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## Arachidonic acid impairs hypothalamic leptin signaling and hepatic energy homeostasis in mice

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### Abstract

Epidemiological evidence suggests that the consumption of a diet high in n-6 polyunsaturated fatty acids (PUFA) is associated with the development of leptin resistance and obesity. We aim to examine the central effect of n-6 PUFA, arachidonic acid (ARA) on leptin sensitivity and leptin-regulated hepatic glucose and lipid metabolism. We found that intracerebroventricular injection of ARA (25 nmol/day) for 2.5 days reversed the effect of central leptin on hypothalamic JAK2, pSTAT3, pAkt, and pFOXO1 protein levels, which was concomitant with a pro-inflammatory response in the hypothalamus. ARA also attenuated the effect of central leptin on hepatic glucose and lipid metabolism by reversing the mRNA expression of the genes involved in gluconeogenesis (G6Pase, PEPCK), glucose transportation (GLUT2), lipogenesis (FAS, SCD1), and cholesterol synthesis (HMG-CoA reductase). These results indicate that an increased exposure to central n-6 PUFA induces central cellular leptin resistance with concomitant defective JAK2-STAT3 and PI3K-Akt signaling.

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## **Arachidonic acid impairs hypothalamic leptin signaling and hepatic energy homeostasis in mice**

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## **Abstract**

Epidemiological evidence suggests that the consumption of a diet high in n-6 polyunsaturated fatty acids (PUFA) is associated with the development of leptin resistance and obesity. We aim to examine the central effect of n-6 PUFA, Arachidonic acid (ARA) on leptin sensitivity and leptin-regulated hepatic glucose and lipid metabolism. We found that intracerebroventricular injection of ARA (25 nmol/day) for 2.5 days reversed the effect of central leptin on hypothalamic JAK2, pSTAT3, pAkt, and pFOXO1 protein levels, which was concomitant with a pro-inflammatory response in the hypothalamus. ARA also attenuated the effect of central leptin on hepatic glucose and lipid metabolism by reversing the mRNA expression of the genes involved in gluconeogenesis (G6Pase, PEPCK), glucose transportation (GLUT2), lipogenesis (FAS, SCD1), and cholesterol synthesis (HMG-CoA reductase). These results indicate that an increased exposure to central n-6 PUFA induces central cellular leptin resistance with concomitant defective JAK2-STAT3 and PI3K-Akt signaling.

**Key words:** Arachidonic acid, leptin resistance, hypothalamus, inflammation, glucose metabolism, lipid metabolism, n-6 PUFA

Evidence suggests that fatty acids act directly on the central nervous system to affect food intake, insulin sensitivity, and leptin sensitivity [1, 2]. Recent studies have shown that the ingestion of a fat-rich diet leads to leptin resistance in the hypothalamus [1, 3]. High-fat diets rich in n-6 polyunsaturated fatty acids (PUFA) have been shown to increase the risk of leptin resistance, diabetes, and obesity in humans and rodents [4, 5]. High-fat diet (HFD)-induced leptin resistance and obesity may be mediated by defects in leptin in the Janus kinase 2 and signal transducer and activator of transcription (JAK2-STAT3) and the phosphatidylinositol 3-kinase (PI3K-Akt) signaling pathways in the hypothalamus [1, 6, 7]. The JAK2-STAT3 pathway has been shown to mediate the effect of leptin on glucose metabolism [8], and the PI3K-Akt pathway is involved in both glucose [9] and lipid metabolism [10]. Recent studies have provided strong evidence for the contribution of hypothalamic inflammation to HFD-induced leptin resistance and/or type 2 diabetes mellitus [11, 12].

A number of studies show that leptin also plays a primary role in the regulation of glucose homeostasis [13]. For instance, leptin administration in rodents enhances insulin mediated suppression of hepatic glucose production [14, 15] and suppress [16] hepatic gluconeogenesis. Moreover, a large amount of evidence supports direct improving function of leptin in peripheral lipid metabolism by regulating lipogenesis,  $\beta$ -oxidation, and cholesterol metabolism [17, 18]. However, the effect of n-6 PUFA on glucose and lipid homeostasis via central leptin way is still unclear.

Arachidonic acid (ARA, 20:4 n-6) is one of the most abundant long-chain PUFA in the brain. It is a key component of cell membranes and serves as a precursor to some eicosanoids (prostaglandins, thromboxanes, leukotrienes) which mediate inflammatory responses [19]. Interestingly, an excess consumption of n-6 PUFA (abundant in the western diet) can increase inflammation and contribute to the pathology of major chronic diseases, including metabolic syndrome, type 2 diabetes mellitus, and obesity [20, 21]. Increased ARA content in adipose tissue is also associated with an increased risk of metabolic syndrome and obesity [21]. Finally, the inhibitory role of ARA has been suggested in both basal and insulin-stimulated leptin expression and production [5].

The aim of the present study was to determine the effect of the direct central infusion of ARA on hypothalamic inflammation, leptin sensitivity, leptin signaling, and hepatic energy homeostasis in mice. In addition, to identify possible molecular mechanisms in which ARA is involved, the hypothalamic expression of tyrosine hydroxylase (TH, a sympathetic activity marker) was assessed.

