future studies will also have to address the potential of hypothalamic IgG as a target for the treatment of diet-induced disorders such as diabetes and obesity.

**METHODS**

**Animals**

All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati.

**Immunohistochemical and immunofluorescent staining**

WT (C57BL/6) and ob/ob mice (Jackson Labs, Bar Harbor, ME) that had received 2 or 16 weeks of HFD (58% fat, Research Diets, D12331, New Brunswick, NJ) or chow diet (ditto) were deeply anesthetized with a lethal dose of sodium pentobarbital and perfused with saline, followed by a solution of 4% paraformaldehyde in 0.1M PBS (pH 7.4) at 4°C. Brains were removed and kept in fixative at 4°C for overnight post-fixation, equilibrated 48 h with 30% sucrose in 0.1 m Tris-buffered saline (TBS; pH 7.2). Brains were coronally cut in a cryostat into 30µm sections; sections used for immunohistochemistry were collected and rinsed in 0.1 M TBS.

General endogenous IgG was detected using anti-mouse IgG (H+L) antibody. IgG isotypes were detected with anti-IgG1, IgG2a, IgG2b and IgG3-specific antibodies. Potential host cells for IgG were detected by co-staining of IgG with microglia activity marker iba1 and the astrocyte marker - GFAP.

For checking total IgG-ir, brain sections were incubated with horse anti-mouse IgG (a general IgG recognizing both heavy and light chains, Vector, USA) for 1 h; rinsed and incubated in avidin–biotin complex (Vector, USA) for 1 h, and the reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide for 7-10min. Sections were mounted on gelatin-coated glass slides, dried, dehydrated in a graded ethanol series, cleared in xylene, and cover-slipped for observation by light microscope.

For co-staining of IgG with iba1 and GFAP, brain sections were first co-incubated with goat anti-iba1 and rabbit anti-GFAP primary antibodies overnight at 4°C, rinsed with TBS
and then incubated with biotinylated horse anti-mouse IgG, and then streptavidin-conjugated Cy3, DyLight 649 conjugated anti-goat IgG and DyLight 488-conjugated anti-rabbit IgG (Jackson ImmunoResearch, USA).

For staining of IgG isotypes, brain sections were co-incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 488, goat anti-mouse IgG2a conjugated with Alexa Fluor 555, goat anti-mouse IgG2b conjugated with Alexa Fluor 350 or goat anti-mouse IgG3 conjugated with Alexa Fluor 594 (Invitrogen, USA). After the last incubation for fluorescent antibodies for 1 h, all sections were rinsed and mounted on gelatin-coated glass slides, dried and covered by polyvinyl alcohol mounting medium with DABCO® (Sigma, USA) and observed by confocal microscopy (Zeiss-LSM710, Germany).

**Analysis of the immunoreactivity profile**

For each mouse with each staining profile, two to three sections in the middle portion of the ARC were selected and images were collected; both sides of the ARC were manually outlined with an area of 0.03mm² on each side. The relative optic densitometry of every instance of immunoreactivity was measured by ImageJ (NIH, USA), and the mean densitometry number from each mouse was calculated. All values were then expressed as the mean ± SEM from each group and data were analyzed using one-way ANOVA. Statistical significance was set at $P < 0.01$.

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**Competing interests statement**

The authors declare that they do not have any competing or financial interests.

