Note

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 effects^{13,14} 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. De-

spite 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 1.¹⁸ 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

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

Primer	Hot start (10min)	Denaturation (10s)	Annealing (10s)	Extension (5s)	Sequence (5'–3')
IL-18	95°C	95°C	53°C	72°C	FOR: CGCAGTAATACGGAGCATAAATGAC REV: GGTAGACATCCTTCCATCCTTCAC
IL-18R α	95°C	95°C	57°C	72°C	FOR: AGCAGAAAGAGACGAGACACTAAC REV: CTCCACCAGGCACCACATC
IL-18R β	95°C	95°C	55°C	72°C	FOR: GACCACAGGATTAAACCATTTCAGC REV: AGCAGGACCTAGTGTGTGATGATG
IL-18BP	95°C	95°C	53°C	72°C	FOR: TTGGTGGGTCCTGCTTCTATATG REV: GGTCAGCGTTCCATTTCAGTG
Caspase-1	95°C	95°C	55°C	72°C	FOR: TGAAGATGATGGCATTAAAGAAGGC REV: CAAGTCACAAGACCAGGCATATTC
GAPDH	95°C	95°C	60°C	72°C	FOR: ACGGCACAGTCAAGGCTGAGA REV: CGCTCCTGGAAGATGGTGAT

other groups [IL-18, IL-18R α , IL-18R β , 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 μ m membrane filter.²¹⁾ The Ca²⁺ concentration of the calcined femur was measured using an inductively coupled plasma-atomic emission spectrometer ICPS-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 20400 $\times g$ 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).²²⁾

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 μ L of solution (200 mM KCl, 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM MgCl₂, 2 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 2 mM ATP, pH 7.4) with or without 2.2 mM CaCl₂ was added to each sample (125 μ L), 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 200 $\times g$ 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 were measured using an Endogen[®] Rat IL-18 ELISA Kit and Endogen[®] Rat

IFN γ ELISA Kit, respectively, according to the manufacturer's instructions (Invitrogen Corp., Camarillo, CA, U.S.A.). The range of detection for IL-18 was 15.6–1000 pg/mL, and the range of detection for IFN- γ was 8–500 pg/mL in the ELISA Kits. 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.

Image Analysis of Lens Opacification The experiment was performed following previous report.¹⁸⁾ The transparency of the rat lenses were monitored by an EAS-1000 equipped with a CCD camera (Nidek, Gamagori, Japan). The outline of the lens image was determined by selecting 4 points on the image, and then the transparent area within the outline and define level were set automatically by the software.

Statistical Analysis All values are presented as the mean $\pm$ standard error of the mean (S.E.M.). Unpaired Student's *t*-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison.

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 $\pm$ 0.8 g/d/rat) and water consumption (35.4 $\pm$ 3.9 mL/d/rat) by OVX rats were similar to those of Sham rats at 3 months after ovariectomy (food intake 23.0 $\pm$ 0.8 g/d/rat, water consumption 36.1 $\pm$ 1.4 mL/d/rat, mean $\pm$ 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 2. Body Weight and Calcium Levels in Bone and Blood of OVX Rats 1–3 Months after Surgery

		1 month	2 months	3 months
Body weight (g)	Sham rats	264±11	307±15	343±17
	OVX rats	307±12*	349±18***	390±13***
Ca level in bone (mg/g)	Sham rats	345±13	359±32	344±54
	OVX rats	257±19*	228±30*	229±39*
Ca ²⁺ level in blood (mg/dL)	Sham rats	262±15	268±13	261±16
	OVX rats	387±12*	321±22***	307±19**

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.

Fig. 1. Ca²⁺ Content (A) and Ca²⁺-ATPase Activity (B) in the Lenses of Sham and OVX Rats 1–3 Months after Surgery

The Ca²⁺ content was measured by the methyl xylene blue colorimetric method, and is expressed as the ratio of the wet weight of lens. The Ca²⁺-ATPase activity was calculated as the difference in the phosphate liberation measured in the presence and absence of Ca²⁺. Open columns, Sham rats; closed columns, OVX rats. The data are presented as the mean±S.E.M. of 4–7 independent rats. * $p<0.05$, vs. Sham rats for each category.

quently decreased. This decrease may be caused by other hormones that regulate Ca²⁺ levels in the body, such as calcitonin and parathyroid hormone, and these changes in the biological characteristics correspond to those seen in postmenopausal women. These results are similar to previous reports using OVX rats,²¹⁾ and show that the OVX rats used in this study were suitable for investigating changes in Ca²⁺ levels and IL-18 expression in lenses after menopause.

