



Obesity induced by estrogen deficiency is associated with hypothalamic inflammation

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ARTICLE INFO

Keywords:

Menopause
Estrogen
Hypothalamus
Inflammation
Microglia
Obesity

ABSTRACT

Occurrence of obesity during the postmenopausal period is closely associated with inflammatory processes in multiple peripheral organs that are metabolically active. Hypothalamic inflammation has been recognized as one of the major underlying causes of various metabolic disorders, including obesity. The association between menopause-related obesity and hypothalamic inflammation remains poorly understood. We observed an elevation in hypothalamic inflammation in the ovariectomized mice, which displayed altered metabolic phenotypes and visceral obesity. Furthermore, we confirmed that ovariectomized mice displayed microglial activation accompanied by the upregulation of multiple genes involved in the inflammatory responses in hypothalamic microglia. Collectively, the current findings suggest that hypothalamic inflammation associated with microglial functioning could be a major pathogenic element in disruption of energy homeostasis during the postmenopausal period.

1. Introduction

Menopausal transition period induces physiological changes in women and, therefore, affects pathogenesis of multiple diseases [1]. In particular, the proportion of women with metabolic disorders is increasing rapidly among women in their postmenopausal period. A growing body of evidence demonstrates that inflammation is a major reason for the development of multiple metabolic disorders such as obesity, diabetes, and cardiovascular diseases [2]. In line with this notion, it is currently accepted that the metabolic disorders revealed in the postmenopausal period are also closely associated with systemic inflammation and related cellular stresses [3,4]. The hypothalamus governs energy homeostasis in the human body by regulating the endocrine and autonomic nervous systems [5]. Thus, the cellular stresses in hypothalamic circuits have been referred to as a pivotal pathogenic component in the deterioration of whole-body metabolism, resulting in obesity. In particular, intensive studies have suggested that hypothalamic inflammation linked to metabolic enrichment is closely associated with the deterioration of hypothalamic circuit activity in the regulation of whole-body energy metabolism [6]. In this context, it is not surprising that evidence has emerged linking enhanced hypothalamic

inflammation and estrogen deficiency during the postmenopausal period. Based on this evidence, we designed the current study to investigate hypothalamic inflammation in response to estrogen deficiency in ovariectomized (OVX) mice. Since it is known that the microglia, which are the resident immune cells in the central nervous system, actively contribute to the obesity pathogenesis by mediating inflammatory processes associated with overnutrition [7,8], we specifically investigated microglial activation accompanied by inflammatory responses in the hypothalamus of OVX mice. In conclusion, the present study reveals the pathological contribution of hypothalamic inflammation triggered by microglial activation in developing obesity and its related secondary complications during the postmenopausal period.

2. Materials and methods

2.1. Animals

Seven weeks old female C57BL/6 J mice, weighing approximately 20 g, were purchased from Daehan Bio link (DBL, Eumseong, South Korea) and maintained in a temperature (23–25 °C) and humidity controlled chamber with a 12 h light/dark cycle (light exposure during

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<https://doi.org/10.1016/j.bbrep.2020.100794>

Received 16 April 2020; Received in revised form 20 July 2020; Accepted 29 July 2020

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7am to 7pm). The mice had access to standard chow (DBL) and water ad libitum. CX3CR1-Cre (#025524) and RPL22-HA (#011029) mice were purchased from Jackson Laboratory. CX3CR1-Cre mice express Cre recombinase under the direction of the *CX3CR1* promoter in microglia. The RPL22-HA mice carry the HA-affinity tag adjacent to the ribosomal protein Rpl22, separated from the natively expressed terminal exon by loxP recognition sites. Hence, the tagged version of Rpl22 is only expressed following Cre mediated recombination. The RPL22-HA mouse was bred into the CX3CR1-Cre mouse line to obtain RPL22-HA expressing homozygous mice. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Incheon National University (permission number: INU-2016-001).

2.2. Ovariectomized mice model

After a week of acclimatization, the mice were randomly assigned to the OVX and sham groups. The mice were anesthetized with tiletamine/zolazepam (80 mg/kg, Zoletil 100, Virbac Corporation, France) and xylazine (10 mg/kg, Rompun, Bayel, Germany) solutions. For the OVX group, anterior uterine horns were cut to remove the ovaries; whereas for the sham group, dorsoventral incisions were made through the skin, muscles, and periosteum without removal of the ovaries. Changes in body weight were monitored for a month. At the end of body weight monitoring, the brain and white adipose tissue were quickly excised from the mice and stored at -80°C for gene expression analysis. For the histological experiments, mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Extracted tissues for histological experiments were post-fixed with 4% paraformaldehyde overnight at 4°C . Hematoxylin and eosin (H&E) staining was performed to analyze the morphologies of white adipose tissue in both groups according to the manufacturer's instructions (H&E staining kit, Sigma-Aldrich, St. Louis, MO, USA).

