Research Article

Effect of lactic fermentation products on human epidermal cell differentiation, ceramide content, and amino acid production

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Short Title: Effect of lactic fermentation products on cell differentiation

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Abstract

Introduction: Lactic fermentation products (LFPs) are thought to affect “good” bacteria in the gut. We previously reported that oral administration of LFPs has beneficial therapeutic effects in a mouse model of atopic dermatitis. However, it is unclear how LFPs affect human epidermal cell differentiation, ceramide and amino acid production.

Objective: The aim of this study was to determine the effects of LFPs on epidermal cell differentiation, by assessing amino acid and ceramide production.

Methods: A three-dimensional cultured human epidermis model and normal human epidermal keratinocytes were used. Cytotoxicity tests were performed using alamar Blue. Transepidermal water loss (TEWL) was used as an index to assess barrier function. Keratin 1 (K1), keratin 5 (K5), keratin 10 (K10), involucrin (INV), calpain 1, and transglutaminase (TGase) (markers of differentiation) and profilaggrin (proFLG) and bleomycin hydrolase (BH) (amino acid synthesis–related genes) expression levels were quantified by RT-PCR. In addition, TGase protein levels were measured by western blotting. The intercellular lipid content of the stratum corneum was measured by high performance thin layer chromatography (HPTLC). Amino acids were quantified using an amino acid analyzer. Finally, bound water content in the stratum corneum was measured by differential scanning calorimetry (DSC).

Results: Cell viability did not change, but TEWL was significantly decreased in the cells treated with LFPs compared with the control cells. Treatment with LFPs significantly increased expression of the late differentiation markers INV and TGase at the RNA level. Furthermore, TGase protein expression was significantly increased by treatment with LFPs. Treating a three-dimensional cultured epidermis model with LFPs significantly increased the intercellular lipid content of the stratum corneum and production of the amino acid Arg. The amount of bound water in the stratum corneum was increased significantly in the LFP application group.

Conclusion: Treatment with LFPs promotes human epidermal cell differentiation and increases the intercellular content of the free fatty acid, Chol, Cer [NS], Cer [AS], and Cer [AP]. This may result in improved skin barrier function. The increased amount of Arg observed in keratinocytes may help improve water retention.

Introduction

Intercellular lipids in the stratum corneum are generated in the stratum granulosum and accumulate in lamellar granules [1]. These lipids are important for maintaining skin barrier function [2, 3]. Ceramide (Cer) accounts for 50% of the intercellular lipids in the stratum corneum and is important for water retention within this epidermal layer [4]. Amino acid production and Cer production, which are closely related to differentiation, are important for moisturizing the epidermis. Normal cell differentiation helps maintain skin barrier and moisturizing functions. Epidermal moisture is maintained in part by stratum corneum lipids, sebum film, and natural moisturizing factor (NMF) [5-8], which is composed of amino acids and their derivatives [9]. In the epidermis, epidermal keratinocytes originate in the basal layer and undergo changes in their morphology as they migrate to the epidermal surface in a process known as differentiation. The epidermis contains four distinct layers formed by keratinocytes at different stages of differentiation: the stratum corneum, the granular layer, the spiny layer, and the basal layer. Epidermal NMF and amino acids are biosynthesized as follows. ProFLG, which is typically contained in keratohyalin granules, is extruded into the stratum corneum during differentiation [10]. The proFLG is converted to filaggrin monomers via dephosphorylation and processing, which is then metabolized by bleomycin hydrolase [11]. Recently, it has been published on the relationship between stratum corneum lipid loss and drying rates [12], ceramide and filaggrin biosynthesis [13], and filaggrin and NMF [14].

Intestinal flora are important for human health [15]. More than 100 species and 100 trillion bacteria make up the intestinal flora in the large intestine [16]. It is generally known that intestinal bacteria comprise both “good” bacteria and “bad” bacteria, as well as opportunistic bacteria that do not belong to either category [17, 18]. A balanced microbiome is important for good health [19], and improving the intestinal environment can improve the condition of the skin [20, 21]. Bifidobacteria and lactic acid bacteria are representative examples of good gut bacteria. Lactic acid bacteria help suppress the growth of bad bacteria such as *Escherichia coli* in the intestine, thereby balancing the intestinal bacterial population. However, lactic acid bacteria are typically acquired by ingestion, and are different from intestinal bacteria, so they can struggle to grow in and colonize the intestine. In this study, we focused on lactic fermentation products (LFPs). LFPs do not act on the intestinal flora; rather, they act directly on the body to promote a balanced intestinal flora population.

Previously, we reported that oral administration of LFPs to a mouse model of atopic dermatitis improves the water content of stratum corneum, reduced transepidermal water loss (TEWL), and decreased epidermal thickness [22]. However, the effects of LFPs on human epidermal cell differentiation and Cer production have not yet been investigated.

In this study, we focused on the effects of LFP on epidermal cell differentiation. Epidermal keratinocytes and a three-dimensional cultured human epidermis model were treated with LFPs, and the expression levels of genes and proteins related to differentiation and NMF were measured. In addition, the effects of LFPs on amino acid, Cer production during differentiation and bound water contents in stratum corneum were also assessed. The overall goal of this study was to clarify the effect of LFPs on human epidermal cell differentiation.

Materials and Methods

Materials

Palmitic acid, cholesterol, and Amino Acids Mixture Standard Solution, Type H were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RNAiso Plus, TB Green Premix EX Taq (Tli RNAaseH Plus), and PrimeScript RT Reagent Kit were purchased from Takara Bio, Inc. (Kusatsu, Shiga, Japan). Cer [NS] and Cer [AS] were obtained from Matreya (Pleasant Gap, PA, USA). Cer [NP] and Cer [AP] were purchased from Evonik (Relinghauser, Essen, Germany). alamar Blue was purchased from Invitrogen, Inc. (Carlsbad, CA, USA). The anti-TGase antibody (12912-3-AP) was purchased from Proteintech, Inc. (Chicago, IL, USA). β-actin (13E5) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The donkey anti-rabbit IgG HRP-labeled secondary antibody was purchased from GE Healthcare, Inc. (Chalfont, UK). Other reagents were of analytical grade and used without further purification.

Preparation of lactic fermentation products (LFPs)

LFPs were prepared by the Koei Science Laboratory Co., Ltd. (Wako, Saitama, Japan); a detailed description can be found in a previous report [22]. Briefly, inoculated with 35 strains of 16 species of lactic acid bacteria were added to soymilk, which was then fermented at 37 °C for 120 hours and sterilized. Next, the solution was centrifuged at 12,000 × g at 4 °C for 15 minutes (in a tabletop micro-cooled centrifuge 3500, Kubota Corporation, Tokyo, Japan) to obtain the LFPs.

Cell culture

Normal human epidermal keratinocytes (NHEK, Thermo Fisher Scientific K. K., Waltham, MA, USA) were cultured in HuMedia-KG2 (KURABO Industries Ltd, Osaka, Japan) at 37 °C with 5% CO_2. LFPs were added to the medium at various concentrations (0.01, 0.1, 1% (v/v)), and the cells were cultured for 24, 48, and 72 hours. The medium was changed daily.

Three-dimensional cultured human epidermis models (LabCyte EPI-MODEL 6D) were purchased from Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan). LFPs were added to the supplied medium at various concentrations (0.01, 0.1, 1.0% (v/v)), and the models were cultured for 7 days. The medium was changed daily.

Cell viability

The three-dimensional cultured human epidermis models were cultured for 7 days in medium containing various concentrations of LFPs. To measure cell viability, an alamar Blue solution diluted 10-fold with the medium was added to the culture medium, the model was re-incubated for 3 hours, and the fluorescence intensity (Ex: 570 nm /Em: 585 nm) was measured using a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA).

