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Adverse effects of excessive nitric oxide on cytochrome *c* oxidase in lenses of hereditary cataract UPL rats

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Abstract

The UPL rat is a newly developed hereditary cataract model. We previously found that the ATP content in UPL rat lenses decreases during cataract development, and the decrease in ATP content causes Ca^{2+}-ATPase dysfunction resulting in an elevation in Ca^{2+} and cataract development. In addition, we reported that the oral administration of disulfiram and aminoguanidine ameliorates the decrease in ATP content and the elevation in Ca^{2+} content in UPL rat lenses. In this study, we demonstrate the effect of nitric oxide (NO) on the expression and activity of cytochrome *c* oxidase (CCO) in normal and UPL rat lenses during cataract development. We also determined the effects of the oral administration of disulfiram and aminoguanidine on the mRNA expression and activity of CCO and NO production in UPL rat lenses. The expression of CCO-1 mRNA in UPL rat lenses, determined by a quantitative real-time RT-PCR method, decreased during cataract development. CCO activity in UPL rat lenses also decreased with aging. On the other hand, the oral administration of disulfiram and aminoguanidine attenuated the decrease in CCO-1 mRNA expression and CCO activity. These results suggest that excessive NO causes the decrease in CCO-1 mRNA expression and CCO activity, and that the decrease in CCO may cause the decrease in ATP production in UPL rat lenses. Disulfiram and aminoguanidine may attenuate the decrease in ATP production, resulting in a delay in cataract development.

Keywords: Cataract; Cytochrome *c* oxidase; Nitric oxide; Anti-cataract drugs; Calcium

1. Introduction

Any alteration in the optical homogeneity of the lens or decrease in its transparency is known as a cataract (Iwata, 1986; Ye and Zadunaisky, 1992), and numerous factors have been implicated in its etiology, including genetic factors, diabetes, smoking, nutrition, the cumulative effect of X-rays, ultraviolet irradiation, and alterations in both endocrine and enzymatic equilibria (Sallman and Locke, 1951; Garland, 1990; Rasi et al., 1992; Ye and Zadunaisky, 1992; Cekic and Bardak, 1998; Dilsiz et al., 2000). Currently, reactive oxygen species, induced by UV rays in sunlight, are considered to be important in perturbing lens homeostasis. Therefore, exposure to reactive oxygen species results in a breakdown of lens homeostasis, and the Ca^{2+} content in the lens becomes elevated. The elevated Ca^{2+} content in the lens has been deduced to activate calpain, a Ca^{2+}-dependent protease. Furthermore, the degradation of lens proteins, such as crystallin proteins, would result in an opaque lens (Shearer et al., 1992; Spector, 1995; Dilsiz

Abbreviations: CCO, cytochrome *c* oxidase; GAPDH, glyceraldehyde-3-phosophate dehydrogenase; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor kappa B.

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et al., 2000). Although, there have been many studies exploring the mechanisms of cataract development, due to uncertainties regarding efficacy and safety, a potent anti-cataract drug for human cataracts has not yet been introduced. Therefore, the development of effective and safe anti-cataract drugs is needed.

In studies aimed at the development of anti-cataract drugs, the selection of the experimental animal is very important. The UPL rat is a dominant hereditary cataract model derived from Sprague–Dawley rats (Tomohiro et al., 1993). Opacification of the lenses of UPL rats starts at 35–42 days of age, and by 50 days of age, the lenses are almost entirely opaque. The incidence of cataract in adult UPL rats is 100% (Tomohiro et al., 1993, 1996, 1997; Nabekura et al., 2001). Previous investigations have revealed that oxidized glutathione concentrations in the lenses of UPL rats are increased, and that reduced glutathione values are decreased (Nabekura et al., 2003). The proteolyses of some crystallins and cytoskeletal proteins are enhanced in the lenses of UPL rats (Tomohiro et al., 1996, 1997). Ca^{2+} content in the lenses of UPL rats rise markedly with aging in comparison with normal rats, and the autolytic product of calpain is also detected in the lenses of UPL rats (Tomohiro et al., 1997). Some changes in the biological characteristics of UPL rat lenses may correspond to those of human cataracts. Therefore, UPL rats may provide a useful model for studies on the mechanism of cataract development and the development of anti-cataract drugs.