Next, we investigated the effect of hypercalcemia on the Ca²⁺ content of lenses in OVX rats. Although, the Ca²⁺ content in the aqueous humor of OVX rats 1 months after ovariectomy (6.80±0.83 mg/L, $n=4$) was higher than that of Sham rats 1 months after ovariectomy (2.15±0.31 mg/L, $n=4$), the Ca²⁺ content in the lenses of OVX rats 1–3 months after ovariectomy did not change (Fig. 1A). It is known that the Ca²⁺ content in lenses is regulated by Ca²⁺-ATPase. Therefore, we measured the Ca²⁺-ATPase activity in the lenses of

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

		1 month	2 months	3 months
IL-18 ($\times 10^{-4}$)	Sham rats	8.91±2.98	8.16±1.93	8.57±2.11
	OVX rats	36.4±4.89*	37.2±5.02*	41.1±5.80*
IL-18R α ($\times 10^{-4}$)	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-18R β ($\times 10^{-3}$)	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 ($\times 10^{-5}$)	Sham rats	7.55±1.80	7.46±1.81	7.95±1.97
	OVX rats	7.16±1.34	7.69±1.90	7.70±1.83
Caspase-1 ($\times 10^{-3}$)	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.

Sham and OVX rats 1–3 months after ovariectomy. The Ca²⁺-ATPase activity in the lenses of OVX rats peaked 1 month after ovariectomy, and the activities 1 and 2 months after ovariectomy were significantly higher than in Sham rats (Fig. 1B). The changes in the Ca²⁺-ATPase activity in the lenses of OVX rats were 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 24-kDa pro-form that is cleaved by caspase-1 to an 18-kDa active form (mature IL-18).^{25,26)} The mature IL-18 exerts its effects upon binding to its cognate receptor (IL-18R).²⁷⁾ IL-18R α , R β are the two subunits of IL-18R.²⁷⁾ IL-18R α is a low-affinity receptor for IL-18,²⁷⁾ while IL-18R β 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.²⁸⁾ The binding of mature IL-18 to IL-18R leads to the production of IFN- γ .²⁹⁾ IL-18 and IFN- γ play an important role in inflammatory action,^{25,28)} and cause lens opacification.^{18,19)} As compared with Sham rats, the lenses of OVX rats showed high expression levels of the mRNAs for IL-18, IL-18R α , IL-18R β and caspase-1 (Table 3), and the levels remained high 3 months after ovariectomy. On the other hand, it was known that the IL-18BP was a specific, functional inhibitor of IL-18, which may act as an endogenous inhibitor of circulating IL-18.³⁰⁾ 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.

Fig. 2. IL-18 (A) and IFN- γ (B) Levels in the Lenses of Sham and OVX Rats 1–3 Months after Surgery

IL-18 and IFN- γ levels were determined using an ELISA method. Open columns, Sham rats; closed columns, OVX rats. The data are presented as the mean $\pm$ S.E.M. of 4–7 independent rats. * $p < 0.05$, vs. Sham rats for each category.

Figure 2 shows the IL-18 and IFN- γ levels determined by the enzyme-linked immunosorbent assay (ELISA) method in the lenses of OVX rats 1–3 months after ovariectomy. No changes in the levels of IL-18 and IFN- γ in the lenses of Sham rats were observed, while the IL-18 and IFN- γ levels in the lenses of OVX rats were both significantly higher than in the lenses of Sham rats 1–3 months. Three months after ovariectomy, the IL-18 and IFN- γ levels in OVX rats were approximately 6.8 and 4.2 times higher than in Sham rats, respectively (Fig. 2).

These results suggest that IL-18 expression was induced in the lenses of OVX rats by the ovariectomy, and that excessive IL-18 and IFN- γ production in the lenses may be related to cataract development in postmenopausal women. It has been reported that excessive IFN- γ production (12–20 pg/mg protein) *via* IL-18 in the lens cause an increase in the Ca^{2+} content and opacification.^{18,19} However, in the OVX rats used in this study, lens opacity was not observed during the period 1–3 months after ovariectomy using EAS-1000.^{18,19} It was known that the IFN- γ production *via* IL-18 caused the lens epithelial cell damage.³¹ On the other hand, Harocopos *et al.* reported that a loss of cells from the lens epithelium, by apoptosis or any other mechanism of cell death, did not seem to play a major role in lens opacification.³¹ In addition, the elevated Ca^{2+} content in lenses cause the degradation of crystallin proteins and lens opacification.^{3,4} Therefore, powerful stimulation or damage by multiple factors (some combination of IL-18 and other factors, such as reactive oxygen species) may be needed to induce the dysfunction of Ca^{2+} regulation and onset of cataracts. Further studies are needed to elucidate

the relationship between IL-18 expression and the development of cataracts in postmenopausal women. In addition, it is important to clarify the mechanism 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,³² on the Ca^{2+} regulation in the lenses of OVX rats. These results support previous studies that have assessed lens opacification in postmenopausal women.^{5–8,33}

Conflict of Interest The authors declare no conflict of interest.

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