Increased Expression of Interleukin-18 in Lenses of Ovariectomized Rats

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Previous studies showed an increased prevalence of cataracts in postmenopausal women. In this study, we investigated changes in the levels of calcium ion (Ca$^{2+}$) and interleukin (IL)-18, which are factors in cataract development, in the lenses of ovariectomized (OVX) rats, a model of postmenopausal woman. Although the Ca$^{2+}$ content in the blood of OVX rats increased 1 month after ovariectomy and subsequently decreased, the Ca$^{2+}$ content in the lenses was unchanged in OVX rats 1–3 months after ovariectomy. The Ca$^{2+}$-ATPase activity in the lenses of OVX rats peaked 1 month after ovariectomy, and the behavior of Ca$^{2+}$-ATPase activity in lenses of OVX rats was similar to that of the Ca$^{2+}$ concentration in the blood. It is possible that hypercalcemia increases the Ca$^{2+}$ inflow into the lens; however, the enhanced Ca$^{2+}$-ATPase activity prevents the Ca$^{2+}$ level from rising. On the other hand, we found that the levels of both IL-18 and interferon (IFN)-γ in the lenses of OVX rats were significantly increased as compared with the lenses of sham (control) rats during the period 1–3 months after surgery. These results suggest that the expression of IFN-γ via IL-18 in the lenses of OVX rats is induced by ovariectomy, and that excessive IL-18 and IFN-γ production in the lenses may be related to cataract development in postmenopausal women. These findings support those of previous studies that assessed lens opacification in postmenopausal women.

Key words interleukin-18; lens; ovariectomized rat; interferon-γ; calcium

Cataracts are defined as any alteration in the optical homogeneity of the lens or a decrease in its transparency.1–2 Exposure to reactive oxygen species, interleukin (IL) or interferon (IFN)-γ induced by UV in sunlight are considered to result in a breakdown of lens homeostasis, resulting in an elevation in the calcium ion (Ca$^{2+}$) content of the lens. This elevated Ca$^{2+}$ content has been deduced to activate calpain, a Ca$^{2+}$-dependent protease, leading to an increased degradation of lens proteins, such as crystallin proteins, resulting in an opaque lens.3–4 Therefore, the regulation of Ca$^{2+}$ levels in lens is important to prevent opacification. Previous studies have shown an increased prevalence of cataracts in postmenopausal women, and estrogen has been reported to be effective in preventing age-related cataracts.5–8

Estrogen is a hormone that participates in regulating calcium (Ca) levels in the body, and postmenopausal women experience a drop in estrogen secretion. Bone resorption is regulated by estrogen, and a decrease in estrogen level leads to a decrease in vitamin D. Since vitamin D enhances Ca$^{2+}$ absorption from the small intestine,9–12 postmenopausal women are at greater risk of osteoporosis and hypercalcemia/hypocalcemia via the dysfunction in Ca$^{2+}$ regulation due to a chronic reduction in circulating estrogen levels.9–12 This reduced capacity to regulate Ca$^{2+}$ levels may be related to the onset of cataracts via an increase in Ca$^{2+}$ levels in the lens. Estrogens are also known to have antioxidant effects13,14 due to direct scavenging of free-radicals15 and upregulation of antioxidative enzymes.16 In postmenopausal patients, peroxidation increases because of the dramatically decreased estrogen levels. In addition, it has been reported that estrogens prevent the expression of IL-18 in mouse uterus.17 On the other hand, we also found that excessive IFN-γ via IL-18 results in cataract development.18,19 From these reports, it is possible that IL-18 levels are also increased in the lenses of postmenopausal women, and that enhanced IFN-γ via IL-18 may lead to the formation and progression of cataracts. Despite these findings, there are, to our knowledge, no reports of concerning Ca$^{2+}$ regulation and IL-18 expression in the lenses of postmenopausal women.

In this study, we investigated changes in Ca$^{2+}$ levels and IL-18 expression in postmenopausal lenses using ovariectomized (OVX) rats, an animal model of postmenopausal woman.

MATERIALS AND METHODS

Animals Female Wistar rats, 5 weeks of age, were ovariectomized (OVX rat) or sham operated (Sham rat). All procedures were performed in accordance with the Kinki University Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNAs were extracted from the lenses of rats at 10, 14, and 18 weeks of age (1, 2, and 3 months after ovariectomy) by the acid guanidium thiocyanate–phenol–chloroform extraction method20 using Trizol reagent (Life Technologies Inc., Rockville, U.S.A.), and the RT reactions were performed using an RNA PCR Kit (AMV Ver 3.0, TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The RT reactions were performed at 42°C for 15 min, followed by 5 min at 95°C. The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer’s instructions (Roche Diagnostics Applied Science, Mannheim, Germany). The conditions for PCR and the primers used are shown in Table 1.21 The quantities of the PCR products were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche Diagnostics Applied Science, Mannheim, Germany). The differences in the threshold cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and
other groups [IL-18, IL-18Ra, IL-18Rβ, IL-18 binding protein (IL-18BP), caspase-1] were used to calculate the levels of mRNA expression.

