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**Original article**

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# The 7-days-exposure to gamma-hexachlorocyclohexane causes resistance to insulin in differentiated mature 3T3-L1 adipocytes

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## Abstract

The object of this study is to elucidate the effect of gamma-hexachlorocyclohexane ( $\gamma$ -HCH), an organochloride pesticide, on glucose uptake in fully differentiated mature 3T3-L1 adipocytes. Differentiated mature adipocytes were treated with or without  $\gamma$ -HCH for 7 days at 37°C in a 5% CO<sub>2</sub> incubator. After that, both cell viability and glucose uptake were measured by colorimetric assay, and generation of intracellular reactive oxygen species (ROS) were measured by fluorescence assay. Levels of mRNA and protein were analyzed using real-time polymerase chain reaction and immunoblotting, respectively. Treatment with sub-toxic doses of  $\gamma$ -HCH decreased insulin-stimulated glucose uptake, enhanced the production of ROS, and upregulated glucose transporter 4 (GLUT4) while reducing its translocation to the plasma membrane. Treatment with  $\gamma$ -HCH also stimulated the expression of markers of ROS production and phosphorylation of c-Jun N-terminal kinase (JNK). Moreover, phosphorylation of serine-307 (Ser307) in insulin receptor substrate-1 (IRS-1), which has an inhibitory effect on insulin-mediated glucose uptake, was increased, whereas the phosphorylation of AKT2 and/or AKT substrate 160 (AS160) proteins decreased. These findings indicate that  $\gamma$ -HCH attenuated insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes by reducing the translocation of GLUT4 protein to the plasma membrane via the phosphorylation of Ser307 in IRS-1 and ROS-stimulated activation of JNK.

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《Key words》 Gamma-hexachlorocyclohexane, mature 3T3-L1 adipocyte, insulin resistance, glucose transporter 4

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## Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has recently increased in developing

countries.<sup>1,2)</sup> In addition to obesity, it has been reported that the exposure to persistent organic compounds, most commonly pesticides, cause

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T2DM.<sup>3-6)</sup> They also cause other metabolic diseases in humans<sup>3-6)</sup> positively associated with T2DM and related metabolic conditions.<sup>5,6)</sup> It has been suggested a correlation between the development of T2DM with chronic exposure to pesticides via direct oral intake and food chain.

Gamma-hexachlorocyclohexane ( $\gamma$ -HCH) is a common insecticide used in agriculture worldwide.<sup>7)</sup> However,  $\gamma$ -HCH has toxic effects on humans, such as in the development of diseases of the central nervous system, blindness, mental confusion, convulsion, and dysarthria.<sup>7)</sup> Therefore,  $\gamma$ -HCH has been banned in several developed countries and it has been included as a persistent organic pollutant in 2009 by the Stockholm Convention. Nevertheless,  $\gamma$ -HCH is popular in developing countries,<sup>8)</sup> although this chemical directly or indirectly damages the ecosystem. The increase in occurrence of T2DM in developing countries<sup>1,2)</sup> may be correlated with chronic exposure to  $\gamma$ -HCH. Indeed, it has been reported that  $\gamma$ -HCH is also associated with dyslipidemia<sup>9)</sup> and accumulates in adipose tissue.<sup>10,11)</sup>

Mature adipocytes constitute the principal part of adipose tissue. Therefore, it is hypothesized that  $\gamma$ -HCH affects adipocytes resulting in the occurrence of T2DM. However, little is known about the toxic effects of  $\gamma$ -HCH on the physiological responses in mature adipocytes. The NOEL (dose, no observable effect level) of  $\gamma$ -HCH is 600 mg/L in L6-derived myotubes.<sup>12)</sup> This concentration corresponds to approximately 2 mM (MW: 290.83). Therefore, to determine the cytotoxic and sub-toxic dose of  $\gamma$ -HCH in mature 3T3-L1 adipocytes, cell viability should be examined at various concentrations of  $\gamma$ -HCH from low levels to 1000  $\mu$ M.

It is also hypothesized that  $\gamma$ -HCH affects the many mechanisms related to the T2DM. Adipocyte uptakes glucose by insulin stimulation. If

this mechanism was impaired, it would result in impaired glucose tolerance. The translocation of glucose transporter 4 (GLUT 4) protein from the cytosol to the plasma membrane plays a key role in insulin-stimulated glucose uptake in adipocytes.<sup>13-15)</sup> Therefore, it is useful to determine the modification of GLUT4, by determination of the mRNA levels of GLUT4 and intracellular localization of GLUT4 protein for elucidation whether  $\gamma$ -HCH affects insulin-stimulated uptake via GLUT4. It is known that excessive intracellular ROS suppresses the translocation of GLUT4 protein in 3T3-L1 adipocytes.<sup>16)</sup> Previous study demonstrated that chronic up-regulation of reactive oxygen species (ROS) induced by  $\gamma$ -HCH caused insulin resistance in 3T3-L1 preadipocytes.<sup>16)</sup> Also,  $\gamma$ -HCH enhances intracellular ROS production in Ehrlich ascites tumor cells<sup>17)</sup> and human lymphocytes.<sup>18)</sup> To elucidate the mechanism related to glucose uptake in adipose cells resulting in insulin resistance in mature adipocytes, it is necessary to examine the modification of ROS production in adipocytes (intracellular ROS production) by  $\gamma$ -HCH.

Many ROS markers have been known such as cytochrome P450 1b1, haptoglobin, ceruloplasmin, and xanthine dehydrogenase. ROS markers are overexpressed upon the generation of intracellular ROS.<sup>19)</sup> In addition, the levels of NADPH oxidase-4 (Nox-4) mRNA and nuclear factor erythroid 2-related factor 2 (Nrf2) have shown to be increased by intracellular ROS generation in 3T3-L1 adipocytes.<sup>20,21)</sup> Therefore, it is useful to examine levels of these markers in adipose tissues exposed to  $\gamma$ -HCH by determination of mRNA expressions of them.

