BPB Reports

Report

Effects of the Interplay between Selenocystine and Methylmercury on Their Cytotoxicity and Glucose-Driven Insulin Secretion from Mouse Insulinoma Cells

Daichi Chida,[1] Takashi Toyama,[1,*] Takanori Chiba, Takayuki Kaneko, Kotoko Arisawa, and Yoshiro Saito[*]

Laboratory of Molecular Biology and Metabolism, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-8578, Japan
Received April 26, 2022; Accepted June 27, 2022

We've previously shown that high levels of selenoprotein P (SeP), a major selenoprotein in plasma, can be a risk factor of type 2 diabetes. It was also thought that inhibition of insulin secretion caused by over-supplementation of selenium by SeP to pancreatic β cells contributed to the progress of diabetes. On the other hand, methylmercury, which is an environmental pollutant, is known to cancel the action of selenium via the covalent modification. Therefore, we thought that the interaction between selenium and methylmercury could be associated with the pathogenesis of diabetes. To address the hypothesis, MIN6 cells, a mouse pancreatic β-cell line, were treated with selenocystine (as a selenium donner) and methylmercury then examined insulin release from the cells. Selenocystine (400–1200 nM), which corresponds to the concentration of selenium in SeP of diabetic patients, shows cytotoxicity and inhibited glucose-driven insulin secretion. Methylmercury rescued the cytotoxicity that induced by selenocystine, however it affected the insulin secretion that is depressed by selenocystine at little intense. These data indicate that the mechanisms underlying inhibition of insulin secretion by selenocystine are independent of cytotoxicity, and methylmercury cannot be expected to restore insulin secretion or suppress diabetes as selenium neutlizer.

Key words Selenocystine, Methylmercury, Diabetes

INTRODUCTION

Insulin is the only hormone that lowers blood glucose levels and plays central role in the pathogenesis of diabetes. Decreasing of insulin releases or progress of insulin resistance makes it difficult to take up glucose into the tissues/cells, and lead continuous hyperglycemic conditions, which leads to serious complications such as diabetic retinopathy and diabetic kidney disease.[1,2] The number of people with diabetes has been rising worldwidely, nearly quadrupling from 108 million in 1980 to 422 million in 2014 and 90% of all diabetic patients have type 2 diabetes. Interestingly, it has become clear that trace biometal elements and trace harmful-metal elements are involved in the pathogenesis of diabetes, respectively. These have been attracting attention as a new research area of metallomics in recent years, but little is understood about the interactions among these trace elements in the pathogenesis of diabetes.

Although selenium is an essential trace element, higher uptake of the element causes risk of human health. A few intervention studies performed in U.S. indicate daily supplementation of selenium enhanced risk of diabetes, however the mechanism underlying is not well understood.[3–7] Selenium is incorporated into the side chain of the amino acid, selenocysteine, and used as selenoproteins in the cells. In the liver, selenoprotein P (SeP) is known as a major selenoprotein and it is released out to the plasma to transfer the selenium to the whole body.[8] We have been reported that increased SeP production in the liver during chronic hyperglycemic conditions such as type 2 diabetes, which causes insulin resistance in the liver and skeletal muscle, and also impaired insulin secretion in pancreatic β cells.[9–11] We've been also found that insulin resistance and insulin secretion are improved by inhibiting SeP uptake and selenium supply of SeP into cells by administering a SeP neutralizing antibody in such an excessive SeP condition.[11] From the above, it has become clear that excess SeP promotes diabetes through excessive supply of selenium.