## **Materials and Methods:**

### **Animals**

Male C57BL/6J mice (10 weeks old, body weight:  $22.74 \pm 3.22$  g) were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22°C, 12 hour light/dark cycle). Mice were maintained on a normal lab chow diet (LC; Vella Stock feeds, Doonside, NSW, Australia) throughout the study. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

### **Experiment protocols**

The mice were randomised into four groups: vehicle+saline, vehicle+leptin, ARA+saline, ARA+leptin (n=12/group). After 1 week of acclimatization, mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study [22]. The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Supplementary Fig. 1). A total of 3 times of ARA icv injections (for 2.5 days each time) were conducted in this study.

### **Central leptin sensitivity test**

Five days after the cannula implantation, a central leptin sensitivity test was performed as described previously [23]. The mice received icv injections of ARA (25 pmol twice daily for 2.5 days, 5 injections in total) or vehicle [24]. At the end of day 2 of the test, the mice fasted overnight. One hour after the last

ARA/ vehicle injection, each group of mice will be treated with an icv injection of either leptin (0.50 µg/mice) or saline. Food intake and body weight were measured 24 hours after the icv leptin/saline injection. ARA (A9673, Sigma-Aldrich, Australia) was dissolved in 96% ethanol, dried using nitrogen gas, and then dissolved in 40% hydroxypropyl-β-cyclodextrin (HPB) (H107, Sigma-Aldrich) and stored at -20°C.

### **Intraperitoneal glucose tolerance test (GTT)**

After a 3 day interval, icv injections were repeated (details as per “Central leptin sensitivity test”). The glucose tolerance test was performed 30 minutes after the leptin/saline injection. Blood glucose was measured at 0, 30, 60, and 120 min after the glucose administration (0.5 g/kg glucose, intraperitoneal) using a glucometer (Alameda, CA).

### **Tissue collection**

After another 3 day interval, the icv injections were repeated (details as per “Central leptin sensitivity test”). Thirty minutes after the leptin/saline injection, the mice were sacrificed by CO<sub>2</sub> asphyxiation. The brain and liver were immediately collected, snap frozen in liquid nitrogen, and stored at -80°C for further processing and analysis. In a cryostat at a temperature of -18°C, 500 µm frozen brain sections were cut from Bregma -0.58 mm to -2.72 mm according to a standard mouse brain atlas [28]. The mediobasal hypothalamus (MBH) and paraventricular nuclei (PVN) were dissected from frozen coronal sections using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) based on previously described coordinates [23].

### **Western blot analysis**

Western blotting was performed on protein extracts from frozen tissue as described in our previous study [10]. The expression of specific proteins was determined using the following antibodies: TNF-α (sc-8301), IL-1β (sc-7884), IL-6 (sc-7920), pIkBα (sc-8404), and pJAK2 (sc-21870) (Santa Cruz Biotechnology, California), and pSTAT3 (Tyr705) (#9145), suppressor of cytokine signaling 3 (SOCS3) (#2932), pAkt (#9271), and phosphor-forkhead box protein O1 (pFOXO1) (#9461) (Cell Signaling Technology Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density was analysed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories,

Hercules, CA, USA). All quantitative analyses were normalized to  $\beta$ -actin, based on our previous studies [25]. Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a previously-described modified multi-strip western blot, which allows the detection of multiple proteins with a smaller sample size than in a standard western blot [23].

### **RNA isolation and RT-PCR**

Total RNA from the liver was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA, USA) and reverse-transcribed to first-strand complementary DNA using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in a 20  $\mu$ l final reaction volume using a SYBR green I master on a Lightcycler 480 Real-time PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. This was repeated for a total of 45 cycles. The mRNA expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula  $2^{-\Delta\Delta Ct}$  (where  $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ reference}$ ) as described previously [26]. The primers used are listed in Supplementary Table.

### **Statistics**

Data were analysed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). The two-tailed student's t-test was used to compare hypothalamic cytokine expression between the ARA and vehicle groups. One-way ANOVA and two-way ANOVA with post hoc Tukey-Kramer honestly significant difference (HSD) test were used to analyse hypothalamic leptin signaling molecules, central leptin sensitivity, and the mRNA expression of genes regulating hepatic glucose and lipid metabolism.  $p < 0.05$  was regarded as statistically significant. Values are expressed as mean  $\pm$  SEM.

## **Results**

### **Central arachidonic acid administration reduces central leptin sensitivity**



To investigate the effect of ARA on central leptin action, we examined food intake and body weight change in response to the central administration of leptin. Compared to the saline group, food intake was reduced in the leptin group at 1 hour (-57.27%,  $p<0.001$ ), 4 hours (-57.55%,  $p<0.001$ ), 16 hours (-45.19%,  $p<0.001$ ), and 24 hours (-50.98%,  $p<0.001$ ) (Fig. 1A). Conversely, there was no significant difference in food intake between the leptin group and the saline group at any of the time points measured (except for 1-hour which shows a 48.59% reduction,  $p<0.01$ , Fig. 1A) after ARA infusion. This suggests that the “leptin-desensitizing” effect of ARA was not evident at this early stage of treatment. Above changes in food intake indicate that the central administration of ARA reduces central leptin sensitivity by inhibiting the anorexigenic effect of leptin. However, 24-hour body weight gain was reduced by leptin in both vehicle groups and ARA groups (by 426.85%,  $p<0.001$  and 95.87%,  $p<0.05$ , respectively), indicating that the ARA groups were less sensitive for leptin compared to the vehicle groups from a body weight gain perspective (Fig. 1B).

