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Research article

Autophagy activation is required for homocysteine-induced apoptosis in bovine aorta endothelial cells

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ABSTRACT

An elevated level of homocysteine (Hcy) in plasma is an independent risk factor for cardiovascular disease and central nervous system disease. Endothelial dysfunction as a result of apoptosis in endothelial cells is involved in the development and progression of these diseases. In this study, we aimed to investigate the effect of autophagy activation by amino acid starvation on Hcy-induced cytotoxicity in bovine aorta endothelial cells (BAECs). Hcy-induced lactate dehydrogenase (LDH) release was promoted by amino acid starvation. In addition, Hcy increased cleaved caspase-3 level, an indicator of apoptosis, by amino acid starvation. We revealed that oxidative stress is not involved in the Hcy-induced cytotoxicity promoted by amino acid starvation. Salazosulfapyridine (SASP), an SLC7A11 inhibitor, protected against the Hcy-induced LDH release promoted by amino acid starvation. SASP decreased the Hcy-induced cleaved caspase-3 level by amino acid starvation. We demonstrate for the first time that autophagy activation by amino acid starvation promotes Hcy-induced apoptosis in BAECs. Moreover, SLC7A11 inhibitor SASP, which is an amino acid transporter, protects against Hcy-induced apoptosis due to autophagy.

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid derived from methionine metabolism [1]. An elevated level of Hcy in plasma, a condition called hyperhomocysteinemia, is an independent risk factor for cardiovascular disease, such as coronary heart disease, and central nervous system disease, such as Alzheimer disease [1, 2]. Although Hcy is involved in the onset and progression of many diseases, studies of the underlying mechanism are lacking. Endothelial dysfunction as a result of apoptosis in endothelial cells is involved in the development and progression of these diseases [3]. However, how endothelial cells are protected against Hcy-induced cytotoxicity has not been investigated.

Autophagy is an intracellular system that delivers cytosolic proteins and organelles to lysosome for degradation. Autophagy is widely implicated in pathophysiological processes (e.g., cancer, metabolic and neurodegenerative disorders, cardiovascular and pulmonary diseases) [4, 5, 6]. Autophagy is typically a stress adaptation mechanism in response to a variety of stress stimuli, including starvation, hypoxia, endoplasmic reticulum (ER) stress, and oxidative stress [7]. Amino acid starvation is a typical inducer of autophagy [8, 9]. However, there are few studies showing that Hcy induces autophagy [10, 11]. The effect of the autophagy inducer itself, such as amino acid starvation, on the cytotoxicity of Hcy is unclear. Autophagy promotes cell survival by eliminating dysfunctional mitochondria that release cell-death-inducing reactive oxygen species (ROS) [12]. It has been reported that Hcy induced cytotoxicity via oxidative stress in endothelial cells [13, 14]. Conversely, like apoptosis, autophagy is a form of programmed cell death. It has been reported that Hcy induces mitochondrial apoptosis in SH-SY5Y cells [15]. However, the effect of autophagy on the Hcy-induced apoptosis is unclear.

The X_c system, which consists of SLC7A11 (also known as xCT), functions as a Na^+-independent electroneutral exchange system for cystine/glutamate [16]. SLC7A11 expression on cell membrane is essential for the uptake of cystine required for the synthesis of intracellular glutathione (GSH), an antioxidant that plays an important role in maintaining intracellular redox balance [17]. Human aortic endothelial cells can import Hcy via at least four of the known cysteine transport systems, namely, X_{AG}, L, ASC, and A [18]. However, the relationship between Hcy and SLC7A11 is unclear.

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Glucose starvation induces SLC7A11 expression in several cancer cells [19]. However, the effect of amino acid starvation on SLC7A11 remains little understood. Moreover, it is not clear whether SLC7A11 plays a role in the protection against Hcy-induced cytotoxicity.

