Study on the effect of S-allyl cysteine on testosterone production and neuroprotection

Ph.D. Dissertation

Study on the effect of S-allyl cysteine on testosterone production and neuroprotection

（S-アリルシステインのテストステロン産生増強、神経細胞保護作用に関する研究）

Md Masud Rana

Laboratory of Nutrition

Department of Science of Food Function and Health

Division of Bioscience and Biotechnology for Future Bioindustries

Graduate School of Agricultural Science

Tohoku University

Supervisor

Professor Hitoshi Shirakawa Ph.D.

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1. Md Masud Rana, Kota Shiozawa, Katsuyuki Mukai, Katsuhiko Takayanagi, Koichi Eguchi, Halima Sultana, Yusuke Ohsaki, Michio Komai, Hitoshi Shirakawa**.** S-allyl Cysteine Enhances Testosterone Production in Mice and Mouse Testis-Derived I-10 Cells. Molecules. 2021 Mar 18;26(6):1697.

Chapter 1: Introduction

General introduction

Due to the advancement of science and technology especially in medical science, the average life span of the population all over the world has been increased. The global population over 60 years old was 382 million in 1980, which increased more than double in 2017 to 962 million. This elderly population will be expected to double again in 2050 to nearly 2.1 billion. On the other hand, the population aged over 80 years will become triple from the present 137 million to 425 million in 2050. At present Japan has the world's largest aged population over 60 years which is 33.4% of Japan's total population and it will also remain in the top country list in 2050 (42.4%) [1]. The process of aging is associated with the onset of many diseases such as cardiovascular diseases, hypertension, diabetes, osteoporosis hypogonadism, and neurodegenerative diseases [2]. As a result of population aging the health cost for elderly people is increasing day by day all over the world. As Japan has the highest number of aged people with respect to its population, per capita health cost for the elderly people is also the highest in Japan. This cost will also increase in the near future [1]. Therefore, effective measures should be taken now to prevent age-related diseases for improving the healthy lifespan.

With aging testosterone synthesis declines in the male that is defined as late onset hypogonadism. Several diseases are also associated with the onset of hypogonadism. Moreover, testosterone exerts many physiological functions in various organs to ameliorate many diseases [3]. Testosterone replacement therapy (TRT) to restore the testosterone level especially for elderly men has been popularly used as a medication. However, TRT has been reported with several adverse effects such as cardiovascular disease, prostate cancer, lower urinary tract symptoms, obstructive sleep apnea, and erythrocytosis [4,5]. Therefore, it is

important to search for safe medications or natural bioactive compounds to boost testosterone levels for elderly men or prevent hypogonadism in healthy men.

Neurodegenerative diseases are a heterogeneous group of disorders involved in progressive loss and damage of neurons in the brain leading to cognitive and functional decline over time. Age is the primary risk factor for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. Currently, there is no medicine to cure Alzheimer's diseases and Parkinson's diseases, only some drugs are effective to ameliorate the symptoms. Oxidative stress has been implicated in these neurodegenerative diseases and several antioxidants have been reported experimentally and clinically to ameliorate these diseases [6,7].

To prevent and cure age-related diseases and reduce the cost burden, the search for bioactive compounds from natural sources with therapeutic potential is gaining research interest. Natural bioactive compounds are very easy to extract and therefore very cheap. Moreover, numerous natural compounds are being used traditionally in many cultures as therapeutic agents which are relatively less toxic and safe [8,9]. To this direction the present study focused on a bioactive compound S-allyl cysteine, exclusively found on garlic, to evaluate its role in testosterone synthesis and protection from neurotoxicity.

Purpose of the study

The principal purpose of the study is to analyze the health beneficial effects of S-allyl cysteine (SAC). To evaluate these effects, two major studies were accomplished:

1. Effect of SAC on testosterone production was explored in cell and animal-based experiments.

2. Neuroprotective effect of SAC was investigated using mouse hippocampal-derived HT-22 cells after glutamate-induced neurotoxicity.

S-allyl cysteine

S-allyl cysteine (SAC) is a bioactive sulfur-containing amino acid derivative found in garlic along with other sulfur-containing compounds (Figure 1). SAC has been reported to have antioxidant, anticancer, and neuroprotective properties in numerous animals and cell-based studies. SAC, both from chemically synthesized and garlic extracted, is a white crystalline powder at room temperature and has a characteristic odor. SAC is soluble in water (68mg/mL), has a melting temperature of 222-224°C, and highly stable compound in various physiological conditions [10].

SAC is found in fresh garlic at a very low amount that is 20-30 µg/g fresh garlic. Its content in garlic can be increased by a chemical process called the aging of garlic. In this process, garlic cloves are soaked in 15-20% aqueous ethanol for several months in a stainless still tank at room temperature. γ-Glutamyl-S-allyl cysteine, present at a high amount in garlic, is completely hydrolyzed to SAC by the enzyme γ-glutamyl transpeptidase (Figure 2). In this process, the amount of SAC can be increased from 0.2 mg/g dry extract to 7.2 mg/g dry extract after 24 months [11]. Black garlic is another form of preparation where the amount of SAC in garlic can be enhanced. Black garlic is prepared by heat treatment of whole bulbs of raw garlic at high temperature and high humidity for several days [12]. Studies showed that the amount of SAC could be increased at least 6 times higher after heating the garlic at 40° C for one month [13].

Potential role of SAC as an antioxidant

The earlier studies on SAC were reported principally on its antioxidant properties. SAC is the most abundant compound of aged garlic extract (AGE) preparation. AGE was initially discovered for the protection of various organs such as heart, liver, and kidney from oxidative injury. Later it was found that the antioxidant property of AGE was mostly due to the abundant presence of SAC on it [14,15]. The earlier studies suggested that SAC can suppress reactive oxygen species, superoxides, hydroxyl free radicals, and peroxynitrite generations. SAC also has been reported to prevent lipid and protein oxidation and nitration. SAC can upregulate the antioxidant enzymes and suppress the prooxidant enzymes via activating the transcription factor Nrf2 [16]. Various animal studies suggested the involvement of antioxidant activity of SAC to ameliorate diabetes, kidney injury, and liver injury [17,18,19]. The protective effects of SAC on male reproductive organs via its antioxidant activity also have been reported in various animal models. In a streptozotocin-induced diabetic rat model, SAC restored erectile dysfunction via reducing the reactive oxygen formation through modulation of NADPH oxidase subunit expression [20]. Another study showed that after long-term supplementation of SAC in aged rats, SAC can improve the age-related reduction of the sperm number, motility, and DNA synthesis through inhibition of oxidized proteins [21]. Furthermore, a recent study on boar spermatozoa revealed that SAC improved motility, plasma membrane integrity, and mitochondrial activity of boar sperm [22].

Role of SAC in neurodegenerative diseases

Numerous studies on animal and cell-based experiments have been reported on the neuroprotective effect of SAC. A comparative study on various sulfur-containing compounds from garlic showed that SAC was the most potent compound to have a neurotrophic effect. SAC at a very low concentration (100 ng/mL) could enhance the survival of neurons isolated from rat brain and increased the branching of axons of neurons [23]. Amyloid β proteins aggregation in the central nervous system is the main cause of pathogenesis of Alzheimer's diseases resulting in neuronal cell loss in the brain [24]. A cell-based study showed that SAC selectively protected differentiated PC12 cells, a neuronal cell model, from amyloid β (Aβ) induced cell death. Interestingly, SAC did not show this effect on undifferentiated PC12 cells, suggesting its selective role in neuroprotection [25]. Similarly, in another study, SAC showed neuroprotective effects on isolated rat hippocampal neurons from Aβ and tunicamycin-induced neurotoxicity [26]. A cell-free system-based study demonstrated that SAC dose-dependently inhibited Aβ fibrillation and destabilized preformed Aβ fibrils [27]. In a streptozotocin-induced mice model, pretreated with SAC showed amelioration of cognitive and neurobehavioral impairments. SAC also reduced DNA fragmentation, apoptosis-inducing protein marker, and oxidative stress in the hippocampus of these mice [28]. In another mice model, SAC showed protective effect on the brain after D-galactose induced brain injury via restoring various antioxidant enzymes and reducing Aβ proteins level in the brain [29]. Furthermore, several studies on animal models of various neurodegenerative diseases such as stroke, Huntington disease, Parkinson's disease have demonstrated that SAC has a similar protective effect on the brain [16].

Testosterone and its function

Testosterone is the principal sex hormone in males responsible for developing primary and secondary sex characteristics. Testosterone plays a key role to maintain male fertility and spermatogenesis. Over aging males develop the late onset of hypogonadism which is characterized by low testosterone synthesis in the Leydig cell of testis [30]. A population-based study showed that male starts to decrease blood testosterone level from their middle-age by 2% per year [31]. Low testosterone level is associated with the development of various diseases such as osteoporosis, type 2 diabetes, and cardiovascular diseases [3]. Testosterone and its metabolite dihydrotestosterone mediate their function via binding to the intracellular androgen receptor (AR) that acts as a transcription factor for many target genes. AR is widely expressed in the tissues of various organs like the brain, muscle, and pancreatic islets [32]. It is well established that in aging men decreased muscle mass and strength are restored by testosterone replacement [33]. Experimental animal models with muscle-specific knock-out of AR demonstrated that testosterone has a role in maintaining muscle mass and strength [34]. More recent data suggests that the maximal action on muscle and physical activity by testosterone is achieved by the AR in the neurons of the hypothalamus [35]. The Association of testosterone and bone mineral density also has been established. Testosterone therapy in elder men improves bone mineral density. Animal and cell base studies revealed that testosterone improves bone mineral density via AR on osteoclast and osteoblast cells. Moreover, estradiol, an aromatized metabolite of testosterone also plays role in bone function via the estrogen receptor on various cell types [36]. Observational studies reported that androgen deprivation therapy in prostate cancer patients causes obesity and type 2 diabetes. A recent study on animal models revealed that testosterone can improve insulin secretion in pancreatic β cells via extranuclear androgen receptor and glucagon-like protein 1 receptor [37]. Low level of testosterone also has been reported to be associated with fatigue, depression, loss of cognition,

and sleep disturbance [3]. Therefore, it is important to maintain physiological testosterone levels, especially in aging men to lead a healthy life.

Mechanism of testosterone synthesis

Testosterone is predominantly produced in the Leydig cell of testis in males (Figure 3). The testosterone synthesis pathway begins with the release of luteinizing hormone (LH) from the pituitary gland to the circulation. LH binds to its receptor on the cell surface of the Leydig cell that is a member of G-protein coupled receptors. Then the intracellular cAMP level is increased, protein kinase A (PKA) becomes activated to its phosphorylated form. Activated PKA targets the transcription factor CREB (cAMP responsive element binding protein) for the transcription of many genes involved in testosterone syntheses such as CYP11A and StAR. PKA also phosphorylates the StAR protein to its more active form. StAR proteins transfer cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, and then CYP11A protein produces pregnenolone from cholesterol. In the smooth endoplasmic reticulum, pregnenolone is converted to progesterone and testosterone by other steroidogenic enzymes. [38].