Excessive ROS stimulates the phosphorylation of c-Jun N-terminal kinase (JNK), thereby activating JNK in 3T3-L1 adipocytes.<sup>22)</sup> The phosphorylation of IRS-1 at Ser307 is a target of JNK. Other targets of JNK are known, which



are related to glucose uptake in adipocytes, such as phosphorylation of AS160, which participates in insulin-stimulated GLUT4 translocation downstream of AKT2,<sup>23)</sup> and phosphorylation of AKT2, a key molecule in the activation of insulin-stimulated glucose uptake.<sup>24)</sup> Previous study reported that the inactivation of IRS-1 upon phosphorylation at Ser307 and subsequent inhibition of insulin-related signal transduction.<sup>25)</sup> It is useful to examine the expressions of these genes to elucidate the possible impaired mechanism of glucose uptake by  $\gamma$ -HCH whether or not  $\gamma$ -HCH affect the phosphorylation. It has shown that TNF- $\alpha$  triggers the activation of JNK, thereby impairing insulin action.<sup>26)</sup> It is also useful to examine determine whether  $\gamma$ -HCH stimulates secretion of TNF- $\alpha$ , or increases the mRNA expression of TNF- $\alpha$ .

The objective of this study is to elucidate the effects of  $\gamma$ -HCH on insulin resistance in mature 3T3-L1 adipocytes and the molecular mechanisms underlying possible  $\gamma$ -HCH-induced modification in signal transduction cascade of glucose uptake by insulin stimulation. For this objective, the effects on  $\gamma$ -HCH on the followings in the mature adipocytes were examined; the cytotoxic level of  $\gamma$ -HCH, insulin-stimulated glucose uptake, translocation event of GLUT 4 protein on the plasma membrane, ROS production, the activation of AKT2 and the phosphorylation of Ser307 in insulin receptor substrate-1 (IRS-1), and the mRNA expression and secretion of TNF- $\alpha$ .

## Materials and Methods

### Culturing 3T3-L1 preadipocyte to mature adipocytes and treatment of $\gamma$ -HCH

3T3-L1 preadipocytes were purchased from KAC Co. Ltd. (Kyoto, Japan). 3T3-L1 preadipocytes were seeded in a 25 cm<sup>2</sup> collagen 1-coated flask (Thermo Scientific, NY) and grown till con-

fluence in 3T3-L1 preadipocyte medium (Zen-Bio, Inc., NC). The cells were maintained in the same medium for 2 days post-confluence at 37°C in a 5% CO<sub>2</sub> incubator. Cellular differentiation was induced by treatment with 3T3-L1 differentiation medium (Zen-Bio, Inc., NC) for 3 days. Cells were cultured for 4 more days in 3T3-L1 adipocyte medium (Zen-Bio, Inc., NC) to be differentiated mature adipocytes.

Subsequently, differentiated mature 3T3-L1 adipocytes were cultured in collagen 1-coated 24-well plates (Corning, Inc., ME) with ( $\gamma$ -HCH group) or without  $\gamma$ -HCH (control group) in 1 ml of medium per well for 7 days in incubator. The medium was changed every 2-3 days.

### Cell viability of the mature adipocytes exposed to $\gamma$ -HCH

Differentiated mature adipocytes were treated with 0  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, and 1000  $\mu$ M  $\gamma$ -HCH for 7 days. At the end of cultivation, cell culture media were collected and cell viability was determined using the LDH cytotoxicity detection kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The subtoxic level was determined based on the results of cell viability. The subtoxic level would be the level in which there was no significant difference in the cell viability compared to the control and there were no big variances in the cell viability in the group. The concentration 50  $\mu$ M of  $\gamma$ -HCH in the following experiments were the subtoxic level determined here.

To adjust the data determined by LDH amount, the remaining cells were harvested using a cell scraper and resuspended in a solution of protease inhibitor cocktail (Nacalai Tesuque, Kyoto, Japan) and phosphatase inhibitor (Nacalai Tesuque, Kyoto, Japan) including M-PER Mammalian protein extraction reagent (Thermo Scientific, NY). Data were calculated by dividing



the total protein concentration of each well.

#### Insulin-stimulated glucose uptake

After 7 days of cultivation with or without  $\gamma$ -HCH, the culture medium was aspirated and cells were cultured in serum-free culture medium for 6 h in CO<sub>2</sub> incubator. After incubation, cells were washed three times with buffer A (Krebs Ringer Phosphate Hepes buffered with 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 118 mM NaCl, 5 mM KCl, 30 mM Hepes, pH 7.5). Buffer A containing 2% BSA (essential fatty acid free, Sigma Ca. No. A0281, Sigma Aldrich, MO). Ten  $\mu$ l of 100  $\mu$ M insulin was added to each well at the final concentration as 1  $\mu$ M and the plates were incubated for 18 min in CO<sub>2</sub> incubator. Next, 2-deoxyglucose (Nacalai Tesque, Kyoto, Japan) was added at the final concentration as 1 mM and incubated for 20 min in CO<sub>2</sub> incubator. The culture medium was removed and cells were washed three times with phosphate-buffered saline containing 200  $\mu$ M phloretin (Sigma-Aldrich, MO), a glucose uptake inhibitor. The cells were harvested using a cell scraper in 10 mM Tris-HCl (pH 8.0). The cellular suspension was homogenized by 30 passages using a 5/8-inch 27-gauge needle attached to a syringe. The homogenate was heated at 80°C for 15 min and centrifuged at 15,000 $\times$ g at 4°C for 20 min. The obtained supernatant was used as the sample for the analysis of glucose uptake. The amount of 2-deoxyglucose was determined using the commercially available 2-Deoxyglucose uptake measurement kit (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

#### Intracellular ROS levels

The cells were seeded and grown in collagen 1-coated 96-well plates and differentiated/treated with or without  $\gamma$ -HCH as described above.

