

Unsaturated Fatty Acids Block the Effects of
Saturated Fatty Acids on
Insulin Signaling in Astrocytoma Cells

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Insulin Signaling in Astrocytoma Cells

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감사의 글

하늘에 계신 하나님 아버지께서 부족한 저를 여기까지 도와주시고 이끌어주셨습니다. 늘 감사드리며 먼저 모든 영광 돌려드립니다.

연구 시작부터 너무 귀한 지도와 도움을 주셨던 박주영 교수님과 최선주 교수님께 진심으로 감사드립니다. 또한 늘 격려를 아끼지 않으시고, 여러 조언을 해주신 이해용 교수님, 차병호 교수님, 그리고 공인덕 교수님께도 깊이 감사드립니다. 바쁘신데도 제게 큰 힘이 되어주신 서희석 선생님과 박현숙 선생님께도 다시 한 번 감사드립니다. 여러분들의 가르침 덕분에 오늘의 결과가 있을 수 있었다고 생각합니다.

먼 거리에서 학업을 마쳐야 하는 과정마다 많은 도움을 주셨던 김경호 교수님과 목동병원 여러 소아청소년과 선생님들께도 이 자리를 통해 감사드립니다.

마지막으로 언제나 옆에서 큰 사랑을 부어주시고 기둥이 되어주시는 아버님과 어머님, 그리고 하나뿐인 동생에게 말로는 부족하지만 진심으로 감사와 사랑을 전합니다.

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국 문 요 약

불포화지방산에 의한 별아교세포종 세포에서 포화지방산의 인슐린 신호 영향에 대한 억제

비만은 많은 급성 및 만성 질환의 발생 위험을 높이는데 연관되어 있다고 알려져 있다. 혈중 지방산 농도는 비만과 대사증후군 환자들에게서 유의하게 높아지는데, 포화지방산은 말초기관과 신경세포에서 염증반응을 유발하는 것으로 알려져 있고, 이는 인슐린 저항성과도 연관이 있다. 그러나 포화지방산이 뇌의 별모양세포와 에너지 항상성에 미치는 영향은 분명하게 밝혀져 있지 않다. 별모양세포에서의 포화지방산의 영향을 알아보기 위해 본 연구에서는 U87MG 사람 별아교세포종 세포주를 배양하여 포화지방산을 처리한 후 인슐린 신호의 변화와 관련 경로들을 조사하였다. 또한 포화지방산의 별모양세포에 미치는 영향에 대해 불포화지방산은 어떤 작용을 하는지 알아보려고 하였다.

U87MG 사람 별아교세포종 세포에 포화지방산인 팔미트산염과 스테아르산염을, 불포화지방산으로는 올레산과 리놀레산을 처리하였다. 지방산을 별아교세포종 세포에 처리한 후, 인슐린 신호 변화는 Akt의 인산화를 통해 살펴보고, 염증반응이 일어나는지 유무는 싸이토카인 IL-1 β , IL-6, IL-8, TNF- α 의 mRNA 발현과 단백질 변화를 통해 측정하였다. 포화지방산을 처리한 후 소포체 스트레스 반응을 Xbp-1, CHOP 그리고 세포괴사는 caspase -3, JNK 경

로의 활성화 유무는 p-JNK를 통해 확인하였다.

실험을 통해 팔미트산염을 처리하였을 때 p-Akt 가 농도에 비례하여 감소하였고, IL-6 과 IL-8 의 mRNA 의 발현과 단백질은 농도에 비례하여 증가하였으나, IL-1 β , TNF- α 의 변화는 없었다. 또한 포화지방산 처리 후, 소포체 스트레스를 나타내는 spliced Xbp-1 과 CHOP mRNA 발현이 증가되었다. 포화지방산을 처리 후 JNK 의 활성화가 확인되었는데, 이런 변화들은 올레산과 리놀레산을 처리하였을 때 포화지방산 처리 이전상태로 회복되는 양상이 관찰되었다.

이상의 결과를 종합할 때 팔미트산염과 스테아르산염이 사람 별아교세포종 세포에서 인슐린 신호를 감소시키고, 이에 소포체 스트레스 및 JNK 경로가 관여할 것으로 생각된다. 또한 불포화지방산이 상기 작용들을 회복시키므로, 불포화지방산이 풍부한 식이를 통해 별모양세포에 미치는 포화지방산의 영향을 상쇄시킬 수 있는 치료적 후보가 될 것으로 생각된다.

핵심되는 말 : 포화지방산, 불포화지방산, 별모양세포, 염증 반응, 인슐린 신호

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I. Introduction

Obesity is a global health problem causing worldwide mortality and morbidity. Globally, there are more than 1 billion overweight adults, with at least 300 million of them being obese (Das UN. 2010, 459–73). Also, 15.8% of children aged from 6 to 11 years and 16.1% of adolescents are overweight in the USA, and 31.8% of school-aged children are overweight or obese in European countries (Chiarelli and Marcovecchio. 2008, 67–74). Obesity is closely related to many diseases such as cardiovascular disease, type II diabetes, sleep apnea, osteoarthritis, cancer, and psychological disturbances (Gale et al. 2004, 295–8) (Guh et al. 2009, 88).

Energy homeostasis is a balance between food intake and energy expenditure involving both the central nervous system (CNS) and the periphery (Martin et al. 2006, 333–9) (Leibowitz and Wortley. 2004, 473–504). The mechanism of energy homeostasis is not clearly revealed, but the current model explains that body adiposity is regulated by “adiposity negative feedback loop” through hormones insulin and leptin which circulate in proportion to body adipose stores (Posey et al. 2009, 1003–12). Insulin and leptin reduce appetite while increasing energy expenditure through actions on NPY/AgRP (neuropeptide Y/agouti-related protein) and POMC/CART (proopiomelanocortin/cocaine and amphetamine regulated transcriptergic) neuron in the arcuate nucleus of hypothalamus

(Millington. 2007, 661–71).

If energy intake exceeds energy expenditure, circulating insulin and leptin levels will rise (Posey et al. 2009, 1003–12), leading to inhibition of NPY/AgRP neurons and stimulation of POMC/CART neurons, which reduce food intake, increase energy expenditure (Schwartz et al. 2000, 661–71), and consequently protect against the pathological expansion of fat mass (Posey et al. 2009, 1003–12). However, CNS resistance to insulin and leptin is observed in obese human and animal models of diet-induced obesity (DIO) (De Souza et al. 2005, 4192–9) (Munzberg et al. 2004, 4880–9). It is expected that the understanding of these mechanisms should lead to effective treatments for the control of obesity and its related problems (Gale et al. 2004, 295–8).

1. Insulin resistance and Inflammation

Insulin is involved in diverse metabolic functions on various tissues such as liver, skeletal muscle, and adipose tissue (Luca and Olefsky. 2008, 97–105). For example, insulin increases glucose uptake via GLUT4 glucose transporter in skeletal muscle, inhibits gluconeogenesis in liver, and reduces lipolysis in adipose tissue (Lee et al. 2006, 1467–74) (Yu et al. 2002, 50230–6) (Luca and Olefsky. 2008, 97–105).

