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Anti-prion activity found in beetle grub hemolymph of *Trypoxylus dichotomus septentrionalis*

Taichi Hamanaka, Keiko Nishizawa, Yuji Sakasegawa, Hiroshi Kurahashi [1], Ayumi Oguma, Kenta Teruya, Katsumi Doh-ura *

Department of Neurochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryocho, Sendai 980-8575, Japan

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ABSTRACT

No remedies for prion disease have been established, and the conversion of normal to abnormal prion protein, a key event in prion disease, is still unclear. Here we found that substances in beetle grub hemolymph, after they were browned by aging for a month or heating for hours, reduced abnormal prion protein (PrP) levels in RML prion-infected cells. Active anti-prion components in the hemolymph were resistant to protease treatment and had molecular weights larger than 100 kDa. Aminoguanidine treatment of the hemolymph abolished its anti-prion activity, suggesting that Maillard reaction products are enrolled in the activity against the RML prion. However, levels of abnormal PrP in RML prion-infected cells were not decreased by incubation with the Maillard reaction products formed by amino acids or bovine serum albumin. The anti-prion components in the hemolymph modified neither cellular or cell-surface PrP levels nor lipid raft or autophagosome levels. The anti-prion activity was not observed in cells infected with 22 L prion or Fukuoka-1 prion, suggesting the anti-prion action is prion strain-dependent. Although the active components of the hemolymph need to be further evaluated, the present findings imply that certain specific chemical structures in the hemolymph, but not chemical structures common to all Maillard reaction products, are involved in RML prion formation or turnover, without modifying normal PrP expression. The anti-prion components in the hemolymph are a new tool for elucidating strain-dependent prion biology.

1. Introduction

Prion diseases, also called transmissible spongiform encephalopathies, are progressive, fatal neurodegenerative illness. They include Creutzfeldt-Jakob disease in humans and scrapie, bovine spongiform encephalopathy, and chronic wasting disease in animals. These diseases are characterized by the accumulation of abnormal prion protein (abnormal PrP), which is a main component of the pathogen that is derived from the normal prion protein (PrPc) through a conformational transformation [1]. Abnormal PrP molecules form insoluble protein polymers, which have protease-resistant cores (PrPres). Both the conversion of PrPc to abnormal PrP and the turnover of abnormal PrP are key events in prion diseases but are still unclear.

As for remedies for these diseases, dozens of compounds or substances have been reported to either inhibit prion formation in prion-infected culture cells or to prolong the incubation period in prion-infected animals [2–4]. Some remedies are effective in a prion-strain dependent fashion [5–8], but the mechanism underlying prion strain-dependent efficacy remains unclear. Although a few anti-prion compounds have been tested in clinical trials against human prion diseases, no sufficiently beneficial effects have ever been reported [9–13].

To obtain clues for the development of remedies as well as to explore the mechanism of the enigmatic PrP conversion, we searched for compounds or substances that modify abnormal PrP formation in prion-infected cells. In earlier studies [14,15], medicinal compounds or natural products approved by the United States Food and Drug Administration had been extensively screened for anti-prion activity. Therefore, we focused on untouched materials obtained from natural sources. We had previously reported anti-prion substances extracted from natural sources, including fucoidan and protein-bound polysaccharide K [16,17]. Insects are under-cultivated natural resources, which exist abundantly in both number and variety. It may be possible to discover new insect-derived substances that are useful either for exploring the PrP conversion mechanism, or for developing the

Abbreviations: PrP, prion protein; PrPc, normal cellular PrP; PrPres, protease-resistant abnormal PrP; AGE, advanced glycation end product

* Corresponding author. Fax: +81 22 717 7656.
E-mail address: doh-ura@med.tohoku.ac.jp (K. Doh-ura).
[1] Laboratory for Protein Conformation Diseases, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

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treatment, or prophylaxis of the illness. In fact, anticancer, antiviral, and antimicrobial compounds are found in insect extracts [18–21].

