

ORIGINAL RESEARCH

Scallop mantle extract inhibits insulin signaling in HepG2 cells

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Funding information

Japan Society for the Promotion of Science,
Grant/Award Number: 16K07865

Abstract

Scallops are important marine products in Hokkaido, Japan. Not only scallop adductor muscle but also mantle is often eaten at sashimi or smoking in Japan. We showed previously that feeding the scallop mantle epithelial cell layer causes an increase in serum glucose concentration and the death of rats. To clarify the mechanism of glucose metabolism disorder by mantle epithelial cell layer, we investigated whether extracts from mantle tissue (mantle extract) induce insulin resistance using HepG2 cells. Mantle extract suppressed insulin-stimulated phosphorylation of Akt, key protein which is involved in insulin signaling. In addition, treatment of HepG2 cells with mantle extract decreased significantly glycogen content and mRNA expression levels of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) involved in gluconeogenesis, suggesting that mantle extract inhibits insulin signaling. These results show that mantle extract inhibits insulin signaling in HepG2 cells, suggesting that an increase in serum glucose concentration in vivo may be due to the inhibition of insulin signaling.

KEYWORDS

HepG2 cell, insulin signaling, scallop mantle, toxin

1 | INTRODUCTION

Shellfish such as scallops, clams, and mussels accumulate marine toxins that are produced by algae such as toxic dinoflagellate. Toxication of shellfish gives large damage to seafood and fishing industries. Two types of toxicity have been detected in shellfish that was harvested in Japan: One is paralytic shellfish poisoning (PSP) and the other is diarrhetic shellfish poisoning (DSP; Farabegoli, Blanco, Rodríguez, Vieites, & Cabado, 2018; Smith & Swoboda, 2018). We reported previously that feeding the scallop mantle epithelial cell layer causes an increase in serum glucose concentration and death of rats (Hasegawa, Itagaki, Konno, & Hasegawa, 2018). In addition, we suggested that the toxic substance is unknown toxic one different from PSP and DSP toxins.

Insulin is a hormone, which is responsible for regulating blood glucose levels and keeping a steady blood glucose level. Insulin

activates insulin receptors and causes tyrosine phosphorylation of insulin receptor substrate (IRS; Valverde et al., 2003). The phosphorylation activates phosphatidylinositol3 (PI3) kinase-Akt pathway. Activated Akt (phosphorylated Akt) induces glycogen synthesis through phosphorylation (inactivation) of glycogen synthase kinase (GSK)-3 β (Beurel, Grieco, & Jope, 2015). Akt also suppresses gluconeogenesis by lowering the expression of its related enzymes in hepatocytes (Liu et al., 2015).

Insulin resistance is a status in which cells do not respond properly to the insulin and causes high blood glucose concentration, leading to type 2 diabetes. Insulin resistance is a deficit in signal transduction from insulin such as inactivation of PI3 kinase-Akt signaling pathway. While the exact cause of insulin resistance is still not understood, factors such as obesity, inflammation, chronic stress, and lack of physical activity have been reported (Sanghez et al., 2016; Shoelson, Herrero, & Naaz, 2007). There have been many studies about the pathway

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causing insulin resistance. Endoplasmic reticulum (ER) stress has been reported to inhibit insulin signaling through pathways such as inactivation of IRS by activation of C-Jun N-terminal kinase (JNK; Castro et al., 2013; Kaneto, Nakatani, & Matsuhisa, 2004). Obesity and inflammation increase the expression of suppressor of cytokine signaling (SOCS) protein 1 and protein 3 in liver and inhibits tyrosine phosphorylation of IRS, leading to the insulin resistance (Rui, Yuan, Frantz, Shoelson, & White, 2002; Ueki, Kondo, & Kahn, 2004).