Methylmercury is a harmful environmental pollutant, and is naturally produced by microorganisms from inorganic mercury and accumulated in large fish through the food chain.[12] Number of studies suggested that methylmercury is a risk factor for the neural development of fetus,[13,14] while some recent epidemiological studies suggested that methylmercury could rise risk of diabetes.[15,16] Studies of cultured cells and *in vivo* have shown that excess methylmercury causes inhibition of insulin secretion in pancreatic β-cells via oxidative stresses and inducing cell death.[17] However, the effects of sub-cytotoxic methylmercury on pancreatic β-cells are not well understood. It has been reported that selenium and methylmercury cancel each other's toxicity *in vivo*, may be due to formation of stable covalent bond.[18,19] Taken together excess selenium and excess methylmercury, which are the factors that exacerbate diabetes, are expected to have some effect on the pathological condition of diabetes by interacting each other *in vivo*. Hence this study aimed to elucidate the effect of the interac-

*To whom correspondence should be addressed. e-mail: yoshiro.saito.a8@tohoku.ac.jp; takashi.toyama.c6@tohoku.ac.jp
[1] These authors contributed equally to the work.

tion between selenium and methylmercury on insulin secretion in pancreatic β cells.

MATERIALS AND METHODS

Reagents L-selenocytine (> 97% pure) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Methylmercuric chloride (> 95% pure, analytical grade) was purchased from Kanto Chemical (Tokyo, Japan). All other reagents used in this study were highest grade available.

Cell Culture and Treatment of Chemicals MIN6 cells, a mouse insulinoma cell line, was used in this study. The cells were maintained at 37°C in a humidified incubator in an atmosphere of CO_2 (5%) and ambient air (95%). The cells were cultured in DMEM (high glucose) supplemented by 10% FBS and 18 μg/mL of streptomycin penicillin. Harvested cells were counted and seeded onto cell culture dish for passage, 12 well plate (2.9×10^5 cells/well) or 96 well plate (2.2×10^4 cells/well) for experiments. Cells were seeded before 24 h exposure of chemicals. When the time of exposures, the culture medium were removed and replaced with a fresh medium containing selenocystine and/or methylmercury, and further cultured for 24 h. In the steady-state insulin secretion analysis, cells and medium were collected at the endpoint.

Cell Viability Cell viability was measured by WST-8 based cell counting kit (Dojindo, Kumamoto, Japan) according to the manufactures protocol. Briefly, the incubation medium of the cells were discarded at the endpoint and added 100 μL of 10% Cell Counting Kit-8 diluted in fresh culture medium. After the incubation for further 2 h, absorbance 450 nm was measured by Spectra Max iD5 (Molecular Devices, CA, USA). Cell viability was shown as 100% of control. Alternatively, cell viability was also evaluated by trypan blue assay. The cells were harvested and stained with trypan blue solution (Sigma-Aldrich, MO, USA), then the white cells were counted as viable cells and blue cells were as dead cells. Dead cells per total cells were shown as cell viability.

Glucose-Driven Insulin Secretion We performed glucose-driven insulin secretion assay as reported previously.[20] After the exposures of chemicals to the MIN6 cells, the incubation medium were changed to 2.8 mM glucose containing Krebs-Ringer bicarbonate-HEPES buffer (KRBH) and incubated for 1 h to set the cells at low glucose condition. Then the buffer changed with 22.4 mM glucose containing KRBH and incubated for 1 h, and harvested the cells or supernatants.

SDS-PAGE and Western Blotting The cells were washed with PBS and harvested by incubating with RIPA buffer (0.1% SDS, 0.5% DOC, 1% NP-40, 150 mM NaCl in 50 mM Tris-HCl (pH 8.0)) for 15 min on ice bath. After centrifuge for 15,000 *g*, 10 min at 4°C, supernatants were collected as cell lysate. Protein concentration of each lysate was examined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). The lysate containing 20 μg of proteins were mixed with 4 × SDS sample buffer and boiled for 5 min to detect pro-insulin. SDS sample buffer without 2-mercaptoethanol (2-ME) were used for the detection of mature insulin. Aliquot of the medium were mixed with 4 × SDS sample buffer without 2-ME and boiled for 5 min to prepare medium sample. These samples were subjected to SDS-polyacrylamide gel electrophoresis. The gel was transferred to nitrocellulose membrane (Fujifilm-WAKO, Osaka, Japan) and reacted with antibodies against anti-insulin mouse mAb (Cell signaling technology, CA, USA) for the detection of mature insulin, and anti-insulin mouse mAb (Sigma-Aldrich) for the detection of pro-insulin. Anti-βactin antibody (Sigma-Aldrich) was used for internal control.