Measurement of transepidermal water loss (TEWL) and conductance of cultured human epidermis

The three-dimensional cultured human epidermis models were allowed to keep on a clean laboratory bench at room temperature for 45 minutes [23-25]. TEWL was measured using a VAPOSCAN AS-VT100RS (Asch Japan Co., LTD, Tokyo, Japan). Conductance was measured using an ASA-MX100 (Asch Japan Co., LTD)

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from NHEK cells and the three-dimensional cultured human epidermis models using RNAiso Plus (Takara Bio). The reverse transcription reaction solution was prepared using a PrimeScript RT Reagent Kit (Takara Bio, Kusatsu, Shiga, Japan), and the reverse transcription reaction was performed using a thermal cycler (Veriti 96-well Thermal Cycler, Applied Biosystems, Foster City, CA, USA). Then, cDNA was prepared. Real time PCR was performed using TB Green Premix EX Taq (Tli RNaseH Plus, Takara Bio). The following primer sets were used: Gapdh forward 5'-GAAGGTGAAGGTCGGAGT -3', Gapdh reverse 5'- GAAGATGGTGATGGGATTTC -3', K1 forward 5'-

CACTTATTCCGGAGTAACCAG -3', K1 reverse 5'- GAATAGGATGAGCTAGTGTAA -3', K5 forward 5'-GAGCTGAGAAACATGCAGGA -3', K5 reverse 5'- TCTCAGCAGTGGTACGCTTG -3', K10 forward 5'-CCATCGATGACCTTAAAAATCAG -3', K10 reverse 5'- GCAGAGCTACCTCATTCTCATACTT -3', INV forward 5'- CGCCAAATCCGCACCAAGGTC -3', INV reverse 5'- GAAGCAGGGTCCAGCTGTGAA -3', TGase forward 5'- TCTGTAACCCAGAGCCTTCGA -3', TGase reverse 5'- TCTTCAAGAACCCCCTTCCC -3', CAPN1 Forward 5'- TCTGTAACCCAGAGCCTTCGA -3', CAPN1 reverse 5'- TCTGTAACCCAGAGCCTTCGA -3', BH forward 5'- GTACTTGTGCTGGGGCCTAG -3', BH reverse 5'- TGGCCCCATAACACCCTTGG -3', proFLG forward 5'-CCATCATGGATCTGCGTGG -3', proFLG reverse 5'- CACGAGAGGAAGTCTCTGCGT -3'. The reactions were performed using a Step One Plus ™ Real Time System (Applied Biosystems). The results were normalized to GAPDH and analyzed using the ΔΔCt method.

Quantification of TGase1 protein by western blotting

After separating the proteins by SDS-PAGE, the gel was inserted into a membrane kit (Trans-Blot Turbo Transfer Pack, BIO-RAD) and transferred using a transfer device (Trans-Blot Turbo System, BIO-RAD). After transfer, the membrane was treated with a blocking buffer, then treated with the a TGase1 antibody (Proteintech Group, Chicago, IL, USA, b-actin (13E5) Rabbit mAb; Cell Signaling Technology Inc., Danvers, MA, USA) for 24 hours. After the primary antibody reaction, the membrane was washed with TBS-T (1% Tween-20, 0.13 M NaCl, 2.0 mM Tris-HCl), and the secondary antibody (ECL Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody from Donkey (GE Healthcare, Inc.) was added and allowed to react with the membrane for 1 hour. After the secondary antibody reaction, the membrane was washed with TBS-T, and the protein bands were detected using a detection reagent (ECL Prime western blotting detection, BIO-RAD). The images were analyzed with Image Lab ™ Software (BIO-RAD).

Lipid extraction

The three-dimensional cultured human epidermis models were collected from the transwell, and a chloroform:methanol solution (2:1, v/v) was added. The solution was sonicated for 10 minutes using a Sonifire 250 Advanced sonicator (Branson, CT, USA) under the following conditions: output control: 3, duty cycle: 100%. The solution was filtered through a filter (SLG033NB, Merck Milipore Ltd, Billerica, MO, USA), the organic solvent was removed under nitrogen gas, and the solution was redissolved in a chloroform:methanol solution (2:1, v/v).

153 Separation and quantification of lipids by high-performance thin-layer chromatography

154 The extracted lipid solution (10 µL) was spotted to HPTLC Silica gel 60 (Merck KGaA, Darmstadt,
155 Germany) using a capillary pipette (Ringcaps, Hirschmann, Eberstadt, Germany). The samples were
156 developed twice: for Cer, using chloroform:methanol:acetic acid (190:9:1, v/v) [4, 26]; for free fatty
157 acid and Chol, hexane:diethyl ether:acetic acid (80:20:1, v/v) [27, 28]. The HPTLC plate was dried and
158 sprayed with a staining solution (10% copper sulfate/8% phosphoric acid reagent). The lipids were
159 visualized by heating on a hot plate (TLC Plate Heater, Camag, Muttenz, Switzerland) at 180 °C for 10
160 minutes. The obtained bands were photographed with ChemiDoc (BIO-RAD) and quantified using
161 Image Lab Software (BIO-RAD). The quantitative value was calculated from a calibration curve for
162 each lipid obtained from the same plate.

163

164 Determination of the amino acid content of the three-dimensional cultured human epidermis models

165 The three-dimensional cultured human epidermis models were collected from the transwell and
166 minced with scissors in PBS (-). An equal amount of 5-sulfosalicylic acid dihydrate solution was added,
167 and the mixture was centrifuged for 15 minutes. The amino acid content of the supernatant was
168 quantified using a JLC-500/V (JEOL Ltd., Tokyo, Japan). Amino acid analysis was performed with the
169 ninhydrin reagent set on an automated amino acid analyzer dedicated to reagent/biological amino
170 acid analysis. Five lithium citrate buffers (P-21, pH 2.98; P-12, pH 3.28; P-13, pH 3.46; P-14, pH 2.83;
171 P-15, pH 3.65) and a lithium hydroxide aqueous solution (P-19) were used. An LCR-7 (4.0 × 75 mm,
172 JEOL Ltd) column was used as the pre-column, an LCR-6 (4.0 × 120 mm, JEOL Ltd) column was used as
173 the separation column, and the flow rate was 0.43 mL/min. The absorbance of each peak was
174 measured at 570 and 440 nm. For the amino acid standard solution, a calibration curve was prepared
175 using Amino Acids Mixture Standard Solution (concentration range= 0.01 to 0.1 µmol/mL), Type H
176 (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the amino acid content was
177 corrected for the weight of the epidermis.

178

179 Isolation of the stratum corneum

180 Three-dimensional cultured skin (LabCyte Epimodel 6D, Japan Tissue Engineering Co., Ltd.) was
181 soaked in a 0.25% trypsin/EDTA solution overnight at 4 °C and the stratum corneum was removed.
182 The stratum corneum was washed with Milli-Q water, decompressed to 100 hPa in a vacuum
183 desiccator, dried until there was no weight loss (4 days), and then placed in a container with a
184 saturated potassium sulphate solution (98% relative humidity) until there was no weight gain (6 days).

The moisture-absorbed stratum corneum was placed in an aluminum pan, weighed, fitted, weighed again, and stored overnight in the container to eliminate the effect of moisture desorption from the DSC measurement.

Differential scanning calorimetry (DSC)

The instrument was a DSC 7020 (Hitachi High-Tech Corp., Tokyo, Japan). DSC measurement was performed as follows. The temperature was lowered from room temperature to -40 °C at -5 °C/min, maintained for 10 min, and then raised to 180 °C at 5 °C/min.