The human hepatoma cell line HepG2 has extensively been used to investigate hyperglycemia and diabetes because these cells exhibit many functions of normal human hepatocytes (Hu et al., 2014; Vidyashankar, Varma, & Patki, Varma & Patki, 2013; Zang et al., 2004). In this study, we studied whether mantle extract causes insulin resistance in HepG2 cells for clarifying the action mechanism increasing serum glucose concentration.

2 | MATERIALS AND METHODS

2.1 | Materials

Scallops (*Patinopecten yessoensis*), which were harvested from Mutsu Bay, Aomori, Japan, were purchased on the market. Mantle including epithelial cell layer was prepared from the scallops. β -Actin, Akt, S473-phosphorylated Akt (p-Akt), JNK, T183-phosphorylated JNK (p-JNK), GSK-3 β , S9-phosphorylated GSK-3 β (p-GSK-3 β), IRS-1, or S307-phosphorylated IRS-1(p-IRS) was purchased from Biorbyt.

2.2 | Extract from the scallop mantle tissue

Extract of mantle tissue including mantle epithelial cell layer was prepared as described previously (Hasegawa et al., 2018). Mantle tissue was lyophilized and homogenized in deionized water. After centrifugation at 12,000 \times g for 15 min, the supernatant was used as the mantle extract.

2.3 | Cell culture and viability assay

Human hepatoma HepG2 cells were purchased from RIKEN Cell Bank. HepG2 cells were maintained in modified Eagle's medium (MEM) and 10% fetal calf serum. Cells were seeded into a 96-well plate at a density of 4×10^3 cells/well and cultured overnight. After cells were treated with various concentrations of mantle extract for

48 hr, cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Manthorpe, Fagnani, Skaper, & Varon, 1986).

2.4 | Glycogen content

Glycogen content in HepG2 cells was examined in the presence or absence of mantle extract according to a slight modification of the method described previously (Hasegawa et al., 2018). Briefly, HepG2 cells were seeded on a 6-well plate at a density of 1×10^5 cells/well. After 24 hr, culture medium was exchanged to serum-free medium and mantle extract was added to the culture medium at the indicated concentration. After cells were treated with 100 nM insulin for 30 min, cells were collected and trichloroacetic acid was added to be 10%. After incubating the solution at 100°C for 20 min, centrifugation at 14,000 g for 5 min at 4°C was performed and the supernatant was collected. Ethanol was added to be 80% and centrifuged at 14,000 g for 15 min at 4°C. The precipitation was dried and dissolved at deionized water, and anthrone reagent was added. After treatment at 100°C for 10 min, absorbance at 620 nm was measured.

2.5 | Semi-quantitative reverse transcription polymerase chain reaction analysis

After HepG2 cells were treated with insulin in the absence or presence of the mantle extract for 30 min, total RNA was prepared using an RNAiso Plus (Takara), as per the manufacturer's protocol. Total RNA was also prepared from liver tissues of mice fed control diet or mantle diet as well. To perform semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), first-strand complementary DNA (cDNA) was synthesized using oligo (dT) primer and PCR was carried out using specific primers. The specific forward and reverse primers are shown in Table 1. The intensities of the bands of the PCR products were quantitated using ImageJ software and normalized with respect to β -actin. The PCR cycles were selected on the basis of the relationship between the number of cycles and amount of PCR product.

2.6 | Western blotting

After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min,

Gene	Forward primer	Reverse primer
β -Actin	5'-CATCCGCAAAGACCTGTACG-3'	5'-CCTGCTTGCTGATCCACATC-3'
G6Pase	5'-ATTGACACCACACCTTTGCG-3'	5'-GACGTAGAAGACCAGCTCGA-3'
SOCS	5'-CACCTACTGAACCCTCCTCC-3'	5'-AGAGATGCT GAAGAGTGGCC-3'
PEPCK	5'-CTGGGAAGGCATTGATGAGC-3'	5'-CGGCCTCCAAAGATAATGCC-3'
CHOP	5'-AGGGAGAACCAGGAAACG GAAACA-3'	5'-TCCTGCTTGAGCCGTTTATTCTCT-3'
Spiced Xbp-1	5'-TGTCACCCCTCCAGAACATC-3'	5'-AAGGGAGGCTGGTAAGGAAC-3'
GRP78	5'-CGGTCTACTATGAAGCCCGT-3'	5'-CATCTGGGTTTATGCCACGG-3'