In this study, we investigated the effect of autophagy on Hcy-induced cytotoxicity in bovine aorta endothelial cells (BAECs). We demonstrated that autophagy activation by amino acid starvation promotes Hcy-induced apoptosis. Moreover, SLC7A11 inhibitor protects against the Hcy-induced apoptosis due to autophagy.

2. Materials and methods

2.1. Cell culture and treatment with Hcy

BAECs were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Cells were grown to 80–90% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Before treating the cells with Hcy (Sigma-Aldrich, St. Louis, MO, USA), the culture medium was replaced with serum-free DMEM in the presence or absence of amino acid. BAECs were treated with 2.5 mM Hcy for 24 h.

2.2. Measurement of protein

p62, LC3, caspase-3, and SLC7A11 protein levels were analyzed by western blotting. The cells were treated with 2.5 mM Hcy or 2.5 μM chloroquine (CQ) for 24 h, washed with Dulbecco's phosphate buffered saline (DPBS), and lysed in lysis buffer [50 mM HEPES (pH 7.4), 5 mM EDTA, 120 mM NaCl, 1% Triton X-100, protease inhibitors (10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate)]. The lysate was centrifuged at 10,000×g for 15 min and 15 μg of protein in the supernatant was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the following primary antibodies: anti-rabbit LC3 polyclonal antibody (Novus Biologicals, Centennial, CO, USA), anti-rabbit p62 (Cell Signaling Technology, Danvers, MA, USA), anti-rabbit caspase-3 (Cell Signaling Technology), anti-rabbit SLC7A11 (Cell Signaling Technology), and anti-mouse β-actin polyclonal antibody (Sigma-Aldrich). Following primary antibody incubation, the membrane was incubated with horseradish-peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was detected with Immobilon (Merck, Darmstadt, Germany).

2.3. Measurement of cytotoxicity

Cell viability was assessed by measuring acid phosphatase activity. Acid phosphatase activity, which is an accurate indicator of the number of endothelial cells in culture, was assayed using the method of Connolly et al. [20]. BAECs in 96-well plates were treated with 2.5 mM Hcy for 24 h. After the treatment with Hcy, acid phosphatase activity was measured. The medium containing detached BAECs was removed. Cells remaining in the 96-well plates were washed with DPBS and incubated with 100 μL of 0.1 M sodium acetate buffer (pH 5.5) containing 0.1% Triton X-100 and 10 mM *p*-nitrophenyl phosphate at 37 °C for 20 min. The reaction was stopped by adding 10 μL of 1 M NaOH. Produced *p*-nitrophenol was measured at 405 nm using a Bio-Rad iMark microplate reader (Tokyo, Japan).

Cell death was assessed by measuring lactate dehydrogenase (LDH) release. After treatment of BAECs in 24-well plates with 2.5 mM Hcy for 24 h, aliquots of the medium were taken to measure the activity of LDH released from cells. The remaining intracellular LDH was released by adding 0.1% Triton X-100 in phosphate-buffered saline (PBS) at pH 7.4. LDH activity was measured spectrophotometrically on the basis of the increase in absorbance at 340 nm with 60 mM lithium lactate in 0.3 M diethanolamine buffer (pH 9.0), after the reaction was initiated with 3 mM (final concentration) NAD^+. Released LDH activity was expressed as percentage of total LDH activity (activities of LDH in the medium and in the remaining cells).

2.4. Measurement of mRNA

Quantitative RT-PCR analysis was used to measure mRNA levels. Total RNA from treated cells was extracted with a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. mRNAs were reverse-transcribed into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems). Primers for bovine SOD1, bovine SOD2, bovine catalase, and bovine SLC7A11 were purchased from Applied Biosystems. mRNA levels were acquired from the values of the threshold cycle (Ct) of SOD1, SOD2, catalase or SLC7A11 normalized to that of GAPDH. Relative mRNA levels were compared and expressed as percentage of control levels. Data are representative of three experiments.