Measurement of Calcium Content Femur, blood, aqueous humor and lens from rats at 10, 14, and 18 weeks of age (1, 2, and 3 months after ovariectomy) were removed. The femur was boiled for 2 h, dried at room temperature, and then calcined in a muffle furnace KDF S-80 (Sansyo Co., Ltd., Osaka, Japan) at 550°C for 48 h. Calcined femur was dissolved in 1% nitric acid, and the sample solution was filtered through a 0.45 µm membrane filter. The Ca²⁺ concentration of the calcined femur was measured using an inductively coupled plasma-atomic emission spectrometer ICP-MS-7500 (ICP-AES, Shimadzu Corp., Kyoto, Japan). The lenses were homogenized in phosphate-buffered saline (pH 7.4) on ice. The lens homogenates and blood were centrifuged at 20,400 g for 30 min at 4°C, and the supernatants were used for measurements of Ca²⁺. The Ca²⁺ concentrations in the blood, aqueous humor and lens were determined by a Ca test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).21)

Measurement of Ca²⁺-ATPase Activity Lenses from rats at 10, 14, and 18 weeks of age (1, 2, and 3 months after ovariectomy) were removed. The Ca²⁺-ATPase activity was analyzed according to our previously report.22) Briefly, 125 µL of solution (200 mM KCl, 100 mM N-(2-hydroxyethyl)pipеразине-N’-2-етансульфонная кислота (HEPES), 10 mM MgCl₂, 2 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetie acid (EGTA) and 2 mM ATP, pH 7.4) with or without 2.2 mM CaCl₂ was added to each sample (125 µL), and the samples were incubated for 1 h at 37°C. The reaction was stopped by the addition of trichloroacetic acid, and the absorbance (660 nm) of the supernatants was measured. Ca²⁺-ATPase activity containing plasma membrane Ca²⁺ ATPase and sarco/endoplasmic reticulum Ca²⁺-ATPase was calculated as the difference in phosphate liberation measured in the presence and absence of Ca²⁺. Protein levels were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, U.S.A.).22)

Measurement of IL-18 and IFN-γ Levels Lenses from rats at 10, 14, and 18 weeks of age (1, 2, and 3 months after ovariectomy) were homogenized in ultrapure water. The lens homogenates were centrifuged at 20,000×g for 10 min at 4°C, and the supernatants were used for the measurement of IL-18 and IFN-γ levels.18) The IL-18 and IFN-γ levels in this study were within the detection range.18) The protein levels were analyzed by the method described above.

RESULTS AND DISCUSSION

In this study, we investigated the changes in Ca²⁺ levels and IL-18 expression in OVX rat, and found that the expression of IL-18 is enhanced by ovariectomy.

In order to predict the changes in Ca²⁺ levels and IL-18 expression in the lenses of postmenopausal women, the selection of the experimental animal is very important. The OVX rat is an accepted female animal model to mimic postmenopausal women.21,23) The OVX procedure promotes bone loss. Following bilateral ovariectomy, serum Ca²⁺ levels rise and bone mass decreases due to the loss of estrogen.23,24) Therefore, we confirmed the changes in body weight and calcium contents in the bone and blood in Sham and OVX rats 1–3 months after ovariectomy (Table 2). Food intake (23.0±0.8 g/d/rat) and water consumption (35.4±3.9 mL/d/rat) by OVX rats were similar to those of Sham rats at 3 months after ovariectomy (food intake 23.0±0.8 g/d/rat, water consumption 36.1±1.4 mL/d/rat, mean±S.E.M., n=5). However, the body weight of OVX rats was higher than that of Sham rats. The Ca²⁺ content in the bones of OVX rats decreased following ovariectomy with a level 3 months after ovariectomy approximately 67% that of Sham rats. On the other hand, the Ca²⁺ contents in the blood of OVX rats peaked 1 month after ovariectomy, and subse-

Table 1. Parameters and Sequences of Primers Used for Quantitative RT-PCR Analysis