2.3. Quantitative real-time PCR

Total RNA was isolated from the hypothalamus of OVX and sham mice and reverse-transcribed to obtain cDNA using a Maxime RT PreMix kit (Intron Biotechnology, Seoul, Korea). Real-time PCR amplification of the cDNA was analyzed with SYBR Green Real-time PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) in a Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The results were analyzed using the CFX Manager software and normalized by the housekeeping genes β -actin and *L19*. The Primers used were as follows:

For β -actin:

Forward: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'
Reverse: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'

For *L-19*:

Forward: 5'-GGT GAC CTG GAT GAG AAG GA-3'
Reverse: 5'-TTC AGC TTG TGG ATG TGC TC-3'

For *IL-1 β* :

Forward: 5'-AGG GCT TCC AAA CCT TTG AC-3'
Reverse: 5'-ATA CTG CCT GCC TGA AGC TCT TGT-3'

For *Iba-1*:

Forward: 5'-AGC TTT TGG ACT GCT GAA GG-3'
Reverse: 5'-TTT GGA CGG CAG ATC CTC ATC-3'

For *TNF- α* :

Forward: 5'-TGG GAC AGT GAC CTG GAC TGT-3'
Reverse: 5'-TTC GGA AAG CCC ATT TGA GT-3'

For *CD11b*:

Forward: 5'-CCA CTC ATT GTG GGC AGC TC-3'
Reverse: 5'-GGG CAG CTT CAT TCA TCA TGT C-3'

For *COX-2*:

Forward: 5'-TGC TGT ACA AGC AGT GGC AA-3'
Reverse: 5'-AGG GCT TTC AAT TCT GCA GCC A-3'

For *mPGES1*:

Forward: 5'-CTG CTG GTC ATC AAG ATG TAC G-3'
Reverse: 5'-TGC CAG ATT TTC TCC ATG TCG-3'

For *Ctnnb1*:

Forward: 5'-ATC CAA AGA GTA GCT GCA GG-3'
Reverse: 5'-TCA TCC TGG CGA TAT CCA AG-3'

For *ER- α* :

Forward: 5'-GTG CCT GGC TGG AGA TTC TG-3'
Reverse: 5'-GAG CTT CCC CGG GTG TTC -3'

For *ER- β* :

Forward: 5'-CAG TCC GTC TGG CCA ACC T-3'
Reverse: 5'-ACC ACA TTT TTG CAC TTC A-3'

For *GFAP*:

Forward: 5'-TCA ATG ACC GCT TTG CTA GC-3'
Reverse: 5'-ACT CGT GCA GCC TTA CAC AG-3'

2.4. Immunohistochemistry

Mice were anesthetized and perfused transcardially with 0.9% saline (w/v), followed by fixation with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains were harvested and post-fixed overnight with 4% paraformaldehyde in PB buffer before preparing the coronal sections (50 μm thickness) using a vibratome (5100 mz Campden Instruments, Leicestershire, UK). After several PB washes, the sections were preincubated with 0.3% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature (RT). They were then incubated with primary antibodies against rabbit Iba-1 (1:1000 dilution, Wako, Osaka, Japan), rabbit COX-2 (1:1000 dilution, Abcam, Cambridge, UK), and mouse hemagglutinin (HA) antibodies (BioLegend, San Diego, CA, USA) overnight at RT. Immunofluorescence was performed with the secondary antibodies (Alexa Fluor 594-labeled anti-rabbit antibody, 1:1000 dilution; Alexa Fluor 488-labeled anti-mouse antibody, Invitrogen Life Technologies, Carlsbad, CA, USA) for 2 h at RT. The sections were then mounted onto glass slides and covered by coverslips with a drop of mounting medium (Dako North America Inc, Carpinteria, CA, USA). The coverslips were sealed with nail polish to prevent desiccation and movement of the samples under the microscope. The images were recorded using fluorescence microscopy (Axioplan2 Imaging, Carl Zeiss Microimaging Inc., Thornwood, NY, USA). The sections containing hypothalamic nuclei (stereotaxic coordinates: between -1.46 and -1.82 mm from the bregma) were matched with the mouse brain atlas book (Paxinos and Franklin, 2001, the Mouse Brain in Stereotaxic Coordinates—second edition, San Diego, CA, USA, Academic Press) and subjected to analyses.

