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# Aminoguanidine-Treatment Results in the Inhibition of Lens Opacification and Calpain-Mediated Proteolysis in Shumiya Cataract Rats (SCR)[1]

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**The Shumiya cataract rat (SCR) is a hereditary cataract model in which lens opacity appears spontaneously in the nuclear and perinuclear portions at 11–12 weeks of age. We found incidentally that the oral administration of aminoguanidine (AG), an inhibitor of inducible nitric oxide synthase (iNOS), strongly inhibits the development of lens opacification in SCR. Since our previous results strongly suggested that calpain-mediated proteolysis contributes to lens opacification during cataract formation in SCR, we examined the calpain-mediated proteolysis in AG-treated SCR lenses in detail. The results show that the calpain-mediated limited proteolysis of crystallins is also inhibited by AG-treatment. However, the administration of AG has no effect on the substrate susceptibility to calpain. On the other hand, the autolytic activation of calpain in AG-treated lenses is strongly inhibited, although AG itself does not inhibit calpain activity *in vitro*. Then, we analyzed the effect of AG-treatment on calcium concentrations in lens, and found that the elevation in calcium concentration that should occur prior to cataractogenesis in lenses is strongly suppressed by AG-treatment. These results strengthen our previous conclusion that calpain-mediated proteolysis plays a critical role in the development of lens opacification in SCR. Moreover, our results indicate that the inhibition of calpain-mediated proteolysis by AG-treatment is due to the suppression of calcium ion influx into the lens cells.**

**Key words: aminoguanidine, calpain, crystallin, hereditary cataract, inducible nitric oxide synthase.**

Calpain is an intracellular cysteine proteinase distributed in various tissues and cells. There are two ubiquitous calpain isozymes, μ- and m-calpains, active at μM and mM $Ca^{2+}$ concentrations, respectively. Both calpains are composed of a large catalytic (80 kDa) and a small regulatory (30 kDa) subunit and each subunit contains a calmodulin-like domain at the C-terminus. Upon $Ca^{2+}$ binding to the calmodulin-like domains, calpains become active and begin to autolyze (*1–4*). In the case of μ-calpain, it is believed that the restricted autolysis in the large subunit that accompanies the increase in calcium sensitivity is itself the activation step (*5, 6*). On the other hand, it has been reported that the large subunit of mammalian m-calpain does not undergo autolysis within the time span of substrate proteolysis (*7*). However, even in the case of m-calpain, activated calpains are degraded autolytically into smaller fragments when their substrate proteins are exhausted, and ultimately lose their proteolytic activity. Thus, the disappearance of intact m-calpain and the generation of its fragments can be evidence of m-calpain activation. Calpain is a major proteinase in lens and m-calpain is the predominant calpain species in rat lens (*8*). Although the exact physiological function of calpain has not yet been established, it has been suggested to play important roles in cellular functions that occur in response to mobilized calcium ions. It seems likely that the enzyme is involved not only in physiological events but also in various pathological states, including the development of cataracts (*1–4, 9*).

The Shumiya cataract rat (SCR) is a new hereditary cataractous rat strain, obtained by cross-breeding a spontaneous hypertensive rat and a Zucker fatty rat. Lens opacity in SCR appears spontaneously in the perinuclear and nuclear portions at 11–12 weeks of age in 2/3 of animals (*10, 11*). Our previous studies indicated that calpain participates in the proteolytic modification of several proteins in cataractous SCR lenses, and strongly suggested that calpain-mediated proteolysis contributes to the development of lens opacification through the insolubilization of lens proteins (*12*).

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Abbreviations: E-64c, L-*trans*-epoxysuccinyl-leucylamido (3-methyl) butane; PVDF, polyvinylidene difluoride.

Recently, we found incidentally that the oral administration of aminoguanidine (AG), an inhibitor of inducible nitric oxide synthase (iNOS), strongly suppresses lens opacification during cataractogenesis in SCR. In the present study, therefore, we examined calpain-mediated proteolysis in AG-treated SCR lenses based on our previous results, as a first step to clarify the mode of AG action. The results show that the autolytic activation of calpain and the calpain-mediated limited proteolysis of crystallins in cataractous lenses are strongly inhibited by AG-treatment. Moreover, our results demonstrate that the elevation in calcium concentration that occurs in cataractous lenses is also suppressed by AG-treatment. The mechanism of calcium ion influx into the lens cells is also discussed.

