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Resveratrol Ameliorates High-fat-induced Metabolic Complications by Changing the Expression of Inflammasome Markers and Macrophage M1 and M2 Markers in Obese Mice

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The purpose of this study was to investigate the effects of resveratrol supplementation on inflammasome, inflammation, and macrophage markers in subcutaneous adipose tissue of high-fat-diet - induced obese mice. C57BL/6 mice were randomly assigned to three groups: normal diet control (NC; n=10), high-fat diet control (HC; n=10), or high fat with resveratrol (HRE; n=10) group. The mice were fed a high-fat diet (60% of calories from fat) or normal diet (18% of calories from fat). Resveratrol dissolved in a 0.1ml solution of dimethyl sulfoxide was supplemented orally at 25 mg/kg body weight. After 15 weeks, the body weight was significantly higher in the high-fat diet group than in the normal diet group. The inflammasome markers NLRP3, ASC, and caspase1 were significantly lower in the HRE group than in the HC group. The levels of an inflammation marker, IL-18, were also significantly lower in the HRE group than in the NC and HC groups. The levels of macrophage markers F480 and CD86 were significantly lower in the HRE group than in the HC group. The levels of the M2 macrophage marker CD206 were significantly decreased in the HC and HRE groups. Resveratrol had a positive effect on ameliorating the complications of high fat diet-induced obesity by reducing inflammasome and M1 macrophage gene expressions. However, resveratrol supplementation did not reduce inflammation gene expression.

Key words : Inflammasome, inflammation, macrophage marker, obese, resveratrol

Introduction

Obesity is a serious problem and has grown fast recent years [9]. Obesity can be defined as excess body fat. Obesity often associated with various chronic diseases such as diabetes, dyslipidemia, and cardiovascular disease [9, 16-18]. Excess of adipose tissue for a long time can be linked to inflammatory processes. Recently, adipose tissue has been known as an endocrine organ that secretes several adipokines and produces some pro-inflammatory cytokines [13, 25].

Our previous study has been reported that high-fat diet induced obese mice can increase the concentration of TLR4, ICAM-1, and VCAM-1 which induce pro-inflammatory cytokine production [16]. To prevent the development of obesity process and alleviate the determination of obese induced complication, many scientists have looked into prospective therapeutic effects of naturally occurring phytochemicals [11, 26, 40].

Resveratrol (3,4_,5-trihydroxystilbene, RSV), one of phytochemical, is a polyphenol compound found in berries, grapes, nuts, and several plants source [5, 6, 43]. Resveratrol mimics the metabolic effects of long-term calorie restriction, and many *in vitro* studies have demonstrated that resveratrol has an anti-obesity potential by inhibiting pre-adipocyte differentiation, decreasing adipocyte proliferation, inducing adipocyte apoptosis, decreasing lipogenesis, and promoting lipolysis and fatty acid β-oxidation [41].

In vitro and pre-clinical studies have greatly facilitated the characterization of molecular targets activated by resveratrol. PGC-1α, SIRT1, NF-kB AMPK, and peroxisome proliferator activated receptor-ɣ (PPARɣ) seem to be related to obesity pathophysiology and affected by specific hormetic mechanisms [31].

There is some group of protein that recognizes the stimulus of inflammation. It controls the production of pro-in-

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flammatory cytokines such as IL-1β and IL-18 called inflammasome [34, 38, 39]. The component of inflammasome proteins is NOD-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase activation recruitment domain (ASC) and caspase 1 (CASPASE 1) [34]. Vandanmagsar et al. [40] have found that high-fat diet induced obese caused the inflammasome marker increased significantly in adipose tissue and liver. Another study has found that there is a strong correlation between obesity, inflammation and NLRP3 inflammasome expression in abdominal subcutaneous adipose tissue [12].

Knockout NLRP3 inflammasome in mice has been reported to protect from obesity-associated macrophage activation in adipose tissue, reducing the M1-like macrophage activation in adipose tissue and increasing the expression of M2-like cytokine [40]. Besides that, mice deficient inflammasome components (NLRP3, ASC, and Caspase 1) are resistant from increasing body weight even with high-fat diet, which correlated with obesity protection [37]. Inflammation is related to response to immune process against pathogens and or pathological condition such as obesity, cancer, and syndrome metabolic [28, 32, 38].