Here we examined insect larva-derived hemolymph, using prion-infected cells. We found that beetle grub hemolymph inhibited abnormal PrP formation in prion-infected cells when the hemolymph was browned by aging or heating. We then studied the mechanism by which the browned hemolymph enacted its anti-prion properties. The Maillard reaction products, also called advanced glycation end products (AGEs), in the browned hemolymph were suggested to contain the active, anti-prion components. These substances are a new type of probe for identifying the prion strain-dependent formation of abnormal PrP.

2. Materials and methods

2.1. Samples and reagents

We obtained grubs of the Japanese horned beetle, *Trypoxylus dichotomus septentrionalis*, at the third instar larval stage, from a local insect shop. Hemolymph from these grubs was obtained as previously described [22,23] with minor modification. Briefly, phosphate buffer saline (PBS, 100 μL) was injected into the body of grubs, and the grubs were reared in leaf mold for 5 h. After surface sterilization and anesthesia on an ice pack, bleeding was done through the amputated prologs. Hemolymph was collected into tubes on ice and immediately heated at 95 °C for 5 min. The hemolymph fluids were subsequently centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant from this step was the source of the hemolymph samples used in the study. The hemolymph samples were stored at −80 °C, after addition of 20 μg/mL aprotinin (Roche, Penzberg, Germany), until use.

Aminoguanidine, glucose, amino acids, and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Maillard reaction products of amino acids or BSA were obtained by incubating 25 mg/mL amino acid or BSA with 125 mg/mL glucose in water at 70 °C for 17 h. Inhibition of the Maillard reaction was performed by the addition of 230 mM aminoguanidine into the reaction solution.

2.2. Analysis of PrP and other factors

We used mouse neuroblastoma N2a cells as well as three types of N2a-derived cells infected with distinct prion strains: ScN2a cells infected with the RML prion, N167 cells infected with the 22 L prion, and F3 cells infected with the Fukuoka-1 prion. Cells were treated for three days with test materials as previously described [6,7,24]. Cell lysates were prepared using lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS, pH 7.4). The amounts of PrPres, PrPc, β-actin, and autophagosomal protein LC3 in the cell lysates were determined using an immunoblotting procedure with a specific antibody for each protein, as previously described [25]. Flow cytometry was performed to determine the level of cell-surface PrPc or lipid raft microdomains, in N2a cells, using anti-PrP monoclonal antibody or fluorescence-conjugated cholera toxin B, respectively, as described previously [25]. Maillard reaction products were assayed by detecting N(6)-carboxymethyl lysine with an immunoblotting procedure using 6D12 monoclonal antibody (1:200; Trans Genic Inc., Kumamoto, Japan) [26].

3. Results

3.1. Anti-prion activity of beetle grub hemolymph

We examined whether beetle grub hemolymph of *Trypoxylus dichotomus septentrionalis* has anti-prion activity in RML prion-infected cells. Cells were incubated with the culture medium containing the hemolymph samples for three days. PrPres levels were subsequently assayed by immunoblotting. Beetle grub hemolymph samples were transparent immediately after collection and heat inactivation, but they gradually changed to a tan color while stored at 4 °C. The non-aged hemolymph samples did not inhibit the formation of abnormal PrP in ScN2a cells. However, the hemolymph samples that had become brown after storage for 1 month at 4 °C inhibited the formation of abnormal PrP in ScN2a cells. The anti-prion activity was never observed in hemolymph samples that had been stored for 2 months at 4 °C (Fig. 1A). These results indicate that beetle grub hemolymph samples contain anti-prion activity that was gained during aging for one month at 4 °C, but was lost during an additional month of storage. To shorten the aging period, the hemolymph samples were heated at 37 °C or 70 °C for 17 h. These heat treatments caused the hemolymph samples to turn brown and to inhibit the formation of abnormal PrP in ScN2a cells (Fig. 1B). These results suggest that temperature is one of the factors that facilitate the production of anti-prion components in hemolymph samples. Anti-prion activity was observed in hemolymph samples heated at 70 °C for only 3 h; the anti-prion activity was coincident with the formation of brown color in the hemolymph samples (Fig. 1C). The anti-prion activity was maintained in samples treated with protease (Fig. 1D). The molecular mass of the anti-prion components in the hemolymph samples was examined by using centrifugal ultrafiltration devices. The anti-prion components were found in the retentate of a filter with a 100 kDa cut-off; the anti-prion activity was not observed in the filtrates of any of the four types of filters (Fig. 1E).

