1. Introduction

Sirtuins are evolutionarily conserved nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases/ADP-ribosyltransferases that modulate many metabolic responses affecting aging. Sirtuins expressed in tissues and organs involved in systemic metabolism have been extensively studied. However, the characteristics of sirtuins in the retina, where local energy expenditure changes dynamically in response to light stimuli, are largely unknown. Here we analyzed sirtuin mRNA levels by real-time PCR, and found that all seven sirtuins are highly expressed in the retina compared with other tissues, such as liver. We then analyzed the sirtuin mRNA profiles in the retina over time, under a 12-h light/12-h dark cycle (LD condition) and in constant darkness (DD condition). All seven sirtuins showed significant daily variation under the LD condition, with all except Sirt6 being increased in the dark phase. The expression patterns were different under the DD condition, suggesting that sirtuin mRNA levels except Sirt6 are affected by light–dark condition. These findings were not obtained in the brain and liver. In addition, the mRNA expression patterns of Nicotinamide phosphoribosyltransferase (Nampt), peroxisome proliferator-activated receptor gamma coactivator (PGC1α), and transcription factor A, mitochondrial (Tfam) in the retina, were similar to those of the sirtuins except Sirt6. Our observations provide new insights into the metabolic mechanisms of the retina and the sirtuins’ regulatory systems.
mRNAs for peroxisome proliferator-activated receptor gamma coactivator (PGC1α) and transcription factor A, mitochondrial (Tfam), which are indicators of mitochondrial biogenesis; mitochondrial biogenesis is enzymatically regulated by Sirt1 (Rodgers et al., 2005). We compared the profiles of sirtuin mRNA levels over time in mice kept under a 12-h light/12-h dark (LD) cycle with their levels in mice kept in constant darkness (DD). Our findings provide basic information about the sirtuins in the retina, which will be essential for future research on retinal metabolism and sirtuin regulation.

2. Materials and methods

2.1. Animals and tissue sampling

Six-week-old male C57BL6/J mice (CLEA Japan, Tokyo, Japan) were used. The mice were kept in an air-conditioned room (22 ± 1 °C) under a 12-h light/12-h dark (LD) cycle, with the light on from 08:00 to 20:00. The light intensity at the surface of the cages was 200 lx. The mice were given food and water ad libitum. After being subjected to the LD cycle for 2 weeks, the mice were randomly divided into an LD group and a constant darkness group (DD group). The LD group was kept under the LD cycle and sacrificed at zeitgeber time (ZT) 0, 4, 8, 12, 16, and 20 h. ZT0 was defined as the time of light onset and ZT12 as the time of dark onset. The DD group was transferred to constant darkness after the dark phase of the last LD cycle, and kept in the dark for two 24-hour DD cycles. During the last and third 24-hour DD cycle, the mice were sacrificed at circadian time (CT) 0, 4, 8, 12, 16, and 20 h. CT0 was defined as the onset of the last 24-hour DD cycle. Five or six mice were sacrificed at each time point, and samples of the retina, brain parietal cortex, heart, liver, and kidney were obtained. For tissue sampling during the dark phase, a dim red light was used, and the samples were placed individually in light-tight containers on ice and dissected under a microscope within 2 min. All the animal experiments were conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. RNA isolation and real-time PCR

The retina or other tissue sample was placed in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) to extract the total RNA. To generate complementary DNA (cDNA), 1 μg of the total RNA was added to the Super Script VILO cDNA Synthesis Kit (Life Technologies) and reverse-transcribed according to the manufacturer’s instructions. PCR was performed using the StepOnePlus Real Time PCR system (Life Technologies), and the mRNA was quantified using the relative delta delta Ct method. To compare the mRNA levels in the retina with those in other tissues, the expression levels were normalized to that for β-actin. To obtain profiles of the mRNA levels over time, the expression levels were normalized to that for 36B4, which showed constant expression under LD and DD conditions (data not shown).