2.5. Measurement of GSH

Intracellular GSH levels were measured by spectrophotometric methods, as described previously [21]. BAECs in 12-well plates were treated with 2.5 mM Hcy for 24 h. Each sample for GSH measurement was mixed with 0.6 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 5 mM ethylenediaminetetraacetic acid (EDTA) in 0.1 M sodium phosphate buffer (pH 7.5). The reaction was initiated by adding glutathione reductase.

2.6. Other procedures

Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

2.7. Statistical analysis

All experiments were performed independently at least three times. Data were combined and expressed as means ± S.D. Statistical significance was determined using two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. A p value of <0.05 was considered significant.

3. Results

3.1. Autophagy activation promotes Hcy-induced cytotoxicity

Amino acid starvation is a well-known inducer of autophagy [8, 9]. LC3 and p62 are known as indicators of autophagy [22]. At first, we confirmed whether amino acid starvation activates autophagy in BAECs. We investigated the effects of CQ on p62 and LC3 protein levels because accurate monitoring of LC3 levels requires a flux assay using an autophagy inhibitor such as CQ [22]. Amino acid starvation decreased p62 and LC3-II protein levels (Figure 1a). Then, we investigated the effect of autophagy on Hcy-induced cytotoxicity. We measured LDH release as an indicator of cell death and acid phosphatase as an indicator of cell viability. Hcy-induced cell death was promoted by amino acid starvation (Figure 1b). Moreover, Hcy decreased cell viability by amino acid