3.2. Characterization of anti-prion components in beetle grub hemolymph

All the findings described above imply the involvement of the Maillard reaction in the production of active anti-prion components in the hemolymph samples. Therefore, we examined whether the Maillard reaction inhibitor aminoguanidine abolished the anti-prion activity of the hemolymph samples, by heating a mixture of hemolymph and aminoguanidine at 70 °C for 17 h and subsequently adding the mixture to ScN2a cells. The presence of Maillard reaction products was confirmed by immunoblotting with 6D12 monoclonal antibody, which specifically detects N(6)-carboxymethyl lysine in Maillard reaction products. As shown in Fig. 2A, no N(6)-carboxymethyl lysine signals were detected in hemolymph samples prepared in the presence of aminoguanidine. Coincidently, anti-prion activity was abolished in hemolymph samples prepared in the presence of aminoguanidine (Fig. 2B). The results indicate that the Maillard reaction process is necessary for the production of anti-prion components in hemolymph samples.

Meanwhile, we examined whether other Maillard reaction products exert anti-prion activity in ScN2a cells. We investigated Maillard reaction products prepared by incubation of amino acids or BSA with glucose at 70 °C for 17 h. The results demonstrate that the levels of abnormal PrP formed in ScN2a cells were not reduced by any of these samples. Rather, the levels of abnormal PrP increased obviously in ScN2a cells treated with samples prepared from lysine or a lysine-containing amino acid mixture (Fig. 2C, Glucose (+)). This increase in PrPres levels was abolished when the samples were prepared in the presence of aminoguanidine (Fig. 2C, Glucose (+) and AG (+)). These results suggest that