Resveratrol has been previously identified as an antioxidation, and anti-inflammation agent also prevents cardiovascular disease, cancer progression, and metabolic syndrome [5, 8, 16, 17, 21, 30, 35]. Chronic resveratrol supplementation may attenuate inflammation and oxidative stress in epididymal WAT of diet-induced obese mice and appears to reduce adipocyte dysfunction by decreasing adipocyte size and increasing SIRT1 activity [24].

Macrophages have frequently been grouped into two categories: the classically activated macrophages (M1 macrophage) and the alternatively activated macrophages (M2 macrophages) [29, 32]. Diet-induced obesity can shift the M2 macrophage to M1 macrophage that produces pro-inflammatory cytokine [3, 16, 23].

Recently, many scientists use variety way to treat obese induced metabolic complication such as functional nutrition diet. Our previous study has been shown the effect of resveratrol on inflammatory process, mitochondrial biogenesis, lipid metabolism in the liver, skeletal muscle, and peritoneal macrophage [16, 17, 21, 30]. So far the effect of resveratrol on the inflammatory and obesity processes have been established, but how the effects on inflammasome and macrophage infiltration markers are not clearly known especially in subcutaneous adipose tissue [16-18, 21, 30].

Therefore, the aim of this study was to investigate the effects of resveratrol supplementation on inflammasome, inflammation, and macrophage marker in subcutaneous adipose tissue of high-fat-diet-induced obese mice.

Material and Methods

Animals and diet

Male C57BL/6 mice 4 weeks old, n=30 from Central Experimental Animal, Korea were housed in cages (5 mice per cage) with standard experimental laboratory, at temperature 22±2˚C, with 60±5% humidity. After one week adaptation period, the mice were feed either a high fat diet (60% of calories from fat, 20% from carbohydrate, 20% from protein, Orient Bio Inc., #D12492) or a normal diet (18% calories from fat, 58% from carbohydrate, 24% from protein, Orient Bio Inc., #2018) ad libitum for 15 weeks.

The mice were randomly assigned to three groups: normal diet control (NC; n=10), high fat diet control (HC; n=10), and high fat with resveratrol (HRE; n=10) groups. The mice were weighed and food intake per cage (5 mice) measured weekly. All experiments were approved by the Animal Care and Use Committee at the Chungnam National University (CNU-00494).

Resveratrol supplements

Resveratrol supplement purchased from Sigma Aldrich Inc. Resveratrol supplement was orally given 25 mg/kg body weight dissolved in a 0.1ml solution of Dimethyl Sulfoxide (DMSO). The supplements were administered orally using a disposable 1 ml syringe at dose 0.1 ml per mouse 4 times a week.

All the mice were sacrificed after fasted for 12 hr under anesthesia using a mixture ketamine (80 mg/kg) and xylazine (10 mg/kg). The subcutaneous adipose tissue was quickly removed, weighed and frozen in liquid nitrogen and stored at -80˚C until analysis.

RNA extraction and semi quantitative reverse transcription-polymerase chain reaction (RT PCR)

Total RNA was extracted from 150 mg subcutaneous adipose tissue homogenate using 1ml Trizol reagent (Ambion, Carlsbad, CA, USA). The total RNA concentration was calculated by measuring the absorbance at 260 nm and 280 nm using an ultraviolet spectrophotometer. For cDNA synthesis,

Maxi RT PreMix kit (iNtRON, Korea) was used according to the manufacturer's instructions.

The PCR was set using the following program: 95°C for 2 min, 95°C for 30sec 38-40 cycle, the appropriate annealing temperature between 55-57°C for 30sec and 72°C for 2 min. After loaded onto 1% of the agarose gel containing ethidium bromide then measured the PCR band density with Image Lab 4.0 (Bio-Rad, USA). The mRNA levels were normalized with β-Actin. The PCR primer sequences for each studied gene are shown in Table 1.

Statistical analysis

Statistical analysis from RT-PCR data was performed by SPSS V22.0 using one-way ANOVA with LSD posthoc tests. Statistical significance was defined as α=0.05.

Results

Characteristic changes

Fig. 1 shows body and subcutaneous adipose tissue (SAT) weight. There was a significant difference between NC and HC group in the body and SAT weight. However, the body and SAT weight of HRE group were not significantly different compared to those of HC group (p<0.05).

Inflammasome marker changes in the subcutaneous adipose tissue

Fig. 2 shows the effect of resveratrol supplementation on mRNA expression of inflammasome marker in high-fat diet induced obese mice. The NLRP3 and ASC mRNA expression of HC group significantly increased compared to those of NC group. There was no significant difference between NC and HC groups on caspase1 mRNA expression.