There are two major signaling pathways in insulin mediated activities: phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein (MAP) kinase signaling. It is generally accepted that the PI3K/Akt pathway is responsible for most of the metabolic actions of insulin (Feng et al. 2012, 1356–61). Within the PI3K pathway, binding of insulin to the receptor triggers the recruitment and phosphorylation of insulin receptor substrate (IRS), which interact with the regulatory p85 subunit of PI3K, resulting in synthesis of phosphatidylinositol phosphate (PIP3). It then activates phosphoinositide-dependent protein kinase (PDK) and recruits Akt to the cell membrane (Hemmings and Restuccia. 2012, a011189). PDK mediates the phosphorylation of Akt, and this results in Akt

activation. Akt is known to mediate the effects of insulin on glucose transport and storage, protein synthesis, inhibition of lipolysis, FOXO-mediated transcription of target genes that promote apoptosis, and cell-cycle arrest (Luca and Olefsky. 2008, 97–105) (Hemmings and Restuccia. 2012, a011189).

Many studies in diabetes or obesity and insulin resistance have reported the association of chronic low grade inflammation and decreased insulin sensitivity (Gregor and Hotamisligil. 2011, 415–45) (Buckman et al. 2013, 1322–33). De Souza reported that DIO induces the expression of proinflammatory cytokines in the rodent hypothalamus, which is associated with attenuated insulin signal transduction (De Souza et al. 2005, 4192–9) (Posey et al. 2009, 1003–12) (Buckman et al. 2013, 1322–33).

2. Endoplasmic reticulum stress and apoptosis

Endoplasmic reticulum (ER) stress is a cellular response triggered by the disruption of ER homeostasis (Pirot et al. 2007, 1069–77). If the normal folding of proteins in the ER is perturbed, consequently unfolded proteins accumulate (Wu and Kaufman. 2006, 374–84). Then, the cells activate the unfolded protein response (UPR) which induces key transcription factors, X-box binding protein-1 (Xbp-1), activating transcription factor 6 (ATF6), and phosphorylation of the eukaryotic initiation factor (eIF2) via the kinase PKR-like endoplasmic reticulum kinase (PERK) (Pirot et al. 2007, 1069–77). The CCAAT/enhancer binding protein (C/EBP) homologous transcription factor (CHOP) also increases markedly in ER stress (Oyadomari and Mori. 2004, 381–9). If the UPR fails to restore normal ER function, apoptosis will be triggered (Wu and Kaufman, 2006, 374–84). The association of insulin signaling and ER stress has been reported in type 2 diabetes mellitus, cultured liver cells, and obese mice (Luca and Olefsky. 2008, 97–105) (Mayer and Belsham.

2010, 576–85). Caspase-3 is a downstream member of the caspase cascade and is essential for the characteristic changes in cell morphology and biochemical events associated with the execution and completion of apoptosis. The active caspase-3 cleaves specific aspartate residues in proteins with various structural, housekeeping, and regulatory functions such as β -actin (Porter and Jänicke. 1999, 99–104) (Yu et al. 2008, 486–95).

3. Saturated fatty acids and its metabolic effects

Obesity is often accompanied by elevations in circulating free fatty acids (FFAs) concentrations (Posey et al. 2009, 1003–12). In vitro, prolonged treatment of primary rat islets and human islets with saturated fatty acid (SFA), palmitate, activates ER stress and induces apoptosis (Mayer and Belsham. 2010, 576–85) (Kharroubi et al. 2004, 5087–96). In vivo, high levels of circulating FFAs activate pro-inflammatory responses in vascular endothelial cells and adipocytes, which leads to systemic inflammation, insulin resistance in skeletal muscle, and pancreatic β -cell dysfunction and death (Luca and Olefsky. 2008, 97–105) (Pirot et al. 2007, 1069–77) (Posey et al. 2009, 1003–12). Posey et al. reported that intracerebroventricular (icv) infusion of palmitate or high fat diet (HFD) in a rat induces hypothalamic inflammation and insulin resistance (Posey et al. 2009, 1003–12).

4. Effects of unsaturated fatty acids on CNS in obesity

Unsaturated fatty acids (UFAs) have well known anti-inflammatory effects in various tissues and cell types (Reynolds et al. 2009, 2351–7), and there are numerous data that show the positive effects of UFAs in metabolic syndromes. In the periphery, ingestion of UFAs lead to decrease in glucose concentration (Suresh and Das. 2003, 213–8). Particularly in the CNS, oleic acid decreased the hypothalamic expression of NPY, suggesting that UFAs control appetite by their action on

hypothalamic centers (Das UN. 2010, 459–73). Cintra et al. also showed that UFAs, oleic (C18:1, v9) and linolenic (C18:3, v3) acids, have anti-inflammatory effects acting either as nutrients or directly in the hypothalamus, correcting hypothalamic dysfunction and reducing body mass in obesity. Moreover, the icv injection of UFAs improved leptin and insulin signal transduction in the hypothalamus (Cintra et al. 2012, e30571).

5. Astrocyte in CNS energy homeostasis

Astrocytes are one of the major types of glial cells, which occupy up to 50% of the total brain volume (Kimelberg and Nedergaard, 2010, 338–53) (Yi et al. 2011, 143–9). Astrocytes are known to have diverse functions such as supportive systems for neurons, modulation of synaptic plasticity, release of neuronal trophic factors and inducing neuronal differentiation, and a component of the neurovascular unit of the blood–brain barrier (BBB) (Kimelberg and Nedergaard, 2010, 338–53) (Buckman et al. 2013, 1322–33) (Gupta et al. 2012, 1060–71). In particular, astrocytes regulate the physical tightness of the BBB and express transporters and enzymes, providing nutrition to neurons and participating in metabolic homeostasis between the periphery and the CNS (Ricci et al. 2009, 317–36) (Gupta et al. 2012, 1060–71).

Although the roles of neurons in energy homeostasis or obesity have been well researched, the roles of astrocytes regulating energy homeostasis in the CNS have not been clarified (Gupta et al. 2012, 1060–71). In obesity, FFAs levels are increased in the plasma and uptaken into the brain across the BBB (Gupta et al. 2012, 1060–71) (Wang et al. 1994, 517–22). There are several data which show that astrocytes could be a target of elevated fatty acids and participate in energy homeostasis in the CNS; Bernoud et al. reported that peripherally administered fatty acids accumulate primarily in astrocytes (Bernoud et

al. 1998, 1816–24); Horvath et al. reported that HFD in rats results in reactive astrogliosis, which prevents NPY and POMC neurons from accessing blood vessels and neuronal–neuronal communication (Horvath et al. 2010, 14875–80) (Buckman et al. 2013, 1322–33) (Yi et al. 2011, 143–9). Recently, Gupta et al. reported that saturated fatty acids (SFAs) and UFAs could modulate inflammatory signaling in cultured rat astrocytes (Gupta et al. 2012, 1060–71). However, it is not well known whether SFAs or UFAs influence the insulin signaling in astrocytes.

In this study, we evaluated the effects of the SFAs and UFAs and its mechanism on insulin resistance in astrocytes.

II. Materials and Methods

1. Reagents

The saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0); the unsaturated fatty acids, oleic acid (C18:1 ω -9) and linoleic acid (C18:2 ω -6), were all purchased from Nu-Check Prep (USA). Fatty acid free albumin (FAFA) was from Roche (Germany) and Human recombinant insulin (Humulin R) was from Lilly (USA). Tumor necrosis factor- α (TNF- α) and JNK inhibitor (SP600115) were from calbiochem (Germany).

2. Cell culture and preparation

The human astrocytoma cell line U87MG was produced from Korean Cell Line Bank (Korea). The cells were propagated in Minimum Essential Medium (MEM, Life technologies, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and maintained at 37 °C and 5% CO₂ humidified incubator. Trypan blue exclusion was used to determine the cell viability.