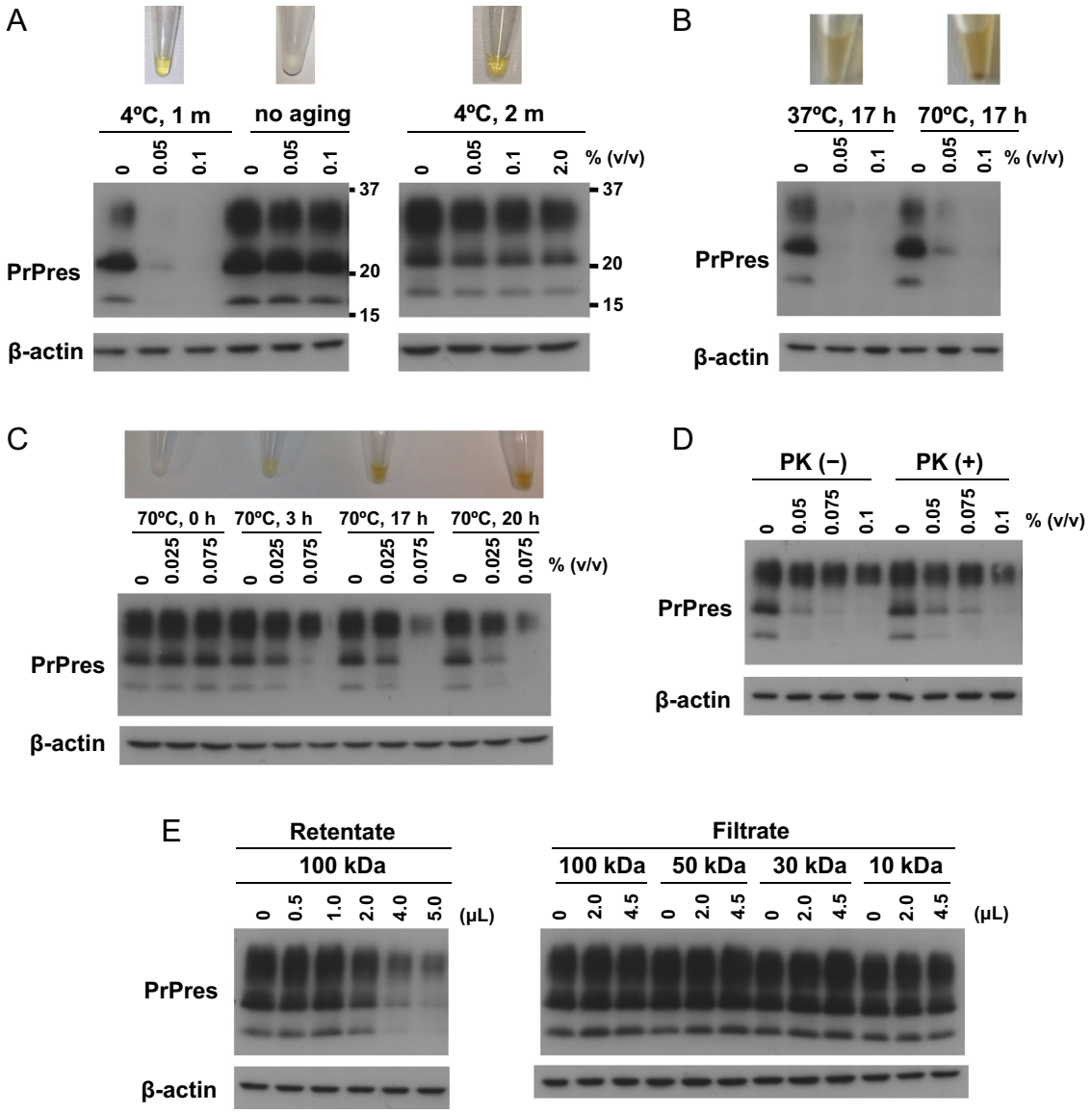

Fig. 1. Anti-prion activities of beetle grub hemolymph. (A) Immunodetection of PrPres in ScN2a cells treated with fresh (no aging) or aged hemolymph (4 °C, 1 or 2 months). Signals for β-actin are shown as controls for the integrity of the samples used for PrPres detection. Molecular size markers on the right indicate sizes in kDa. (B) Immunodetection of PrPres in ScN2a cells treated with the hemolymph that had been heated at 37 °C or 70 °C for 17 h. (C) Immunodetection of PrPres in ScN2a cells treated with the hemolymph that had been heated at 70 °C for various lengths of time. (D) Immunodetection of PrPres in ScN2a cells treated with the browned hemolymph that had been treated with proteinase K [PK (+)] or that had been left untreated [PK (−)]. Browned hemolymph was prepared by heating hemolymph at 70 °C for 17 h. (E) Immunodetection of PrPres in ScN2a cells treated with the retentate or filtrate of browned hemolymph after ultrafiltration through membranes with different molecular pore sizes. Designated amounts of filtrates or filled-up retentates with PBS to the original volumes were added into 10 mL of each cell culture medium.

certain specific components in the hemolymph, rather than the common structures of Maillard reaction products, are needed for anti-prion activity against the RML prion.

3.3. Effects on PrPc expression and other prion strains

We examined the influence of anti-prion hemolymph components on PrPc expression. We found that the browned hemolymph samples, prepared by heating the hemolymph at 70 °C for 17 h, modified neither the total cellular PrPc levels (Fig. 3A) nor the cell surface PrPc levels in N2a cells (Fig. 3B). The lipid raft microdomain of the cell membrane, which is reportedly one of the possible sites of PrP conversion or of interaction between PrPc and abnormal PrP [27–31] was also examined. The cholera toxin B-binding lipid raft levels were not modified in N2a cells treated with the browned hemolymph samples (Fig. 3B). We examined the anti-prion activity of the browned hemolymph samples in two other cells infected with distinct prion strains: N167 cells infected with 22 L prion and F3 cells infected with Fukuoka-1 prion. The browned hemolymph samples showed no anti-prion activity in these two cell cultures (Fig. 3C). These data suggest that the browned hemolymph samples exert anti-prion activity in a prion-strain dependent manner without any effects on PrPc expression.