### **Central arachidonic acid administration inhibits leptin signaling in the MBH and PVN of the hypothalamus**

To investigate the effect of ARA on central leptin signaling, the key markers of the JAK2-STAT3 and PI3K-Akt signaling pathways were examined in the MBH and PVN of the hypothalamus. The central leptin injection significantly increased the levels of pJAK2 and pSTAT3 in the vehicle groups in both the MBH (pJAK2:  $p<0.01$ , pSTAT3:  $p<0.05$ , Fig. 2A) and the PVN (both  $p<0.05$ , Fig. 2B) of the hypothalamus. However, these effects were eliminated in the ARA groups. Similarly, SOCS3 was also increased after the leptin icv injection in both the MBH and PVN in the vehicle groups (both  $p<0.05$ , Fig. 2A & 2B) but not the ARA groups (Fig. 2A & 2B). Additionally, compared to the vehicle+saline group, the ARA+saline group showed an increased level of SOCS3 in the PVN ( $p<0.05$ , Fig. 2F) but not in the MBH. In the PI3K-Akt pathway, leptin significantly increased pAkt and pFOXO1 in both the MBH (both  $p<0.05$ , Fig. 3A) and the PVN (both  $p<0.05$ , Fig. 3B) in the vehicle groups but not in the ARA groups. In addition, compared to the vehicle+saline group, the ARA+saline group increased the level of pFOXO1 in the PVN ( $p<0.05$ , Fig. 3B) but not in the MBH. These results suggest that ARA blunts both the leptin JAK2-STAT3 and PI3K-Akt signaling pathways in the MBH and the PVN.

### **Central arachidonic acid administration stimulates pro-inflammatory responses in the PVN of the hypothalamus**

Previous studies show that a high consumption of n-6 PUFA can induce inflammation *in vitro* and *in vivo* [27, 28]. To investigate the effect of ARA on hypothalamic inflammation, we measured the protein levels of pro-inflammatory molecules (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and pI $\kappa$ B $\alpha$ ) in the MBH and PVN of the hypothalamus. The central administration of ARA significantly increased TNF- $\alpha$  in the MBH ( $p < 0.05$ , Fig. 4A) and TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and pI $\kappa$ B $\alpha$  in the PVN (TNF- $\alpha$ :  $p < 0.05$ , IL-1 $\beta$ :  $p < 0.01$ , IL-6:  $p < 0.05$ , and pI $\kappa$ B $\alpha$ :  $p < 0.05$ , Fig. 4B) (ARA+saline vs vehicle+saline). These results indicate that the central administration of ARA induce an obvious pro-inflammatory response in the PVN of the hypothalamus.

### **Central arachidonic acid administration attenuates central leptin action on hepatic glucose metabolism**

After the central infusion of ARA and leptin, a glucose tolerance test was used to determine whether the short-term ARA infusion altered the peripheral glucose production in response to the central leptin administration. There was no significant difference in the fasting blood glucose levels between the vehicle and ARA groups, which indicates that the central leptin administration failed to affect the glucose levels. To further investigate the effects of ARA on the central leptin regulation of hepatic glucose metabolism, the mRNA expression of the markers involved in gluconeogenesis (Glucose 6-phosphatase, G6Pase, and Phosphoenolpyruvate carboxykinase, PEPCK), glucose transportation (glucose transporter 2, GLUT2), and glycolysis (glucokinase, GK) were measured in the liver. As depicted in Fig 5, the mRNA expression of G6Pase ( $p < 0.01$ , Fig. 5), PEPCK ( $p < 0.01$ , Fig. 5), and GLUT2 ( $0.05 < p < 0.1$ , Fig. 5) in the vehicle group decreased significantly after the icv leptin injection, suggesting that central leptin can inhibit hepatic gluconeogenesis and glucose transportation. However, icv leptin did not produce this effect following the ARA treatment, indicating that the inhibitory effect of leptin was blunted by ARA. In addition, the ARA treatment alone significantly decreased the mRNA expression of G6Pase ( $p < 0.01$ , Fig. 5) and PEPCK ( $p < 0.05$ , Fig. 5) in the liver. Finally, the icv injection of leptin tended to decrease hepatic GK mRNA expression ( $p = 0.079$ , Fig. 5) in the vehicle groups but not the ARA groups, suggesting that

the down-regulatory effect of leptin on hepatic glycolysis was inhibited by ARA. The icv injection of ARA increased the mRNA level of PEPCK in response to central leptin ( $p < 0.01$ , Fig. 5).