3. Preparation of fatty acid-albumin complex

The fatty acids were dissolved in 0.1 N NaOH at 70 °C and then conjugated with 10% FAFA using a 3:1 ratio. This solution was added to cells to obtain final fatty acid concentrations ranging from 50, 100, 200 to 500 μ M. Serum-free MEM containing 10% FAFA was used as the negative control (Mayer and Belsham. 2010, 576-85) (Feng et al. 2012, 1356-61).

4. RNA isolation and real-time PCR

Total cellular RNA was extracted using Trizol reagent (Invitrogen, USA),

and the amount of RNA was determined using a spectrophotometer. One μg of RNA was reverse transcribed according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems, UK) using an ABI 7900. Gene expression levels were normalized to GAPDH expression. Quantification was performed using the critical threshold (CT) method. The target amount of each mRNA sample was subsequently divided by the control gene amount (which was assigned a value of 1 arbitrary unit) to obtain a normalized target value. The C(t) for each gene from the different samples was standardized to the sample GAPDH value [$C(t)_{\text{gene}} - C(t)_{\text{GAPDH}}$], and this value was then compared using the $2^{-\Delta C(t)}$ method to determine the fold change {fold change [$2^{-\Delta C(t)}$]}. To determine X-box binding protein-1 (XBP-1) mRNA splicing, PCR was performed using cDNA as a template. The PCR product was separated with 4% agarose gel with a PCR product of 446 bp for unspliced and 416 bp for spliced XBP-1. PCR products were imaged by image analyser (Bio-Rad, USA) and analysed by Quantity One Software (Bio-Rad, USA) (Gallego-Sandín et al. 2011, 469–75).

5. Western blot

U87MG cells were stimulated with fatty acid or fatty acid-free albumin and then treated with 100 nM insulin for 15 min before the cell lysates were prepared. The concentration of the protein was determined using the BCA method, and the same amount of proteins were used in each lane for Western blotting. Equal proteins (20–40 μg) were resolved on SDS-PAGE (10–12%) and transferred to immobilon-P polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 3% BSA for 2 hours at room temperature and then probed with the primary antibodies against phospho-Akt antibody, phospho-SAPK/JNK antibody, SAPK/JNK antibody, cleaved caspase-3 antibody (1:1,000; Cell Signaling Technology), Akt antibody, and β -Actin antibody (1:2,000; Cell Signaling

Technology) overnight at 4°C. After three washes, the blots were subsequently incubated with the secondary anti-rabbit IgG HRP-linked antibody (1:2,000; Cell Signaling Technology) for 1 hour at room temperature. The protein-antibody complexes were visualized by exposure to X-ray film after reacting with ECL detection reagent (Santa Cruz Biotechnology, USA) and densitometric analysis of bands was performed using image analyzer (Bio-Rad) and Quantity One Software (Bio-Rad) (Feng et al. 2012, 1356-61) (Wang et al. 2012, 6325-30).

6. ELISA

U87MG cells (1×10^5 /ml) cultured in 24-well plates were stimulated with or without fatty acid for 24 hours. IL-6 and IL-8 productions in the culture media were quantified by using the commercially available IL-6 or IL-8 specific ELISA kits (R & D system, USA) according to the manufacturer's instructions. The plates were read at 490 nm.

Table 1. Primer Sequences for RT-PCR

Gene	Forward	Reverse	Target Sequence Size
TNF-α	TGGCCAATGGCGTGGAGCTG	GTAGGAGACGGCGATGCGGC	152
IL-1β	TGCTGGTTCCTGCCACAGA	TGCTGTGAGTCCCGGAGCGT	163
IL-6	AGAGGCACTGGCAGAAAAC	TGCAGGAACTGGATCAGGAC	219
IL-8	TTGGCAGCCTTCCTGATT	AACTTCTCCACAACCCTCTG	248
Xbp-1	CCTTGTAGTTGAGAACCAGG	GGGGCTTGGTATATATGTGG	446/416
CHOP	ACCAAGGGAGAACCAGGAAACG	TCACCATTGGTCAATCAGAGC	201
GAPDH	CGGATTTGGTCGTATTGGG	CTCGCTCCTGGAAGATGG	215

TNF- α : tumor necrosis factor- α , IL-1 β : interleukin-1 β ,

IL-6: interleukin-6, IL-8: interleukin-8, Xbp-1: X-box binding protein -1,

CHOP: CCAAT/enhancer-binding protein homologous protein,

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

7. Statistical analyses

All data are presented as means \pm standard deviation (SD). The statistical significance of the differences among groups was performed with one-way analysis of variance (ANOVA), followed by least significant difference (LSD) for multiple comparison as a post hoc test. Statistical significance for all analyses was accepted at $p < 0.05$.

III. Results

1. Saturated fatty acids attenuated insulin signaling

To explore whether SFAs cause insulin resistance on astrocytoma cells, we evaluated the effect of palmitate or stearate on insulin signaling via the phosphorylation of Akt. U87MG cells in 6-well plates were treated with palmitate or stearate in serum free DMEM for 6 h and then incubated with 100 nM insulin for 15 min. In the absence of insulin and SFAs, the phosphorylation of Akt was not induced (Cont) (Fig. 1). Insulin stimulation led to increase in the phosphorylation of Akt. However, palmitate or stearate pre-treatment for 6 h significantly reduced the insulin-stimulated phosphorylation of Akt. These data indicate that palmitate and stearate decrease the phosphorylation of Akt by the treatment of insulin (Fig. 1) (Feng et al. 2012, 1356–61).

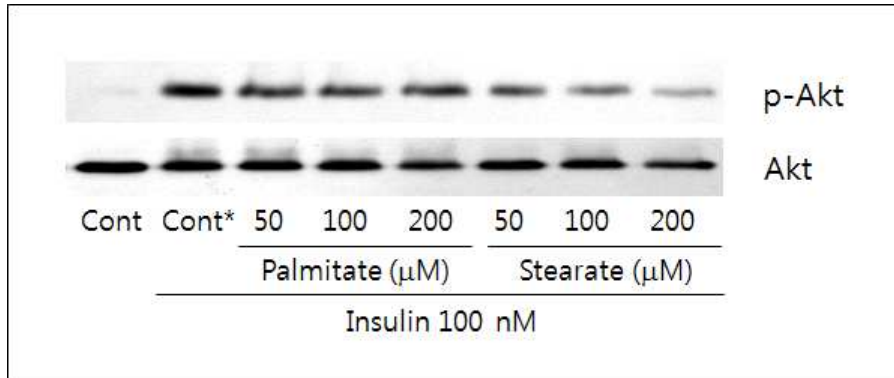


Fig. 1. Saturated fatty acids attenuated insulin signaling in astrocytoma cells. U87MG human astrocytoma cells were treated with insulin for 15 min followed by 50, 100, or 200 μ M of palmitate or stearate for 6 h. Phosphorylated-Akt and Akt protein were measured using Western blot analysis. Cont: negative control with FAFA treatment, Cont*: positive control treated with FAFA and insulin.

2. Saturated fatty acids increased IL-6 and IL-8 but not TNF- α and IL-1 β

We evaluated whether palmitate or stearate increased the mRNA expression and release of the inflammatory cytokine. Palmitate increased the mRNA expression of IL-6 and IL-8 in a dose-dependent manner but did not significantly increase the expression of TNF- α and IL-1 β . The secretion of IL-6 and IL-8 was checked using ELISA. FAFA was used as a negative control and TNF- α as a positive control for the cytokine induction. The secretion of IL-6 and IL-8 was dose-dependently increased by palmitate (Fig. 2A). Stearate also increased the mRNA expression and secretion of IL-6 and IL-8 in a dose-dependent manner, relatively more than palmitate (Fig. 2B).