Mechanism of glutamate-induced neurotoxicity in HT-22 cell

Glutamate is one of the most important excitatory neurotransmitters and the most abundant amino acid in the brain. Glutamate participates in neural transmission, development, differentiation, and plasticity. Glutamate mediates its action via two types of receptors on neuronal cells named ionotropic receptors and metabotropic receptors. Ionotropic receptors include NMDA, AMPA, or kainate receptors that act as ion channels. On the other hand, metabotropic receptors are G-protein coupled receptors that activate second messenger cascades. Glutamate also can act on CySS/glutamate antiporter. It has been reported that in neurodegenerative diseases like Alzheimer’s disease and Parkinson's disease, the extracellular concertation of glutamate increases. In neuronal cells, overexcitation by glutamate mediates

the influx of Ca^{2+} ions leading to cell death via activation of various cellular events such as up-regulation of nNOS, mitochondrial dysfunction, ROS production, ER stress, and release of lysosomal enzymes. [39].

HT-22 cells are mouse hippocampal-derived cell lines that are widely used as a model for glutamate-induced neurotoxicity studies. Though HT-22 cells do not have any functional glutamate receptors, excessive glutamate causes both necroptotic and apoptotic cell death in this cell. In HT-22 cells excessive intracellular glutamate blocks the influx of cystine via the cystine/glutamate transporter resulting in depletion of cysteine in the cell (Figure 4). Consequently, glutathione synthesis is compromised and cellular reactive oxygen species level increases both in the cytoplasm and mitochondria. This type of cell death is associated with mitochondrial fission. Mitochondria releases apoptosis-inducing factor and cytochrome c to the nucleus resulting in nuclear fragmentation and ladder formation. A previous comprehensive study suggested that this type of cell death is independent of caspase activation, although involvement of caspase 3 activation has been reported. Extracellular signal-related kinases (ERK) and other mitogen activated protein kinases (MAPK) like p-38 and JNK play a key role in glutamate-induced cell death in HT-22 cells. Prolonged activation of these MAPK proteins has been reported to cause cell death in HT-22 cells by glutamate challenge. [40,41,42,43].

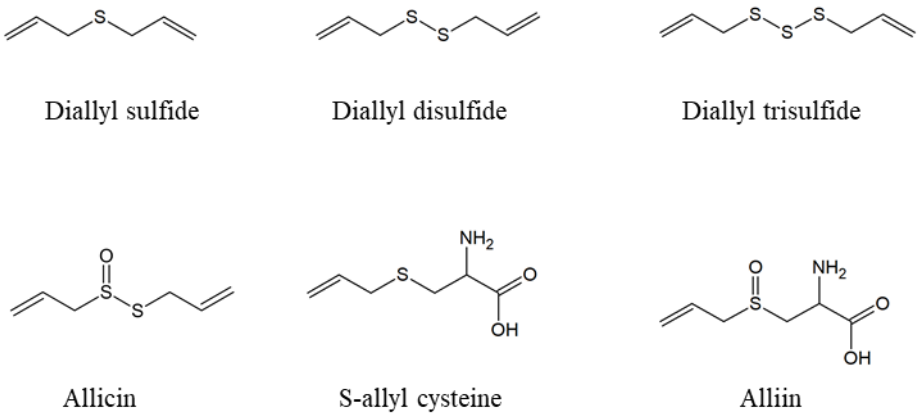

Figure 1. Chemical compounds commonly found in garlic.

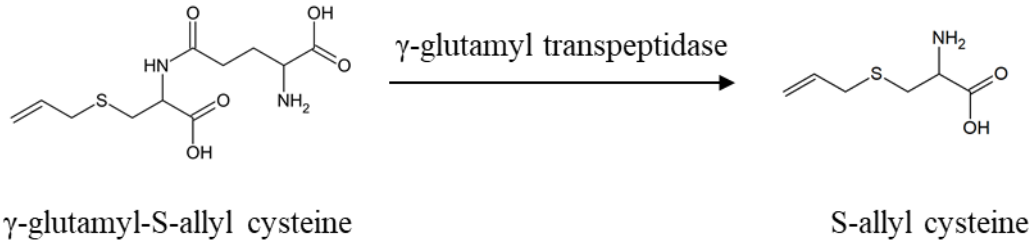

Figure 2. Synthesis of S-allyl cysteine during the aging process.

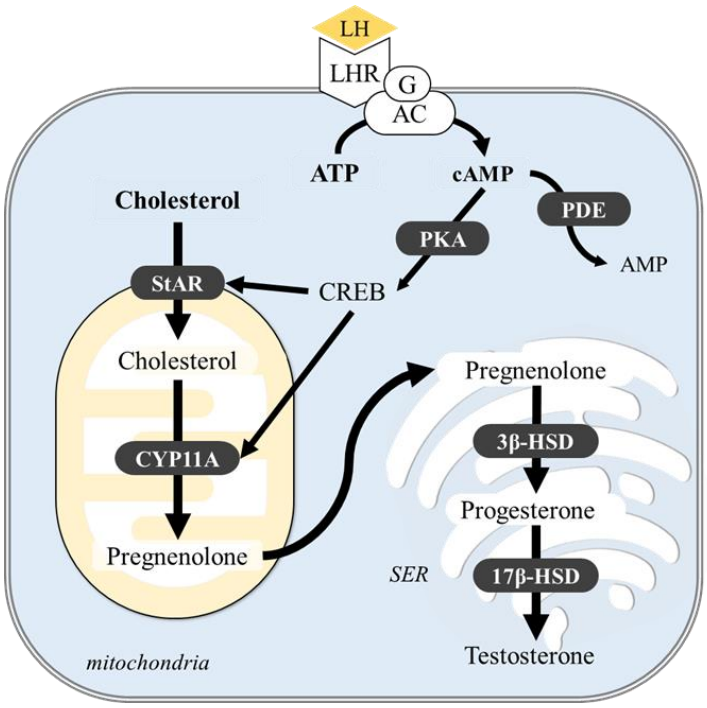

Figure 3. Testosterone synthesis pathway in Leydig cell of the testis.

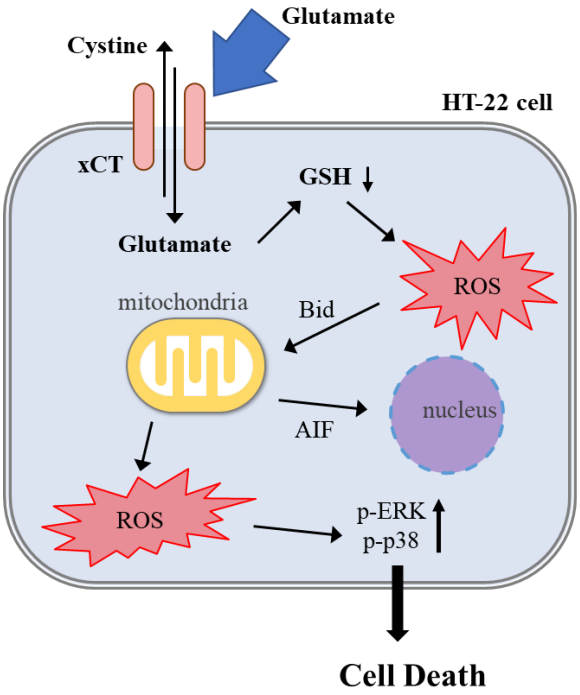

Figure 4. Mechanism of glutamate-induced neurotoxicity in HT-22 cells.

Chapter 2: Effect of S-allyl cysteine on testosterone production

To confirm the effect of SAC on testosterone production, animal and cell-based experiments were conducted. For a single dose experiment, BALB/c mice and chronic dose experiment, Wistar rats were used. For cell-based experiment mouse testis-derived I-10 tumor cells were investigated.

Study 1: Effect of S-allyl cysteine on testosterone production in mice after single dose administration

Methods and materials

SAC

SAC was purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). SAC was stored at room temperature.

Animal experiment

Six-week-old male BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were then maintained for 1 week in a 12 h/12 h dark/light cycle for acclimation. The mice had free access to the F-2 laboratory diet (Funabashi Farm Co., Funabashi, Japan) and tap water. After one week of acclimation, mice were divided into two groups (n=8 in each group). Mice of the treatment group were administered SAC intraperitoneally at a concentration of 50 mg/kg body weight in normal saline, whereas the mice of the control group were administered normal saline only. The final volume of the vehicle was at a ratio of 20 µL to 1 g of body weight of the mice. After 6 h of SAC administration, the mice were sacrificed. Blood from the heart was collected by heparinized 26.5G needles and testis were dissected for further analysis. For plasma collection, the blood was centrifuged at

2,000 x g for 15 min at 4°C. The supernatant was collected and stored at -65°C for further analysis.

Testosterone extraction and measurement from plasma and testis

Testosterone was measured from the testis and plasma by ELISA method according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA). At first, testes were homogenized in phosphate-buffered saline (PBS) at a ratio of 100 mg testes to 5 mL PBS using a polytron homogenizer. Next, 1 mL of the testis homogenate or 200 µL of plasma was mixed with 5 times volume of diethyl ether in a glass test tube and centrifuged at 1500 × g for 3 min. Approximately 90% of the upper ether layer was collected in another tube. This procedure was repeated three more times. The collected ether layer was evaporated using a vacuum evaporator (Spin Dryer Light VC-36R, TAITEC Corp., Saitama, Japan) at 1800 rpm, 30°C. After evaporation, the ELISA buffer supplied with the kit was added to the residue, and testosterone was measured from this solution. Testosterone level in testes was normalized with the amount of protein.

Lowry method for protein quantification from testis

Testis homogenate was centrifuged at 13,000 rpm for 5 min at 4°C. From the supernatant, protein amount was calculated by the Lowry method. Bovine serum albumin was used as a protein standard. 80 µL sample or protein standard were mixed with 400 µL alkaline solution (2% Na_2CO3 in 0.1 N NaOH and $CuSO_4 \cdot 5H_2O$ at a ratio of 50:1) and incubated at 37°C for 10 min. Next, 40 µL of Follin reagent was mixed with each sample and incubated at 37° C for 30 min. Absorbance was taken at 750 nm.

Luteinizing hormone measurement

Luteinizing hormone (LH) levels in the plasma of BALB/c mice were measured by rodent LH test kit according to the manufacturer's instructions (Endocrine Technologies, Newark, NJ, USA). Briefly, 50 µL LH standard or plasma was mixed with 100 µL enzyme conjugate in well supplied with the kit. and incubated for 2 h at 37°C. The mixture was removed, and the wells were washed by wash buffer five times. 100 µL of TMB solution was added to the wells and incubated for 20 min in the dark. 50 µL of 2 N HCl was added to stop the reaction. Then the absorbance was taken at 450 nm using an ELISA plate reader.