2.5. Translating ribosome affinity purification

Translating ribosome affinity purification (TRAP) was performed as described by a previous reporter [9] with some modifications. The hypothalamus was collected and weighed before homogenization (10 % wt/vol) in homogenization buffer [50 mM Tris, pH 7.4 (Lugen Sci, Gyeonggi-do, South Korea), 100 mM KCl (Lugen Sci), 12 mM MgCl₂ (Lugen Sci), 1% NP-40 (Lugen Sci), 1 mM DTT (Lugen Sci), 200 U/mL RNasin (Promega, Madison, WI, USA), 1 mg/mL heparin (Sigma-Aldrich), 100 µg/mL cycloheximide (Sigma-Aldrich), protease inhibitor (Roche, Basel, Switzerland)]. Samples were allowed to settle on ice for 10 min and then centrifuged at 10,000×g for 10 min. One microliter of HA antibody (BioLegend, San Diego, CA, USA) was added to the supernatant (400 µL) and the solution was incubated for 5 h at 4 °C. For immunoprecipitation of polysomes detected by a monoclonal antibody against HA reporter protein, protein G magnetic beads (Thermo Scientific, Waltham, MA, USA) were added to the supernatant and rotated overnight at 4 °C. The following day, samples were spun at 2500 rpm for 30 s to collect the pellet. The pellet was washed thrice with high salt buffer (50 mM Tris, pH 7.4, 300 mM KCl, 12 mM MgCl₂, 1% NP-40, 1 mM DTT, 100 µg/mL cycloheximide). To isolate total RNA, 350 µL of RLT buffer (Qiagen, Germantown, MD, USA) was added to the pellets. Total RNA was extracted using a RNeasy Mini kit (Qiagen), according to the manufacturer's instructions, and quantified with NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). To evaluate the levels of ribosome-associated mRNA in microglia, we synthesized cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and performed quantitative real-time PCR (qRT-PCR).

2.6. Statistical analysis

Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± SEM. An unpaired *t*-test was performed to analyze the significance between the two experimental groups. Two-way repeated-

measures ANOVA analysis was used to detect interaction between time and groups. Significance was set at *p* < 0.05.

3. Results

3.1. Estrogen deficiency triggers obesity development

Since it is known that menopausal women display visceral obesity [10], we evaluated body weight gain and adiposity utilizing ovariectomized (OVX)-mice, an experimental mouse model of menopause. Consistent with the previous findings, we observed that OVX mice displayed a significant increase in the body weight gain when compared with sham-control mice (Fig. 1A). Additionally, the weights of pre-ovarian and perirenal fat pads in OVX-mice were significantly higher than those observed in the sham-control mice (Fig. 1B). In accordance with increased body weight and fat pads, the average size of adipocytes in OVX-mice was larger than that seen in sham-control mice (Fig. 1C and D). Consequently, a fewer number of adipocytes was observed in pre-ovarian fat pad of OVX-mice (Fig. 1E). These data verified that the OVX-mice used in the present study are suitable for investigating the underlying causes of visceral obesity during the postmenopausal period.

3.2. Estrogen deficiency leads to an elevation of inflammatory gene expression in the hypothalamus

Although previous studies have reported that estrogen deficiency causes systemic inflammation which is linked to multiple metabolic disorders such as obesity and diabetes [3,4], the relationship between menopause-induced estrogen deficiency and hypothalamic inflammation is remains poorly understood. In this study, we observed elevated mRNA levels of inflammatory cytokines, including *TNF-α* (Fig. 2A) and *IL-1β* (Fig. 2B) in the hypothalamus of OVX-mice when compared with sham-control mice. Furthermore, we found that the absence of estrogen led to an increase in *cyclooxygenase-2* (*COX-2*) gene expression, the rate-limiting enzyme involved in the synthesis of prostaglandin E₂,