## MATERIALS AND METHODS

*Reagents*—m-Calpain was purified from bovine lung as previously described (*13*). Aminoguanidine sulfate was purchased from Wako Chemicals. Keyhole limpet hemocyanin (maleimide-activated) was obtained from Boehringer Mannheim. EDTA and diaminobenzidine were purchased from Dojindo Laboratories. E-64c was a kind gift from Taisho Pharmaceutical. PVDF membranes were obtained from Bio-Rad. Peroxidase-conjugated anti-mouse IgG (Fab)′ and anti–rabbit IgG were purchased from Medical & Biological Labs and Seikagaku, respectively. The Western blotting detection system (ECL Plus) was a product of Amersham Pharmacia Biotech. Other chemicals were obtained from Nacalai Tesque and Wako Chemicals.

*Animals*—SCR strain rats were maintained in this institute. Lens opacity in SCR appears at 11–12 weeks of age in exactly 2/3 of animals; the remainder have normal clear lenses. In these experiments, therefore, the rats were divided into two groups, *i.e.*, normal (clear) and cataractous (opaque) rats. The judgment as to whether individual rats would be opaque or clear was based on observation with an Anterior Eye Segment Analysis System (EAS-1000, Nidek, Aichi) at 6 weeks of age. Both groups of rats were treated with aminoguanidine (AG) orally (150 mg/kg, twice per day) from 5 to 15 weeks of age. Controls were age-matched rats without AG-treatment. Daily oral administration of AG caused no apparent side effects in normal and cataractous rats up to 15 weeks of age.

*Image Analysis for Cataract Development*—The pupils of SCR were dilated with 0.1% pivalephrine (Santen Pharmaceutical, Osaka) without anesthesia 5 min before taking slit images with an EAS-1000. The lens images were obtained using an EAS-1000 equipped with a CCD camera. The area of opacity, in pixels, was analyzed using computerized image analysis software connected to the EAS-1000 as described previously (*14*).

*Antibodies*—A monoclonal antibody against the large subunit of calpain ($1D_{10}A_7$) was isolated as described before (*15*). Polyclonal antibodies against the native forms and calpain-generated fragments of crystallins (αA-, αB-, and βB1-) were produced by immunizing rabbits with synthetic peptide-carrier protein (KLH, keyhole limpet hemocyanin) conjugates (*12*). Polyclonal anti-iNOS antibody (NOS2, M-19) was obtained from Santa Cruz Biotechnology.

*Preparations of Water-Soluble and Water-Insoluble Lens Proteins*—AG-treated and non-treated SCR were sacrificed by an overdose of pentobarbital and the eyes were enucleated. The decapsulated lenses from each age group were homogenized in 10 volumes (v/w) of 20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A) using a glass-glass Dounce homogenizer. The homogenates were centrifuged at 10,000 ×*g* for 15 min at 4°C and the recovered supernatants were designated as the water-soluble fraction (WSF). The insoluble pellets were washed three times with buffer A and the resulting pellets were resuspended in buffer A to the original volume of the whole homogenate and designated as the water-insoluble fraction (WIF).

*Western Blot Analysis*—SDS-PAGE was carried out by the method of Laemmli (*16*) in 13.5% polyacrylamide slab gels. The proteins in the gels were transferred onto PVDF membranes and the blotted proteins were incubated with first antibodies followed by incubation with peroxidase-conjugated anti–mouse IgG (for m-calpain) or anti–rabbit IgG (for native and calpain-generated fragments of crystallins, and for iNOS) as the second antibody. The antigens were visualized using diaminobenzidine as the substrate except for m-calpain and iNOS. m-Calpain and iNOS antigens were detected by enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech). The amounts of iNOS protein were determined by densitometric scanning, Shimadzu CS-9000 (Kyoto).

*Assay of Calpain Activity*—Calpain activity was measured using alkali-denatured casein as a substrate according to the procedure described previously (*13*).

*Calcium Analysis*—The calcium contents were analyzed by the methods of Bunce *et al.* (*17*) with minor modification. The lenses were dried in a vacuum at 100°C for 8 h and then digested in 60% nitric acid (100 μl/lens) at 80°C. One milliliter of distilled water was added to the digests and the tubes were centrifuged at 300 ×*g* for 10 min. One-tenth milliliter of 100 μg/ml lanthanum chloride was added to the supernatant fraction. The calcium contents in the supernatant fractions were determined by atomic absorption spectrophotometry, Shimadzu AA-6400.

