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Modulation of pro-inflammatory adipokines and myokines by capillarisin and alantolactone in the metabolic disorders

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ABSTRACT

Modulation of pro-inflammatory adipokines and myokines by capillarisin and alantolactone in the metabolic disorders

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Skeletal muscle and white adipose tissue are the largest organs in the human body and considered as endocrine organs. Skeletal muscle produces cytokines and chemokines that contribute to further immune system. Cytokines and chemokines that are produced and released by muscle fibers expressing paracine/endocrine effects are termed ‘myokines’. Adipocytes also release metabolites, lipids, and bioactive peptides; so-called ‘adipokines’. Released adipokines regulate biological process, such as inflammation, fat distribution, and insulin sensitivity. Both myokines and adipokines have profound effects on inflammation and metabolism, contributing to the pathogenesis of inflammation, obesity, insulin resistance, diabetes, and other diseases. In fact, many myokines are also produced by adipocytes and vice versa. Therefore, recent reports proposed the term ‘adipo-

myokines'. IL-6, TNFα, and MCP-1 are the most studied pro-inflammatory adipo-myokines released by both skeletal muscle and adipose tissue. Therefore, it is likely that adipo-myokines may crosslink between the inflammation and metabolic homeostasis in the skeletal muscle and adipose tissues.

Herein, this study investigated first, eccentric (downhill) exercise, second, cytokine (IL-6), and third, fatty acid (palmitate) induced metabolic disorders in the skeletal muscle and adipocytes in association with commonly expressed pro-inflammatory adipo-myokines IL-6, TNFα and MCP-1.

First, eccentric (downhill) exercise can lead to leucocyte infiltration, inflammation and reactive oxidative stress (ROS) in the skeletal muscle. Capillarisin isolated from *Artemisia capillaris* Thunberg is known to have antioxidant and anti-inflammatory effects. Eccentric exercise was conducted to induce muscle damage in mice skeletal muscle. Intense exercise increased the level of ROS production in the skeletal muscle, but capillarisin administration reduced these levels in a dose dependent manner ($p<0.05$). Muscle damage markers, CPK and LDH were also attenuated at plasma level from capillarisin treated groups. Exercise activated MAPK (ERK 1/2 and JNK but not p38) and NF-κB (nuclear p50 and p65, and cytosolic p-IκBα) subunits at protein level but capillarisin suppressed these increase. At the mRNA level, inflammation-associated chemokines CINC-1 and MCP-1, and cytokine IL-6 and TNFα in gastrocnemius muscle were increased by exercise, whereas capillarisin showed protective and inhibitive effects against these changes. Overall, our results indicate that capillarisin can attenuate muscle damage

by exerting antioxidant and anti-inflammatory effects by regulating pro-inflammatory adipo-myokines.

Second, cytokines, such as IL-6 and TNFα are reported as a bridge between inflammation and insulin resistance. IL-6 has been proposed to be one of the mediators that link chronic inflammation to glucose intolerance and insulin resistance. In the present study, we observed protective effects of alantolactone, a sesquiterpene lactone isolated from *Inula helenium* against IL-6 induced inflammation and glucose intolerance in association with myokine expressions. Alantolactone has been reported to have anti-inflammatory and anti-cancer effects through IL-6-induced STAT3 signalling pathway. Prolonged IL-6 exposure also increased expression of TLR4, involved in inflammation in the skeletal muscle, thus the underlying mechanisms were investigated. We observed dysregulation of glucose uptake and suppression of AKT phosphorylation after prolonged IL-6 treatment; however, pretreatment with alantolactone activated AKT phosphorylation and improved glucose uptake. Alantolactone also attenuated IL-6-stimulated STAT3 phosphorylation, followed by an increase in expression of negative regulator SOCS3. Furthermore, IL-6-induced expression of pathogen recognition receptor, TLR4, was also suppressed by alantolactone pretreatment. Post-silencing of STAT3 using siRNA approach, IL-6-stimulated siRNA-STAT3 improved glucose uptake and suppressed TLR4 gene expression. Taken together, we propose that, as a STAT3-SOCS3 inhibitor, alantolactone, improves glucose uptake in the skeletal followed by inhibition of the TLR4 gene expression. Lastly,

alantolactone suppressed IL-6 induced pro-inflammatory myokines, IL-6, TNFα and MCP-1. Therefore, alantolactone can be a promising candidate for the treatment of inflammation-associated glucose intolerance and insulin resistance.

Third, adipocytes, together with macrophages create a crosstalk between inflammation and insulin resistance. Resident macrophages are surrounded by adipocytes that constantly release free fatty acids (FFAs) via lipolysis. FFAs such as palmitate activate macrophages and pro-inflammatory adipokines such as IL-6, TNFα and MCP-1, consequently alter their function. This research aimed to evaluate the potency of alantolactone in reducing palmitate-induced glucose intolerance, fat accumulation, and inflammation in 3T3-L1 adipocytes. In vitro obese model was constructed by adipocyte-macrophage co-culture system (3T3-L1-RAW264.7). This study observed that palmitate and co-culture system reduced glucose uptake and increased fat accumulation, which indicated dysfunctional adipocytes. Alantolactone pretreatment reversed these changes by increasing glucose uptake and attenuating fat accumulation in a dose-dependent manner ($P < 0.05$). Palmitate and co-culture model activated JNK and IKKβ/α phosphorylation, and increased the levels of pro-inflammatory adipokines (TNFα, IL-6, and MCP-1). Alantolactone treatment selectively reduced JNK and TLR4 gene expression, suggesting inhibition of TLR4-JNK signalling. Alantolactone also reduced macrophage infiltration associated chemkines MCP-1 and cytokine IL-6 in both adipocyte and adipocyte-macrophage co-culture system. Our study showed that palmitate treatment led to adipocyte dysfunction and inflammation; however,

alantolactone improved palmitate-induced glucose intolerance and inflammation. These findings suggest that alantolactone may inhibit obesity-induced insulin resistance and improve glucose homeostasis and inflammation in the adipose tissues.

Overall, this study suggests capillarisin and alantolactone, as pro-inflammatory adipokines and myokines regulators, are promising therapeutic agents for inflammation associated metabolic disorders in the skeletal muscle and adipose tissue.

Keywords: Adipokines, Myokines, Adipo-myokines, Inflammation, Glucose intolerance, Obesity, Insulin resistance, Capillarisin, Alantolactone

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I. INTRODUCTION

1. Adipokines, myokines and adipo-myokines

1.1. Adipokines

Adipocytes release metabolites, lipids, and bioactive peptides; so-called ‘adipokines’ that signal the functional status of adipose tissue to the brain, liver, pancreas, immune system, muscle, and other tissues [1]. Adipose tissue is recognized as an endocrine organ that is responsible for the triglyceride storage, thermoregulation, and organ protection [2,3]. Adipokines contribute to the regulation of biological process such as inflammation, fat distribution, insulin secretion and sensitivity, energy expenditure, and homeostasis [3]. Adipokines that exert specific effects include: inflammation (e.g., interleukin (IL)- 1β, -6, -8, and -10, tumor necrosis factor α (TNFα), monocyte chemotactic protein-1 (MCP-1), and resistin); glucose metabolism e.g., leptin, adiponectin, DPP-4, FGF21, vaspin, and angiopoietin-like protein 8 (Angptl8); insulin sensitivity (e.g., leptin, adiponectin, chemerin, RBP4, and omentin); lipid metabolism (e.g., soluble CD36 and apelin); (Table 1) [1]. Increasing body weights and energy expenditure alters adipose tissue size, distribution, and function [3]. Expansion of adipocytes, or adipocyte hypertrophy impairs metabolic function of individuals, and initiates predisposition of adipose tissue to immune cell infiltration and adverse adipokine secretion [2,4]. Adipocyte hypertrophy is typically featured with elevated pro-inflammatory factors, including leptin, IL-6, IL-8, and MCP-1 with reduced levels of the insulin sensitivity [2,5]. In obesity, adipokine secretion may change metabolic and inflammatory diseases linking adipose tissue dysfunction and even malignant comorbidities [1].

Table 1 List of main actions of adipokines

Main actions	Adipokines
Inflammation	IL-1β, -6, -8, and -10, TNFα, monocyte, MCP-1, and resistin
Glucose metabolism	leptin, adiponectin, DPP-4, FGF21, vaspin, and Angptl8
Lipid metabolism	Soluble CD36 and apelin

1.2. Myokines

Skeletal muscle produces cytokines and chemokines as an endocrine organ and further immune system [6]. Cytokines and chemokines that are produced and released by muscle fibers expressing paracine and endocrine effects are termed 'myokines' [7]. Production of multiple myokines by myotubes has been reported to be regulated by exercise, electrical stimulation, and external and internal stimuli [8-10]. Leukemia inhibitory factor and IL-15 are paracrine effectors, while other myokines, such as myonectin, IL-6, irisin, calprotectin, and oncostatin M, are endocrine effectors, involved in pathologic conditions [11]. In this research, myokines that result in paracrine manner were mainly studied. Loss of muscle mass is a common feature of metabolic disorders and frequently associated with increased production of pro-inflammatory myokines such as TNF-α, IL-1, IL-6, IFN-γ and MCP-1 [11]. In this regard, healthy animals exposed to pro-inflammatory myokines, increased both ubiquitin expression and proteasome enzymatic activity and developed muscle wasting [12]. Circulating nitric oxide levels were reported to be associated with increased IL-6 levels after eccentric exercise-induced muscle damage [13]. It was also noted that TNFα production and secretion were increased in insulin resistant individuals [14]**.** Consistent with this, recent evidence reported the observations of MCP-1 mRNA elevated in type 2 diabetes (T2D) human skeletal muscle, after TNFα treatment [15]. In addition, secretion of TNFα, IL-6 and MCP-1 were all elevated in T2D myotubes [8]. It is interesting to note that these myokines not only act locally in the muscle in an paracrine manner, but also act as auto/endocrine factors to influence other tissues in the body [16].

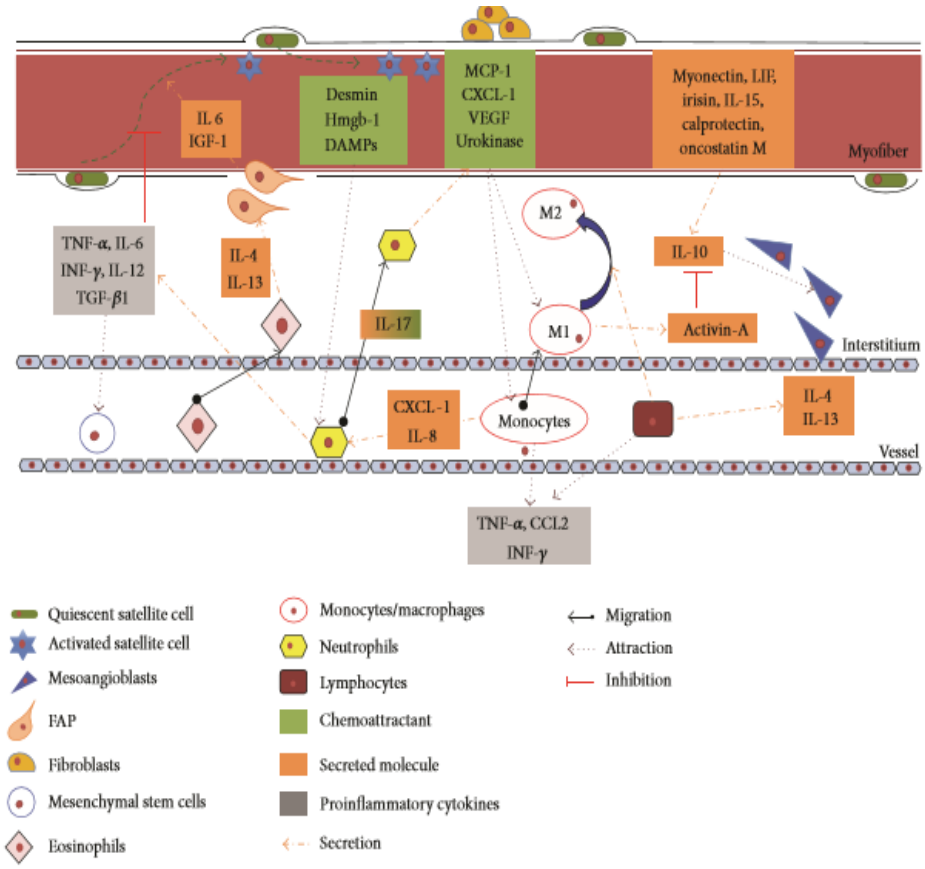


Figure 1 Myokines involved in skeletal muscle inflammation [11]

1.3. Adipo-myokines

Skeletal muscle and white adipose tissue are the largest organs in the human body and considered to be endocrine organs that secrete cytokines [7,17]. Both myokines and adipokines have profound effects on inflammation and metabolism, contributing to the pathogenesis of obesity, diabetes, and other diseases [17]. In fact, many myokines are also produced by adipocytes and vice versa. Therefore, recent reports proposed the term 'adipo-myokines' [7]. Certain cytokines and chemokine such as IL-6, TNFα, and MCP-1 are adipo-myokines released by skeletal muscle or adipose tissue [18]. Therefore, it is likely that adipo-myokines may contribute in the metabolic homeostasis and crosstalk between skeletal muscle and adipose tissues [7]. By using the adipo-myokine concept, this review aims 1) to gain a view on roles of adipo-myokines, IL-6, MCP-1 and TNFα, that mediate chronic inflammation and metabolic disorders, 2) to examine effects of natural compounds, capillarisin and alantolactone as regulators of adipo-myokines, 3) to investigate underlying molecular events.

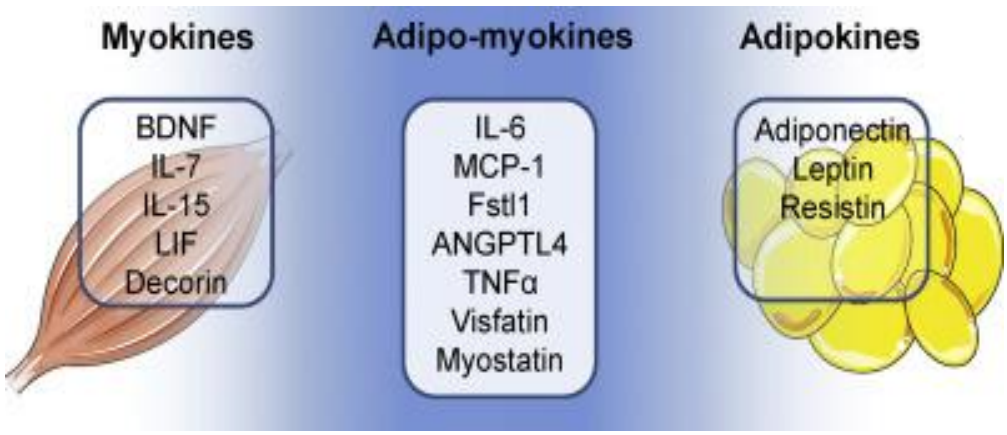


Figure 2 Myokines, adipo-myokines and adipokines [7]

1.3.1 IL-6

IL-6 is a pleiotropic cytokine that modulates immune response produced by various cells, including skeletal muscle and adipose tissue [19]. Accumulating evidence indicates that IL-6 has both pro-inflammatory and anti-inflammatory effects. However, the nature of this role still remains controversial [20-22]. Low levels of IL-6 can promote myotube regeneration acting as anti-inflammatory effects, while high levels of IL-6 promote skeletal muscle wasting and metabolic disorders as a pro-inflammatory cytokine [21,22]. The pro-inflammatory effects of IL-6 can be explained by a crosstalk of the IL-6/IL-6 receptor and gp130 trans-signalling pathway that oppose to anti-inflammatory classical IL-6 receptor signalling pathway [22] (Figure 3). As a myokine, IL-6 is synthesized by contracting muscle after strenuous exercise and increased in relation to other pro-inflammatory cytokines, such as TNFα and IL-1β [22]. It is well established that IL-6 plays an important role in the metabolic regulation of not only muscle itself but also in other organs such as liver, adipose tissue and pancreatic ß-cells [19]. Recent study demonstrated that IL-6 induced hepatic insulin resistance by increasing SOCS3 expression which may directly inhibit the insulin receptor [23]. Moreover, the prevention of IL-6 signalling by genetic deletion of IL-6 markedly reduces insulin-induced phosphorylation of hepatic STAT3 [6]. As an adipokine, IL-6 is also highly expressed in adipose tissue and positively correlated with obesity in humans [24]. Short administration of IL-6 was reported to enhance energy expenditure and decreased obesity, while prolonged exposure induced obesity through a central nervous system mechanism [24]. Pathologic effects of IL-6 were evidenced by IL-6 overexpression in adipose tissues and plasma of obese animals and humans [25].

Chronically elevated levels of IL-6 was observed in patients with rheumatoid arthritis, indicating a pathogenic role of IL-6, and by the fact that blocking IL-6 has positive effects on arthritis [6]. FDA approved IL-6 blocker, which is a biological drug for rheumatoid arthritis secondary treatment for TNFα blockers resistant patients [6]. Therefore, IL-6 suppression by natural products may contribute in treatment of chronic inflammatory diseases.

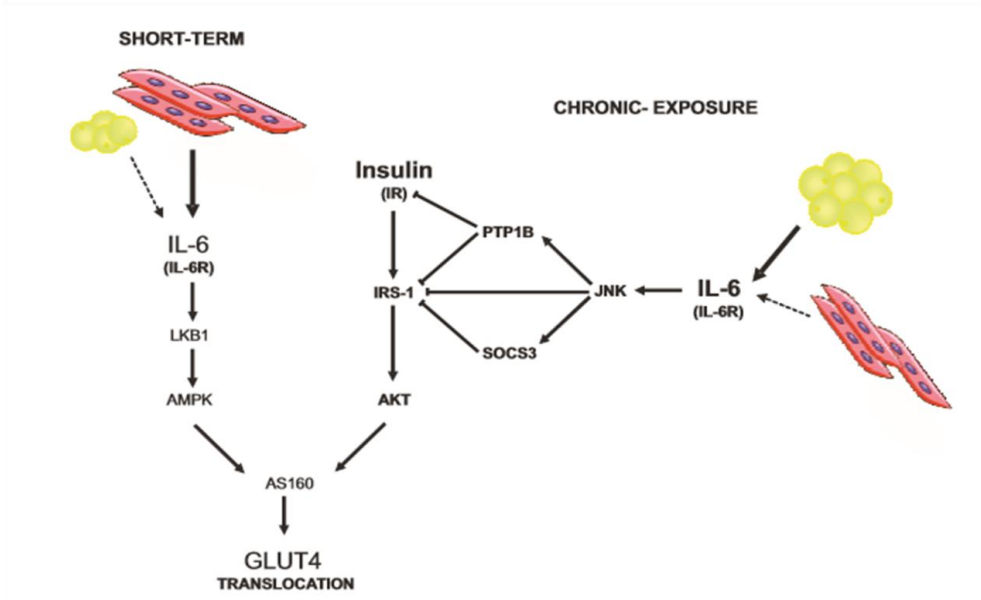


Figure 3 Dual role of IL-6 signalling pathway [21]

1.3.2 TNFα

TNFα is a typical pro-inflammatory cytokine and an adipo-myokine that is increased in obese humans and rodents and plays an important role in the pathogenesis of metabolic syndroms. TNFα mediates biological effects on two distinct cell surface receptors TNFα receptor (TNFR) 1 and TNFR2 [26]. Both receptors are transmembrane glycoproteins that release soluble forms that involved in the neutralization and excretion of TNFα [26]. TNFα act directly on muscle through nuclear factor-κB (NF-κB) and increase reactive oxygen species (ROS) production via mitochondrial electron transport [27]. NF-κB increase activity of the ubiquitin/proteasome pathway, which accelerates the regulated degradation of muscle proteins and promotes muscle weakness [27]. TNFα levels in the skeletal muscle are increased during an eccentric exercise in response to muscle damage via TNFα/NF-κB signalling [7]. Many reports suggest that TNFα provides crosstalk between inflammation and metabolic signalling through c-Jun N-terminal kinase (JNK) and I kappa beta kinase (IKK) pathways [28]. TNFα level in the plasma is positively correlated with insulin resistance, but clinical effects of TNFα are still controversial**.** It was reported that long-term treatment of TNFα blocker in obese patients with rheumatoid arthritis improved insulin sensitivity [24]. It was noted that TNFα inhibits the insulin signalling cascade through the insulin receptor substrate (IRS) and AKT substrate 160 in human skeletal muscle in vitro and in vivo [26]. This indicates that TNFα plays a direct pathogenic role in glucose homeostasis [6]. Immune function is closely linked to fatty acids (FA) that can alter cytokine production, which plays a significant role in diet-induced obesity and inflammation [6]. Intriguingly, calorie restriction and weight loss are associated

with decreased cytokine production [26]. Adipose tissue is a metabolism regulator and glucose sensor. This is supported by the fact that mice lack of glucose transporter GLUT4 in their adipose tissue develop insulin resistance in liver and skeletal muscle, resulting in glucose intolerance and hyperinsulinaemia [26].