Intracellular ROS levels were measured using the ROS-Glo™ H<sub>2</sub>O<sub>2</sub> Assay kit (Promega, WI) and fluorescence microplate reader. After the measurement, cell numbers were counted in each well using the Cell Count Normalization Kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

#### Quantitative real-time polymerase chain reaction (PCR)

After 7 days treatment of 3T3-L1 cells with or without  $\gamma$ -HCH, total RNA in the cytoplasm was extracted for following experiment. The total RNA was extracted using TRI Reagent, Direct-zol™ RNA Miniprep kit (Zymo Research, CA). Complementary DNA was synthesized using the Super Script® IV First-Strand cDNA synthesis reaction system using the OligodT primer (Invitrogen) and RNase inhibitor (Toyobo, Tokyo, Japan). For real-time PCR, RNA was reverse transcribed using the PowerUp™ SYBER Green Master Mix (Thermo Fisher Scientific, CA) and amplified using the Applied Biosystems StepOne® Real-Time PCR system (Waltham, MA). The amplification protocol included a hold step for 2 min at 50°C and pre-denaturation for 2 min at 95°C followed by 40 cycles of the following: denaturation for 15 s at 95°C and annealing for 1 min at 60°C. Relative gene expression was normalized to  $\beta$ -actin expression using the 2<sup>- $\Delta\Delta$ Ct</sup> method. Amplification of specific transcripts was confirmed based on the melting curves between 60°C and 95°C following PCR. Primers have been listed in the Table 1.

#### Protein extraction from the plasma membrane and cytosol

After 7 days treatment of 3T3-L1 cells with or without  $\gamma$ -HCH, the protein of both plasma membrane and cytosol were extracted by modifying a previously described protocol.<sup>27)</sup> Cells



**Table 1 Primer sequences for RT-qPCR**

Primer name	Primer Length (mer)	Amplification Size (bp)	Sequence
$\beta$ -actin	21 20	237 bp	Sense: 5'-TCGTACCACAGGCATTGTGAT-3' Antisense: 5'-TGCTCGAAGTCTAGAGCAAC-3'
GLUT4	20 20	99 bp	Sense: 5'-GCCTGCCCCGAAAGAGTCTAA-3' Antisense: 5'-CGCTCTCTCTCCAACCTCCG-3'
Cytochrome P450,1b1	22 20	234 bp	Sense: 5'-TATTACGGACATCTTCGGAGCC-3' Antisense: 5'-TGTGGAATGGTGACAGGCAA-3'
Haptoglobin	20 21	196 bp	Sense: 5'-GTGGAGCACTTGGTTCGCTA-3' Antisense: 5'-CCATAGAGCCACCGATGATGC-3'
Ceruloplasmin	20 24	226 bp	Sense: 5'-CCTGGAGTCTGGATGCTCAG-3' Antisense: 5'-CTGAATCACTTCCAGAGGCTGTTA-3'
Xanthine Dehydrogenase	20 20	120 bp	Sense: 5'-GGATGCTAATCGCAGAATAC-3' Antisense: 5'-GCTTCTGGTTGAAGTGAGTC-3'
Nox-4	20 20	196 bp	Sense: 5'-GTGTCTGCATGGTGGTGGTA-3' Antisense: 5'-AGACTAATGCAGCCAGGAGG-3'
Nrf2	20 20	178 bp	Sense: 5'-AGCTGGCTGATACTACCGCT-3' Antisense: 5'-CTGGCACATCAGTGGAGAGG-3'

Abbreviations: GLUT4: glucose transporter 4; Nox-4; NADPH oxidase-4; Nrf2: Nuclear factor erythroid 2-related factor 2

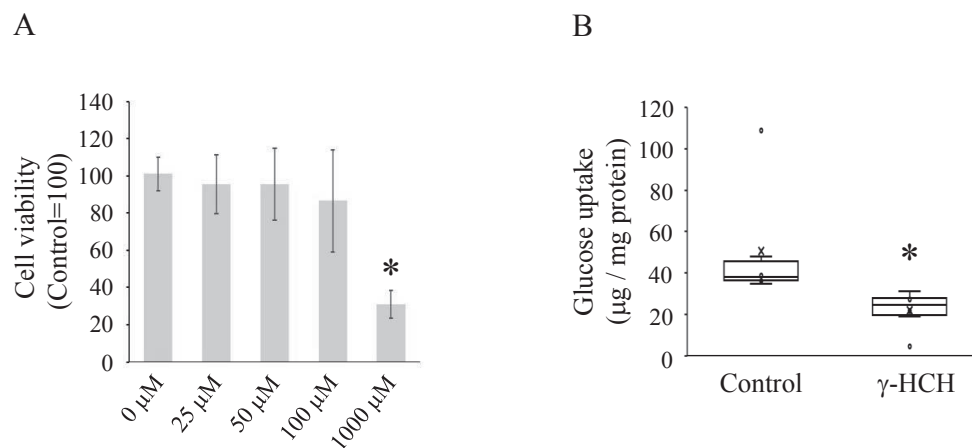
were homogenized in ice-cold buffer A, i.e., M-PER mammalian protein extraction reagent (Thermo Scientific, IL) containing 0.1% (v/v) NP-40 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) by passing ten times through a 5/8-inch 27-gauge needle attached to syringe at 4°C. The homogenate was centrifuged at 1,000×g for 10 min at 4°C and the supernatant was collected and comprised the cytosolic fraction 1. The obtained precipitate was resuspended in NP-40-free buffer A and incubated on ice for 10 min with gentle mixing. Subsequently, the resuspended participates was centrifuged at 1,000×g for 10 min at 4°C and the supernatant was collected as cytosolic fraction 2. This precipitate was resuspended in buffer A containing 1.0% (v/

v) NP-40 and incubated on ice for overnight with gentle mixing followed by centrifugation at 16,000×g for 30 min at 4°C. The supernatant was collected as the plasma membrane fraction. In this fraction, we confirmed that expression of ATPase protein, a marker of plasma membrane fraction, by immunoblotting. Finally, cytosolic fractions 1 and 2 were centrifuged at 16,000×g for 30 min at 4°C. The supernatants were collected as the final cytosolic fractions. All samples were stored at -80°C until further use.

### Immunoblotting

The protein samples, which were described as above, were mixed with Laemmli's sample buffer and incubated in a heat block at 100°C for 3 min (cytosol fraction) and 37°C for 10 min (plasma membrane fraction). Cooled samples at room temperature were loaded onto a 9%–12% sodium





**Figure 1. Effect of  $\gamma$ -HCH on cell viability and glucose uptake in mature 3T3-L1 adipocytes**

(A) The cell viability. Bars and vertical lines indicate mean  $\pm$  standard deviation (SD).