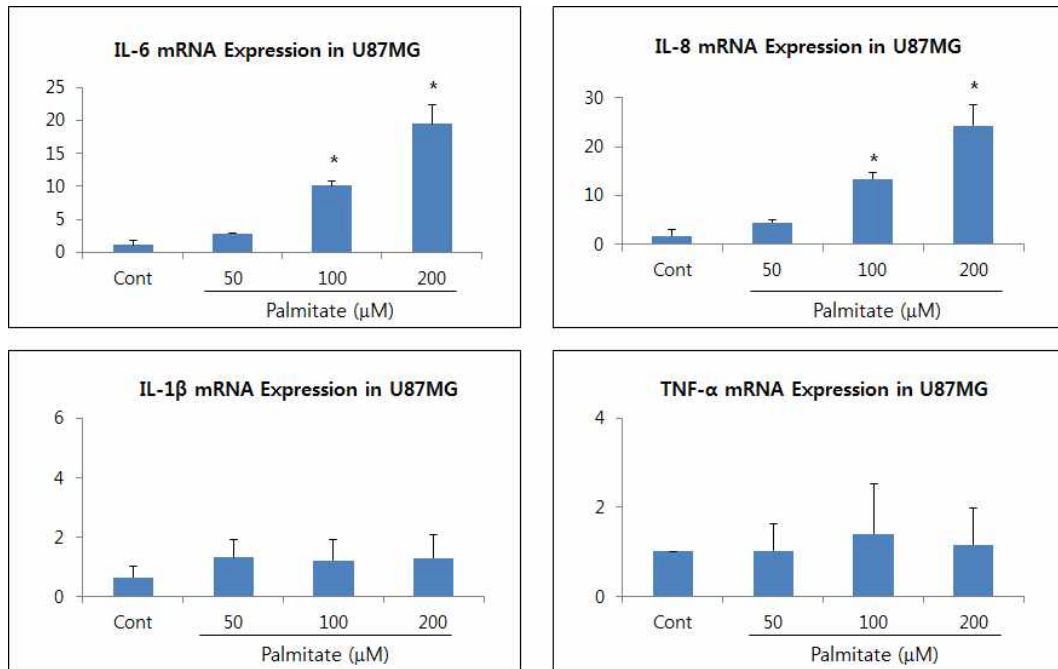


Fig. 2A. Palmitate induced IL-6 and IL-8 mRNA expression. The cells were treated with 50, 100, or 200 μM of palmitate for 6 h. Expression of mRNA was measured using real time PCR. Cont: FAFA treated negative control, The data shown as relative to FAFA treated control with standard deviation (n=3, independent experiments). * p<0.05

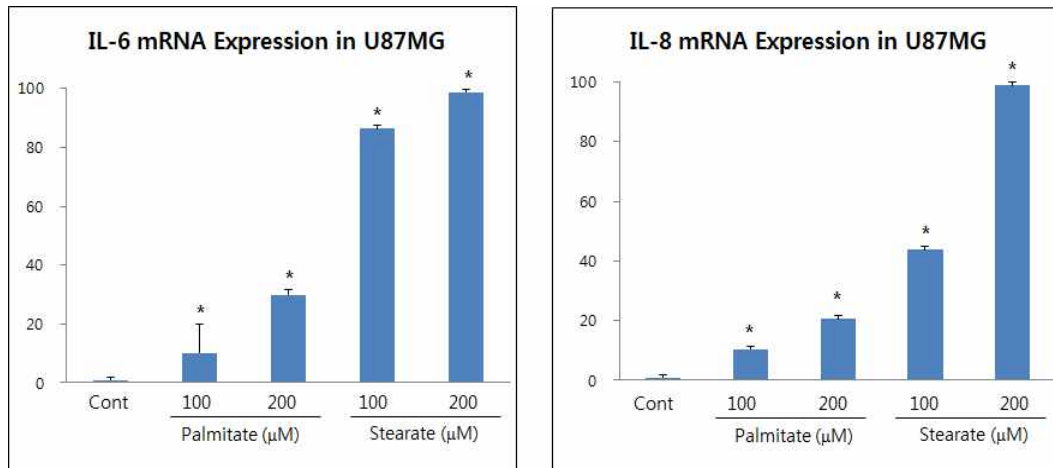


Fig. 2B. Palmitate and stearate induced IL-6 and IL-8 mRNA expression. The cells were treated with 50, 100, or 200 μM of palmitate for 6 h. Expression of mRNA was measured using real time PCR. Cont: FFAA treated negative control, The data shown as relative to FFAA treated control with standard deviation ($n=3$, independent experiments). * $p<0.05$

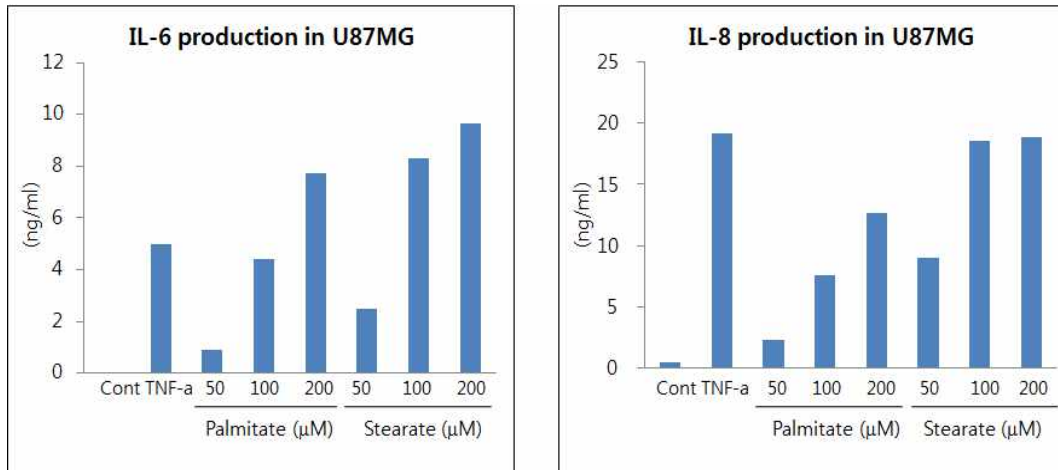


Fig. 2C. Palmitate and stearate induced IL-6 and IL-8 secretion. The cells were treated with 50, 100, or 200 μ M of palmitate or stearate for 24 h. Cytokine level was measured using ELISA. Cont: FAFA treated negative control, TNF- α : Tumor necrosis factor- α for positive control.

3. Saturated fatty acids induced ER stress

We next investigated whether palmitate or stearate induces ER stress in astrocytoma cells. U87MG cells were treated with 50, 100, or 200 μ M of palmitate or stearate for 6 h. Among the ER stress markers, we investigated the spliced Xbp-1 and CHOP gene expression. We found that palmitate and stearate induced Xbp-1 splicing and CHOP expression in a dose-dependent manner (Fig. 3). Stearate induced more Xbp-1 splicing and CHOP expression than palmitate. These results demonstrate that palmitate and stearate induce ER stress in astrocytoma cells.