Disulfiram, a dimer of diethyldithiocarbamate, has long been used to treat alcoholic syndrome without severe side effects (Rall, 1990). Disulfiram is an inhibitor of nuclear factor kappa B (NFκB, Schreck et al., 1992; Mulsch et al., 1993), and is a powerful antioxidant that scavenges reactive oxygen species (Ito et al., 1999). We previously reported that the oral administration of disulfiram and aminoguanidine, a selective inhibitor of inducible nitric oxide synthase (iNOS, Holstad et al., 1996), produces anti-cataract activity in UPL rats (Nabekura et al., 2003). We also showed that the ATP content in UPL rat lenses decrease during cataract development, and the decrease in ATP content causes Ca^{2+}-ATPase dysfunction resulting in an elevation in Ca^{2+} (Nabekura et al., 2004). The oral administration of disulfiram and aminoguanidine can prevent the decrease in the ATP content and the elevation in Ca^{2+} content in UPL rat lenses (Nabekura et al., 2004). Therefore, it is very important to elucidate the precise mechanisms of the decrease in ATP during cataract development. It is known that cytochrome *c* oxidase (CCO) in the mitochondrial respiratory pathway plays an important role in ATP production. In the present study, we determined the expression and activity of CCO in lenses of UPL rat during cataract development. Moreover, the preventive effects of disulfiram and aminoguanidine on the decrease in CCO activity were examined in the lenses of UPL rats.

2. Materials and methods

2.1. Animals and materials

The rats used were normal and late onset type UPL rats aged 15–53 days. They were housed under standard conditions (12 h/day fluorescent light (07:00–19:00); 25 °C room temperature) and given a commercial diet (CE-2, Clea Japan Inc., Osaka, Japan) and water. The drugs used were disulfiram (Ouchi Shinko Chemical, Tokyo, Japan) and aminoguanidine (Sigma–Aldrich Japan, Tokyo, Japan). Disulfiram was suspended in saline solution containing 0.5% methylcellulose; aminoguanidine was dissolved in saline. Disulfiram (200 mg/kg body weight/day) and aminoguanidine (300 mg/kg body weight/day) were administered orally to the rats twice a day (9:00 am and 7:00 pm) beginning when the rats were 18 days of age. All animal experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research. The ATP Assay kit, Mitochondrial Isolation kit and Cytochrome *c* Oxidase Assay kit were obtained from Sigma–Aldrich Japan (Tokyo, Japan). The Ca test kit was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methylcellulose was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals used were of the highest purity available.

2.2. RNA preparation

Lenses were removed from normal and UPL rats at 25, 32, 39, 46 and 53 days of age, and snap-frozen in liquid nitrogen. Total RNA was prepared from each individual lens by the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) using Trizol reagent (Life Technologies Inc., Rockville, USA). The purity and concentrations of RNA were determined spectrophotometrically. The OD_{260}/OD_{280} values of all RNA used were greater than 1.8, which indicates low protein contamination and high purity RNA.

2.3. Quantitative real-time RT-PCR

The RT reaction was performed using an RNA PCR kit (AMV Version 2.1, Takara Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 μl of 10 mM Tris–HCl buffer (pH 8.3) containing 5 mM $MgCl_2$ and 50 mM KCl. The following components were then added to give a final volume of 10 μl: 1 unit/μl RNase inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 units/μl reverse transcriptase, and 0.125 μM oligo dT-adaptor primer. The RT reaction was performed at 42 °C for 15 min, followed by 5 min at 95 °C. The PCR reactions were performed using LightCycler FastStart

Table 1
Sequences of primers used for quantitative RT-PCR analysis

Primer		Sequence (5′-3′)	GenBank Accession No.
CCO-1	FOR	TCACAGTAGGGGGCCTAACA	X14848 (Mitochondrial genome)
	REV	GGCTTTTGCTCATGTGTCATT	
CCO-2	FOR	TGGCTTACAAGACGCTACATC	X14848 (Mitochondrial genome)
	REV	GGAGGGAAGGGCAATTAGAA	
CCO-3	FOR	TCTTCTTTGCCGGATTTTTC	X14848 (Mitochondrial genome)
	REV	ATGGTTTCGGTTGCCTTCTA	
iNOS	FOR	GGAGAGATTTTTCACGACACCC	NM_012611
	REV	CCATGCATAATTTGGACTTGCA	
GAPDH	FOR	ACGGCACAGTCAAGGCTGAGA	NM_017008
	REV	CGCTCCTGGAAGATGGTGAT	

DNA Master SYBR Green I according to the manufacturer's instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 μl of cDNA was mixed with 2 μl of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 μl containing specific primers for CCO-1, 2, 3 and iNOS, or glyceraldehyde-3-phosophate dehydrogenase (GAPDH, 10 pmol each). The primers used are summarized in Table 1, and the conditions of the PCR are shown in Table 2. 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). After completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect nonspecific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and other groups (CCO-1, 2, 3 and iNOS) were used to calculate the levels of mRNA expression in the UPL rats.

