Chapter-III

Evaluation of the effect of ellagic acid on lenticular calcium homeostasis in selenite-induced cataractogenesis

1. INTRODUCTION

Intracellular free calcium ($\text{Ca}^{2+}$) has long been recognized as an important regulator of many physiological and cellular events by acting as a second messenger; it is also known to trigger pathological events in cell injury and death (Missiaen et al., 2000). The lens is no exception to these functions; in human cataractous lenses, total calcium is elevated 3 to 3000-fold when compared to clear human lenses (Jedziniak et al., 1976; Hightower and Reddy, 1982b). Elevated levels of intracellular calcium have been found to modify the relative expression of cytochrome c oxidase-I (COX-I), the terminal enzyme of the electron transport chain that plays a major role in the process of oxidative dephosphorylation, as well as that of the early growth response protein-1 (EGR-1), an immediate-early gene. EGR-1, in myocytes, has been reported to inhibit the transcription of sarco/endoplasmic reticulum $\text{Ca}^{2+}$-ATPase 2 (SERCA2), which actively transports calcium ions from the cytosol into the endoplasmic reticulum and thus restores the intracellular calcium pools (Arai et al., 2000).

The involvement of lenticular calcium metabolism in the development of experimental and human cataract has been explored in several studies (Bunce et al., 1984; Sanderson et al., 2000; Nabekura et al., 2001; Tang et al., 2003). Selenite cataract in rats is an extremely rapid and convenient model of human nuclear cataract. Although the precise mechanisms remain to be elucidated, selenite cataractogenesis has been shown to involve oxidation of critical sulfhydryl groups, leading to the inactivation of $\text{Na}^{+}$, $\text{K}^{+}$, $\text{Ca}^{2+}$-ATPase or to the opening of ion channels in epithelial membranes. Influx of calcium then causes activation of the calcium-dependent protease, calpain II, which partially degrades crystallin proteins. This activated calpain
leads to increased degradation of lenticular proteins, such as crystallins, resulting in an opaque lens (Shearer et al., 1997). Therefore, malfunctioning of the Ca$^{2+}$-regulatory mechanisms is thought to be one of the causes for the development of selenite cataract.

In the lens, the intracellular calcium level is regulated by the activities of SERCA, plasma membrane Ca$^{2+}$-ATPase (PMCA) and plasmalemmal Na$^+$/Ca$^{2+}$ exchanger (Rhodes and Sanderson, 2009). Several investigators have reported that lenticular transparency is maintained if alterations in calcium homeostasis are prevented (Marcantonio et al., 1986; Mathur et al., 2000; Shridas et al., 2001; Nabekura et al., 2004). The previous chapters demonstrated that ellagic acid, a scavenger of free radicals, delays cataract development in Wistar rat pups challenged with selenite. It was also shown that this anticataractogenic effect was because ellagic acid prevented the depletion of antioxidant enzymes and the acceleration of lipid peroxidation accompanying selenite cataract formation. In addition, the possible role for ellagic acid in modulating the redox homeostasis in lenses of Wistar rats during selenite cataractogenesis has also been documented. In the present chapter, an attempt to test the hypothesis that ellagic acid prevents selenite cataractogenesis by maintaining calcium homeostasis in a normal state, therein modulating calcium-mediated calpain activity is described.

2. MATERIALS AND METHODS

2.1. Experimental animals and morphological examination

The rat pups, grouping of animals and treatment regimen used in this phase of the study were as described in the General Materials and Methods. Morphological examination of both eyes of each rat pups was performed on the 16th postnatal day by a slitlamp biomicroscope at 12X magnification and alterations in lenticular transparency were recorded. The animals were then sacrificed by cervical dislocation. The lenses from each rat were dissected out for further analysis.
2.2. **Determination of calcium concentration in the lens**

The lenses from Group I, Group II and Group III rats were analyzed for Ca$^{2+}$ concentration. The dry weight of the lens was measured after heating at 100ºC for 20 hours. The lenses were then digested with 0.2 ml concentrated HCl at room temperature overnight and adjusted to 1.0 ml with deionized water. The mixtures were centrifuged at 10,000xg for 10 minutes to remove insoluble material, if any. The calcium concentrations in the supernatant fractions were then measured by an atomic absorption spectrophotometer (model Spectra AA-220, Varian), operated with a slit width of 0.5 nm, with the wavelength set at 422.7 nm. Standard solutions were prepared from CaCO$_3$ and deionized water. The results were expressed as μmol of calcium/gm dry weight of the lens.