## RESULTS

*Inhibition of Lens Opacification by Oral Administration of Aminoguanidine to SCR*—To examine the effect of aminoguanidine (AG) on lens opacification, SCR were treated orally with AG. In the case of non-treated cataractous rats, lens opacity appeared first in the perinuclear region of the lens at 11 weeks of age and spread to the entire nuclear region of the lens with age (Fig. 1). In AG-treated cataractous lenses, however, lens opacification was strongly inhibited, although slight opacity was observed at 12 and 13 weeks of age. These results indicate that AG acts to prevent lens opacification during cataractogenesis in SCR.

*Proteolysis of Lens Proteins in AG-Treated and Non-Treated SCR*—Figure 2 shows the electrophoretic patterns of the water-soluble and water-insoluble lens proteins from 15-week old AG-treated and non-treated SCR. As previously reported (*12*), the proteolyses of several proteins are enhanced in cataractous SCR lens. Namely, in the water-soluble fraction from non-treated cataractous lens (C-NT), the intensities of the 32, 24.5, 23.5, and 22 kDa lens crystallin bands are significantly lower than in normal control lenses (N-NT), and new polypeptide bands at 26, 21.5, 20.5 kDa accumulate. These changes observed in non-treated

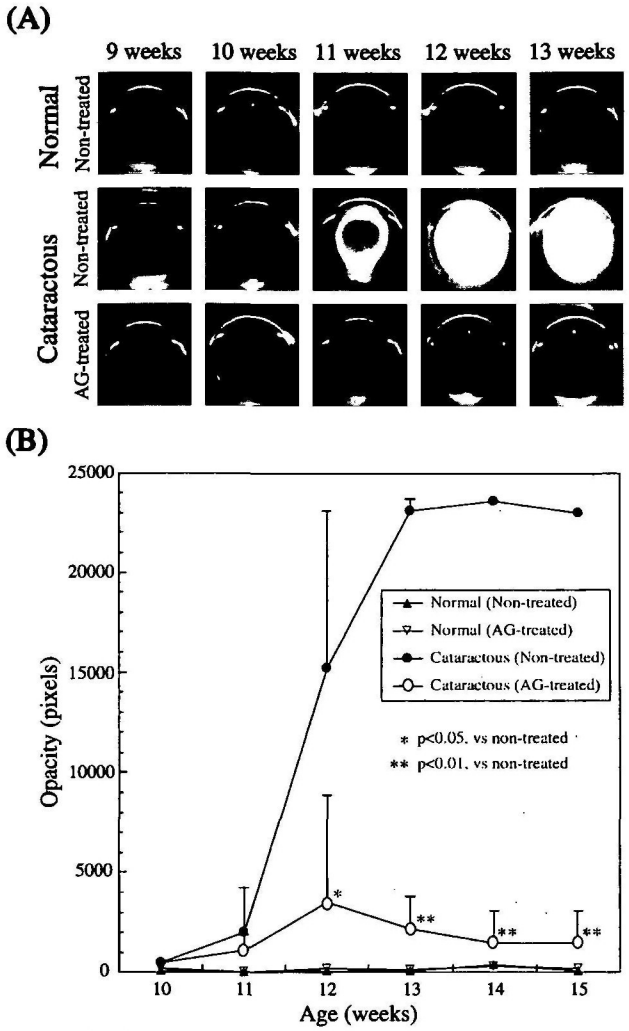



Fig. 1. **Effect of the oral administration of aminoguanidine on lens opacification in SCR.** (A) Lens opacification was estimated by observation with an Anterior Eye Segment Analysis System. (B) The area of opacity, in pixels, was analyzed using computerized image analysis software. Data are means ± SE ($n$ = 4).

cataractous lens were strongly inhibited by the oral administration of AG (C-AG).