2.4. Assay of nitric oxide levels in UPL rat lens

Lenses from normal and UPL rats at 25, 32, 39, 46 and 53 days of age were homogenized in saline on ice. The lens homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used for measurements of nitric oxide (NO) levels. A concentric microdialysis probe (A-1-20-05: 5 mm length; Eicom Corp., Kyoto, Japan) was placed in the supernatant and perfused with Ringer's solution (140 mM NaCl, 4 mM KCl, 1.26 mM $CaCl_2$, and 1.15 mM $MgCl_2$, pH 7.4) at a constant flow rate of 2 μl/min using a microsyringe pump (ESP-64, Eicom Corp., Kyoto, Japan). NO_2^- and NO_3^- in the supernatant were separated on a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 mm × 50 mm, Eicom Corp., Kyoto, Japan), and NO_3^- was reduced to NO_2^- in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom Corp., Kyoto, Japan). NO_2^- was mixed with a Griess regent to form a purple azo dye in a reaction coil and placed in a column oven set at 35 °C. The absorbance of the color product dye at 540 nm was determined using a flow-through spectrophotometer (NOD-10, Eicom Corp., Kyoto, Japan). The mobile phase consisted of 10% methanol containing 0.15 M NaCl–NH_4Cl and 0.5 g/l EDTA-4Na, and was delivered by a pump at a rate of 0.33 ml/min. The Griess reagent (1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l *N*-naphthylethylenediamine) was delivered at a rate of 0.1 ml/min. In this study, NO levels are expressed as the level of the NO_2^- metabolite, which is the product of NO.

2.5. Measurement of CCO activity

CCO activity was determined using a Mitochondrial Isolation kit and Cytochrome *c* Oxidase Assay kit (Sigma–Aldrich Japan, Tokyo, Japan). Lenses from normal and UPL rats at 25,

Table 2
Parameters used for quantitative RT-PCR analysis

Primer	Hot start (10 min)	Denaturation (10 s)	Annealing (10 s)	Extension (5 s)
CCO-1	95 °C	95 °C	59 °C	72 °C
CCO-2	95 °C	95 °C	59 °C	72 °C
CCO-3	95 °C	95 °C	59 °C	72 °C
iNOS	95 °C	95 °C	60 °C	72 °C
GAPDH	95 °C	95 °C	60 °C	72 °C

32, 39, 46 and 53 days of age were used for measurements of CCO activity levels. Mitochondria were isolated from the lenses according to the following procedure. The lenses were homogenized individually in 1 ml of isolation buffer 1 (10 mM HEPES, 200 mM mannitol, 70 mM sucrose and 1 mM EDTA, pH 7.5) containing 2 mg/ml albumin. The lens homogenates were centrifuged at 2600 rpm for 5 min at 4 °C and the supernatants were centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatants were removed, and the pellets were each resuspended with 1 ml of isolation buffer 1. The samples were again centrifuged at 2600 rpm for 5 min at 4 °C, and the supernatants were centrifuged at 11,000 rpm for 10 min at 4 °C. The resulting pellets were vortexed with 40 μl of storage buffer (10 mM HEPES, 200 mM mannitol, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4 and 1 mM DTT, pH 7.4). Determination of cytochrome *c* oxidase activity was based on a colorimetric assay that quantifies the oxidation of ferrocytochrome *c* to ferricytochrome *c* *via* cytochrome *c* oxidase, a reaction that results in a decrease in absorbance at 550 nm. The decrease in absorbance at 550 nm was monitored by a spectrophometer (UV 2200, Shimadzu, Kyoto, Japan) calibrated to zero using assay buffer (10 mM Tris–HCl and 120 mM KCl, pH 7.0). In a cuvette, 0.95 ml of assay buffer was combined with 70 μl of enzyme buffer (10 mM Tris–HCl and 250 mM sucrose, pH 7.0) and 30 μl of isolated mitochondria. The reaction was initiated by the addition of 50 μl of ferrocytochrome *c* (reduced with 0.1 M dithiothreitol), and the decrease in absorbance at 550 nm was measured for 1 min. Activity was calculated based on the following equation. Units/ml = [(ΔAbs550/min for the sample − ΔAbs550/min for the blank) × dilution factor × total reaction volume]/[mitochondria isolate volume—the difference in extinction coefficients between ferro- and ferricytochrome *c* at 550 nm (21.84)]. One unit will oxidize 1 μM reduced cytochrome *c* per min at pH 7.0 and 25 °C.

