論文の内容の要旨

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Studies on the effects of calorie restriction on the gene expression in *Brachionus plicatilis*
(シオミズツボワムシの遺伝子発現に及ぼすカロリー制限の影響に関する研究)

The beneficial effects of calorie restriction (CR) have been known to occur in a wide range of organisms that are evolutionarily developed from their ancestors. Studies on changes in life histories of CR animals have disclosed similar physiological refinements. These similarities finally lead to a general conclusion that the molecular mechanisms by which CR is acting on organisms are similar or the same and thus these mechanisms might be evolutionarily conserved across various species. The rotifer *Brachionus plicatilis* (Ishikawa strain) has been widely used as a model organism for physiological, ecological and ecotoxicological studies. Because of the availability of parthenogenetic mode of reproduction as well as its versatility to be used as live food in aquaculture, studies on population dynamics using the rotifer have become more important and acquired the priority over those using other species. Many studies have been conducted to identify environmental factors that influence rotifer populations. Each individual of the rotifer population at exponential phase can access limitless amounts of food reproduces exponentially, whereas population at stationary phase, where an increased population size limits the amounts of food accessible to each individual. Actually CR extends life span and during CR, a trade-off between reproduction and life span, has been proposed as an alternative life history strategy of rotifers. However, the molecular mechanisms involved in the effects of CR on reproduction still remain to be elucidated.

The present study was undertaken to identify gene(s) differentially expressed by CR in the rotifer. Efforts were also made to compare expression levels of selected genes between the well fed (WF) and CR groups containing individuals of various ages, and between populations at the exponential and stationary phases mimicking WF and CR, respectively. Finally, DNA synthesis and expression levels of selected genes were examined in the WF and CR groups with the same ages.

1. Identification of genes differentially expressed in CR rotifers

CR-induced differentially expressed genes were investigated in the parthenogenetic rotifer *B. plicatilis* (Ishikawa strain). Eggs were collected from stock cultures and hatched out. Neonates were

divided into two groups: one fed *ad libitum* (WF) as the control and the other fed 3 h day^{-1} (CR). cDNA subtraction was carried out by a suppression subtractive hybridization (SSH) technique using the CR group for forward subtraction as follows. Briefly, cDNAs synthesized from the CR group were used as testers, whereas cDNAs constructed from the WF group were used as drivers. Tester cDNAs, but not driver cDNAs, were ligated to the adaptor sequences that provided binding sites for primers during PCR and the tester cDNAs were subtracted from the driver cDNAs. The subtracted PCR products were ligated into pGEM-T plasmid vectors and the plasmids were transformed into *Escherichia coli*. Subsequently, plasmid DNAs were purified and sequenced and expressed sequence tags (ESTs) were submitted to the NCBI database for searching homologous sequences. Among 163 ESTs submitted, 109 ESTs (67%) retrieved their homologous sequences, whereas other 54 ESTs (33%) showed no significant similarity to any known genes in the database. ClustalW multiple sequence alignment program sorted the ESTs with the same gene products into 38 different genes, which were differentially expressed in the CR group. Gene ontology study showed one group of genes having unknown functions (3%) and 6 functional groups of genes related to cellular structure, transport and division (24%), DNA replication (11%), metabolism (36%), other functions (18%), transcription (5%), and RNA biosynthesis (3%) among 38 genes.

SSH is a powerful technique to isolate differentially expressed genes, but sometime it yields false results and thus additional methods are necessary to verify the results from SSH. Therefore, 38 differentially expressed genes were further validated by semi-quantitative reverse transcription (RT)-PCR (RT-PCR) using the elongation factor-1α gene (*EF-1α*) as an internal standard. Two different CR regimes were employed: CR groups used in the first and second RT-PCR experiments were fed *ad libitum* whole day long every 2 and 3 days, respectively, whereas their WF counterparts were fed *ad libitum*. cDNAs were synthesized from all CR and WF groups and subjected to RT-PCR. Gene-specific primers used in RT-PCR were designed from 38 differentially expressed genes. Accumulated mRNA levels were compared between the CR and WF groups based on the amount of PCR products in gel electrophoresis. RT-PCR confirmed that while 29 genes were consistently up-regulated in the two CR groups, 8 genes were consistently down-regulated in the respective groups among 38 genes. One gene showed its unchangeable mRNA levels in the CR and WF groups.

2. Comparison in expression levels of selected genes between WF and CR groups containing individuals of various ages as well as between populations at exponential and stationary phases

Seventeen out of 38 differentially expressed genes were selected based first on their putative functions and second on their sequence lengths desirable for designing primers to analyze by more accurate quantitative real-time RT-PCR (qRT-PCR). The selected genes encompassed 4 groups according to Gene ontology: 1) DNA synthesis, 2) cellular structure, transport and division, 3) metabolism, and 4) other functions. Group 1 comprised 4 genes encoding mismatch repair protein (*Msh6p*), DNA polymerase epsilon (*Pole*), CDT1 proteins (*CDT1*) and DNA polymerase sigma (*Pols*). Group 2 consisted of 5 genes

encoding BRCA2 and CDKN1A-interacting protein isoform beta (*BCCIPβ*), lissencephaly-1 (*Lis1*), dynein heavy chain domain 3 (*Dnahc3*), spectrin beta (*Spnb1*) and calmodulin synthetic construct (*CaM64B*). Group 3 included 4 genes encoding stom protein (*stom*), succinate dehydrogenase complex subunit D (*SDHD*), iron regulatory protein (*Irp*) and amylase 2 (*Amy2*). Group 4 had 4 genes encoding tissue factor pathway inhibitor (*TFPI*), transposase (*Tsase*), mitochondrial 16S rRNA (*16S rRNA*) and multifunctional 14-3-3 family chaperone (*14-3-3*).