The data showed there was no significant difference among all groups in IL-1β level. However, the IL-18 mRNA expression of resveratrol group was significantly reduced compared to NC and HC groups (p<0.05).

Macrophage infiltration marker changes in subcutaneous adipose tissue

Fig. 3 shows macrophage infiltration marker mRNA expression. The M1 macrophage marker including F480 and CD86. There was significantly different in F480 and CD86 mRNA expression between NC and HC groups. The HRE group also significantly decreased in CD86 mRNA expression compared with HC group.

The M2 macrophage infiltration marker CD206 was significantly decreased in HC group compared with NC group. However, there was no significant difference in HRE group

Table 1 Reverse transcription-polymerase chain reaction primer sequences

Gene	Forward	Reverse
NLRP3	GCTCCAACCATTCTCTGAC	AGTTACACTGTGGGTCCTT
ASC	CAGGTATTGCCATCATTCAGT	TTCCATAGGTAGGACCATAAATAA
Caspase 1	GCAAAGAGGAAGCAATTTATCA	GCCTTGTCCATAGCAGTAAT
IL-1β	TCACAAGCAGAGCACAAG	GAAACAGTGCAGCCCATAC
IL-18	AATCTGTAATGTTCACTCTCACTA	GCCTCGGGTATTCTGTTATG
F480	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
CD86	TCTCCACGGAAACAGCATCT	CTTACGGAAGCACCCATGAT
CD206	CAGGTGTGGGCTCAGGTAGT	TGTGGTGAGCTGAAAGGTGA
β-actin	TCACCCACACTGTGCCCATCACGA	CAGCGGAACCGCTCATTGCCAATGG

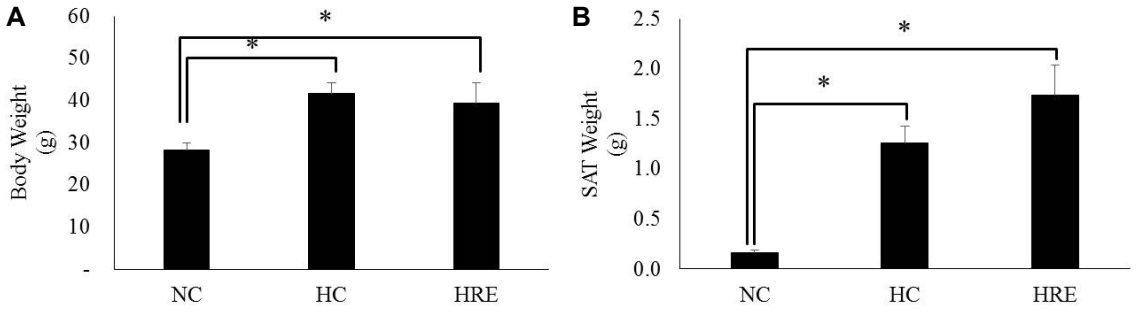

Fig. 1. The change of (A) Body Weight ; (B) Subcutaneous Adipose Tissue (SAT) weight of high-fat-diet -induced obese mice. Values represent means±SEM. * p<0.05 significant difference from NC group.

Table 2. Inflammasome and inflammation marker mRNA expression in subcutaneous adipose tissue

Gene	Group	M±SD	F	p	Post hoc
NLRP3	NC	0.58±0.06	13.186	0.003	0.003
	HC	0.95±0.08			0.003
	HRE	0.58±0.19			0.973
ASC	NC	0.57±0.08	7.99	0.01	0.006
	HC	0.85±0.15			0.006
	HRE	0.59±0.07			0.804
Caspase1	NC	0.94±0.20	2.348	0.166	0.437
	HC	1.14±0.15			0.070
	HRE	0.65±0.40			0.251
IL-1β	NC	0.94±0.20	1.419	0.304	0.256
	HC	0.76±0.09			0.768
	HRE	0.72±0.20			0.148
IL-18	NC	0.94±0.20	4.832	0.056	0.980
	HC	0.94±0.12			0.035
	HRE	0.59±0.14			0.037