2.6. Measurement of protein

Protein levels in the lenses of normal and UPL rats were determined according to the method of Bradford (1976) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

2.7. Measurement of ATP

Lenses from UPL rats at 25, 32, 39, 46 and 53 days of age were homogenized in 200 μl of 10 mM HEPES/KOH buffer (pH 7.4), and centrifuged at 15,000 rpm for 15 min at 4 °C. The ATP contents of the supernatants were determined using a Sigma ATP Bioluminescent Assay Kit (Sigma–Aldrich Japan, Tokyo, Japan) and a luminometer AB-2200 (Atto Corporation, Tokyo, Japan) according to the manufacturers' instructions.

2.8. Measurment of Ca^{2+} content

Lenses from UPL rats at 25, 32, 39, 46 and 53 days of age were homogenized in homogenized in phosphate-buffered saline (pH 7.4) on ice. The lens homogenates were centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatants were used for measurements of Ca^{2+} concentration. The Ca^{2+} concentrations of the lenses were determined by the methyl xylenol blue colorimetric method using the Ca test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and CL-770 (Shimadzu Corp., Kyoto, Japan).

2.9. Image analysis for cataract development

Experiments were performed according to the method of Ito et al. (1999). The pupils of rats were dilated by the instillation of 0.1% pivalephrine (Santen Pharmaceutical Co., Osaka, Japan) without anesthesia. Changes in the transparency of the lenses were monitored using an EAS-1000 equipped with a CCD camera (Nidek, Aichi, Japan). The outline of the lens image was determined by selecting four points on the image, and then the transparent area within the outline and thread level were set automatically by the software. The total area of opacity of the lenses, expressed as pixels, was calculated by the following equation.Pixels within opacity (Pixel) = pixels within outline − pixels within transparent area

2.10. Statistical analysis

All data are expressed as the mean ± standard error (S.E.) of the mean. Unpaired Student's or Aspin–Welch's *t*-test was used to evaluate statistical difference, and multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. *P* values less than 0.05 were considered significant.

3. Results

3.1. Changes in NO levels in UPL rat lenses during cataract development

Fig. 1 shows iNOS mRNA expression in the lenses of 25- to 53-day-old normal and UPL rats as deter-

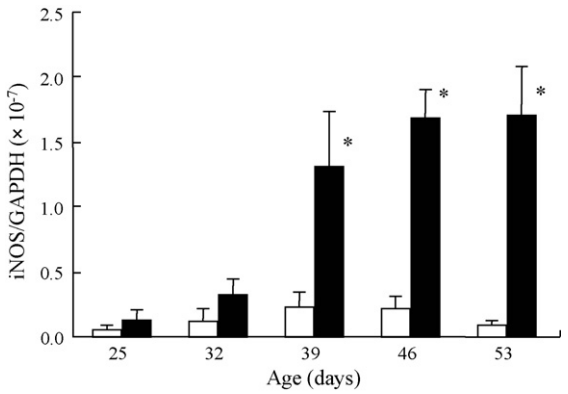

Fig. 1. Expression of iNOS mRNA in the lenses of 25- to 53-day-old normal and UPL rats. iNOS mRNA expression was determined using the quantitative real-time RT-PCR method. Open columns, normal rat; closed columns, UPL rat. The data are presented as means ± S.E. of 7–10 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

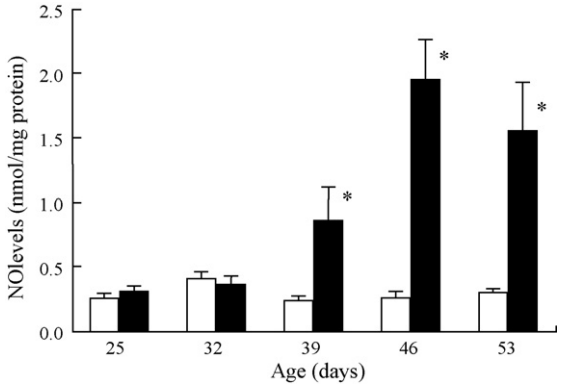

Fig. 2. NO levels in the lenses of 25- to 53-day-old normal and UPL rats. NO levels were measured by the microdialysis method. Open columns, normal rat; closed columns, UPL rat. The data are presented as means ± S.E. of 4–7 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