Analysis and calculation of bound water

The analysis was based on a previous report [29, 30]. Endothermic peaks detected around 0 °C corresponded to the melting of free water. The measured endothermic peak area was divided by 334.6 J/g to obtain free water. Bound water was determined from the weight of the stratum corneum before DSC to the difference in weight of the stratum corneum after DSC and then from the difference in the weight of free water. Bound water in the stratum corneum was calculated as the percentage of the weight of the stratum corneum.

Data analysis

All results are shown as mean and standard deviation. Statistical analysis was performed by Dunnett's multiple comparison test using JMP Pro 14 (SAS Institute, Cary, NC, USA).

Results

Evaluation of the effects of LFP on cell viability and epidermal barrier function

Three-dimensional cultured human epidermis was cultured in medium containing various concentrations of LFPs (0, 0.01, 0.1, 1.0%) for 7 days, and cell viability was evaluated using alamar Blue. There was no difference in cell viability between the tissues that were treated with LFPs and the control tissue (Fig. 1a). Next, the effect of LFPs on epidermal barrier function was evaluated by measuring the TEWL (Fig. 1b). The TEWL was significantly decreased in tissue treated with 1% LFPs compared with the control tissue ($p < 0.05$). Conductance increased in an LFP concentration-dependent manner (Fig. 1c), and was significant in the 0.1% and 1% addition groups ($p < 0.05$).

215

216 Effect of LFP on the expression of differentiation- and amino acid production–related genes in three-
217 dimensional cultured human epidermis

218 The expression of *K1*, *K10*, *K5*, *INV*, *TGase*, and *CAPN1* in the epidermis were measured by real time
219 PCR. Treatment with LFPs decreased expression of the early differentiation markers *K1* and *K10*
220 compared with the control tissue, while expression of the undifferentiated marker K5 did not change.
221 The expression of the late differentiation markers *INV* and *TGase* increased with increasing LFP
222 concentration, and was significantly higher in the tissue treated with 1.0% LFP compared with the
223 control tissue ($p < 0.05$). The expression of *CAPN1*, an amino acid production–related gene, was
224 significantly increased in the tissue treated with 1.0% LFP with the control tissue (Fig. 2a, $p < 0.05$).

225

226 Effect of LFPs on amino acid production-related gene expression in normal human epidermal
227 keratinocytes

228 The *proFLG* and *BH* gene expression levels were measured by real time PCR. *ProFLG* expression was
229 significantly higher in cells that had been treated with LFP for 72 hours compared with the control
230 cells (Fig. 2b, $p < 0.05$).

231

232 Effect of LFPs on epidermal differentiation-related protein expression in three-dimensional cultured
233 human epidermis

234 The level of TGase expression was determined by western blotting. TGase expression was
235 significantly higher in the tissue treated with 1.0% LFP compared with the control tissue (Fig. 3, $p <$
236 0.05).

237

238 Effect of LFPs on lipid content in three-dimensional cultured human epidermis

239 Free fatty acid content was significantly increased in the tissues treated with 0.1% or 1.0% LFPs, and
240 the cholesterol content was significantly higher in the tissue treated with 1.0% LFPs. Furthermore,
241 Cer [NS], Cer [AS], and Cer [AP] contents were significantly higher in the tissue treated with 1.0% LFPs
242 (Fig. 4 and 5).

243

244 Effect of LFPs on amino acid content in three-dimensional cultured human epidermis

The serine (Ser), glycine (Gly), and alanine (Ala) contents did not change compared with the control group. In contrast, the histidine (His) content of the tissue treated with 0.01% LFPs was significantly higher, and the arginine (Arg) content increased significantly in an LFP concentration–dependent manner (Fig. 6).

Effect of LFPs on the bound water content in the stratum corneum of three-dimensional cultured skin

The bound water content in the stratum corneum of three-dimensional cultured skin was increased with the concentration of LFPs. In particular, the bound water content was increased significantly in the 1% LFP application group compared with the control group (Fig. 7).

Discussion/Conclusion

This study showed that LFPs affect epidermal cell differentiation and are involved in Cer and amino acid production. Our results also confirmed that LFPs are not cytotoxic, as the three-dimensional cultured human epidermis treated with 0.01 to 1.0% LFPs exhibited no signs of cytotoxicity.

Three-dimensional cultured human epidermis was cultured in medium containing LFP, and the expression levels of differentiation-related genes and proteins were measured. The stratum corneum is formed by the proliferation and differentiation of epidermal keratinocytes [31, 32]. In the epidermis, K5 is expressed in the basal layer [33]. As cells enter their differentiation program, the transcription of these keratins is diminished and the pro-differentiation markers K1 and K10 are expressed in the spinous layer [34], and the differentiated granule layer expresses the late-differentiation markers INV and loricrin (LOR) [35, 36]. Late differentiation involves TGase expression and activation, and is essential for the formation of the corneocytes and cornified envelope in the stratum corneum. A balance between epidermal keratinocyte proliferation and differentiation is essential for maintaining the integrity of the skin barrier and preventing skin disease [31, 35, 37]. Treating cells with LFPs decreased the expression of *K1* and *K10* compared with the control group. In contrast, the *K5* gene expression level did not change. *INV* and *TGase* gene expression levels increased in a concentration-dependent manner, and were significantly higher in cells treated with 1.0% LFPs ($p < 0.05$). In addition, *CAPN1* expression was significantly higher in cells treated with 1.0% LFP ($p < 0.05$) (Fig. 2a). TGase protein expression was significantly higher ($p < 0.05$) in cells treated with 1.0% LFP 1 compared with the control cells (Fig. 3). The increases in *INV* gene expression and TGase gene and protein expression suggest that treatment with LFP for 7 days transitioned proliferation to differentiation, eventually increasing expression of differentiation factors near the

granular layer. TGase has been reported to be partially degraded by intracellular calcium-dependent proteases (CAPNs), increasing its sensitivity to activation by calcium, which is required for catalytic reactions [38]. Thus, it is possible that the LFP-induced increase in CAPN1 expression activated TGase, resulting in enhanced cell differentiation and improved barrier function.

Transepidermal water loss (TEWL) was measured after treating a three-dimensional cultured human epidermis model with LFPs. TEWL is a well-established index of skin barrier function [39, 40], and improved skin barrier function correlates with lower TEWL. Stratum corneum lipids such as Chol and Cer are important for maintaining this barrier. The loss of some or all of these lipid components destroys the lamellar structure between stratum corneum cells. As a result, water molecules can move freely between stratum corneum cells, and the TEWL increases. We found that the TEWL was significantly lower in tissue treated with 1.0% LFP compared with the control tissue ($p < 0.05$) (Fig. 1b). These results suggest that treatment with 1.0% LFP improves epidermal barrier function. It is known that the conductance of the stratum corneum is related to the water content of the stratum corneum [41]. In the present study, the conductance of the stratum corneum was improved following the application of LFP to the three-dimensional cultured skin. This suggests that LFP increased the water content of the stratum corneum. Furthermore, the amount of Chol was significantly higher in the tissues treated with 0.1% or 1.0% LFP, and the amount of free fatty acid was significantly higher in the tissue treated with 1.0% LFP. Cer [NS], Cer [AS], and Cer [AP] were present in significantly higher amounts in the tissue treated with 1.0% LFP (Fig. 4 and 5). These results suggest that LFP enhanced epidermal differentiation, increased the lipid content of the stratum corneum, and resulted in improved skin barrier function.