2.3 **Calpain activity assay**

The combined activity of calpains was determined fluorimetrically with casein as the substrate (Banay-Schwartz et al., 1998) with minor modifications as described by Elanchezhian et al. (2009). For this assay, rat lenses from each group were homogenized thoroughly in 10 vol of 10 mM sodium borate buffer, pH 8.0, containing 5 mM EDTA, 3 mM sodium azide, 1 mM dithiothreitol and 0.1% Triton X-100. After centrifugation of the homogenate at 10,000xg for 10 minutes, the supernatant was collected for estimation of calpain activity. To prepare the substrate, an aqueous solution of casein (60 mg/ml) was subjected to boiling at pH 9.5 (maintained by addition of 0.1 N NaOH [20 ml]), cooled, adjusted to pH 5.9 with 0.1 N HCl, and then centrifuged to collect the supernatant. Aliquots of lenticular homogenate corresponding to 500 mg of protein were added to a mixture containing 3.57 mg of alkali-treated casein, 25 mM of sodium borate, 1 mM of 2-mercaptoethanol, and 5 mM of CaCl$_2$ to a total volume of 0.35 ml. The pH of the final incubation mixture was 7.5. Incubation was for 1 hour at room temperature (incubation mixture without calcium contained 1 mM ethylene glycol tetraacetic acid [EGTA]). Zero-time incubation blanks were
subtracted. The enzyme reaction was stopped by the addition of 200 µl of 36% (w/v) trichloroacetic acid. The tubes were stored at 4°C overnight. After centrifugation, 200 µl of trichloroacetic acid supernatant were mixed with 1.5 ml of 0.1 M sodium borate buffer (pH 8.5) and 200 µl fluorescamine solution (3.4 mg/10 ml of acetone) were added to each tube and the contents were mixed thoroughly. The quantum of liberated amino groups was measured fluorimetrically in a spectrofluorimeter and compared with standards of known amounts of glutamate in 10% trichloroacetic acid. One unit of calpain activity was defined as the amount that caused the release of 1 µmol of amino groups/hour under the conditions described. Results were expressed as µmol of glutamic acid equivalents released/mg protein/hour.

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA expression

Lenses for this experiment, after removal from rat eyes, were immediately homogenised in Trizol (Sigma-Aldrich, St. Louis, MO) reagent (1 ml/100 mg tissue) and RNA was isolated per the manufacturer’s protocol. RNAs were quantified using a spectrophotometer at 260 nm and checked for RNA integrity via agarose gel electrophoresis by assessing 18S and 28S band intensities.

RT-PCR was performed using a one-step RT-PCR kit (Qiagen, Germany), per the manufacturer’s instructions; 1 µg of template RNA and 0.6 µM of each of the forward and reverse primers specific to the PMCA1, EGR-1, COX-I, m-calpain and GAPDH genes were added to the reaction mixture (Table 3.1). Amplification was done in an Eppendorf thermal cycler (Germany). The RT-PCR conditions were as described in Chapter II (Page 47). After completion of the PCR reaction, a 10 µl portion of the PCR product was electrophoresed in a 2% agarose gel. The ethidium bromide-stained gel was photographed with a DS-34 type Polaroid camera and the band was scanned with an imaging densitometer (Bio-Rad, Model GS-670). The GAPDH gene was used
Table 3.1. Primer sequences and expected product sizes for the genes amplified in samples from lenses of Wistar rats