mined by the quantitative real-time PCR method. The expression of iNOS mRNA did not change with aging in normal rats. In contrast to the results in normal rats, iNOS mRNA expression in UPL rat lenses increased with aging. Although the lenses of 39-day-old UPL rats were transparent, iNOS mRNA expression was elevated. Fig. 2 shows NO levels in the lenses of 25- to 53-day-old normal and UPL rats. No changes in the NO levels in lenses of 25- to 53-day-old normal rats were observed. On the other hand, the NO levels in UPL rat lenses increased with aging, and the increase in NO levels in lenses of 39-day-old UPL rats was about 3.6-fold compared with normal rat lenses.

3.2. Changes in CCO mRNA expression and activity in UPL rat lenses during cataract development

Fig. 3 shows CCO-1, 2 and 3 mRNA (mitochondrial subunits) expression in the lenses of 25- to 53-day-old normal and UPL rats as determined by the quantitative real-time PCR method. The expressions of CCO-1, 2 and 3 mRNAs did not change with aging in normal rats. The expression of CCO-2 and -3 mRNAs in the lenses of 25- to 53-day-old UPL rats were lower than in normal rats. On the other hand, CCO-1 mRNA expression in the lenses of 25-day-old UPL rats was similar to that of normal rats. The expression of CCO-1 mRNA in UPL rat lenses decreased with aging, and CCO-1 mRNA expression in the lenses of 39-day-old UPL rats was approximately 60% that of normal rat lenses. Fig. 4 shows CCO activity in the lenses of 25- to 53-day-old normal and UPL rats. CCO activity in normal rats did not change with aging. CCO activity in the lenses of 25-day-old UPL rats was similar to that of normal rats.

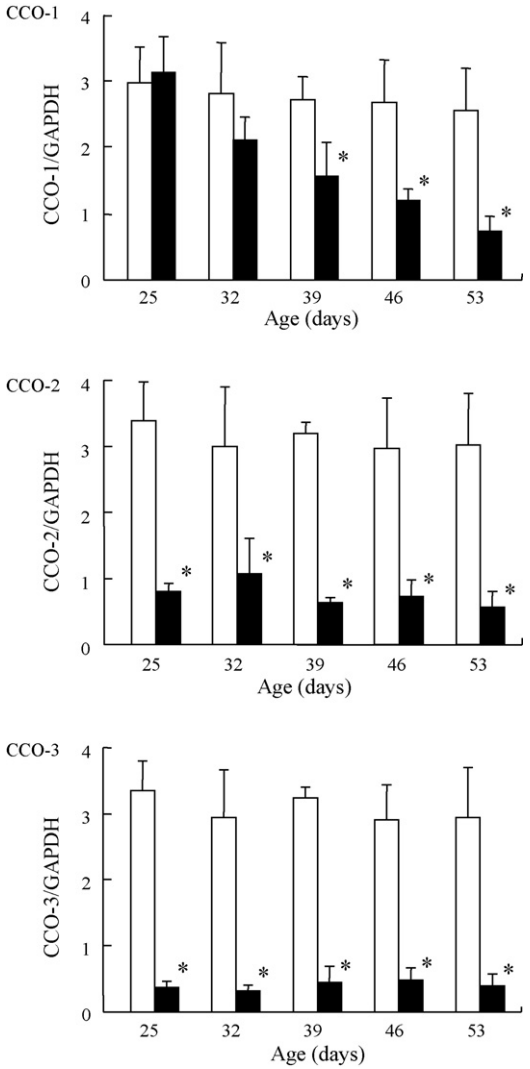

Fig. 3. Expression of CCO-1, 2 and 3 mRNAs in the lenses of 25- to 53-day-old normal and UPL rats. The expressions of the CCO-1, 2 and 3 mRNAs were determined using the quantitative real-time RT-PCR method. Open columns, normal rat; closed columns, UPL rat. The data are presented as means ± S.E. of 4–5 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

On the other hand, the CCO activity in UPL rat lenses decreased with aging, with the activity in the lenses of 39-day-old UPL rats significantly lower than that in normal rat lenses.