### **Central arachidonic acid administration attenuates central leptin action on hepatic lipid metabolism**

To examine the effect of icv ARA on hepatic lipid metabolism in response to leptin, the mRNA expression of the genes involved in hepatic lipogenesis, lipid beta-oxidation, and cholesterol metabolism were examined by quantitative RT-PCR. In the vehicle group, icv leptin significantly decreased the level of fatty acid synthase (FAS, a multifunctional enzyme that catalyzes fatty acid synthesis) and Stearoyl-CoA desaturase (SCD1, a rate-limiting enzyme that converts saturated fatty acids to monounsaturated fatty acids) (both  $p < 0.05$ , Fig. 6A), indicating a reduced lipogenic effect was induced by leptin in the liver. However, leptin failed to decrease FAS and SCD1 mRNA expression after the ARA treatment (ARA+leptin vs ARA+saline). Unexpectedly, the mRNA expression of Acetyl-CoA carboxylase a (ACCa, an enzyme to provide the malonyl-CoA substrate for the biosynthesis of fatty acids) was enhanced by central leptin administration ( $p < 0.05$ , Fig. 6A) in both the vehicle and the ARA groups.

Acyl-CoA oxidase (ACOX) and acetyl-CoA acetyltransferase 1 (ACAT1) are two markers of lipid beta-oxidation in the liver. No significant difference was found in the mRNA levels of ACOX and ACAT1 after the central leptin injection in both the vehicle and the ARA groups. Moreover, the mRNA expression of key enzymes involved in cholesterol metabolism was also analyzed. The mRNA expression of 3-hydroxy-3-methylglutaryl-coenzyme reductase (HMG-CoA reductase; the key enzyme involved in the de novo synthesis of cholesterol) was down-regulated after the central leptin injection ( $p < 0.05$ , Fig. 6B), while disappeared in the ARA (ARA+leptin vs. ARA+saline) groups. No significant difference was observed in the mRNA levels of Apo lipoprotein A1 (APoA1; the main component of high-density lipoprotein) in both the vehicle and the ARA treatment groups.

### **Central arachidonic acid administration attenuates the effect of leptin on the expression of TH**

To examine the hypothalamic sympathetic response to central leptin and ARA administration, the protein levels of TH were measured in the MBH and PVN. The central leptin injection significantly elevated the TH protein levels in the vehicle groups in the MBH and PVN (both  $p < 0.05$ ). This elevation effect of

leptin was eliminated in the ARA groups in both the MBH and the PVN (both  $p < 0.05$ , Fig. 7). Moreover, the icv injection of ARA alone significantly enhanced the TH protein levels in both the MBH and the PVN (both  $p < 0.05$ , Fig. 7).

## **Discussion**

Diets rich in n-6 PUFA have been implicated in regulating insulin resistance, leptin resistance, metabolic syndrome, and obesity [29-31]. However, the role of n-6 PUFA in regulating leptin signaling in specific regions of the hypothalamus and energy homeostasis in the liver has not been systematically addressed. We demonstrate for the first time that central exposure to ARA, an n-6 PUFA present in high levels in the western diet, results in central leptin resistance, leading to impaired regulatory effects of leptin on food intake, hypothalamic JAK2-STAT3 and PI3K-Akt signaling pathways, and hepatic glucose and lipid metabolism.

Firstly, our purpose is to explore the effect of ARA on central leptin resistance. Previous study from Nuernberg et al. demonstrated that a high-fat diet rich in n-6 PUFA for 8 weeks induced peripheral leptin resistance with high fat deposition in mice [5]. In the current study, food intake at 4, 16, and 24 hours were significantly decreased after a central leptin injection. However, these effects were absent in the ARA treatment groups, indicating that the icv injection of ARA may inhibit the ability of central leptin to regulate food intake. Although the body weight change is not significant, the reduction trend still show a sensitivity inhibited effect of ARA on leptin, and longer time test for body weight gain may be suggested. To our knowledge, this is the first study to report such a suppressive effect of n-6 PUFA on leptin sensitivity in the central nervous system.

To elucidate the mechanism involved in the effect of ARA on central leptin resistance, we also investigated two leptin signaling pathways involved in two sub-regions of the hypothalamus. Suppression of the JAK2-STAT3 signaling pathway in the hypothalamus has been reported in genetically-modified (*db/db*) [32] and HFD-induced [1, 33] obese mice, indicating leptin resistance in these animals.

Interestingly this suppression effect resembles the ARA injection mouse model in our present study, in which central ARA administration blunted the leptin-induced activation of the JAK2-STAT3 signaling pathway in the MBH and the PVN of the hypothalamus. However, in another study this effect was only observed in the arcuate nucleus (ARC) and not in the ventromedial hypothalamus (VMH) or dorsomedial hypothalamus (DMH) [1]. In addition, SOCS3 (a negative regulator of the leptin JAK2-STAT3 signaling pathway) has been suggested as a possible mediator of central leptin resistance in obesity. Leptin-induced STAT3 tyrosine phosphorylation in the hypothalamus is enhanced in SOCS3-deficient mice [34]. In the present study, central ARA infusion alone enhanced the expression of SOCS3 in the PVN, suggesting that ARA may inhibit leptin's effect on the JAK2-STAT3 signaling pathway via a direct effect on SOCS3. Further studies are warranted to investigate the direct effect of ARA on SOCS3 and the JAK2-STAT3 signaling pathway via pharmacological inhibition or the genetic ablation of SOCS3, as well as other possible targets of ARA upstream of the JAK2-STAT3 signaling pathway.