Autophagy has been reported to regulate abnormal PrP clearance [32]. Therefore, we examined whether autophagosome formation is enhanced in ScN2a cells treated with browned hemolymph samples. We found that the browned hemolymph samples did not enhance autophagosome formation in these cells (Fig. 3D). Similarly, compounds such as the tetracycline group compounds [33] and polycationic compounds [34] turn abnormal PrP molecules into less protease-resistant PrP molecules when cell lysates containing abnormal PrP molecules are incubated with these compounds. Therefore, we tested whether browned hemolymph samples modify the protease sensitivity of abnormal PrP

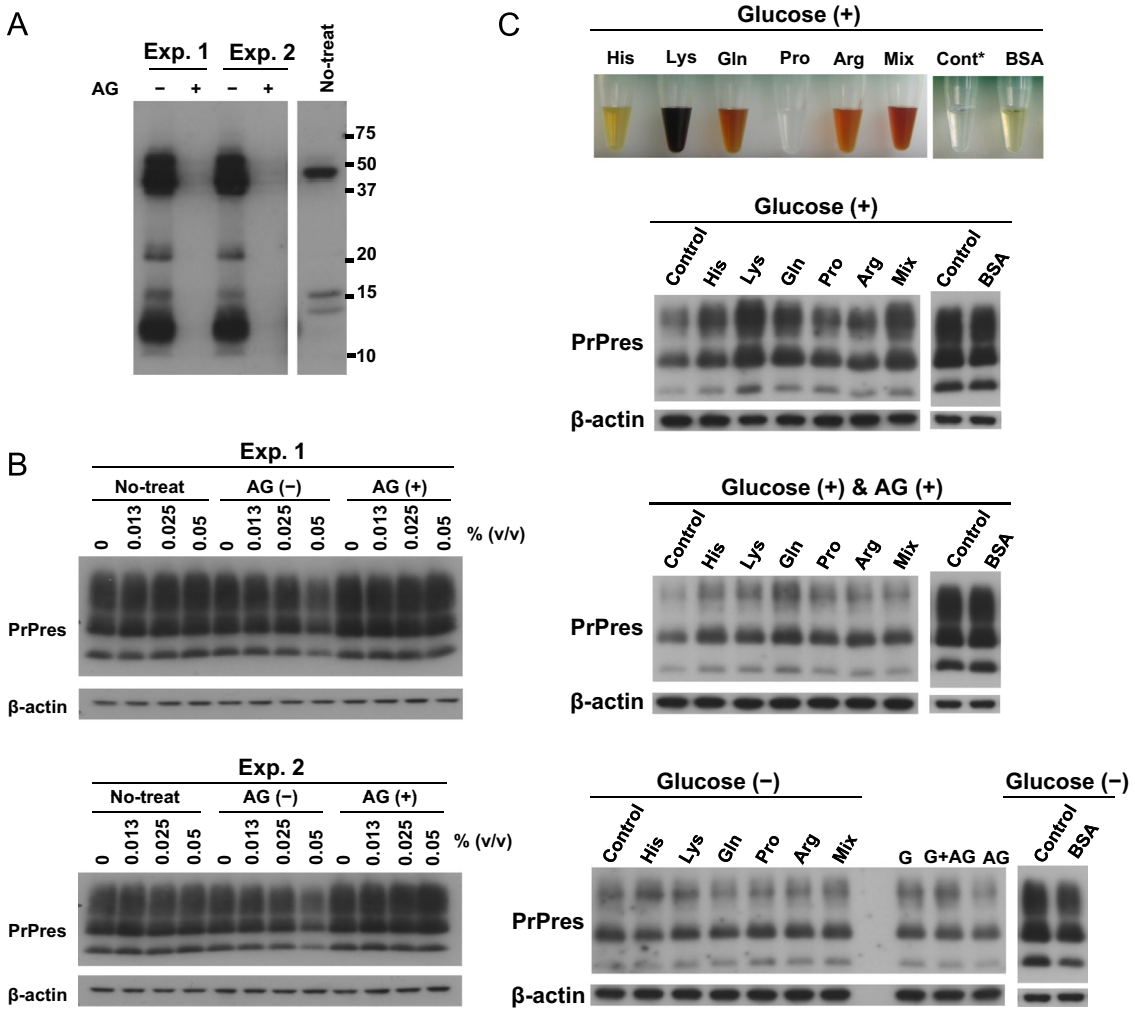

Fig. 2. Characterization of anti-prion components in beetle grub hemolymph. (A) Immunodetection of N(6)-carboxymethyl lysine in hemolymph that was heated at 70 °C for 17 h in the presence (+) or absence (−) of aminoguanidine (AG). Untreated hemolymph (No-treat) is also shown. Weak immunoreactive signals in the untreated hemolymph may represent a certain amount of N(6)-carboxymethyl lysine, presumably produced during the heat inactivation treatment, which was done immediately after sample collection. The data from two independently collected hemolymph samples are shown. (B) Immunodetection of PrPres in ScN2a cells treated with the hemolymph samples shown in (A). (C) Maillard reaction products of amino acids and BSA, and immunoblot data of PrPres in ScN2a cells treated with the Maillard reaction products [Glucose (+)]. Immunoblot data are also shown for PrPres in ScN2a cells treated with the Maillard reaction products made in the presence of aminoguanidine [Glucose (+) & AG (+)]. As negative controls, immunoblot data are shown for PrPres in ScN2a cells treated with respective amino acids or BSA in the absence of glucose [Glucose (−)] or treated with glucose alone (G), glucose plus aminoguanidine (G+AG), or aminoguanidine alone (AG). Cont* indicates a non-heated sample containing both BSA and glucose. ScN2a cells were treated by adding 0.2% (*v*/*v*) of each reaction solution to the culture medium.