(B) Glucose uptake. \* $P < 0.05$ .

dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). The PVDF membrane was first incubated for 5 min in Bullet Blocking One (Nacalai Tesque, Kyoto, Japan) at room temperature. After blocking, the PVDF membrane was incubated overnight with specific primary antibodies in Tris-buffered saline supplemented with Tween 20 at 4°C. We used the following primary antibodies at a 1:1,000 dilution:  $\beta$ -actin, ATPase (Abcam, Cambridge, UK), GLUT4 (Cell Signaling Technology, Inc., Danvers, MA), JNK, JNK phosphorylated at tyrosine-183/185 (Cell Signaling Technology), IRS-1 (Abcam), IRS-1 phosphorylated at serine-307 (Ser307) (Merck KGaA, Darmstadt, Germany), AKT-2, AKT-2 phosphorylated at serine-474 (Ser474), AS160, AS160 phosphorylated at serine-588 (Ser588) (Abcam). After washing, the membranes were incubated for 60 min with anti-rabbit or anti-mouse immunoglobulin G (1:2,000 or 5,000 dilution) antibodies conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) and TidyBlot western blot

detection reagent:HRP (Bio-Rad Laboratories Inc., CA). The membranes were washed and immunoreactive bands were detected using ChemiLumi One Super (Nacalai Tesque, Kyoto, Japan) with the ChemiDoc imaging system (Bio-Rad Laboratories Inc., CA).

#### Enzyme-Linked Immuno Sorbent Assay of TNF- $\alpha$

The protein levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the medium and cell lysate, were determined by commercially available kit in accordance with the manufacturer's instructions (R and D systems, MN).

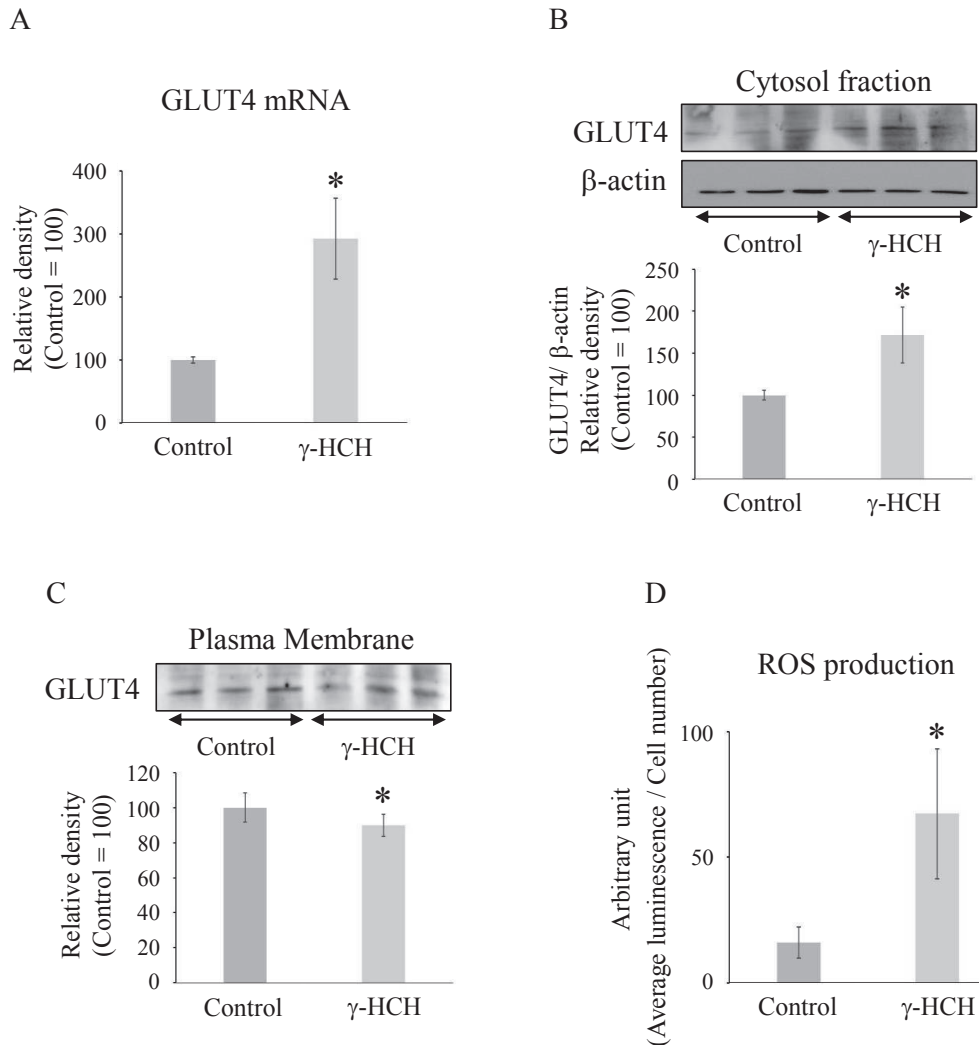
#### Statistical analysis

Parametric data were represented as mean  $\pm$  standard deviation. The mean values were compared by the Dunnett's test and Student's t-test. Non-parametric data was shown with boxplot. The values were compared by Mann-Whitney U test.  $P < 0.05$  was considered statistically significant.