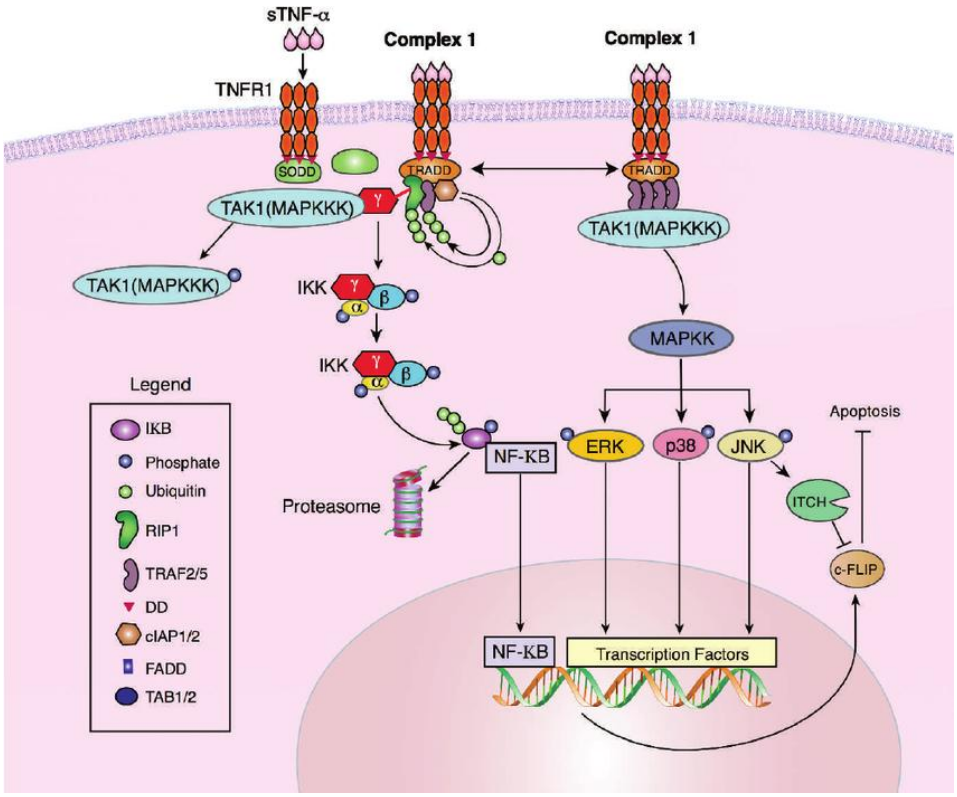


Figure 4 TNFα signalling pathway [29]

1.3.3 MCP-1

The chemokine MCP-1 is another adipo-myokine and plays an important role in the recruitment of monocytes and T lymphocytes into tissues [7]. MCP-1 is a typical chemokine that mediate inflammation and metabolic disorders. Accordingly MCP-1 receptor, CC-chemokine receptor 2 (CCR2)-deficient mice showed suppressed macrophage infiltration, inflammation, and insulin resistance [24].

In glucose deprivation, MCP-1 has been shown to be increased in adipose tissues. In addition, genetically obese (ob/ob) and diet-induced obese mice have high levels of MCP-1 expression in their white adipose tissue [30,31]. High levels of circulating MCP-1 are reported to induce macrophage recruitment and inflammation, as well as promote glucose intolerance and insulin resistance [30]. Recent study observed high fat diet MCP-1 deleted mice gaining similar weight as their wild-type but suppressed inflammation and improved insulin sensitivity. This result suggests that there may be other factors for the weight gain, but MCP-1 is an effective regulator of inflammation and metabolic disorders [31].

It was also found that the chemokine MCP-1 impaired insulin signalling and reduced insulin-stimulated glucose uptake in muscle cells, suggesting that MCP-1 may represent a molecular link in the negative crosstalk between adipose tissue and skeletal muscle [32].

2. Inflammation

2.1 Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinase (MAPK) signalling cascades are made up of three kinases, a MAPK kinase kinase (MAP3K); that activates a MAPK kinase (MAP2K); that activates following the MAPK by dual phosphorylation [33]. The classical MAPKs are comprised of three families, extracellular signal-regulated kinase (ERK) family, ERK1 and ERK2, the p38 MAPK family of four isoforms (p38α, p38β, p38γ and p38δ), and the Jun N-terminal kinase (JNK) family of three isoforms (JNK1, JNK2 and JNK3) [33]. Many MAP3Ks, including MAPK/ERK kinase kinases (MEKKs), TAO1, TAO2, apoptosis signal-regulating kinase 1 (ASK1), mixed-lineage kinase 2 (MLK2), MLK3 and TGFβ-activated kinase 1 (TAK1), is known to activate the p38α and JNK cascades [33]. However, little is known about the regulation and the physiological function of the remaining MAPKs.

In the immune system, deletion of JNK1 and JNK2 are involved with embryonic lethality [34]. However, this has recently been overcome by selective deletion of JNK1 and JNK2 mice, showing the regulation involved in insulin function [35]. In the high-fat diet study, JNK deficiency protected against the development of insulin resistance, and lower number of adipose tissue macrophages in the knockout mice [36]. These results indicate that there is a pro-inflammatory role for JNK signalling in macrophages, which suggests that inhibition of JNK could be a potential therapeutic approach in inflammation and obesity.

The ERK1 and ERK2 pathway has been shown to regulate cytokine production. ERK1 and ERK2 activate TPL2 downstream and affect inflammatory responses, inducing the production of TNFα, IL-1β and IL-10 following TLR stimulation [33]. TLR4 mediate induction of TNFα production and that this is ERK1 and ERK2 independent [37]. The identification of the substrates that TPL2 phosphorylates to regulate TNF production independently of ERK1 and ERK2 will be an important area of future research.

P38 is a critical target in protecting tmultiple autoimmune and inflammatory models [38]. The conditional deletion of p38α in macrophages reduces the TLR-mediated induction of TNFα and showed endotoxin resistance [39]. The importance of p38α in pro-inflammatory cytokine production has been confirmed by using mice in which either p38α or p38β are resistant to p38 inhibitors [33]. This work showed that the effects of p38 inhibitors on TLR-induced TNFα production. In addition to its roles, p38α has important cell type-specific roles, including activating feedback pathways that downregulate inflammation; for example, p38α signalling decreased ultraviolet B (UVB) induced skin inflammation in myeloid cells, whereas p38α signalling increased UVB-induced skin inflammation in epithelial cells [40].

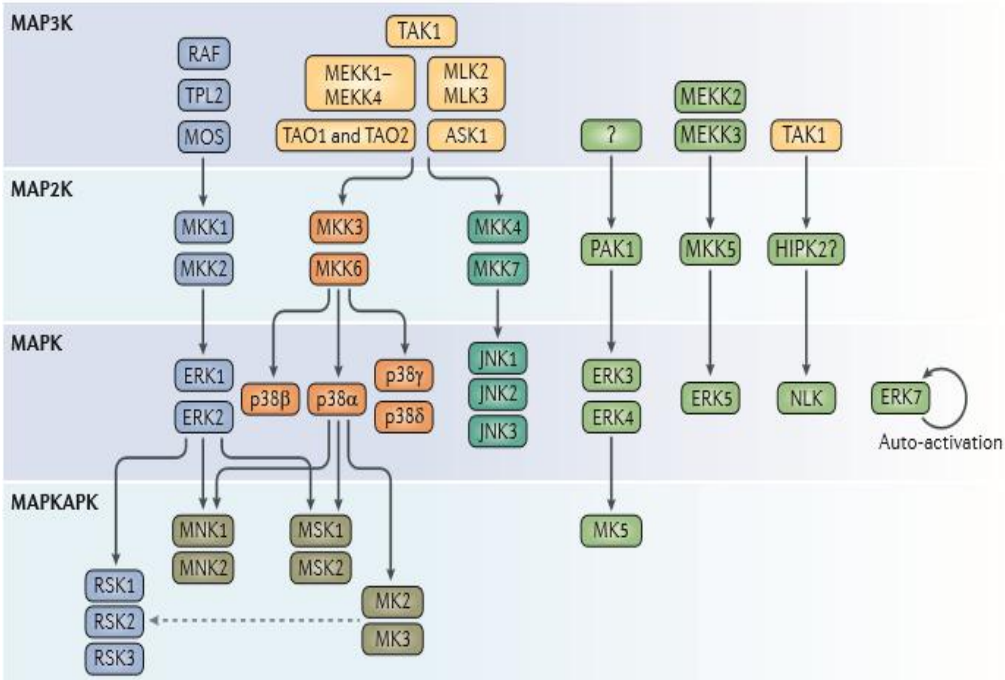


Figure 5 MAPK signalling pathway [33]

2.2 NF-κB

NF-κB is a major inflammatory regulator, activated due to increased cellular levels of ROS and pro-inflammatory cytokines [20]. Cytokines and pathogen-associated molecular patterns (PAMPs) stimulate cell surface receptors such as TLRs to initiate the signalling [41]. NF-κB is known to involve in both external and internal infection or damage and respond to inflammation characterized by macrophage infiltration, swelling and organ dysfunction.

Activation of NF-κB involves the phosphorylation and degradation of the inhibitory protein IκB by specific IκB kinases [42]**.** NF-κB heterodimer p50 and p65 passes into the nucleus and bind the promoter regions of pro-inflammatory cytokine genes [43]. It was reported that inhibition of IκB/NF-κB signalling improves insulin sensitivity, and diet-induced obesity in rats leads to a decrease in muscle IκB content [44]. These results suggest that activation of IκB/NF-κB pathway and subsequent low-grade inflammation impair insulin action [20].

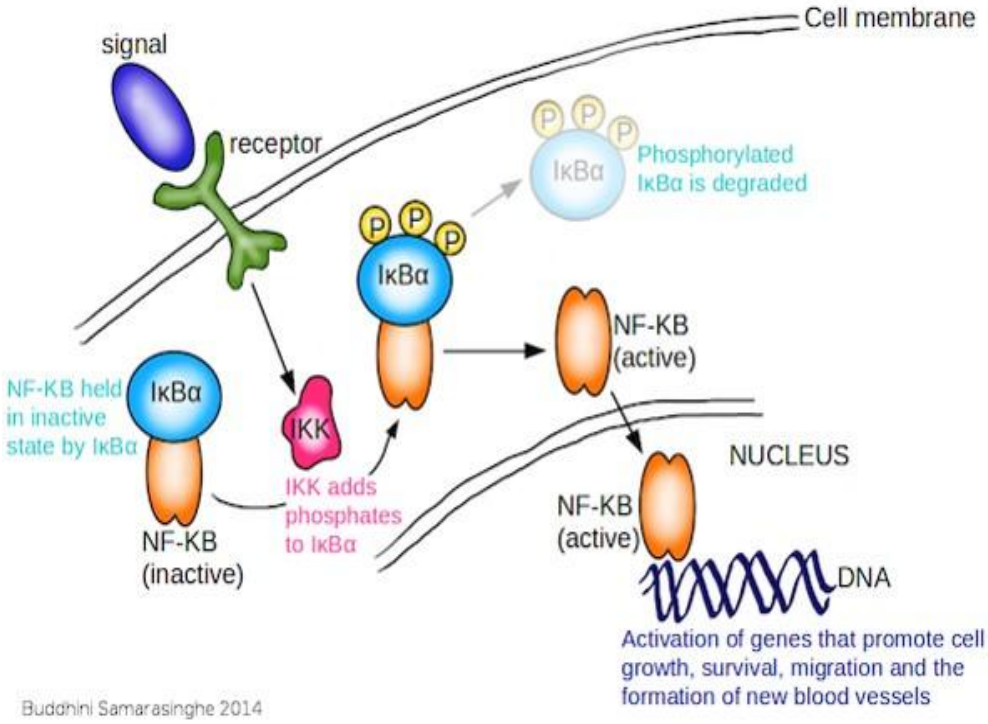


Figure 6 NF-κB pathway [45]

2.3 STAT3 and SOC3

Signal transducer and activator of transcription 3 (STAT3) is predominantly activated by IL-6 through a transmembrane receptor complex containing glycoprotein gp130 and other gp130-acting cytokines (e.g., IL-11, LIF, oncostatin-M) [46]. IL-6 signals activates Janus activated kinase (JAK) tyrosine kinases that downregulates STAT3 through Tyr705 and Ser 727 phosphorylation [23]. Recent study suggested that insulin resistance involves the activation of STAT3 and subsequent induction of suppressor of cytokine signalling 3 (SOCS3), a negative regulator of cytokine signalling [23]. In obesity, the level of inflammation increases pro-inflammatory cytokine levels, such as IL-6, which affect glucose regulation. IL-6 activates the STAT3/SOCS3 signalling pathway, which suppresses insulin receptor signalling (IRS) and glucose metabolism. Inflammation and IL-6 also inhibit AMPK activity, which reduces basal glucose control [47]. Impaired glucose utilization during physiological stress, such as ischemia, might contribute to cell death, cardiomyopathy and diabetic heart failure [47].

SOCS family of proteins (SOCS1–7) bind via their SH2 domains to tyrosine phosphorylation sites on cytokine receptors inhibit inflammatory signal transduction [48]. SOCS signalling is the downstream of JAK/STAT signalling pathways, and deletion of SOCS3 showed a substantial protection against obesity-induced hyperinsulinemia and hyperglycemia [20,48]. Overexpression of SOCS3 in liver caused insulin resistance and hepatocyte-specific deletion of the SOCS3 gene improved insulin sensitivity in mice [49]. Furthermore, SOCS3 expression in skeletal muscle may contribute to the exercise-induced increase in IL-6 expression

through NF-κB activation [50]. SOCS1 and SOCS3 can bind insulin receptor and inhibit IRS-1 and -2 [20].

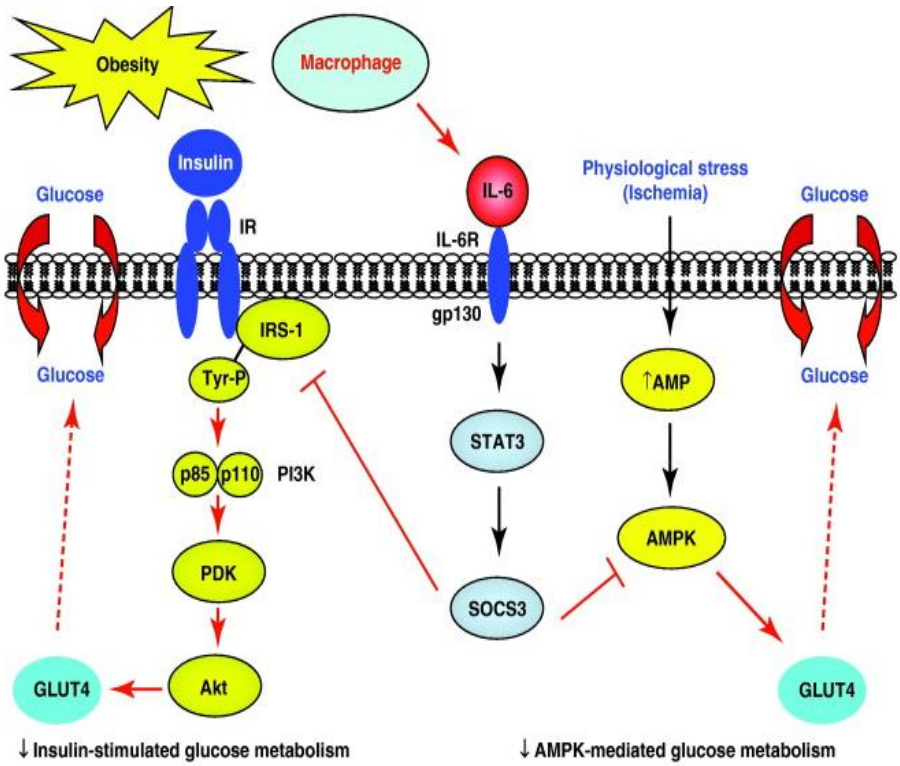


Figure 7 STAT3/ SOCS3 signalling pathway [47]

3. Metabolic disorders.

3. 1 Skeletal muscle

Skeletal muscle is the most abundant tissue that accounts more than 40% in the human body, and is involved in several physiological functions. Beyond aging, muscle wasting is a common feature of several pathological states and chronic diseases such as cancer, COPD, muscular dystrophies, AIDS, sepsis, and immune disorders [11]. Muscle depletion has important implications, such as exercise intolerance and inability of daily activity that eventually lead to poor quality of life. Most of the pathological conditions are associated with chronic inflammation that could play a relevant role in the onset of muscle wasting [22]. Indeed, inflammation is considered one of the diagnostic features of cachexia, a wasting condition that often occurs in chronic diseases [11].

Inflammation and skeletal muscle metabolic disorders have recently been established. This is supported by 1) increased inflammatory molecule levels, such as inducible nitric oxide synthase (iNOS), fibrinogen, and C-reactive protein (CRP) in the skeletal muscle-associated metabolic disorders and type 2 diabetes; 2) skeletal muscles generate and secrete several pro-inflammatory myokines, such as TNFα and IL-6; 3) skeletal muscles involve in innate immune system through cytokine receptors and TLRs [20].

3.1.1. Exercise induced muscle damage

When a muscle shortens, it involves a concentric action, whereas when it lengthens, it results in an eccentric action. At the same power, eccentric action causes much greater muscle damage than concentric exercise [51]. Symptoms include muscle delayed onset muscle soreness, structural damage, proteolysis and muscle wasting. It should be noted that eccentric exercise can cause not only muscle damage but activate inflammatory transcription factors and oxidative stress, decrease glucose control resulting glucose intolerance and insulin resistance [51]. Muscle can be affected by inflammatory cytokines from other tissues, such as liver and adipose tissue, even though it may not be a site of significant production of inflammatory mediators [41].