Because LFPs appeared to promote differentiation, we expected the amount of amino acids present in the epidermis to change, and therefore assessed the amount of amino acids present in three-dimensional cultured human epidermis. The amino acids present in the greatest amounts in the stratum corneum are Ser, Gly, Arg, and Ala [42]. Therefore, in this study we assessed these four amino acids as indicators of NMF. Glutamate (Glu), a precursor of PCA, and His, a precursor of transurocanic acid, were also measured using the same method. We found that the Ser, Gly, and Ala contents of tissues treated with LFPs did not change compared with the control group. In contrast, His was present in significantly greater amounts in the tissues treated with 0.01% or 0.1% LFP. The Arg content also increased significantly in an LFP concentration–dependent manner. Arg and ornithine (Orn), a major component of NMF [42], is metabolized from Arg to Orn by arginase, and the presence of this enzyme has been detected in the epidermis [43]. Arginine has been reported to be the major amino acid component of NMF [44] and in the synthesis of urea [45]. Therefore, we

hypothesize that arginine is likely important for improving the water retention of the stratum corneum.

Because the amount of Arg, an amino acid that is important for skin water retention, increased, we investigated the expression of genes related to the production of this amino acid.

ProFLG expression was significantly higher ($p < 0.05$) in the tissues treated with LFPs for 72 hours compared with the control tissue (Fig. 2b). These results suggest that treatment with LFP enhanced epidermal differentiation and increased the amount of Arg.

Finally, the bound water content was measured in the stratum corneum. The water content of the untreated group was approximately 30%. Takenouchi et al. reported that the water content in the stratum corneum is about 32.7%, which was similar to our results [29]. The increase in the bound water content after the application of LFPs may be due to increases in ceramides and amino acids.

Taken together, our results suggest that treatment with LFP promotes differentiation and increases the intercellular free fatty acid, Chol, Cer [NS], Cer [AS], and Cer [AP] contents. These changes are likely to lead to improved skin barrier function and water retention by increasing the amount of Arg in keratinocytes (Fig. 6).

Statement of Ethics

This study did not involve human subjects or experimental animals, and as such did not require ethical approval.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work: Moe Otsuka, Tsuyoshi Tamane, Yoshihiro Tokudome

Drafting the work or revising it critically for important intellectual content: Yoshihiro Tokudome

Final approval of the version to be published: Moe Otsuka, Tsuyoshi Tamane, Yoshihiro Tokudome

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure Legends

Fig.1. Effect of lactic fermentation products on (a) cell viability and (b) TEWL, c) Conductance in a three-dimensional cultured human epidermis model. Value are the mean ± S.D. of n = 3 replicates, *p < 0.05, Dunnett's test (versus control).

Fig. 2. Effect of lactic fermentation products on the expression of genes related to differentiation in a three-dimensional cultured human epidermis model (a) and the expression of genes related to amino acid production in normal human epidermal keratinocytes (b). Values are the mean ± S.D. of n = 3 replicates, *p < 0.05, **p < 0.01, Dunnett's test (versus control).

Fig. 3. Effect of lactic fermentation products on TGase protein expression in a three-dimensional cultured human epidermis model. Protein expression levels were determined by western blotting. TGase and β-actin western blot bands (a) and TGase/β-actin expression (b). Values are the mean ± S.D. of n = 3 replicates, *p < 0.05, Dunnett's test (versus control).

Fig.4. HPTLC image of a LabCyte Epi-model after application of lactic fermentation products. Quantification of ceramide [NS], [NP], [AS] and [AP] (a), free fatty acid and cholesterol (b).

Fig. 5. Changes in epidermal ceramide, free fatty acid, and cholesterol content in a three-dimensional cultured human epidermis model. Lipid contents of a three-dimensional cultured human epidermis model after 7 days of treatment with lactic fermentation products, as determined by HPTLC. Value are the mean ± S.D. of n = 3 replicates, *p < 0.05, **p < 0.01, ***p < 0.001, Dunnett's test (versus control).

Fig. 6. Amino acid (Ser, Ala, Gly, Glu, Arg, and His) content of a three-dimensional cultured human epidermis model. Values are the mean ± S.D. of n = 3 replicates, *p < 0.05, **p < 0.01, ***p < 0.001, Dunnett's test (versus control).

Fig. 7. Bound water content in stratum corneum of a three-dimensional cultured human skin. Values are the mean ± S.D. of n = 3 replicates, *p < 0.05, Dunnett's test (versus control).