Western blot method

Testes were homogenized with PBS (at a ratio of 100 mg testes to 1 mL PBS) containing complete proteinase inhibitor cocktail, and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science, Mannheim, Germany) using polytron homogenizer. The homogenates were centrifuged at 13,000 rpm, 4°C for 5 min. Protein concentration from the supernatant was measured by the Lowry method as mentioned earlier in this section. Proteins from the supernatant were denatured in SDS gel loading buffer. 15 µg of proteins were resolved in 10–20% SDS-polyacrylamide gel (Wako Pure Chemical Industries, Osaka, Japan) by electrophoresis at 150 V for 150 min. Proteins separated from the gel were transferred into polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in TBS-T buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk or 3% bovine serum albumin for 2 h. The membranes were then incubated overnight with blocking buffer containing an antibody against PKA, phosphorylated PKA (Cell Signaling Technology, Danvers, MA, USA), and CYP11A (Chemicon, Temecula, CA, USA) followed by horseradish peroxidase (HRP)-tagged secondary antibody incubation for 1 h. Antibodies against α-tubulin (Sigma-Aldrich) or β-actin (Abcam, Tokyo, Japan) were incubated for 1 h followed by an HRP-tagged secondary antibody incubation for 1h. HRP

substrate (Immobilon Western Detection Reagent, Millipore) was incubated with the membrane at room temperature to detect the immunoreactive bands. The bands were visualized using a LAS-4000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan). Relative protein expression levels were measured by normalizing with the expression of α-tubulin or β-actin.

Statistical analysis

All the data presented in the figures are shown as mean ± standard error (SE). For the comparison of means between two groups, student's t-test was performed. In the case of comparing the means of more than two groups, data were analyzed by one-way ANOVA or Dunnett's test, or Tukey test. All the statistical analyses were performed using SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA) with a significance level of $\alpha = 0.05$. $P<0.05$ is considered statistically significant.

Results:

After the single dose of SAC administration in BALB/c mice, the plasma testosterone level was significantly increased compared to the control group (Figure 5.A). Testosterone level in the testis was also measured. Like the plasma result, it was observed that the testosterone level in the testis of BALB/c mice was significantly higher in the SAC treated group compared to the control group (Figure 5.B). However, the Plasma level of LH hormone did not change significantly after SAC treatment (Figure 5.C).

Next, the proteins involved in testosterone synthesis in the testis like PKA and CYP11A were analyzed by the western blot method. It was found that SAC significantly upregulated the phosphorylated PKA (p-PKA) where the total PKA was unchanged. Moreover, the ratio of p-PKA to PKA was also significantly changed in the SAC treated group compared to the control group. On the other hand, the level of CYP11A protein was found unchanged after SAC treatment (Figure 6).

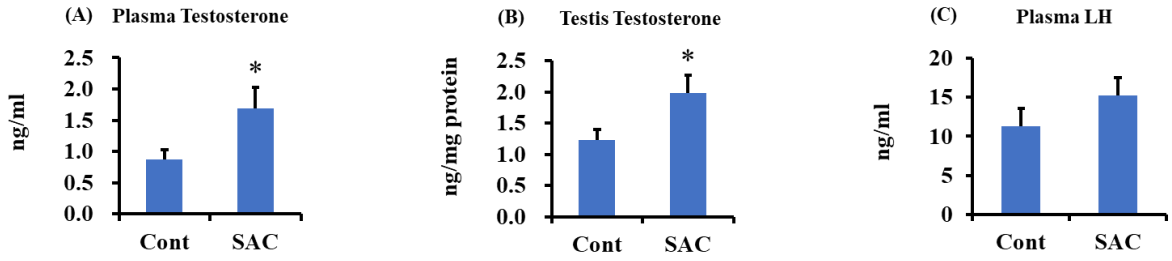

Figure 5. Effect of SAC on testosterone production in BALB/c mice. (A) plasma testosterone (n = 6–7), (B) testis testosterone (n = 7–8) and (C) plasma luteinizing hormone (LH) (n = 6) were measured by ELISA. Data are presented as the mean ± SE. Data were analyzed by Student's t-test. * $p < 0.05$ compared to the control group.

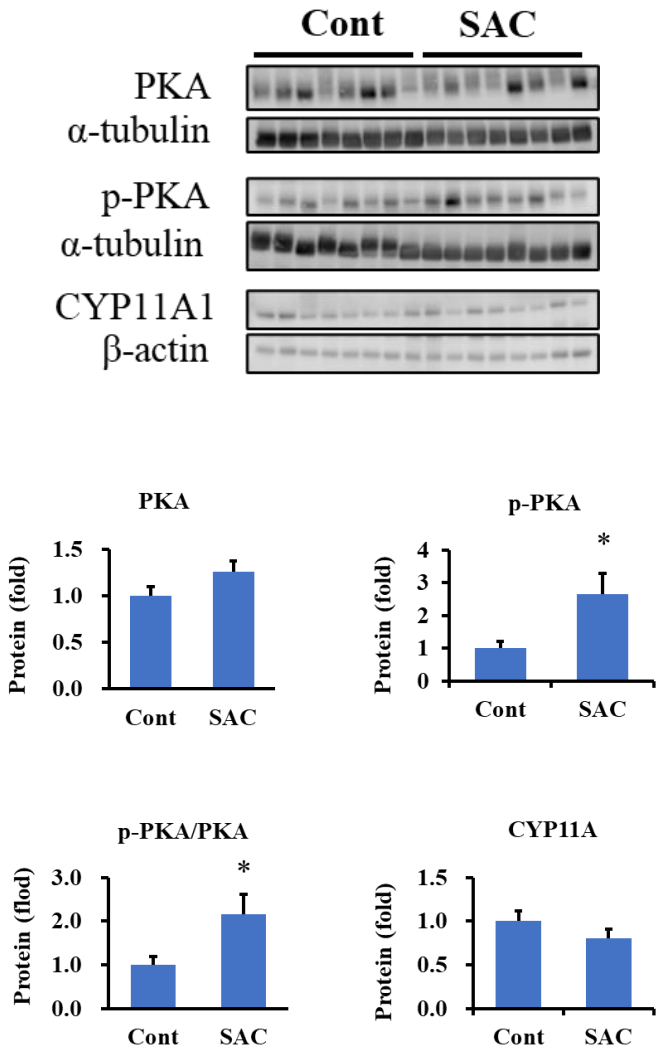

Figure 6. Effect of SAC on the level of PKA, p-PKA, and CYP11A proteins in the testis of BALB/c mice. Proteins were measured by the western blot method. Data are shown as mean±SE. n=8. Data were analyzed by student's t-test. *P<0.05.

Study 2: Effect of S-allyl cysteine on testosterone production in I-10 cells.

In this section, the molecular mechanism of testosterone synthesis stimulated by SAC was investigated on testosterone-producing I-10 cells.

Methods and materials

Cell culture method

Mouse testis-derived I-10 tumor cells were purchased from the Health Science Research Resource Bank (Osaka, Japan). For the maintenance, cells were cultured in Ham's F-10 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Cosmo Bio Co., Ltd., Tokyo, Japan), 50 U/L of penicillin, and 50 mg/mL of streptomycin (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) in 10 cm dish in a humidified chamber at 37 °C and 5% CO_2. For the passaging of cells, the culture medium was removed by using an aspirator followed by washing the culture dish with PBS twice. Cells were detached from the surface using 0.25% trypsin-EDTA for 5 min. Cells were resuspended with fresh medium and transferred to new dishes. Cells were used in the experiment when they reached 70–80% of confluence. SAC was dissolved in water at a concentration of 200 mM and kept at −20 °C (stock solution). The stock solution was further diluted with water before mixing with the medium. The final concentration of water was 0.1% in the medium used for the experiments.

Cell proliferation assay

I-10 cells (3.6×10^4 cells) in 100 µL medium per well in 96-well plate were incubated overnight, followed by replacing the medium with 100 µL fresh medium containing 1, 10, and 100 µM of SAC. After 24 h of incubation, 10 µL WST-1 reagent (Takara Bio Inc., Shiga, Japan) was added to each well. Absorbance at different time intervals was taken at 450 nm with a reference wavelength of 630 nm using a microplate reader XR (Bio-Rad, Hercules, CA, USA). The proliferation rate of the cells was calculated from the absorbance data.

Testosterone measurement from I-10 cells

I-10 cells at a density of 3.6×10^4 per well in 800 µL medium in a 12 well plate were incubated overnight. The next day the medium was replaced with fresh medium containing SAC. After 24 h of incubation with SAC, 600 µL medium was collected and centrifuged at 1000 x g for 5 min. Testosterone was measured directly from the supernatant by ELISA method according to the manufacturer's instruction. (Cayman Chemical Co., Ann Arbor, MI, USA).

Western blot method

I-10 cells (2×10^6 cells) in 5 mL medium in 6 cm dish were incubated overnight, followed by replacing the medium with 5 mL fresh medium containing SAC for 1.5 h or 6 h. After the indicated incubation time the medium was removed, and cells were washed with ice-cold PBS. Next, 1 mL of ice-cold PBS was added to the dish placed on ice, and cells were collected in a tube using a rubber scraper. Cell suspension in the PBS was centrifuged at 1,000 x g, 4°C for 2 min. After removing the PBS, 250 µL lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% SDS, 5 mM EDTA) containing phosphatase inhibitor and protease inhibitor (Roche Applied Science, Mannheim, Germany) was added to the pellets and mixed thoroughly by pipetting. After mixing, cells with the lysis buffer were kept on ice for 20 min followed by centrifuging at 13,000 rpm, 4°C for 20 min. Supernatant from the cell lysate was collected in another tube. From the supernatant total protein concentration and different protein expression level was measured by Lowry method and western blot method respectively as described on study 1 of this chapter.

RNA extraction method

I-10 cells (10^6 cells) in 5 mL medium in 6 cm dish were incubated overnight, followed by replacing the medium with fresh medium containing 100 µM SAC. After 3 h of incubation, the medium was removed and 500 µL Isogen reagent (Nippon Gene, Tokyo, Japan) was added

to the dish. Isogen reagent was mixed with the cells by pipetting. The total cell lysate was collected using a rubber scraper to a tube. 100 µL chloroform was added to the tube and mixed by a vortex mixer for 15 s. This solution was centrifuged at 13,000 rpm, 4°C for 15 min. The upper aqueous layer was collected to another tube and an equal volume of isopropanol was added. After mixing with isopropanol the solution was centrifuged at 13,000 rpm, 4°C for 10 min. The supernatant was removed by decantation and the RNA pellet was washed with 500 µL 75% ethanol at 13,000 rpm, 4°C for 5 min twice. After decanting the ethanol, the RNA pellet in the tube was air-dried for 30 min. Next DEPC treated water was mixed with the RNA by pipetting. RNA purity and quantity were measured spectrophotometrically using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA) from the absorbance at 260 nm in relation to that at 280 nm.

c DNA synthesis and mRNA quantification

4 µg RNA was used for cDNA synthesis as a template. RNA was denatured for 5 min with 2.5 µM oligo-dT primer (Hokkaido System Science Co., Sapporo, Japan) and 0.5 mM dNTP (GE Healthcare, Tokyo, Japan) at 65 °C. From the denatured RNA, cDNA was synthesized using 50 U SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 20 U RNaseOUT RNase inhibitor (Invitrogen) in RT buffer (50 mM Tris-HCl at pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 5 mM dithiothreitol) at 50 °C for 60 min. After completion of cDNA synthesis, cDNA was diluted 10 times and stored at -20°C for further analysis. 30 times diluted cDNA was used as a template to amplify the target sequence using gene-specific primers (shown in Table 1). SYBR Premix Ex Taq solution (Takara Bio, Otsu, Japan) was used for the quantitative RT-PCR using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The mRNA levels were then normalized to the levels of eukaryotic elongation factor 1α1 (Eef1a1).

