The Induction of Heat Shock Protein 70 after Oral Administration of Concentrated Brewed Rice Vinegar Kurozu in Mice

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Summary Heat shock protein 70 (HSP70) is induced by various stresses. Since HSP70 has a protein refolding activity and an anti-inflammatory activity, the HSP70 induction will help cells from harmful acute stresses. Feeding a diet containing concentrated brewed rice vinegar Kurozu (CK) diet for 5 wk resulted in an increase of HSP70 in the brains of mice. In the present study, we evaluated whether oral feeding of 25 μL CK induces HSP70 mRNA in brain and other tissues. HSP70 mRNA was significantly increased in the esophagus, small intestine, liver, and brown adipose tissue within 1 h after the oral administration of CK. A weaker induction of HSP70 mRNA was demonstrated in the stomach, large intestine, and brain. HSP70 mRNA induction returned to basal levels within 3 h after feeding. We doubted that the induction of HSP70 mRNA was caused by manual restraint of the mice during CK administration. Manual restraint of the mice did not influence HSP70 mRNA expression in intestine 1 h after these treatments. Our results suggest that transient HSP70 mRNA induction by oral feeding of CK was not caused by retention stress. There are some compounds in CK that increase HSP70 mRNA in various tissues.

Key Words HSP, chaperone, RT-PCR, acetate, lactate

Heat shock proteins (HSPs) protect cells against various stresses, such as heat shock, cytotoxic agents, and hypoxia (1, 2). The primary function of HSPs is folding of unfolded or denatured proteins. HSPs are also immune modulators that counteract inflammation (3). Based on these functions, the proper induction of HSPs bring beneficial effects, such as ameliorating heat shock responses (4), liver injury (5), inflammatory responses (6), and Alzheimer’s disease (7). HSP70 is one of the most ubiquitous HSPs in all species examined, except for a number of archaea, and is a component in all eukaryote cells (8). Some specific HSP70 inducers that have been previously described include geranylgeranylacetone (9), carbenoxolone (10), and ebamipide (11). Recently, we reported that the 5 wk ad libitum feeding of the diet containing 2.5% (w/w) concentrated brewed rice vinegar Kurozu induced HSP70 in the brains of senescence accelerated mice-prone 8 (12). Kurozu is a traditional Japanese vinegar produced using semi-grained rice which provides organic compounds and minerals. Kurozu is composed of 94% water, 4% acetic acid, and 2% soluble solids. Acetate sometimes demonstrates therapeutic effects and may act like a drug in several disease (13). Therefore, to study the specific effects of Kurozu, not acetate, we have used 10 times concentrated Kurozu (CK) in the study described here. The acetate is eliminated during the freeze drying process that is used to produce CK. In the present study, CK was made from Kurozu liquid (Sakamoto Kurozu, Fukuuyama, Kagoshima, Japan) by freeze drying. The chemical composition of CK was 82.5% water, 9% crude protein (calculated as mineral nitrogen×6.25), 2.5% organic acid, 5% ash, and 1% carbohydrate. We measured the concentrations of short-chain acids in CK using high performance liquid chromatography. The quantification method was performed in the manner described in a previous study (14). CK demonstrated high lactic acid concentrations (Table 1). Previous reports describe that the oral feeding of CK induces HSP70 expression in the brain (11), but HSP70 induction in other organs has not been reported. In the present study, we investigated the induction of HSP70 in various tissues after the oral administration of CK.

Eight-week-old C57BL/6N mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). We obtained 24 mice. Four mice each were housed in a total of six cages and were maintained at 24±2°C and 60±5% humidity with a 12-h light/dark cycle. CLEA Rodent Diet CE-2

Note

Recently, we reported that the 5 wk ad libitum feeding of the diet containing 2.5% (w/w) concentrated brewed rice vinegar Kurozu (CK) diet for 5 wk resulted in an increase of HSP70 in the brains of mice. In the present study, we evaluated whether oral feeding of 25 μL CK induces HSP70 mRNA in brain and other tissues. HSP70 mRNA was significantly increased in the esophagus, small intestine, liver, and brown adipose tissue within 1 h after the oral administration of CK. A weaker induction of HSP70 mRNA was demonstrated in the stomach, large intestine, and brain. HSP70 mRNA induction returned to basal levels within 3 h after feeding. We doubted that the induction of HSP70 mRNA was caused by manual restraint of the mice during CK administration. Manual restraint of the mice did not influence HSP70 mRNA expression in intestine 1 h after these treatments. Our results suggest that transient HSP70 mRNA induction by oral feeding of CK was not caused by retention stress. There are some compounds in CK that increase HSP70 mRNA in various tissues.