Intense exercise leads to muscle damage, followed by protein degradation, oxidative stress, and inflammation. Previous studies demonstrated that such muscle damage usually occurs after strenuous exercise due to eccentric action in the muscle [52,53]. Eccentric exercise increases the generation of reactive oxygen species (ROS), leading to a hypermetabolic state [13]. Free radicals are known to exacerbate muscle pathologies and cause secondary damage in the first 24 h after muscle injury [53]. Oxidative stress and inflammation are associated with skeletal muscle wasting in various disease states including cancer cachexia, muscular dystrophy, sepsis and diabetes [43]. ROS produced during exercise may increase the expression of mitogen-activated protein kinase (MAPK), the nuclear translocation of nuclear factor-κB (NF-κB), chemokines, and cytokines, leading to inflammation [52]. Soluble muscle enzymes, such as creatinine phosphokinase

(CPK), lactate dehydrogenase (LDH) are commonly tested in muscle damage markers [13]. The MAPK family is composed of four signalling modules in the skeletal muscle 1) ERK 1 and 2 (ERK 1/2); 2) p38 MAPK; 3) JNK; and 4) ERK5 or big MAPK. Apart from ERK5, all MAPK are known to be activated by exercise [54]. ROS activate MAPK signalling and the transcription factor nuclear factor-κB, thereby linking signal transduction to transcriptional processes. In this study, MAPK signalling was studied along with NF-κB pathway. Throughout unaccustomed exercise, one of the signalling pathways that could be activated by ROS is NF-κB [52]. NF-κB/Rel transcription factors in the cytoplasm bind to the subunit of the IκB protein [52].The subsequent phosphorylation of IκB initiates the dissociation and nuclear translocation of the active p50/p65 complex [13,52]. Many studies have demonstrated that an acute bout of exercise in animals increased NF-κB binding [13,52]. In the present study, NF-κB pathway was studied in both nucleus and cytosol of muscle protein. When muscles are damaged, muscle-release factors help promote muscle repair. The overlapping functions of chemokines involved in inflammatory responses have become a muscle damage evaluating marker [55]. Although the regulatory mechanism underlying the expression of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and monocyte chemotacticprotein-1 (MCP-1) is not clear, several studies have confirmed that redox-sensitive transcription factors, such as NF-κB, mainly regulate chemokine expression [55]. IL-6 and TNFα are one of the first cytokines secreted by pro-inflammatory macrophages at the site of injury [56]. The causative effect of IL-6 increase and muscle damage is still unclear, but IL-6 is used for muscle damaging and regeneration markers. Overall, the hypotheses of this study were: 1) intense

exercise would generate ROS and cause muscle damage, followed by inflammation and production of pro-inflammatory adipo-myokines, and 2) the antioxidant effect of capillarisin may have a protective effect against exercise-damaged muscle.

3.1.2. IL-6 induced myocyte dysfunction

Skeletal muscle tissue accounts for over 80% of insulin-mediated glucose uptake and fatty acid oxidation [57]. Skeletal muscle plays a fundamental role in glucose homeostasis through interactive cross-talk with hepatic and adipose tissue [23]. Therefore, the decreased glucose transport in muscle tissue may lead to insulin resistance [57,58]. Many studies have demonstrated insulin resistance in association with low-grade chronic inflammation [59,60]. In the state of chronic inflammation, macrophage infiltration alters metabolic properties of muscle cells that produce inflammatory cytokines such as IL-6 and TNFα [61]. A recent study described the dual effects of IL-6 on insulin action in the skeletal muscle [21]. Specifically, short-term treatment with IL-6, improved glucose control and insulin sensitivity, whereas long-term treatment with IL-6 contributed to glucose intolerance and insulin resistance [21,61]. It is generally accepted that depletion of IL-6 improves glucose regulation and obesity in mouse model and obesity-associated insulin resistance in type 2 diabetes in humans [25,62]. In human skeletal muscle, it has been reported that long-term exposure of IL-6 downregulates expression of STAT3 and SOCS3 proteins [23,62]. STAT3 is implicated in the development of IL-6-induced insulin resistance in cultured skeletal myotubes obtained from patients with impaired glucose tolerance [61]. SOCS3 is associated with the IL-6–STAT3 pathway in insulin signalling and has been reported to be increased in the skeletal muscle of severely obese or type 2 diabetes patients [48]. In addition, recent studies have suggested that SOCS3 blocks phosphorylation of IRS1 and downregulates PI3K complexes and phosphorylation of AKT [7,48]. Therefore, this study evaluated phosphorylation levels of AKT to validate glucose

control in association with SOC3 expression. Chronic inflammation partly impairs insulin action via TLR activation, specifically, TLR2 and TLR4 [59]. TLR4 is expressed in insulin target tissues, including the liver, adipose tissue, and skeletal muscle [59,60]. Thus, activation of TLR4 may directly exacerbate insulin sensitivity, through activation of inflammatory kinases [63]. Previous data suggest that TLR signalling may also link chronic inflammation to insulin resistance in the skeletal muscle [59,63,64]. Herein, this study hypothesized that increased levels of IL-6 may be associated with increased levels of TLR4. This study aimed to examine the mechanism underlying IL-6-induced inflammation and glucose intolerance in L6 skeletal muscle and protective effects of alantolactone by STAT3-TLR4 signalling inhibition.

3.2. Adipose tissues

Adipose tissue has been considered as energy storage organ and releases fatty acids in times of systemic energy need. However, over the last two decades adipose tissue has been established as a highly active endocrine organ that modulates energy expenditure and glucose homeostasis. In rodents, brown adipose tissue plays an essential role in thermogenesis and serves to protect against diet-induced obesity. White adipose tissue is responsible for the secretion of an array of signalling molecules, adipokines [24].

In rodents and humans, inflammation in adipose tissues is one mechanism to induce insulin resistance. Hyperlipidemia and hyperglycemia caused by excess nutrients, lipolysis, and gluconeogenesis induce mitochondrial dysfunction, ER stress and oxidative stress to stimulate stress responsive signalling molecules such as JNK and IKKβ. The phosphorylation of JNK and IKKβ signalling pathways increase inflammatory gene expression in target tissues amplifying systemic inflammation [35,65]. Saturated free fatty acid also binds to TLR4 to activate NF-κB and JNK and mediate inflammation and insulin resistance [66]. Furthermore inflammation in adipose tissue is mediated by pro-inflammatory adipokines such as IL-6, TNFα, and MCP-1.

For example, elevated levels of TNFα and IL-6 have all been reported in various diabetic and insulin-resistant states [26,67,68]. One theory is the expansion of adipose tissue leads to hypertrophic adipocytes leading to cell autonomous hypoxia with activation of cellular stress pathways. This causes cell inflammation and the release of pro-inflammatory adipokines. As part of the chronic inflammatory

process, locally secreted chemokines such as MCP-1 attract pro-inflammatory macrophages into the adipose tissue where they form crown-like structures around large dead or dying adipocytes. These tissue macrophages then release cytokines that further activate the inflammatory program in neighboring adipocytes, exacerbating inflammation and insulin resistance [67].

3.2.1. Palmitate-induced adipocyte dysfunction

Adipose tissue is an important endocrine organ, and it affects many metabolic disorders [69,70]. The activation of inflammatory pathways in the adipose tissue is a key mediator of obesity-associated insulin resistance [71]. Plasma free fatty acid (FFA) levels are elevated in obesity and diabetes in both humans and animals, wherein lowering of FFA levels leads to increased insulin sensitivity [72]. Dysregulation of lipolysis because of adipocyte dysfunction and increased levels of adipose inflammatory cytokines are the main causes of systemic insulin resistance [42]. Generally, the expansion of the adipose tissue in obesity increases macrophage infiltration, which create a crosstalk between inflammation and insulin resistance [69,72]. Macrophages can be classified into classically activated (M1) macrophages, predominant in obese individuals, or alternatively activated (M2) macrophages, predominant in lean individuals [69,73]. In progressive obesity, macrophages undergo a phenotypic switch from an anti-inflammatory M2 polarization state (lean) to a proinflammatory M1 polarization state (obese) [72]. At present, it is believed that M2 macrophages contribute to the maintenance of insulin sensitivity via secretion of interleukin (IL)-4 and IL-10, whereas obesity initiates M1 polarization, which induces insulin resistance via secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, and monocyte chemoattractant protein (MCP)-1 [73]. Because obesity-induced insulin resistance is characterized by macrophage infiltration and immune cell activation in the tissues, this study investigated the factors that regulate fat accumulation and the involved intracellular signalling cascades in an adipocyte-macrophage co-culture system [73]. Many studies have shown that excessive FFA consumption leads to

obesity-associated insulin resistance via toll-like receptor-4 (TLR4)-dependent pathway [66,74]. TLR4 is known to be involved in innate immunity, and act as an important mediator of insulin resistance and inflammation 9. The main two TLR4-dependent pathways, c-Jun Nterminal kinases (JNK) and IκB kinase β/α (IKKβ/α) are known to be involved in desensitizing insulin signalling [75-77]. JNK and IKKβ are activated resulting in the production of inflammatory cytokines via the transcription factors, activator protein (AP)-1 and NF-κB, respectively [75]. In the obese state, the enlarged adipocytes release MCP-1, which promotes the infiltration of monocytes that differentiate into adipose tissue macrophages (ATMs) [69,78]. ATMs secrete additional chemokines and cytokines, including MCP-1, IL-6, and TNFα [69,71,78]. Recent experimental evidence suggests that FFA-induced JNK-1 leads activation of insulin resistance through the inhibition of the insulin receptor substrate (IRS-1)/phosphoinositide (PI) 3kinase (PI3K)/protein kinase B (AKT); (IRS1-PI3K-AKT) pathway that regulates glucose control in the adipocytes. Herein, we investigated glucose uptake and fat accumulation caused by palmitate treatment, and alantolactone's protective effects against excessive FFA in adipocytes. Given that the adipose tissue is an important source of inflammatory mediators that induce glucose intolerance and insulin resistance, this research aimed to investigate the effects of alantolactone on palmitate-induced glucose intolerance and low-grade chronic inflammation, which is the main cause of insulin resistance and type 2 diabetes.

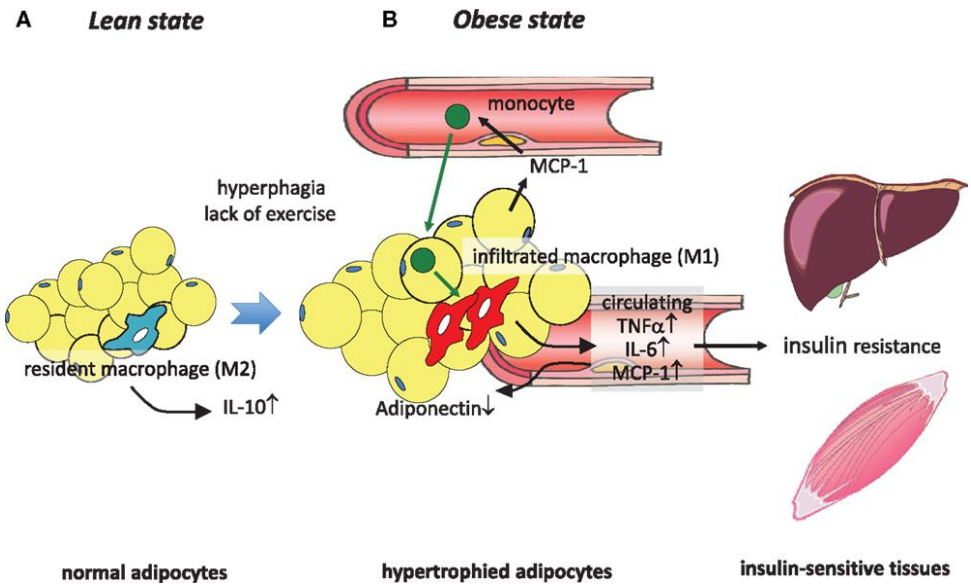


Figure 8 Progression of obesity [73]

4. Capillarisin from *Artemisia capillaries*

The aerial parts of Artemisia capillaris Thunberg (Compositae) has been widely used as a liver protective agent, diuretic, analgesic, lipid digestive agent, antimicrobial agent [79]. Capillarisin is a naturally occurring chromone isolated from the plant species *A.capillaris* and has been studied for anti-inflammatory [80], anti-hyperalgesic[81], anti-obesity [82], and liver protective effects [83].

As *A. capillaris* is known to exhibit diuretic activity, capillarisin was hypothesized to have vasodilating and muscle-relaxing abilities. Due to its anti-inflammatory and antioxidant effects and its potential as a muscle relaxant, capillarisin was further studied and applied for muscle damaging exercise. Eccentric exercise in mice was conducted to investigate ROS generation as mediators of skeletal muscle damage. If ROS were involved in muscle damage, capillarisin would be expected to exert an antioxidant effect to protect muscle from exercise-induce damage.

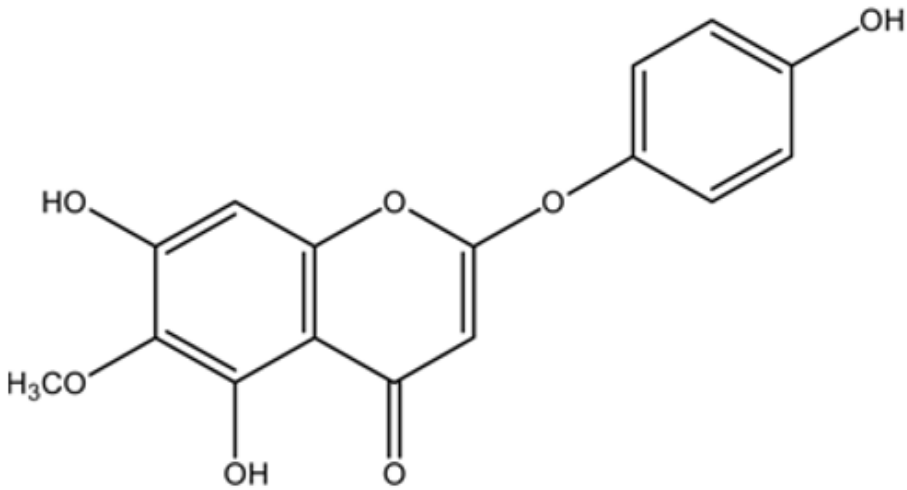


Figure 9 Structure of capillarisin

5. Alantolactone from *Inula helenium*

Inula helenium is a widely occurring perennial plant distributed in Asia and Europe. Traditionally, its roots have been used, and as agents of tuberculosis in China and native Americans, chronic enterogastritis and bronchitis and a preservative in China, and a diuretic expectorant agent in Europe [84]. Many sesquiterpenes were isolated from *I. helenium*. Alantolactone is a major constituent of *I. helenium* [85]. Alantolactone is a major sesquiterpene lactone of *I. helenium* and has recently been reported to have various pharmacological activities such as anti-inflammation [86], antifungal [87], induction of detoxifying enzymes [88], and apoptosis of hepatoma cells [89], colon cancer cells [90], glioblastoma cells [91], and doxorubicin-resistant cells [92].

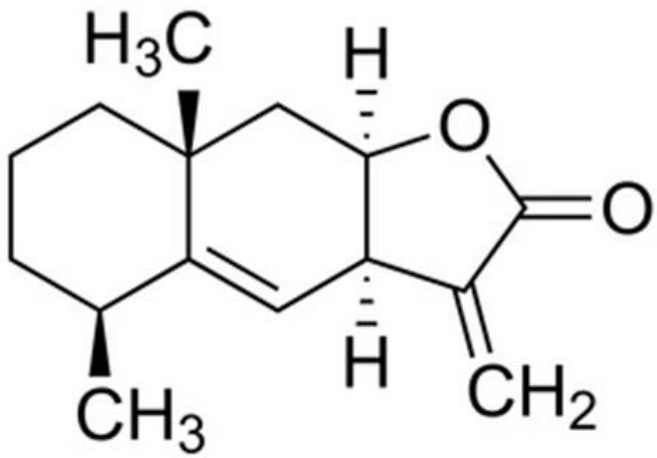


Figure 10 The chemical structure of alantolactone

II. STATE OF THE PROBLEM

Skeletal muscles and white adipose tissues are the largest endocrine organs in the human body. Both organs mutually secrete pro-inflammatory cytokines and chemokines, IL-6, TNFα and MCP-1 termed ‘adipo-myokines’ that cross-link between inflammation and metabolism. Few studies have focused on pro-inflammatory effects of adipo-myokines as recent studies focus mainly on the positive effects of adipokines and myokines. Therefore, this research aimed 1) to investigate the pathological effects of pro-inflammatory adipokines and myokines 2) crosstalk between adipo-myokine-associated inflammation and metabolic disorders in the skeletal muscle and adipose tissue 3) to examine pro-inflammatory adipokines and myokines regulating effects of capillarisin and alantolactone and underlying molecular mechanism.

Eccentric exercise may cause detrimental effects other than just muscle damage. The pre-liminary study of anti-inflammatory and anti-oxidative activities of capillarisin and alantolactone, was taken place in the plasma and skeletal muscle tissue of mice. Both capillarisin and alantolactone were previously studied in our lab; capillarisin for its anti-inflammatory, anti-hyperalgesic and anti-allodynic effects, and alantolactone for its anti-inflammatory and anti-cancer effects. Capillarisin was chosen for eccentric exercise study, due to its anti-oxidative effects, which was not shown in alantolactone from our preliminary study. This research aimed to investigate eccentric exercise increased inflammatory signalling pathway, MAPK and NF-κB, along with adipo-myokines, IL-6, TNFα and MCP-1, and anti-inflammatory effects of capillarisin against exercise-induced muscle damage. Muscle damaging factors in plasma, CPK and LDH from exercise were observed to confirm muscle damage produced by eccentric exercise. ROS studies,

DCFH-DA and TBARS assay were examined to observe ROS scavenging effects of capillarisin.

Mounting evidence suggest that chronically elevated IL-6 leads to metabolic dysfunction in the skeletal muscle. IL-6 activates transcriptional factor, STAT3 and downregulates SOCS3 genes which inhibits insulin receptor (IRS)-PI3K-AKT signalling that regulates glucose control. Alantolactone was hypothesized to present protective effects against prolonged IL-6 exposure, due to its STAT3 inhibiting factors reported in the previous study. This research aimed to investigate inflammation in association with metabolic disorder, by examining pro-inflammatory myokines, IL-6, TNFα and MCP-1. Alantolactone was hypothesized to have anti-inflammatory effects against prolonged IL-6 exposure by inhibiting TLR4 signalling and pro-inflammatory myokines. Alantolactone was also expected to suppress IL-6-induced glucose intolerance by inhibiting STAT3/SOC3 signalling pathway.

Adipose tissue macrophages (ATMs) together with other immune cells produce chemokines, inflammatory cytokines, and metabolites that induce amplified inflammatory process in obesity. In the adipose tissue, resident macrophages constantly release free fatty acids (FFAs) via lipolysis. FFAs activate ATMs, and cause activation of JNK and IKKβ inflammatory pathways, leading to cellular inflammation and insulin resistance. FFAs promote the expression of various pro-inflammatory adipokines, IL-6, TNFα and MCP-1. in part through interaction with the adipocyte TLR4 downstream pathway. Herein, ATM *in vitro* model was conducted by coculturing differentiated 3T3-L1 adipocytes and RAW 264.7 macrophages. This study aimed to observe palmitate-induced adipocyte metabolic

disorder, such as obesity and glucose intolerance and alantolactone's effect against it in both adipocyte only and adipocyte-macrophage coculture system.

It is now evident that pro-inflammatory adipo-myokines are important contributors to the etiology of inflammation and metabolic dysfunction. Overall, this study aimed to unravel pro-inflammatory adipokines and myokines involved signalling pathway in association with inflammation and metabolic disorder, and examine protective effects of capillarisin and alantolactone against these events.