Statistical Analysis Statistical significance was assessed by Graphpad Prism using one-way ANOVA post-hoc Tukey test.

RESULTS

Selenocystine Inhibits Insulin Release from MIN6 Cells, Accompanied by Cytotoxicity We first confirmed whether excessive selenium supply inhibits insulin secretion using MIN6 cells, which are pancreatic β-cell lines that secrete insulin. After being taken up into cells, SeP is degraded by lysosomes and metabolized to selenocysteine.[21] Assuming that the plasma SeP concentration of diabetic patients is approximately 10 μg/mL and 10 residues of selenocysteine are encoded per 1 molecule of SeP,[10] thus the concentration of selenocysteine corresponding to the amount of selenium in SeP in this plasma is approximately 1600 nM. On the other hand, selenocysteine cannot be obtained due to its instability. Therefore, selenocystine, which is an oxidized selenocysteine was used as a selenocysteine donor (800 nM of selenocystine is comparable to 1600 nM selenocysteine and 10 μg/mL of SeP). As a result of treating MIN6 with selenocystine, the cell viability was significantly reduced at 400 nM or higher concentration (Fig. 1A). In this condition, static insulin secretion was partially decreased and intracellular pro-insulin levels were also lowered compared with control (Fig. 1B). We evaluated glucose-driven insulin secretion in the same condition with above, and found that selenocystine inhibited glucose-responsive release of insulin at cytotoxic concentration (Fig. 1C). These results suggest that over-supplementation of selenium to MIN6 cells causes inhibition of insulin secretion, and this is associated with cytotoxicity.

Methylmercury Did Not Inhibit but Promote Insulin Secretion from MIN6 Cells at Sub-Cytotoxic Concentrations As mentioned above, methylmercury has been reported to damage pancreatic β-cells and exacerbate diabetes at *in vivo*.[17] Thus, we examined the effects of methylmercury on the cell survival and insulin release form MIN6 cells. Cell viability was significantly reduced at 1200 nM of methylmercury compared with the control (Fig. 2A). Then MIN6 was treated with indicated concentrations of methylmercury for 24 h and intracellular pro-insulin, intracellular matured insulin, and extracellular matured insulin were detected. Intracellular pro-insulin and βactin levels were decreased by cytotoxic concentration of methylmecury (1200 nM), although methylmercury did not alter intracellular and extracellular mature insulin levels (Fig. 2B). Sub-cytotoxic concentration of methylmercury did not alter pro-insulin and mature insulin levels. Glucose-driven insulin release was partially enhanced by 200–400 nM of methylmercury and this effect was decreased to basal levels by the higher concentration (Fig. 2C). These data indicate that methylmercury may alter insulin production via depression of pro-insulin at cytotoxic dose, however sub-cytotoxic concentration of methylmercury may involve in enhancement of insulin secretion in a part.

Although Methylmercury Canceled the Cytotoxicity of Selenocystine, It Affected Little Intense on the Inhibition of Insulin Secretion by Selenocystine Toxicity of selenium is known to be canceled by methylmercury, thus we thought that inhibition of insulin secretion by selenocystine over-sup-