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CLEA Japan and water were provided ad libitum. At 9-wk-of-age, the mice, except for the 0 h group, were fasted for 8 h following the oral administration of 25 µL of CK using a pipette. Four mice each were then euthanized by cervical dislocation at 0, 1, 3, 5, 8, and 24 h after administration. Tongue, esophagus, stomach, small intestine, large intestine, liver, brain, and brown adipose tissue were collected and immediately stored in RNAlater solution (Thermo Fisher Scientific, Rockford, IL). Total RNA was isolated using TRizol reagent (Thermo Fisher Scientific) and was later used as template for real-time PCR (RT-PCR). The RT-PCR reaction was performed using the PrimeScript RT-PCR Kit (Takara Bio, Kusatsu, Shiga, Japan) working on StepOnePlus (Thermo Fisher Scientific). Primer sets for HSP70 as a target gene and glyceraldehyde 3-phosphate dehydrogenase (GAPD) as a control gene were purchased from Takara Bio Perfect Real Time support system (Takara Bio). The primer sequences were 5'-CAGAGGCCAGGGCTTGATTA-3' (HSP70 forward), 5'-ACACATGCTGGTTGTCACTTC-3' (HSP70 reverse), 5'-GGATGCAGGGATGTGTTC-3' (GAPD forward), and 5'-AAGTTGGCATTGTGGAAGG-3' (GAPD reverse). The results were evaluated with the \( \Delta \Delta C_t \) method and presented as mean ± standard errors of the mean (SE). The difference between \( \Delta C_t \) values were evaluated for statistical significance using Tukey’s comparison test. All statistical analyses were performed using JMP version software (SAS Institute Inc., Cary, NC). A p value of <0.05 was considered statistically significant. All animal studies were performed in strict accordance with the recommendations in the guide for the humane treatment and management of animals of the Japanese Law (No. 105) and Notification (No. 6). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kagoshima University Committee for Animal Experiment (Permit Number: VM16053).

Table 1. The composition of the short-chain acids in CK.

| Organic acids          | Concentration (mM) |
|------------------------|--------------------|
| Lactic acid            | 487.0              |
| α-Ketoglutaric acid    | 53.8               |
| Pyroglutamic acid      | 44.8               |
| Succinic acid          | 14.2               |
| Acetic acid            | 4.7                |
| Formic acid            | 1.7                |
| Malic acid             | 0.5                |

Data were shown in Fig. 1. HSP70 induction occurred within 1 h after administration in all organs except the tongue; significant increases were demonstrated in the esophagus, small intestine, liver and brown adipose tissue (0 h vs. 1 h). The induction ratios between 0 h and 1 h were 8.0-fold (esophagus), 5.3-fold (stomach), 3.7-fold (small intestine), 2.2-fold (large intestine), 3.5-fold (liver), and 2.0-fold (brown adipose tissue). The induction of HSP70 mRNA in each organ was quantified by real-time PCR. The results are expressed as the relative level compared with 0 h. Data are presented as the mean ± SE (n=4). Values with different letters are significant differences between groups (p<0.05, Tukey–Kramer HSD test). CK, concentrated Kurozu.

Fig. 1. HSP70 mRNA expression after CK oral administration. Organs were excised at each time after the oral administration of CK. The quantification of HSP70 mRNA was evaluated using real-time PCR. Data are expressed as the relative value compared with 0 h. Data are presented as the mean ± SE (n=4). Values with different letters are significant differences between groups (p<0.05, Tukey–Kramer HSD test). CK, concentrated Kurozu.
The induction level was the highest in the brown adipose tissue. The induction of HSP70 after CK administration returned to basal levels by 3 h post administration. Samples taken at 8 h post administration indicated a tendency towards the increase of HSP70 induction in the liver; the increase was statistically significant only in the liver (0 h vs. 8 h). A previous report demonstrated that continuous CK feeding increased HSP70 mRNA 1.8-fold in brain (11). The present study also shows that CK increased the HSP70 mRNA by 1.6-fold in brain, but the effect was transient. CK may need to be administered several times a day to stimulate the continuous induction of HSP70.

We sought to investigate whether HSP70 induction was induced by stress triggered by the manual restraint of the mice during CK administration. To evaluate whether the HSP70 induction was caused by stress from restrained manually for CK administration, the expression level of HSP70 mRNA in the intestine was compared among non-treated mice (no manual restraint), 25 μL water fed mice, and 25 μL short-chain acids containing solution (487 mM lactic acid, 54 mM α-ketoglutaric acid, and 14.2 mM succinic acid) fed mice 1 h after oral treatment. HSP70 expression levels, however, did not show any differences among groups (data not shown). Therefore, we conclude that HSP70 induction by CK did not result from retention stress. The data also suggested that the mixture of short-chain acids (lactic acid, α-ketoglutaric acid, and succinic acid) which were major components of CK could not affect HSP expression.

Because the induction was recognized within 1 h after administration and the event occurred simultaneously in multiple tissues, we suggest two hypotheses. First, some compounds in CK quickly reach target organs through the blood stream, which directly stimulate HSP70 expression. Second, some compounds stimulate the gastrointestinal tract resulting in the production of hormone molecules and/or nervous system activation following HSP70 induction. Further experiments are needed to reveal the mechanism by which CK induces HSP70.

In summary, the oral feeding of CK significantly increased HSP70 mRNA expression in the esophagus, small intestine, liver, and brown adipose tissue. The increase was temporary and decreased within 1 h after administration.

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