Evidence suggests that PI3K-Akt signaling plays an important role in transducing leptin action in the hypothalamus and contributing to the development of leptin resistance [33, 35], however the effect of central n-6 PUFA administration on the hypothalamic PI3K-Akt signaling pathway remains poorly understood. Consistent with previous studies [36], central leptin activated the PI3K-Akt signaling pathway through the phosphorylation of Akt and FOXO1 in the present study. However, ARA treatment eliminated this effect in both the MBH and PVN, suggesting that ARA impairs leptin signaling. Apart from leptin resistance, n-6 PUFA has also been reported to eliminate the effect of insulin on the phosphorylation of Akt in the hypothalamus [37]. It is possible that ARA may affect this pathway by a synergistic effect on both leptin and insulin, since both insulin and leptin regulate energy homeostasis through the PI3K-Akt signaling pathway. However, further studies are required to confirm this hypothesis.

It has been suggested that hypothalamic inflammation could be an important mediator of HFD-induced leptin resistance [11, 38]. In the present study, the pro-inflammatory markers TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and

pI $\kappa$ B $\alpha$  were up-regulated at the hypothalamus by central ARA administration, suggesting that central ARA administration is sufficient to induce hypothalamic inflammation. Interestingly, this up-regulatory effect was absent in the MBH of the hypothalamus. This indicates that a possible nucleus-specific action of ARA on inflammation may be responsible for its central leptin resistance effect. In addition, it has been suggested that the pro-inflammatory effects of n-6 PUFA may be involved in the reduction in anti-inflammatory molecules [39] or the activation of cyclooxygenase-2 (COX-2). Future studies investigating the nucleus-specific effect of ARA via alterations of anti-inflammatory molecules and COX-2 activities are warranted. It has been reported that ARA is rapidly incorporated into neural membranes, and more than 90% of brain radioactivity is esterified within 1 minute in conscious rats [40]. Therefore, we postulate that the high food intake in the western diet may result in an elevated level of ARA in the blood and brain. This will increase the release of COX-2, and subsequently increase the production of the pro-inflammatory eicosanoids (prostaglandins, thromboxanes and leukotrienes), and finally induce the inflammatory response in the hypothalamus. Furthermore, the activation of intracellular inflammatory signals has been proven to induce hypothalamic leptin resistance [41]. Overall, these findings (including ours) imply that the n-6 PUFA-induced activation of the pro-inflammatory molecules in the PVN of the hypothalamus may induce central leptin resistance. However, cautions should be taken when extrapolating these results onto oral feeding studies, which warrant future studies.

Metabolic studies have shown that the central administration of leptin [42], insulin [43, 44], and fatty acids [43, 45] are also involved in the regulation of hepatic glucose and lipid metabolism. However, it is unclear whether the acute central administration of n-6 PUFA modulates the ability of central leptin to regulate hepatic glucose and lipid metabolism. Consistent with previous studies [16, 46], hepatic gluconeogenesis, glucose transportation, and glycolysis were inhibited by central leptin administration in the present study. Furthermore, this study shows that ARA treatment reversed the effect of leptin in suppressing these hepatic glucose metabolism processes. Many mechanisms have been proposed to explain the effect of leptin on peripheral glucose metabolism. Buettner et al. proved that the cellular mechanisms underlying the hypothalamic regulation of hepatic glucose efflux depend on intact leptin

STAT3 signaling [9]. A study by Morton et al. indicates that hypothalamic leptin PI3K signaling is an important neuronal mechanism of glucose metabolism [10]. These reports, combined with our findings, suggest that the impairment of central leptin on glucose metabolism is likely to be a major contributor to hepatic glucose metabolic disruption in leptin resistance and obesity. Finally, it appears that some markers of metabolic syndrome (eg. fasting blood glucose levels and glucose tolerance) were not affected by ARA treatment in the present study. This is due to the fact that this is an acute study designed to focus on the detection of acute molecular changes upon central ARA administration. Future studies are required to study the chronic effect of ARA on these metabolic markers.

It has been suggested that central leptin also regulates the expression of key genes encoding lipid metabolism [18, 47]. Specifically, leptin has been reported to suppress hepatic lipogenesis and  $\beta$ -oxidation [7]. Consistent with previous studies, the mRNA expression of FAS and SCD1 was significantly down-regulated by central leptin administration. Expectedly, the present study shows that ARA pre-treatment blunts the suppressive effect of leptin on hepatic lipogenic metabolism, which indicates that ARA may act as an impaired mechanism intended to induce leptin resistance and obesity. However, the expression of ACOX and ACAT1 were unaltered after the central leptin injection in the present study, which is inconsistent with a previous report using *ob/ob* mice [17]. This discrepancy may be due to different genetic backgrounds, diet composition, environmental factors, and experimental protocols. Our results suggest that the mRNA expression of key metabolic enzymes involved in hepatic glucose metabolism after n-6 PUFA treatment can be partly explained by the central leptin resistant effect of n-6 PUFA.