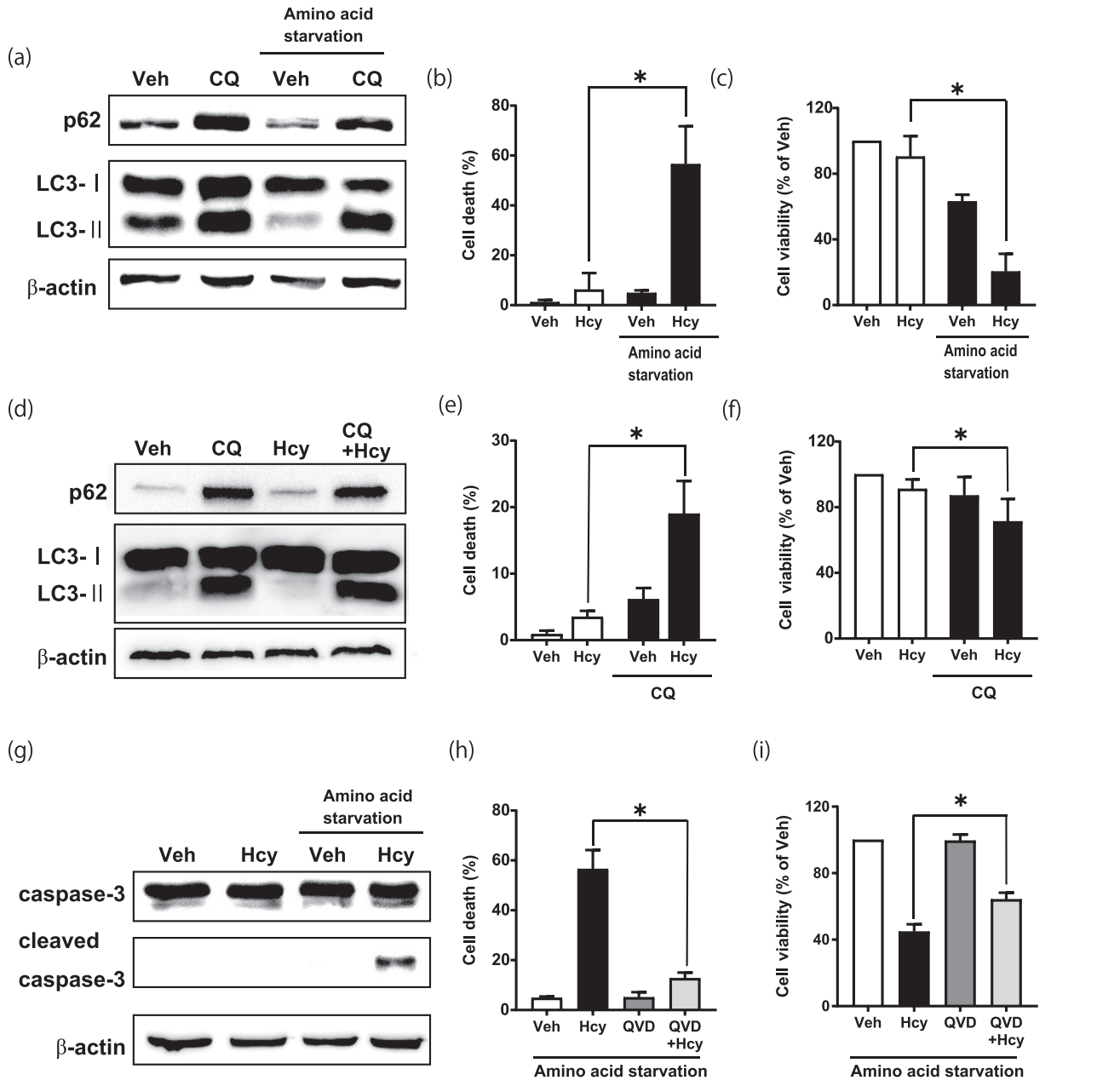


Figure 1. Autophagy activation promoted Hcy-induced cytotoxicity. (a) BAECs were treated with 2.5 μM CQ for 24 h in amino-acid-free medium and cell lysates were analyzed by western blotting. Uncropped images are provided in Supplemental Fig. 1. (b, c) BAECs were treated with 2.5 mM Hcy in amino-acid-free medium for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay. Data are means ± S.D. of three independent experiments. *Significant difference from the value of vehicle (Veh) treated with Hcy alone ($p < 0.05$). (d) BAECs were treated with 2.5 μM CQ for 2 h and then treated with 2.5 mM Hcy for 24 h, and cell lysates were analyzed by western blotting. Uncropped images are provided in Supplemental Fig. 2. (e, f) BAECs were pretreated with 2.5 μM CQ for 2 h and then treated with 2.5 mM Hcy for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay. Data are means ± S.D. of three independent experiments. *Significant difference from the value of Veh treated with Hcy alone ($p < 0.05$). (g) BAECs were treated with 2.5 mM Hcy in amino-acid-free medium for 24 h and cell lysates were analyzed by western blotting. Uncropped images are provided in Supplemental Fig. 3. (h, i) BAECs were treated with 2.5 mM Hcy and 10 μM QVD in amino-acid-free medium for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay. Data are means ± S.D. of three independent experiments. *Significant difference from the value of Veh treated with Hcy alone ($p < 0.05$).

starvation in BAECs (Figure 1c). These results suggest that the Hcy-induced cytotoxicity was promoted by amino acid starvation.

Next, we investigated whether Hcy alone induces autophagy. Figure 1d shows that autophagy was not induced by Hcy treatment in BAECs. Further, we investigated the effect of autophagy inhibition on the Hcy-induced cytotoxicity. CQ promoted Hcy-induced cell death significantly (Figure 1e). Figure 1f shows that the combination of CQ and Hcy decreased cell viability in BAECs. These results suggest that autophagy inhibition increased the Hcy-induced cytotoxicity significantly.

We also investigated whether Hcy induces apoptosis by amino acid starvation. Cleaved caspase-3 is an indicator of apoptosis. Hcy increased cleaved caspase-3 level by amino acid starvation (Figure 1g). QVD, a caspase inhibitor, inhibited Hcy-induced cell death promoted by amino acid starvation (Figure 1h). Moreover, QVD recovered cell viability

decreased by Hcy in BAECs. These results suggest that autophagy promoted Hcy-induced apoptosis.