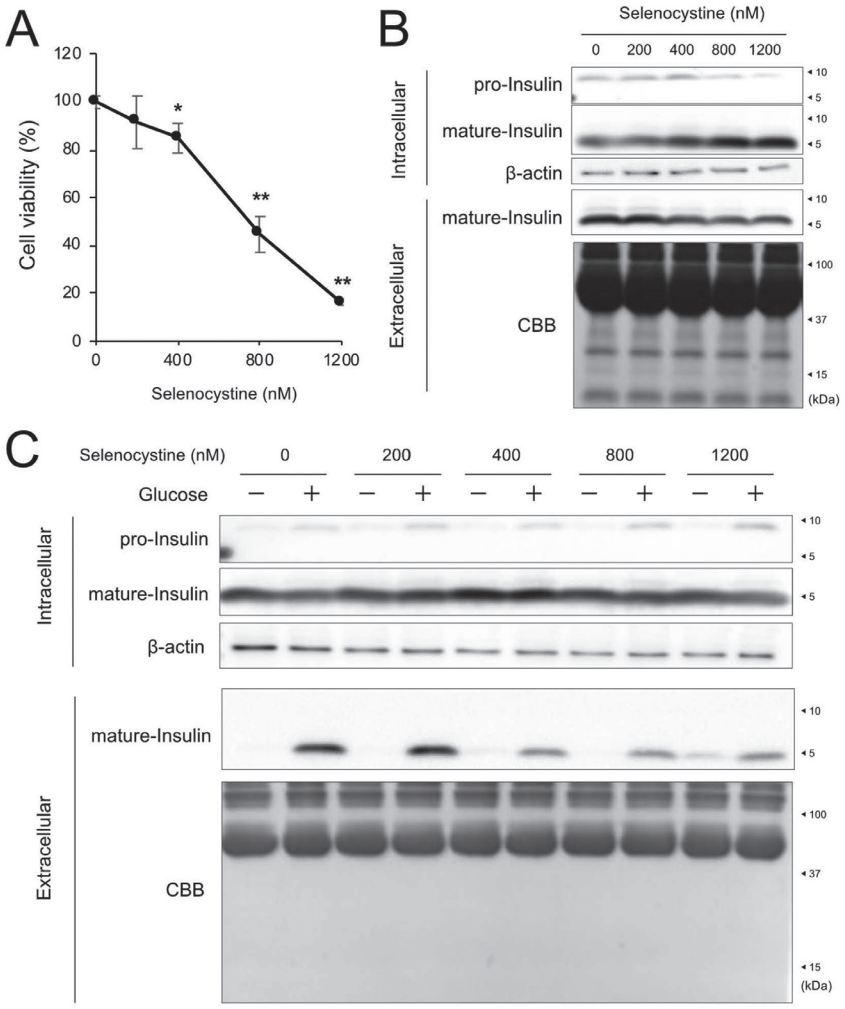

Fig. 1. Effects of Selenocystine on Cytotoxicity and Glucose-Driven Insulin Release from MIN6 Cells

(A) MIN6 cells were cultured for 24 h. The cells were treated with the indicated concentration of selenocystine for 24 h. After that, cell viability was measured by WST-8 assay according to MATERIALS AND METHODS section (n = 4, mean ± S.D. were shown). * $P < 0.05$ vs control, ** $P < 0.01$ vs control. (B) The cells were cultured for 24 h and exposed to the indicated concentrations of selenocystine for 24 h. Cell lysate and medium were collected and subjected to Western blotting. (C) Glucose-driven insulin secretion assay was performed according to materials & methods section, and collected samples were subjected to Western blotting.

plementation would be rescued by methylmercury. To address that, the effect of methylmercury on selenocystine-induced cell death was measured. The cell viability was decreased by 800 nM of selenocystine, and this is completely canceled by addition of 800 nM of methylmercury (Fig. 3A, B). In this condition, methylmercury itself was not affected the cell viability. Interestingly, despite of the recovery of cytotoxicity by selenocystine, methylmercury affected little intense on the inhibition of glucose-driven insulin release by selenocystine (Fig. 3C). These result suggest that methylmercury-selenocysteine adduct might be not toxic but still has the capability to inhibit the glucose-driven insulin secretion.

DISCUSSION

In this study, we found that excessive selenocystine inhibits insulin secretion from pancreatic β-cells and this is concomitant with cell death. Methylmercury canceled the cytotoxicity induced by selenocystine, while it alter little against inhibition of insulin release by selenocystine. These data clearly indicate that interplay of selenocysteine and methylmercury is important for neutralization of their toxicity, while it may be independent from insulin secretion responded to glucose. Detailed mechanism are obscure, while these data present new insight into metal-interactions in the pathogenesis of diabetes.