Fig. 1a

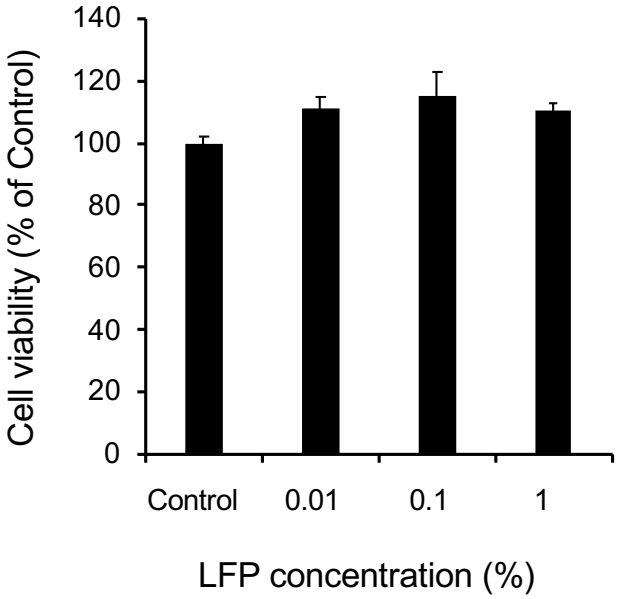


Fig. 1b

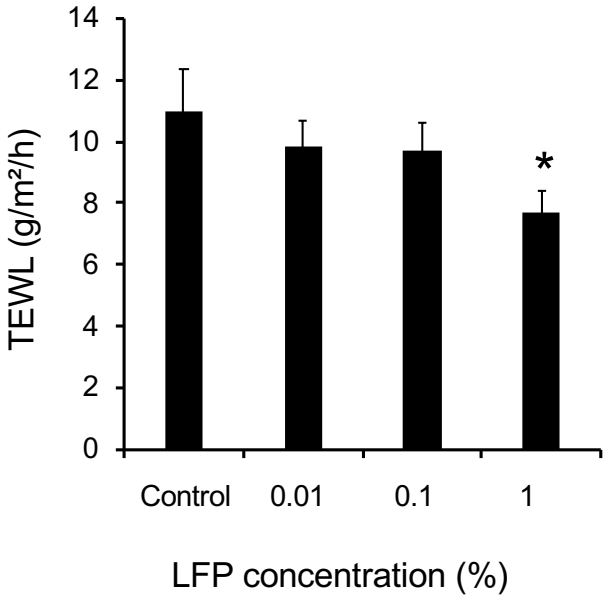


Fig. 1c

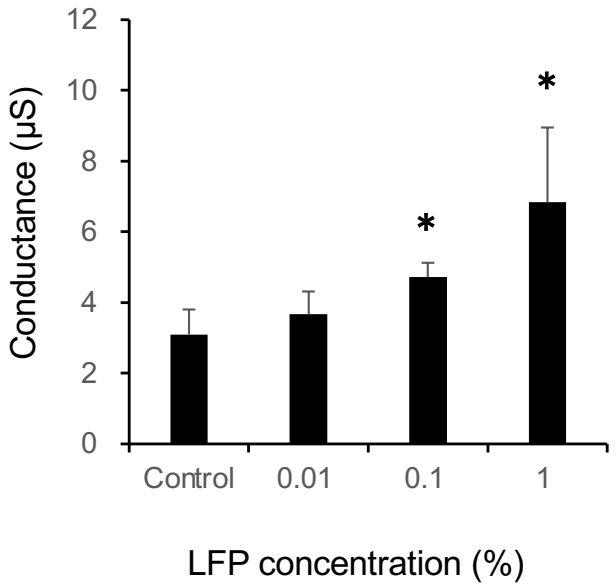


Fig. 2a

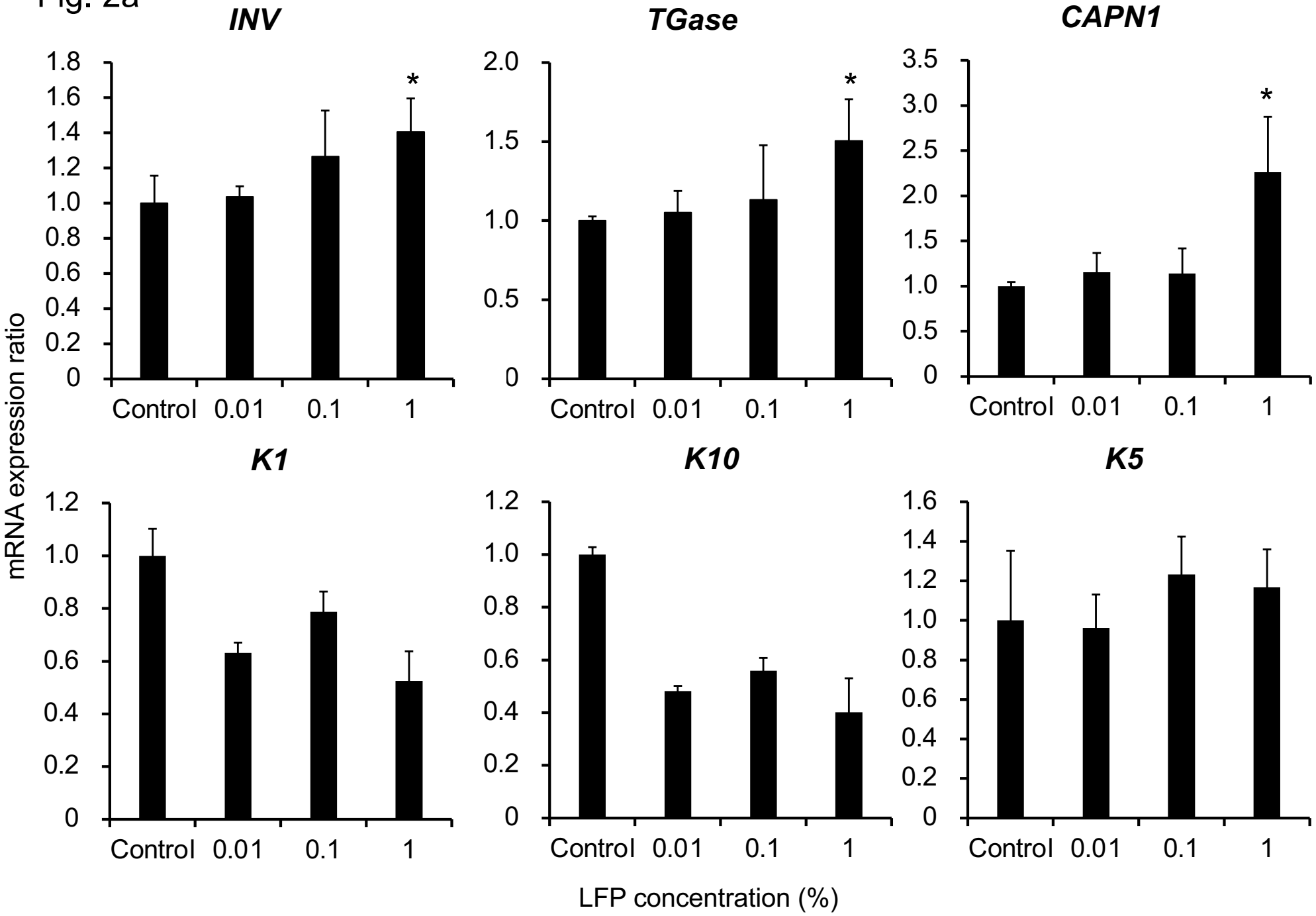

INV
TGase
CAPN1
K1
K10
K5
mRNA expression ratio
LFP concentration (%)
Control
0.01
0.1
1
*

Fig. 2b

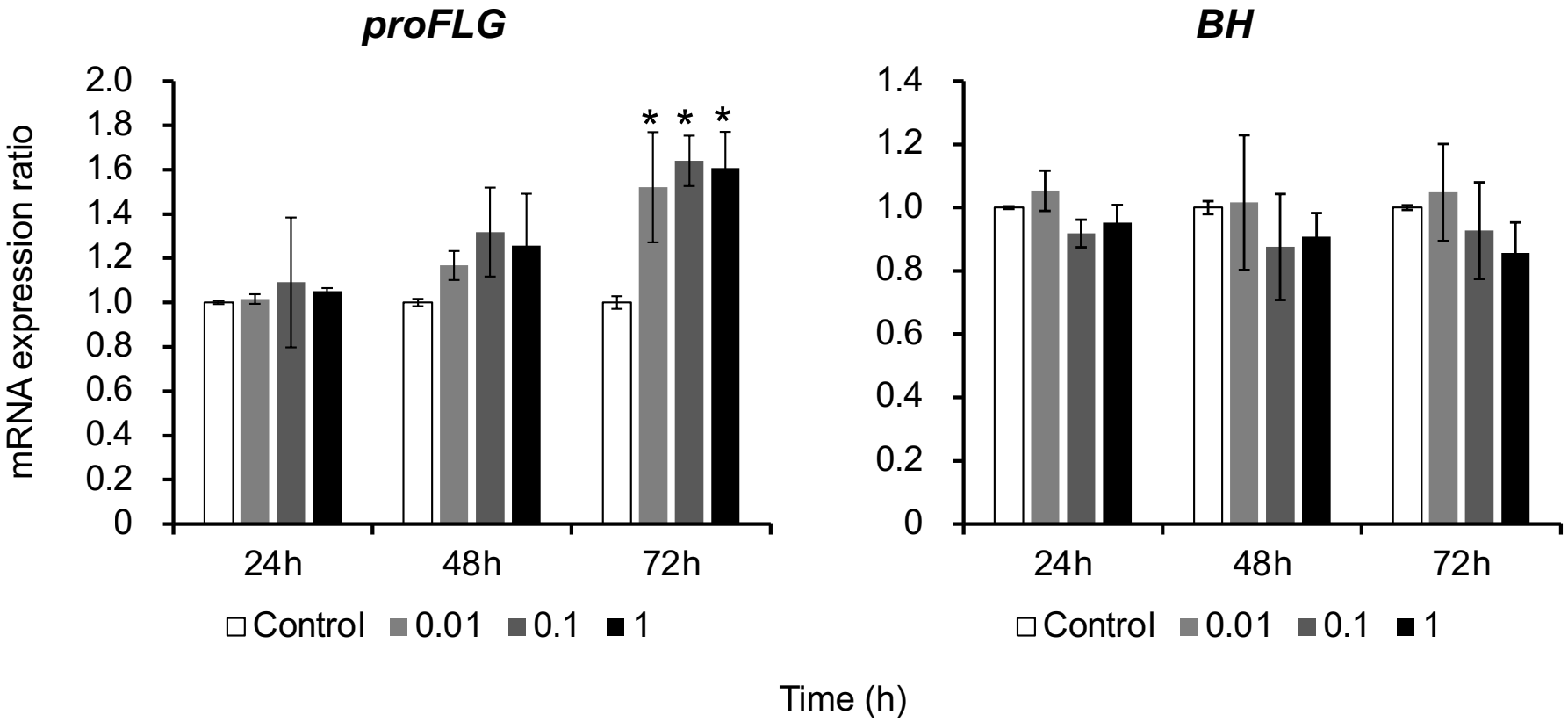

proFLG
BH
mRNA expression ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
24h
48h
72h
Control
0.01
0.1
1
Time (h)