III. RESULTS

1. Eccentric exercise: Protective effects of capillarisin against eccentric exercise-induced muscle damage

1.1. Reduction of ROS production and attenuation of peroxidative damage from eccentric exercise by capillarisin treatment in the skeletal muscle

The production of ROS in the isolated gastrocnemius muscle was measured with a DCFH probe. The level of ROS production was increased in the exercised group (peak 169 ± 1.24% of the control), but decreased in the capillarisin-treated groups (CAP 20 mg/kg 109 ± 4.67 and CAP 80 mg/kg 105 ± 7.28%, respectively, **P<0.01 of exercise group). This result indicates that capillarisin can reduce oxidative stress and protect skeletal muscle from exercise-induced muscle damage (Figure 11A). Thiobarbituric acid-reactive substances (TBARS) assay was also conducted to observe tissue oxidation as a part of ROS study. After the exercise, the level of tissue oxidation was increased (peak 1.30 ± 0.02 mU/mg protein), but attenuated by capillarisin treatment (CAP 20 mg/kg 0.85 ± 0.07 and CAP 80 mg/kg 0.70 ± 0.03 mU/mg protein, respectively, **P<0.01 of exercise group) (Fig 11B).

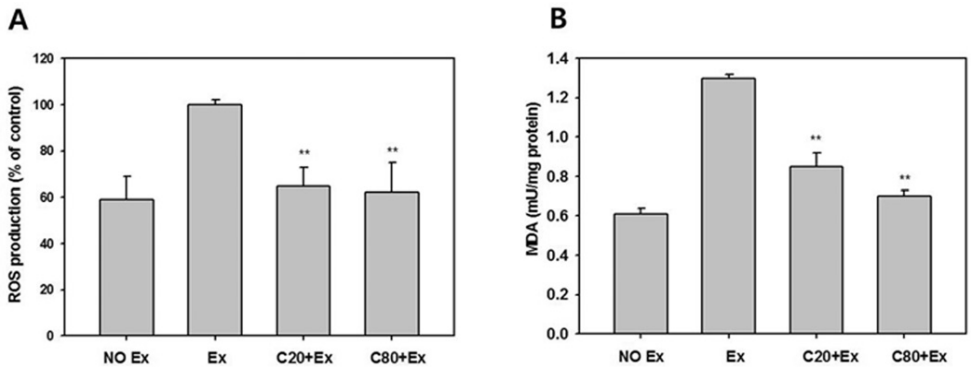


Figure 11 Antioxidative effects of capillarisin in the skeletal muscle

The antioxidant effect of CAP treatment after exercise. Values are mean ±SEM of data conducted three times. (A) The levels of ROS production evaluated by DCFH-DA assay. The one-way ANOVA test was used to compare activity in the C20 and C80 to that in the EX group (**$P < 0.01$). (B) The levels of lipid peroxidation were measured by TBARS assay. The one-way ANOVA was used to compare activity in the C20 and C80 to that in the Ex group (**$P < 0.01$).

1.2. Reduction of muscle damage markers from exercise by capillarisin treatment in the plasma

The damage markers, CPK and LDH were measured from the plasma at 24 h after the exercise. Intense exercise caused muscle damage by increasing the level of CPK, but decreased in the CAP 20 mg and CAP 80 mg groups, respectively (Figure 12A). The level of LDH also increased in the EX group but reduced to near control level in the CAP 20 mg and CAP 80 mg groups, respectively (Figure 12B).

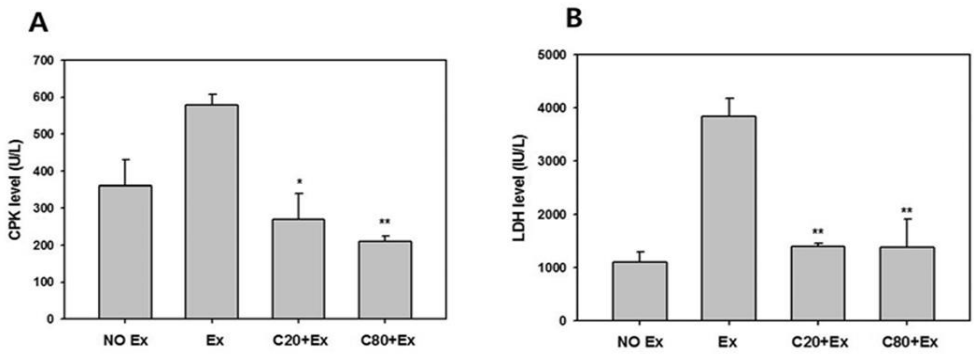


Figure 12 (A) CPK and (B) LDH plasma level

Values are mean ±SEM of six mice. The plasma levels of both CPK and LDH were examined. The one-way ANOVA and Fisher PLSD test was used to compare activity in the C20 and C80 to that in the EX group (**P* < 0.05, ***P* <0.01).

1.3. Improvement of muscle histological morphology by capillarisin treatment in the skeletal muscle

The gastrocnemius muscle morphology was observed and quantitative analysis was performed by using the Image J program. The number of centrally nucleated myofibers (CNFs) were prominent in the interstitial spaces of the gastrocnemius muscle at both 0 h and 24 h (Figure 13A(B and E)) compared to the rested control (Figure 13A(A)). It was clearly evident that the both capillarisin (CAP) 20 and 80 mg/kg-treated groups showed the attenuated structural damage, reduced central nucleation, and showed protected morphologies compared to the rested control (Figure 13A(D and G) versus Figure 13A(A)). From the quantitiative analysis, the number of CNFs peaked 24 h after the exercise but protected by capillarisin treatments.

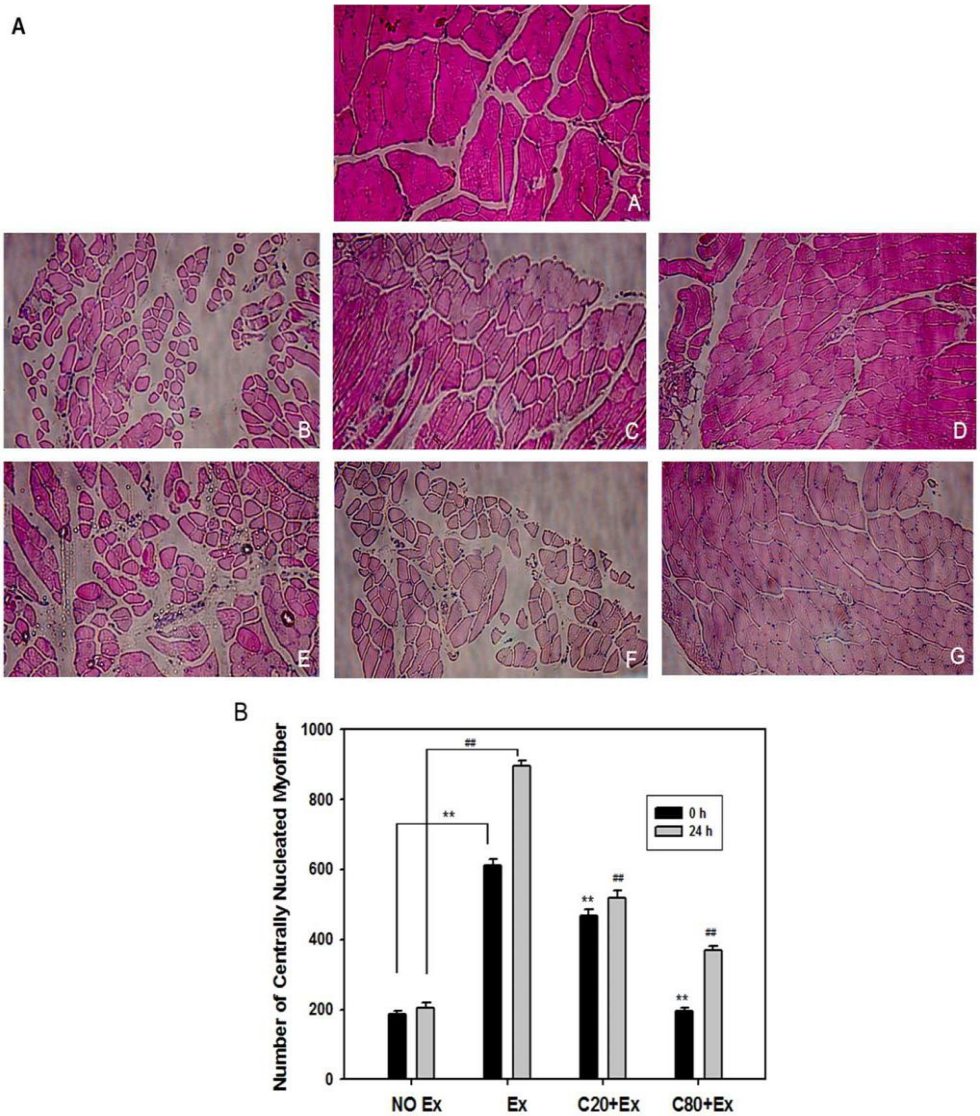


Fig 13 H&E-stained histology of gastrocnemius muscle

The rested, exercised and CAP treated groups (20 mg/kg, 80 mg/kg): 0 h (immediately) and 24 h after exercise (400x H&E). Values are mean ±SEM of six mice. Both CAP treated groups reduced significant number of degenerative fibers (peak 610±20, 895±15 reduced to CAP 20 mg/kg as 467±17.5, 520±20 and CAP 80 mg/kg as 196±9, 370±12.5, 0 h and 24 h respectively) The one-way ANOVA was used to compare C20 and C80 to that in the Ex group in both 0 h and 24 h (** and ## $P < 0.01$) and Ex group to CON group in both 0 h and 24 h (** and ## P <

0.01) A: Rested control; B: Exercised (0 h, immediately); C: C20+Ex (0 h); D: C80+Ex (0 h); E: Exercised (after 24 h); F: C20+Ex (24 h); G: C80+Ex (24 h)

1.4. Suppression of anti-inflammatory pathways, MAPK and NF-κB by capillarisin in the skeletal muscle

Muscle damaging exercise leads to inflammation. MAPK and NF-κB signalling pathway was observed in this study. The subfamily of MAPK, ERK, JNK, and p38 phosphorylation were measured. Exercise activated all sub-members of MAPK, but only ERK and JNK phosphorylations were suppressed by capillarisin treatment (Figure 14A). The transcription factor NF-κB factors, IκBα in cytosol, and p65 and p50 proteins in nucleus were also measured. Exercise activated cytosolic IκBα phosphorylation (Figure 14B), and activated p50 and p65 subunits (Figure 14C), but capillarisin treatment attenuated these increase in a dose-dependent manners. Overall, these results indicate that intense exercise triggered both MAPK and NF-κB pathways, but suppressed by capillarisin treatment. The quantification of western blot bands was performed by using image J program (**$P<0.01$ of exercise group).

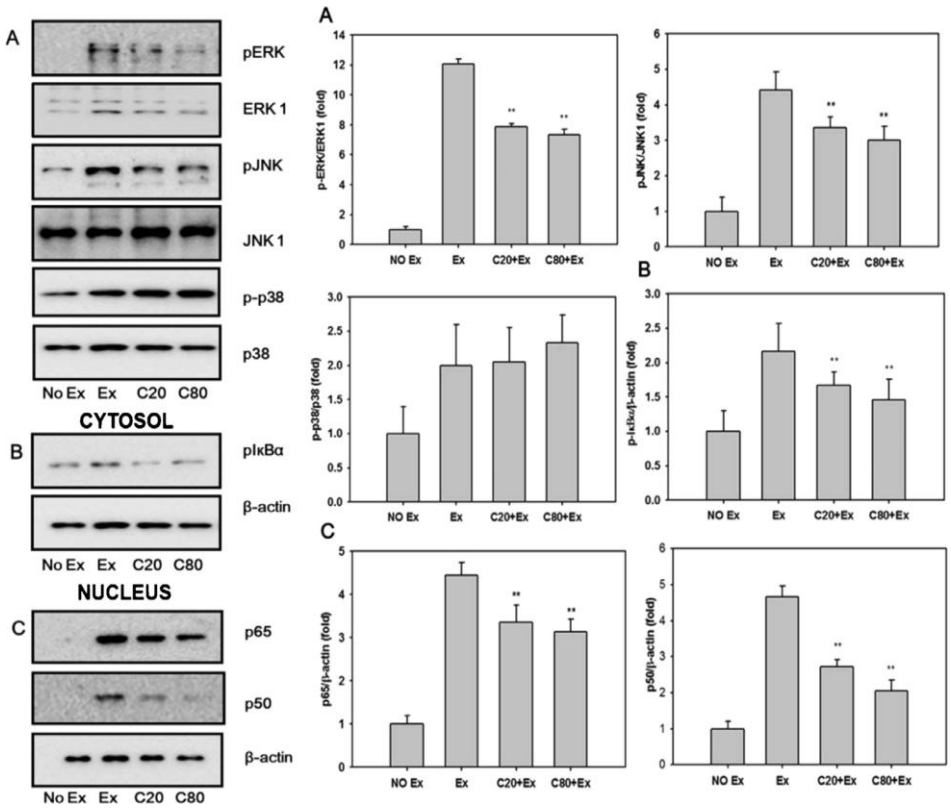


Figure 14 Western blot analysis of MAPK and NF-κB subunits

(A) MAPK subunits (pERK, ERK1, p-JNK, JNK, p-p38 and p38), (B) NF-κB (p-IκBα in cytosol) and (C) NF-κB (p50 and p65 in nucleus) (**$P < 0.01$ of exercise group). NO Ex: Rested control; Ex: Exercised; C20+Ex: Capillarisin 20 mg/kg and exercised; C80+Ex: Capillarisin 80 mg/kg and exercised

1.5. Suppression of pro-inflammatory chemokines and cytokines by capillarisin treatment in the skeletal muscle

Muscle pro-inflammatory chemokines and cytokines, MCP-1, CINC-1, IL-6 and TNFα expression were examined. All factors were increased by exercise. However, capillarisin administration suppressed these increase in a dose dependent manner. These results indicate that capillarisin's anti-inflammatory effects by inhibiting MCP-1, IL-6 and TNFα, and anti-oxidative effects by suppressing CINC-1. Therefore this data supports the hypothesis of capillarisin's protective effects against exercise-induced inflammation and oxidative stress.

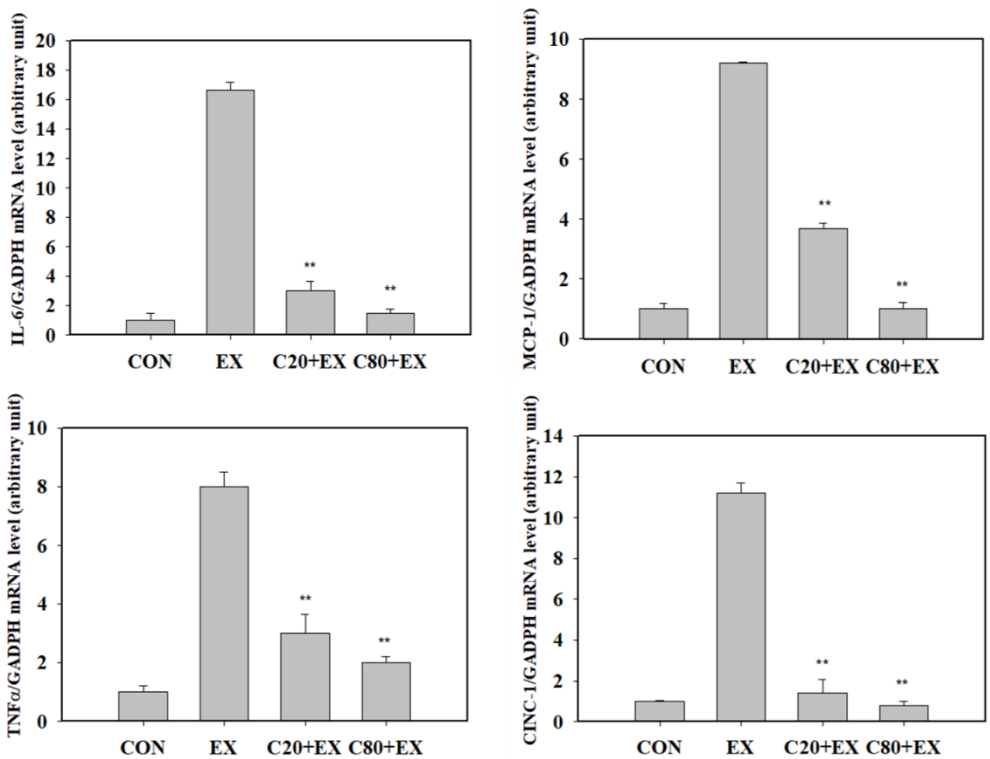


Figure 15 The levels of IL-6 MCP-1, TNFα and CINC-1 mRNA in the gastrocnemius muscle

(n=6, ***P* < 0.01, significant difference compared to Ex group by ANOVA test)

2. Cytokine IL-6: Protective effects of alantolactone against skeletal muscle dysfunction

2.1. Improvement of glucose uptake by alantolactone after prolonged exposure of IL-6 in the L6 skeletal muscle cell

A 2-NBDG glucose uptake assay was conducted to evaulate the difference between the treatment time, the acute (2 h) and chronic (24 h) IL-6 treatment on glucose homeostasis. Insulin-stimulated glucose uptake peaked at a concentration of 40 ng/ml after 2 h, whereas after 24 h of treatment, decreasing trend of IL-6 was observed from 40 ng/ml (Figures 16A,B). Therefore, 40 ng/ml IL-6 was chosen for the concentration inducing glucose intolerant state at 24 h. IL-6 treatment was further treated for 48 and 72 h for 40 ng/ml as to confirm glucose intolerance in the skeletal muscle cell. The level of glucose uptake was remain decreased 24 h onward (Figure 16C). Alantolactone was pre-treated for 4 h, and increased the suppressed glucose level by IL-6 stimulation, indicating its protective effect against chronic IL-6 treatment (Figure 16D). The level of glucose uptake was determined by using fluorescence microscopy (x200 original magnification) by using 2-NBDG (Figure 17). Stattic, a chemical STAT3 inhibitor (5 mM), was used as a positive control, first, as alantolactone was reported to be a strong STAT3 inhibitor, and second, the main mechanism in this study was hypothesized to involve STAT3 downstream. The fluorescent intensity was most significant in the control group, and diminished after IL-6 exposure (Figures 17A,B). These changes were significantly reversed by 0.5 mM alantolactone pre-treatment (Figure 17C), but no significance was observed with Stattic pretreatment (Figure 17D). Alantolactone improved glucose uptake after IL-6 exposure, measured by using a microplate

reader, but remained unchanged with Stattic pre-treatment, which corresponds to the previous result (Figure 17E). This is an important result to note that inhibiting STAT3 solely does not have an impact on glucose control, and other downstream factors must have involved to increase glucose uptake level. The quantification of the fluorescent cell counts were evaluated using the image J program. A similar trend with significant increase in alantolactone was observed in glucose uptake assay (Figure 17F).