TABLE 1 Primer sequences used in semi-quantitative RT-PCR

cells were homogenized in a solution containing 0.2% SDS, 20 mM Tris-HCl (pH 7.5), and bromophenol blue. After SDS polyacrylamide gel electrophoresis (Laemmli, 1970), the proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking the membrane with 5% skim milk (w/v) in a solution containing 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.05% Tween 20 (solution A) for 2–6 hr at room temperature, antibodies against β -actin, Akt, p-Akt, JNK, p-JNK, GSK-3 β , p-GSK-3 β , IRS-1, or p-IRS-1 were incubated overnight. After washing with solution A, the membrane was treated with an alkaline phosphatase-conjugated secondary antibody for 2 hr and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The band intensities were estimated using Image J.

2.7 | Statistical analysis

Each experiment was performed at least three times. Data were expressed as the mean and the standard deviation (SD). The data were analyzed using Student's *t* test.

3 | RESULTS

3.1 | Effects of mantle extract on insulin signaling in HepG2 cells

We showed previously that intake of mantle epithelial cell layer tissue finally increases serum glucose concentration (Hasegawa et al., 2018). In this study, we investigated the effect of mantle extract on insulin signaling using HepG2 cells for clarifying the cause of increased serum glucose concentration.

Insulin stimulation of HepG2 cells induces tyrosine phosphorylation of Akt through binding to insulin receptor (Figure 1). We investigated whether treatment with mantle extract suppresses the phosphorylation of Akt in HepG2 cells. The phosphorylation of Akt was significantly inhibited in the presence of mantle extract at

concentrations of 0.02 and 0.1 mg/ml (Figure 2). On the other hand, mantle extract did not show any toxicity at a concentration of 0.1 mg/ml. These results show that mantle extract inhibits insulin signaling.

Insulin stimulation is known to suppress gluconeogenesis and promote glycogen synthesis in hepatocyte through phosphorylation of Akt (Figure 1). Therefore, inhibition of insulin signaling will promote gluconeogenesis and decrease glycogen content. In order to confirm that mantle extract inhibits insulin signaling pathway, we investigated the mRNA expression levels of two key enzymes of gluconeogenesis, G6Pase and PEPCK, by semi-quantitative RT-PCR. Mantle extract increased significantly G6Pase and PEPCK mRNA expression levels to about 2.5–3.5-fold compared to the control (Figure 3), suggesting that mantle extract inhibits insulin signaling. Next, we investigated glycogen content in HepG2 cells. Treatment with mantle extract significantly decreased glycogen content to about 70% compared to that of the control (Figure 4). To confirm further this result, we measured the expression levels of phosphorylated GSK-3 β which regulates glycogen synthesis using Western blot. Mantle extract increased significantly the expression of unphosphorylated GSK-3 β level (Figure 4). Increase in unphosphorylated GSK-3 β decreases glycogen content through inactivation of glycogen synthase. This result also supports that mantle extract inhibits insulin signaling in HepG2 cells.