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5'-3'</th>
<th>PCR cycle #</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-cal – up</td>
<td>GGGCAGACCAACATCCACCTCAGCAAAAAAC</td>
<td>30</td>
<td>404</td>
</tr>
<tr>
<td>m-cal-down</td>
<td>GTCTCGATGCTGAAGCCATCTGACTTGAT</td>
<td>30</td>
<td>454</td>
</tr>
<tr>
<td>PMCA-up</td>
<td>GACTCGCCACTGAAAGGCGGT</td>
<td>30</td>
<td>454</td>
</tr>
<tr>
<td>PMCA-down</td>
<td>GGCTTCCCCGCAAACCTGCAC</td>
<td>30</td>
<td>454</td>
</tr>
<tr>
<td>COX-I - up</td>
<td>ATGTAGACACCGAGGCTAATTAA</td>
<td>30</td>
<td>288</td>
</tr>
<tr>
<td>COX-I - down</td>
<td>CGAAGCAAGCTATGATGGCGAATA</td>
<td>30</td>
<td>288</td>
</tr>
<tr>
<td>EGR-1-up</td>
<td>TGCAGGCACAGCCTTGAGGTATTAAGAGCC</td>
<td>30</td>
<td>257</td>
</tr>
<tr>
<td>EGR-1-down</td>
<td>GGGACTGGTGAGGTGTTATAAGAGCC</td>
<td>30</td>
<td>257</td>
</tr>
<tr>
<td>GAPDH-up</td>
<td>TCAAGAAGGTTGGTAAGCCAGGC</td>
<td>30</td>
<td>207</td>
</tr>
<tr>
<td>GAPDH-down</td>
<td>GGTCACCACCCCTGTGGTGTA</td>
<td>30</td>
<td>207</td>
</tr>
</tbody>
</table>

**Abbreviations**
- m-cal – m-calpain
- PMCA – Plasma membrane calcium ATPase
- COX-I – Cytochrome c oxidase-I
- EGR1 – Early growth responsive protein 1
- GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
as an internal standard for the RT-PCR reaction. To quantitate the transcript level, the ratio of the study gene product to the GAPDH gene product was calculated. Experiments were performed in triplicate.

2.5. Immunoblotting

The lenses were homogenized in 10 times their mass of 50 mM phosphate buffer (pH 7.2) and centrifuged at 12,000xg for 15 minutes at 4°C. Total protein in each sample of the supernatant obtained was estimated by the method of Bradford (1976) using bovine serum albumin as a standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels using the Tris–glycine buffer system. Immunoblotting for Lp82 and COX-I were performed by electrotransferring proteins from SDS-PAGE gel to nitrocellulose membrane at 30 V (constant) for 100 minutes at an ice-cold temperature using Tris–glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol). The monoclonal anti-Lp82 (Sigma-Aldrich, USA) and anti-COX-I (Hayman, USA) antibodies were used at 1:1000 dilution, and immunoreactivity was visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody (Genei, Bangalore, India).

2.6. Statistical analysis

Statistical analysis was performed with SPSS software (version 11.5) (SPSS Corporation, Chicago, IL). The mean value of each biochemical parameter assessed in each individual group of rats was calculated from at least six individual values and the results were expressed as mean±SD. For expression studies, experiments were performed at least three times with duplicate samples and the results were expressed as mean±SD. For Lp82 protein expression, non-parametric analysis was used and the results were expressed as median (min-max). To test for correlations between two parameters, Spearman analysis was performed. \( P \) values <0.05 (two-tailed) were considered statistically significant.
3. RESULTS

3.1. Morphological examination and calcium level

Morphological examination of both eyes of each rat pup revealed dense lenticular opacification in all (100%) Group II (selenite-challenged, untreated) rats but complete lenticular transparency in all Group I (normal) pups (Fig. 3.1). In selenite-challenged rats treated with ellagic acid (Group III), lenticular opacification did not occur in 60% of rats. The mean lenticular calcium concentrations in the three groups of rats on the 16th postpartum day were estimated (Fig. 3.1). The actual mean calcium concentration (1.96 ± 0.22 μmol) was significantly (P < 0.001) higher in the lenses of Group II (selenite challenged, untreated) rats than that in the lenses of Group III (selenite-challenged, ellagic acid-treated) rats (0.81 ± 0.13 μmol) and that in Group I (normal) rats (0.64 ± 0.08 μmol).

3.2. Calpain activity

With reference to the mean calpain activity in the supernatant of lenses removed on the 16th postpartum day from the different groups of rats (Fig. 3.2), the mean activity in Group II (selenite-challenged, untreated) rat lenses (0.58 ± 0.04 μmol) was significantly (P < 0.01) lower than that in the lenses of Group III (selenite-challenged, ellagic acid-treated) rats (0.79 ± 0.03 μmol) and that in Group I (normal) rats (0.94 ± 0.03 μmol).