Measurement of cAMP

I-10 cells were seeded at a density of 10^6 cells into 6 cm dish overnight followed by changing the medium containing 100 μM SAC for 1.5 h. After removing the medium, 1 mL 0.1 M HCl was added to the dish and incubated at room temperature for 20 min. Cells with the HCl solutions were collected using a rubber scraper into a tube and centrifuged at 1000 x g for 10 min. KOH and acetic acid anhydride were added to the supernatant for the acetylation of cAMP. Acetylated cAMP concentration in the cell lysate supernatant was determined by cAMP ELISA kit (Cayman Chemical).

Results

To investigate the direct effect of SAC on steroidogenesis, mouse testis-derived I-10 cell line was used. First, the cytotoxic or proliferative effect of SAC was checked on I-10 cells using the water-soluble tetrazolium salts-1 (WST-1) assay. It was observed that SAC has no cytotoxic or proliferation effect at a concentration of 1–100 µM on I-10 cells after 24 h of incubation (Figure 7). To confirm the testosterone synthesis in I-10 cells, forskolin was used as a positive stimulator for steroidogenesis. After 12 h of incubation with 10 µM forskolin, the testosterone level in the culture medium was around 5 times higher than that of the control group in I-10 cells (Figure 8.A). Next, testosterone levels in I-10 cells were measured after SAC treatment. SAC at 10 µM significantly enhanced the testosterone level in I-10 cells. After 100 µM SAC treatment the testosterone level was approximately two times higher than that of the control group (Figure 8.B).

Next, the expression levels of PKA and p-PKA in SAC-treated I-10 cells were measured by western blot method to elucidate the effect of SAC on the activation of PKA in I-10 cells after 1.5 h of incubation. It was observed that SAC did not change the PKA level but significantly enhanced p-PKA expression in I-10 cells (Figure 9). Additionally, other steroidogenic proteins like CYP11A and CREB levels were measured after 6 h of incubation with SAC in I-10 cells. It was found that at this time point, SAC significantly increased the phosphorylated CREB compared to the control group whereas the total CREB level was not changed. On the other hand, the CYP11A protein level was not changed at this time point by SAC treatment (Figure 10).

Furthermore, after 3 h of incubation with SAC, the mRNA expression levels of *StAR* and *Cyp11a* were measured in I-10 cells by quantitative RT-PCR. SAC significantly increased the mRNA level of *Cyp11a* but did not change the *StAR* mRNA level after the indicated incubation time (Figure 11). In addition, the cAMP level was measured by ELISA method after

1.5 h of incubation with SAC in I-10 cells. However, it was found that SAC did not change the level of cAMP in I-10 cells (Figure 12).

Table 1. Primer sequence used in the qRT-PCR. Sequences are shown as 5'→3' direction.

Gene Name	Forward Primer	Reverse Primer
Cyp11a	CGTGACCTTGCAGAGGTACACT	GCTGGAATCTTGTAATTACGAAGCA
StAR	GGAGCTCTCTGCTTGGTTCTC	TTAGCACTTCGTCCCCGTTC
Eef1a1	GATGGCCCCAAATTCTTGAAG	GGACCATGTCAACAATTGCAG

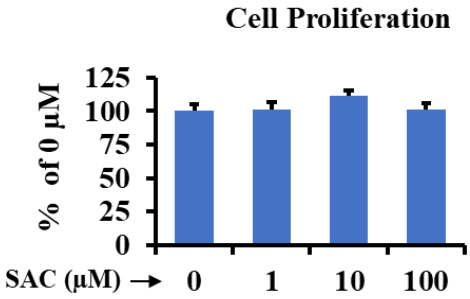

Figure 7. Effect of SAC on cell proliferation of I-10 cells by WST-1 method. n=4-5. Mean ± SE. Data were analyzed by one-way ANOVA.

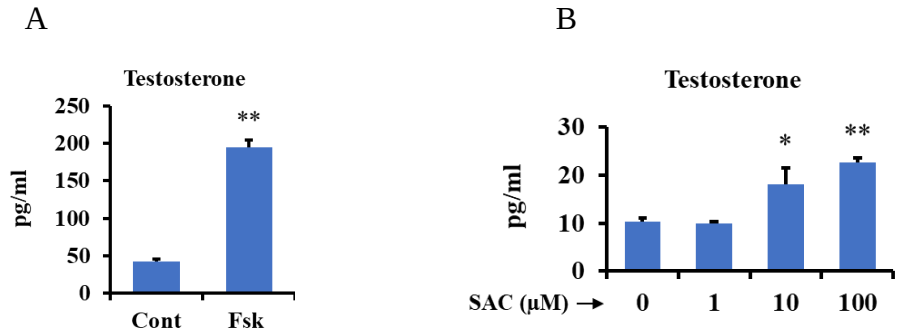

Figure 8. Effect of SAC on testosterone production in I-10 cells. A. Testosterone level after forskolin (Fsk) treatment for 12 h. Testosterone level after SAC treatment for 24 h. Data are shown as mean ± SE. n=3. Data were analyzed by student's t-test or Dunnett test. *P<0.05, **P<0.01 vs 0 µM SAC.

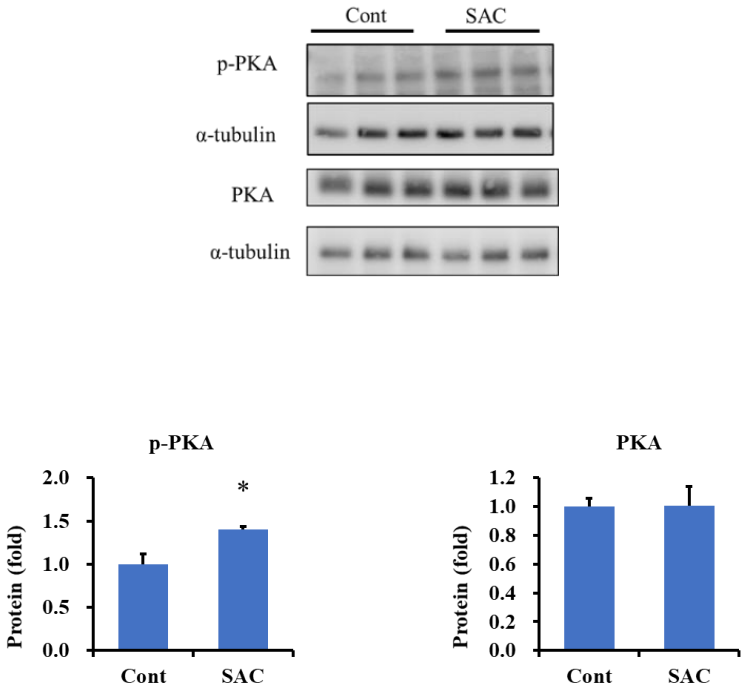

Figure 9. Effect of SAC on PKA, p-PKA protein level in I-10 cells. 100 μM SAC was treated on I-10 cell for 1.5 h. Proteins were measured by the western blot method. n=3. Mean ± SE. Student's t-test. *P<0.05.

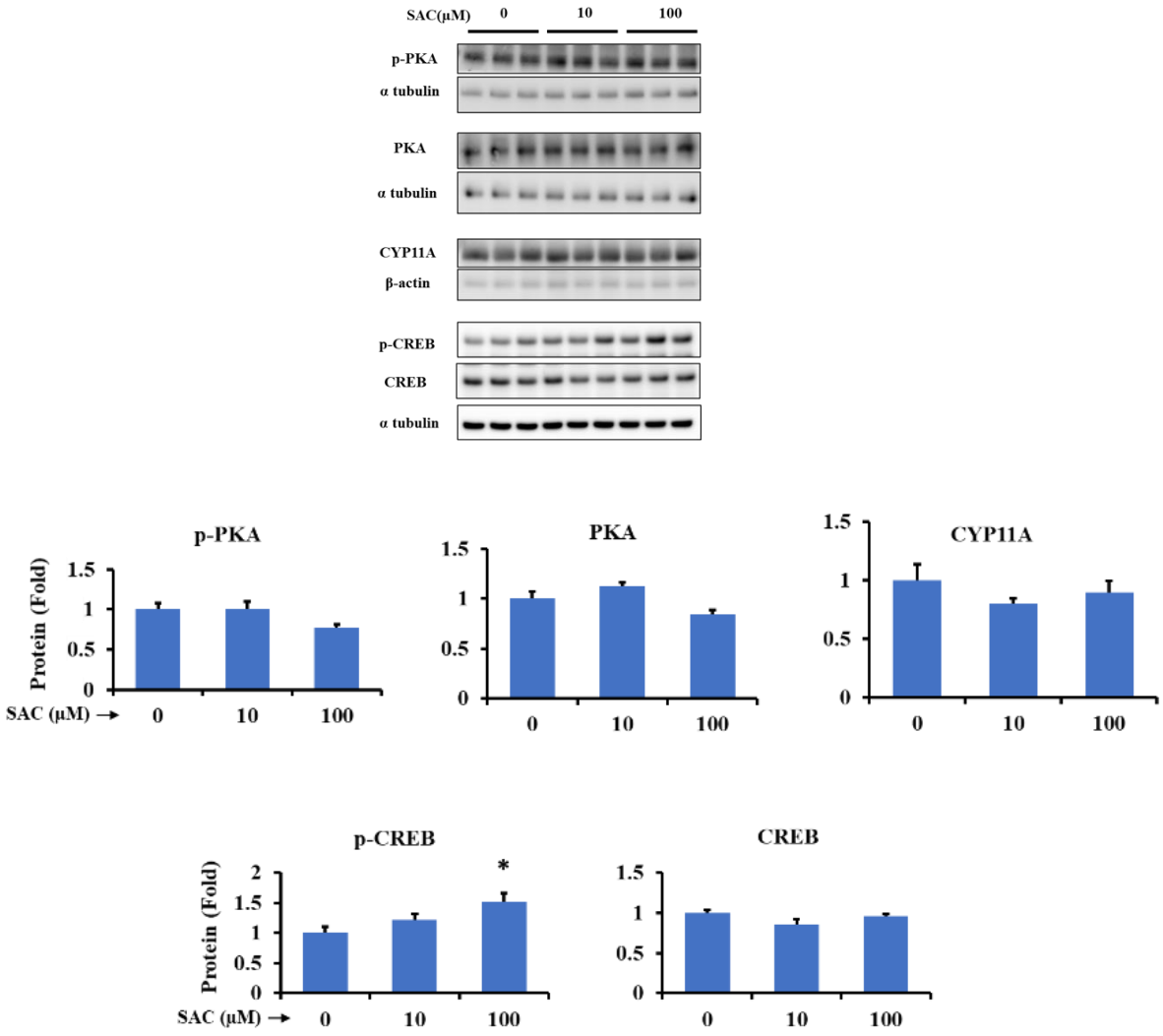

Figure 10. Effect of SAC on PKA, p-PKA, CYP11A. CREB and p-CREB protein level in I-10 cells. SAC was treated on I-10 cells for 6 h. Proteins were measured by the western blot method. n=3. Mean ± SE. Tukey test. *P<0.05 vs 0 μM.