3.3. Effect of disulfiram and aminoguanidine on NO and CCO in UPL rat

Fig. 5 shows the effects of disulfiram and aminoguanidine on iNOS mRNA expression (A) and NO levels (B) in the lenses of 46-day-old normal and UPL rats. Both disulfiram and aminoguanidine significantly suppressed

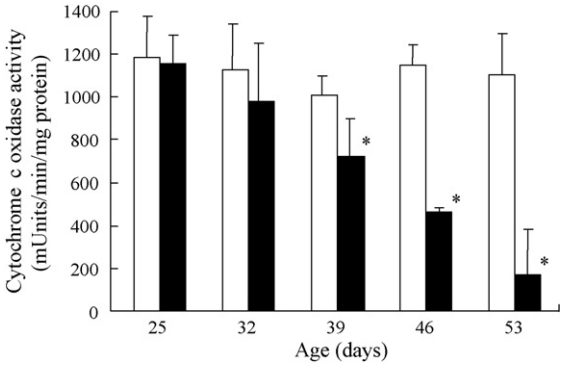

Fig. 4. CCO activity in the lenses of 25- to 53-day-old normal and UPL rats. CCO activity was determined by the colorimetric method. Open columns, normal rat; closed columns, UPL rat. The data are presented as means ± S.E. of 4–10 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

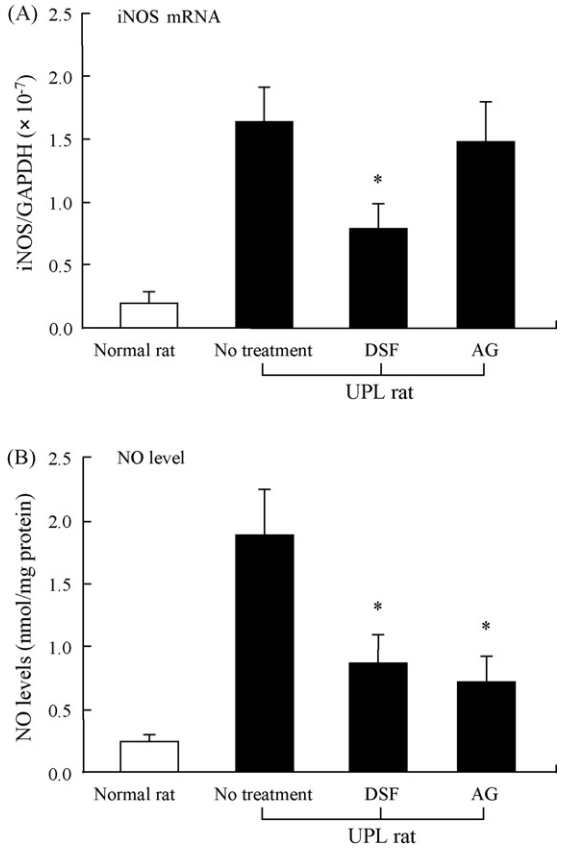

Fig. 5. Effects of disulfiram and aminoguanidine on iNOS mRNA expression (A) and NO levels (B) in the lenses of 46-day-old UPL rats. The expression of the iNOS mRNA was determined using the quantitative real-time RT-PCR method. The NO level was measured by the microdialysis method. Open columns, normal rat; closed columns, UPL rat. Normal, normal rats; UPL, UPL rats; DSF, disulfiram (200 mg/kg/day) administered UPL rats; AG, aminoguanidine (300 mg/kg/day) administered UPL rats. The data are presented as means ± S.E. of 5–10 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