| Primer     | Hot start (10 min) | Denaturation (10 s) | Annealing (10 s) | Extension (5 s) | Sequence (5’–3’)       |
|------------|--------------------|---------------------|------------------|-----------------|-------------------------|
| IL-18      | 95°C               | 95°C                | 53°C             | 72°C            | FOR: CGCAATATACCCGAGGCTATAGTC<br>REV: GGTTGACATCTCCTTCCATCTTCAC |
| IL-18Ra    | 95°C               | 95°C                | 57°C             | 72°C            | FOR: AGCCAAAAGACGAGACAGCTACTAC<br>REV: CTCCACGGCCAGCACCACATC |
| IL-18Rβ    | 95°C               | 95°C                | 55°C             | 72°C            | FOR: GACACAGGATTTAACCATTCA<br>REV: AGCAGAGCATGTTGTGATGATG |
| IL-18BP    | 95°C               | 95°C                | 53°C             | 72°C            | FOR: TTGGTGGCTGCTGTCTATATG<br>REV: GGTCACGGTCCATTCCAGTG |
| Caspase-1  | 95°C               | 95°C                | 55°C             | 72°C            | FOR: TGAAAGATGGTGGTACATAG<br>REV: CAAGTCAAGACAGCGCATATTC |
| GAPDH      | 95°C               | 95°C                | 60°C             | 72°C            | FOR: ACGGCACAGTCAAGGCTGAGA<br>REV: CGCTCTGGGAAGATGTTGAT |
Sham rats 1 months after ovariectomy (2.15 ± 0.56* vs. Sham rats 1.90 ± 0.71* p < 0.05)

After ovariectomy, the amount of Ca2+ in the aqueous humor of OVX rats 1 months after surgery was similar to those of the Ca2+ concentration in the blood. From these results, we hypothesize that hypercalcemia may increase the Ca2+ inflow into the lens; however, the enhanced Ca2+-ATPase activity prevents the Ca2+ level from rising.

In this study, we also investigated the expression of IL-18 in the lenses of OVX rats. IL-18, which is a pleiotropic cytokine belonging to the IL-1 family, is expressed as an inactive 25-kDa pro-form that is cleaved by caspase-1 to an 18-kDa active form (mature IL-18). The mature IL-18 exerts its effects upon binding to its cognate receptor (IL-18R). IL-18Rα, IL-18Rβ are the two subunits of IL-18R. IL-18BP is a low-affinity receptor for IL-18 that does not bind IL-18 directly; instead the β chain increases the IL-18 binding affinity and is necessary for initiating signal transduction in target cells. The binding of mature IL-18 to IL-18R leads to the production of IFN-γ and IL-18 and IFN-γ play an important role in inflammatory action.

As compared with Sham rats, the lenses of OVX rats showed high expression levels of the mRNAs for IL-18. The expression of IL-18BP in OVX and Sham rats was similar 1–3 months after ovariectomy. This result shows that the IL-18BP does not affect the IL-18 activity.

Table 2. Body Weight and Calcium Levels in Bone and Blood of OVX Rats 1–3 Months after Surgery

| Body weight (g) | Sham rats | 1 month | 2 months | 3 months |
|-----------------|-----------|---------|----------|----------|
| Ca level in bone (mg/g) | Sham rats | 264±11 | 307±15 | 343±17 |
| | OVX rats | 307±12* | 349±18*** | 390±13*** |
| Ca2+ level in blood (mg/dL) | Sham rats | 345±13 | 359±32 | 344±54 |
| | OVX rats | 257±19* | 228±30* | 229±39* |

The data are presented as the mean±S.E.M. of 4–7 independent rats. *p<0.05, vs. Sham rats for each category. **p<0.05, vs. OVX rats 1 month after surgery.

Table 3. Expression of IL-18 mRNA in Lenses of OVX and Sham Rats 1–3 Months after Surgery

| IL-18 (×10^-4) | Sham rats | 1 month | 2 months | 3 months |
|----------------|-----------|---------|----------|----------|
| IL-18Rα (×10^-4) | Sham rats | 1.65±0.51 | 1.53±0.49 | 1.59±0.54 |
| | OVX rats | 3.26±0.56* | 3.95±0.71* | 4.18±0.77* |
| IL-18Rβ (×10^-5) | Sham rats | 1.97±0.71 | 2.19±0.72 | 1.91±0.70 |
| | OVX rats | 2.44±0.68 | 2.95±0.74 | 3.65±0.71* |
| IL-18BP (×10^-5) | Sham rats | 7.55±1.18 | 7.46±1.81 | 7.95±1.97 |
| | OVX rats | 7.16±1.34 | 7.69±1.90 | 7.70±1.83 |
| Caspase-1 (×10^-3) | Sham rats | 4.61±1.17 | 4.56±1.05 | 4.67±1.09 |
| | OVX rats | 8.93±1.26* | 10.4±1.31* | 11.7±1.29* |

The data are presented as the mean±S.E.M. of 4–7 independent rat lenses. *p<0.05, vs. Sham rats for each category.
tallin proteins and lens opacification. However, in the OVX rats used, elevated Ca$^{2+}$ may be needed to induce the dysfunction of Ca$^{2+}$ regulation in the lenses of OVX rats. Therefore, we are now planning to investigate the effects of estrogen on IL-18 expression in the lenses of OVX rats. Moreover, we will demonstrate the effects of calmodulin, which is related to the activity of Ca$^{2+}$-ATPase, on the Ca$^{2+}$ regulation in the lenses of OVX rats. These results support previous studies that have assessed lens opacification in postmenopausal women.

**Conflict of Interest** The authors declare no conflict of interest.

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