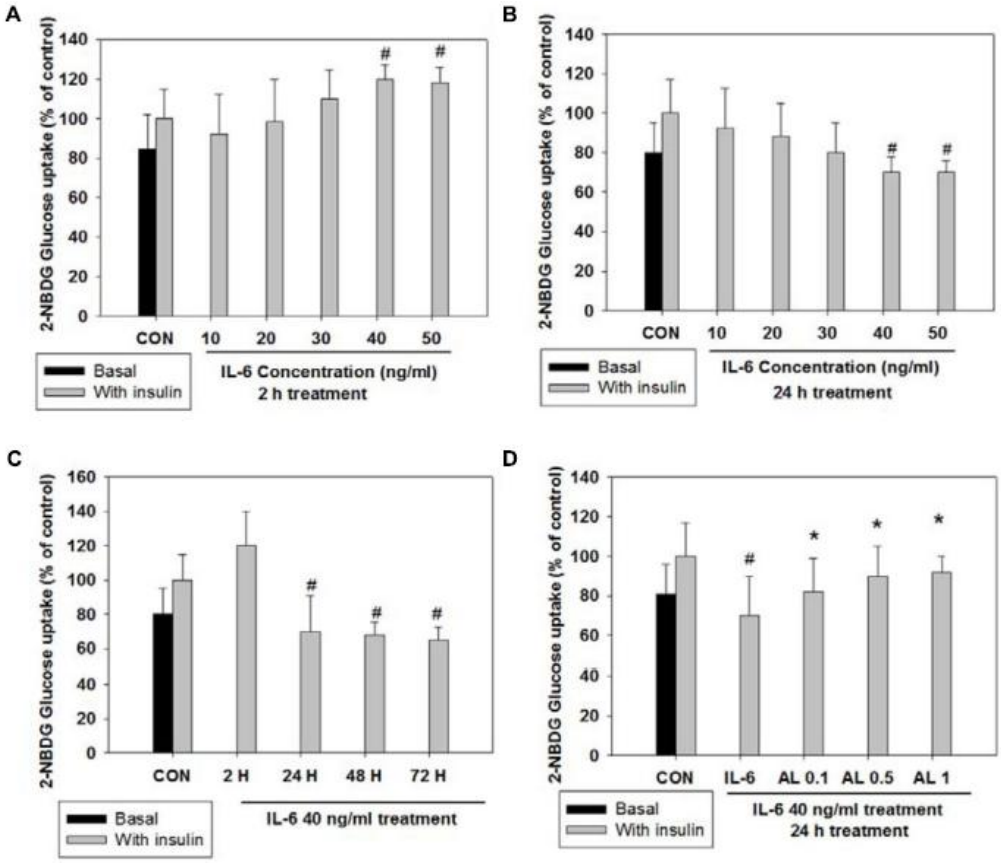


Figure 16 2-NBDG glucose uptake after acute (2 h) and chronic (24 h) IL-6-induced insulin-stimulated exposure in L6 skeletal muscle cells

Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3). (A) 2 h IL-6 treatment in a different concentration (10-50 ng/ml) (n=3, *, $p < 0.05$ vs. insulin-stimulated control). (B) 24 h IL-6 treatment in a different concentration (n=3, *, $p < 0.05$ vs. insulin-stimulated control). (C) IL-6 treatment (40 ng/ml) in different timeline (2, 24, 48, 72 h) with or without insulin 100 nM for 30 min (n=3, *, $p < 0.05$ vs. insulin-stimulated control, #, $p < 0.05$ vs. insulin-stimulated IL-6). (D) Effects of alantolactone on 2-NBDG glucose uptake after chronic (24 h) IL-6 exposure (n=3, #, $p < 0.05$ vs. insulin-stimulated control, *, $p < 0.05$ vs. insulin-stimulated IL-6).

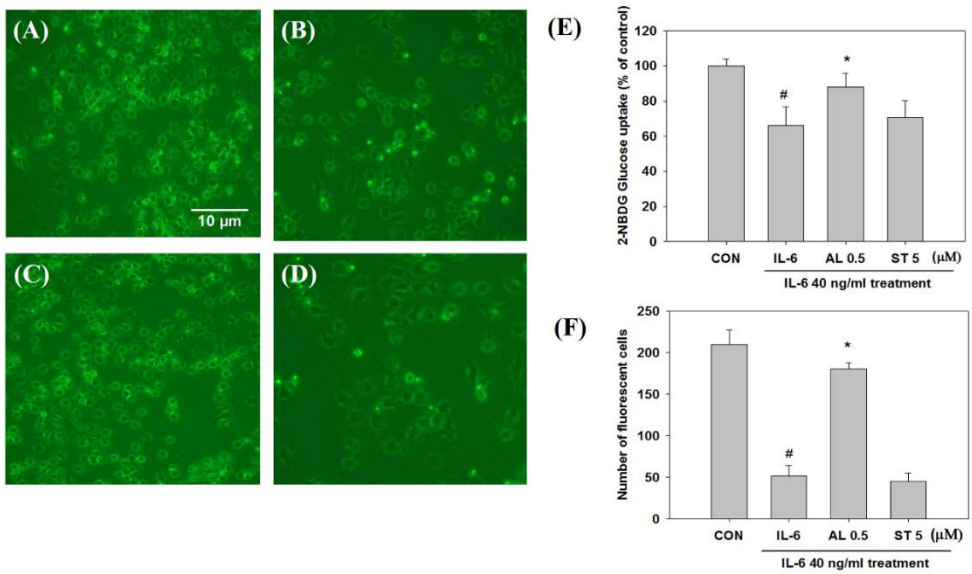


Figure 17 Glucose uptake observation by fluorescence microscopy after staining with 2-NBDG (x200 original magnification)

(A) CON+insulin (control with insulin treatment);); (B) IL-6+insulin (40 ng/ml IL-6 24 h exposure with insulin treatment) Scale bar: 10 μm; (C) AL 0.5+IL-6+insulin (0.5 μM alantolactone pretreatment for 4 h and IL-6 24 h exposure with insulin treatment).; (D) ST 5+IL-6+insulin (5 μM Stattic pre-treatment for 4 h and IL-6 24 h exposure with insulin treatment).; AL: alantolactone, ST: Stattic (E) Effects of alantolactone and Stattic on glucose uptake level after IL-6 exposure (n=3, #, $p < 0.05$ vs. insulin-stimulated control, *, $p < 0.05$ vs. insulin-stimulated IL-6). (F) Quantification of fluorescent cells after 2-NBDG glucose uptake

2.2. Suppression of STAT3/SOC3 pathway by alantolactone after IL-6-induced insulin-stimulated L6 cells

Previous result showed that only alantolactone had impact on glucose control. Therefore, further mechanism at protein level was examined by using western blots. Increased STAT3 phosphorylation after 24 h IL-6 exposure was suppressed by both alantolactone and Stattic pretreatment. Thus, the downstream of STAT3, SOCS3 was examined. SOCS3 was reported to play a critical role in the development of insulin resistance in type 2 diabetes. Expression of SOCS3 increased after IL-6 exposure, this increase was attenuated by alantolactone pretreatment (Figure 18A). Pretreatment with Stattic resulted in reduction of STAT3 phosphorylation as expected, but no change was observed in SOCS3 expression, indicating selective STAT3 inhibition of Stattic in the skeletal muscle. This result shows the importance of SOCS3 inhibition in the glucose control, and supports the idea that alantolactone may have protected 24 h IL-6 expousre by inhibiting STAT3/SOCS3 signalling.

2.3. Activation of IL-6-induced insulin-stimulated AKT phosphorylation by alantolactone

AKT is an important protein involved in glucose homeostasis, thus phosphorylation was evaluated to examine glucose uptake regulation. IL-6 suppressed AKT phosphorylation, but alantolactone reversed the suppression to the control level (Figure 18B). However, no significance was observed with Stattic treatment compared to IL-6- stimulated group, which corresponds to the previous glucose uptake result. Overall, alantolactone blocks STAT3/SOCS3 signalling and affects AKT phosphorylation in glucose control.

2.4. Suppression of IL-6-induced insulin-stimulated TLR4 gene expression by alantolactone

The activation of TLR4 may directly exacerbate insulin sensitivity, through activation of inflammatory kinases. Therefore, TLR signalling may link chronic inflammation to insulin resistance in the skeletal muscle. TLR4 expression was evaluated to observe impact of IL-6 on inflammation. IL-6-insulin stimulation increased the expression level of TLR4 gene. However, this increase was suppressed by both alantolactone and Stattic pretreatment (Figure 18C).

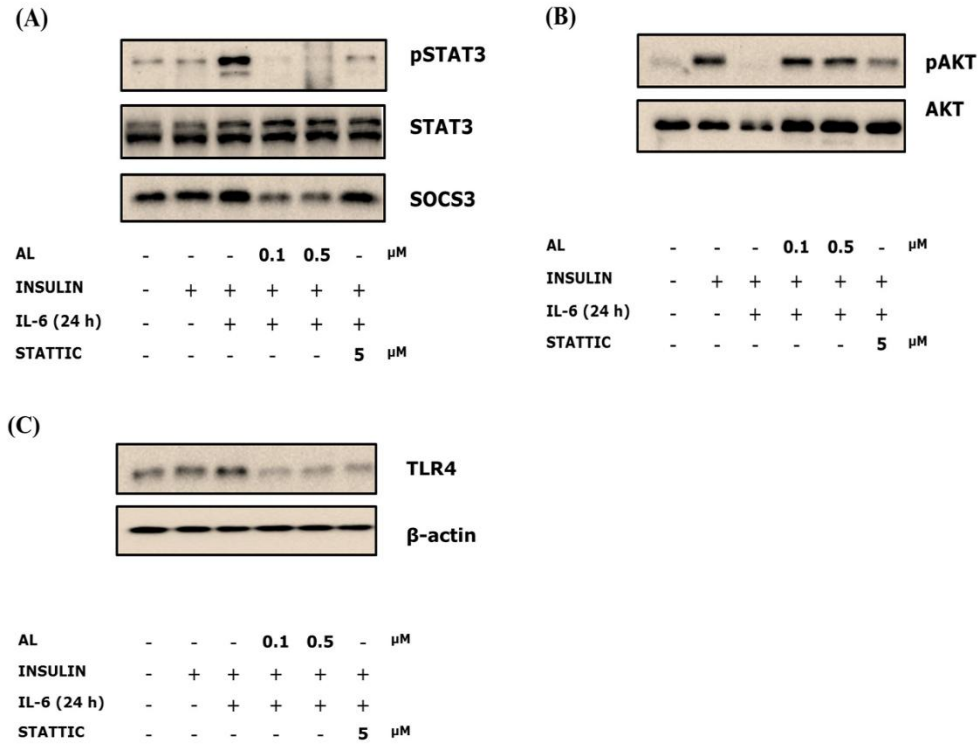


Figure 18 Western blots of alantolactone and Stattic in IL-6-induced insulin-stimulated L6 cells

All western blots were triplicated (A) Effects of alantolactone and Stattic on IL-6-induced insulin-stimulation on STAT3 phosphorylation and SOCS3 activation. (B) Effects of alantolactone and Stattic on IL-6-induced insulin-stimulated AKT phosphorylation. (C) Effects of alantolactone and Stattic on IL-6-induced insulin-stimulated TLR4 gene expression.

2.5. Improvement of glucose uptake by siRNA-based gene silencing of STAT3

To examine the impact of IL-6/STAT3/SOCS3 signalling on inflammation in association with glucose homeostasis, IL-6-induced insulin stimulated uptake of 2-NBDG in the skeletal muscle was observed after silencing STAT3 and TLR4. After 72 h of transfection with siRNA-STAT3, followed by 24 h of IL-6 treatment, the glucose level in the insulin-stimulated siRNA-STAT3 was reversed to that of the IL-6 non-treated scrambled siRNA group. Overall, siRNA-STAT3 showed a more improved glucose uptake. Therefore, it was chosen to study further signalling pathway (Figure 19A). After silencing of STAT3, alantolactone was treated before IL-6 treatment and showed improved glucose uptake level (Figure 19B).

2.6. Diminished TLR4 gene expression in myotubes treated with siRNA-STAT3

To evaluate IL-6-induced TLR4 gene expression via STAT3 signaling, STAT3 was silenced with or without alantolactone treatment. After siRNA-STAT3 transfection, alantolactone was pre-treated for 4 h, followed by IL-6 treatment for another 24 h. Expression of the TLR4 gene was suppressed with siRNA-STAT3. The similar trend was also confirmed after alantolactone pretreatment (Figures 19C,D).

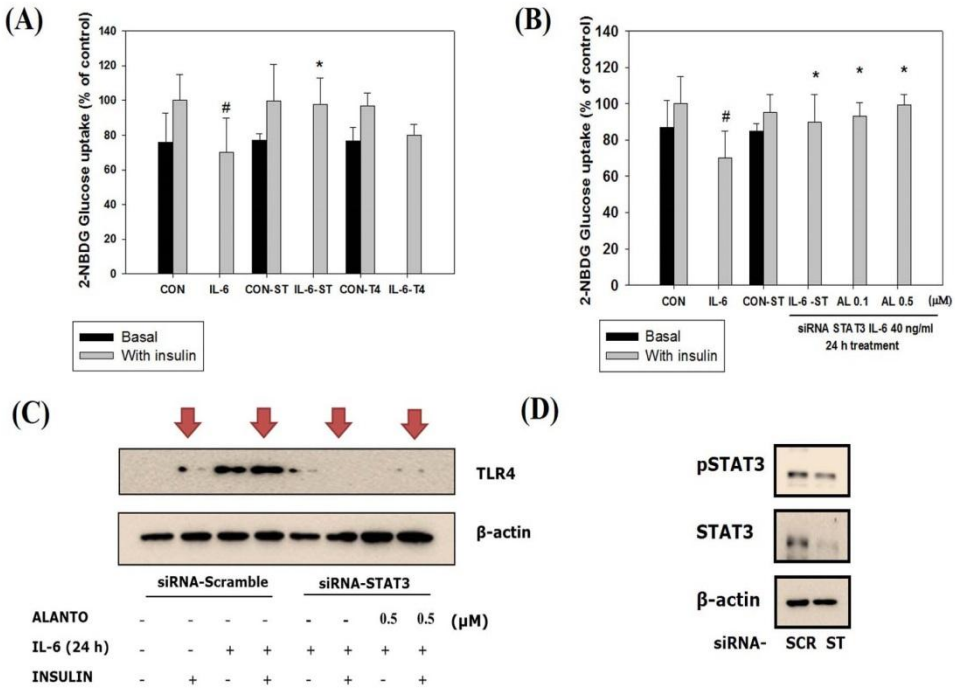


Figure 19 Silencing of STAT3

Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3). (A) L6 transfection with siRNA-STAT3 for 72 h followed by 40 ng/ml IL-6 for 24 h. n=3, *, $p < 0.05$ vs. insulin-stimulated IL-6-SC).; CON-SC (control-scrambled-siRNA); IL-6-SC (IL-6 treated-scrambled siRNA); CON-ST (control-siRNA STAT3); IL-6-ST (IL-6 treated-siRNA STAT3); CON-T4 (control-siRNA TLR4); IL-6-T4 (IL-6 treated-siRNA TLR4). (B) 2-NBDG glucose uptake of siRNA-STAT3 and siRNA-TLR4. SiRNA-STAT3 reversed IL-6 induced insulin-stimulated glucose uptake level to the control (n=3, *, $p < 0.05$ vs. insulin-stimulated IL-6-SC).; A; alantolactone. (C) Western blot of IL-6 induced insulin-stimulated scrambled-siRNA on TLR4 gene expression (n=3). (D) Western blot of scrambled-siRNA and siRNA-STAT3.

2.7. Suppressed myokines expression in myotubes

To examine, alantolactone's effect on IL-6-induced skeletal muscle inflammation, pro-inflammatory myokines were measured in mRNA level using qRT-PCR. Alantolactone suppressed IL-6 induced pro-inflammatory myokines, IL-6, TNFα, and MCP-1 in a dose dependent manner ($P<0.05$) (Figure 20).

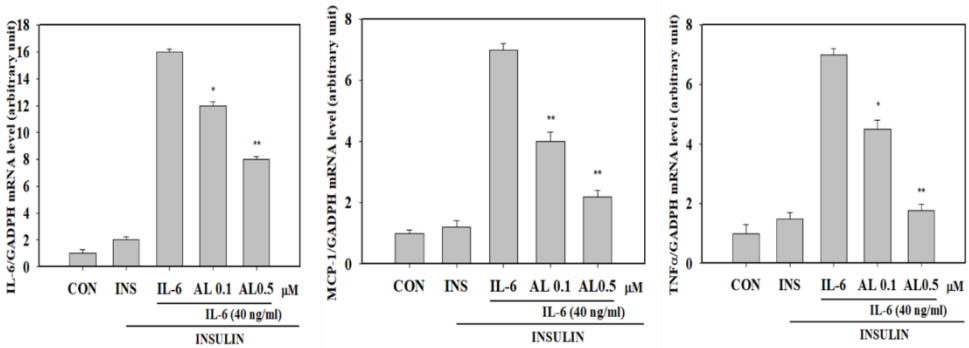


Figure 20 The levels of IL-6 MCP-1, and TNFα mRNA in the L6 skeletal muscle

(n=3, *P < 0.05 and **P < 0.01, significant difference compared to IL-6 group)

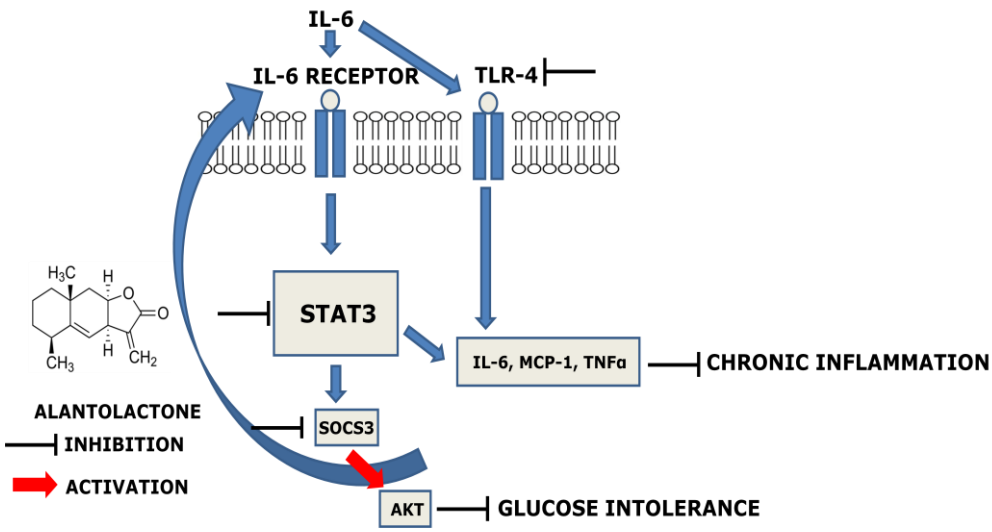


Figure 21 Alantolactone pathway scheme

Alantolactone suppresses IL-6-stimulated TLR4 expression via STAT3 phosphorylation and SOCS3 expression, which activates AKT phosphorylation.

3. Protective effect of alantolactone on adipocyte dysfunction

3.1. Improved palmitate-induced glucose intolerance by alantolactone in 3T3-L1 adipocytes

The effects of palmitate on glucose homeostasis in the differentiated 3T3-L1 adipocytes using 2-NBDG glucose uptake assay was evaluated. After 24 h of incubation, glucose uptake was significantly reduced from 0.5 mM palmitate onwards ($P < 0.05$) (Figure 22A). However, palmitate-induced glucose uptake reduction was retrieved by alantolactone pretreatment in a dose-dependent manner ($P < 0.05$) (Figure 22B).

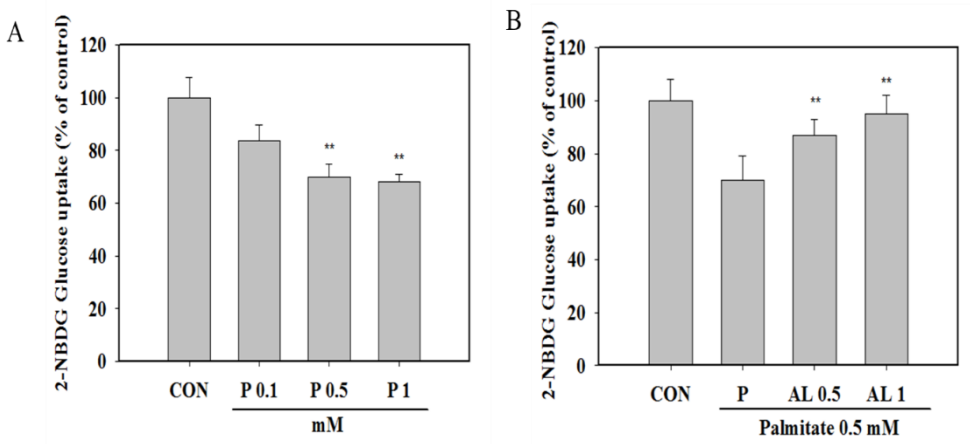


Figure 22 Glucose uptake in 3T3-L1 adipocytes

(A) 2-NBDG glucose uptake in 3T3-L1 adipocytes with the indicated concentrations by using a microplate reader at 540 nm. (B) 2-NBDG glucose uptake in AL-pretreated cells by using a microplate reader.