3.2. Oxidative stress is not involved in cytotoxicity induced by a combination of Hcy and autophagy inducer

We investigated whether amino acid starvation promotes Hcy-induced cytotoxicity via oxidative stress. Amino acid starvation decreased intracellular GSH levels in BAECs (Figure 2a). However, superoxide dismutase (SOD) and catalase (CAT) mRNA levels were not affected by amino acid starvation (Figure 2b). In addition, amino acid starvation had no significant effect on SOD and catalase activities (data not shown). Although we had expected that GSH would protect against the Hcy-induced cytotoxicity promoted by amino acid starvation, N-acetylcysteine did not protect against the Hcy-induced cytotoxicity promoted by amino acid starvation (Figure 2c, d). Moreover, L-buthionine-(S,R)-sulfoximine (BSO), a GSH depleting agent, did not promote Hcy-induced LDH release and Hcy-decreased cell viability (Figure 2e, f). These results suggest that GSH did not play a protective role in the Hcy-induced cytotoxicity promoted by amino acid starvation.

3.3. SLC7A11 inhibitor protects against Hcy-induced cytotoxicity promoted by amino acid starvation

SLC7A11 is a component of a plasma membrane transporter that mediates the cellular uptake of extracellular cystine in exchange for intracellular glutamate [23]. Amino acid starvation did not increase SLC7A11 mRNA and protein levels (Figure 3a, b). Salazosulfapyridine (SASP), an SLC7A11 inhibitor, protected against the Hcy-induced LDH release promoted by amino acid starvation (Figure 3c). Moreover, SASP decreased Hcy-induced cleaved caspase-3 level with amino acid starvation (Figure 3d). These results suggest that the SLC7A11 inhibitor protected against the Hcy-induced apoptosis promoted by amino acid starvation.

4. Discussion

We demonstrated for the first time that autophagy activation by amino acid starvation promotes Hcy-induced apoptosis in BAECs. Moreover, we found that SLC7A11 is involved in the Hcy-induced apoptosis promoted by amino acid starvation.

Hyperhomocysteinemia is a pathological condition that is characterized by exceedingly high plasma homocysteine levels (normal homocysteine levels range from 4 to 12 μM) [24]. Evidence has emerged that elevated plasma homocysteine levels in the range of 15–25 μM are correlated with coronary heart disease [25], stroke [26], peripheral artery stenosis [27], and venous thrombosis [28]. A meta-analysis study reported a seven-fold increase in mortality rate in patients with elevated plasma homocysteine levels compared with healthy subjects [29]. Hyperhomocysteinemia, or increased serum concentration of total homocysteine, is a risk factor for cardiovascular disease and seems to be an independent risk factor for dementia [30, 31]. We used 2.5 mM Hcy in