molecules. The results showed that the browned hemolymph samples did not change the protease sensitivity of abnormal PrP molecules and did not modify the stability of PrPc molecules (Fig. 3E).

4. Discussion

In the present study, we found that beetle grub hemolymph of *Trypoxylus dichotomus septentrionalis*, when it is browned at 4 °C for a month or heated for hours, inhibits abnormal PrP formation in RML prion-infected cells. Anti-prion components in the browned hemolymph were protease resistant and had a molecular weight greater than 100 kDa. Treatment of the hemolymph with aminoguanidine abolished the production of anti-prion activity, which strongly suggests that the anti-prion components are Maillard reaction products. Conversely, Maillard reaction products of amino acids or BSA did not inhibit abnormal PrP formation in RML prion-infected cells. Therefore, although the active components of browned hemolymph need to be further evaluated, certain specific chemical structures in the hemolymph, but not the common structures of Maillard reaction products, are likely to be involved in inhibiting abnormal PrP formation in cells infected with the RML prion.

Although many compounds or biological materials have been reported to inhibit the formation of abnormal PrP in prion-infected cells, this type of Maillard reaction product has never been reported to have anti-prion activity. High-molecular-weight substances, including polycationic polymers [34], polyanionic glycans [35], and the protein-polysaccharide complex substance PSK [17] have potent anti-prion activity in prion-infected cells. However, the anti-prion components in the hemolymph are different from these high-molecular-weight substances. The anti-prion components in the hemolymph exhibit prion-strain dependent efficacy, whereas those high-molecular-weight substances are effective not only in RML prion-infected cells but also in cells infected with other prion strains.