#### Results

As shown in Fig. 1A, cell viabilities were sig-





**Figure 2. Effect of  $\gamma$ -HCH on the mRNA and protein levels of GLUT4 and ROS generation in mature 3T3-L1 adipocytes**

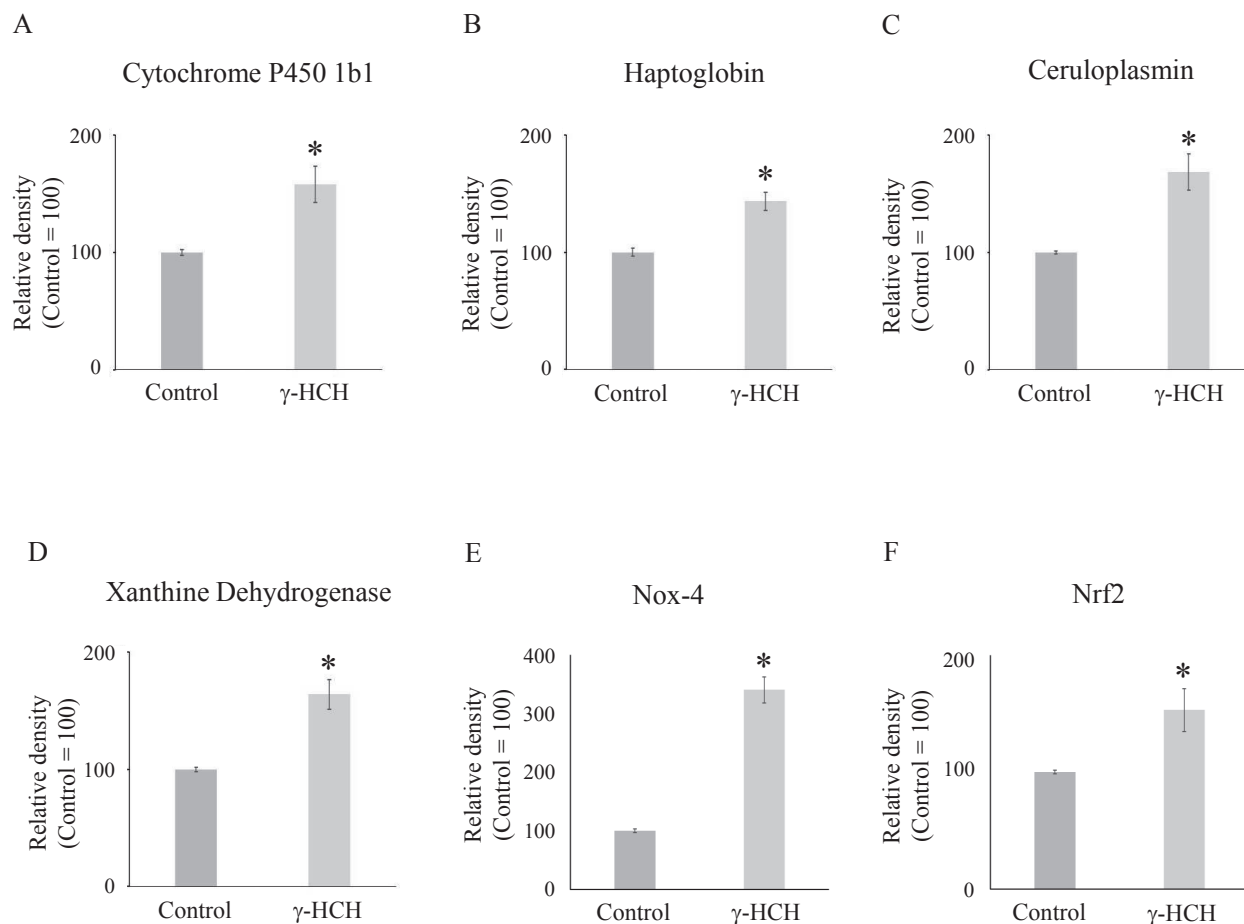
(A) The mRNA and (B) protein levels of GLUT4 in the cytosol fraction, (C) protein levels of GLUT4 in the plasma membrane, (D) intracellular reactive oxygen species (ROS) levels. Results were representative of three independent experiment ( $n = 5$  for each group). Bars and vertical lines indicate mean  $\pm$  SD. \* $P < 0.05$ .

nificantly reduced at 1,000  $\mu$ M of  $\gamma$ -HCH compared with that in non-treated control cells (0  $\mu$ M). No significant changes were observed at 25  $\mu$ M, 50  $\mu$ M and 100  $\mu$ M of  $\gamma$ -HCH. In 100  $\mu$ M of  $\gamma$ -HCH, however, the cell viability had large variation compared with 50  $\mu$ M of  $\gamma$ -HCH. We determined, therefore, 50  $\mu$ M of  $\gamma$ -HCH as a sub-toxic dose of  $\gamma$ -HCH in this study. Under these conditions,  $\gamma$ -HCH significantly reduced insulin-

stimulated glucose uptake as compared to that in control cells (Fig. 1B).

As shown in Fig. 2A, GLUT4 mRNA levels were significantly higher in  $\gamma$ -HCH-treated cells than that in control cells. The protein levels of GLUT4 were significantly increased and decreased in the cytosolic (Fig. 2B) and plasma membrane fractions (Fig 2C), respectively of  $\gamma$ -HCH-treated cells as compared to the levels in





**Figure 3. Effect of  $\gamma$ -HCH on the mRNA levels of ROS markers**

(A) Cytochrome P450 1b1, (B) haptoglobin, (C) ceruloplasmin 350, (D) xanthine dehydrogenase. (E) NADPH oxidase-4 (Nox-4), and (F) nuclear factor erythroid 2-related factor 2 (Nrf2). Bars and vertical lines indicate mean  $\pm$  SD. \* $P < 0.05$ .