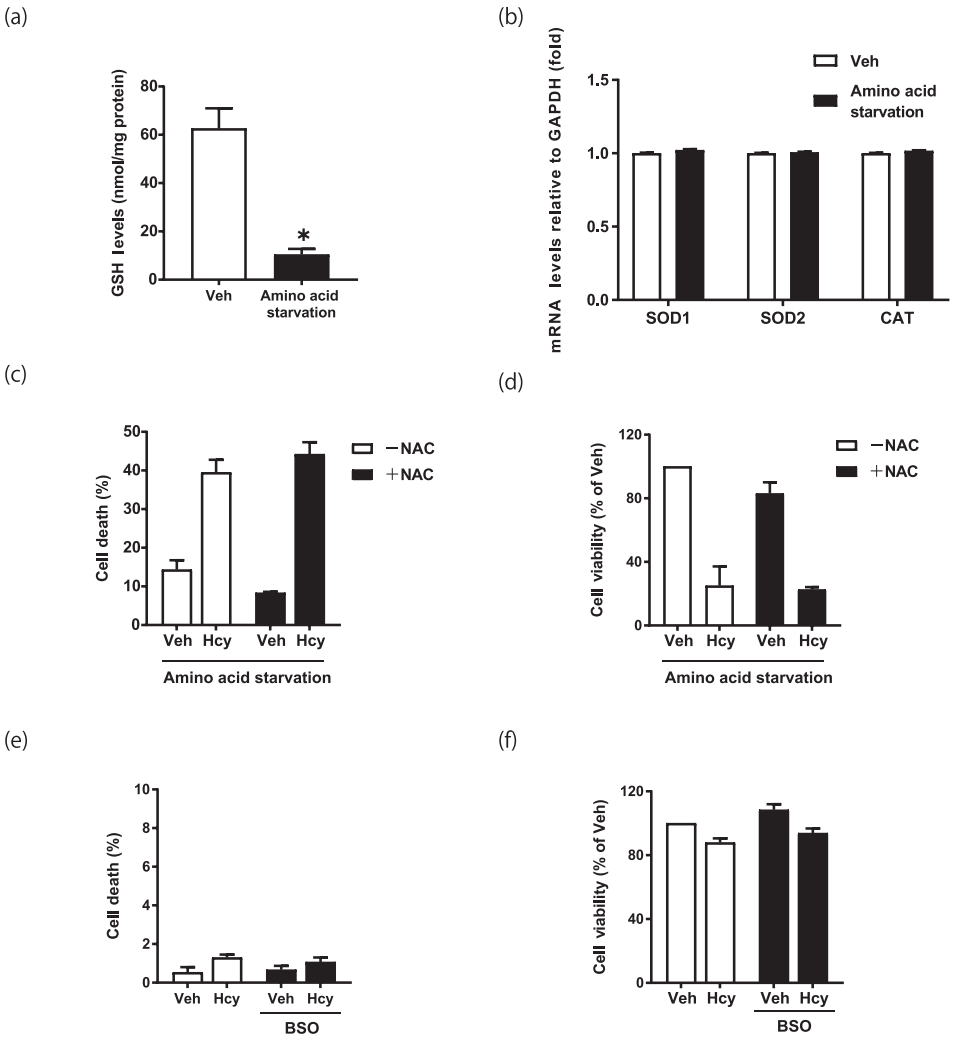


Figure 2. Combination of Hcy- and autophagy-induced cytotoxicity does not involve oxidative stress. (a) BAECs were treated with amino-acid-free medium. Intracellular GSH levels were measured by spectrometric methods, as described previously [17]. Data are means ± S.D. of three independent experiments. *Significant difference from the value of Veh ($p < 0.05$). (b) BAECs were treated with amino-acid-free medium. SOD1, SOD2, and CAT mRNA levels were measured by qPCR. (c, d) BAECs were treated with 2.5 mM Hcy and 1 mM N-acetylcysteine in amino-acid-free medium for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay (e, f) BAECs were treated with 2.5 mM Hcy and 1 mM BSO for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay.