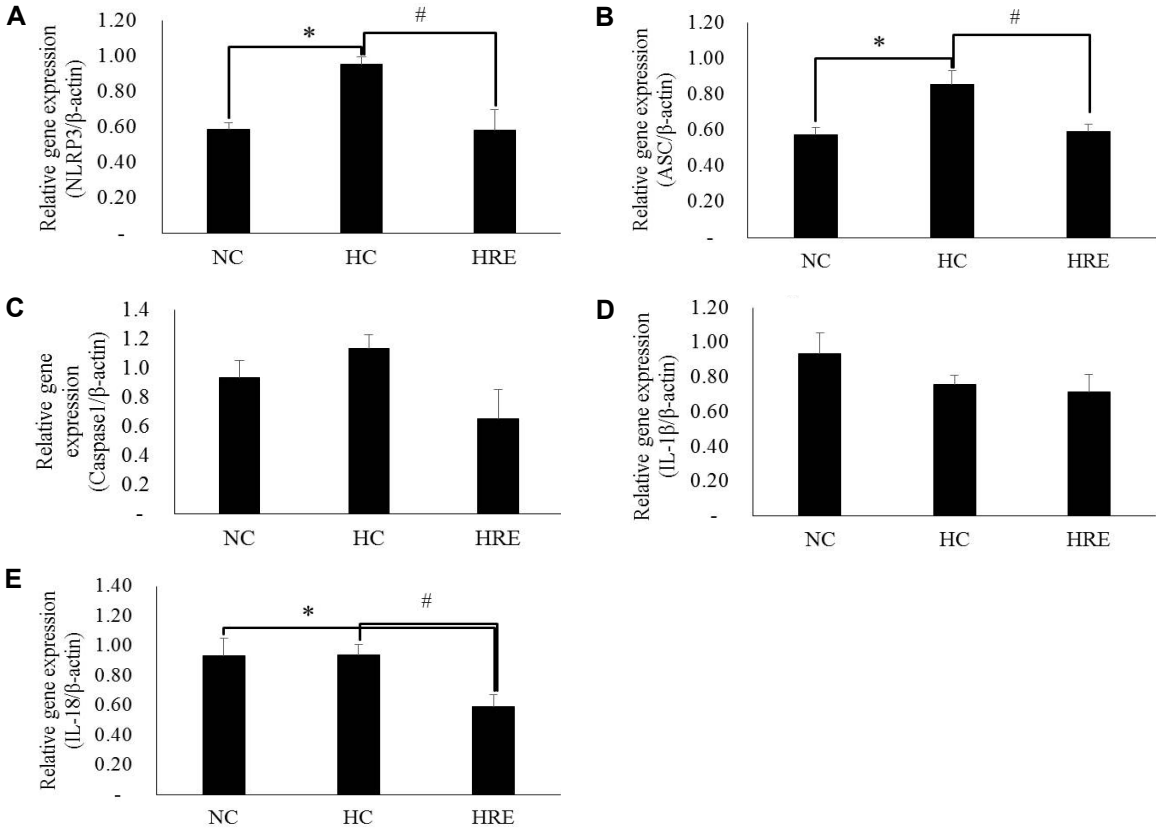

Fig. 2. Effect of resveratrol supplementation on inflammasome marker in high-fat diet induced obese mice. (A) NLRP3 Inflammasome; (B) ASC; (C) Caspase 1; (D) IL-1β; (E) IL-18. Data represent means±SEM. *p<0.05 significant difference from NC group. #p<0.05 significant difference from HC group.

compared with HC group.

Discussion

A high-fat diet can induce obesity and other metabolic dysfunction related [9]. It was reported that resveratrol stimulated anti-obese effects by inhibition of adipogenic differentiation and lipid droplet accumulation as well as by stimulation of cytotoxic effects on adipocytes [5]. Also, resveratrol improved dyslipidemia and steatohepatitis in-

Table 3. Macrophage infiltration mRNA expression in subcutaneous adipose tissue

Gene	Group	M±SD	F	p	Post hoc
F480	NC	0.46±0.09	4.174	0.057	0.021
	HC	0.68±0.11			0.048
	HRE	0.53±0.09			0.395
CD86	NC	0.66±0.04	18.910	0.002	0.002
	HC	0.95±0.10			0.001
	HRE	0.61±0.07			0.466
CD206	NC	1.16±0.34	9.470	0.008	0.003
	HC	0.54±0.07			0.519
	HRE	0.64±0.15			0.008