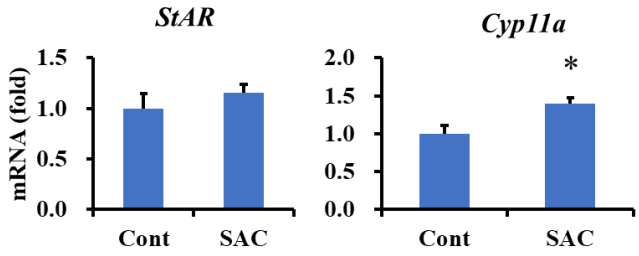

Figure 11. Effect of SAC on the mRNA level of *StAR* and *Cyp11a* genes in I-10 cells by the qRT-PCR method. 100 μM SAC was treated in I-10 cell for 3 h. n=3. Mean ± SE. Student's t-test. *P<0.05.

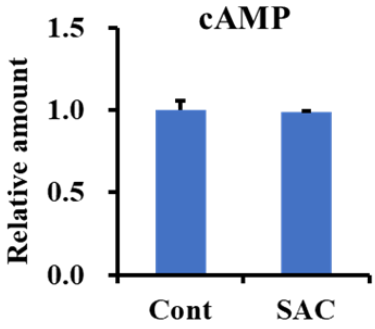

Figure 12. Effect of SAC on the intracellular cAMP level in I-10 cells by ELISA method. 100 μM SAC was treated in I-10 cells for 1.5 h. n=3. Mean ± SE. Student's t-test. *P<0.05.

Study 3: Effect of SAC on testosterone production and liver function in Wistar rat.

In this section, the effect of SAC on Wistar rats after chronic supplementation was investigated. Primarily testosterone level in the plasma was analyzed, later some parameters associated with liver function were analyzed.

Methods and materials

Animal experiment

7 week old male Wistar rats were purchased from Japan SLC, In. The rats were maintained in steel cages with free access to laboratory F2 diet (Funabashi Farm Co., Funabashi, Japan.) and tap water. After one week of acclimation, rats were separated into 2 groups (n=8 in each group). Rats from the treatment group were given SAC by oral gavage in distilled water at 50 mg/kg body weight daily for 10 weeks. Rats from the control group were treated with distilled water only. The final volume of distilled water given by oral gavage was 2 mL/kg body weight. Testosterone level from the plasma was measured weekly for up to 8 weeks. After 10 weeks of SAC supplementation, rats were sacrificed by decapacitation. Whole blood was immediately collected and kept at room temperature for around 15-20 min to coagulate. Serum was prepared by centrifuging the blood at 2,000 x g, 4°C for 15 min. Serum was stocked at -65°C for further analysis. Markers associated with liver function were assessed by Oriental Yeast Co.

Weekly blood collection and plasma preparation

Before collecting blood, rats were anesthetized with isoflurane. Rats were placed on a heating pad at 39°C for 3-4 min. In the heating pad, the tail veins were observed for dilation. Blood was collected from the dilated lateral vein of the tail using a pre heparinized 26.5 G needle. Plasma preparation method was the same as described in study 1 of this chapter.

Testosterone extraction and measurement

Testosterone extraction and measurement methods from the plasma are the same as described in study 1 of this chapter.

Western blot method

Western blot method for determining the liver phosphorylated AMPK and total AMPK (Millipore) are the same as described in study 1 of this chapter.

Results

The mean plasma testosterone levels for the rats of the control up to 8 weeks were 2.0, 2.8, 1.8, 1.7, 0.9, 1.2, 1.8, and 1.3 ng/mL, respectively. On the other hand, plasma testosterone levels for the SAC treated rats were 1.6, 1.9, 2.3, 2.1, 1.2, 1.2, 1.4, 1.0 ng/mL, respectively (Figure 13). Testosterone level for every week was analyzed by students t-test. It was found that there was no significant change in testosterone level after chronic administration of SAC.

The body weight of the Wistar rat was taken every day during the experimental period. The weekly body weight changes are shown in Figure 14. After sacrificing the rats, the weight of the liver and testis were also taken (Figure 15). It was found that after chronic administration, SAC did not change the body, liver, and testis weight of the Wistar rat.

Serum parameters associated with liver function were analyzed in Wistar rats after chronic supplementation of SAC (shown in Table 2). It was found that SAC significantly reduced serum levels of glucose, TG, and ALP. However, the AST level in serum was increased after SAC treatment. Moreover, the liver AMPK protein level was measured by western blot method. It was found that SAC can significantly enhance the phosphorylation of AMPK whereas the total AMPK was unchanged (Figure 16).

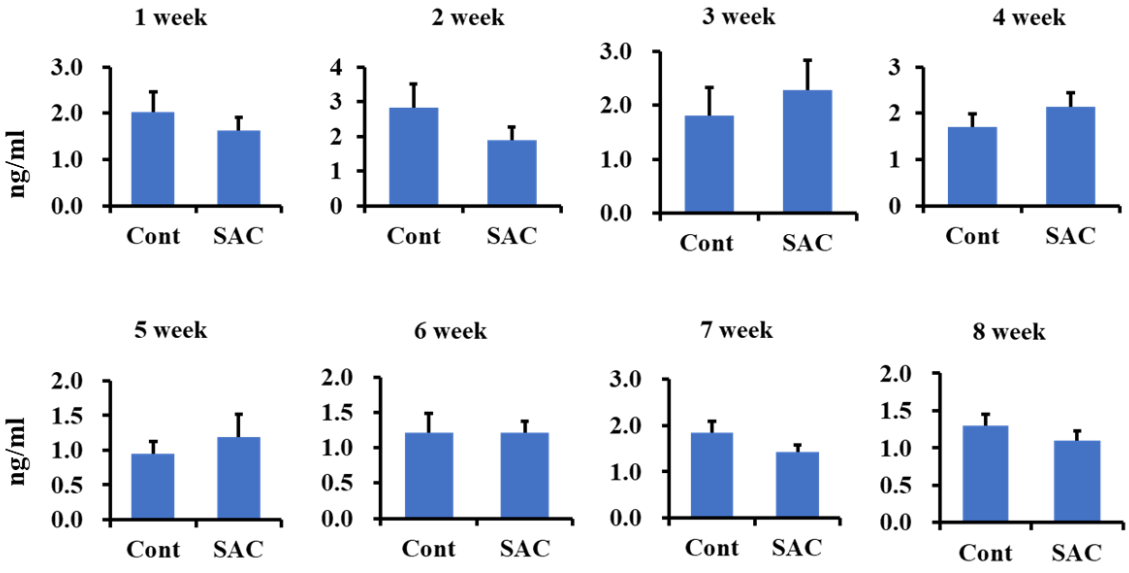

Figure 13. Effect of SAC on weekly testosterone level in the plasma of Wistar rat by ELISA method. Data are presented as the mean ± SE. n=8.

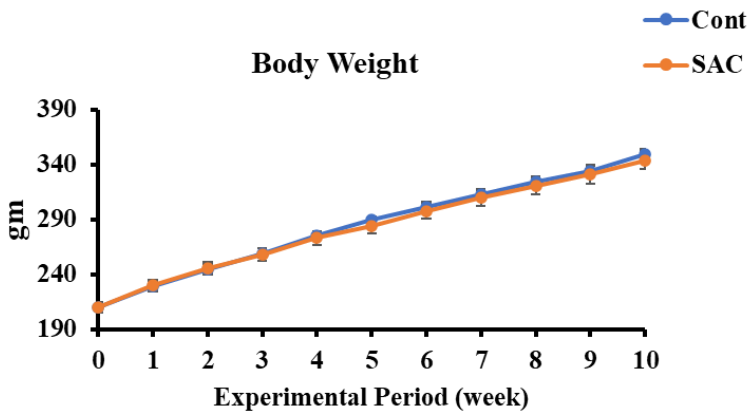

Figure 14. Effect of SAC on weekly body weight change of Wistar rat. Data are presented as the mean ± SE. n=8.

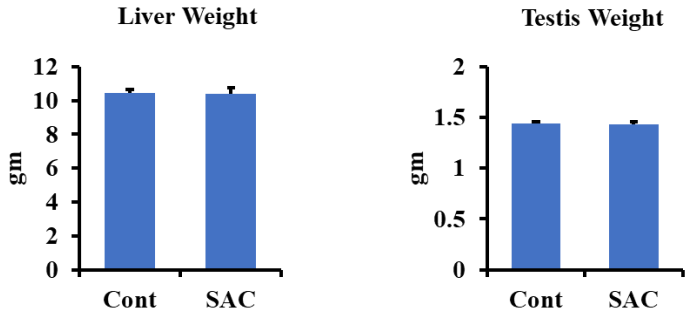

Figure 15. Effect of SAC on testis and liver weight of Wistar rat. Data are presented as the mean ± SE. n=8.

Table 2. Serum profiling of some parameters associated with liver function.

Parameters	Control	SAC
Total Protein (g/dL)	6.75±0.105	6.53±0.056
Albumin (g/dL)	4.688±0.069	4.662±0.026
Albumin to globulin ratio	2.288±0.079	2.512±0.077
Fe (µg/dL)	159.625±7.66	157.375±4.355
AST (IU/L)	253.25±16.3	336.88±25.671(*)
ALT (IU/L)	75.5±2.196	88.4±8.185
ALP (IU/L)	778.00±26.349	636.25±34.341(*)
LDH (IU/L)	1933.75±135.751	2275.00±172.047
Leucine aminopeptidase (IU/L)	59.75±1.146	58.63±0.905
Choline Esterase (IU/L)	12.13±1.156	11.25±0.675
Total cholesterol(mg/dL)	78.00±2.679	78.63±2.5
Free cholesterol (mg/dL)	19.3±0.648	18.5±0.707
Esterified cholesterol (mg/dL)	58.75±2.085	60.13±1.95
TG (mg/dL)	159.375±22.329	96.125±12.62 (*)
LDL-Cholesterol(mg/dL)	4.50±0.267	4.25±0.164
HDL-C cholesterol(mg/dL)	33.375±0.844	32.625±1.017
Total bilirubin (mg/dL)	0.028±0.006	0.026±0.005
Direct bilirubin (mg/dL)	0.014±0.003	0.01±0.003
Indirect bilirubin (mg/dL)	0.014±0.005	0.016±0.005
Total bile acid (µmol/L)	9.375±1.322	8.375±0.981
Glucose (mg/dL)	152.34±3.474	143.00±1.35(*)

Data are shown as mean ±SE. n=8. Data were analyzed by student's t-test. *P <0.05 vs control.