3.2 | Mantle extract induces ER stress

To clarify further the inhibitory mechanism of the insulin signaling by the mantle extract, we investigated mRNA expression levels of ER stress-induced genes (Samali, FitzGerald, Deegan, & Gupta, 2010), which have been reported to causes insulin resistance (Figure 1). Addition of mantle extract increased mRNA expressions of ER stress markers, CHOP, GRP78, and spliced Xbp-1 (Figure 5). In addition, the mantle extract increased the level of phosphorylated JNK, which is phosphorylated by ER stress, and increased serine phosphorylation of IRS (Figure 6). Phosphorylated JNK is known to promote serine

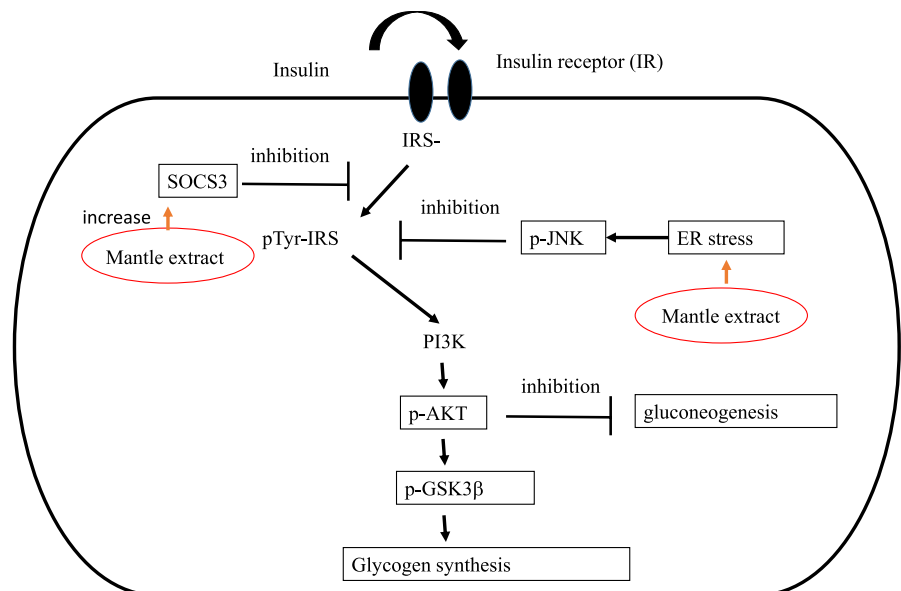


FIGURE 1 Signaling pathways after insulin stimulation. Inhibition of signaling was shown by lines ending in bars. Arrows show activation of pathways

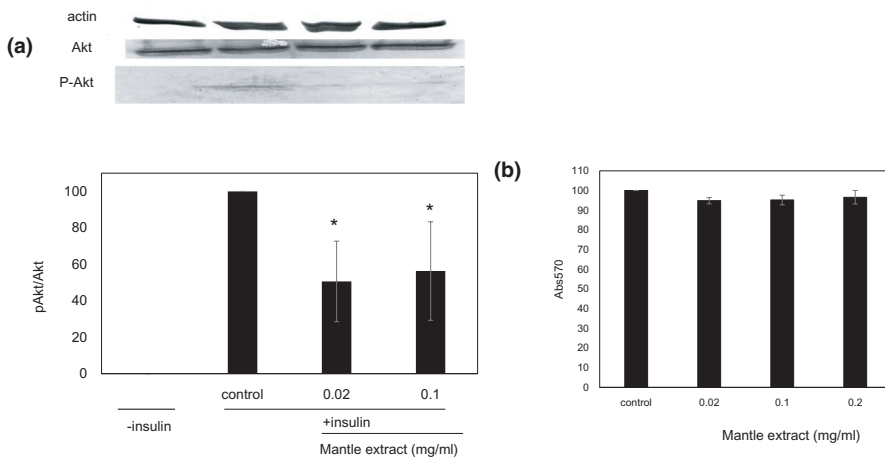


FIGURE 2 Effect of mantle extract on Akt phosphorylation after insulin stimulation. (a) HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min. Amount of nonphosphorylated and phosphorylated Akt was measured using Western blotting. Bars show SD. * $p < 0.05$ relative to control. (b) Toxicity of the mantle extract at the indicated concentrations was measured by MTT assay

phosphorylation of IRS which suppresses insulin signaling (Figure 1). These results suggest that mantle extract induces ER stress and leads to activation of JNK, resulting in inhibition of the insulin signaling.