As substances similar to Maillard reaction products, melanin, which is similarly brown-colored and of high molecular weight, is considered because melanin is easily produced even in collected

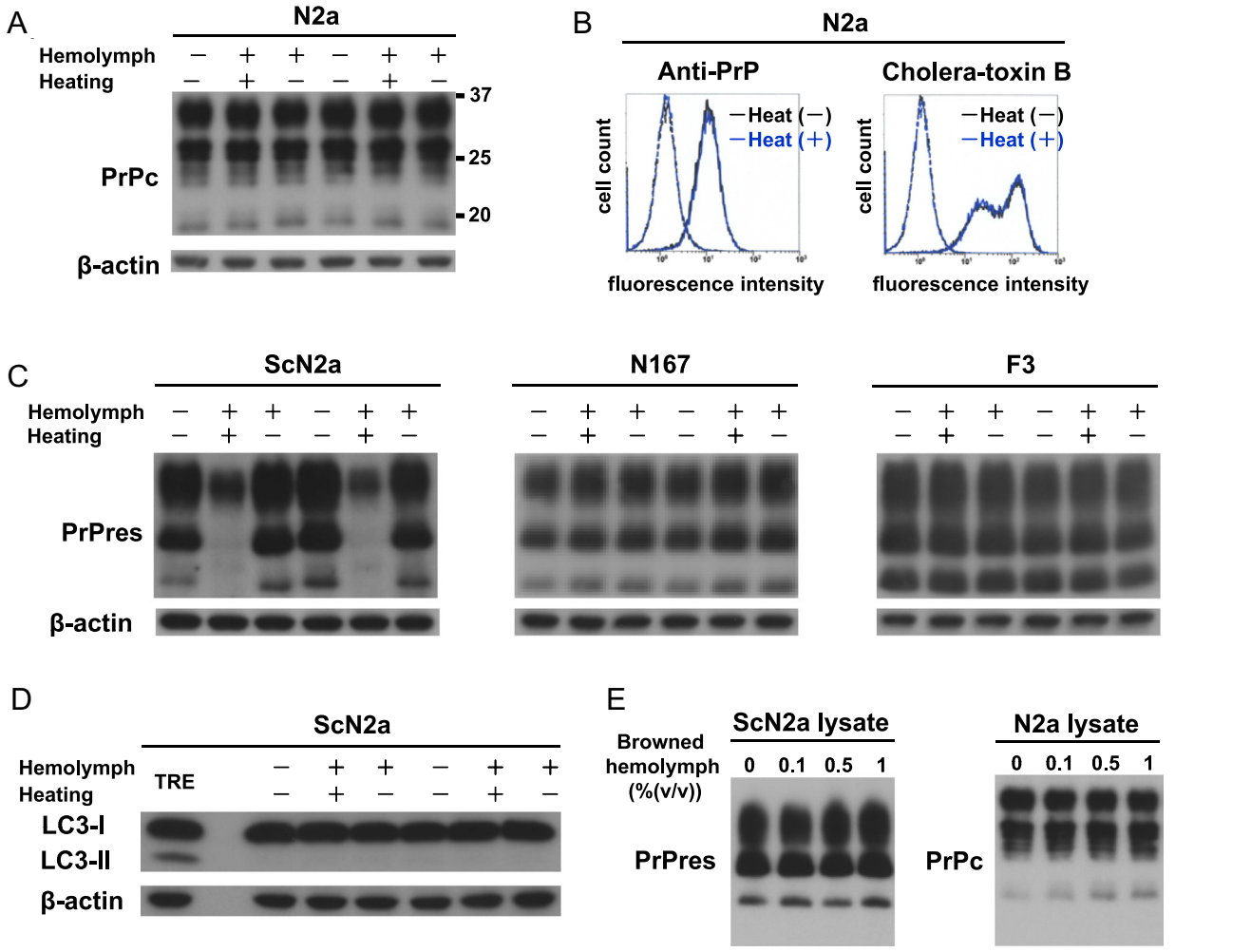

Fig. 3. Effects of browned hemolymph on PrPc expression and other prion strains. (A) Immunodetection of total cellular PrPc in N2a cells treated with the hemolymph that had been heated at 70 °C for 17 h, or not heated. Cells were treated by adding 0.1% (*v*/*v*) of each reaction solution to the culture medium. (B) Flow cytometry of cell surface PrPc (Anti-PrP) and lipid raft microdomains (Cholera-toxin B) in N2a cells treated with hemolymph that had been heated at 70 °C for 17 h, or not heated. The broken line peaks on the left show their respective isotype controls. Cells were treated as described in (A). (C) Immunodetection of PrPres in three distinct prion-infected cell lines treated with hemolymph that had been heated at 70 °C for 17 h, or not heated. Cells were treated as described in (A). (D) Immunodetection of autophagosome-related LC3-II in ScN2a cells treated with hemolymph that had been heated at 70 °C for 17 h, or not heated. A trehalose-treated cell sample (TRE) is shown as a positive control. Cells were treated as described in (A). (E) Immunodetection of PrPres and PrPc in the cell lysates treated with the browned hemolymph. ScN2a and N2a cell lysates were treated with the browned hemolymph at designated concentrations at 37 °C for 1 h, and subsequently ScN2a cell lysates were digested with proteinase K. Then, PrPres in ScN2a cell lysates and PrPc in N2a cell lysates were detected. Browned hemolymph was prepared as described already.

hemolymph fluids by endogenous phenoloxidase enzymes. However, it is unlikely that melanin was produced in the hemolymph samples we used, because they were collected in an ice cold condition and were immediately heated at 95 °C to inactivate phenoloxidase enzymes. In fact, the phenoloxidase enzymes in fly larvae hemolymph are reportedly inactivated by heating at 50 °C or higher [36].