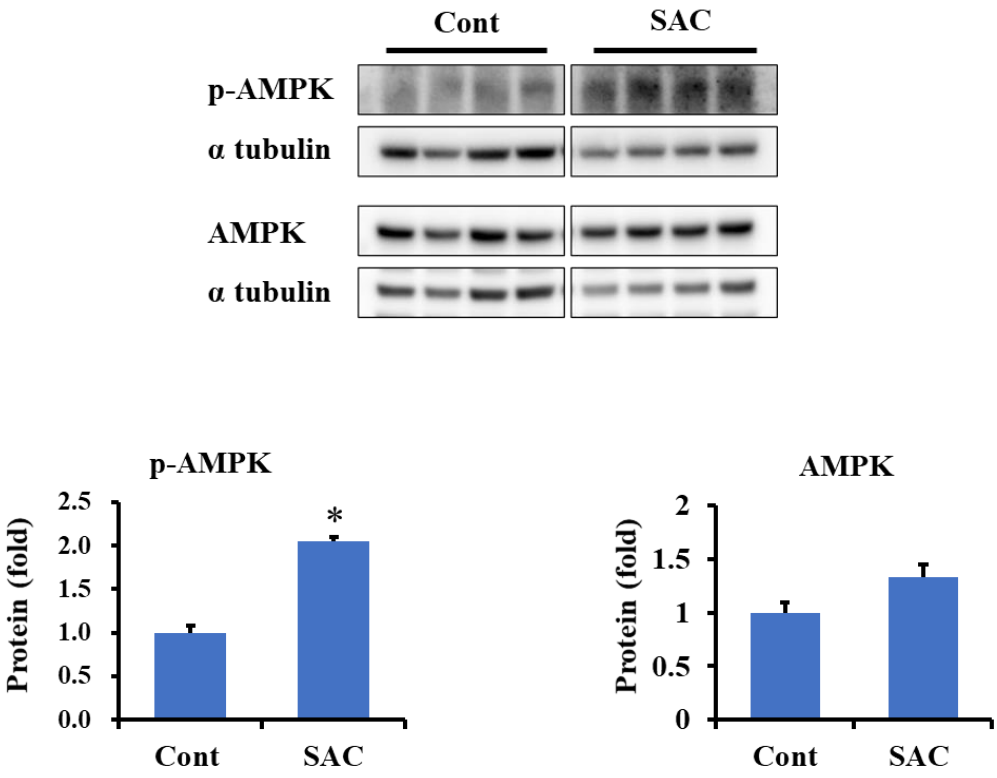

Figure 16. Effect of SAC on the level of p-AMPK and AMPK protein in the liver of Wistar rat. Western blot method. Data are presented as the mean ± SE. n=4, Data were analyzed by Student's t-test. * $p < 0.05$ compared to the control group.

Discussion

In this study, BALB/c mice were used as an animal model to study the steroidogenic effect by SAC in a single dose experiment. Previous study in our lab showed that biotin and other food ingredients can stimulate testosterone production in BALB/c mice in a single dose experiment. Therefore BALB/c mice were chosen as an animal model in this study. It was found that after single dose administration, SAC can enhance testosterone levels both in plasma and testis. Testosterone synthesis depends on the secretion of luteinizing hormone to the circulation (LH) from the pituitary gland [38]. Therefore, LH level in the plasma of BALB/c mice was also measured. However, SAC did not change the LH level in plasma, suggesting the direct role of SAC on the testis rather than the hypothalamus-pituitary axis.

To analyze the direct effect of SAC on steroidogenic cells, mouse testis derived I-10 cell was used for the cell-based experiment. I-10 cell produces steroid hormones and secretes the hormones to the culture medium. Various natural compounds have been reported to stimulate steroidogenesis in I-10 cells [44,45]. Therefore, in this study I-10 cell was chosen as a cell model for testosterone synthesis. In this study testosterone synthesis by I-10 cells was confirmed by using forskolin, a positive control for steroidogenesis. It was found that SAC can enhance testosterone production in I-10 cells without changing cell proliferation. Collectively these result from animal and cell-based experiment suggest that SAC directly stimulate the testosterone synthesis on steroidogenic cells of the testis.

Testosterone synthesis is firmly regulated by complex mechanisms in Leydig cells. PKA plays a key role in steroidogenesis as activated PKA phosphorylates various regulator proteins involved in steroidogenesis such as CREB and StAR. Therefore, in this study, the role of SAC on the level of PKA in testis and I-10 cells was investigated. It was found that SAC activates PKA in its phosphorylated form both in testis and I-10 cells without changing the

total PKA level. Classically, activation of PKA is mediated by the intracellular cAMP. However, for steroidogenesis, both cAMP-dependent and independent activation of PKA have been reported from previous studies [46]. Therefore, in this study, the intracellular cAMP level was measured after SAC treatment. It was found that SAC did not change the intracellular cAMP level after indicated incubation time. Therefore, the activation of PKA in I-10 cells by SAC might follow the cAMP-independent mechanism. For steroidogenesis, activated PKA phosphorylates CREB. Activated CREB is a transcription factor for other steroidogenic proteins. This study also found that SAC enhanced the level of phosphorylated CREB protein without changing the level of total CREB. mRNA level of *Cyp11a* and *StAR* gene were also measured by the qRT-PCR method. It was found that SAC can upregulate the mRNA level of *Cyp11a* but not the *StAR* after indicated incubation time.

CYP11A protein (the other name is P450 cholesterol side-chain cleavage enzyme) catalyzes the conversion of cholesterol to pregnenolone in the mitochondria, which is the rate-limiting step of all steroid hormone synthesis. Moreover, pregnenolone is the precursor of all steroid hormones. Pregnenolone and its other metabolites are considered as neurosteroids. Neurosteroids in the brain are essential for neuronal cell survival, proliferation, memory function, and cognition. Moreover, testosterone has been reported to be synthesized in the hippocampus [47]. As this study found that SAC affects to upregulate *Cyp11a* gene expression, therefore it is conceivable that SAC might affect the neurosteroids production in the brain.

In the I-10 cell, this study found that SAC did not change the *StAR* gene expression. StAR is the first regulatory protein in the steroidogenic pathway that transfers cholesterol to the inner mitochondria. StAR protein is phosphorylated for its maximal action by PKA. A previous study on the steroidogenic cell model demonstrated that both phosphorylation and transcription of StAR are regulated by different subtypes of PKA [48]. As PKA was found to be upregulated in this study, it is probable that in I-10 cells, StAR protein may be regulated by

the phosphorylation by PKA. However, measuring the phosphorylated StAR after SAC treatment in I-10 cells could clarify the mechanism of how StAR is regulated by PKA in I-10 cells for steroidogenesis.

A recent study showed that some cysteine sulfoxides (cyclo-alliin, propenyl cysteine sulfoxide) from onion extract could stimulate progesterone secretion in I-10 cells via activating the PKA signaling pathway [45]. Similar results were also found in this study by SAC treatment in I-10 cells. SAC shares some structural similarities with cysteine sulfoxides. Therefore, it is probable that SAC could be metabolized to cysteine sulfoxides or vice versa for the steroidogenic effect of these compounds. In the previous study, a relatively very high concentration of cysteine sulfoxides (100 to 1000 μM) showed the steroidogenic effect. On the contrary in this study low concentration of SAC (10 to 100 μM) showed the steroidogenic effect. So, the chance to metabolize SAC to cysteine sulfoxides in the present cell-based study could be very low. Previous pharmacokinetic studies in rats and dogs revealed that a very little amount of SAC could be metabolized to S-allyl cysteine sulfoxide (alliin) among cysteine sulfoxides compounds. Moreover, alliin has not been reported yet to have a steroidogenic effect. Therefore, in the present animal study the chance that cysteine sulfoxides contributing to steroidogenesis is also very low. However, it is also probable that some cysteine sulfoxides and SAC could be metabolized to a common metabolite to show steroidogenic effect and acting as prodrugs.

For the steroidogenic effect of SAC, PKA activation could be the most upstream regulator in this study. The direct activation of PKA has been suggested by various flavonoids for their insulinotropic effect [49]. Whether the activation of PKA by SAC is mediated by direct activation needs further study. As SAC is a potent antioxidant it is assumable that reducing the reactive oxygen species by SAC could activate PKA in this study. However, the opposite phenomenon was established in several studies as activation of PKA could reduce reactive

oxygen species. PKA has been reported to phosphorylate and inhibit the NADPH oxidases (NOX) in several studies to reduce the reactive oxygen species [50].

In the chronic dose experiment, SAC did not change the plasma testosterone level in the Wistar rats. However, it was found that SAC can modulate some markers associated with liver function. Testosterone and other steroid hormones are metabolized in the liver by phase 1 and phase 2 reactions [51]. Therefore, it is probable that enhanced testosterone could undergo metabolism in the liver after chronic administration of SAC.

Several studies on animal diseases models showed that SAC could ameliorate hyperglycemia and hyperlipidemia [52,53]. In this study, SAC reduced the serum TG level and glucose level after 10 weeks of chronic supplementation. Elevated serum level of TG is a risk factor for metabolic syndromes like cardiovascular diseases and hypertension. The liver is the primary site for lipid and glucose metabolism. AMPK is the master regulator of glucose and lipid metabolism via sensing the energy status. Therefore, the AMPK level in the liver was evaluated. SAC activated AMPK in the liver of Wistar rat without changing the total AMPK level. It is noteworthy that SAC and garlic extract already have been reported to activate AMPK in cell and animal-based studies [54,55]. Therefore, this study further strengthens the previous findings of the ameliorative effect of SAC on diabetes, hypertension, and cardiovascular diseases.

In this study, SAC at 50 mg/kg body weight was used in both single and chronic-dose animal experiments. As the content of SAC in fresh garlic is very low (20-30 µg/g fresh garlic), consuming garlic that is usually added to food may not exert the beneficial effect suggested in this study. To get 50 mg/kg bodyweight concentration, a male with 70 kg body weight needs at least 100 kg of garlic that is not possible to consume. However, as the content of SAC in garlic can be increased by several chemical processing it is better to use the extracted pure

SAC. Moreover, SAC is already sold in many countries as a dietary supplement including in Japan.

Several pharmacokinetic studies on SAC were extensively investigated on animal models like rats and dogs. SAC has high bioavailability, renal reabsorption, and limited metabolism. Furthermore, SAC from garlic in a clinical trial showed no apparent toxicity. In this study, it was found that long-term supplementation of SAC had not affected the body, liver, and testis weight. Therefore, SAC can be safely used for the treatment and prevention of hypogonadism and metabolic syndrome.