3.2. Improved palmitate-induced fat accumulation by alantolactone

Oil Red O staining was conducted to examine the level of fat accumulation by palmitate induction in 3T3-LI adipocytes. After 24 h of palmitate treatment, the level of lipid accumulation was increased 1.8 fold. However, alantolactone pretreatment significantly reduced the level of lipid accumulation in a dose dependent manner. This data suggests protective effects of alantolactone against lipid accumulation (Figure 23).

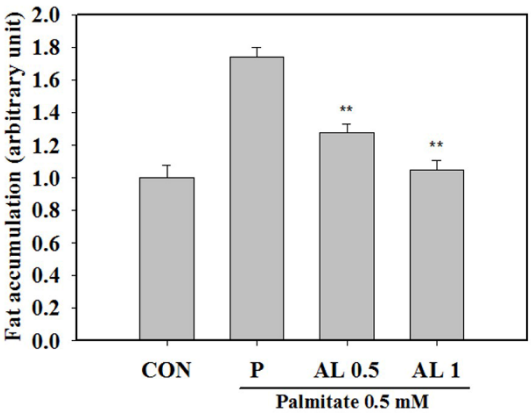


Figure 23 Fat accumulation in 3T3-L1 adipocytes

Fat accumulation examined *via* Oil Red O using a microplate reader.

(1) Control, (2) Palmitate 0.5 mM (3) AL 0.5 μM + palmitate, and (4) AL 1 μM + palmitate.

3.3. Inhibition of JNK activation in both 3T3-L1 adipocytes and co-culture system through inhibition of TLR4-JNK pathway

The molecular mechanism was examined in both palmitate-treated adipocytes and co-culture system. Western blotting was performed to investigate the inflammation associated signalling at protein level (Figs. 24A and 25B). MAPK- and NF-κB proteins, and upregulating factor, TLR4 are known inflammation associated genes. In both 3T3-L1 adipocytes and co-culture system, palmitate increased MAPK-associated proteins and TLR4 in a dose dependent manner. However, alantolactone significantly decreased palmitate-induced increase in p-JNK, p-ERK, and TLR4 gene expression in adipocytes, and p-JNK and TLR4 in co-culture system (Figure 24A, 25B). This result suggested that the main pathway of obesity associated inflammation involved JNK phosphorylation. All western blots were triplicated and quantified by using Image J program.

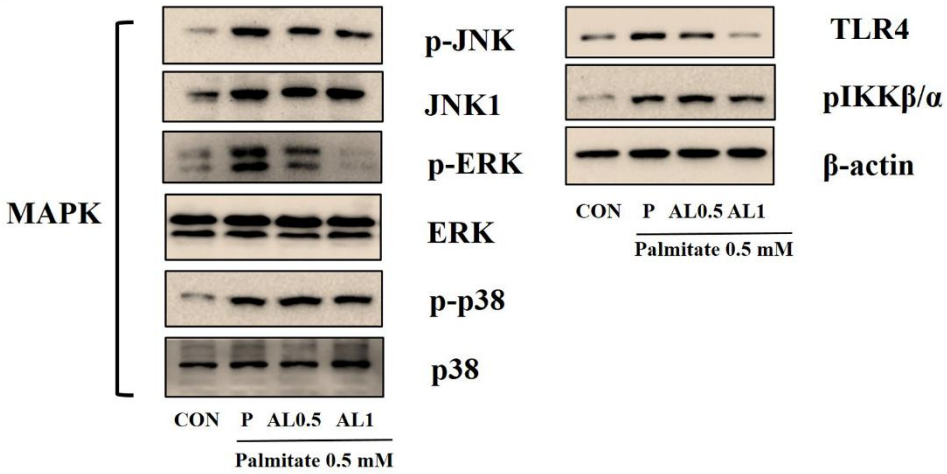
3.4. No significance with NF-κB pathway by alantolactone

NF-κB is one of the key inflammation factors and its sub protein, p-IKKβ/α, was activated by palmitate treatment in both 3T3-L1 adipocytes and co-culture system. The macrophage co-culture and palmitate increased the gene expression of p-IKKβ/α along with TLR4. However, alantolactone pretreatment did not affect NF-κB signalling but selectively inhibit JNK phosphorylation through TLR4 expression (Figures. 24A and 25B).

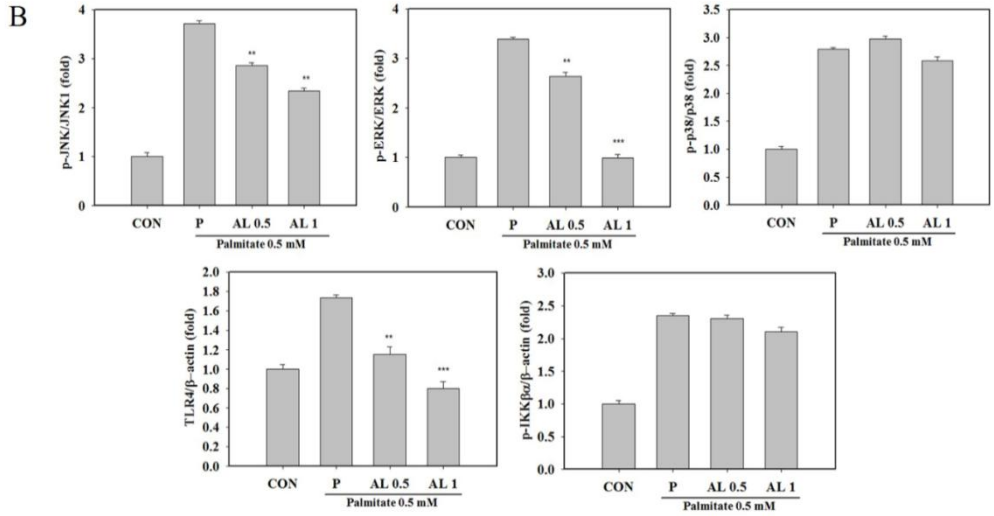
3.5. Reduction of pro-inflammatory cytokines and chemokines (IL-6 and MCP-1)

The pro-inflammatory cytokines and chemokine were investigated by using qRT-PCR (IL-6, TNFα, and MCP-1) (Figures 24B, 25D). Palmitate increased the mRNA level of the pro-inflammatory cytokines, IL-6 and TNFα, and chemokine, MCP-1. In both adipocytes and co-culture system, alantolactone inhibited the increase in IL-6 and MCP-1 levels in a dose dependent manner, but no significance was observed in TNFα levels (Figures 24B, 25D).

A


MAPK
p-JNK
JNK1
p-ERK
ERK
p-p38
p38
CON P AL0.5 AL1
Palmitate 0.5 mM
TLR4
pIKKβ/α
β-actin
CON P AL0.5 AL1
Palmitate 0.5 mM

B


p-JNK/JNK1 (fold)
p-ERK/ERK (fold)
p-p38/p38 (fold)
TLR4/β-actin (fold)
p-IKKβα/β-actin (fold)
CON P AL 0.5 AL 1
Palmitate 0.5 mM

C

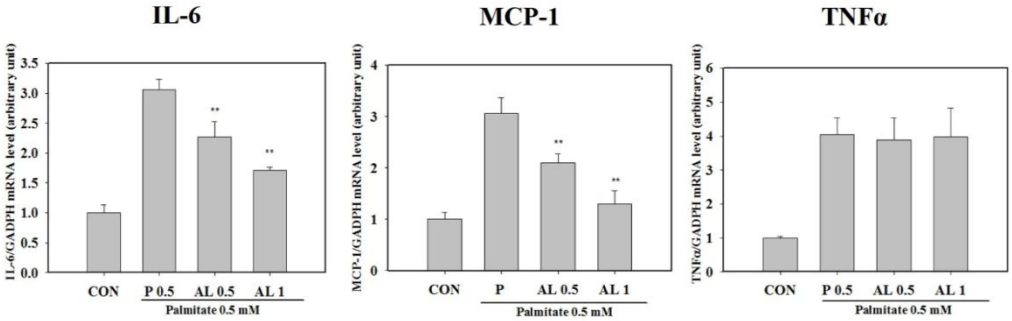
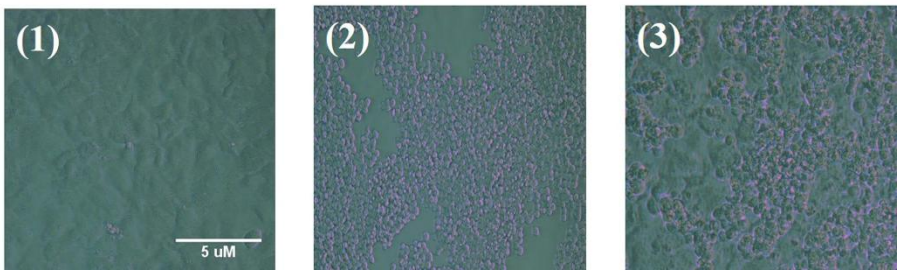

IL-6
MCP-1
TNFα
IL-6/GADPH mRNA level (arbitrary unit)
MCP-1/GADPH mRNA level (arbitrary unit)
TNFα/GADPH mRNA level (arbitrary unit)
CON P 0.5 AL 0.5 AL 1
Palmitate 0.5 mM

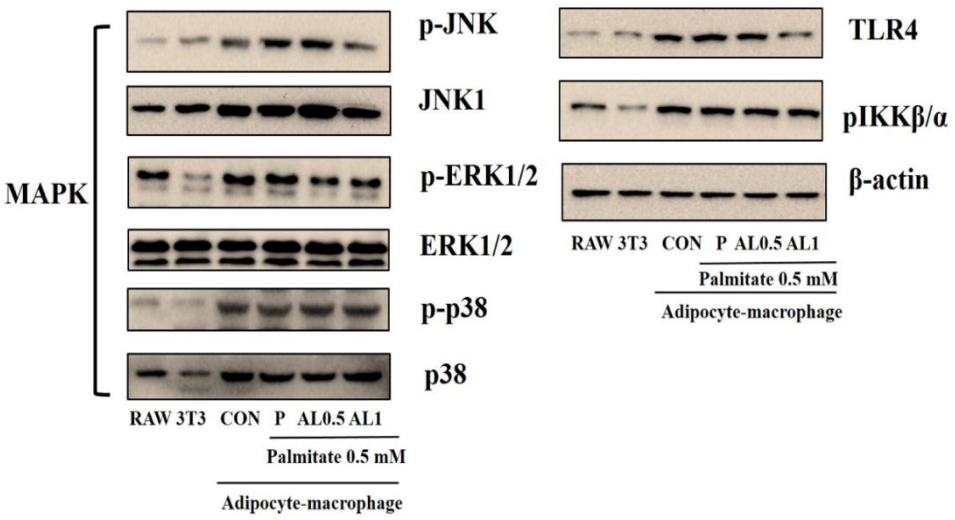
Figure 24 Inflammation associated proteins and RNA levels in 3T3-L1 adipocytes (lean state)

(A) Western blots of MAPK pathway-associated proteins, NF-κB, and its upregulating factor, TLR4 gene expression levels (B) Quantification of western blots (**$P < 0.05$, ***$P < 0.01$) (C) Cytokines IL-6, MCP-1, and TNFα mRNA expression levels evaluated by using real-time PCR. Asterisks indicate a significant difference (n=3) (**$P < 0.05$), compared to palmitate-treated cells.

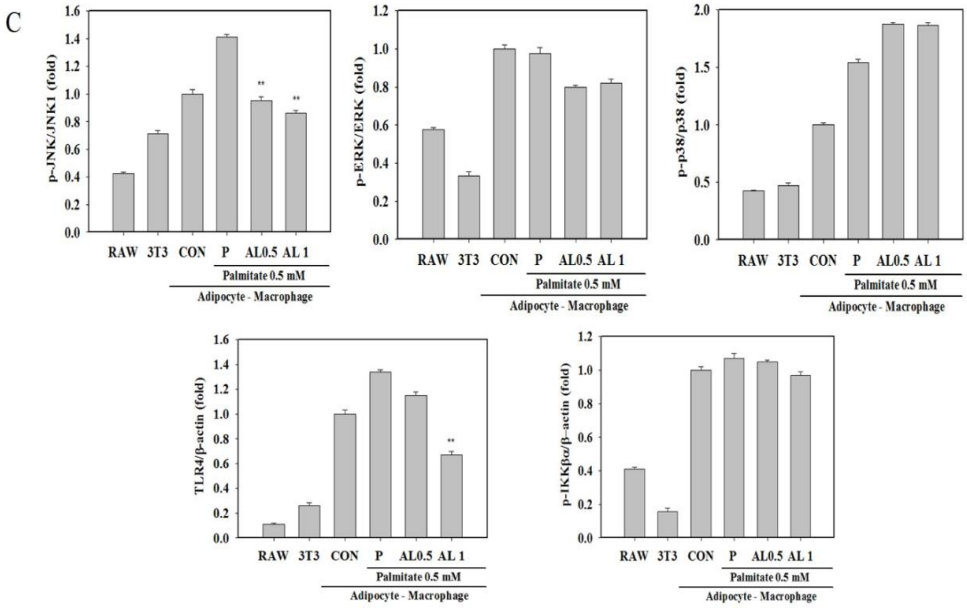
A


(1)
5 uM
(2)
(3)

B


MAPK
p-JNK
JNK1
p-ERK1/2
ERK1/2
p-p38
p38
RAW 3T3 CON P AL0.5 AL1
Palmitate 0.5 mM
Adipocyte-macrophage
TLR4
pIKKβ/α
β-actin
RAW 3T3 CON P AL0.5 AL1
Palmitate 0.5 mM
Adipocyte-macrophage

C


p-JNK/JNK1 (fold)
p-ERK/ERK (fold)
p-p38/p38 (fold)
TLR4/β-actin (fold)
p-IKKβα/β-actin (fold)
RAW 3T3 CON P AL0.5 AL 1
Palmitate 0.5 mM
Adipocyte - Macrophage

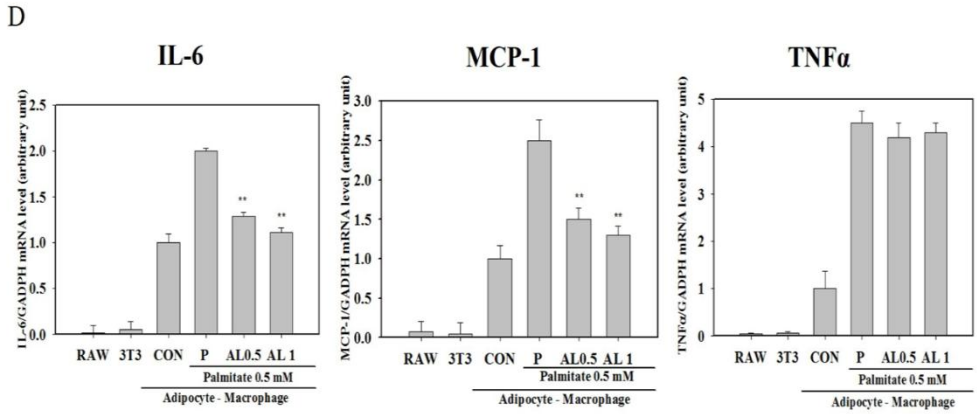


Figure 25 Inflammation associated protein and RNA levels in coculture system (obese state)

(A) Adipocyte-macrophage contact co-culture system (x200) (B) JNK, ERK and P38 phosphorylation levels, pIKKb/a,and upregulating factor TLR4 gene expressions were determined by Western blot analysis. (C) mRNA levels of IL-6, MCP-1, and TNFa were evaluated by real-time PCR. Asterisks indicate a significant difference (**P < 0.05).

IV. DISCUSSION

1. Exercise-induced muscle damage

This study investigated the protective effecst of capillarisin against eccentric exercise-induced inflammation and ROS in the skeletal muscle and subsequent damage in mice. Previous studies showed that capillarisin had anti-inflammatory and antihyperalgesic activities. [80,81,83] The goal of this study was to investigate the effects of capillarisin on two main inflammatory pathways, MAPK and NF-κB pathway, and suppression of exercise-induced inflammation, in association with adipo-myokines, IL-6, TNFα and MCP-1. Capillariisn administration suppressed the exercise induced muscle inflammation by inhibiting MAPK subgroups ERK 1/2 and JNK phosphorylation, but not p38 activation. This result implies the existence of other mechanisms involved in association with p38 phosphorylation. Possible pathways could be the TRX and ASK1 signalling that regulate p38-MAPK, although this hypothesis remains unclear [83]. The MAPKs mediate inflammatory and mitogenic signals to activate transcription factors, particularly NF-κB [80]. NF-κB is a dimeric transcription factor, which is activated by a variety of external stimulants, such as H_2O_2, pro-inflammatory cytokines, and LPS. Many studies have reported that the NF-κB pathway regulated exercise-induced inflammation [52,54]. A previous study demonstrated that capillarisin inhibited the NF-κB pathway in RAW 264.7 cells, which corresponds to the result of this study in the skeletal muscle [80]. Capillarisin treated exercise groups showed the lowering of NF-κB translocation in the muscle nuclear extraction p65 and p50, as well as reduced the level of exercise-induced IκBα phosphorylation. This result suggests that capillarisin downregulated the inflammation associated NF-κB signalling pathway. Although this study did not demonstrate MAPK- NF-κB

pathway stream in order, but mounting evidence suggests that MAPKs are the upstream of NF-κB pathway in the rat skeletal muscle [52]. In addition, MAPK and NF-κB signalling pathways are critical factors in mediating gene expression of MnSOD and iNOS in response to exercise stress [54].

In the course of an inflammation, immune cells such as neutrophils and macrophages arrive at an injured site. Monocyte infiltration and macrophage conversion are necessary to induce inflammation and activate the surrounding cells to support muscle regeneration [93]. The most studied chemokine released by injured muscle is the chemokine and adipo-myokine MCP-1, which has an important function in initiating and maintaining inflammation [30]. The muscle-damaging exercise resulted increased level of MCP-1, but attenuated by capillarisin treatment. The secretion of the pro-inflammatory myokines IL-6 and TNFα are associated with the immune responses to tissue damage [56]. When pro-inflammatory monocytes/macrophages are infiltrated in the junction of injured muscle tissues, a high level of cytokine IL-6 is produced. The secreted IL-6 activates the gp130/STAT3 pathway in macrophages, which is also essential for the production of the chemokines MCP-1 and CINC-1 [93]. In the present study, mRNA level of IL-6 in the gastrocnemius muscles after exercise. IL-6 was significantly increased in the exercised group, but attenuated by capillarisin administration. This result supports the idea that capillarisin inhibits the secretion of IL-6 and the STAT3 pathway, thus decreasing the level of MCP-1 and the production of CINC-1. Pro-inflammatory adipo-myokine TNFα was also increased by eccentric exercise, indicating muscle inflammation, but suppressed by capillarisin treatment, supporting the pro-inflammatory effects of capillarisin.