Fig. 3

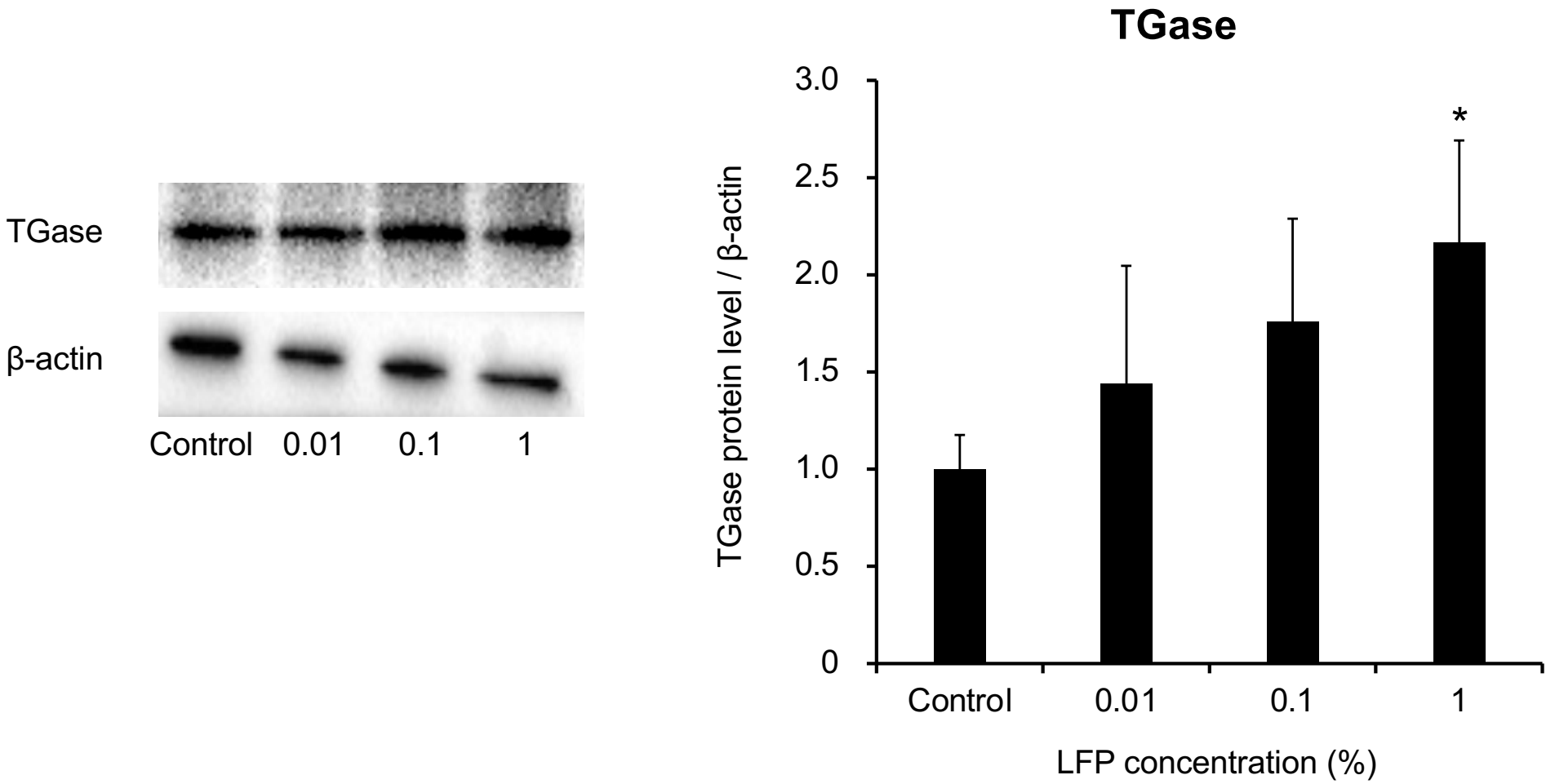
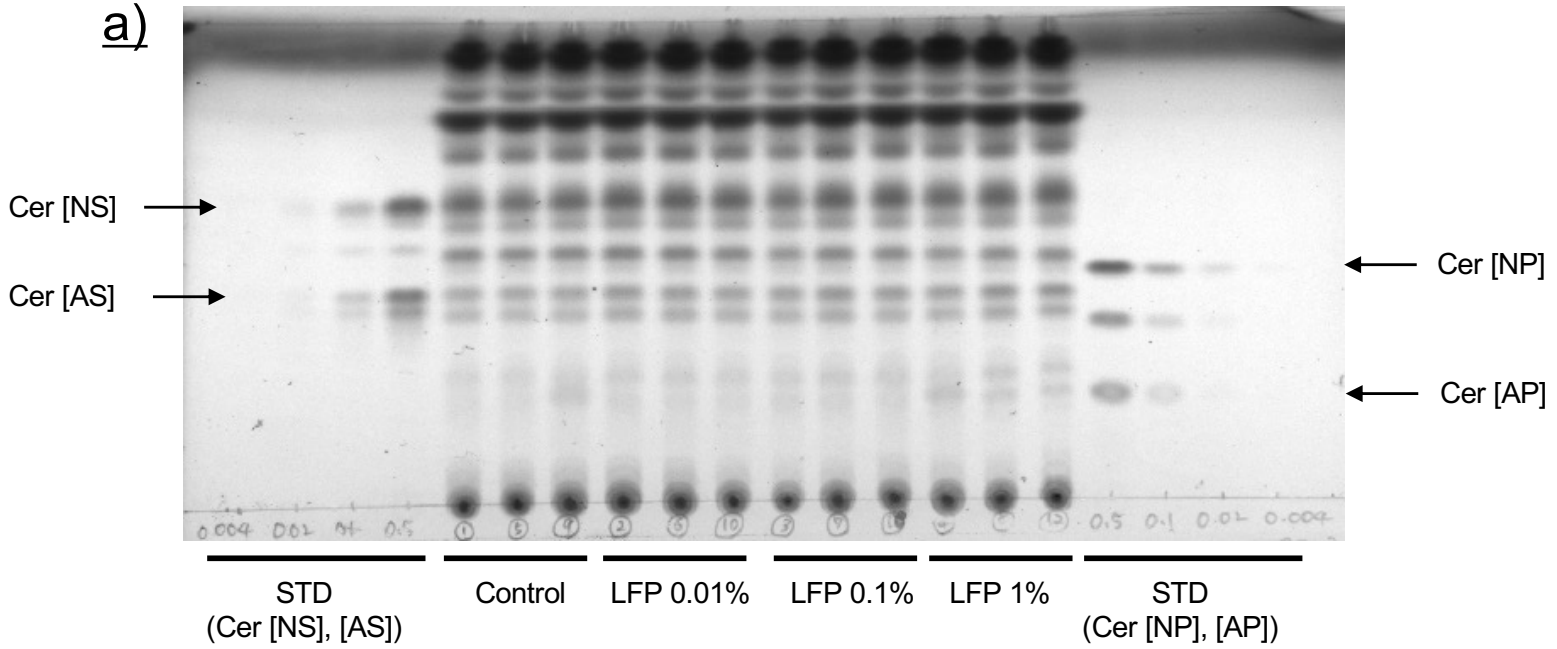