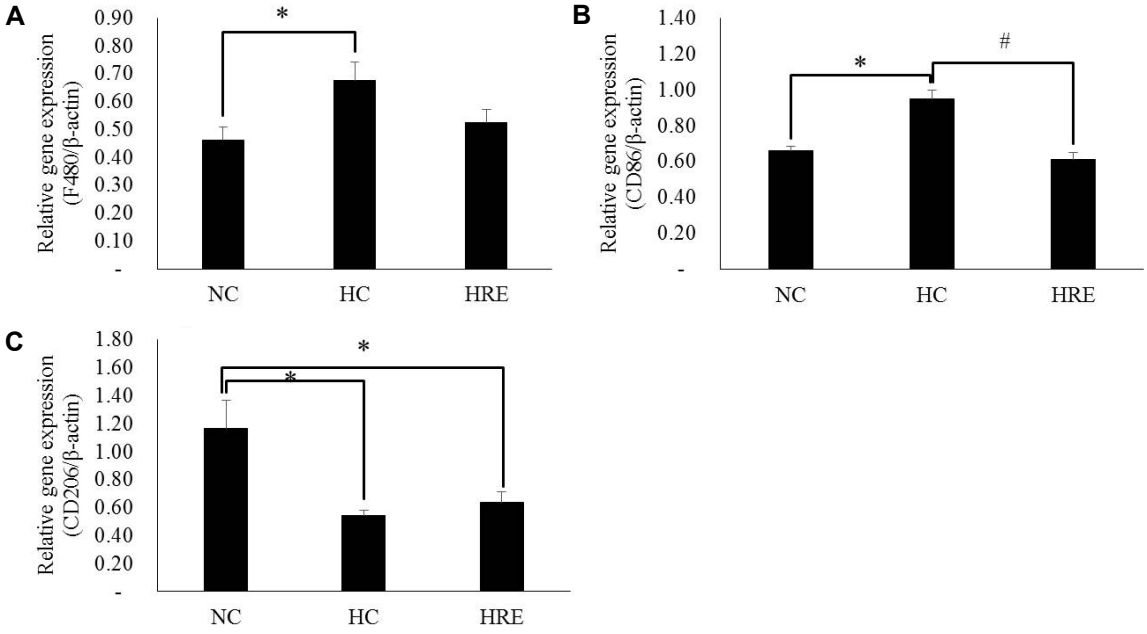

Fig. 3. The effect of resveratrol supplementation on macrophage marker in high-fat diet induced obese mice. (A) F480, (B) M1 macrophage marker CD86, (C) M2 macrophage marker, CD206. Data represent means±SEM. *p<0.05 significant difference from NC group. $^{\#}p$<0.05 significant difference from HC group.

duced by the atherogenic diet [1]. The previous study confirmed that resveratrol has a role in the promotion of mitochondrial activity in the brown adipose tissue, both *in vitro* and *in vivo*.

In the *in vivo* study, resveratrol re-established the impaired mitochondrial activity that was induced by the high-fat diet, increased the expression of endoplasmic reticulum-α, and improved insulin sensitivity [19]. Thus, it was important to know the effect of resveratrol on obesity-induced metabolic complications.

In this study, we found that body weight was not significantly reduced by resveratrol supplementation. The previous study reported that there was significant weight reduction with resveratrol supplementation (200-400 mg/kg/day) in obese mice [20]. However, our previous results with 10mg/kg also showed no significant effect on body weight [17]. In this research, we use 25mg/kg body weight of mice that were given orally but the weight of resveratrol supplementation group was not significant difference compared to the high-fat diet group. The effect of resveratrol on the obese model suggested that weight reduction can differ based on the level of obesity, the duration, and concentration of resveratrol administration [5, 30]. Thus, further studies are needed in the future

NLRP3 inflammasome is a novel protein complex that integrates multiple exogenous and endogenous danger signals into immediate secretions of IL-1β and IL-18 [2]. Our study found there were not significant difference in IL-1β and

IL-18 mRNA expressions between high fat diet and normal diet groups. However, IL-18 mRNA expressions were significantly decreased only in resveratrol group. Previous study demonstrated that NLRP3 inflammasome is spesifically activated in response to high fat diet and controls the production of IL-1β in adipose tissue and IL-18 in obesity [40].

Schmidt (2012) [33] found that processing of IL-1β and IL-18 are independently regulated by activation of NLRP3 inflammasome. This process was related with the production of reactive oxygen species (ROS) and modification of the thioredoxin interacting protein, TXNIP. It was found that inhibitor of ROS production inhibited secretion of IL-1β, but did not in IL-18 [33].