*Calpain-Mediated α-Crystallin Proteolysis in AG-Treated and Non-Treated SCR Lens*—Since our previous results strongly suggest that the calpain-mediated proteolysis of lens proteins is involved in lens opacification, we examined the calpain-mediated proteolysis of some crystallins in AG-treated and non-treated SCR lens. Calpain-mediated proteolysis was analyzed by Western blotting using specific antibodies against calpain-generated fragments of αA- and αB-crystallins. As shown in Fig. 3, antibody-reactive fragments were detected predominantly in the water-soluble and water-insoluble fractions of non-treated cataractous lens (C-NT). However, the generation of these fragments was strongly inhibited in AG-treated lens (C-AG). The oral administration of AG also inhibited the generation of calpain-mediated fragments of βB1-crystallin (data not shown). These results clearly demonstrate that the calpain-mediated limited proteolysis in cataractous lens is blocked by AG-treatment.

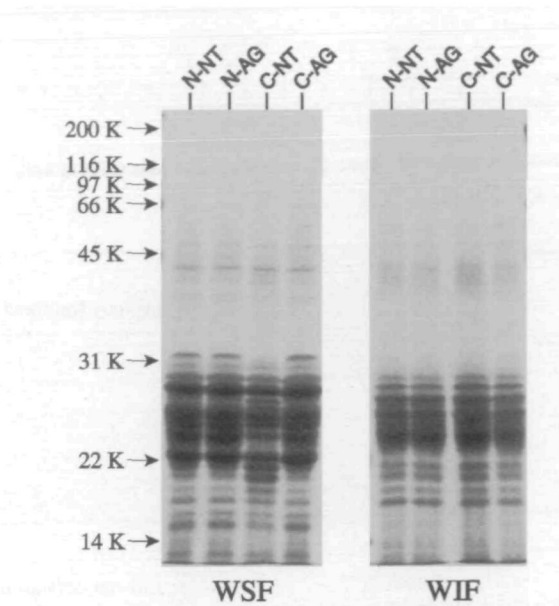
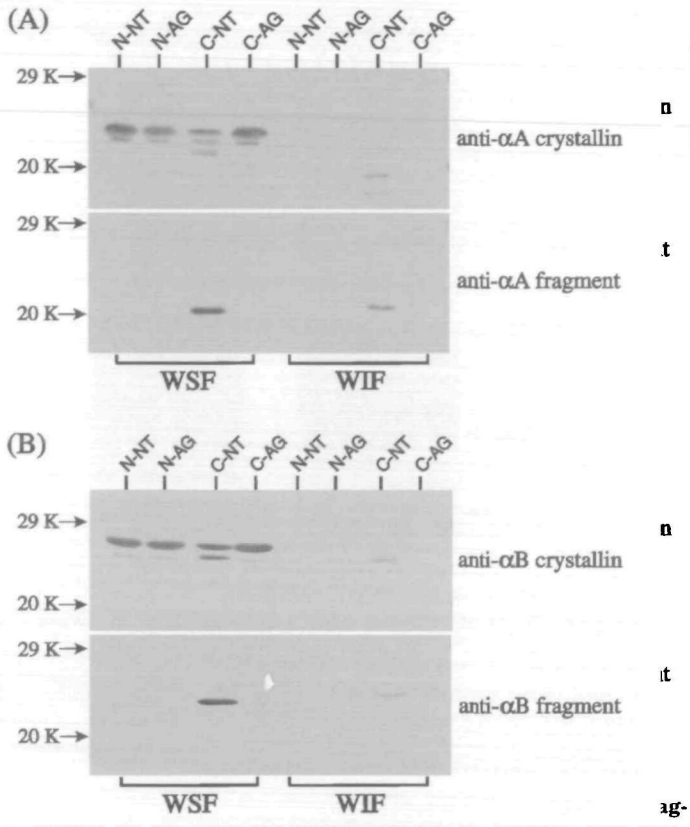



Fig. 2. **proteins from AG-treated and non-treated SCR lenses.** The water-soluble (WSF) and water-insoluble fractions (WIF) prepared from 15-week-old AG-treated and non-treated SCR lenses (equal in volume) were electrophoresed in 13.5% SDS–polyacrylamide gels and stained with Coomassie Brilliant Blue. N-NT, non-treated normal lens; N-AG, AG-treated normal lens; C-NT, non-treated cataractous lens; C-AG, AG-treated cataractous lens.