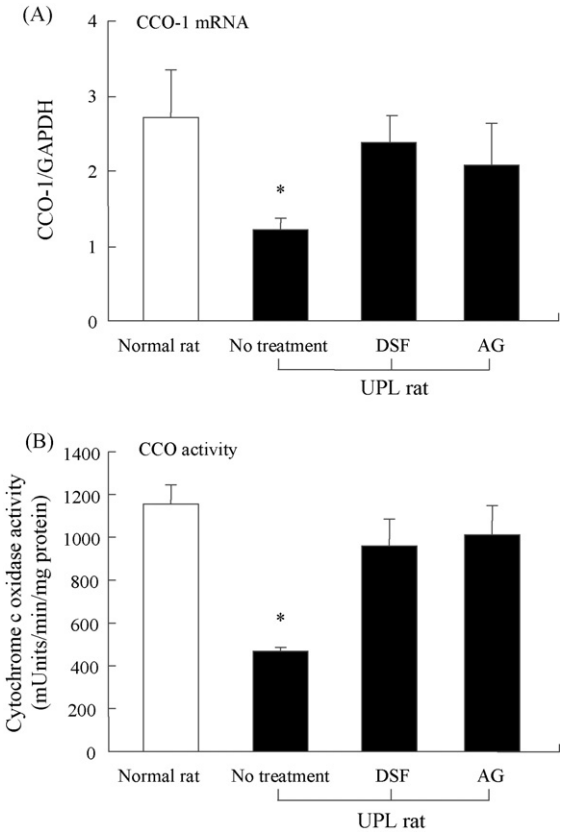

Fig. 6. Effects of disulfiram and aminoguanidine on CCO-1 mRNA expression (A) and CCO activity (B) in the lenses of 46-day-old UPL rats. The expression of the CCO-1 mRNA was determined using the quantitative real-time RT-PCR method. The CCO activity was determined by the colorimetric method. Open columns, normal rat; closed columns, UPL rat. Normal, normal rats; UPL, UPL rats; DSF, disulfiram (200 mg/kg/day) administered UPL rats; AG, aminoguanidine (300 mg/kg/day) administered UPL rats. The data are presented as means ± S.E. of 4–7 independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

the increase in NO levels in the lenses of 46-day-old UPL rats. In addition, the administration of disulfiram also attenuated the increase in iNOS mRNA expression in the lenses of 46-day-old UPL rats. Fig. 6 shows the effects of disulfiram and aminoguanidine on CCO-1 mRNA expression (A) and CCO activity (B) in the lenses of 46-day-old normal and UPL rats. The decrease in CCO-1 mRNA and CCO activity in the lenses of 46-day-old UPL rats was also prevented by the oral administration of disulfiram and aminoguanidine.

4. Discussion

We previously reported that the elevation in Ca^{2+} levels and the decrease in ATP contents in UPL rat lenses take place during cataract development, and that these

changes can be prevented by the oral administration of iNOS inhibitors (Nabekura et al., 2003, 2004). The results suggested that the induction of iNOS is related to cataract development in UPL rats. In the present study, we investigated the changes in iNOS gene expression and NO levels in UPL rat lenses, and clarified the mechanisms of the decrease in ATP content in UPL rat lenses during cataract development. Moreover, the preventive effects of disulfiram and aminoguanidine on the decrease in CCO activity were examined in the lenses of UPL rats.

NO is synthesized from the guanidino-nitrogen of L-arginine and molecular oxygen by NOS. iNOS, one of the three isoforms of NOS (Baek et al., 1993), is usually not present under normal conditions, is Ca^{2+} independent, and is induced by cytokines, such as interferon-γ and tumor necrosis factor α. The induction of iNOS results in the sustained and unregulated release of excessive amounts of NO, which is cytotoxic to neighboring cells (Yun et al., 1997; Nathan and Xie, 1994). Inomata et al. (2001) reported that the induction of iNOS mRNA and protein occur prior to the elevation of Ca^{2+} content in Shumiya cataract rat lenses. We also show that the iNOS mRNA expression and NO levels are increased in UPL rat lenses, and that the increases in iNOS mRNA expression and NO levels are observed prior to the elevation in Ca^{2+} content and lens opacity in UPL rats. On the other hand, both disulfiram and aminoguanidine suppressed the increase in NO levels in the UPL rat lenses, and disulfiram, which is an inhibitor of NFκB (Schreck et al., 1992; Mulsch et al., 1993), also attenuated the increase in the iNOS mRNA expression in UPL rat lenses. These results suggest that the increase in iNOS mRNA expression and NO levels in the UPL rat lenses may be caused by NFκB activation.