3.3. Effect of ellagic acid on mRNA transcript levels

The mean levels of mRNA transcripts of PMCA1, EGR-1, COX-I and m-calpain were analysed by semi quantitative RT-PCR with GAPDH as an internal control. In the lenses of Group II (selenite-challenged, untreated) rats, the mean levels of PMCA1 and EGR-1 mRNA transcripts were found to be significantly (P < 0.05) higher than those in normal (Group I) rats (Figs. 3.3 and 3.4). In Group III rats (selenite-challenged, ellagic acid-treated), the mean levels of the mRNA transcripts of
Fig. 3.1. Calcium levels in lenses of normal (Group I), selenite-challenged, untreated (Group II) and selenite-challenged, ellagic acid-treated (Group III) 16-day-old Wistar rat pups. The mean calcium level in Group II rat lenses, where selenite cataract developed, was higher than that in normal and ellagic acid-treated rat lenses. Results are presented as box plots, where the box represents the interquartile range, which contains 50% of values. The whiskers extend from the box to the maximum and minimum values. The median is indicated by a line across the box. The pictures depicted are slit-lamp photographs of the respective groups.

* Group I vs Group II values ($P < 0.001$). # Group II vs Group III values ($P < 0.001$).
Fig. 3.2. Calpain activity in lenses of 16-day-old Wistar rat pups. (Group I = normal; Group II = selenite-challenged, untreated; Group III = selenite-challenged, ellagic acid-treated). GA- Glutamic acid. Values are expressed as mean (n=6)±SD.

* Group I vs Group II values ($P < 0.001$).
# Group II vs Group III values ($P < 0.01$).
**Fig. 3.3.** (a) RT-PCR for plasma membrane Ca\(^{2+}\)-ATPase (PMCA) in rat lenses, visualized on an ethidium bromide-stained agarose gel. M - 100 bp DNA ladder, GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated). (b) The results (the corresponding band intensity) depicted are normalized to control levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are mean values (experiment run in triplicate) of ratios of intensity for gene of interest divided by that for GAPDH.

* Group I vs Group II values ($P < 0.01$). # Group II vs Group III values ($P < 0.05$).
Fig. 3.4. (a) RT-PCR for early growth response protein-1 (EGR-1) in rat lenses, visualized on an ethidium bromide-stained agarose gel. M - 100 bp DNA ladder, GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated). (b) The results (the corresponding band intensity) depicted are normalized to control levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are mean values (experiment run in triplicate) of ratios of intensity for gene of interest divided by that for GAPDH.

* Group I vs Group II values ($P < 0.001$). # Group II vs Group III values ($P < 0.01$).
lenticular PMCA1 and EGR-1 were found to be lower than those in selenite-challenged untreated (Group II) rats. The mean levels of mRNA transcripts of COX-I and m-calpain were found to be significantly ($P < 0.05$) lower in Group II (selenite-challenged, untreated) rat lenses then those in Group I (normal) rat lenses and were at near normal levels in Group III (selenite-challenged, ellagic acid-treated) rat lenses (Figs. 3.5 and 3.6).

### 3.4. Immunoblot for COX-I and Lp82

To further validate the data on mRNA transcript level of COX-I, an immunoblot was performed with COX-I antibody to detect COX-I protein. Immunoblot analysis revealed significantly lowered band intensity in the lenses of selenite-challenged untreated (Group II) rats than that in normal (Group I) rat lenses. However, the anti-COX-I antibody detected appreciable amounts of COX-I protein in the lenses of selenite-challenged, ellagic acid-treated (Group III) rats compared to that detected in selenite-challenged untreated (Group II) rat lenses (Fig. 3.7).

The relative amounts of Lp82 in the soluble fractions of the lenses were determined by immunoblotting with lens-specific Lp82 antibodies. Immunoblot analysis revealed that there was a smaller amount of Lp82 in the soluble fraction of Group II (selenite-challenged, untreated) rat lenses than that in lenses of Group I (normal) and Group III (selenite-challenged, ellagic acid-treated) rats (Fig. 3.8a). Spearman correlation analysis was also performed to determine whether the intensity of Lp82 expression correlated with lenticular calcium concentration. In Fig. 3.8b, the band intensity is depicted as box plots, the box representing the interquartile range, which contains 50% of the values. The whiskers extend from the box to the maximum and minimum values, and the median value is indicated by a line across the box. Fig. 3.8c shows that there was a significant negative correlation ($r=-0.593; P<0.01$) between the intensity of the band corresponding to Lp82 and lenticular calcium concentration.
Fig. 3.5. (a) RT-PCR for cytochrome c oxidase-I (COX-I) in rat lenses, visualized on an ethidium bromide-stained agarose gel. M - 100 bp DNA ladder, GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated). (b) The results (the corresponding band intensity) depicted are normalized to control levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are mean values (experiments run in triplicate) of ratios of intensity for gene of interest divided by that for GAPDH.