TGase
β-actin
Control
0.01
0.1
1
TGase
TGase protein level / β-actin
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Control
0.01
0.1
1
LFP concentration (%)

Fig. 4

a)


Cer [NS]
Cer [AS]
Cer [NP]
Cer [AP]
STD
(Cer [NS], [AS])
Control
LFP 0.01%
LFP 0.1%
LFP 1%
STD
(Cer [NP], [AP])

b)

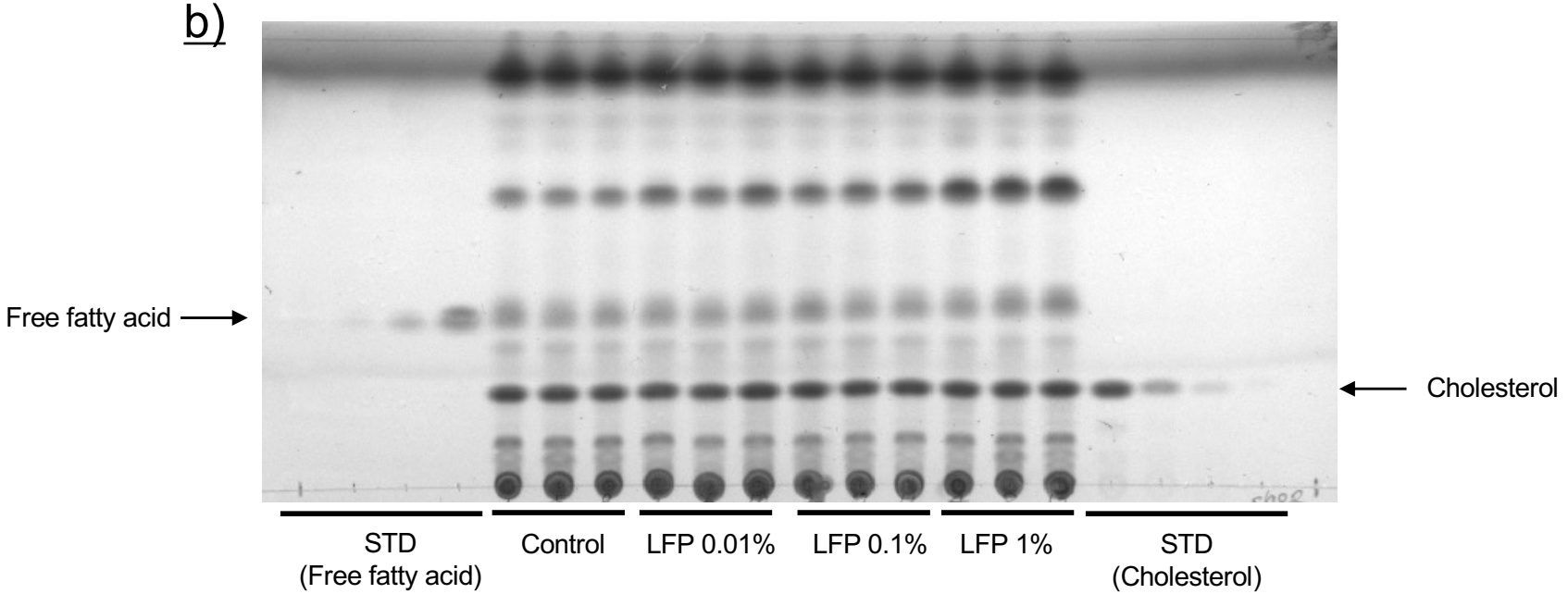

Free fatty acid
Cholesterol
STD
(Free fatty acid)
Control
LFP 0.01%
LFP 0.1%
LFP 1%
STD
(Cholesterol)

Fig. 5

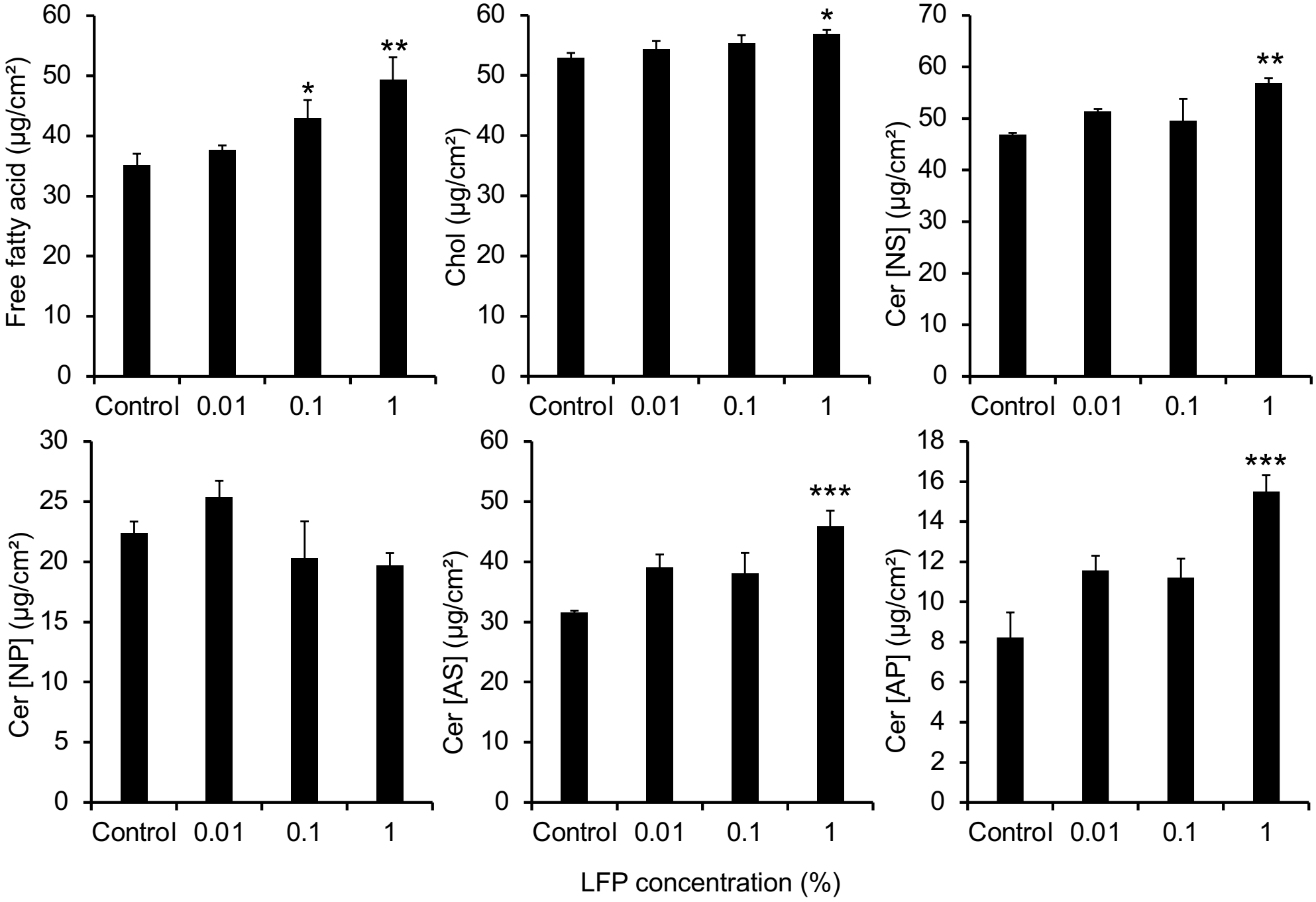

Free fatty acid (μg/cm²)
Chol (μg/cm²)
Cer [NS] (μg/cm²)
Cer [NP] (μg/cm²)
Cer [AS] (μg/cm²)
Cer [AP] (μg/cm²)
Control
0.01
0.1
1
LFP concentration (%)