Regarding the mechanism of the action of the anti-prion components of the browned hemolymph in prion-infected cells, they had no effect on PrPc expression and localization; total cellular and cell surface PrPc levels were not modified; and cell membrane lipid raft microdomain levels were unchanged. In addition, the anti-prion components of the browned hemolymph did not modify autophagosome levels, and they were active in a prion strain-dependent manner. Considering all these together, these results suggest that the anti-prion components of the browned hemolymph reduce abnormal PrP levels by directly modifying abnormal PrP formation or turnover. We previously experienced similar results with amyloidophilic compounds such as compound B, BSB, and styrylbenzoazole derivatives [37]. We speculate that the prion strain-dependent efficacy of these compounds is attributable to strain-specific structure, biosynthesis, or turnover of abnormal PrP molecules. However, none of these mechanisms of prion strain-dependency have previously been reported. The present findings of a new class of anti-prion materials might provide a clue to understanding the mechanisms of strain-dependent anti-prion activity.

There are a few reports describing the roles of Maillard reaction products or AGEs in prion biology or prion disease pathogenesis [38–40]. Choi and colleagues found that one or more lysine residues and one arginine residue of abnormal PrP are specifically modified with AGEs [38]. They suggest the Maillard reaction is responsible for one of the post-translational modifications of abnormal PrP, and that it might facilitate protection of abnormal PrP molecules against cellular degradation. Sasaki and colleagues found that both PrP-positive granules and AGE- or AGE receptor-immunopositive granules are colocalized in the astrocytes of human brains with prion disease, and they proposed an AGE receptor-mediated PrP degradation pathway in prion-infected brains [39]. Taken together, these results suggest that the Maillard reaction plays an important role in the biosynthesis and turnover of abnormal PrP. Meanwhile, the findings of this study suggest that

certain Maillard reaction products inhibit abnormal PrP formation in prion-infected cells. Thus, although their chemical structure needs to be further elucidated, the anti-prion components of the browned hemolymph of beetle grubs are a new tool for resolving an enigma of prion formation and turnover. These findings may also provide a clue to as to why some compounds are effective in a prion strain-dependent manner.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.07.009.

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