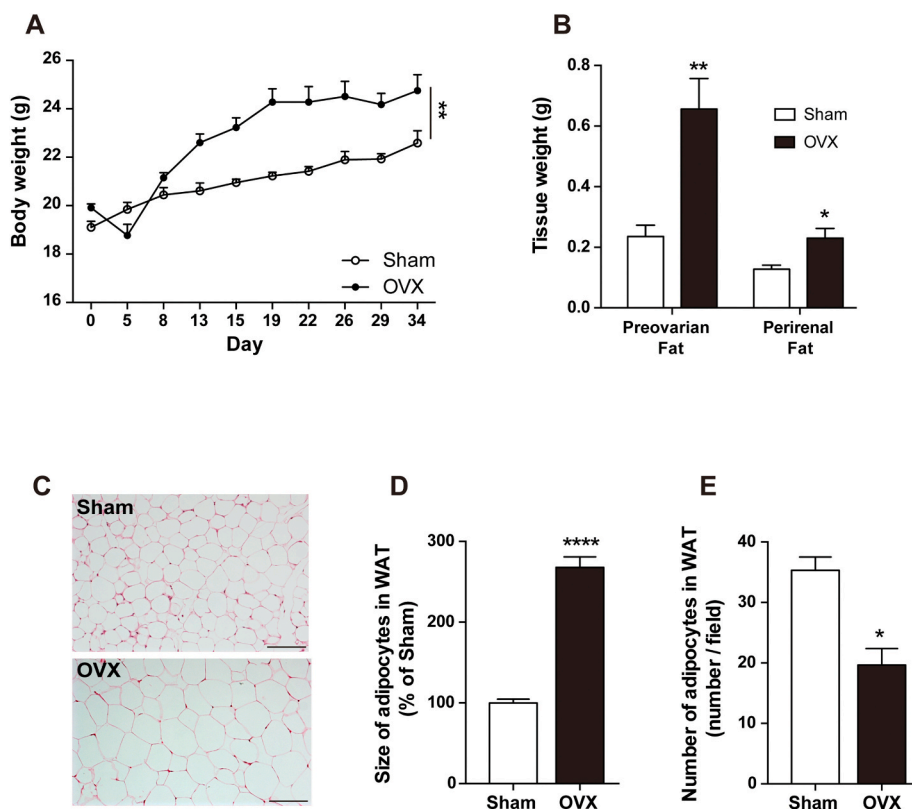


Fig. 1. Ovariectomized mice displayed visceral obesity. Ovariectomized (OVX) mice were maintained on a regular diet for 5 weeks and the changes in body weight and adiposity were measured. (A) The body weight gain in OVX mice was higher than that observed in sham-control mice. (B) OVX mice displayed elevated levels of preovarian and perirenal adipose tissue mass as compared with sham-control mice. (C) Representative images showing the morphology of preovarian adipose tissue stained with H&E. (D) The size of preovarian fat in OVX mice was greater than that observed in sham-control mice. (E) The number of adipocytes per field was reduced in OVX mice when compared with sham-control mice. *n* = 4–6 mice per group. **p* < 0.05, ***p* < 0.01, *****p* < 0.001 for effects of OVX versus sham-control group. Scale bar = 100 µm.

which triggers the cellular inflammatory process (Fig. 2C). We further observed that OVX-mice revealed an elevated number of COX-2 positive cells in the arcuate nucleus (ARC) of the hypothalamus, as determined by immunohistochemistry (IHC) (Fig. 2D and E). From these data, we demonstrated the effects of estrogen deficiency on the development of hypothalamic inflammation.

3.3. Estrogen deficiency triggers microglia activation in the hypothalamus

Hypothalamic gliosis coupled with inflammation is the hallmark of degenerating hypothalamic functions during overnutrition and negative energy balance [6,7]. In particular, microglia are recognized as master cells that mediate the adverse effect of hypothalamic inflammation induced by metabolic stresses [6,8,11,12]. Thus, we evaluated the patterns of microglial activation in the hypothalamic ARC. Immunostaining with an antibody against Iba-1 (Fig. 3A), a molecular marker for microglia, showed an increased number of microglia cells in the hypothalamic ARC of the OVX-mice (Fig. 3B) as well as an increase in the size of the soma (Fig. 3C), indicating microglial activation. Consistent with these findings, we also found higher mRNA levels for *CD11b* (Fig. 3D), another marker for microglial activation, and *Iba-1* (Fig. 3E) in the hypothalamic ARC of the OVX-mice. Since astrocytes are also involved in the inflammatory processes, we further confirmed whether activation of

astrocytes occurs in hypothalamus of the OVX mice by analyzing the mRNA expression of *GFAP*, a molecular marker for astrocyte and *catenin β -1* (*Ctnnb1*), which is also participates in reactive astrogliosis [13]. As shown Fig. 3 F and G, the mRNA levels of *GFAP* and *Ctnnb1* were not altered in hypothalamus of the OVX-mice, when compared with sham-control mice. These findings suggest that estrogen deficiency enhances inflammatory responses in the hypothalamus, and is associated with microglial activation.

3.4. Estrogen deficiency gives rise to an increased expression of inflammatory genes in the hypothalamic microglial cells

To further verify the inflammatory effect of estrogen deficiency on hypothalamic microglial cells, we tested the expression of genes involved in the inflammatory responses from isolated microglia-specific mRNA by immunoprecipitating the polysomes. We crossed *CX3CR1 Cre* mice with *Rpl 22* floxed mice and generated a line that enabled the purification of the microglia-specific RNA after *Cre* recombination. We first validated successful microglia-specific *Cre* recombination determined by the presence of Iba-1 immunolabeling in the cells that express *CX3CR1*-driven HA reporter protein (Fig. 4A). We also validated the purification of mRNA isolated from the microglia by confirming a predominant expression of *Iba-1* mRNA in purified sample compared with