* Group I vs Group II values \( P < 0.001 \). # Group II vs Group III values \( P < 0.01 \).
**Fig. 3.6.** (a) RT-PCR for m-calpain in rat lenses, visualized on an ethidium bromide-stained agarose gel. M - 100 bp DNA ladder, GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated). (b) The results (the corresponding band intensity) depicted are normalized to control levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are mean values (experiment run in triplicate) of ratios of intensity for gene of interest divided by that for GAPDH. * Group I vs Group II values ($P < 0.001$). # Group II vs Group III values ($P < 0.01$).
Fig. 3.7. (a) 10% SDS-PAGE. (b) Western blots demonstrating expression of cytochrome c oxidase-I (COX-I) in the lenses of normal, selenite-challenged, untreated and selenite-challenged, ellagic acid-treated groups. The β-actin antibody was used as loading control. Results are representative of three repetitions for each protein examined. (c) Bar graphs of mean normalised densitometry for these data. GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated).
Fig. 3.8. (a) Representative Western blot for Lp82 in soluble fraction of lens, with β-actin as the loading control. Two biological replicates are presented. (b) Box-plot showing the level of Lp82 as relative levels of at least 3 biological replicates with experimental duplicates, normalized to the band intensity of β-actin. The band intensity is depicted as box plots, where the box represents the interquartile range, which contains 50% of values. The whiskers extend from the box to the maximum and minimum values. The median is indicated by a line across the box. (c) Negative correlation noted between Lp82 and the lenticular calcium level. GI - Group I (normal), GII - Group II (selenite-challenged, untreated), GIII - Group III (selenite-challenged, ellagic acid-treated). (○) Values in Group I; (x) Values in Group II; (●) Values in Group III.

* Group I vs Group II values ($P<0.001$). # Group II vs Group III values ($P<0.001$).
4. DISCUSSION

Recent evidence suggests that unregulated Ca\(^{2+}\)-mediated proteolysis of essential lenticular proteins by calpains is a major contributor to some forms of cataract in both animals and humans (Biswa et al., 2004). Moreover, elevated levels of Ca\(^{2+}\) associated with certain forms of injury to the lens appear to induce proteolysis of crystallins by calpains (Biswas et al., 2001). Shearer et al. (1997) hypothesized that early changes in the lenticular epithelium may result from oxidative damage caused by selenite, possibly to critical sulfhydryl groups on molecules such as Ca\(^{2+}\)-ATPase or to ion channels.

In the experiments described in the current chapter, the observed increase in lenticular calcium was possibly due to oxidation of sulfhydryl groups or other changes in the membrane caused by selenite. These results are consistent with those obtained in a study on human colonic carcinoma cells in which selenite was found to increase intracellular calcium (Wang et al., 2003). There was also a significant increase in the expression of the calcium-regulating protein, PMCA1 in selenite-challenged, untreated (Group II) rat lenses. The upregulation of PMCA1 after selenite administration suggests a protective mechanism in the lenticular epithelium to overcome a deleterious, oxidation-induced, increase in lenticular calcium. Similar observations on upregulation of PMCA1, as well as increased calcium level, have been reported in human lenticular epithelial cells after exposure to H\(_2\)O\(_2\) and thapsigargin (Marian et al., 2008) and also in hereditary cataract formation (Nagai et al., 2008).