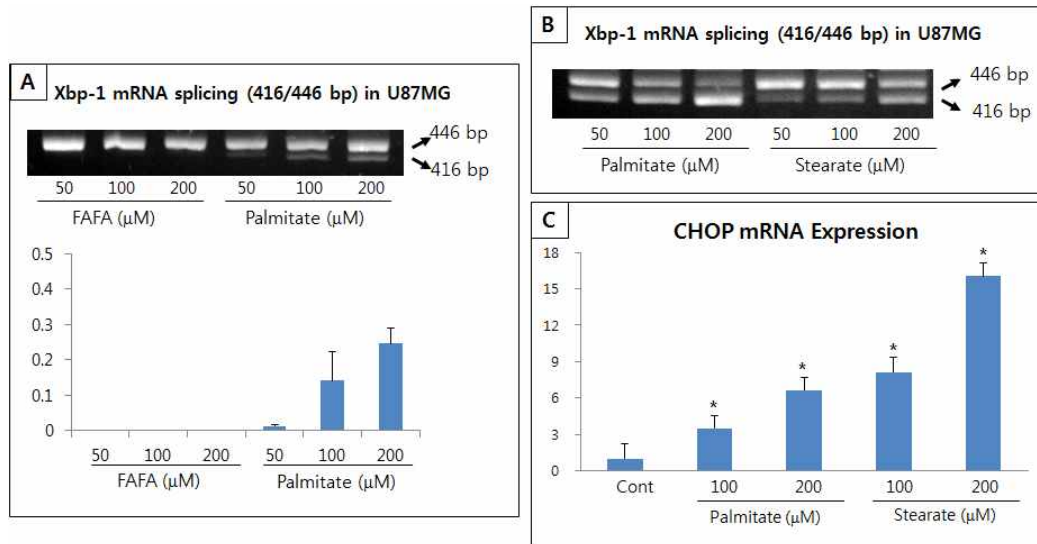


Fig. 3. Saturated fatty acids induced Xbp-1 mRNA splicing and CHOP mRNA expression. The cells were treated with FAFA and 50, 100, or 200 μ M of palmitate (A,C) or stearate (B,C) for 6 h. Relative spliced Xbp-1 levels and CHOP mRNA expression were analyzed using RT-PCR (A-C). Unspliced Xbp-1 is detected at 446 bp, while spliced Xbp-1 at 416 bp (A,B). The data shown as fold increase to FAFA treated control with SD (n=3 independent experiments). The figures are representative of two to three similar experiments. * p<0.05

4. Saturated fatty acids did not induce apoptosis

We investigated whether palmitate and stearate could induce apoptosis by using cleaved caspase-3 as an apoptotic marker. The cells were treated with 100, 200, or 500 μ M of palmitate or stearate, but no significant increase of caspase-3 protein was noted in both palmitate and stearate-treated cells as well as in control cells (Fig. 4). Therefore, these results indicated that palmitate and stearate do not induce apoptosis of astrocytoma cells in these concentrations.

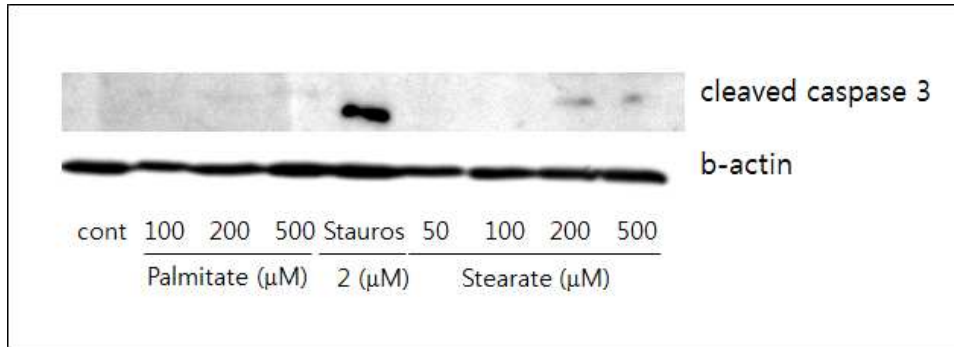


Fig. 4. Effect of saturated fatty acids on caspase-3 activation. The cells were treated with 50, 100, 200, or 500 μM of palmitate or stearate for 24 h, but no significant increase of caspase-3 protein was noted in both palmitate and stearate-treated cells as well as in control cells. Cleaved caspase-3 and β -actin were measured using Western blot analysis. Cont: Treatment with FAFA as a negative control. Stauros (Staurosporin): Treatment with 2 μM of staurosporin as a positive control.

5. Saturated fatty acids exposure increased p-JNK

We investigated whether palmitate and stearate increase p-JNK in astrocytoma cells. Relative p-JNK and JNK were measured using Western blot analysis. The cells were treated with 100 or 200 μ M of palmitate from 15 min to 6 h. When treated with 200 μ M of palmitate, p-JNK increased in the 15 and 30 min, but decreased by the 60 min. P-JNK began to rise again in the 3 h and 6 h (Fig. 5A). When the cells were treated with 100 μ M and 200 μ M of palmitate and stearate, p-JNK was increased in a dose-dependent manner (Fig. 5B).

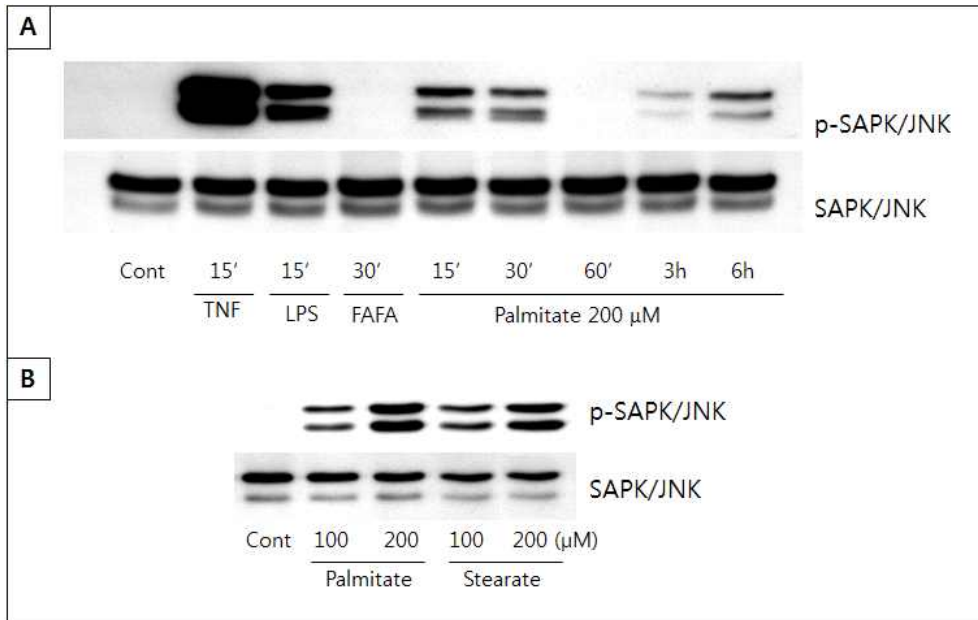


Fig. 5. Palmitate and stearate increased p-JNK in astrocytoma cells. The cells were treated with 200 μ M of palmitate (A) or 100 or 200 μ M of palmitate or stearate (B) for 6 h. Relative p-JNK and JNK were measured using Western blot analysis. Cont: FFAA treated cells as a negative control for JNK activation, TNF- α (10 ng/ml) and LPS (100 ng/ml) treatments were used as positive control for JNK activation.

6. Unsaturated fatty acids blocked the effects of saturated fatty acids on insulin signaling

To explore whether UFAs could block the effect of saturated fatty acids attenuating insulin signaling, we investigated the effect of linoleic acid or oleic acid on insulin signaling. U87MG cells in 6-well plates were pretreated with 50, 100, or 200 μM of linoleic or oleic acid for 1 h before application of FAFA or 100 μM of palmitate or stearate. The cells were then treated with palmitate or stearate in serum free DMEM for 6 h and finally incubated with 100 nM of insulin for 15 min. As in the results of the previous experiment, in the absence of palmitate or stearate, insulin led to increase in the phosphorylation of AKt (Cont), and palmitate or stearate treatment significantly reduced the insulin-stimulated phosphorylation of Akt (Fig. 6). However, pretreatment with UFA for 1 h before application of palmitate or stearate significantly and dose-dependently restored the insulin signaling. These data indicate that linoleic and oleic acids can block the decreasing insulin signaling effect of SFAs on astrocytoma cells (Fig. 1) (Feng et al. 2012, 1356–61).