**Author contributions**

Conceived and designed the experiments: C.X.Y., S.C.W., M.H.T., S.M.H. Performed the experiments: C.X.Y. Analyzed the data: C.X.Y. Wrote the paper: C.X.Y., M.H.T., S.M.H. Corrected manuscript drafts: S.C.W. M.H.T., S.M.H.
Figure 1 Sixteen weeks of HFD induces intensive IgG deposition in the ARC (B) in comparison to Chow-fed control mice (A). This deposition pattern is not apparent in chow-fed obese ob/ob mice ARC (C), but can be induced by 2 weeks exposure to the HFD (D). Scale bar: 100μm.
Figure 2 Relative densitometry measurement of IgG-ir in the ARC of mice fed chow and HFD for 16 weeks, and in the ARC of ob/ob mice fed with chow or HFD for 2 weeks. *: p<0.01 vs. WT Chow; #: p<0.01 vs. ob/ob Chow.
Figure 3 Immunofluorescent co-staining of IgG, iba1 and GFAP in the ARC of mice fed chow for 16 weeks (A-D) or HFD for 16 weeks (E-H). In comparison to chow fed mice ARC, IgG, iba1 and GFAP immunoreactivity are all increased in the ARC of HFD mice, and only iba1 and not GFAP is co-localized with IgG. Arrows point to the microglia co-stained with IgG. Scale bar: 20 μm.
Figure 4 Immunofluorescent staining of IgG1, IgG2a and IgG3 in the ARC of mice fed chow (A-C) for 16 weeks or HFD (D-F). Only IgG1 but not IgG2a or IgG3 immunoreactivity is increased in the ARC of HFD mice. III: third ventricle. Scale bar: 50μm.
Figure 5 Relative densitometry measurement of IgG1-ir IgG2-ir and IgG3-ir in the ARC of mice fed chow or HFD for 16 weeks. ∗: p<0.01 vs. Chow.
References


Translational impact box

Clinical Issue

Our modern society is suffering more and more from obesity and other associated metabolic diseases in the last decades. With numerous efforts made by education and medication, the obesity epidemic shows no sign of reversing course. One of the reasons might be due to the uncompleted understanding of the pathology of metabolic syndrome, which obstructs the development of effective therapeutic strategy. Among the bulk of hypotheses, hypothalamic neuropathy is one of the current leading theories. However, the causes of neurons malfunction are largely unknown. What we have known is the normal function of neurons can only be maintained by a clean local microenvironment, which dynamically communicates with general circulation and comprises not only neurons but also other cell types like vascular cells as well as the glial cells (mainly astrocytes and microglia). In our very recent studies, during early state of metabolic syndrome induced by consumption of calorie-dense high fat diet (HFD, to mimic the calorie-dense western diet which is considered to be one of the major causes of human obesity), in the arcuate nucleus of the mediobasal hypothalamus of mice, which is identical to the human infundibula nuclei, we observed reactive microglia as a sign of inflammatory reaction. The very recent finding from other lab shows an infiltration of IgG into the white adipose tissue (WAT) rends us to hypothesize that HFD could also lead to IgG infiltration into the ARC, and can be involved in activation of microglia which is the “cleaning worker” of the local microenvironment. Consequently, these inflammatory changes will impair the normal neuronal function and break the energy homeostasis.

Results

After 16 weeks of HFD, we observed a significant increase of IgG accumulation specifically in the ARC. Moreover, as we expected, all the IgG deposits inside the microglia. This IgG accumulation is very likely caused by HFD, but not obesity, since there is not such accumulation pattern seen in the obese ob/ob mice fed with chow diet, but as soon as we feed the ob/ob mice with HFD, even within two weeks, the IgG deposition pattern in the ARC is comparable to those HFD fed WT mice. We also investigated the subtypes of the IgG, and found the deposited subtype is IgG1, which is different from the IgG2 found in the WAT.
Implications and Future Directions

This finding extends our knowledge about the pathological changes inside the central nervous system in development of metabolic syndrome. The similarity between the brain and the WAT regarding the IgG infiltration indicates the therapeutic strategy in treating obesity has similar target in both brain and periphery; moreover, the early onset of IgG accumulation in the ob/ob mice means IgG infiltration into the brain might be belonged to one of the first steps in developing other inflammatory changes, therefore to reduce IgG level in the brain could be an effective therapeutic approach to prevent obesity or treating obesity at early-stage, this hypothesis will be tested in our future study.