Studies have shown that the sympathetic nervous system is involved in the transduction of leptin signals from the central nervous system to regulate peripheral glucose and lipid metabolism [9, 48]. TH is a rate-limiting enzyme which synthesizes catecholamine. Hypothalamic TH has been shown to mediate leptin-induced sympathetic activity and energy expenditure [49]. In line with above previous studies, our study showed that the icv central injection of leptin significantly increased the TH protein levels in the MBH

and PVN. Furthermore, we found that this effect was inhibited by central ARA administration, indicating that ARA suppresses the leptin-activated sympathetic outflow. It has also been reported that the decrease of sympathetic tone is attributed to the attenuated hypothalamic leptin-mediated PI3K signaling in the obese mice [7]. Therefore, these findings provide a mechanism to connect the action of ARA on hepatic glucose and lipid metabolism with the central leptin signaling pathway via the sympathetic nervous system.

In summary, the acute central injection of ARA induces decreased leptin sensitivity in the central nervous system and blunts the effect of leptin on the suppression of food intake and body weight in mice. The hypothalamic leptin JAK2-STAT3 and PI3K-Akt signaling pathways were impaired after the ARA treatment, and followed by a significant pro-inflammatory response in the PVN. Impaired hypothalamic leptin signaling stimulated by ARA leads to a pronounced attenuation in the gene expression involved in hepatic glucose and lipid metabolism. A better understanding of these mechanisms, and the further characterization and role of high n-6 PUFA consumption in physiological and pathological processes of leptin resistance, might lead to the identification of novel therapeutic targets for the prevention and treatment of diabetes and obesity. In further studies, some unsolved questions need further exploration, such as the interactions of neuronal/glial cells to modulate the fatty acid sensing, and the response of the leptin signaling pathways in other brain regions.

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## Figure legends:

### **Fig. 1. Icv ARA induces central leptin resistance.**

Food intake for 1, 4, 16, and 24 hours (A) and body weight gain (B) for 24 hours was significantly decreased after the icv injections of leptin compared to the icv injections of saline in the vehicle mice, but not in the ARA icv treated mice.  $**p<0.01$  vs vehicle+saline;  $^{\#}p<0.05$ ,  $^{\#\#}p<0.01$  vs ARA+saline. ARA: arachidonic acid.

### **Fig. 2. Icv ARA attenuates leptin JAK2-STAT3 signaling in the hypothalamus.**

Phospho-JAK2 (pJAK2), phospho-STAT3 (pSTAT3), and SOCS3 in the MBH (A) and PVN (B) of the hypothalamus were detected by western blot in mice treated with icv injections of leptin or saline following an icv injection of ARA/vehicle.  $*p<0.05$ ,  $**p<0.01$  vs vehicle+saline. Vehi: vehicle. ARA: arachidonic acid.

### **Fig. 3. Icv ARA attenuates leptin Akt-FOXO1 signaling in the hypothalamus.**

Phospho-Akt (pAkt) and phospho-FOXO1 (pFOXO1) in the MBH (A) and PVN (B) of the hypothalamus were detected by western blot in mice treated with icv injections of leptin or saline following an icv injection of ARA/ vehicle.  $*p<0.05$  vs vehicle+saline. Vehi: vehicle. ARA: arachidonic acid.

### **Fig. 4. Effect of icv ARA on the inflammatory response in the hypothalamus.**

TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and pI $\kappa$ B $\alpha$  in MBH (A) and PVN (B) of the hypothalamus were detected by western blot in mice treated with an icv injection of ARA/vehicle.  $*p<0.05$ ,  $**p<0.01$  vs vehicle+saline.

### **Fig. 5. Effect of icv ARA on mRNA expression of genes involved in gluconeogenesis, glucose transportation, and glycolysis in the liver.**

The mRNA levels of G6Pase, PEPCK, GLUT2, and GK in the liver were measured by quantitative real-time PCR in mice treated with icv injection of leptin or saline following an icv injection of ARA/vehicle.

\* $p < 0.05$ , \*\* $p < 0.01$  vs. vehicle+saline. <sup>+</sup>  $0.05 < p < 0.10$  vs vehicle+saline. Vehi: vehicle; ARA: arachidonic acid.