Upregulation of EGR-1 mRNA and down-regulation of mitochondrial genes have been shown to be induced by increased intracellular Ca\(^{2+}\) (Frevissonet et al., 2004). EGR-1 is an immediate-early gene, which is upregulated by mitogenic stimulation and during membrane depolarization and diverse exogenous stimuli (Gashler and Sukhatme, 1995). EGR-1 is reported to partly inhibit the transcription of
sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase2, which actively transports calcium ions from the cytosol into the endoplasmic reticulum and restores intracellular calcium pools in myocytes (Arai et al., 2000). In the experiments described in the current chapter, the mRNA transcripts of EGR-1 were found to be increased in selenite-challenged, untreated (Group II) rat lenses, compared to levels in normal (Group I) rat lenses; this increase was possibly a consequence of the elevated intracellular calcium pool following loss of the epithelial barrier function (Nakajima et al., 2006a). Previous studies have suggested that selenite induces DNA damage through reactive oxygen species (Zhou et al., 2003). Ionizing and ultraviolet radiation also induce the expression of EGR-1. Thus, DNA damage might be one of the factors inducing expression of EGR-1. Interestingly, the results of the present experiments showed that ellagic acid treatment maintained mRNA transcripts of EGR-1 at near normal levels in lenses of selenite-challenged rats (Group III). Although the present experiment did not elucidate the precise mechanism by which ellagic acid modulates EGR-1 mRNA, induction of EGR1 mRNA may involve ERK1/2 and, potentially, SAPK/JNK pathways, as has been reported in U87 glioma cells (Lim et al., 1998). A recent report also suggests that epigallocatechin gallate (EGCG) protects against oxidative stress-induced, mitochondrial-dependent apoptosis in human lenticular epithelial cells by inhibiting the down-regulation of mitogen-activated protein kinases and Akt (Yao et al., 2008). The operation of a similar signal cascade may underlie the protective role of ellagic acid in the lens.

It is known that COX-I in the mitochondrial respiratory pathway plays an important role in ATP production; dysfunction of this enzyme results in decreased ATP synthesis. In the experiments described in the current chapter, the significantly ($P < 0.05$) lower levels of COX-I (Fig. 3.5) gene transcripts and protein levels (Fig. 3.7) in Group II (selenite-challenged, untreated) rat lenses may have resulted in decreased synthesis of ATP, leading sequentially to decreased Ca\textsuperscript{2+}-ATPase function,
elevated lenticular Ca\textsuperscript{2+} and, finally, to lenticular opacification (Fig. 3.1). A similar pattern of decreased expression of COX-I has been noted in selenite-cataractous lenses (Nakajima et al., 2002; Elanchezhian et al., 2010) and in Upjohn Pharmaceuticals Limited (UPL) rats (Nabekura et al., 2004). Selenite-induced \(\alpha\)-TN4 cell death has been found to be accompanied by preferential down-regulation of mitochondrial RNAs, such as COX-I, and release of cytochrome c from mitochondria into cytosol (Belusko et al., 2003). During cell death, mitochondrial calcium overload has been shown to activate the permeability transition pore (PTP), resulting in transient mitochondrial depolarization and decreased synthesis of ATP; this PTP gating may also cause release of mitochondrial proteins that activate apoptotic pathways (Strasser et al., 2000). In the present set of experiments, it was interesting to note that there was increased cytochrome c oxidase activity following treatment with ellagic acid, suggesting a protective role for this compound.

One of the many cellular proteins involved in Ca\textsuperscript{2+} signaling in mammalian cells is the Ca\textsuperscript{2+}-activated neutral protease, calpain. Calpain has been implicated in a wide range of human pathologies, including cataracts (Biswas et al., 2001). Thus, the quest for non-surgical approaches to cataract treatment, coupled to the increased recognition of the role of calpain in cataractogenesis, has prompted research into the possible use of calpain inhibitors as anticataract agents. Hence, it was hypothesized that ellagic acid could modulate calcium homeostasis and the calpain cascade in lenses of Wistar rats therein preventing or retarding selenite cataractogenesis, an aspect that has hitherto not been studied. In the experiments described herein, decreased mean calpain activity was observed in the lenses of selenite-challenged, untreated (Group II) rats when compared to the mean activity in lenses of normal age-matched (Group I) rats. However, in the lenses of selenite-challenged, ellagic acid-treated (Group III) rats, the mean calpain activity was essentially similar to that in Group I rat lenses suggesting that ellagic acid treatment possibly prevented an increase in lenticular Ca\textsuperscript{2+} levels in
Group III rats. Decreased lenticular calpain activity appeared to occur concomitantly with increased lenticular Ca\(^{2+}\) levels, presumably due to the well-known autolysis of calpain that results from exposure of the enzyme to an elevated calcium concentration (Suzuki, 1987). Loss of lenticular calpain activity after formation of cataract in the Nakano mouse model is also postulated to be due to autolysis of calpain (Yoshida et al., 1985). Decreased levels of lenticular calpain-2 have been observed during selenite cataractogenesis in Sprague-Dawley rat pups (Hightower et al., 1987).