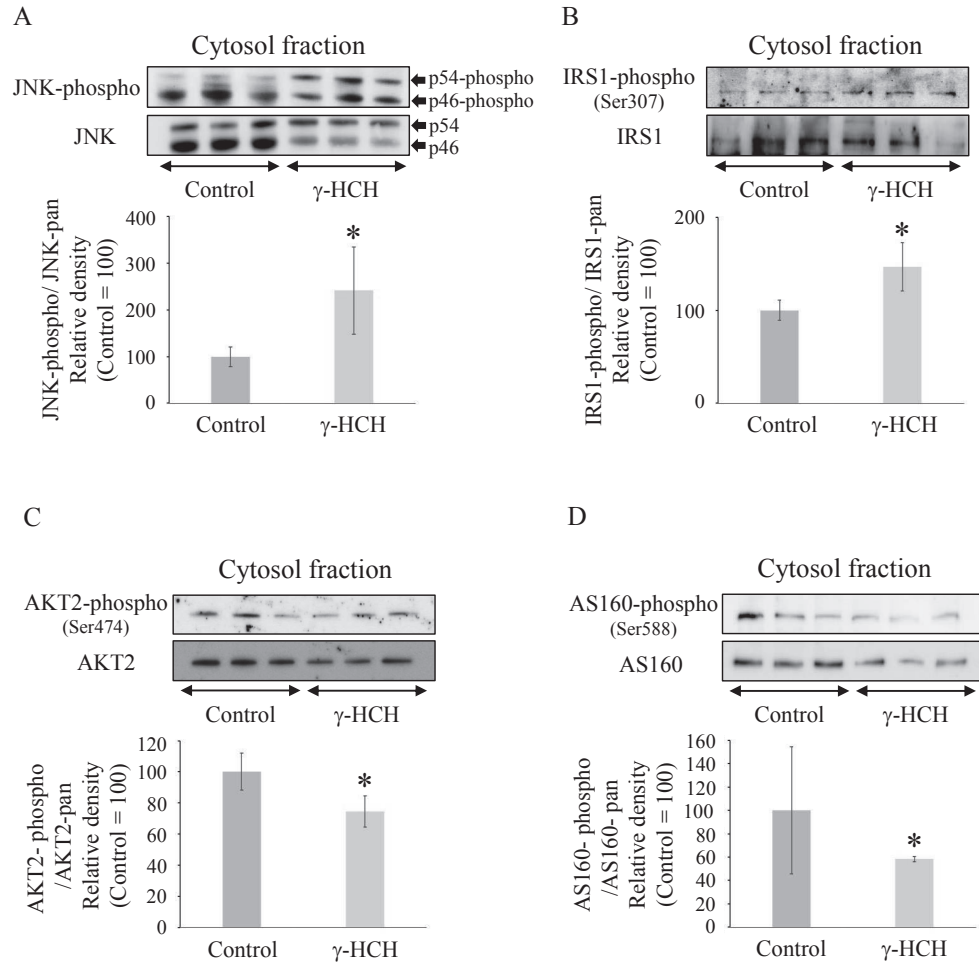
control cells. Intracellular ROS production was enhanced in  $\gamma$ -HCH-treated cells as compared to that in control cells (Fig. 2D). The exposure to  $\gamma$ -HCH enhanced the levels of ROS markers, such as cytochrome P450 1b1 (Fig. 3A), haptoglobin (Fig. 3B), ceruloplasmin (Fig. 3C), and xanthine dehydrogenase (Fig. 3D), as compared to those in the control cells. The levels of NADPH oxidase-4 (Nox-4) mRNA (Fig. 3E) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Fig. 3F) were also significantly augmented in  $\gamma$ -HCH-treated cells.

Treatment with  $\gamma$ -HCH enhanced intracellular ROS levels and phosphorylation of JNK (Fig.

4A). Under this condition,  $\gamma$ -HCH also enhanced phosphorylation of IRS-1 at Ser307, a target of JNK (Fig. 4B). The phosphorylation of AKT2 was suppressed upon treatment with  $\gamma$ -HCH (Fig. 4C). Phosphorylation of AS160 was also downregulated by  $\gamma$ -HCH (Fig. 4D).

In the incubation medium, there were no change in the protein levels of TNF- $\alpha$  with or without  $\gamma$ -HCH (Fig. 5A), while significant decrease was observed at the levels of both TNF- $\alpha$  mRNA (Fig. 5B) and protein (Fig. 5C) in the lysate of the cells exposed to  $\gamma$ -HCH compared to non-treated control cells.





**Figure 4. Effect of  $\gamma$ -HCH on the expression and phosphorylation of JNK, IRS-1, AKT2, and AS160 in mature 3T3-L1 adipocytes**

Phosphorylation of (A) JNK and (B) IRS-1, (C) AKT2 and (D) AS160. Results were representative of three independent experiment ( $n = 5$  for each group). Bars and vertical lines indicate mean  $\pm$  SD. \* $P < 0.05$ .