Finally, we investigated the expression level of SOCS-3 which suppresses tyrosine phosphorylation of IRS (Figure 1). Mantle extract increased significantly the expression of SOCS-3 (Figure 7). These results suggest that mantle extract inhibits insulin signaling through ER stress and the increase in SOCS-3 expression in HepG2 cells.

4 | DISCUSSION

We previously found that feeding diets containing mantle epithelial cell layer caused an increase in serum glucose concentration and death of rats (Hasegawa et al., 2018). In this study, we studied whether mantle extract causes insulin resistance in HepG2 cells for clarifying the action mechanism increasing serum glucose concentration. We showed that mantle extract inhibited insulin

signaling pathway by reducing phosphorylation of Akt and led to a decrease in glycogen content through GSK-3 β . In addition, the inhibition of Akt phosphorylation promoted gluconeogenesis by increasing the expression of PEPCK and G6Pase in HepG2 cells. Insulin resistance has been reported to be induced by several substances such as palmitate and glucosamine. Treatment of HepG2 cells with palmitate (0.5 mM; 0.12 mg/ml) induced inhibition of Akt phosphorylation, inactivation of GSK-3 β , and increased expression of PEPCK (Cang et al., 2016; Ishii, Maeda, Tani, & Akagawa, 2015; Tang et al., 2015; Yadollah, Kazempour, Bakhtiyari, & Nazifi, 2017). Exposure of hepatocyte to high concentration of glucosamine (10 mM, 1.79 mg/ml) also suppressed the insulin response for Akt phosphorylation and stimulated the expression of G6Pase and PEPCK (Liu et al., 2015). The water-soluble mantle extract inhibits insulin signaling at a concentration of 0.1–0.2 mg/ml. Mantle extract does not seem to contain high concentration of palmitate or glucosamine, suggesting that mantle extract contains the insulin signaling-inhibiting substance different from palmitate and glucosamine.

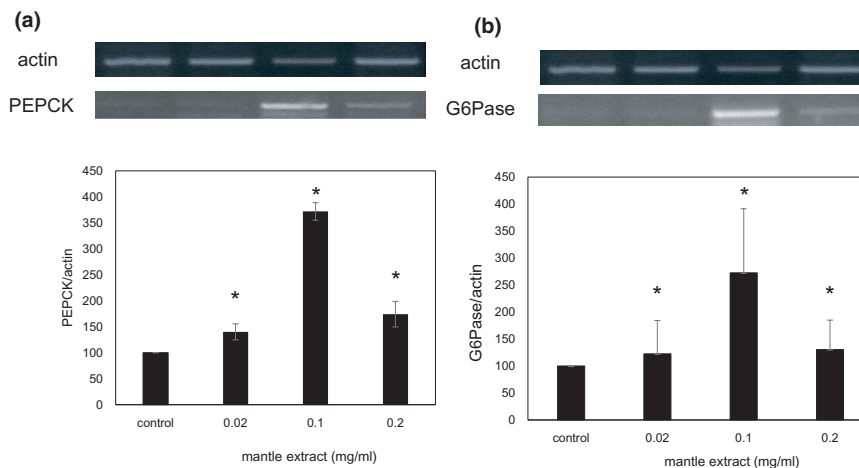
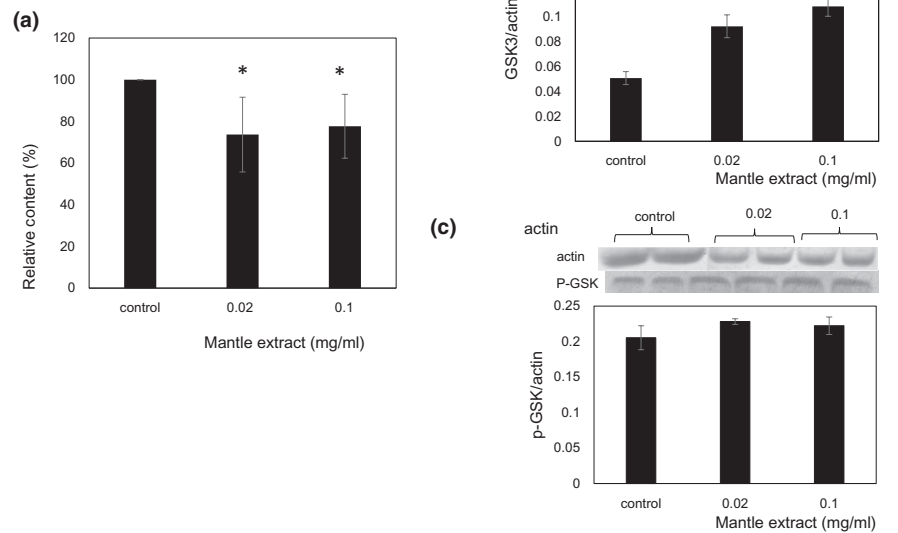