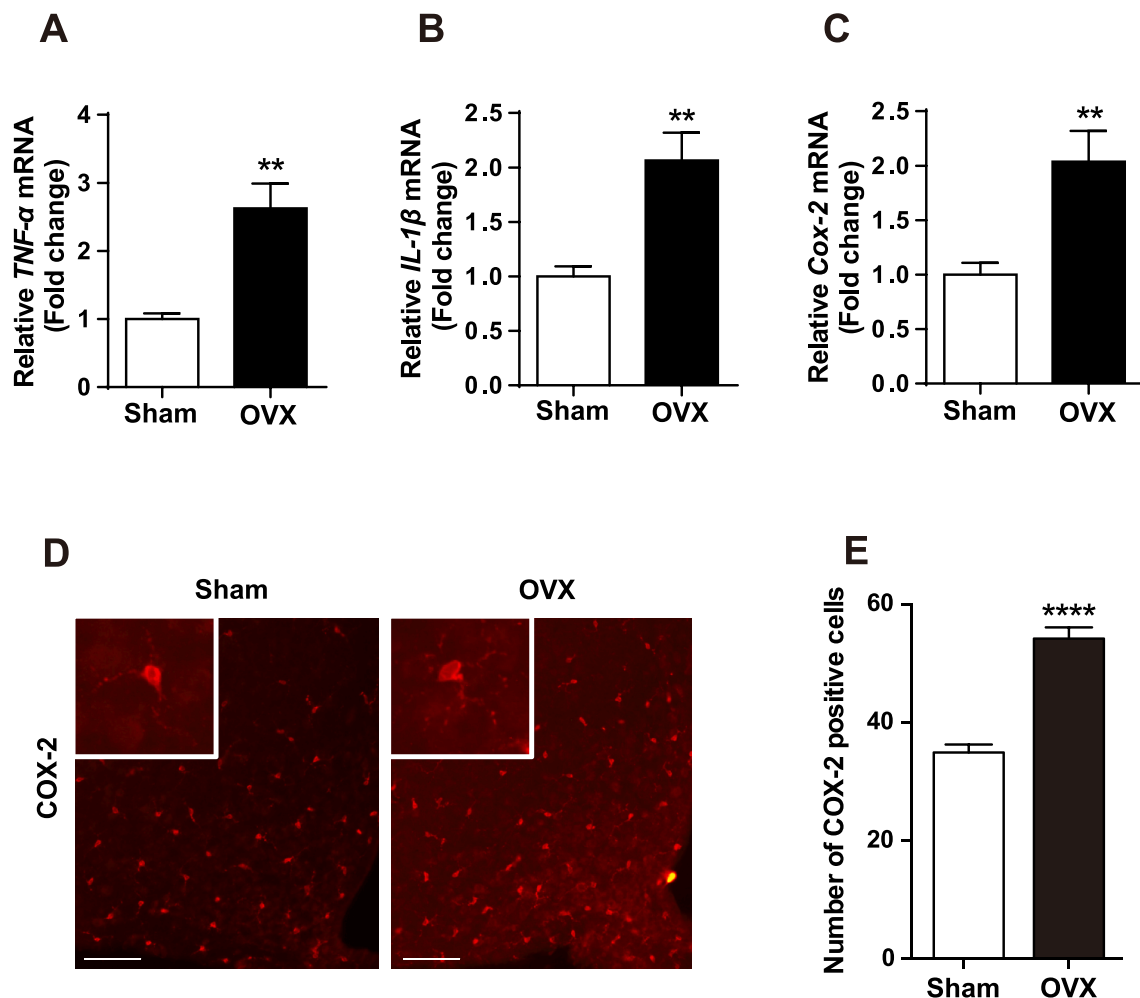


Fig. 2. Estrogen deficiency leads to an elevation in expression of inflammatory genes in the hypothalamus. Whole hypothalamus was harvested from both OVX and sham-control mice and analyzed for mRNA expression involved in the inflammatory responses. The elevated mRNA levels of hypothalamic genes involved in inflammatory processes such as (A) *TNF- α* , (B) *IL-1 β* , and (C) *Cox-2*, were observed in OVX-mice ($n = 5$ mice per group). (D) Representative images showing immunosignals of COX-2 protein. (E) The number of COX-2-positive cells in the hypothalamus was higher in OVX mice when compared with sham-control mice ($n = 5$ mice per group and 3 sections per mouse). Results are presented as the mean \pm SEM. ** $p < 0.01$, **** $p < 0.001$ for effects of OVX versus sham-control group. Scale bar = 100 μ m.