2.3. Statistics

Results are presented as the mean ± SD, and p < 0.05 was considered statistically significant. To compare the sirtuin expression levels among tissues, we used Tukey’s HSD test or the Games–Howell test, depending on Levine’s test for equality of variance. The mRNA expressions over time were compared by one-way ANOVA within the LD or DD group, followed by Tukey’s HSD test or the Games–Howell test. The expression levels between LD and DD groups at each time point for 24 h were compared by Student’s t-test. All statistical tests were performed using IBM SPSS statistics Ver.19 (IBM, Armonk, NY).

3. Results

3.1. High sirtuin mRNA levels in the retina

We first analyzed the mRNA levels of sirtuins in the retina and other tissues (brain parietal cortex, heart, liver, and kidney) at ZT20 by real-time PCR (Fig. 1). The mRNA levels of Sirt1, 2, 4, 6, and 7 were significantly higher in the retina than in all the other tissues examined. The Sirt3 mRNA level in the retina was the same as in the liver, and significantly higher than in the rest of the tissues. The Sirt5 mRNA level in the retina was significantly higher than in all the other tissues examined except the heart (relative mRNA levels were: retina: brain: heart: liver: kidney, Sirt1 2.3 fold; Sirt2 2.5 fold; Sirt3 2.0 fold; Sirt4 2.2 fold; Sirt5 2.0 fold; Sirt6 1.9 fold at ZT20). As for daily variation, those in other tissues, the expression levels were normalized to that for 36B4, which showed constant expression under LD and DD conditions (data not shown).

3.2. The mRNA profiles of sirtuins in the retina under LD and DD conditions over time

We next analyzed the mRNA profiles of sirtuins in the retina, brain, and liver over the course of a day under LD and DD conditions, by real-time PCR (Fig. 2). In the retina, all the sirtuins (Sirt1–7) showed significant daily variation under the LD condition (ANOVA: Sirt1–7 p < 0.01). The mRNA levels of all the sirtuins except Sirt6 were significantly increased in the dark phase compared with ZT0, peaking at ZT16–ZT20 (Sirt1 1.6 fold at ZT20; Sirt2 1.7 fold at ZT 20; Sirt3 1.8 fold at ZT 16; Sirt4 1.9 fold at ZT 20; Sirt5 1.7 fold at ZT20; Sirt7 1.9 fold at ZT20).

In contrast, under the DD condition, none of the sirtuins was elevated or peaked during CT16–CT20, the comparative peak time under the LD condition. Moreover, the mRNA levels of all the sirtuins except Sirt6 were significantly higher at each time point from CT0 to CT12 under the DD condition than under the LD condition from ZT0 to ZT12 (relative mRNA levels of CT0 compared to ZT0; Sirt1 2.3 fold; Sirt2 2.5 fold; Sirt3 2.0 fold; Sirt4 2.2 fold; Sirt5 2.0 fold; Sirt7 2.2 fold). Regarding the daily variations under the DD condition, those of Sirt2–5 were significant (ANOVA: Sirt2, 4, and 5 p < 0.01; Sirt3 p = 0.028), but those of Sirt1, 6, and 7 were not.
In the brain under the LD condition, there were significant daily variations in Sirt3 and Sirt5 (ANOVA: Sirt3 $p = 0.036$; Sirt5 $p = 0.036$), but there was no increase in the dark phase. Under the DD condition, Sirt1–5 and Sirt7 showed significant daily variations in the brain (ANOVA: Sirt1–5, $7 p < 0.01$), but Sirt6 did not. The differences in the data obtained from the brain between the LD and DD groups were not as obvious as in the retina.

In the liver under the LD condition, there were significant daily variations in all the sirtuins (ANOVA: Sirt1–3–6 $p < 0.01$; Sirt2 $p = 0.019$; Sirt7 $p = 0.013$), but there was no increase in the dark phase. Under the DD condition, Sirt1–6 showed significant daily variations in the liver (ANOVA: Sirt1, 3, 6 $p < 0.01$; Sirt2 $p = 0.019$; Sirt4 $p = 0.031$; Sirt5 $p = 0.043$), but Sirt7 did not. The differences in the data obtained from the liver between the LD and DD groups were not as obvious as in the retina, although there were shifts in the timing of some peaks.