Chapter 3: Protective effect of S-allyl cysteine in HT-22 cells from glutamate-induced toxicity

In this chapter, the neuroprotective effect of SAC was investigated using mouse hippocampal-derived HT-22 cells.

Methods and materials

Cell culture method:

H-22 cells were purchased from Merck (KGaA, Darmstadt. Germany). For the maintenance, cells were cultured in high glucose DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biosera, Nuaillè, France), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma-Aldrich) in 10 cm dish in a humidified chamber at 37°C and 5% CO_2. The other cell culture conditions are the same as mentioned in study 2 of chapter 2. Cells were used for the experiment from the passage number between 5 to 10. Sodium glutamate (Wako Pure Chemical Corporation, Japan) was dissolved in water at 1M concentration and kept at 4°C for further use.

Cell proliferation assay

HT-22 cells were seeded in a 96 well plate at a density of 5×10^3 cells/well with 100 µL medium. Next, cells were incubated overnight followed by changing the medium with various concentrations of SAC or glutamate or both for 24 h. The other methods for cell proliferation assay were the same as described in study 2 of chapter 2.

Western blot method

HT-22 cells (5 x10^5 cells) were incubated into 6 cm dish overnight followed by changing the medium containing SAC or vehicle. After 3 h of incubation with SAC, glutamate. was added to the culture medium at 1.5 mM concentration and incubated for 8 h. Next, the medium was removed, and cells were washed with ice-cold PBS. 250 µL lysis buffer (10 mM HEPES at pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% SDS) containing phosphatase inhibitor and protease inhibitor was added directly to the dish. Cells with the lysis buffer in the dish were incubated on ice for 30 min. The other methods for protein extraction and western blot are the same as described in study 2 of chapter 1. Membranes were incubated with antibodies against ERK 1/2, p-ERK1/2, p38, p-p38, cleaved caspase 3, caspase-3 (Cell Signaling Technology), and BDNF (Abcam, Cambridge, UK).

Results

First, the cell proliferation of HT-22 cells by SAC was investigated by WST-1 method. SAC at 0, 1, 5,10, 20, 40 and 80 µM concentration was incubated for 24 h in HT-22 cell. The relative mean cell proliferation for the groups were 100.00, 109.67, 116.58, 113.33, 114.32,113.00, 104.90, and 108.21%, respectively (Figure 17.A). It was observed that SAC at these concentrations did not significantly change the cell proliferation of HT-22 cells. Cell proliferation was also measured after only glutamate treatment for 24 h incubation. The cell proliferation after 0, 1, 2, 3 mM glutamate treatment were 100.00, 60.46, 22.12, and 3.62% respectively (Figure 17.B). It was found that glutamate significantly decreased the cell proliferation dose-dependently. Next, HT-22 cells were co-treated with 1.5 mM glutamate and 20 to 80 µM SAC for 24 h. After only the glutamate challenge the cell proliferation significantly decreased from100.00% to 29.30%. The reduced cell proliferation is further enhanced by 20, 40, and 80 µM SAC to 34.70, 45.55, and 51.97% respectively (Figure 18).

The MAPK proteins like ERK1/2 and p38 proteins were analyzed by western blot method after 8 h of glutamate challenge in HT-22 cell. 1.5 mM glutamate significantly upregulated the phosphorylated ERK1/2, whereas 3 h preincubation with SAC at 40 and 80 μM significantly downregulated this level. A similar result was also found for the p-38 protein level (Figure 19). Furthermore, brain-derived neurotrophic factor (BDNF) protein level by western blot was measured. Glutamate significantly enhanced the pre-BDNF level and decreased the mature BDNF level. SAC significantly reversed the BDNF level in HT-22 cells (Figure 20).

Glutamate-induced cell death was reported to be mediated by the caspase 3 dependent apoptosis pathway. Therefore, the caspase 3 level was measured to clarify the type of cell death after 8 h of glutamate challenge in HT-22 cells. Cleaved caspase 3 protein was not detected at this period. Besides the level of caspase 3 protein was not changed after glutamate treatment (Figure 21).

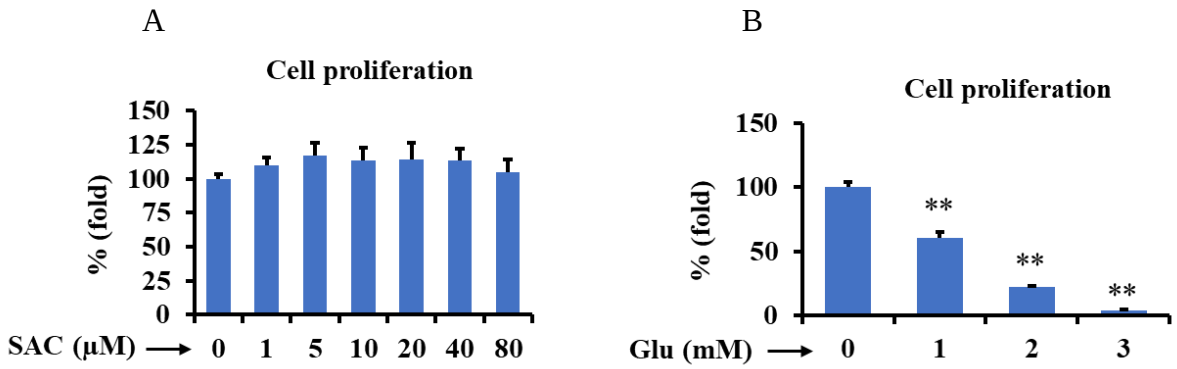

Figure 17. Cell proliferation assay by WST-1 method in HT-22 cells. SAC or glutamate was treated in I-10 cells for 24 h. A. n=6. B. n=4. Data are shown as mean ± SE. Data were analyzed by Tukey test. **P<0.01 vs 0 mM glutamate.

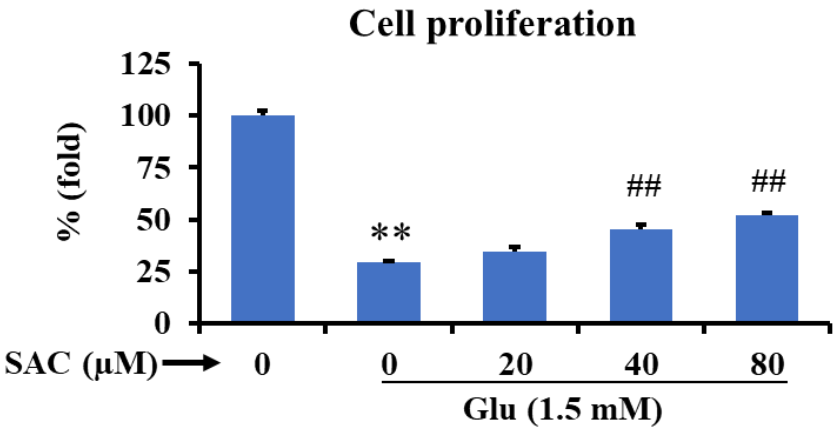

Figure 18. Cell proliferation of HT-22 cells. WST-1 method. SAC and glutamate were treated in I-10 cells for 24 h. n=6. Mean ± SE. Tukey test. **P<0.01 vs 0 mM glutamate, ##P<0.01 vs glutamate only.

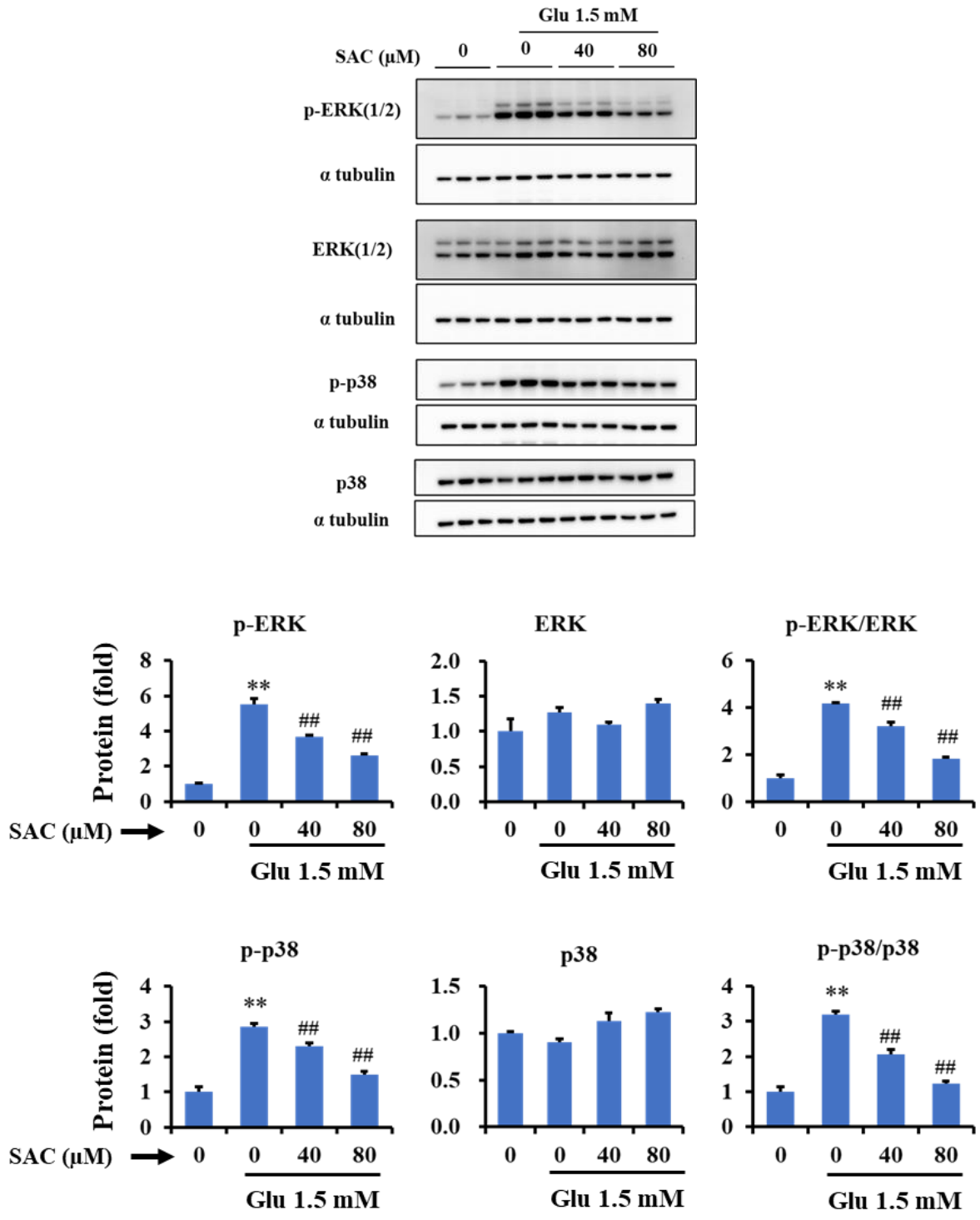

Figure 19. MAPK protein level in HT-22 cells. Western blot method. n=3. Mean ± SE. Tukey test. **P<0.01 vs 0 mM glutamate, ##P<0.01 vs glutamate only.