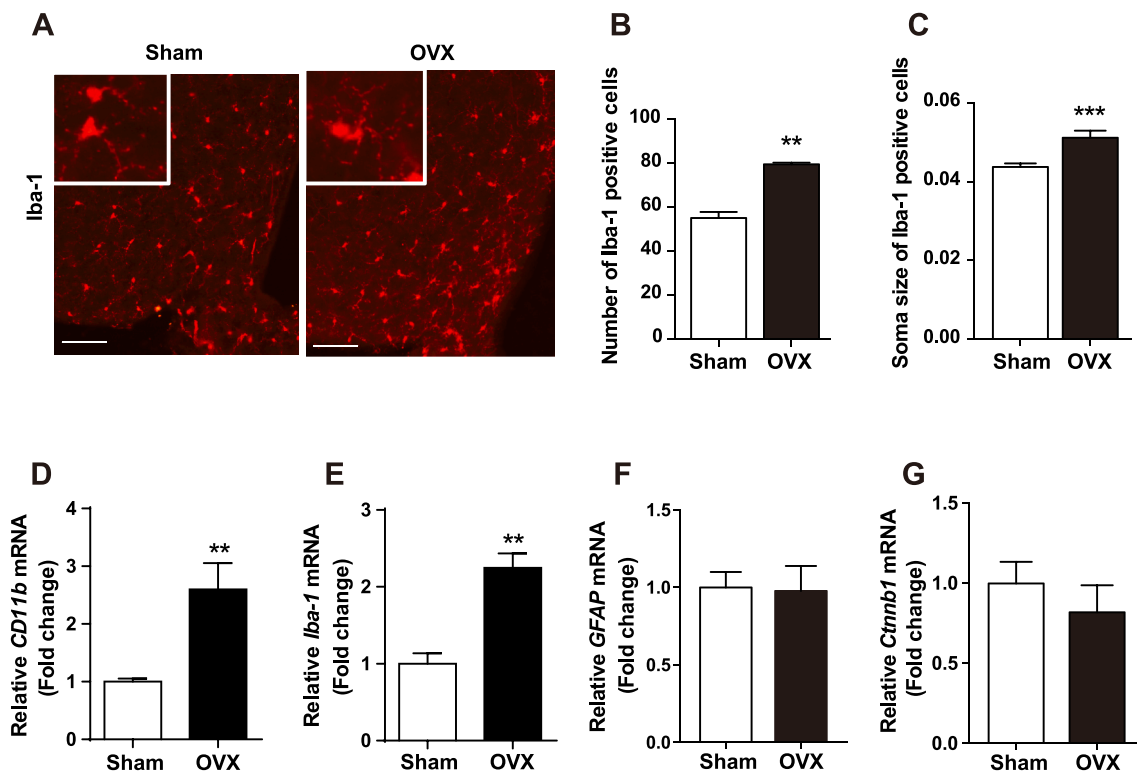


Fig. 3. Estrogen deficiency triggers microglial activation in the hypothalamus. (A) Representative images showing immunosignals of Iba-1 in the sections of the hypothalamus from each group. Increased (B) number and (C) soma size of microglia were observed in the hypothalamus of OVX-mice when compared with sham-control mice ($n = 5$ mice per group and 3 sections per mouse). The mRNA levels of hypothalamic (D) *CD11b* and (E) *Iba-1*, markers for microglia activation, were significantly elevated in OVX mice when compared with sham-control mice ($n = 5$ mice per group). The mRNA levels of hypothalamic (F) *GFAP* and (G) *Ctnnb1*, markers for astrocyte activation, were not altered in OVX mice when compared with sham-control mice ($n = 5$ mice per group). Results are presented as the mean \pm SEM. ** $p < 0.01$, *** $p < 0.005$ for effects of OVX group versus sham-control group. Scale bar = 100 μ m.

the input control sample (Fig. 4B). In polysome immunoprecipitation, we observed that OVX mice displayed elevated levels of inflammatory cytokines such as *TNF- α* (Fig. 4C), *IL-1 β* (Fig. 4D), and genes involved in prostaglandin E_2 synthesis, such as *COX-2* (Fig. 4E) and *microsomal prostaglandin E synthase-1 (mPGES-1)* (Fig. 4F) in hypothalamic microglial cells, when compared with sham-control mice. Additionally, increased *Iba-1* and *CD11b* mRNA levels were found in hypothalamic microglia of the OVX-mice (Fig. 4G and H). Furthermore, we confirmed the presence of *estrogen receptor- α* (*ER- α*) and *estrogen receptor- β* (*ER- β*) without changes of their expression levels in hypothalamus of the OVX-mice, when compared with sham-control mice (Fig. 4I and J). These observations confirmed that the estrogen deficiency gives rise to microglial activation and its related inflammatory responses in the hypothalamus.