3.3. The mRNA profiles of Nampt, PGC1α, and Tfam in the retina under LD and DD conditions over time

Next, we analyzed the profiles of the Nampt, PGC1α, and Tfam mRNA levels over time in the retina, brain, and liver under the LD and DD conditions, by real-time PCR (Fig. 3). In the retina under LD conditions, all three mRNAs showed significant daily variations (ANOVA: Nampt, PGC1α, and Tfam $p < 0.01$), peaking at ZT16 (Nampt 1.6 fold; PGC1α 1.8 fold; Tfam 1.4 fold). Under the DD condition, the daily variations were significant for the PGC1α and Tfam (ANOVA: PGC1α $p = 0.023$; Tfam $p = 0.025$), but not the Nampt. The mRNA level at each time point from CT0 to CT12 in the DD group was significantly higher than that from ZT0 to ZT12 in the LD group, for all three molecules (relative mRNA levels of CT0 compared to ZT0; Nampt 2.6 fold; PGC1α 2.6 fold; Tfam 2.6 fold).

In the brain under LD conditions, the daily variations were significant for the Nampt and Tfam (ANOVA: Nampt $p = 0.023$; Tfam $p < 0.01$), but not the PGC1α. Under the DD condition, the daily variations were significant for the Nampt and PGC1α (ANOVA: Nampt and PGC1α $p < 0.01$), but not the Tfam. In the liver, significant daily variation was observed for the Nampt and Tfam in both the LD (ANOVA: Nampt and Tfam $p < 0.01$) and DD groups (ANOVA: Nampt, PGC1α, and Tfam $p < 0.01$), but the PGC1α only in the DD group. As seen for the sirtuins in the brain and liver, the differences between the LD and DD groups were not obvious compared with the retina.

4. Discussion

We demonstrated that the mRNAs of all seven sirtuins were expressed at high levels in the retina. All of the sirtuin mRNAs except Sirt6 were increased in the dark phase, and the daily expression patterns were different between the LD and DD conditions. These findings were not obtained in the brain and liver. We also examined the expression patterns of Nampt and mitochondria-related gene transcripts in the retina over time, and found that they mirrored the expression pattern of the sirtuins except Sirt6.

We confirmed that all seven sirtuin mRNAs were expressed in the retina. Interestingly, the expression levels in the retina were very high, overall, compared with other tissues. Sirtuins are deeply involved in energy metabolism in cells and tissues, and the high level of sirtuin mRNAs in the retina is consistent with the retina being one of the highest energy-consuming tissues in the body (Niven and Laughlin, 2008). The photoreceptors, which represent the largest population of retinal neuronal cells, convert light stimuli to electric impulses by modulating rhodopsin, and this system requires adenosine triphosphate (ATP) (Okawa et al., 2008). Moreover, the impulse is transmitted to and processed in the inner layer of the retina, where energy must constantly be expended to maintain the concentration gradient of ions that regulates synaptic function (Niven and Laughlin, 2008).

Alternatively, the high sirtuin expression levels may be involved in retinal cell survival. For example, Sirt1 was previously reported to contribute to cell survival in the retina. That is, the loss of activating E2f transcription factors downregulates the p53 deacetylase Sirt1, causing p53 hyperacetylation and elevated apoptosis; thus, a E2f-Sirt1-p53 pathway promotes cell survival in the retina (Chen et al., 2009). This function can explain the retinal phenotype of Sirt1 conditional knockout mice, in which p53 hyperacetylation and elevated apoptosis reduce the number of retinal neuronal cells during development (Cheng et al., 2003). In addition, the nuclear-cytoplasmic translocalization of Sirt1 is observed in...
the photoreceptor cells of rd10 mice (Jaliffa et al., 2009), a model of hereditary retinal degeneration, which might also be associated with the cell survival system, although the details were not reported. Considering that the retina generates reactive oxygen species (ROS) upon its exposure to light (Sasaki et al., 2012), sirtuins might also be expressed at high levels to protect retinal cells from light-induced apoptosis.
Besides light exposure, the process of ATP generation in mitochondria produces intracellular ROS, which can cause mitochondrial dysfunction and cell apoptosis. However, an age-related change (hearing loss) is diminished by calorie restriction in the presence of Sirt3, which reduces mitochondrial oxidative stress in the inner ear (Someya et al., 2010). ROS are involved in age-related retinal degenerative disorders such as diabetic retinopathy (Sasaki et al., 2010), glaucoma (Yuki et al., 2011), and age-related macular degeneration (Barot et al., 2011). Thus, sirtuins may also contribute to the neuroprotection of the retina by regulating ROS.