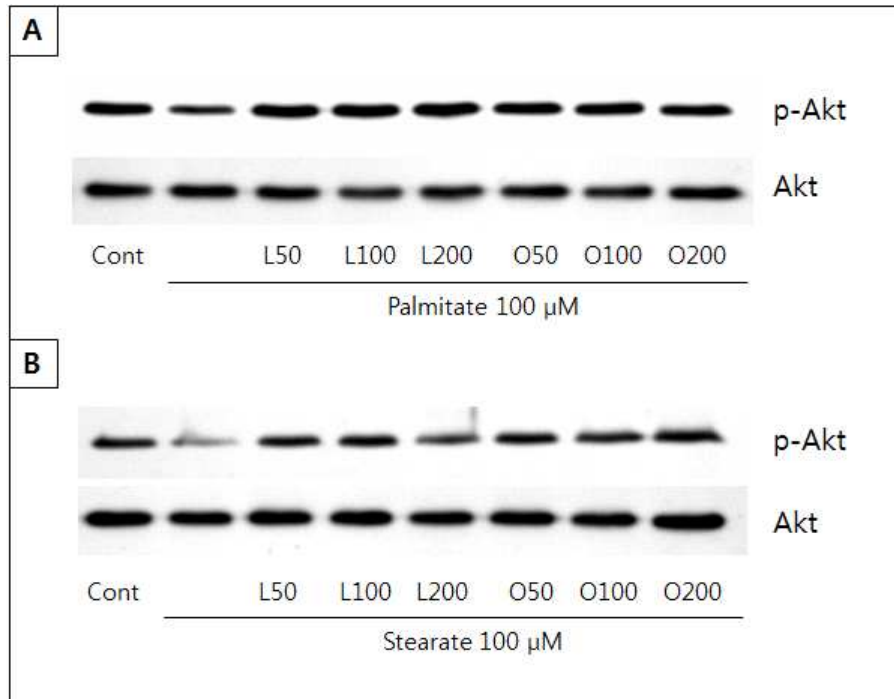


Fig. 6. Unsaturated fatty acids blocked the effects of saturated fatty acids on insulin signaling. The cells were treated with 50, 100, or 200 uM of linoleic acid (L) or oleic acid (O) for 1 h followed by 100 uM of palmitate (A) or stearate (B) for 6 h. Phosphorylation of Akt and Akt protein were measured using Western blot analysis. Cont: FAFA treated cells as a negative control.

7. Unsaturated fatty acids blocked the effects of saturated fatty acids on cytokine release

To determine if UFAs could block the effect of SFAs on cytokine expression, astrocytoma cells were pretreated with 100 or 200 μ M of linoleic or oleic acid for 1 h before application of palmitate or stearate. The data show that pretreatment with UFAs significantly and dose-dependently attenuated palmitate or stearate-induced cytokine expression and release (Fig. 7A and B).

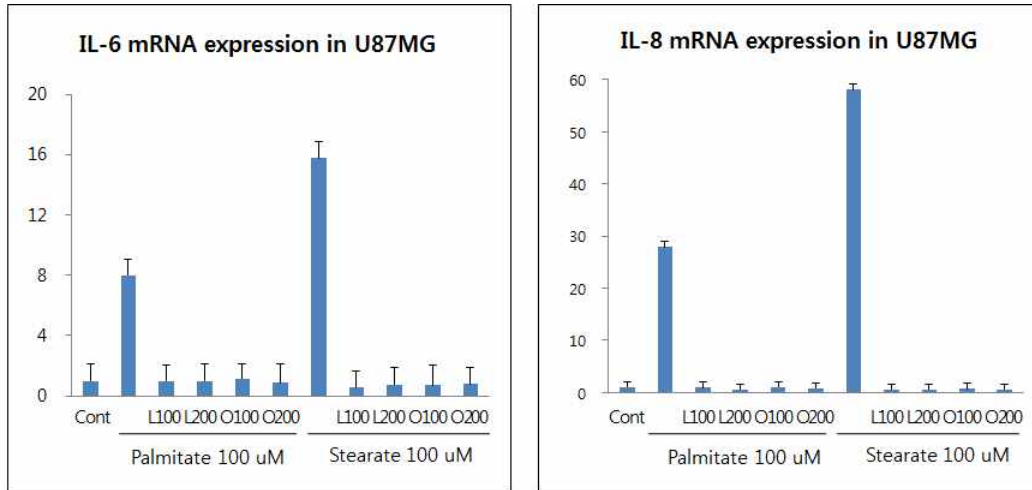


Fig. 7A. Unsaturated fatty acids blocked the effects of saturated fatty acids on IL-6 and IL-8 mRNA expression. The cells were treated with linoleic acid (L) or oleic acid (O) for 1 h and followed by 100 uM of palmitate or stearate for 6 h. Expression of mRNA was measured using real time PCR. Cont: FAFA treated cells as a negative control.

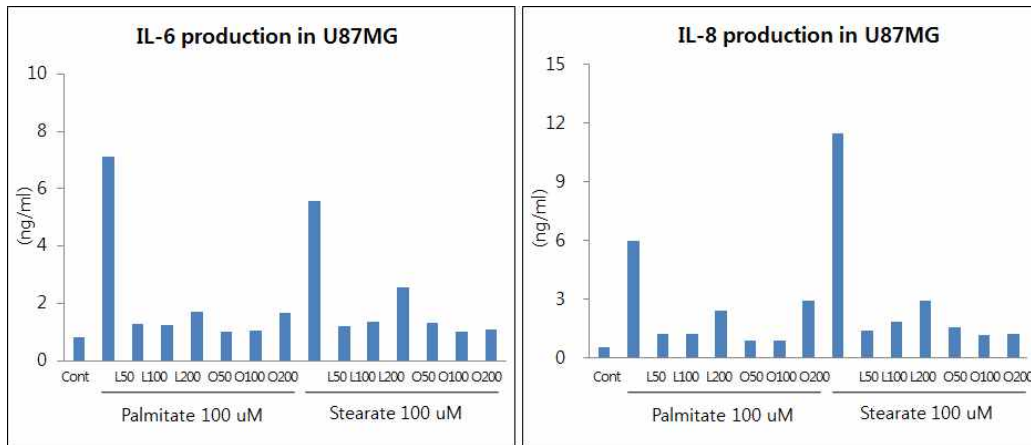


Fig. 7B. Unsaturated fatty acids blocked the effects of saturated fatty acids on IL-6 and IL-8 release. The cells were treated with linoleic acid (L) or oleic acid (O) for 1 h and followed by 100 uM of palmitate or stearate for 24 h. Cytokine level was measured using ELISA. Cont: FAFA treated negative control, The data shown as relative to FAFA treated control with standard deviation (n=3 independent experiments).

8. Unsaturated fatty acids blocked the effects of saturated fatty acids on ER stress

We next investigated whether UFAs attenuate the effect of palmitate or stearate on ER stress in astrocytoma cells. U87MG cells were pretreated with 100 or 200 μ M of linoleic acid or oleic acid for 1 h before application of FAFA (as a negative control) or 100 μ M of palmitate or stearate. The data show that pretreatment with UFAs significantly and dose-dependently attenuated Xbp-1 splicing (Fig. 8A) and CHOP expression (Fig. 8B). This results represent that UFAs attenuate the effect of SFAs-induced ER stress (Fig. 8A and B).