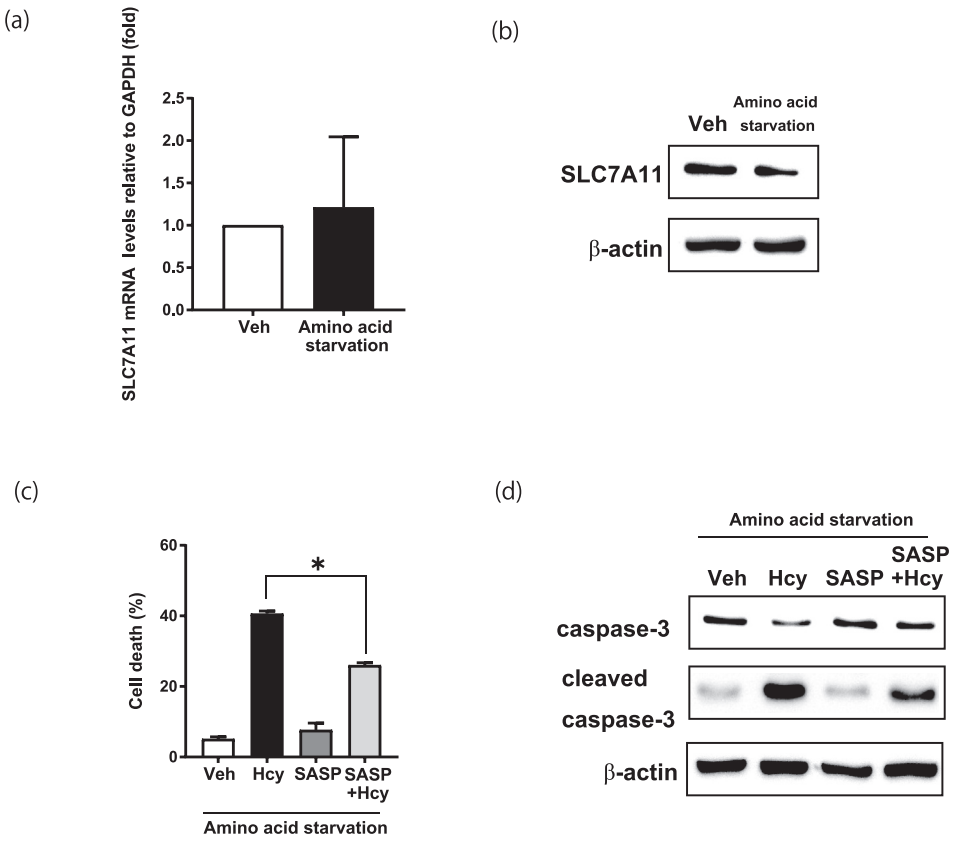


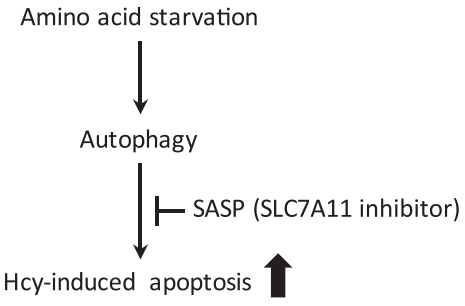
Figure 3. SLC7A11 inhibitor protects against Hcy-induced cytotoxicity promoted by amino acid starvation (a, b) BAECs were treated with amino-acid-free medium. SLC7A11 mRNA levels were measured by qPCR. SLC7A11 protein levels were measured by western blot analysis. Data are means ± S.D. of three independent experiments. Uncropped images are provided in Supplemental Fig. 4. (c) BAECs were treated with 2.5 mM Hcy and 50 μM SASP in amino-acid-free medium for 24 h. Cell death was assessed by LDH release assay. Cell viability was assessed by acid phosphatase assay. Data are means ± S.D. of three independent experiments. *Significant difference from the value of Veh treated with Hcy alone ($p < 0.05$). (d)BAECs were treated with 2.5 mM Hcy and 50 μM SASP in amino-acid-free medium for 24 h and cell lysates were analyzed by western blotting. Uncropped images are provided in Supplemental Fig. 5.

this study, which is higher than normal plasma homocysteine levels. The short-term exposure (24 h) to high Hcy concentration may reflect lifelong exposure to moderately elevated levels of Hcy as found in patients.