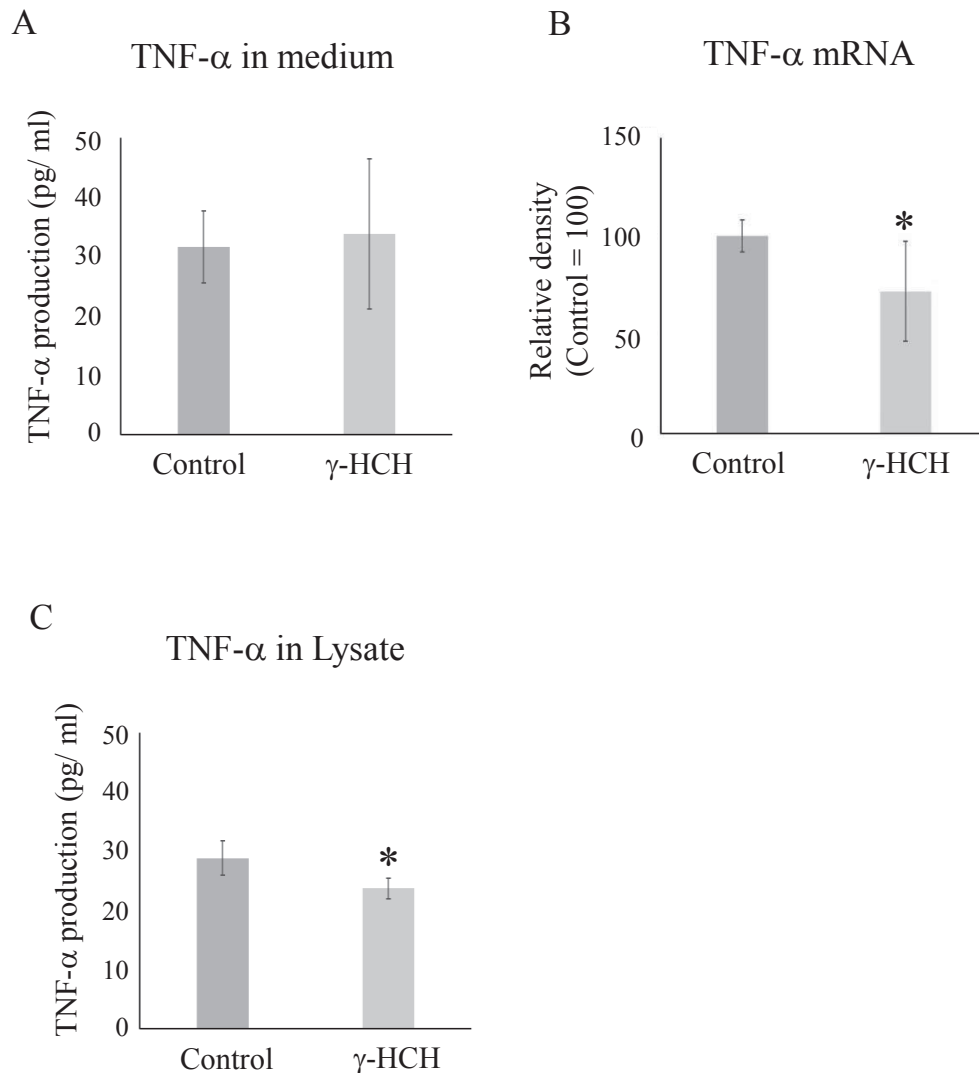
## Discussion

We firstly confirmed sub-toxic dose of  $\gamma$ -HCH in mature 3T3-L1 adipocytes as 50  $\mu$ M, and applied the concentration for whole experiments in this study. Thereby the results of this study are thought to be occurred under the normal situation without cell damage.

This study demonstrated that the addition of  $\gamma$ -HCH significantly reduced glucose uptake by fully differentiated mature 3T3-L1 adipocytes via the increased phosphorylation of IRS-1 at Ser307 following JNK activation that impaired

insulin-related signal transduction and resulted in insulin resistance. This was supported by the following experimental data. GLUT4 protein levels in the plasma membrane decreased in  $\gamma$ -HCH-treated cells as compared with that in untreated control cells, although the mRNA and protein levels of GLUT4 in the cytosol fraction were higher in  $\gamma$ -HCH-treated cells than those in the untreated control cells. In addition,  $\gamma$ -HCH augmented intracellular ROS generation with elevation in the mRNA levels of ROS markers as compared to those in the control cells.





**Figure 5.** Effect of  $\gamma$ -HCH on secretion of TNF- $\alpha$  into the cell culture medium and TNF- $\alpha$  mRNA and its protein in the lysate of mature 3T3-L1 adipocytes.

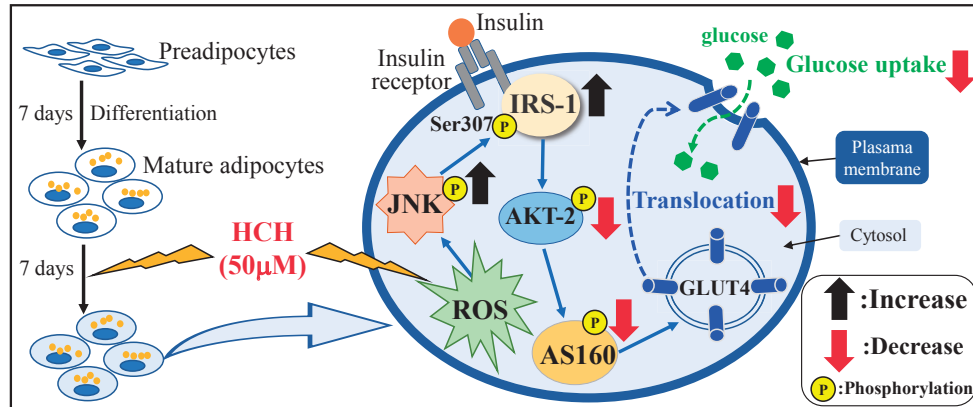
(A) secretion levels of TNF- $\alpha$  into the cell culture medium, (B) TNF- $\alpha$  mRNA and (C) its protein in the lysate. Bars and vertical lines indicate mean  $\pm$  SD. \*  $P < 0.05$  vs control values.

Moreover,  $\gamma$ -HCH hindered the phosphorylation of AKT2 and AS160, downstream molecules of phosphorylated IRS-1 at Ser307, suggesting a negative role of  $\gamma$ -HCH on glucose uptake via the inhibition of GLUT4 translocation from the cytosol to the plasma membrane, thereby causing insulin resistance in mature 3T3-L1 adipocytes.

$\gamma$ -HCH enhanced intracellular ROS produc-

tion in differentiated 3T3-L1 mature adipocytes; this is in accordance with the enhanced generation of ROS in other cells, such as renal distal tubule cells,<sup>28)</sup> dopaminergic neurons,<sup>29)</sup> and Ehrlich ascites tumor cells.<sup>30)</sup> NADPH oxidase-1 (Nox-1), a key enzyme involved in the production of cellular ROS, has a prominent role in  $\gamma$ -HCH-induced ROS generation in dopaminergic neurons.<sup>31)</sup> Interestingly, Nox-4, a member of the





**Figure 6. The schematic model of the current study**

Intracellular ROS generation is increased by treatment of  $\gamma$ -HCH, in turn, JNK is activated via its phosphorylation. Activated-JNK provokes phosphorylation of IRS-1 at Ser307. Consequently, translocation of GLUT4 from the cytosol to the plasma membrane is impaired, thereby attenuating the glucose uptake into mature 3T3-L1 adipocytes.

NADPH oxidase family of proteins, is only expressed in 3T3-L1 adipocytes.<sup>32)</sup> A dramatic increase in the ROS content of adipocytes has shown to be related with elevation of Nox-4 mRNA and protein.<sup>20)</sup> Moreover, Nrf2 mRNA levels are associated with the increased ROS generation in rat white adipocytes.<sup>21)</sup> Consequently, the  $\gamma$ -HCH-induced increase in ROS generation might be regulated by Nox-4 along with elevated levels of Nrf2 mRNA in 3T3-L1 adipocytes. This is in accordance with the findings of this study:  $\gamma$ -HCH induced the overexpression of Nox-4 and Nrf2 mRNAs.