FIGURE 3 Effect of mantle extract on the mRNA expressions of G6Pase (a) and PEPCK (b). After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min, semi-quantitative RT-PCR was performed. Bars show SD. * $p < 0.05$ relative to control

FIGURE 4 Effect of mantle extract on glycogen synthesis. (a) After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min, cells were recovered and glycogen content was estimated. (b, c) Nonphosphorylated and phosphorylated GSK-3 β levels were measured using Western blotting. Bars show SD. * $p < 0.05$ relative to control



Many studies have shown the link between ER stress and insulin resistance. ER stress causes insulin resistance through several pathways. ER stress activates JNK, leading to serine phosphorylation of IRS (Ozcan et al., 2004), which inhibits Akt phosphorylation and insulin signaling downstream in vivo. Lee et al. (2010) showed that transcription factor (CREBH) which is activated by ER stress induces transcription of G6Pase and PEPCK. Treatment with the mantle extract increased the expression of GRP78, CHOP, and spliced Xbp-1, which are ER stress markers. In addition, the mantle extract

increased phosphorylation of JNK and expression levels of G6Pase and PEPCK, suggesting that the mantle extract inhibits insulin signaling through ER stress and CREBH activation.

Insulin resistance is also caused by inflammatory mediators such as interleukin-6 (IL-6) (Senn et al., 2003; Ueki et al., 2004). When HepG2 cells were treated with IL-6, the expression of SOCS-3 mRNA is induced. Upregulation of SOCS-3 in the liver of diabetic mice causes insulin resistance by inhibiting tyrosine phosphorylation of IRS. We found that treatment with the mantle extract increased