We used WST-8 assay, an assay for evaluate cellular reductive activity as cell survival, for determine cytotoxicity of methylmercury and selenocystine. Thus this is limitation of our study because it is difficult to assess whether the effect of methylmercury and selenocystine on WST-8 value is dysfunction of cells (impairment of cellular reductive systems) or cell death. We also examined LDH activity in culture medium, however methylmercury inhibited LDH enzymatic activity directly and it was failed to determine exact values (data not shown). At least trypan blue assay, which can evaluate cell membrane permeability, shows almost same tendency with WST-8 assay (Fig. 3B).

Sub-cytotoxic does of methylmercury induced insulin secretion from pancreatic β-cells. Oxidative stresses are a typical toxic effect of methylmercury,[22] but it has been reported that physiological hydrogen peroxide is required for glucose-responsive insulin secretion.[23] Thus oxidative stresses induced

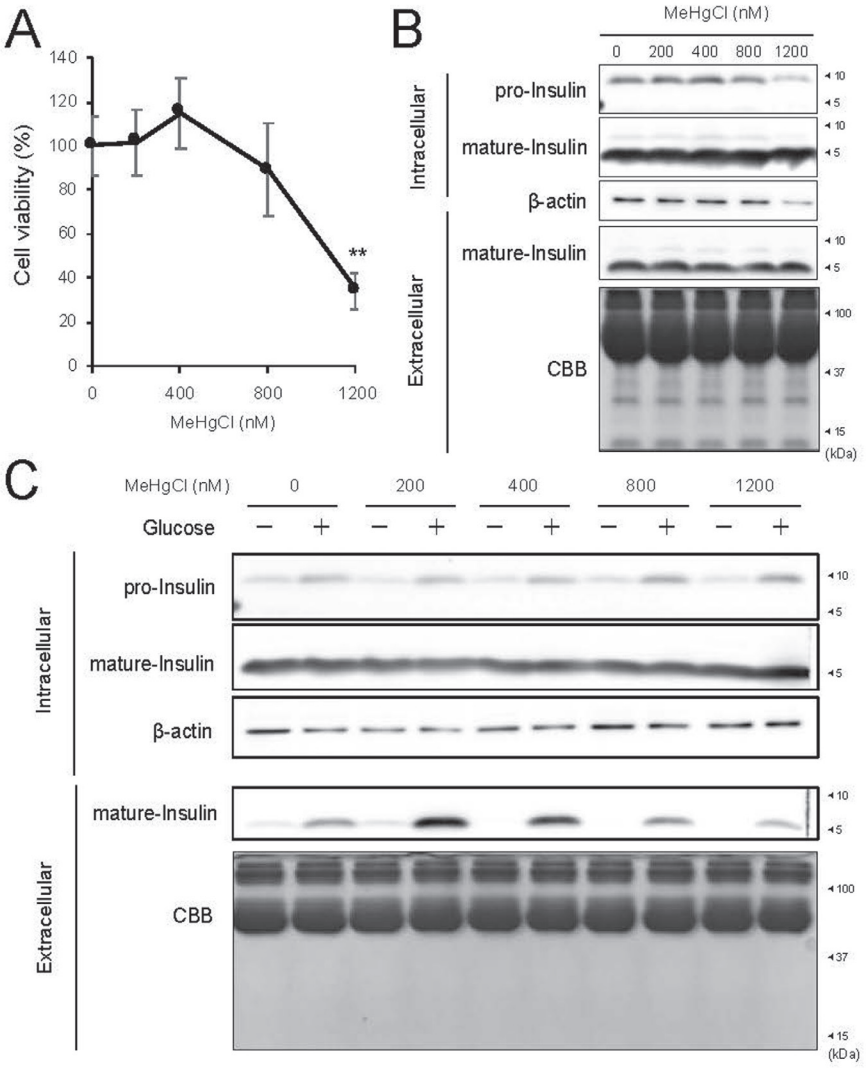

Fig. 2. Effects of Methylmercury on Cytotoxicity and Glucose-Driven Insulin Release form MIN6 Cells