*Effect of AG on Calpain Activity and Its Substrate Proteins*—A few possible explanations are considered for the inhibition of calpain-mediated proteolysis by AG-treatment. One is that AG, which is incorporated into the lens cells, interacts directly with calpain and inhibits its activity. Another possibility is that AG decreases the susceptibility of substrate proteins to calpain as a chemical modifier. In order to explore the former possibility, we examined the effect of AG on the activity of m-calpain *in vitro*. As shown in Fig. 4, however, AG at concentrations up to 10 mM does not inhibit the m-calpain activity at all. To examine the latter possibility, we compared the susceptibility of αA-crystallin in AG-treated cataractous and non-treated normal lenses to calpain (Fig. 5). No appreciable difference in calpain susceptibility was observed between the two preparations. We obtained almost the same results for αB- and βB1-crystallins (data not shown). From these results, the possibilities that the interruption of calpain-mediated proteolysis by AG-treatment is due to the inhibition of calpain activity and/or a decrease in the susceptibility of substrates to calpain can be excluded.

*Effect of AG-Treatment on the Autolytic Degradation of m-Calpain*—Next, we examined the autolytic degradation of m-calpain in AG-treated and non-treated SCR lenses (Fig. 6). As described in the Introduction, activated calpain undergoes autolytic degradation to smaller fragments. Therefore, whether or not m-calpain is activated in tissues can be evaluated by the detection of autolytic products. We thus tried to detect autolytic products in SCR lenses by Western blot analysis using a specific antibody against the large subunit of calpain. In this experiment, we only analyzed the water-soluble fraction since the m-calpain in SCR lenses exists predominantly in that fraction (*12*). In non-




**ments of αA- and αB-crystallins in AG-treated and non-treated SCR lenses.** WSFs and WIFs prepared from AG-treated and non-treated 15-week-old SCR lenses (equal in volume) were electrophoresed in 13.5% SDS–polyacrylamide gels and immunostained. (A) Immunostaining with anti-αA crystallin antibody (upper panel) and anti-αA fragment antibody exclusively specific to the calpain-generated fragment of αA-crystallin (lower panel). (B) Immunostaining with anti-αB crystallin antibody (upper panel) and anti-αB fragment antibody exclusively specific to the calpain-generated fragment of αB-crystallin (lower panel). N-NT, non-treated normal lens; N-AG, AG-treated normal lens; C-NT, non-treated cataractous lens; C-AG, AG-treated cataractous lens.

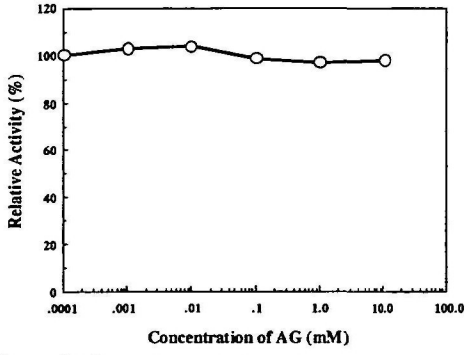



Fig. 4. **Effect of AG on the activity of m-calpain.** m-Calpain was assayed in the presence of aminoguanidine at the indicated concentrations. The assay was carried out in the presence of 4 mM $CaCl_2$ at 30°C for 10 min. The activity in the absence of aminoguanidine was taken as 100%.

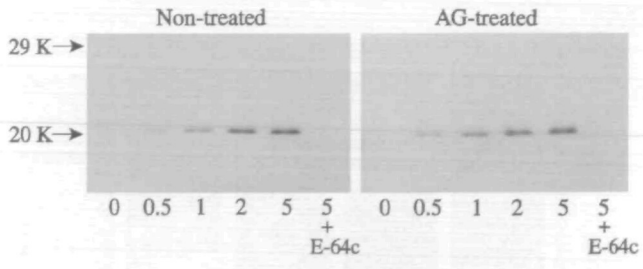



Fig. 5. **Effect of AG-treatment on the susceptibility of αA-crystallin to calpain proteolysis.** The water-soluble proteins from 15-week-old AG-treated cataractous and non-treated normal lenses were subjected to proteolysis by purified m-calpain for various periods in the presence or absence of E-64c, a calpain inhibitor, and the degradation products were analyzed by Western blotting. Immunostaining was performed with an anti-αA fragment antibody exclusively specific to the calpain-generated fragment of αA-crystallin.