JNK activation is enhanced by oxidative stress in 3T3-L1 adipose cells<sup>33,34)</sup> and ROS-mediated activation of JNK promotes insulin resistance in adipose cells.<sup>35,36)</sup> Previous studies have demonstrated that the mechanism underlying JNK-mediated insulin resistance is closely associated with the phosphorylation of Ser307 in IRS-1.<sup>25,37)</sup> The knockdown of JNK rescues insulin resistance in 3T3-L1 adipocytes<sup>38)</sup> and the inactivation of JNK attenuates Ser307 phosphorylation of IRS-1 and confers insulin resistance in 3T3-L1 adipocytes.<sup>39)</sup> Our data also demonstrat-

ed that the  $\gamma$ -HCH-stimulated phosphorylation of Ser307 in IRS-1 inactivated AKT2 and AS160, resulting in the reduced translocation of GLUT4 from the cytosol to the plasma membrane. Therefore,  $\gamma$ -HCH-induced phosphorylation of Ser307 in IRS-1 might play a critical role in the attenuation of insulin-stimulated glucose uptake, which is mediated by the translocation of GLUT4 protein. These results indicate that ROS-mediated activation of JNK upon treatment with  $\gamma$ -HCH inhibits the insulin-stimulated translocation of GLUT4 protein via an increase in the phosphorylation of IRS-1 at Ser307, thereby reducing insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

On the other hand, it has been widely accepted that TNF- $\alpha$  also attenuates insulin-induced glucose uptake via impaired insulin signaling.<sup>40-42)</sup> One of the mechanisms underlying TNF- $\alpha$ -induced insulin resistance has shown to involve the phosphorylation of Ser307 in IRS-1.<sup>43,44)</sup> Therefore, it was hypothesized that  $\gamma$ -HCH increases the secretion of TNF- $\alpha$  from adipocytes, thereby stimulating the phosphorylation of Ser307 in IRS-1 in adipose cells via autocrine



and paracrine signaling. However,  $\gamma$ -HCH had no effect on the secretion of TNF- $\alpha$  from mature 3T3-L1 adipocytes into the culture medium, although the levels of both TNF- $\alpha$  mRNA and protein was significantly reduced in  $\gamma$ -HCH-treated cells as compared to those in control cells. Thus, the ability of  $\gamma$ -HCH at phosphorylating Ser307 in IRS-1 through enhanced JNK activation via increase in ROS generation might be independent on TNF- $\alpha$ -induced phosphorylation event of IRS-1. These results suggest that  $\gamma$ -HCH-induced reduction of insulin-stimulated glucose uptake is independent of secreted TNF- $\alpha$  from cell themselves.

In conclusion,  $\gamma$ -HCH exhibited inhibitory effects on the insulin-stimulated uptake of glucose by mature 3T3-L1 adipocytes via ROS generation, JNK-induced phosphorylation of Ser307 of IRS-1, impaired translocation of GLUT4 protein from the cytosol to the plasma membrane, and inhibition of insulin signaling. Therefore, the findings of this study indicate that sub-toxic exposure to  $\gamma$ -HCH causes insulin resistance in mammalian mature adipocytes, and that removal of  $\gamma$ -HCH from the environment prevents  $\gamma$ -HCH-induced diabetes and other metabolic disorders.

## Conclusion

The present study demonstrated that  $\gamma$ -HCH attenuated glucose uptake in fully differentiated 3T3-L1 adipocytes through enhanced intracellular ROS and activated JNK. Under this condition, JNK-stimulated phosphorylation of Ser307 in IRS-1 impaired the activity of downstream molecules, thereby inhibiting the translocation of GLUT4 protein on the plasma membrane.

## Abbreviations

$\gamma$ -HCH: gamma-hexachlorocyclohexane; ROS: reactive oxygen species; GLUT4: glucose trans-

porter 4; JNK: c-Jun N-terminal kinase; IRS-1: insulin receptor substrate-1; AS160: Akt Substrate of 160 kDa; TNF- $\alpha$ : tumor necrosis factor alpha

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## Competing interests

The authors declare that they have no competing interests.

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## 抄録

本研究の目的は、有機塩素系農薬であるガンマ型ヘキサクロロシクロヘキサン（ $\gamma$ -HCH）が成熟型脂肪細胞へおよぼす影響について検討することである。成熟型に分化した3T3-L1脂肪細胞を  $\gamma$ -HCH の存在下と非存在下で37℃、5 %濃度の二酸化炭素の環境において7日間培養した。培養終了後に細胞の生存率、インスリン添加によるグルコースの取り込み能、細胞内活性酸素の発現量を蛍光アッセイ法にて測定した。mRNA とタンパク質の発現は、それぞれ定量 PCR 法と免疫ブロッティング法にて測定した。 $\gamma$ -HCH の添加は、非添加と比較してインスリン刺激による細胞へのグルコースの取り込みを有意に低下させ、細胞内の活性酸素量を有意に増加させた。この状況下では、細胞膜上における GLUT4タンパク質の発現が有意に低下した。 $\gamma$ -HCH の添加によって、活性酸素の発現マーカーである分子群の mRNA 発現と JNK タンパク質のリン酸化も有意に増加した。さらに、 $\gamma$ -HCH の添加はインスリン刺激による糖取り込みを抑制する IRS-1タンパク質の307番目にあるセリン残基のリン酸化を有意に増加させ、その下流に存在する AKT2タンパク質と AS160タンパク質のリン酸化を有意に抑制した。一連の結果より、 $\gamma$ -HCH の添加は細胞内の活性酸素量を増加させ、それに伴い活性化された JNK タンパク質が IRS-1の307にあるセリン残基のリン酸化を増加させることによって AKT2タンパク質と AS160タンパク質の活性が有意に低下し、これに伴い GLUT4タンパク質の細胞膜への移行が阻害されることによってインスリン刺激によるグルコースの取り込みが低下する、インスリン抵抗性が生じることが明らかとなった。

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《キーワード》 ガンマ型ヘキサクロロシクロヘキサン、成熟型白色脂肪細胞、インスリン抵抗性、グルコース輸送単体4

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