We showed that the daily variations in the mRNAs for all seven sirtuins except Sirt6 in the retina were clearly affected by the LD condition. The retina is a photoreceptive tissue, whose energy consumption changes depending on light exposure. Retinal cells expend more energy in the dark, as shown by their higher O2 consumption and lactate production (Ames et al., 1992; Niven and Laughlin, 2008). This is explained by the mechanism of visual signal transmission. Photoreceptor cells become hyperpolarized in response to light by closing the ion channels in the cytoplasmic membrane, to generate light-induced neuronal activity; thus, in the dark, the channels are kept open, consuming ATP, to keep the cells depolarized and prepared for the next light stimulus. Therefore, the expression patterns of sirtuins in the retina may reflect the relative energy consumption levels occurring in conditions of light exposure or darkness. In the brain and liver, the expression pattern of sirtuins over time was different from that in the retina. Interestingly, the brain and retina are both a part of the central nervous system, but the sirtuin mRNAs behaved differently. Light had a significant effect on the sirtuin mRNA levels in the retina but not in the brain and liver, suggesting that sirtuins are regulated in a tissue- or organ-specific manner.

Because sirtuins are NAD-dependent enzymes, we also analyzed mRNA levels of NAD-related molecules. Interestingly, Nampt, an enzyme in NAD synthesis that plays a central role in the NAD salvage pathway, had a similar daily expression pattern as the retinal sirtuins except Sirt6, and was, moreover, similarly influenced by the LD condition. The transcription of Nampt is regulated by CLOCK-BMAL1, and Sirt1 is recruited to the Nampt promoter to contribute to its circadian synthesis (Imai, 2011; Nakahata et al., 2009). Thus, it is reasonable that Nampt and Sirt1 show similar mRNA expression patterns. Such a regulatory mechanism, involving the concerted changes in the expression of sirtuins and Nampt would efficiently control metabolism in the retina.

The time-course of sirtuin mRNA expressions except Sirt6 under LD and DD conditions was consistent with the metabolism in the retina, as described above. We were, therefore, also interested in whether mRNAs of other metabolism-related molecules would show a similar expression pattern. PGC1α is a metabolic coactivator that induces mitochondrial biogenesis and respiration by interacting with transcription factors, and Tfam is a key activator of mitochondrial transcription of the mitochondrial genome. Interestingly, the expression patterns of these mRNAs were also very similar to those of the sirtuins except Sirt6. The patterns of PGC1α and Tfam expression in the retina over time were also related to the LD condition, indicating that mitochondrial biogenesis is altered by light–dark condition, and that it increases in the dark. This is consistent with previous reports that the retina consumes large amounts of oxygen in the dark (Ames et al., 1992). Neuronal activity dictates the cytochrome activity in mitochondria (Wong-Riley, 2012); thus, mitochondrial biogenesis would be essential for retinal neuronal cell activity, and sirtuins may be involved in the regulatory system for neuronal activity in response to light stimuli. To know the underlying mechanisms, expression patterns of mRNAs which we reported in this study would be very informative, although protein analyses of sirtuins and the related molecules in the retina would be required to progress the future study.
In summary, all seven sirtuin mRNAs were highly expressed in the retina, and their expression patterns except Sirt6 were closely associated with light–dark conditions. Given that the energy metabolism in the retina is related to light stimuli, and that mitochondria contribute to retinal energy metabolism, our observations of sirtuin expression provide new insights into both the mechanism for metabolism in the retina, and the regulation of sirtuins. The mechanism by which the sirtuins are regulated in response to light, and the sirtuins’ involvement in the pathogenesis of age-related retinal diseases will be examined in future studies.

Conflict of interest

The authors have no conflicts of interests.

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