4. Discussion

The present study highlights the relationship between hypothalamic inflammation and obesity pathogenesis associated with menopausal transition. Women going through menopause experience a series of physiological changes, which are linked to the decline of estrogen production and decrease in circulating estrogen [14]. A great deal of attention has been paid to investigate the pathological components linked to a variety of menopause-related degenerative diseases, which include cancer, neurodegenerative diseases, osteoporosis, and metabolic disorders [15–18]. It has also been identified that multiple cellular stresses such as oxidative stress, ER stress, and inflammation are deeply involved in the development of postmenopausal diseases [19,20]. In particular, chronic inflammation triggered by estrogen deficiency is tightly coupled to degeneration of metabolic organs and is therefore directly associated with metabolic diseases such as diabetes and obesity

[21]. Indeed, the proportion of women with metabolic diseases increases rapidly among menopausal women. In particular, these women display an imbalance of body composition, as seen in their elevated visceral fat content and decreased muscle mass [22,23]. Since visceral fats release multiple substances that lead to cellular inflammatory responses [24], it is widely believed that the initiation of pathological events involved in obesity development during the postmenopausal period is directly coupled to systemic inflammation. Multiple studies have been performed to understand the underlying mechanisms that are responsible for the inflammatory properties of metabolic organs and their impact on the perturbation of metabolic control during the postmenopausal period [25,26]. However, the impacts of hypothalamic inflammation on the development of menopause-induced obesity and its related secondary complications remains poorly understood. It is widely accepted that the hypothalamus is the central unit that controls energy homeostasis of the body by mediating the metabolic signals originating from the peripheral organs [5]. Furthermore, multiple lines of evidence have suggested that hypothalamic cells respond to circulating estrogen through their own estrogen receptors [27,28]. Thus, it is noteworthy to identify the relationship between disrupted hypothalamic circuit activity triggered by chronic inflammation and obesity during the postmenopausal period. Previous studies and our study both observe that OVX mice, an experimental mouse model for menopause, displayed visceral obesity showing increased body weight and adiposity [29]. Moreover, numerous studies have reported that sustained elevation of substances derived from visceral fats triggers hypothalamic inflammation concomitant with reactive gliosis [30,31]. In agreement with this, we successfully confirmed that OVX mice showed elevated hypothalamic inflammation by analyzing the expression of genes involved in cellular inflammatory responses. Hypothalamic reactive gliosis is recognized as an important cellular event for both acute and chronic