CCO, the terminal enzyme of the mitochondrial respiratory chain, reduces oxygen to water and pumps protons across the inner mitochondrial membrane (Liang et al., 2006). It is an important energy-generating enzyme and plays an important role in ATP production. CCO contains 13 subunits per monomer. Its largest three catalytic subunits (1, 2 and 3) are encoded in the mitochondrial genome, while the other 10 subunits (4, 5a, b, 6a, b, c, 7a, b, c and 8) are encoded in the nuclear DNA, synthesized on cytoplasmic ribosomes (Kadenbach et al., 1983; Kuhn-Nentwig and Kadenbach, 1985), and imported into the mitochondria (Slonimski and Tzagoloff, 1976; Lewin et al., 1980; George-Nascimento and Poyton, 1981; Capaldi, 1990a,b; Taanman, 1997; Lenka et al., 1998). Recently, Liang et al. (2006) reported that in response to functional inactivaton, the three mitochondrial subunits are down-regulated earlier and more severely than the nuclear subunits. Therefore, we investigated the effect of NO on the expression of the CCO-1, 2 and 3 mRNAs as well as CCO activity in UPL rat lenses. The expression levels of the CCO-2 and -3 mRNAs in the lenses of 25- to 53-day-old UPL rats were significantly lower than in normal rats. On the other hand, the expression of the CCO-1 mRNA in the lenses of 25-day-old UPL rats was similar to that in normal rats. However, the expression of the CCO-1 mRNA in the lenses of UPL rats decreased with aging, and CCO-1 mRNA expression was significantly lower at 39 days of age in UPL rats than in normal rats. CCO activity in the lenses of UPL rat also decreased with aging, and was significantly lower at 39 days of age as compared with normal rats. These decreases in CCO-1 mRNA expression and CCO activity were observed prior to lens opacification, and were prevented by the oral administration of disulfiram and aminoguanidine. Fariss et al. (2005) reported that reactive oxygen species cause damage to the mitochondrial genome. The present report also shows that the oral administration of disulfiram and aminoguanidine attenuates the decrease in CCO-1 mRNA expression in UPL rat lenses. Therefore, the excessive NO production in UPL rat lenses may cause mitochondrial genome damage resulting in a decrease in ATP content. However, the ATP content of UPL rat lenses decreases rapidly with aging, and the ATP content in the lenses of 39-day-old UPL rats is approximately 10% that found in 25-day-old UPL rats (Fig. 7). NO is known to bind CCO, and it has

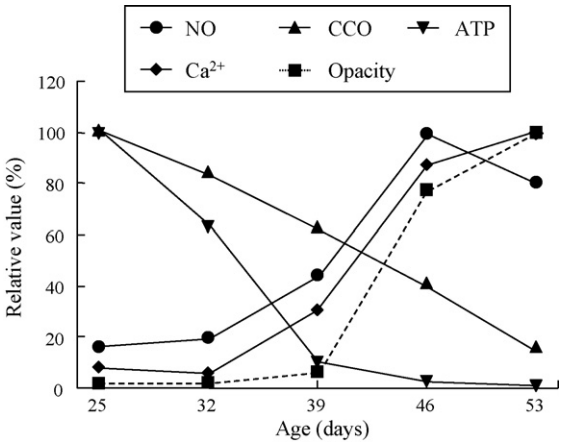

Fig. 7. Temporal relationships of NO level, CCO activity, ATP content, calcium content, and lens opacity during cataract development in UPL rat. NO levels (●) in UPL rat lens were measured by the microdialysis method. CCO activity (▲) in UPL rat lens was determined by the colorimetric method. ATP content (▼) in UPL rat lens was measured by the luciferin-luciferase bioluminescent method. Calcium content (♦) was measured by the methyl xylenol blue colorimetric method. Lens opacity (■) was analyzed using computerized image analysis software connected to the EAS-1000. For each parameter, the highest value attained was taken as 100%.

been reported that NO inhibits CCO function potently and reversibly by reducing the affinity of the enzyme for oxygen (Moncada and Erusalimsky, 2002). In addition, we reported that Ca^{2+}-ATPase gene expression and activity, which play a central role in Ca^{2+} transport and the maintenance of low internal Ca^{2+} concentrations using, increase in UPL rat lenses with aging (Nabekura et al., 2004). Therefore, CCO dysfunction by reducing the affinity of the enzyme for oxygen and high consumption of ATP also may relate to the decrease in the ATP content of UPL rat lenses. Taken together, we hypothesize that in the UPL rat lenses, iNOS induction *via* NFκB is first produced, leading to the production of NO; then, excessive NO may cause mitochondrial genome damage and CCO dysfunction resulting in a decrease in ATP production. The decrease in ATP content may cause the decrease in Ca^{2+}-ATPase function, resulting in the elevation in lens Ca^{2+}, which leads finally to lens opacification. Disulfiram and aminoguanidine inhibit iNOS induction and scavenge reactive oxygen species, thus attenuating damage to the mitochondrial genome and ameliorating the dysfunction in ATP production. Attenuating the decrease in Ca^{2+}-ATPase function may prevent the elevation in Ca^{2+}, resulting in a delay in cataract development in UPL rats.

In conclusion, the present study demonstrates that iNOS induction *via* NFκB produces excessive NO, and that this excessive NO causes the decrease in CCO-1 mRNA expression and CCO activity in UPL rat lenses during cataract development. Disulfiram and aminoguanidine have the ability to attenuate the decrease in CCO-1 mRNA expression and CCO activity, resulting in a delay in cataract development. These findings provide significant information that can be used to design further studies aimed at developing anti-cataract drugs.

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