(A) MIN6 cells were cultured for 24 h. The cells were treated with the indicated concentration of methylmercuric chrolide (MeHgCl) for 24 h. After that, cell viability was measured by WST-8 assay according to materials & methods section (n = 4, mean ± S.D. were shown). ** $P < 0.01$ vs control. (B) The cells were cultured for 24 h and exposed to the indicated concentrations of MeHgCl for 24 h. Cell lysate and medium were collected and subjected to Western blotting. (C) Glucose-driven insulin secretion assay was performed and collected samples were subjected to Western blotting.

by sub-cytotoxic methylmercury may contributed to the promotion of glucose-responsive insulin secretion (Fig. 2C).

It has been thought that methylmercury is covalently modified with selenocysteine residues to form MeHg-Sec to inhibit selenoprotein activity such as glutathione peroxidases and thioredoxine reductase.[24,25] In the present condition, methylmercury could bind with selenocysteine that is produced by intracellular-reducing system from selenocystine. This complex is thought to relatively stable and undergo further metabolize to inorganic mercury HgSe, a less-toxic complex.[26] However, the physiological/toxicological role of HgSe is not well known. Our present data indicate MeHg-Sec or HgSe are less toxic than selenocystine or methylmercury themselves, although these non-toxic complexes seems still have inhibitory effects against glucose-driven insulin releases. Insulin is released out of the cells responded to incorporation of glucose and production of ATP in mitochondria, and following activation of potassium channel/calcium channel cascade that drives calcium-induced calcium release (CICR) from ER and exocytosis.[27] We are trying to elucidate the inhibitory mechanism of insulin secretion due to excess selenium, and found that CICR or exocytosis pathway could be involved in inhibition of insulin release by selenocystine (unpublished observation). Thus the complex of methylmercuy and selenocysteine would be affected as same as selenocysteine at that points. Further studies are needed to elucidate the precise molecular mechanisms.

Acknowledgments This work was supported by a Grant-in-Aid (20H00488 and 20H05493) for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Muoio DM, Newgard CB. Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat. Rev. Mol. Cell Biol.*, **9**, 193–205 (2008).