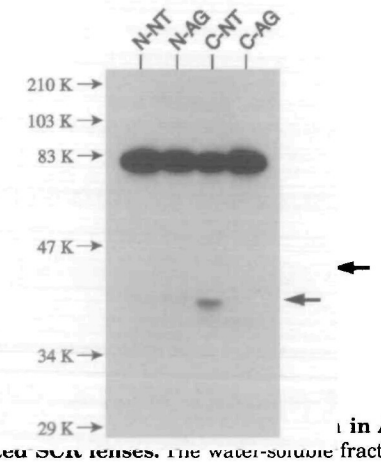



Fig. **in AG-treated and non-treated SCR lenses.** The water-soluble fractions (WSFs) prepared from 15-week-old AG-treated and non-treated SCR lenses (equal in volume) were electrophoresed in 12.5% SDS–polyacrylamide gels and immunostained with a monoclonal antibody ($1D_{10}A_7$) against the large subunit of calpain. The band indicated by the arrow is the autolytic degradation product of m-calpain. N-NT, non-treated normal lens; N-AG, AG-treated normal lens; C-NT, non-treated cataractous lens; C-AG, AG-treated cataractous lens.

treated cataractous lens (C-NT), not only did the amount of m-calpain large subunit (80 kDa) decrease compared with non-treated normal lens (N-NT), but also a new antibody-reactive band appeared at approximately 35 kDa as indicated by the arrow. On the other hand, the autolytic fragment of m-calpain in normal lenses (N-NT and N-AG) appears negligible. These results indicate that the unphysiological activation of m-calpain occurs in cataractous lens. These autolytic changes in m-calpain observed in non-treated cataractous lens were almost completely blocked by the oral administration of AG (C-AG).

*Effect of AG-Treatment on the Calcium Concentrations in Lens*—In order to explore the mechanism for the inhibition of m-calpain activation by AG-treatment, we analyzed the calcium contents in AG-treated and non-treated lenses by atomic absorption spectrophotometry (Fig. 7). The calcium contents in non-treated normal lenses were determined at 10 and 15 weeks of age only, since we have previously reported that the age-dependent changes in the calcium con-

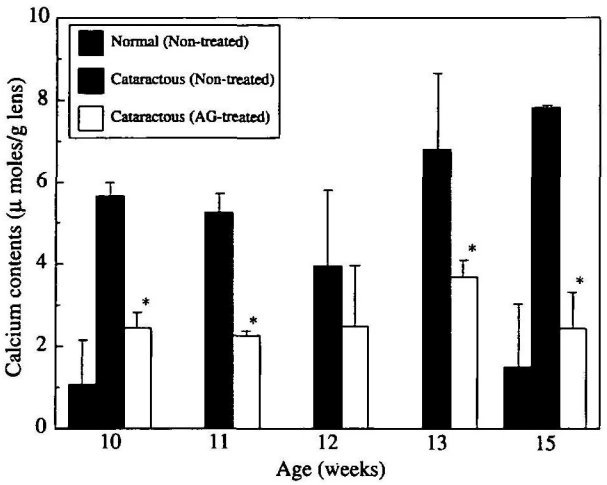



Fig. 7. **Effect of the oral administration of aminoguanidine on calcium contents in SCR lenses.** The calcium contents were determined by atomic absorption spectrophotometry. In the case of normal control lens, the calcium contents were not determined from 11 to 13 weeks of age. Each value represents the mean ± SE of four experiments. $^*p < 0.01$, compared with non-treated (cataractous).

centration in normal lenses are very slight (*12*). In non-treated cataractous lenses, increased calcium contents are observed compared with normal lenses. In contrast, the increase in the calcium content is markedly suppressed in AG-treated cataractous lenses at all ages, although the levels are still slightly higher than in normal lenses.

*Expression of iNOS Protein in Cataractous SCR Lens*—Since aminoguanidine (AG) is an inhibitor of inducible nitric oxide synthase (iNOS), it is of interest and significance to confirm whether iNOS is highly expressed in cataractous lenses. So we investigated the expression of iNOS protein in normal and cataractous SCR lenses by Western blot analysis. As shown in Fig. 8, the amount of iNOS protein in cataractous lens is about 12 times higher than in normal lens.

## DISCUSSION

In this paper, we show that the oral administration of aminoguanidine (AG) strongly inhibits the lens opacification in hereditary cataract rats (SCR). As far as we know, this is the first report that aminoguanidine acts to prevent lens opacification, except for the cataracts in moderately diabetic rats in which the authors used AG as an inhibitor of advanced glycation (*18*). We analyzed calpain-mediated proteolysis in AG-treated cataractous lenses based on our previous results that calpain-mediated proteolysis is closely related to lens opacification (*12*). The results demonstrate that the calpain-mediated proteolysis of crystallins is strongly inhibited in AG-treated cataractous lenses in which opacity has been suppressed (Fig. 3). These results strengthen our earlier conclusion that the calpain-mediated proteolysis of crystallins plays a critical role in the development of lens opacification during cataractogenesis in SCR.