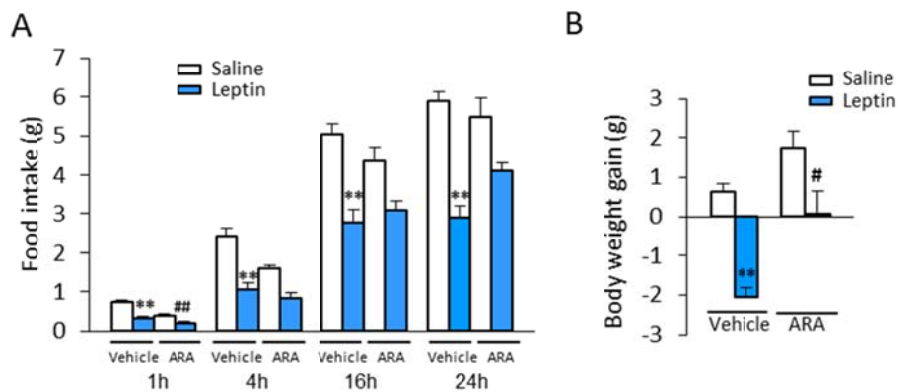
**Fig. 6. Effect of icv ARA on the mRNA expression of the genes involved in lipogenesis, lipid beta-oxidation, and cholesterol metabolism.**

The mRNA levels of FAS, SCD1, ACC, ACOX, ACAT1 (A), and HMG-CoA reductase and APoA1 (B) in the liver were measured by quantitative real-time PCR in mice treated with icv injections of leptin or saline following an icv injection of ARA/vehicle. \* $p < 0.05$  vs vehicle+saline; # $p < 0.05$  vs ARA+saline. Vehi: vehicle; ARA: arachidonic acid.

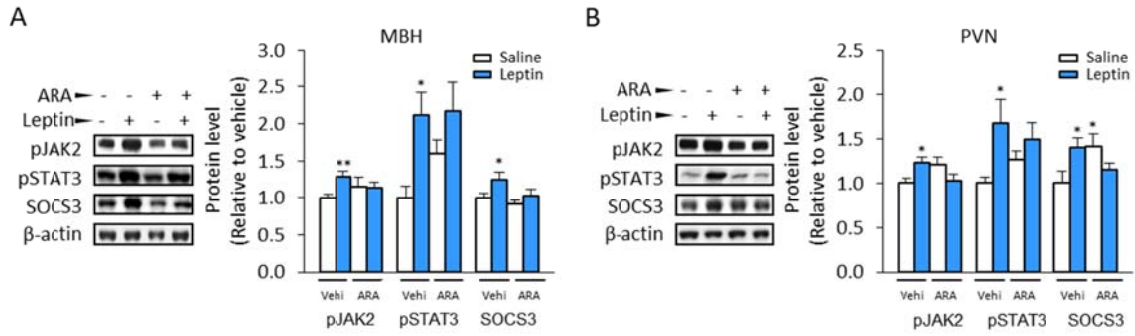
**Fig. 7. Effects of icv ARA on the TH level in the hypothalamus in response to leptin.**

The level of TH in the MBH and PVN of the hypothalamus was detected by western blot in mice treated with icv injection of leptin or saline following an icv injection of ARA/vehicle. \* $p < 0.05$ , vehicle+saline. Vehi: vehicle; ARA: arachidonic acid.

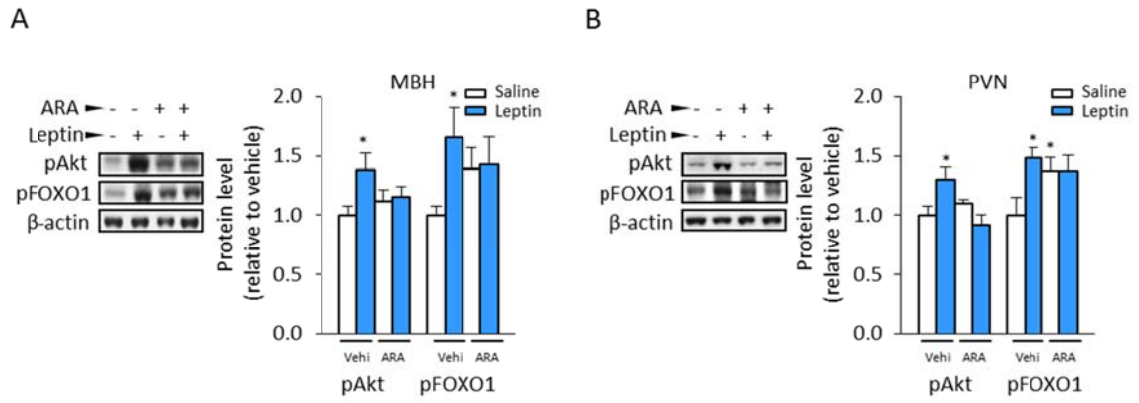
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**