Autophagy is a double-edged sword. Autophagy has been widely investigated in cell death studies [32] and is often thought of as a cell survival mechanism. Inhibiting autophagy can effectively enhance apoptosis [33, 34, 35]. Conversely, autophagy can induce apoptosis [36, 37, 38]. Amino acid starvation, which is a well-known inducer of autophagy, is involved in apoptosis [39, 40]. Autophagy activation is represented by p62 levels reduction and LC3-II levels induction. However, LC3-II level was decreased under our experimental conditions (amino acid free and serum free) (Figure 1a). It is possible that amino acid and serum starvations for 24 h had an effect on LC3-II levels. Prolonged culture of cells in amino acid and serum starvation conditions had an effect on LC3-II levels [22]. Autophagy activation was required for Hcy-induced apoptosis (Figure 1g). Hcy-induced cytotoxicity was promoted by amino acid starvation (Figure 1b, c). Autophagy inhibition increased Hcy-induced cytotoxicity significantly (Figure 1e, f). Our results indicate that the activation of autophagy had greater effects than the inhibition of autophagy on Hcy-induced cytotoxicity.

There are many reports that Hcy induces oxidative stress in cultured cells [13, 14, 41, 42]. Hcy-induced oxidative stress results in cytotoxicity [14, 41, 42]. Hcy-induced neuronal damage is protected by pre-incubation in SOD and CAT [41]. However, N-acetylcysteine and BSO had no effect on the Hcy-induced cytotoxicity promoted by amino acid starvation (Figure 2c, e, 2f). SOD and CAT mRNA levels were not affected by amino acid starvation (Figure 2b). In addition, amino acid starvation prevented H_2O_2-induced cytotoxicity (data not shown). Our results suggest that oxidative stress is not involved in the Hcy-induced cytotoxicity promoted by amino acid starvation and oxidative stress is not the main factor contributing to the Hcy-induced cytotoxicity. What is involved in Hcy-induced apoptosis remains to be determined. ER stress mediates Hcy-induced endothelial dysfunction [43]. Hcy-induced apoptosis is mediated by not oxidative stress but ER stress [44]. As one of the possibilities, ER stress may be involved in Hcy-induced apoptosis in our study. Further studies are needed to resolve this issue.

Hcy transport in endothelial cells is poorly understood. One study has indicated that human aortic endothelial cells can import Hcy via at least four of the known cysteine transport systems, namely, X_{AG}, L, ASC, and A [18]. Meanwhile, L-homocystine is imported through transport systems X_{AG}, L, ASC, and X_C [18]. SLC7A11 is a component of the X_C system, which plays an important role in GSH synthesis [45, 46]. Hcy induced endothelial cytotoxicity via inhibition of amino acid transporter [47]. SASP, an SLC7A11 inhibitor, is an anti-inflammatory drug approved for the treatment of inflammatory bowel disease and rheumatoid arthritis [48]. SASP is expected to be effective against colorectal cancer; in fact, SASP increased cisplatin-induced cytotoxicity in colorectal cancer [48]. However, our results showed that SASP can protect against the Hcy-induced cytotoxicity with amino acid starvation (Figure 3c).



Scheme 1. Autophagy activation promotes Hcy-induced apoptosis and SLC7A11 inhibitor protects against the Hcy-induced apoptosis due to autophagy.

Moreover, SASP reduced Hcy-induced cleaved caspase-3 level with amino acid starvation (Figure 3d). SASP depleted GSH through SLC7A11 [49]. Nevertheless, N-acetylcysteine and BSO had no effect on the Hcy-induced cytotoxicity (Figure 2c, d, e, f). We speculate that the mechanism of SASP protection against the Hcy-induced cytotoxicity does not involve oxidative stress.

In summary, our findings showed for the first time that autophagy activation promotes Hcy-induced apoptosis and SLC7A11 inhibitor protects against the Hcy-induced apoptosis due to autophagy (Scheme 1). However, how SLC7A11 is involved in the Hcy-induced apoptosis remains to be determined. Other steps in this pathway need to be elucidated before we can fully understand how SLC7A11 inhibitor protects against the Hcy-induced apoptosis.

Declarations

Author contribution statement

K. Sato: Performed the experiments; Analyzed and interpreted the data.

T. Nishii and A. Sato: Performed the experiments.

R. Tatsunami: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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