To clarify the mechanism for the inhibition of calpain-mediated proteolysis by AG-treatment, we examined the effect of AG on calpain activity *in vitro* and the effect of the oral administration of AG on the susceptibility of endogenous substrate proteins to calpain. As shown in Figs. 4 and 5, AG had no effect on either. On the other hand, the autolytic degradation of m-calpain that occurs in cataractous lenses was strongly inhibited by AG-treatment (Fig. 6). It seems very unlikely that AG blocks the autolytic degradation of m-calpain, since AG, *per se*, has no effect on calpain activity *in vitro* as described above. Calpain is activated in response to elevated intracellular calcium ions. Therefore, it is believed that the elevation in intracellular calcium ions, which should occur in cataractous lenses, is suppressed by AG-treatment. Indeed, our experimental results demonstrate that AG-treatment inhibits the elevation of calcium content in cataractous lenses (Fig. 7). Although we did not determine free lens $Ca^{2+}$ concentrations, it can be expected that these levels would also rise in proportion to the increase in total calcium concentration, as observed in the case of selenite cataracts (*19*). From these findings, we conclude that the inhibition of calpain-mediated proteolysis by AG-treatment is due to the suppression of $Ca^{2+}$ influx into lens cells.

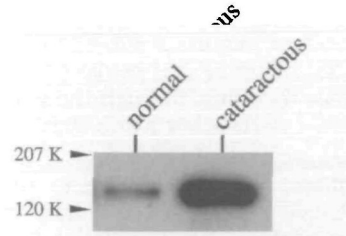



Fig. 8. **Expressi s SCR lens.** The water-soluble fractions (WSFs) prepared from 14-week-old normal and cataractous lenses (equal in volume) were electrophoresed in 13.5% SDS–polyacrylamide gels and immunostained with anti-iNOS antibody.

It has been generally considered that AG is a selective inhibitor of inducible nitric oxide synthase (iNOS) (*20*). iNOS is a transcriptionally regulated isoform of nitric oxide synthase and is involved in protective mechanisms of host defense being induced during infection and chronic inflammation (*21*). However, iNOS generates much larger quantities of the free radical nitric oxide (NO) over longer periods of time than other isoforms. NO generated by iNOS is apparently extremely cytotoxic if produced in excess, contributing to the profound cellular damage observed in a number of pathological conditions (*22*). Moreover, NO reacts readily with the oxygen free radical, superoxide anion, to form the strong oxidant species peroxynitrite ($ONOO^-$), whose cytotoxic potential is greater than that of NO (*23*, *24*). It has been considered that peroxynitrite causes the oxidation of proteins, nitration of tyrosine residues, oxidation of non-protein thiols and membrane lipids, and disruption of the membrane (*25*). There are reports that the administration of AG reduces the pathogenesis of autoimmune and inflammatory diseases, such as experimental allergic encephalomyelitis in rats and lipopolysaccharide (LPS)-induced acute lung injury in dogs, both of which are closely related to iNOS induction (*26*, *27*). Therefore, the preventive effect of AG on lens opacification suggests that iNOS is involved in cataract formation. Actually, in the present study, we found a very high amount of iNOS protein expressed in cataractous lens compared with normal lens (Fig. 8). Therefore, it is conceivable that NO and/or peroxynitrite produced by iNOS will induce membrane

disruption resulting in enhanced $Ca^{2+}$ influx into lens cells. Ishida *et al.* (*28*) reported that peroxynitrite increases $Ca^{2+}$ influx in cardiac myocytes through the plasma membrane. However, further work is needed to clarify the relationship between the expression of iNOS and $Ca^{2+}$ influx into lens cells. Experiments on the changes in the expression of iNOS mRNA and iNOS protein during cataractogenesis are underway in this laboratory. It is also of interest and significance to examine the effect of AG on their expressions, since there are reports that AG inhibits not only iNOS activity but also the expression of the iNOS protein (*26, 27, 29*). These data may provide further support for the hypothesis that iNOS is involved in cataract formation.

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