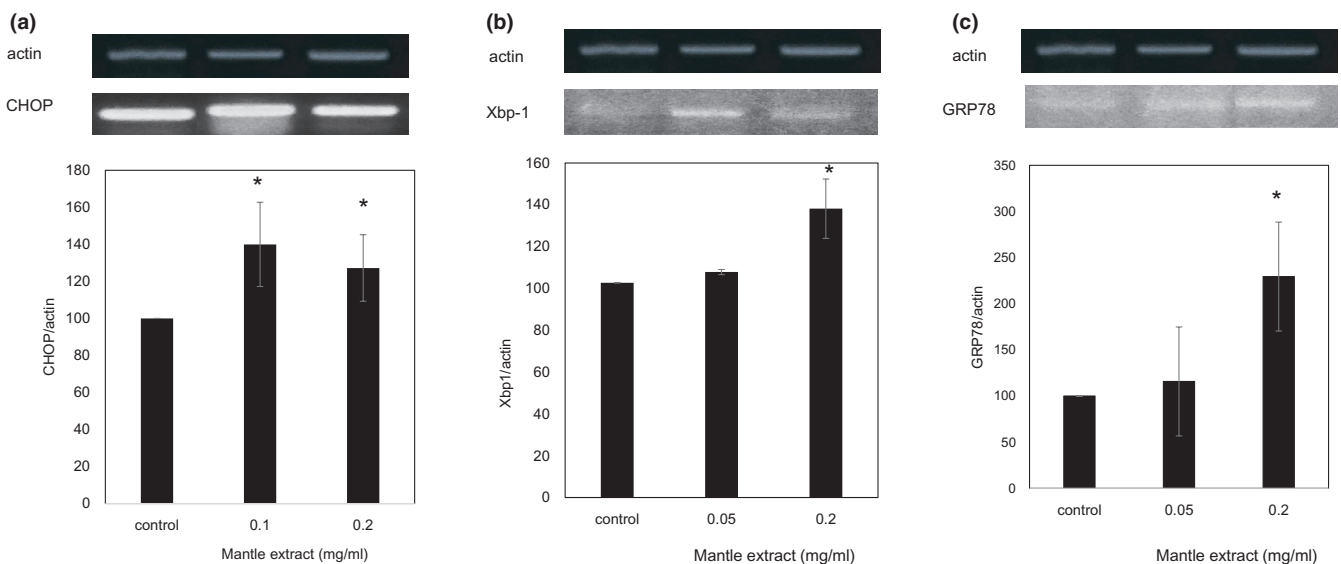


FIGURE 5 ER stress (a–c) by mantle extract. After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min, semi-quantitative RT-PCR was performed using specific primers of CHOP (a), spliced Xbp-1 (b), and GRP78 (c). Bars show SD. * $p < 0.05$ relative to control

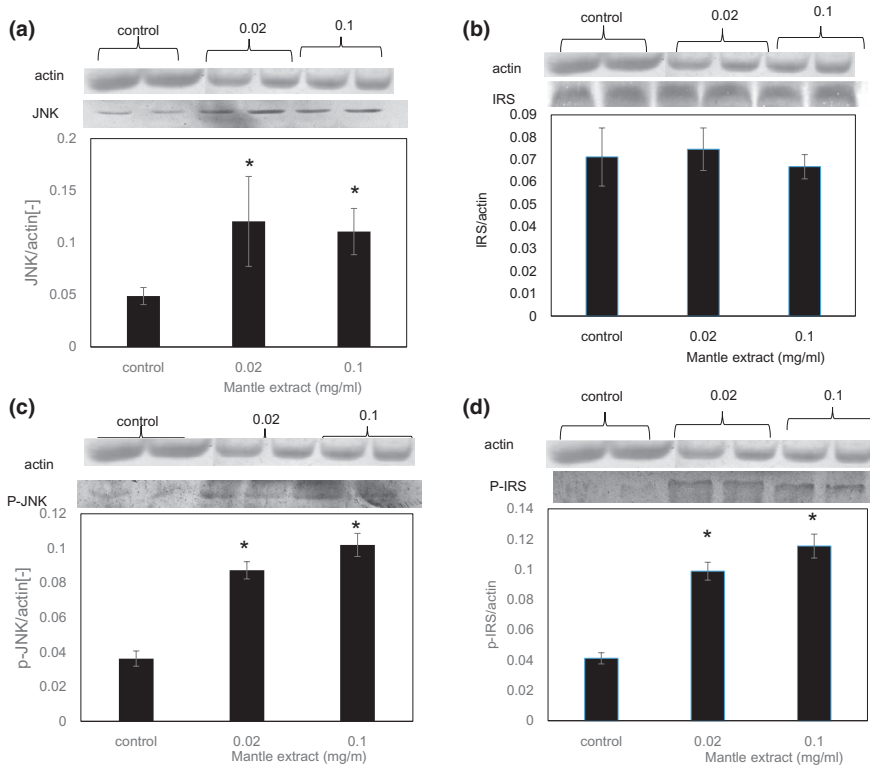


FIGURE 6 Expression levels of nonphosphorylated and phosphorylated JNK (a, b) and IRS-1 (c, d). After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min, Western blotting was performed. Bars show SD. * $p < 0.05$ relative to control

the expression of SOCS-3, suggesting that treatment with mantle extract may induce inflammatory cytokines in liver. Mantle extract may also inhibit insulin signaling through inflammation in addition to ER stress.