The muscle damage markers CPK and LDH were also evaluated as systemic indicators of muscle pathology. The administration of capillarisin attenuated the exercise-induced CPK and LDH level, in agreement with previous findings [13,52]. Based on the histological analysis, the muscle damage was most prominent at 24h after the exercise showing increased number of degenerative fibers within These results suggest that capillarisin treatment has a protective effect against exercise-induced muscle damage

During intense exercise, ROS are produced within myofibers. The redox sensitive signalling pathways use ROS to transport signals in the skeletal muscle to stimulate apoptosis [52]. In this study, three parameters were adopted to assess ROS production after eccentric exercise. First, DCFH-DA assay was conducted to measure intracellular ROS levels, second, TBARS to observe lipid peroxidation, and third, CINC-1, a redox-sensitive chemokine was measured at mRNA level. To investigate the impact of capillarisin on ROS scavenging after exercise, the level of DCF was measured in isolated gastrocnemius muscles. The increased level of ROS by eccentric exercise was attenuated by capillarisin treatments in a dose dependent manner. In addition, TBARS assay was conducted to observe the level of lipid peroxidation as an index of oxidative damage. The level of lipid peroxidation was increased by the muscle damaging exercise, but reversed by capilarisin treatment, indicating that capillarisin exerted antioxidative effect and exhibited protective effects in the skeletal muscle. This result is also supported by other research group's DCFH-DA result in ROS generating intense exercise [52]. It is likely that MAPK and NF-κB are coordinately activated in response to exercise-induced ROS as redox-sensitive signalling [13]. Therefore, as an antioxidant, capillarisin could

have removed ROS that may have activated MAPK and NF-κB pathway in the skeletal muscle. Although, the regulatory mechanism involved has not been fully determined, the decrease of CINC-1 level would suggest less intracellular oxidation in muscle tissue due to elevation of antioxidant activity. CINC-1 is known to be a redox-sensitive chemokine and a damage marker in association with ROS and inflammation [13]. These findings indicate that capillarisin has protective effects against exercise-induced muscle damage by diminishing the redox-sensitive inflammatory cascade by ROS scavenging. Taken together, this study provided strong evidence that capillarisin exerted a protective effect against exercise-induced skeletal muscle damage and ROS production by regulating pro-inflammatory myokines via MAPK and NF-κB pathway in mice.

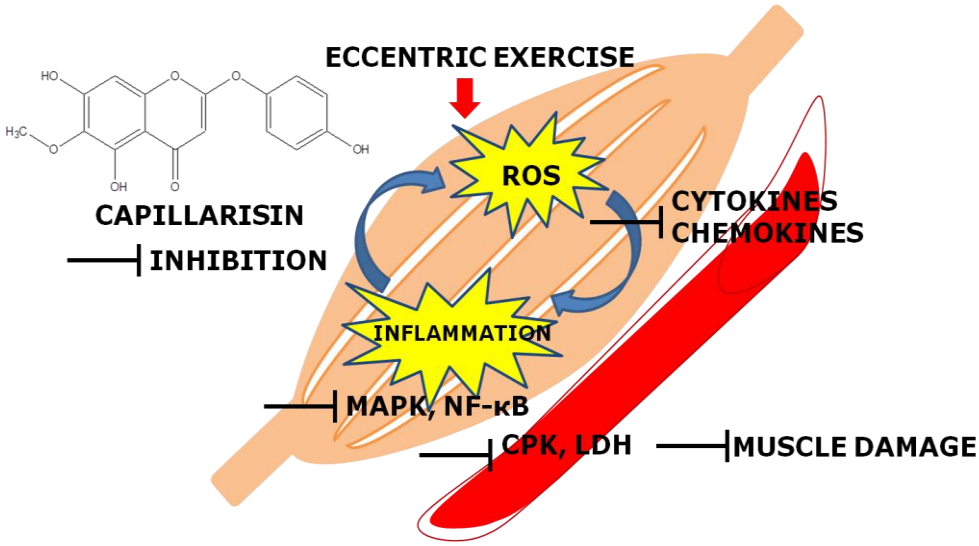


Figure 26 Exercise-induced muscle damage

2. IL-6 induced myocyte dysfunction

Chronically elevated IL-6 levels in the body are generally accepted as a negative regulation in the metabolism [61]. IL-6 level was reported to be elevated more than 25 times compared to that in the normal individuals [68]. The IL-6 signalling downstream STAT3 and SOC3, are reported to be associated with IL-6-induced glucose intolerance in the liver and muscle [61]. This study focused on the glucose intolerance induced by prolonged (24 h) exposure of IL-6. IL-6 activated STAT3/SOCS3 pathway and inhibits glucose regulating AKT phosphorylation. IL-6 also mediated TLR4 gene expression in association with inflammation in the skeletal muscle via STAT3/SOC3. Pro-inflammatory myokines, IL-6, TNFα and MCP-1 were also increased supporting muscle inflammation together with metabolic dysfunction. The main finding of this study is the protective effects of alantolactone against chronically elevated IL-6 levels in the L6 myocytes. Alantolactone was previously reported to have STAT3 inhibiting factor in cancer cells, thus expected to have positive effects on IL-6-induced signalling pathway [94].

Recent studies in the liver and skeletal muscle observed SOCS3 over-expression, by blocking IRS1, PI3K, and AKT phosphorylation (IRS-1–PI3K–AKT pathway) [48,61]. One study observed genetically deleted SOCS3 from the skeletal muscle in obese mouse, enhanced glucose control [48]. This result supports our data that chronic IL-6 exposure activated SOCS3 expression. Both alantolactone and Stattic suppressed STAT3 phosphorylation, but in case of SOC3, only alantolactone pretreatment suppressed SOCS3 and activated AKT phosphorylation, which is

associated with glucose regulation. This result indicates that alantolactone possesses positive effects in improving glucose uptake through SOCS3-AKT signalling. Interestingly, AKT phosphorylation was not changed by Stattic pre-treatment, indicating that Stattic selectively inhibits STAT3 phosphorylation and does not affect glucose regulation. This result supports SOCS3 involvement in insulin signalling, which results in improving glucose intolerance in the skeletal muscle.

Prolonged IL-6 exposure increased TLR4 gene expression. SiRNA was conduncted to determine the mechanisms and the crosstalk between IL-6 and TLR4 expression via STAT3 in the development inflammation and glucose intolerance in the skeletal muscle. After STAT3 silencing, the level of TLR4 gene expression was suppressed, supporting STAT3 as a mediator between IL-6 and TLR4. Previous reports suggested that obese and type 2 diabetes patients have increased the level of TLR4 expression and involvement of NF-κB in human myotubes and p-STAT3 protein activation [64]. Both alantolactone and Stattic attenuated TLR4 expression, indicating the protective effect of alantolactone against muscle inflammation mediated through the IL-6–STAT3–TLR4 pathway.

Accumulating clinical evidence suggest that monocytes/macrophages play a critical role in the pathogenesis of insulin resistance by infiltrating insulin target tissues [60,64]. Cytokines such as TNFα and IL-6 secreted by multiple tissues, are recognized as the inflammatory mediators that cause insulin resistance by reducing the expression of glucose transporter4 (GLUT4) and IRS-1 [60]. These effects are reported to exert JAK–STAT signalling pathway activation followed by SOCS3 expression [60]. IL-6 is also reported to induce insulin resistance by blocking PI3K

and AKT pathway and impair glycogen synthesis by downregulating microRNA200s and upregulating friend of GATA 2 (FOG-2) [60]. Recent study reported alantolactone's inhibitory effect on inducible and constitutively activated STAT3, nuclear translocation suppression, and the DNA binding activity of STAT3 in vitro [94]. This result supports the hypothesis that the anti-inflammatory effect of alantolactone may have suppressed chronic inflammation by inhibiting STAT3 activation, followed by TLR4 expression induced by IL-6 exposure.

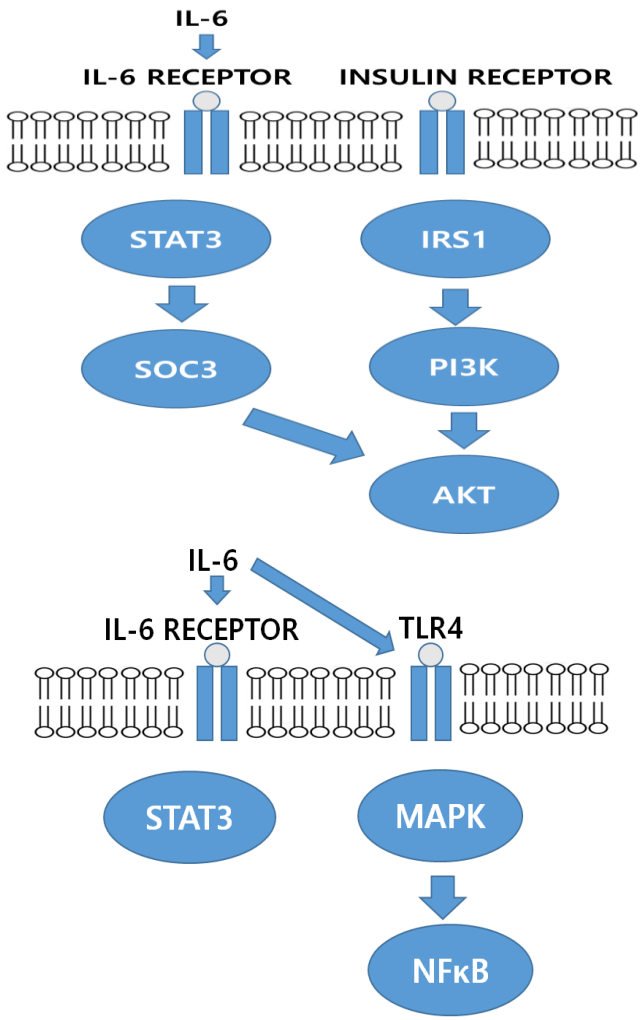


Figure 27 STAT3/SOCS3 signalling pathway and IL-6 induced TLR4 expression

3. Palmitate-induced adipocyte dysfunction

Obesity is one of the inflammation associated metabolic disorders contributing to insulin resistance [66,78]. However, the mechanisms involved for obesity associated inflammation and insulin resistance are poorly understood [70]. This study aimed to investigate protective and preventive effects of alantolactone against palmitate-induced inflammation and metabolic dysfunction in vitro. The adipocytes and adipocyte-macrophage co-culture system were applied in this study. In the obese state, macrophage infiltration initiates proportional to the fat mass, and resident macrophages change from the anti-inflammatory M2 to the pro-inflammatory M1 type [69,78,95]. M1 infiltration induces inflammation and insulin resistance by producing pro-inflammatory cytokines, such as IL-6 and TNFα [78,96]. This study hypothesized that adipocytes represented as 'lean state', and the macrophage co-culture system as 'obese state'. High levels of free fatty acid (FFA) in obesity have been implicated in obesity and insulin resistance [66,97,98]. FFAs are known to inhibit glucose production and cause glucose intolerance in both skeletal muscles and adipose tissue [99]. However, pro-inflammatory signalling activated by FFAs in adipocytes and other tissues remains uncertain. In the present study, the impact of palmitate in the lean (adipocytes) and obese (adipocyte-macrophage co-culture system) states in vitro was examined. The aim of this study was to elucidate the link between adipocyte dysfunction and inflammation leading to insulin resistance. Palmitate treatment resulted in glucose intolerance and increased fat accumulation in the lean state, followed by elevated pro-inflammatory adipokines. Activation of inflammation-associated pathways in both the lean and obese states in vitro was also observed. However, alantolactone pretreatment

prevented these changes. Adipokines, such as IL-6, MCP-1, and TNFα, play a major role in obesity-induced inflammation [72]. In this study, palmitate treatment increased the level of adipokines in both the lean and obese states in vitro. Alantolactone pretreatment significantly decreased the levels of IL-6 and MCP-1; however, it did not affect TNFα expression. The expression of IL-6 in the adipose tissue and plasma is related to the body mass index (BMI) and upregulated by insulin; thus, IL-6 may result in dysregulation of lipid metabolism in the adipocytes via inhibition of adipogenesis [78,100]. MCP-1 is involved in macrophage M1 polarization in adipose tissue, which increases the number of macrophages, promoting an inflammatory response in diet-induced obese mice [30]. These results suggested that alantolactone might prevent the dysregulation of lipid metabolism and inflammation via reduction of IL-6 levels, and regulate macrophage infiltration by reduction of MCP-1 concentrations.

Elevated FFAs lead to increased lipid accumulation in the peripheral tissues. Saturated fatty acids may act as a ligand of the innate immune receptors, such as TLR4 [66,78]. TLR4 is a key receptor for LPS that plays a critical role in the immune system [59,66]. It was reported that a major component of LPS lipid, lauric acid, as well as. other saturated fatty acids, such as palmitate and oleate, activate TLR4 signalling in vitro [63]. In line with this, others observed that TLR4−/− mice with lipid infusion maintained normal glucose homeostasis, whereas TLR4−/− high-fat diet (HFD)-fed mice exhibited insulin resistance and white adipose tissue inflammation [66,101]. In this regard, FFAs can act through TLR4 to induce chronic inflammation and suppress insulin signalling [59,66]. Thus, TLR4 can be an important mediator for endogenous lipids that may contribute

glucose intolerance and insulin resistance. In agreement with previous findings, this study observed palmitate-induced TLR4 expression, and inhibition by alantolactone pretreatment in both adipocytes and adipocytes-macrophages co-culture system. This result indicates that alantolactone suppresses palmitate-induced inflammation through TLR4 signalling and thus suppresses insulin signalling. TLR4 expression has been proposed to involved in JNK and IKKβ signalling; thus, we evaluated the activation of MAPK and NF-κB pathways [76]. JNK and the IKK complex play a central role in inflammation and innate immunity. Palmitate and macrophage co-culture increased JNK and IKKβ phosphorylation; however, alantolactone selectively attenuated the increase in JNK and other MAPK proteins. It is well accepted that p-JNK activates the oncoprotein, c-Jun, which forms a part of AP-1 transcription factor, thereby resulting in glucose intolerance and inflammation [71,76]. In this study, we suggested that the anti-inflammatory effects of alantolactone in palmitate-treated adipocytes and macrophage coculture were dependent, in part, on JNK and AP-1, particularly regarding IL-6 and MCP-1 expression. Although both JNK and NF-κB are involved in TNFα expression, it was suggested that NF-κB is a more potent and independent regulator of TNFα expression [71,74]. Alantolactone pretreatment did not significantly alter TNFα expression, possibly because of its minimal effect on NF-κB [71]. Recent experimental evidence suggests that endoplasmic reticulum (ER) stress and mitochondrial dysfunction caused by FFA has a critical impact on insulin resistance [3]. ER stress activates JNK and IKKβ kinases implicated in obesity and insulin resistance through the inhibition of glucose regulating pathway IRS1-PI3K-AKT in adipocytes[102]. Consistent with these results, this study results observed

palmitate-induced adipocyte dysfunctional features, such as glucose intolerance, fat accumulation, and increased adipo-myokines. However, alantolactone attenuated these adverse changes and showed protective effects against palmitate stimulation. This data suggests that alantolactone regulated inflammation and pro-inflammatory adipo-myokines via inhibition of the TLR4-JNK pathway in adipocytes and adipocyte-macrophage co-cultures.

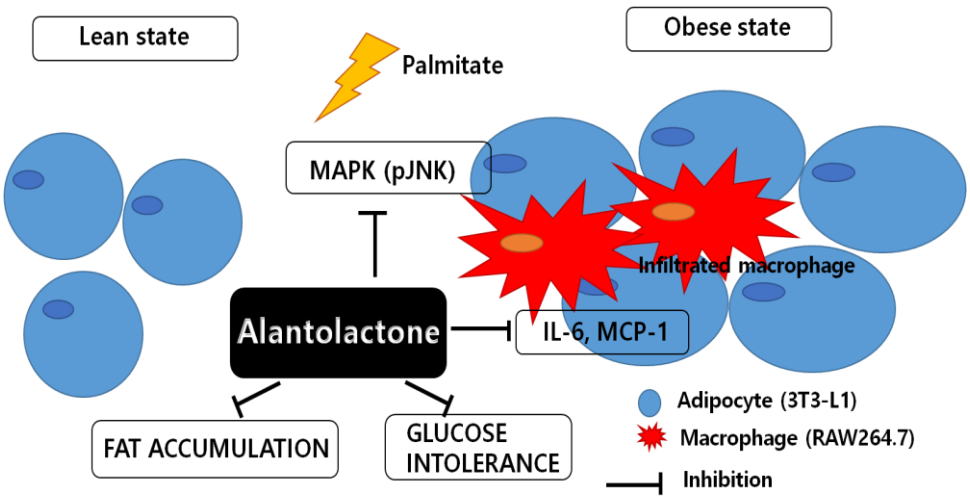


Figure 28 Adipocyte and adipocyte-macrophage coculture system

V. CONCLUSION

This research aimed to demonstrate eccentric exercise, IL-6 and palmitate induced metabolic disorders in the skeletal muscle and adipocytes in association with pro-inflammatory myokines and adipokines regulation.

Eccentric exercise-induced muscle damage study provided strong evidence that capillarisin exerted protective effects against muscle damage in mice. In this research, intense exercise produced ROS and tissue oxidation followed by muscle damage. The level of ROS was greatly attenuated by administration of capillarisin and suppressed both MAPK and NF-κB subunits in accordance with pro-inflammatory adipo-myokine inhibitions. Overall, capillarisin attenuated exercise-induced muscle damage by exerting antioxidant and anti-inflammatory effects. This result points to the potential of capillarisin in muscle treatment, mainly in exercise-induced muscle damage and further, may suggest guideline for cancer cachexia, muscular dystrophy, sepsis and diabetes treatments.

IL-6 –induced myocyte dysfunction indicates that alantolactone exerts its anti-inflammatory effects by inhibiting IL-6-induced insulin-stimulated glucose intolerance and insulin resistance in the skeletal muscle. This study reports that alantolactone suppresses IL-6-stimulated TLR4 expression via STAT3 phosphorylation and SOCS3 activation. Therefore, alantolactone may have a great potential for the treatment of chronic inflammation-associated metabolic disorders, such as insulin resistance and type 2 diabetes. Taking into account, glucose-intolerant and insulin-resistant state was induced using IL-6, which activates STAT3 phosphorylation in the skeletal muscle. Therefore, alantolactone may exert anti-inflammatory effects in association with glucose intolerance. IL-6-induced glucose intolerance by RNAi mediated silencing of STAT3 was examined to

observe impact of STAT3 on glucose regulation and TLR4 expression in association with inflammation. Considering the importance of skeletal muscle in the regulation of glucose control and insulin resistance, alantolactone may be a potent candidate for the treatment of glucose intolerance and insulin resistant treatment in the future.

In case of palmitate-induced adipocyte dysfunction, palmitate increased pro-inflammatory adipokine production, leading to inflammation, glucose intolerance, and insulin resistance through JNK and NF-κB activation. This study reports efficacy of alantolactone in palmitate-induced inflammation and glucose intolerance in both the lean (adipocytes) and obese (adipocyte-macrophage) states in vitro. Therefore, these results suggest that alantolactone may be a novel therapeutic agent for obesity-induced insulin resistance and type 2 diabetes.

In conclusion, capillarisin and alantolactone suppressed pro-inflammatory adipokines and myokines effectively and exerted positive and protective against inflammation and metabolic disorders. Therefore, this research suggests capillarisin and alantolactone as promising therapeutic agents for inflammation associated-metabolic disorders in the skeletal muscle and adipose tissues.

VI. EXPERIMENTAL SECTION

1. Materials

1.1. Capillarisin from *Artemisia capillaris*

The whole plants of *Artemisia capillaris* Thunberg (Asteraceae) were purchased and authenticated by Prof. J. H. Lee (Dongguk University, Korea). The voucher specimen (No. 20090920) was deposited at Prof. J. S. Choi's laboratory, Pukyong National University, Korea. The entire plant was dried and grinded to powder. The dried powder was extracted with 70 % ethanol and water. Capillarisin was isolated and analyzed by high-performance liquid chromatography (HPLC). Then the dried powder was extracted with 70% aqueous ethanol and water, to give the 70% ethanol extract (AR) and water extract, respectively, and they were used for the pharmacological study.