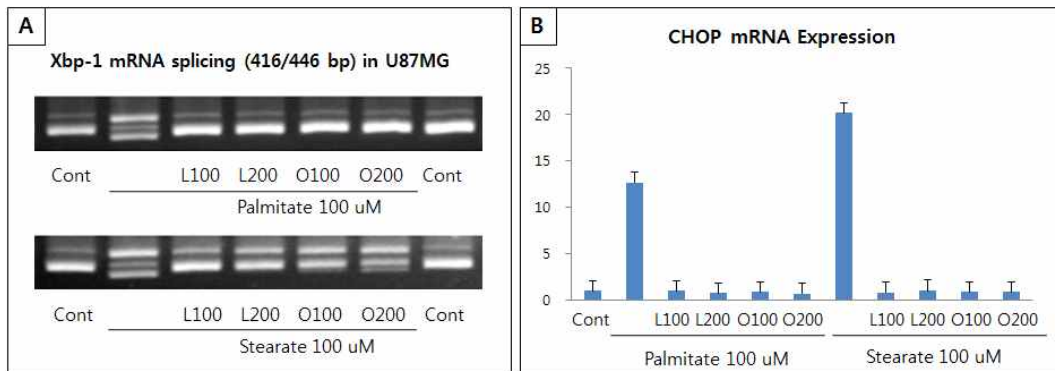


Fig. 8. Unsaturated fatty acids blocked the effects of saturated fatty acids on Xbp-1 splicing (A) and CHOP mRNA expression (B). The cells were pretreated with 100 or 200 uM of linoleic acid (L) or oleic acid (O) for 1 h followed by 100 uM of palmitate or stearate for 6 h. Expression of Xbp-1 mRNA was measured using RT-PCR (A). Expression of CHOP mRNA was determined using real time PCR (B). Cont: FAFA treated negative control.

9. Unsaturated fatty acids blocked the effects of saturated fatty acids on JNK pathway signaling

To determine if UFAs could block the JNK activation caused by SFAs, U87MG cells were pre-treated with FAFA or 50, 100, or 200 μ M of linoleic acid or oleic acid for 1 h before application of FAFA or 100 μ M of palmitate or stearate. The data show that pretreatment with UFAs significantly and dose-dependently attenuated SFAs-induced phosphorylation of JNK (Fig. 9A and B) (Wong et al. 2009, 27384–92).

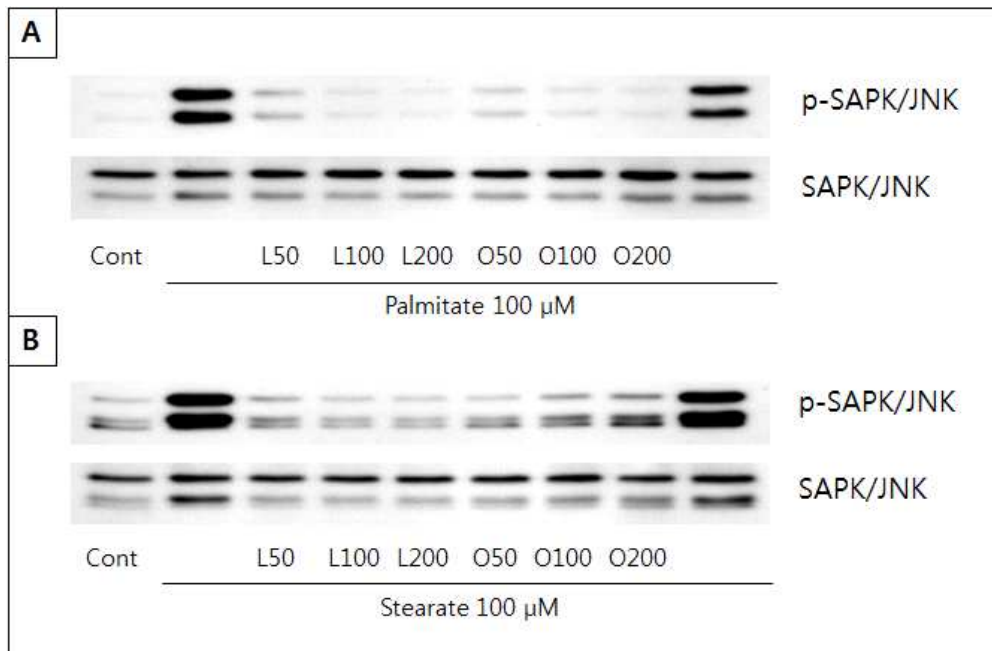


Fig. 9. Unsaturated fatty acids blocked the effects of saturated fatty acids on JNK signaling. The cells were treated with 50–200 μM of linoleic acid (L) or oleic acid (O) for 1 h, followed by 100 μM of palmitate (A) or stearate (B) for 6 h. Phosphorylation of JNK and phospho-JNK protein were measured using Western blot analysis. Cont: FAFA treated negative control.

IV. Discussion

In this study, to evaluate the effects of saturated and unsaturated fatty acids on astrocytes, human astrocytoma cell line U87MG was used. It would be best to use primary cultured human astrocytes for this investigation; however, as to ethical and practical issues, it is not easy to obtain and use them in experiments. Although the U87MG is established from human astrocytoma, these cells are known to maintain the common characteristics of normal astrocytes, especially that of immature astrocytes (Turner and Adamson. 2011, 167–76). Moreover, the adult human brain is known as the most complex organ, and this complexity cannot be well modelled by simple nervous systems of other species (Gibbons and Dragunow. 2010, 844–56).

We investigated whether SFAs affect the insulin signaling in astrocytes. Insulin has diverse functions such as glucose transport, regulation of many gene expressions, enzymatic activity, and especially important in maintaining energy homeostasis (Sharma et al. 2008, 373–80) (Luca and Olefsky. 2008, 97–105). In the CNS, insulin works as a adiposity negative signal which leads to decrease in appetite and plasma glucose levels, and insulin receptors are abundant in the hypothalamus (Das UN. 2010, 459–73). Knowing the factors that regulate insulin action in the brain is significant for the novel approaches to the treatment of common and costly metabolic disorders such as obesity and type 2 diabetes mellitus (Vogt and Brüning. 2013, 76–84).

Western diet commonly contains excessive SFA (Cordain et al. 2005, 341–54) (Iggman and Risérus. 2011, 209–23). The most common dietary SFA are palmitic acid and stearic acid which are also known as the major SFAs in human plasma and tissues (Iggman and Risérus. 2011, 209–23). The plasma concentration of FFA is known to be below 0.7

mM in the postprandial state and may rise to 1000 μ M (1 mM) after a fatty meal or during fasting (Weigert et al. 2004, 23942–52). The fatty acid level in serum is also significantly increased in association with obesity and metabolic syndrome (Reaven et al. 1988, 1020–4). Diets high in saturated fat may actually increase brain uptake of fatty acids from the plasma (Wang et al. 1994, 517–22). These fatty acids penetrate the blood–brain barrier (BBB) mostly by simple diffusion in the unbound form, which indicate that entering of circulating FFAs to the CNS is usually proportional to their plasma concentration (Miller et al. 1987, 1507–14) (Smith and Nagura. 2001, 167172) (Gupta et al. 2012, 1060–71), but direct uptake of lipoprotein particles mediated by lipoprotein receptors may also occur (Rapoport. 2001, 243–61). Posey et al. reported that cellular exposure to dietary fat induces cellular inflammation and insulin resistance in the hypothalamus, which in turn contribute to the pathogenesis of obesity (Posey et al. 2009, 1003–12). The mechanism of insulin resistance in the brain has been relatively well investigated in neurons rather than in astrocytes. Cintra et al. reported that saturated fatty acids induce hypothalamic inflammation by activating signal transduction through Toll-like receptor (TLR) 4, which also leads to ER stress, expression of inflammatory cytokines, and apoptosis of neurons, which consequently lead to insulin or leptin resistance (Cintra et al. 2012, e30571) (Milanski et al. 2009, 359 –70).