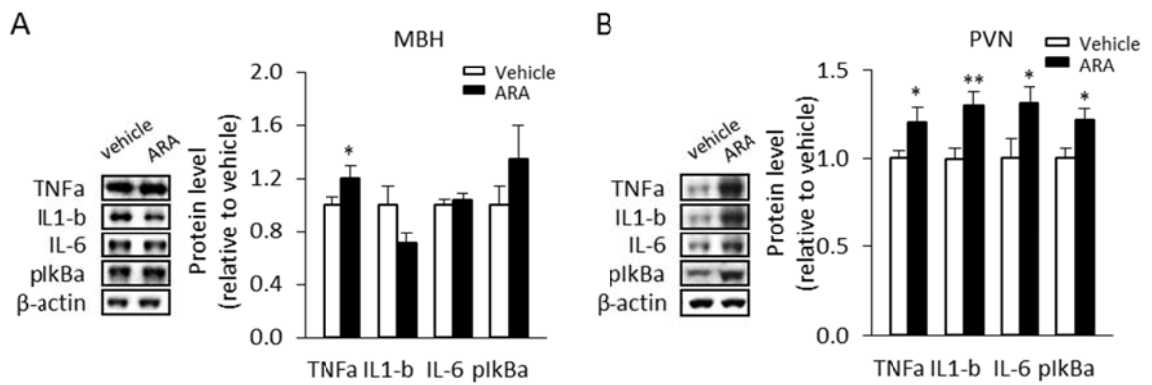


Fig.5

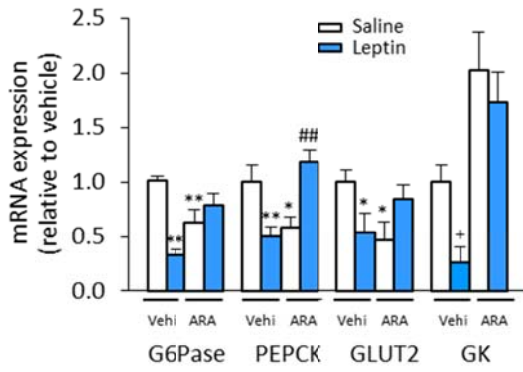
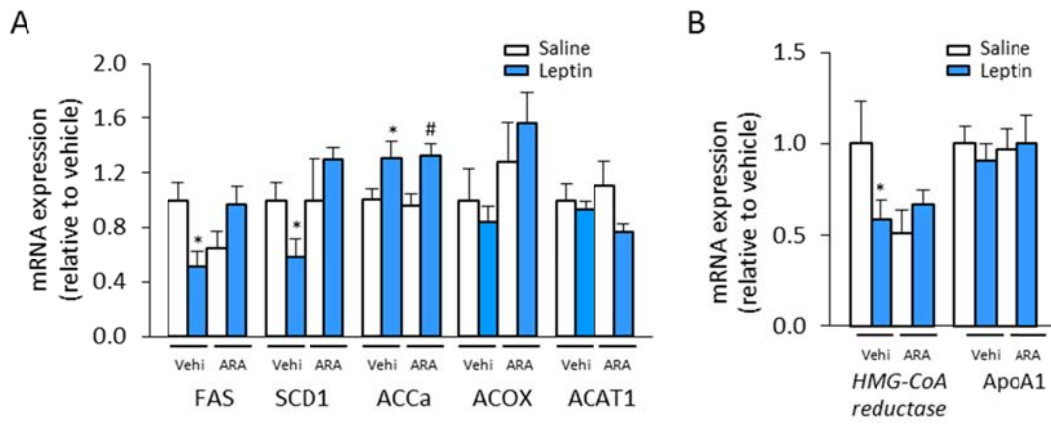
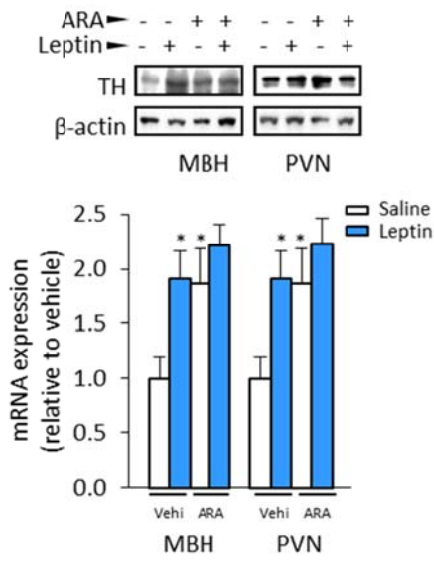


Fig. 6



**Fig. 7**



**Tables:**

**Table 1. Influence of icv ARA on central leptin's effect on the glucose tolerance test.**

Blood glucose level (mM)					
Group	N	0 min (M $\pm$ S)	30 min (M $\pm$ S)	60 min (M $\pm$ S)	120 min (M $\pm$ S)
Vehi+Saline	6	7.15 $\pm$ 1.06	9.08 $\pm$ 1.23	8.95 $\pm$ 0.93	7.73 $\pm$ 1.45
Vehi+Leptin	5	7.22 $\pm$ 1.50	10.72 $\pm$ 2.41	9.00 $\pm$ 2.41	7.44 $\pm$ 1.71
ARA+Saline	5	6.84 $\pm$ 0.51	10.26 $\pm$ 2.36	9.22 $\pm$ 1.71	7.42 $\pm$ 0.93
ARA+Leptin	5	8.62 $\pm$ 0.90	10.70 $\pm$ 2.12	9.21 $\pm$ 1.44	8.02 $\pm$ 1.20

Blood glucose increased at 0 min and 30 min in mice with icv injection of leptin following icv injection of ARA in the glucose tolerance test. Vehi: vehicle; ARA: arachidonic acid.