1.2. Alantolactone from *Inula helenium*

The root of *Inula helenium* (Compositae) was purchased from the herb market in Jechun, Chungbuk, South Korea. The roots (200 g) were extracted with methanol (2 L), and sonicated for 2 h. The extract was filtered through filter paper, and evaporated with a rotary evaporator. Two L of distilled water was added to the dried extract, and fractionated with 2 L of n-hexane. Alantolactone was isolated from the n-hexane fraction by countercurrent chromatography (Tauto Biotech). Alantolactone was determined by HPLC using an Agilent 1100 series (Agilent, INNO C18 column (50 mm× 2.0 mm, 3.0 μm)). HPLC conditions were as follows: eluent A, water with 0.1% formic acid; eluent B, acetonitrile with 0.1% formic acid. The purity of isolated alantolactone was over 98%, by HPLC-PDA (photodiode array detector), and the chemical structure identification was performed by ^{1}H and ^{13}C NMR. alantolactone was dissolved in DMSO for further pharmaceutical test.

1.3. Chemicals and reagents

Dulbecco's phosphate buffered saline (DPBS), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), thiobarbituric acid (TBA), n-butanol, pyridine, 1,1,3,3-tetramethoxypropane (TMP), Isobutylmethylxanthine (IBMX), dexamethasone, and insulin for 3T3-L1 differentiation, palmitate, fatty acid-free bovine serum albumin (BSA), Oil red O were purchased from Sigma Aldrich (St. Louis, MO). All materials, unless specified, were from Sigma Aldrich. Glucose uptake assay fluorescent 2-NBDG, RNAiMAX were from Invitrogen (Carlsbad, CA), and IL-6 was from Thermo fisher scientific (Waltham, MA). The primary antibodies for p-p38, p-JNK, p-ERK, p38, JNK, ERK, p65, p50, piKKβ, iKKβ, iNOS, COX-2, TLR4, SOCS3 and β-actin, as well as all secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX). The primary antibodies for p-Akt, Akt, p-STAT3 (Tyr705), STAT3 were from Abcam (Cambridge, UK). Penicillin, streptomycin, DMEM (high glucose), fetal bovine serum (FBS) and horse serum were obtained from GenDepot (Barker, TX). Small interfering RNAs (siRNAs) STAT3 and TLR4 were created from Bioneer (Daejeon, South Korea).

1.4. L6 cell culture and co-culture of adipocytes and macrophages

L6 rat myotubes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in DMEM supplemented 10% FBS, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Differentiation was induced by 2% FBS medium for 6-7 days after seeding.

3T3-L1 pre-adipocytes (ATCC) were maintained in DMEM (10% calf serum and antibiotics). Differentiation was induced 2 days after seeding, by changing the medium to DMEM (10% FBS and antibiotics) containing 0.5 mM 2-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 1 μg/mL insulin. Two days after induction, the medium was replaced with DMEM (10% FBS and antibiotics) containing 1 μg/mL insulin for every other day for the following 8 days. RAW 264.7 (ATCC) macrophages were grown in DMEM (10% FBS and antibiotics).

The adipocyte-macrophage co-culture used the contact system. 3T3-L1 adipocytes were cultured and differentiated, and day 7 of differentiation, RAW264.7 macrophages were seeded and cultured for 24 h.

1.5. Free fatty acid (palmitate) induction

Palmitic acid was dissolved in 50% ethanol, and heated to 60℃ to obtain a clear 100 mM stock solution. Fatty acid free-BSA was dissolved in PBS, and 50% ethanol was used to dissolve palmitic acid due to its poor solubility. Conjugation of 100 mM palmitate stock solution with 2% fatty acid-free BSA solution in PBS to make final 0.5 mM concentration. Serum-free medium was replaced for 4-24 h and palmitate was induced with different concentrations for 24 h.

1.6. Animals

All experiments and protocols were conducted in accordance with Seoul National University Institutional Animal Care and Use Committees approval and guideline. Male C57BL/6 mice (6 weeks) were purchased from Koatec (Seoul, Korea). Total 24 mice were examined, 6 mice each per group (rested control, exercise, CAP20+exercise, and CAP80+exercise).

2. Methods

2.1. ROS assay

2.1.1. DCFH-DA

The level of ROS was quantified using the fluorescent probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). Tissue homogenates from each group were diluted in ice-cold Locke's buffer to obtain a concentration of 5 mg tissue/ml. The homogenates were pipetted into 96-well plates and allowed to warm to room temperature for 5 min. DCFH-DA was added (10 μM) to each well, and the plates were incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. The converted DCF product was measured using the multiplate reader with a fluorescence spectrophotometer, excitation at 485 nm and emission at 530 nm. The measured fluorescence values were expressed as a percentage of the fluorescence with respect to those of the exercised control.

2.1.2. Thiobarbituric acid-reactive substances (TBARS) assay

Peroxidative damage was determined by measuring malondialdehyde (MDA). The gastrocnemius muscle was homogenized with 10mM potassium phosphate buffer containing 30mM KCl. The samples less than 0.2 ml of 10% (w/v) tissue homogenate were added 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). The mixture was heated in 95 ℃ for 60 min. When cooled, 1.0 ml of distilled water and 5.0 ml of

the n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer (upper layer) was taken and measured its absorbance at 540 nm. MDA contents were calibrated from 1,1,3,3,-tetramethoxypropane (TMP) standard curve.

2.2. 2-NBDG Glucose Uptake Assay

Cells were seeded and serum deprived for 4-24 h. The compounds and inducers were treated at indicated concentrations and time. Culture medium was removed and replaced with the medium with 500 mM florescent 2-NBDG (Invitrogen), a fluorescent derivative of glucose, for 3 h. Supernatants were then removed and PBS buffer was added to each well. The fluorescent 2-NBDG images were determined by fluorescence microscopy (Olympus CKX41) and uptake levels were compared to control by using multiplate reader.

2.3. Oil Red O assay

Differentiated 3T3-L1 adipocytes were stained with Oil Red O (Sigma-Aldrich) at day 8. The cells were washed twice with PBS and fixed with 10% formaldehyde in PBS for 1 h. The cells were later washed, followed by 0.5% Oil Red O staining (in 60% isopropanol) for 2 h at room temperature. After 2 h, the stained cells were washed. Oil Red O stain was solubilized with 4% NP-40 in isopropanol (v/v), and the absorbance was measured at 490 nm using a microplate reader. The relative lipid content was compared to that of the control adipocytes.

2.4. Western blots

The cells were seeded in 6-well plates and attached for 24 h. After incubation and sample treatment, the adherent and floating cells were washed with D-PBS and centrifuged for collection. The pellets were extracted in lysis buffer (20 mM HEPES pH 7.6, 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 50 mM NaF, 0.1 mM DTT, 0.1 mM PMSF, and protease inhibitor cocktail) for 30 min on ice. The extract was centrifuged at 15,000 rpm for 10 min at 4℃. The protein concentration was estimated using a Bradford reagent (Bio-Rad Laboratories Inc., CA). An equal amount (20-30 μg) of protein was loaded on SDS-polyacrylamide gels and transferred using a wet method to a nitrocellulose membrane. After being blocked with 5% skim milk, the membrane was incubated at 4℃ overnight with primary antibodies. The membrane was washed and incubated at room temperature for 1 h with secondary antibodies conjugated with horseradish peroxidase (HRP). Finally, the blot was developed using a chemiluminescence kit (AbFrontier) and the immunoreactive bands on the blot were visualized using a LAS-1000 image analyzer (Fujifilm).

2.4.1 Preparation of cytosolic extracts

Cytosolic extracts were isolated after treatment with compounds. Cells were collected, washed, and resuspended in 100 μl of ice-cold buffer (250 mM sucrose, 20 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). After 30 min incubation on ice, the protein in the isolated cytosolic fractions was analyzed by Western blotting .

2.4.2 Preparation of nuclear extracts

For the nuclear extracts, the cells were suspended in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) and allowed on ice for 15 min. Then, 10% NP-40 was added and centrifuged for 5 min. The nuclear pellets were resuspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) and centrifuged for 10 min at 15,000 rpm. The concentration of nuclear protein was estimated using the Bradford reagent (Bio-Rad, Hercules, CA).

2.4.3 RNA interference (siRNA)

Cells were transfected with small interfering RNAs (siRNA) targeting STAT3 and TLR4 (Bioneer, Daejeon, Korea). AccuTarget Negative control siRNA (Bioneer) was used for the scrambled siRNA. The cells were seeded in the serum- and antibiotic-free media and transfected with siRNA-STAT3 and TLR4 (100 nM of each oligonucleotide sequence), or 50 nM scramble sequence according to the manufacturer's instruction (RNAiMax, Invitrogen). After transfection, the cells were washed and treated with or without compound and inducer at the indicated concentration.

2.5. Real-time RT-PCR analysis

The cells were seeded into 6-well plates and maintained at 37 ℃ CO_2 incubator. The cells were exposed to the compounds and inducers, and total RNA was extracted with TRIzol (Ambion, Austin, TX) according to the manufacturer's directions. RNA (1 μg) was converted to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time RT-PCR analysis were performed using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA). Quantitative PCR was conducted with forward and reverse primers as well as the iTaq™ Universal SYBR Green Supermix (BIO-RAD, Hercules, CA), with the following conditions: 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for15 s, 55 ℃ for 20 s, and 72 ℃ for 35 s. The primers used were as follows: IL-6 (forward, 5′-AGG CTT AAT TAC ACA TGT TCT CTGG; reverse, 5′-TTA TAT CCA GTT TGG TAG CAT CCAT), TNFα (forward, 5′-GAT TTG CTA TCT CAT ACC AGG AGAA; reverse, 5′-AAG TCT AAG TAC TTG GGC AGA TTGA), MCP-1 (forward, 5′-ATG CAG TTA ATG CCC CAC TC; reverse, 5′-TTC CTT ATT GGG GTC AGC AC), and GAPDH (forward, 5′-GCC ATC AAT GAC CCC TTC ATT; reverse, 5′-GCT CCT GGA AGA TGG TGA TGG), CINC-1 were 5'-ACA GTG GCA GGG ATT CAC TT (sense) and 5'-CTA GCA CAG TGG TTG ACA CT (antisense), β-actin were 5′-CCC ACT CCT AAG AGG AGG ATG (sense) and 5′-AGG GAG ACC AAA GCC TTC AT (antisense). A melt curve analysis was employed, and the expression levels were normalized to those of the GAPDH or β-actin internal control. All qPCR measurements were examined more than three times.

2.6. Animal study

The mice were randomly assigned to four different groups (n=6 in each group): Group 1 (rested control), Group 2 (exercise), Group 3 (CAP 20 mg/kg + exercise), and Group 4 (CAP 80 mg/kg + exercise). Mice were adjusted to exercise three times a week prior to the actual experiment. Each mouse was weighed and treated interperitoneally (ip) one day before the actual exercise. A 10-lane motorized rodent dual treadmill (Daejong, Korea) was used in the downhill running. The treadmill had an electrical shock grid at the end of the rail to keep the mice running. The exercise groups (Group 2–4) were experimented on the treadmill 24 h after CAP administration according to the eccentric exercise protocol (13 m/min for 60 min, −16° downhill) (Table 2).

Table 2 Animal model protocol

EXERCISE ADJUSTMENT	ECCENTRIC EXERCISE
10 m/min for 10 min -16° downhill exercise Three times a week	13 m/min for 1 hr -16° downhill exercise DAY 3 (one day)

DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Sample preparation	Sample IP injection	Exercise Sacrifice (0h)	Sacrifice (24h)	Sacrifice (48h)

2.7. Statistical analysis

All of the data are presented as the means±standard deviations (SD) from at least three independent experiments. Statistical analysis was performed using Microsoft Excel 2010. Statistically significant differences between the control and experimental groups were calculated by Student's *t*-test and ANOVA. A p value < 0.05 was considered to be statistically significant.

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국문요지

대사성 질환에서 캐필라리진 및 알란토락톤에 의한 염증성 아디포카인과 마이오카인 조절 억제 연구

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2013-31130 김민지

본 연구는 근육에서 분비되는 염증성 사이토카인과 케모카인인 마이오카인, 지방세포에서 분비되는 아디포카인이 공통적으로 염증 및 대사성 질환에서 증가됨에 주목하였다. 최근 연구는 마이오카인과 아디포카인의 긍정적 효과 내용이 주류를 이루는 반면, 병태생리적 원인으로서 부정적인 효과에 대한 연구가 많이 진행되어 있지 않은 실정이다. 따라서, 본 연구는 염증성 마이오카인과 아디포카인이 염증과 대사성 질환에서 중개자로서의 역할을 할 것이라는 가설에 주력하였다. 체내 염증이 증가하면 염증 반응을 유도하는 대식 세포가 증가하게 되고 근육과 지방세포 모두 공통적으로 마이오카인과 아디포카인인 MCP-1, IL-6 와 TNFα 를 증가시킨다. 본 연구는 천연물질인 캐필라리진과 알란토락톤이 근육과 지방세포 염증성 대사질환에서 증가된 마이오카인과 아디포카인의 발현을 억제하고 보호 효과를 나타낼

것이라고 예상하였다. 또한 관련 매커니즘과 글루코즈 조절능을 확인 연구를 통하여 캐필라리진과 알란토락톤의 효과를 확인하였다.

염증과 대사 질환 유도에 있어서 본 연구는 크게 세 가지 첫째, 신장성 운동 (eccentric exercise), 둘째, 사이토카인 (IL-6), 그리고 셋째, 팔미테이트 지방산을 이용하여 근육과 지방세포에서 염증을 유도하고 글루코즈 조절 기능을 저하시킴을 확인하였다.

첫째, 근육에서 과도한 신장성 운동은 염증 그리고 산화적 스트레스를 유도한다. 신장성 운동은 근육의 길이가 늘어나는 운동으로, 장시간 운동시 근육에 손상을 주게된다. 사철쑥에서 분리한 캐필라리진 (capillarisin)은 항산화와 함염증 작용을 가지고 있다고 알려져 있다. 따라서, 본 연구에서는 캐필라리진의 이러한 효과에 주목하여 쥐 근육에 손상을 입히는 운동 모델을 구축하였다. 캐필라리진 투여 그룹에서 얻은 혈액과 근육에서 운동으로 인해 증가된 산화적 스트레스를 감소를 확인하였고, 근육 손상 지표인 CPK (creatinine phosphate kinase)와 LDH (lactate dehydrogenase) 모두 감소시켰다. 운동으로 인해 증가된 MAPK 와 NF-κB 관련 인자들 또한 캐필라리진 투여 그룹에서 억제하는 것을 확인하였으며 염증성 마이오카인 CINC-1, MCP-1, IL-6 그리고 TNFα 모두 감소하는 것을 확인하였다. 따라서, 본 연구 결과는 캐필라리진이 손상된 근육에서 항산화와 항염증 효과를 나타내며 염증성 마이오카인 또한 억제함을 밝혔다.

둘째, 근육에서 대식세포의 침윤은 염증성 사이토카인을 유도하고 인슐린 반응성에 영향을 준다. IL-6 또는 TNFα 와 같은 사이토카인은 염증과 인슐린 저항성 모두에서 증가한다. 특히 IL-6 는 염증과 글루코즈 과민증의 중개 역할로도 알려져 있다. 본 연구는 IL-6 를 장시간 근육세포 (L6)에 처리하여 글루코즈 흡수 저하와 인슐린 저항성 in *vitro* 모델을 구축하였다. 장시간 IL-6 에 노출된 근육세포는 하위 기전인 STAT3 의 인산화를 증가시키는 동시에 염증과 관여하는 는 TLR4 수용체 발현을 증가시킨다. 따라서, 본 연구는 IL-6 가 근육에서 STAT3 와 TLR4 에 미치는 영향과 STAT3 와 TLR4 의 관계성에 주목하여 연구를 진행하였다. 토목향에서 분리된 세스퀴터핀락톤인 알란토락톤은 항염증과 항암 작용이 있다고 알려져 있고, 특히 IL-6 로 유도된 STAT3 기전을 저해한다. 실험 결과 알란토락톤은 IL-6 에 의해 저해된 글루코즈 조절에 관여하는 AKT 인산화를 증가시킴으로 글루코즈 흡수를 증가시켰다. IL-6 에 의해 증가된 STAT3 인산화와 그 하위단계인 SOCS3 발현 및 TLR4 발현 또한 알란토락톤에 의해 억제되었다. STAT3 유전자 억제 모델에서 (siRNA-STAT3) 글루코즈 흡수가 증가하고 TLR4 발현이 억제되는 것을 확인함으로써 STAT3 가 TLR4 발현에도 관여한다는 사실을 확인하였다. 또한, 염증성 마이오카인 IL-6, TNFα 그리고 MCP-1 모두 감소함을 확인하여 IL-6 가 염증성 마이오카인 발현에 영향을 준다는 것을 확인하였다. 따라서, 본 연구는 장시간 IL-6 을 근육에 투여 시 염증성 마이오카인을 증가시키고 염증 및 글루코즈 조절 장애를

야기함을 확인하지만, 알란토락톤이 STAT3-SOC3 억제제로서 글루코즈 흡수를 증가시키고 염증과 관여하는 TLR4 수용체 발현을 억제함을 밝혔다.

셋째, 비만은 특징적으로 비만세포 주변으로 대식세포의 침윤이 일어나면서 염증성 아디포카인이 증가한다고 알려져 있다. 비만세포는 비만이 진행됨에 따라 지방산을 분비하고 혈중 과도한 지방은 글루코즈 과민성과 염증을 야기하며 인슐린 저항성 상태에 이르게 한다. 본 연구에서는 분화된 지방 세포 (3T3-L1)에서 팔미테이트 지방산 투여로 글루코즈 과민성 상태 유도시 알란토락톤이 지방 축적과 염증을 감소하는지를 확인하였다. 또한, 지방세포와 대식세포 (RAW 264.7) 공동배양으로 대식세포 침윤 모델을 구축하였으며 알란토락톤이 글루코즈 흡수율을 증가시키고 염증에 대해 보호 효과를 나타냄을 확인하였다. 알란토락톤은 염증과 관여하는 TLR4 수용체 발현 억제와 MAPK 관련 단백질인 JNK 인산화를 감소시켰으며 대식세포 침윤과 관여하는 염증성 아디포카인 IL-6 와 케모카인 MCP-1 을 감소시켰다. 본 연구는 알란토락톤이 팔미테이트 지방산과 공동배양으로 인한 글루코즈 과민성 및 염증 상태에서 글루코즈 흡수율을 증가시키고 TLR4-JNK 기전을 통해 염증으로부터 보호함을 확인하였다.

결론적으로 본 연구는 신장성 운동, 사이토카인 IL-6 와 팔미테이트 지방산으로 유도된 염증성 대사질환에서 캐필라리진과 알란토락톤의 효능과 보호 효과를 확인하고자 하였다. 캐필라리진과 알란토락톤 모두

염증성 마이오카인과 알란토락톤을 효과적으로 감소하면서 근육과 지방세포 보호효과를 보였다. 캐필라리진은 신장성 운동으로 손상된 근육에서 항산화와 항염증 효과를 보였고 알란토락톤은 사이토카인과 팔미테이트 지방산에 의해 기능이 저하된 근육과 지방세포에서 글루코즈 흡수를 증가 시키고 항염증 효과를 보였다. 따라서 본 연구는 캐필라리진과 알란토락톤이 염증성 마이오카인과 아디포카인의 효과적인 조절제로서 염증성 대사질환에 효과적인 천연물 치료제로서의 가능성을 제시한다.