Fig. 6

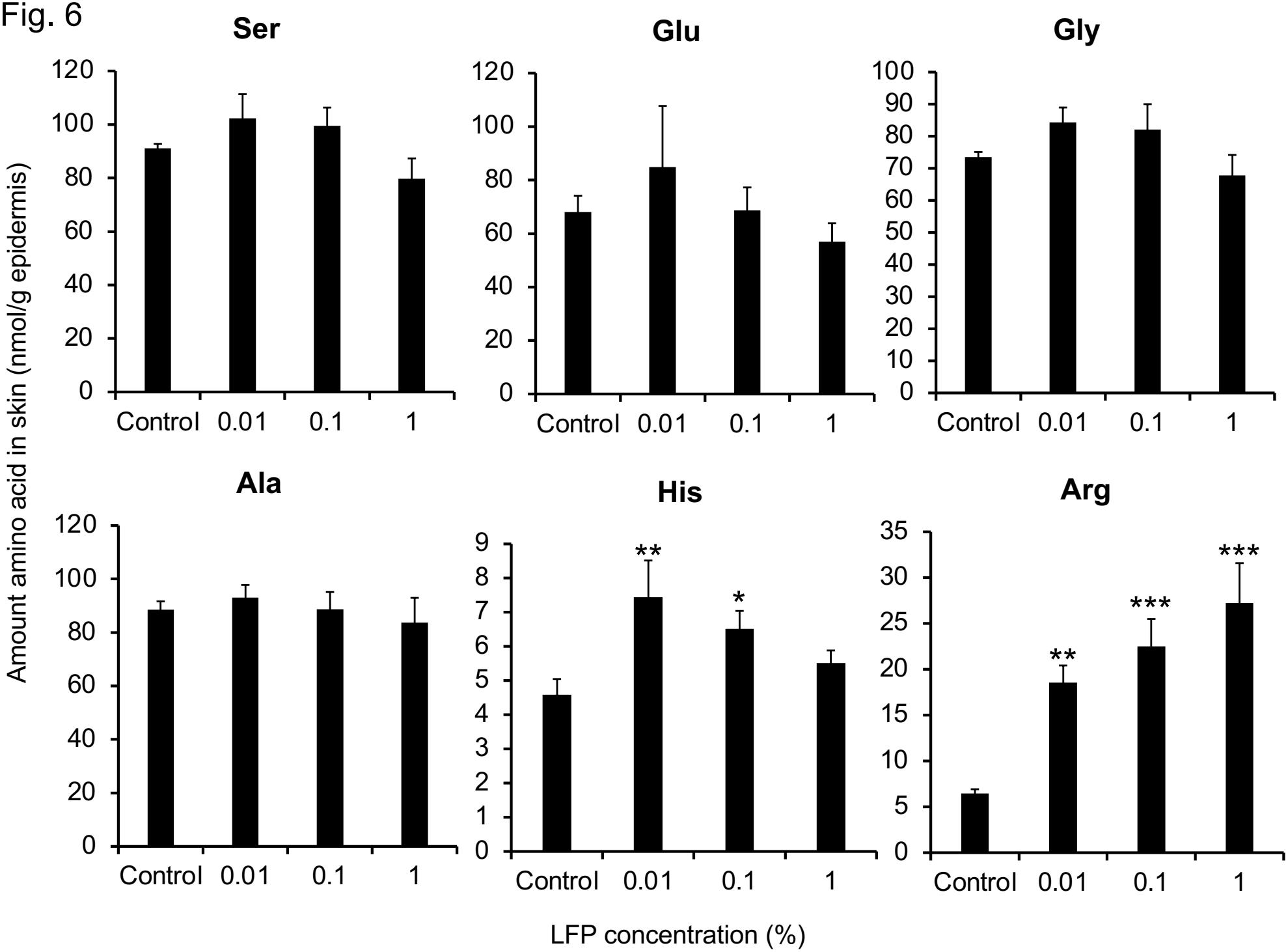
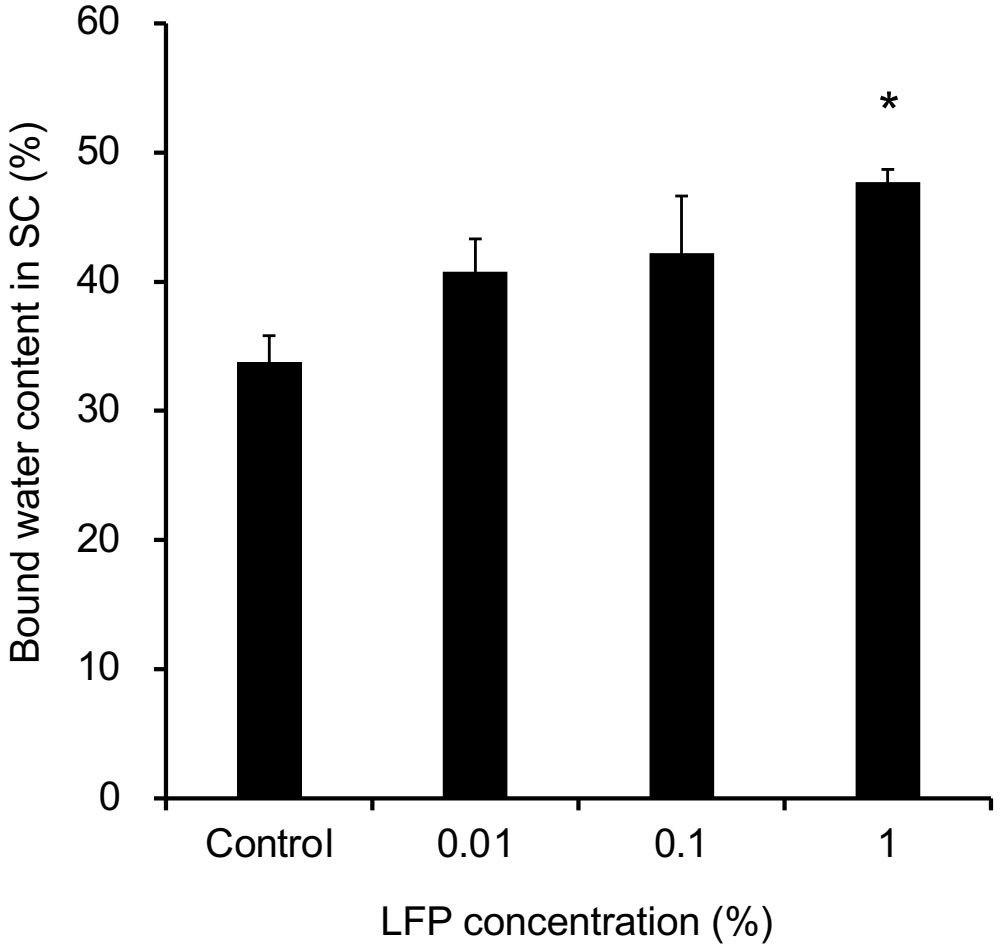

Ser
Glu
Gly
Ala
His
Arg
Amount amino acid in skin (nmol/g epidermis)
LFP concentration (%)
Control
0.01
0.1
1
**
*
***

Fig. 7


60
50
40
30
20
10
0
Bound water content in SC (%)
*
Control
0.01
0.1
1
LFP concentration (%)