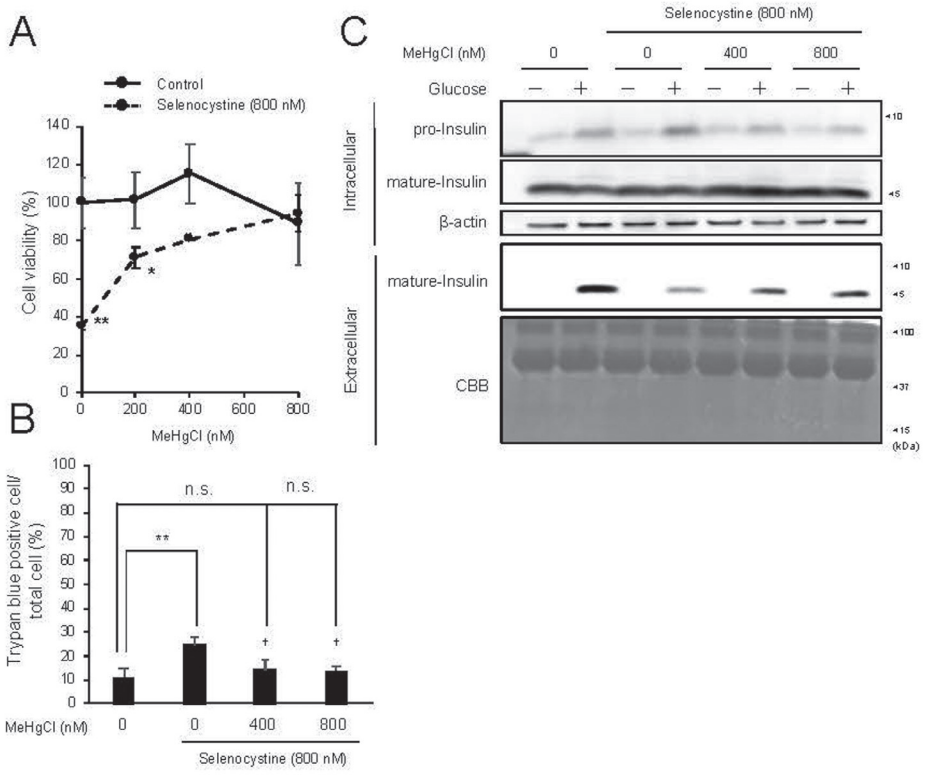

Fig. 3. Effects of Methylmercury on the Cytotoxicity of Selenocystine and Inhibition of Glucose-Driven Insulin Secretion by Selenocystine

MIN6 cells were cultured for 24 h. The cells were treated with 800 nM of selenocystine with the indicated concentration of methylmercuric chrolide (MeHgCl) for 24 h. After that, cell viability was measured by WST-8 assay (A; n = 4, mean ± S.D.) or trypan blue assay (B; n = 3, mean ± S.D.) according to materials & methods section. * $P < 0.05$ vs control, ** $P < 0.01$ vs control. †$P < 0.05$ vs selenocystine 800 nM. n.s indicates not significant. (C) The cells were cultured for 24 h and exposed to 800 nM of selenocystine with the indicated concentrations of MeHgCl for 24 h. Then glucose-driven insulin secretion assay was performed, and collected samples were subjected to Western blotting.

2) Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat. Rev. Endocrinol.*, **14**, 88–98 (2018).
3) Bleys J, Navas-Acien A, Guallar E. Serum selenium and diabetes in U.S. adults. *Diabetes Care*, **30**, 829–834 (2007).
4) Zhou J, Huang K, Lei XG. Selenium and diabetes--evidence from animal studies. *Free Radic. Biol. Med.*, **65**, 1548–1556 (2013).
5) Stranges S, Sieri S, Vinceti M, Grioni S, Guallar E, Laclaustra M, Muti P, Berrino F, Krogh V. A prospective study of dietary selenium intake and risk of type 2 diabetes. *BMC Public Health*, **10**, 564 (2010).
6) Laclaustra M, Navas-Acien A, Stranges S, Ordovas JM, Guallar E. Serum selenium concentrations and diabetes in U.S. adults: National Health and Nutrition Examination Survey (NHANES) 2003-2004. *Environ. Health Perspect.*, **117**, 1409–1413 (2009).
7) Stranges S, Marshall JR, Natarajan R, Donahue RP, Trevisan M, Combs GF, Cappuccio FP, Ceriello A, Reid ME. Effects of long-term selenium supplementation on the incidence of type 2 diabetes: a randomized trial. *Ann. Intern. Med.*, **147**, 217–223 (2007).
8) Saito Y. Selenoprotein P as an *in vivo* redox regulator: disorders related to its deficiency and excess. *J. Clin. Biochem. Nutr.*, **66**, 1–7 (2020).
9) Saito Y. Selenoprotein P as a significant regulator of pancreatic beta cell function. *J. Biochem.*, **167**, 119–124 (2020).
10) Misu H, Takamura T, Takayama H, Hayashi H, Matsuzawa-Nagata N, Kurita S, Ishikura K, Ando H, Takeshita Y, Ota T, Sakurai M, Yamashita T, Mizukoshi E, Yamashita T, Honda M, Miyamoto K, Kubota T, Kubota N, Kadowaki T, Kim HJ, Lee IK, Minokoshi Y, Saito Y, Takahashi K, Yamada Y, Takakura N, Kaneko S. A liver-derived secretory protein, selenoprotein P, causes insulin resistance. *Cell Metab.*, **12**, 483–495 (2010).
11) Mita Y, Nakayama K, Inari S, Nishito Y, Yoshioka Y, Sakai N, Sotani K, Nagamura T, Kuzuhara Y, Inagaki K, Iwasaki M, Misu H, Ikegawa M, Takamura T, Noguchi N, Saito Y. Selenoprotein P-neutralizing antibodies improve insulin secretion and glucose sensitivity in type 2 diabetes mouse models. *Nat. Commun.*, **8**, 1658 (2017).
12) Hintelmann H. Organomercurials. Their formation and pathways in the environment. *Met. Ions Life Sci.*, **7**, 365–401 (2010).
13) Grandjean P, Weihe P, White RF, Debes F, Araki S, Yokoyama K, Murata K, Sorensen N, Dahl R, Jorgensen PJ. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicol. Teratol.*, **19**, 417–428 (1997).
14) Debes F, Budtz-Jorgensen E, Weihe P, White RF, Grandjean P. Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years. *Neurotoxicol. Teratol.*, **28**, 536–547 (2006).
15) He K, Xun P, Liu K, Morris S, Reis J, Guallar E. Mercury exposure in young adulthood and incidence of diabetes later in life: the CARDIA Trace Element Study. *Diabetes Care*, **36**, 1584–1589 (2013).
16) Tsai TL, Kuo CC, Pan WH, Wu TN, Lin P, Wang SL. Type 2 diabetes occurrence and mercury exposure - From the National Nutrition and Health Survey in Taiwan. *Environ. Int.*, **126**, 260–267 (2019).
17) Chen YW, Huang CF, Tsai KS, Yang RS, Yen CC, Yang CY, Lin-Shiau SY, Liu SH. Methylmercury induces pancreatic beta-cell apoptosis and dysfunction. *Chem. Res. Toxicol.*, **19**, 1080–1085 (2006).
18) Ralston NV, Ralston CR, Blackwell JL 3rd, Raymond LJ. Dietary and tissue selenium in relation to methylmercury toxicity. *Neurotoxicology*, **29**, 802–811 (2008).
19) Naganuma A, Kojima Y, Imura N. Interaction of methylmercury and selenium in mouse: formation and decomposition of bis (methylmercuric) selenide. *Res. Commun. Chem. Pathol. Pharmacol.*, **30**, 301–316 (1980).
20) Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes*, **53**, 3142–3151 (2004).
21) Kurokawa S, Hill KE, McDonald WH, Burk RF. Long isoform mouse selenoprotein P (Sepp1) supplies rat myoblast L8 cells with selenium via endocytosis mediated by heparin binding properties and apolipoprotein E receptor-2 (ApoER2). *J. Biol. Chem.*, **287**, 28717–28726 (2012).

22) Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. *Neurotoxicology*, **13**, 637–648 (1992).
23) Plecita-Hlavata L, Jaburek M, Holendova B, Tauber J, Pavluch V, Berkova Z, Cahova M, Schroder K, Brandes RP, Siemen D, Jezek P. Glucose-Stimulated Insulin Secretion Fundamentally Requires H2O2 Signaling by NADPH Oxidase 4. *Diabetes*, **69**, 1341–1354 (2020).
24) Hirota Y, Yamaguchi S, Shimojoh N, Sano KI. Inhibitory effect of methylmercury on the activity of glutathione peroxidase. *Toxicol. Appl. Pharmacol.*, **53**, 174–176 (1980).
25) Wagner C, Sudati JH, Nogueira CW, Rocha JB. In vivo and *in vitro* inhibition of mice thioredoxin reductase by methylmercury. *Biometals*, **23**, 1171–1177 (2010).
26) Khan MA, Wang F. Chemical demethylation of methylmercury by selenoamino acids. *Chem. Res. Toxicol.*, **23**, 1202–1206 (2010).
27) Islam MS, Rorsman P, Berggren PO. Ca(2+)-induced Ca2+ release in insulin-secreting cells. *FEBS Lett.*, **296**, 287–291 (1992).