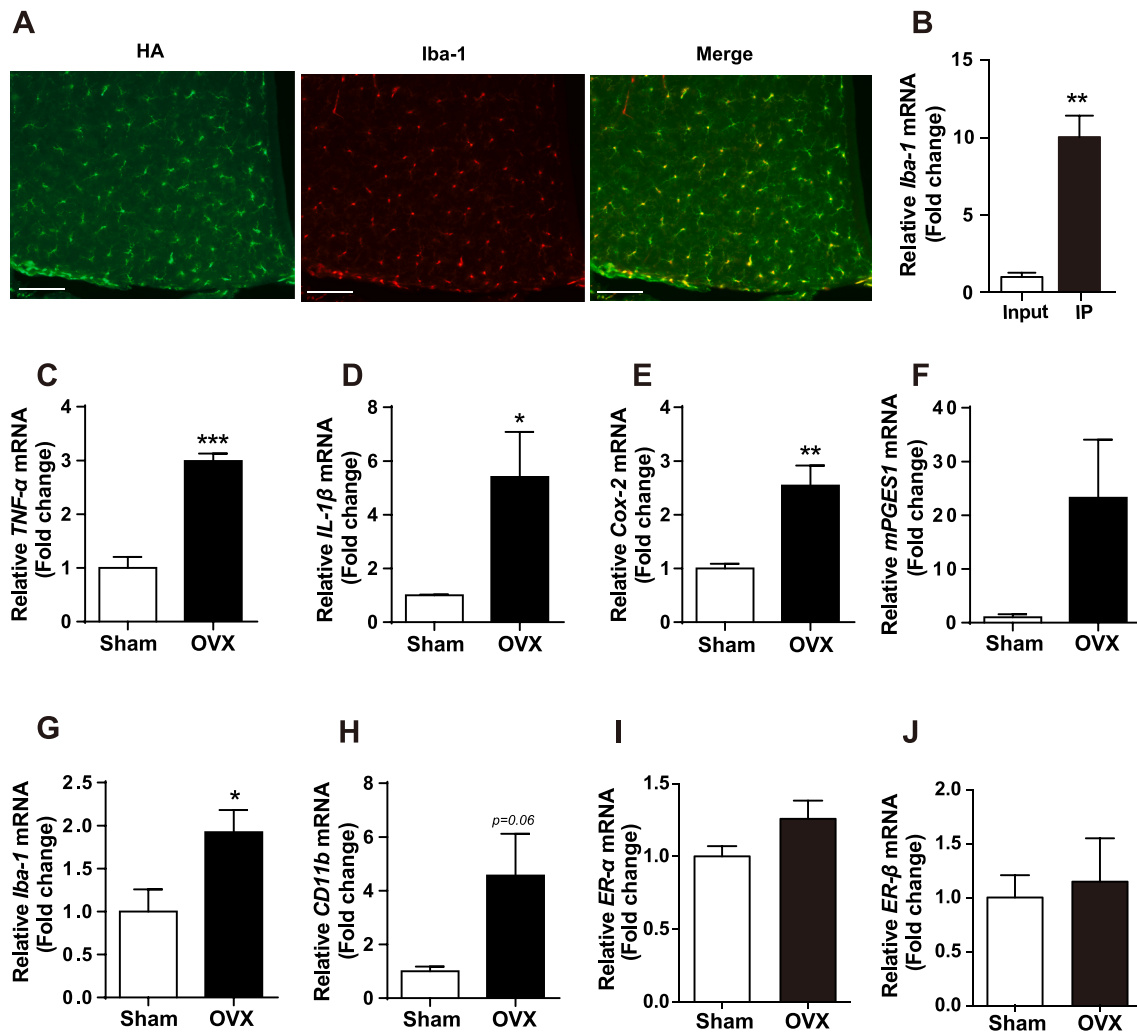


Fig. 4. Estrogen deficiency gives rise to an increased expression of inflammatory genes in the hypothalamic microglial cells. (A) Representative images showing immunosignals of the HA reporter and Iba-1 proteins in the sections from the hypothalamus of *CX3CR1 Cre; RPL22^{flA}* mice. (B) Predominant expression of *Iba-1* mRNA was observed in the purified microglial mRNA compared to the input control mRNA. The mRNA levels of inflammatory cytokines such as (C) *TNF-α* and (D) *IL-1β* were increased in hypothalamic microglial cells of the OVX-mice when compared with sham-control mice. The levels of (E) *Cox-2* and (F) *mPGES1* mRNA were enhanced in hypothalamic microglial cells of the OVX-mice when compared with sham-control mice. Increased mRNA levels of (G) *Iba-1* and (H) *CD11b* were observed in hypothalamic microglial cells of the OVX-mice when compared with sham-control mice. The levels of (I) *ER-α* and (J) *ER-β* mRNA were not changed in hypothalamic microglial cells of the OVX-mice when compared with sham-control mice. Results are presented as the mean \pm SEM. $n =$ at least 4 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ for effects of OVX group versus sham-control group.

inflammation triggered by overnutrition and is directly associated with obesity pathogenesis [8,32]. In particular, microglia actively participates in hypothalamic inflammation by mediating an alteration of multiple metabolites in obesity [11,12,33]. Furthermore, it has been reported that microglial activation accompanied by inflammatory responses is elevated in the models for estrogen deficiency [34,35]. In line with this evidence and our own data showing an elevation of hypothalamic inflammation in OVX mice, we further investigated the possibility of microglial activation in the hypothalamus of OVX mice. Our observations in a mouse model of estrogen deficiency revealed microglial activation in the hypothalamus, with histological data showing an increased number of Iba-1 protein, which is a hallmark of microglial activation, and molecular data presenting an increase in mRNA expression involved in microglial activation. These observations support the hypothesis that obesity development during the postmenopausal period is closely associated with the hypothalamic inflammation, which is in turn linked to microglial activation.

Previous studies have shown that estrogen plays a role as an active substance leading to lipolysis and thereby offering protection against obesity development [36,37]. Thus, it is important to investigate

whether estrogen deficiency is the primary factor in the development of hypothalamic inflammation revealed in the postmenopausal model or the alteration of metabolites associated with an expansion of fat tissues is also responsible for the same. Notably, multiple lines of evidence have shown that deficiency of estrogen leads to inflammatory responses in multiple sites of the brain such as cortex and hippocampus [38,39]. Moreover, estrogen exerts neuroprotective effects in various brain pathologies through the signaling of estrogen receptors [40–42]. Therefore, further studies are required to interrogate whether the coupling of estrogen and its receptor signaling directly participates in mitigating hypothalamic inflammation in association with metabolic disorders in menopause. Collectively, the current study suggests the potential impact of hypothalamic inflammation linked to microglial activation, on obesity pathogenesis during the postmenopausal period.

CRediT authorship contribution statement

Hye Rim Yang: Conceptualization, Investigation, Writing - original draft. **Thai Hien Tu:** Conceptualization, Investigation, Data curation. **Da Yeon Jeong:** Investigation. **Sunggu Yang:** Conceptualization,

Writing - original draft, **Jae Geun Kim**: Conceptualization, Data curation, Writing - original draft, Writing - review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Thai Hien Tu was supported by an Incheon National University (Foreign post-doctor program) Research Grant.

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