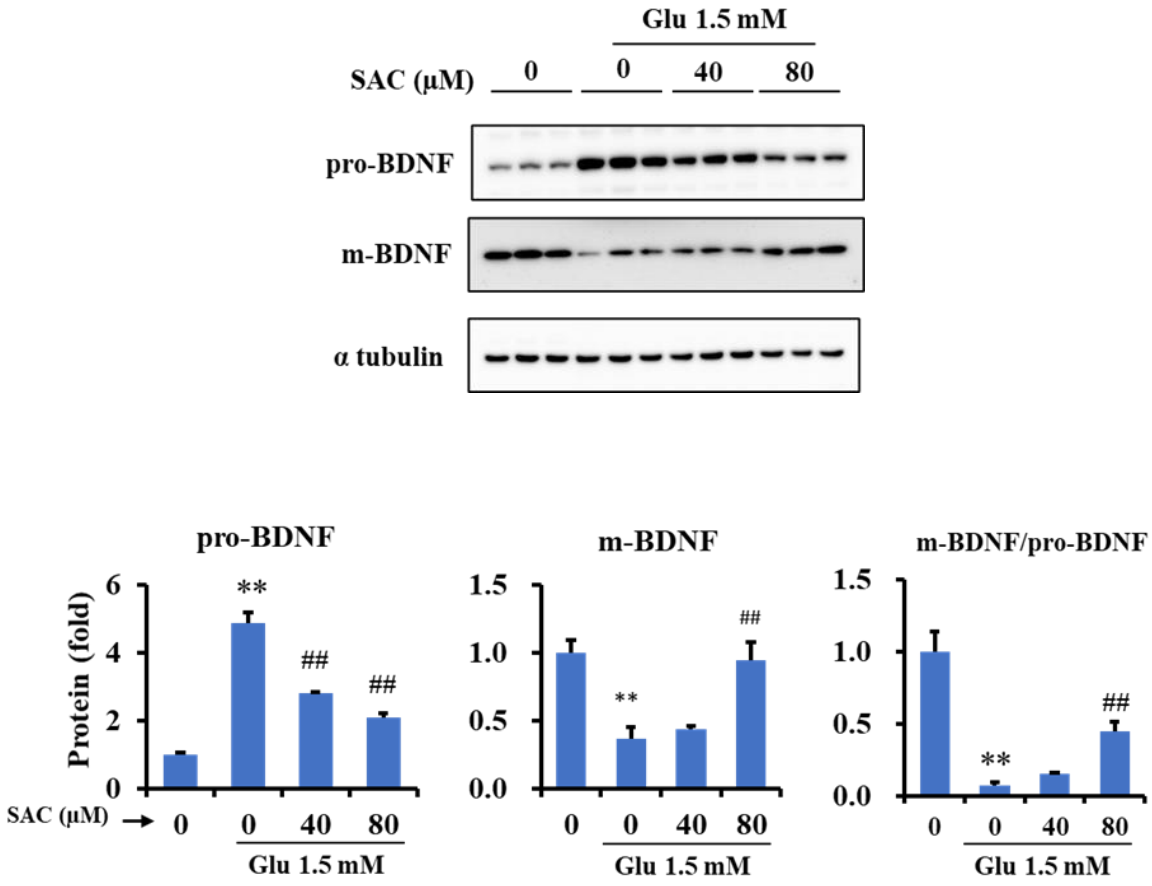

Figure 20. BDNF protein level in HT-22 cells. Western blot method. n=3. Mean ± SE. Tukey test. **P<0.01 vs 0 mM glutamate, ##P<0.01 vs glutamate only.

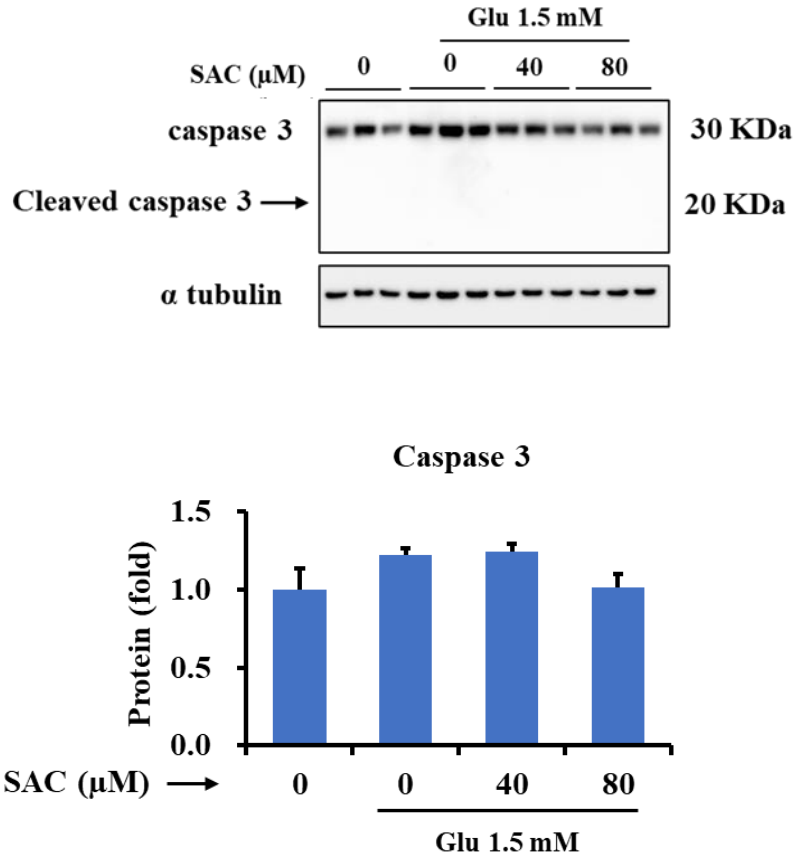

Figure 21. Caspase 3 protein level in HT-22 cells. Western blot method. n=3. Mean ± SE.

Discussion

HT-22 cell is a popular neuronal cell model to investigate glutamate-induced neurotoxicity. Therefore, in this study HT-22 cell was chosen as a cell model to investigate the role of SAC on neuronal cell survival. Similar to the previous studies this study also found that glutamate dose-dependently reduced the cell proliferation of HT-22 cells. Furthermore, this study found that SAC protected the HT-22 cell from glutamate-induced toxicity where SAC did not show any cytotoxic effect.

Mitogen-activated protein kinases (MAPK) like ERK, p38, and JNK are serine-threonine protein kinases that are activated by various stimuli like growth factors or stress in the neuronal cells of the central nervous system. Both the survival and apoptosis of neural cells by MAPKs were reported [56]. In HT-22 cells several studies demonstrated that glutamate stimulated the MAPKs and persistent activation of MAPKs induces cell death [39]. In agreement with the previous findings, this study also found that glutamate upregulated the phosphorylation of ERK1/2 and p38 proteins. Furthermore, SAC inhibited the activation of these proteins. Previous studies already demonstrated that SAC could inhibit MAPKs proteins in culture neurons or brain tissues upon various stresses [57]. Reactive oxygen species were reported as a stimulator of MAPKs in HT-22 cells. As SAC is an antioxidant, inhibition of MAPKs is probably attained by reducing the reactive oxygen species in HT-22 cells.

Brain-derived neurotrophic factor (BDNF) is a key regulatory protein in the central nervous system to promote the cell survival, differentiation, and plasticity of neurons during development. The level of BDNF has been reported to be reduced in various pathological conditions such as Alzheimer’s disease, Parkinson's disease, and Huntington's disease. Hippocampus is the major site for BNDF synthesis in the brain and participates in memory function and cognition. BDNF protein undergoes several translational modifications after

synthesizing transcriptionally and translationally. The active or mature form of BDNF (mBDNF) is produced from its precursor pro-BDNF. While mBDNF has a protective effect on neurons, the pro-BDNF is involved in neuronal apoptosis [58]. Previous study on HT-22 cells showed that glutamate-induced oxidative stress suppressed mBDNF production [59,60]. Therefore, in this study, the protein level of BDNF was measured. It was found that the pro-BDNF level was increased after glutamate treatment where the mBDNF level was decreased. Treatment with SAC significantly reversed this effect, Therefore, the protection of HT-22 cells by SAC might be attributed to the processing of BDNF. A recent study also suggested that SAC could restore BDNF in rat cortical slices after oxidative stress [61].

A previous pharmacokinetic study on rats revealed that SAC can readily cross the blood-brain barrier [62]. A previous study also suggested that SAC can enter to cell via alanine serine cysteine transporter 1 [63]. Taken together, from this study it can be concluded that SAC can be safely used as a supplement for neuroprotection.

Chapter 4: Conclusion

The present study aimed to identify novel beneficial effects of a garlic-derived compound, S-allyl cysteine (SAC), for the amelioration of age-related diseases. For this purpose, testosterone synthesis and neuroprotective effect by SAC were investigated by using animals and cell models.

For the study of testosterone synthesis by SAC, BALB/c mice and Wistar rats were used as animal models whereas mouse testis-derived I-10 cells were used as cell models. In BALB/c mice, SAC enhanced testosterone level both in plasma and testosterone-producing organ testis after a single dose administration. SAC also enhanced testosterone production in I-10 cells without any cytotoxicity. SAC activated the PKA, essential for the steroidogenesis, in both I-10 cells and testis of BALB/c mice although SAC did not affect the intracellular cAMP level in I-10 cells. SAC also activated the CREB, the target of PKA, in I-10 cells. Furthermore, SAC enhanced the expression of the *Cyp11a* gene in I-10 cells. CYP11A protein is the first rate-limiting enzyme for all steroid hormone synthesis. Thus, the cAMP-independent activation of PKA might be responsible for the enhancement of testosterone by SAC. However, after chronic supplementation of SAC in Wistar rats, the testosterone level in the plasma remained unchanged. Additionally, SAC showed reducing the level of glucose and TG in the serum of these rats, activated liver AMPK, suggesting its role to improve age-related metabolic syndrome.

For the study of neuroprotection by SAC, mouse hippocampal-derived HT-22 cell model, widely used for glutamate-induced neurotoxicity, was investigated. Glutamate dose-dependently reduced the cell viability of HT-22 cells. This cytotoxicity was further improved by SAC. SAC also inhibited the glutamate-induced activation of ERK1/2 and p38 MAPK proteins that are responsible for the cell death of HT-22 cells. Furthermore, SAC upregulated

the mBDNF protein after glutamate-induced suppression, suggesting its role to protect neuronal cells via the BDNF signaling pathway.

In conclusion, this study shows that SAC has the potential to improve testosterone decline in males and to prevent glutamate-induced toxicity. SAC has a therapeutic potential to use as a dietary supplement to treat age-related diseases and neurodegenerative diseases as well.

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