First, the expression levels were studied in the CR and WF groups containing individuals of various ages. Six experimental cultures, 3 each for the CR and WF groups, were begun with about 100 rotifers/ml randomly collected from the stock culture and reared in 100 ml beakers for 8 consecutive days. The CR group was fed 3 h day^{-1} as in the cDNA subtraction experiment whereas the WF group was fed *ad libitum*. Samples were collected every day for cDNAs synthesis and subjected to qRT-PCR. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of *EF-1α* used as the internal control and normalized Ct values were compared between the CR and WF groups. Expression patterns of up- or down-regulation under the CR and WF conditions were considered significant if at least $p<0.05$ significance level was observed on two consecutive culture days. While 16 selected genes were verified for their up-regulation by this assessment, one gene, *Spnb1*, showed a propensity for both up- and down-regulation in the CR group.

The second experiment was intended to detect mRNA expression levels of the selected genes in rotifers at exponential and stationary phases mimicking WF and CR, respectively. Ten experimental cultures, 5 each for the two phases, were commenced with adult 100 rotifers without eggs and the rotifers were reared in 100 ml beakers. Samples were collected when they entered the exponential or stationary phase during population growth. The mRNA levels were determined by qRT-PCR and the levels were compared between the two phases as described above. As a result, 14 selected genes were significantly up-regulated at the stationary phase, whereas other three genes, *CDT1*, *Pols* and *Spnb1*, showed down-regulation at this phase, although a significance level was observed only for *CDT1*.

After assembling the expression patterns, 14 out of 17 selected genes showed up-regulated patterns for both under CR and at the stationary phase. Of other 3 genes, *CDT1* and *Pols* contradicted their expressions between rotifers under CR and at the stationary phase. The expression of *Spnb1* remained ambiguous.

3. Comparison in DNA synthesis and expression levels of selected genes between CR and WF groups with the same ages

Rotifers are eutelic (animals with about 1,000 total cells in the adult form) and have been reported to be retarded for organ-specific (gastric glands and vitellarium) nuclear division rate by dietary restriction. Therefore, DNA synthesis and expression patterns of selected genes were compared between the CR and WF groups with the same ages.

Deposited eggs were collected from stock cultures and hatched out. Neonates of 3 hours post hatching (hph) were transferred into a new medium and fed for 3 h. After feeding, the CR group was subjected to CR for the following 21 h, whereas the WF group was fed *ad libitum*. Since feeding for 3 h day^{-1} was employed for the CR group, this group was fed again at 30 hph. Three experimental cultures each for the two groups were raised. Each culture was begun with about 400 rotifers which were reared in 12-well cell culture plates with the culture volume of 5 ml. DNA synthesis was detected in rotifers at 3 - 39 hph by bromo-deoxyuridine (BrdU) labeling. BrdU labels were gradually increased in association with the increase of the vitellarium volume in the WF group from the first feeding period at 6 hph to the following stages, indicating that DNA was increasingly synthesized in the vitellarium of the WF group. In contrast, the CR group showed no apparent BrdU labels nor the increase of the vitellarium volumes. After the CR group was again fed for 3 h at 30 hph, rapid increases in the BrdU labels and the vitellarium volume were observed thereafter and these changes were maintained until 39 h, but without any egg production. It was noted that DNA synthesis rate in the CR group after the second 3 h feeding was much higher than that in neonates of the WF group at 6 or 9 hph.

The expression levels of the selected genes were analyzed for rotifers from 0 hph (egg) to 33 hph, the period encompassing the reproductive age for the WF group. Thirteen genes showed significant up-regulation in the CR group. Three genes, *Pols*, *Lis1* and *14-3-3*, showed their up-regulation in the CR group at stages from 9 to 21 hph, then their levels were down-regulated. *CDT1* was significantly up-regulated in the WF group in an age-dependent manner, especially during reproduction.

Conclusion

Thirty-eight differentially expressed genes were observed in the CR group by SSH. RT-PCR validations confirmed that 29 and 8 genes were up- and down-regulated in the CR group, respectively, whereas other one gene showed unchangeable expression. Comparative studies between the WF and CR groups containing individuals of various ages as well as between populations at exponential and stationary phases mimicking WF and CR, respectively, showed that 14 selected genes were up-regulated both during CR and at the stationary phase. Studies on DNA synthesis in comparison with expression levels of selected genes in the CR and WF groups containing individuals with the same ages revealed that DNA synthesis rate in the WF group was much higher than that in the CR group and DNA synthesis was increased with the progress of age in the WF group. Thirteen selected genes were clearly up-regulated in the CR group. Three genes were up-regulated during 9-21 hph and then down-regulated in the CR group. Other one gene, *CDT1*, was progressively up-regulated in the WF group, especially during reproduction. Based on their putative functions and expression patterns in the present study, *CDT1* is assumed to be most responsible for the reproductive performance of the rotifer in response to the variability of food resources.