Currently, five calpains are known to occur in the lens, including \(\mu\)-calpain (calpain 1), which is expressed only at low levels (Huang and Wang, 2001), Lp85 (Shih et al., 2006), and the major enzymes, m-calpain (calpain 2) (Shearer et al., 2000), calpain 10 (Ma et al., 2001) and Lp82 (Ma et al., 1998b). m-calpain is ubiquitous in mammalian cells and the most extensively studied member of the calpain superfamily. Over the past two decades, increasing evidence from several studies has suggested that m-calpain is involved in cataractogenesis in animals. Studies done in the late 1990s and 2000 established that m-calpain proteolysed lenticular crystallins and that calpain inhibitors could prevent the progression of rodent cataract induced by Ca\(^{2+}\)-mediated activation of m-calpain (Anderson et al., 1996; Azuma et al., 1997; Shearer et al., 2000). More recent studies have shown that the enzyme is the major calpain activated in murine diabetic cataractogenesis (Sakamoto-Mizutani et al., 2002; Thampi et al., 2002). m-calpain has also been shown to induce cataractogenesis and cleave crystallins in the lenses of a variety of other mammalian species, including mice and guineapigs (Fukiage et al., 1997; Udea et al., 2002), monkeys and rabbits (Nakajima et al., 2001), and calves (Udea et al., 2001). m-calpain is reported to be the major calpain in the epithelial cells of human lenses showing age-related cortical cataract (Andersson et al., 1994).
The recently-deciphered crystal structures of rat (Hosfield et al., 1999) and human (Strobl et al., 2000) m-calpain reveal that binding of Ca$^{2+}$ is essential in the realignment of domain IIa and IIb, which together contain the amino acid residues necessary to form the active state of the enzyme and to facilitate proteolytic activity. Taken together with more recent studies (Thampi et al., 2002; Goll et al., 2003), it is now generally accepted that m-calpain plays a major role in the opacification of the rodent lens. Taking into account the observations made in these previous studies, RT-PCR analysis was also performed as one of the experiments described in this chapter to determine the m-calpain mRNA expression in the lenses of the rats (all three groups). The level of the m-calpain gene transcript was clearly lower in selenite-challenged, untreated (Group II) rat lenses (Fig. 3.6) than that in the lenses of selenite-challenged, ellagic acid-treated (Group III) rats and normal (Group I) rats. Interestingly, these findings are consistent with the observations made on total calpain enzyme activity (Fig. 3.2). Again, these original findings suggest that ellagic acid treatment is able to modulate lenticular calpain activity to near normal levels.

Lens preferred 82 (Lp82) is a calpain enzyme that is found only in rodent lenses and does not exist in human or monkey lenses. Immunoblot analysis was performed to assess Lp82 protein expression level in relation to calpain activation, since activation of calpain is usually inferred by observing degradation of calpains on zymograms and immunoblots (Nakajima et al., 2006b). The level of Lp82 protein was found to be lower in the selenite-challenged, untreated group (this group also exhibited autolysis and lower calpain activity), while the lenses of the selenite-challenged, ellagic acid-treated group exhibited protein levels that were almost similar to those in the normal lenses of Group I rats. This novel finding suggests that ellagic acid is able to modulate lenticular Lp82 activity at near normal levels even in the presence of selenite-induced oxidative stress.
5. **CONCLUSION**

In summary, these results support the hypothesis that ellagic acid modulates calcium homeostasis and the calpain cascade in the lenses of Wistar rats exposed to selenite, therein preventing cataractogenesis. The effect on calcium homeostasis appears to occur by maintaining PMCA1, EGR-1 and COX-I mRNA transcripts at near normal levels, while the effect on the calpain cascade appears to occur by preventing Ca\(^{2+}\)-dependent activation of calpain. Further studies may help to elucidate the potential role of ellagic acid in the pharmacological management of cataract.