Inflammasomes have been highlighted in the area of innate immunity. It has been hypothesized that obesity may be caused by activated innate immunity as well as by acquired immunity. Thus, extensive research has been shown to link obesity and inflammasome as regulatory components of innate immunity. Among inflammasomes, the NLRP3 inflammasome is a sensor of obesity-associated inflammation and a key regulator linking metabolism and inflammation [14, 15, 27]. Once the NLRP3 inflammasome is activated, activated caspase1 cleaves a variety of protein precursors, including pro-IL-1β and pro-IL-18, subsequently inducing excessive production of pro-inflammatory cytokines [36].

We found that induction of high‐fat-diet-induced obesity caused NLRP3, ASC and Caspase 1 activation in subcutaneous adipose tissue. This finding was relevant to previous research that obesity condition can activate the inflammasome such as NLRP3, ASC and Caspase 1. This inflammasome activation was specific for several tissues including adipose tissue and liver but not in the kidney [40]. Furthermore, resveratrol had a significant effect on reduced the expression of the inflammasome markers.

The Macrophage can be divided into two types, M1 as classically activated macrophage phenotype and M2 as alternatively activated macrophage [4, 7, 32]. In the development of obesity, adipose tissue mass is increased by hyperplasia and hypertrophy also increased macrophage infiltration in adipose tissue [7]. The increased accumulation of macrophages in adipose tissue may increase production of a proinflammatory cytokine in the adipose tissue and contribute to the pathophysiological effects of obesity [43].

Our study found the macrophage marker F480 was significantly increased in high-fat diet group compared to that in normal diet group. Resveratrol has a significant effect that reduces the F480 mRNA expression in high-fat diet group (p<0.05). This result is consistent with our previous study [16].

M1 macrophage marker CD86 was significantly reduced in resveratrol supplementation group compared to that in high-fat diet induced obese mice group (p=0.001). The previous results demonstrated that resveratrol is a potent inhibitor of the anti-pathogen responses of rat macrophages and suggest that this agent may have applications in the treatment of diseases involving macrophage hyper responsiveness [22].

In recent study M2, macrophage marker CD206 was significantly decreased in high-fat diet group compared with that in normal diet group (p<0.05). In contrast, resveratrol supplementation increased the expression of CD206 compared with high-fat diet induced obese group but not significant. This result also similar with our previous study M2 macrophage specific marker CD163 was not significantly increased with resveratrol supplement [16]. In the condition of obesity, proinflammatory M1 macrophage markers are predominantly presented in adipose tissue whereas anti-inflammatory M2 macrophage markers are less found in obese adipose tissue [7]. This result suggests that resveratrol has an effect to ameliorate the M1 macrophage marker CD86 and controlled the M2 macrophage marker CD206.

In conclusion, resveratrol has a positive effect on inflammasome marker, M1, and M2 macrophage marker. However, resveratrol supplementation cannot change the expression of IL-1β as an inflammatory cytokine that activated by caspase1.

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초록 : 라스베라트롤 투여가 고지방식이 비만쥐의 지방조직에서의 inflammasome과 대식세포 마커에 미치는 영향

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본 연구 목적은 고지방식이 유도 비만 쥐의 피하지방조직에서 라스베라트롤 투여가 대식세포 침윤관련 염증인자에 미치는 영향을 규명하고자 하였다. 본 연구를 위해 정상식이군, 고지방식이군, 고지방식이+라스베라트롤 투여군으로 분류한 후, 라스베라트롤 투여군은 15주간 25 mg/kg 농도로 Dimethyl Sulfoxide에 용해하여 투여하였으며, 비교군은 Dimethyl Sulfoxide 용액만을 투여하였다. 연구결과 고지방식이군은 정상식이군에 비하여 체중이 유의하게 증가하였고, 라스베라트롤 투여군에서 고지방식이 군보다 NLRP3. ASC, Casepase1 mRNA 발현이 감소하였다. 또한 염증마커로 알려진 IL-18 mRNA 발현이 라스베라트롤 투여군에서 정상식이군과 고지방식이군보다 낮게 나타났다. 대식세포 침윤 마커인 F480, CD86 mRNA 발현에서도 라스베라트롤 투여군에서 고지방식이 군보다 유의한 감소를 보였다. 따라서 라스베라트롤 투여는 고지방식이 유도 비만 상황에서 대식세포 침윤 염증과 inflammasome에 긍정적인 영향을 미치는 것으로 보여진다.