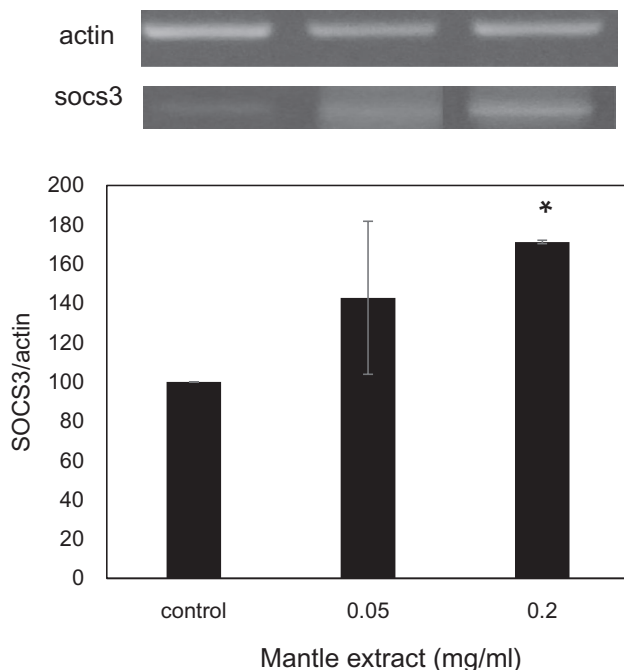


FIGURE 7 Effect of mantle extract on a mRNA expression of SOCS-3. After HepG2 cells were treated with insulin in the absence or presence of the indicated concentrations of mantle extract for 30 min, semi-quantitative RT-PCR was performed using specific primers of SOCS-3

We showed that treatment of HepG2 cells with the mantle extract decreased their glycogen content and increased the mRNA levels of G6Pase and PEPCK. We had previously reported that feeding rats with the mantle tissue decreased the glycogen content in their livers (Hasegawa et al., 2018). We also observed that their livers had increased mRNA levels of G6Pase and PEPCK (T, Kariya, K, Takahashi, Y, Hasegawa, unpublished data). Taken together, these observations suggest that feeding rats with the mantle tissue may also inhibit insulin signaling pathway in their livers. However, it is unclear whether such toxicity also occurs in humans upon ingestion of the mantle tissue. Before investigating this possibility, we first need to isolate the toxic substance and clarify the dose-toxicity relationship and the underlying mechanism in rats. Currently, we are in the process of identifying this toxic substance and its action mechanism.

5 | CONCLUSION

In this study, we studied whether mantle extract causes insulin resistance in HepG2 cells for clarifying the action mechanism increasing serum glucose concentration in vivo and showed that the mantle extract inhibits insulin signaling in HepG2 cells, suggesting that an increase in serum glucose concentration in vivo may be due to the inhibition of insulin signaling. Now, it remains unclear whether an insulin signaling-inhibiting substance is a causal substance causing death of rat. Now, we are trying to identify the causal substance causing death of rat and then clarify whether the substance inhibits the insulin signaling.

ACKNOWLEDGMENTS

This work was financially supported by the Japan Society for the Promotion of Science KAKENHI Grant no. 16K07865 to Yasushi Hasegawa.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

This study does not involve any human nor animal testing.

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How to cite this article: Kariya T, Takahashi K, Itagaki D, Hasegawa Y. Scallop mantle extract inhibits insulin signaling in HepG2 cells. *Food Sci Nutr*. 2019;7:2159–2166. <https://doi.org/10.1002/fsn3.1061>