Astrocytes construct the BBB by contacting both the vessel and neuronal synapse and act as a feasible link between the peripheral and neuronal responses (Yi et al. 2011, 143–9) (Gupta et al. 2012, 1060–1071). Thus, astrocytes may play a crucial role in developing metabolic disorders on the brain, although the role and physiology of astrocytes in metabolic disorders have not been clearly investigated. In this study, astrocytoma cells were treated with saturated or unsaturated fatty acids, and the resulting responses were evaluated. When the astrocytes were pre-treated with palmitate or stearate at concentrations

of 50–200 μM as in physiologic levels (Cintra et al. 2012, e30571), phosphorylation of Akt induced by insulin treatment was reduced (Fig. 1). These data showed that palmitate and stearate inhibit insulin signaling in the astrocytoma cells.

Many reports indicate that inflammation induces insulin resistance in hepatocytes, muscle cells, endothelial cells, and neuron. Therefore, we also examined the induction of inflammatory cytokine release by palmitate or stearate in astrocytoma cells. In our studies, palmitate increased the expression and secretion of IL-6 and IL-8 in a dose-dependent manner (Fig. 2A, B). However, TNF- α and IL-1 β expressions were not significantly increased by palmitate treatment (Fig. 2A). Stearate also dose-dependently increased the secretion of IL-6 and IL-8, relatively more than palmitate. TNF- α and IL-1 β expressions were not checked in stearate (Fig. 2B). Gupta et al. reported that stimulation of rat primary astrocyte cells with the SFAs, palmitate and stearate, induced TNF- α and IL-6 release in a dose-dependent manner (Gupta et al. 2012, 1060–71). The mechanisms of how SFAs increased inflammatory cytokine release and also how stearate increased more inflammatory cytokines than palmitate in astrocytoma cells are not clear. Many researches reported that SFAs induce inflammation by activating signal transduction through TLR (Milanski et al. 2009, 359–70); however we did not investigate the activation of TLR, so further investigation should be needed.

Next, we evaluated the effect of fatty acid on ER stress and apoptosis, known to be associated with insulin resistance. We found that palmitate and stearate induced ER stress in astrocytoma cells in a dose-dependent manner. Stearate induced higher levels of Xbp-1 splicing and CHOP expression than palmitate (Fig 3A, B, C). However, palmitate and stearate did not induce the apoptosis of astrocytoma cells in these SFAs concentrations (Fig. 4).

The saturated fatty acids also activated JNK pathway in astrocytoma

cells (Fig. 5). The JNK pathway has been known to contribute to the development of insulin resistance in obese and diabetic states (Luca and Olefsky. 2008, 97–105). Hirosumi et al. found elevated JNK activity in the liver, adipose tissue, and skeletal muscle of obese, insulin-resistant mice (Hirosumi et al. 2002, 333–6). Treatment of palmitate and stearate increased phosphorylation of JNK in astrocytoma cells in a dose-dependent manner (Fig. 5A and B). These data indicate that the JNK pathway could contribute to the effect of palmitate on the attenuation of insulin-stimulated phosphorylation of Akt.

Finally, our data show that UFAs prevent the actions of SFAs on insulin signaling, expression and secretion of inflammatory cytokines, ER stress, and JNK pathway signaling in a dose-dependent manner in the astrocytoma cells (Fig. 6, 7, 8, 9). UFAs, oleic acid and linoleic acid, did not cause cytokine release or attenuation of insulin signaling (data not shown). In other published reports, UFAs have shown potent anti-inflammatory actions and can improve glucose intolerance, insulin resistance, lipidemia, and other associated inflammations (Fedor and Kelley. 2009, 138–46) (Hassanali et al. 2010, 139–47). In astrocytes, UFAs prevent the pro-inflammatory actions of SFAs via limiting the inflammatory signaling by altering TLR4 presentation (Wong et al. 2009, 27384–92) (Lee et al. 2003, 479–86). TLR4 disruption is known to prevent HFD-induced insulin resistance selectively (Davis et al. 2008, 27384–92). Cintra et al reported that UFAs can reproduce a number of the anti-inflammatory effects of TLR4 or TNF- α inhibition, which may be mediated by the GPR120 receptor (Cintra et al. 2012, e30571). In addition, we pretreated astrocytoma cells with UFAs 1 h before SFAs treatment, so receptor competition might be another cause for the reverting effects of UFAs on SFAs. However, there has been no clear explanation for the UFAs actions on insulin signaling and inflammation, ER stress, and JNK pathway in astrocytes. Therefore, further researches are needed regarding TNR4 and GPR120 receptor in astrocytes or

astrocytoma cells.

This study shows that SFAs attenuated the insulin signaling, increased production of inflammatory cytokines, and induced ER stress in astrocytoma cells. UFAs blocked the effects of SFAs, which indicated that UFAs could attenuate high fat diet related metabolic disturbance in the brain.

V. Conclusions

SFAs attenuated the insulin signaling, increased production of inflammatory cytokines, and induced ER stress in astrocytoma cells. UFAs blocked the effects of SFAs, which showed that UFAs rich diet can be a useful candidate for attenuating high fat diet related metabolic disturbance.

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ABSTRACT

Unsaturated Fatty Acids Block the Effects of Saturated Fatty Acids on Insulin Signaling in Astrocytoma Cells

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Objective: Obesity is associated with an increased risk of many diseases. Plasma concentrations of fatty acids are significantly increased in obese and metabolic syndrome patients, and saturated fatty acids (SFAs) can activate inflammatory responses in the periphery and neurons and are also associated with insulin resistance. However, their effects on astrocyte and their role in energy homeostasis of CNS have not been clearly evaluated. To find out the effects of SFAs on astrocyte and energy metabolism, we investigated the effects of SFAs on insulin signaling and associated pathways in the human astrocytoma cell line U87MG. Also, we investigated how the unsaturated fatty acids (UFAs) affected the effects of the SFAs.

Methods and Results: We used the human astrocytoma cell line U87MG, treated with SFAs (palmitate, stearate) and UFAs (oleic acid, linoleic acid). The exposure to SFAs attenuated insulin signaling in astrocytoma cells, which was revealed by the Akt phosphorylation. SFAs induced IL-6 and IL-8 mRNA expression but did not affect IL-1 β and TNF- α mRNA expression. SFAs induced ER stress, which was assessed by Xbp-1 splicing and CHOP mRNA expression; however, it did not induce

apoptosis. SFAs also activated JNK dependent pathway. These changes were reversed by treatments of UFAs.

Conclusions: SFAs attenuated the insulin signaling, induced the inflammatory cytokines, and generated ER stress in astrocytoma cells. UFAs reversed the effects of SFAs, which showed that UFAs rich diet can be a useful candidate for attenuating the effects of SFAs in astrocytes.

Key words : Saturated fatty acid, Unsaturated fatty acid, Inflammation, Insulin signaling, Astrocyte.