Aggregation of β-crystallin through covalent binding to 1,2-naphthoquinone is rescued by α-crystallin chaperone

Yasuhiro Shinkai¹,²*, Yunjie Ding²*, Takashi Miura² and Yoshito Kumagai¹,²

¹Environmental Biology Laboratory, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
²Doctoral Program in Biomedical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

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ABSTRACT — Cataract induced by exposure to naphthalene is thought to mainly involve its metabolic activation, forming 1,2-naphthoquinone (1,2-NQ), which can modify proteins through chemical modifications. In the present study, we examined the effect of 1,2-NQ on aggregation of crystallins (cry) associated with cataract. Incubation of bovine β-cry with 1,2-NQ caused covalent modification of β-cry at Cys117 and Lys125 accompanied by reduction in its thiol content, resulting in a concentration- and temperature-dependent aggregation of β-cry, whereas only little aggregation of α-cry induced by 1,2-NQ was seen. Interestingly, addition of α-cry to the reaction mixture of β-cry and 1,2-NQ markedly blocked β-cry aggregation induced by 1,2-NQ in a concentration-dependent manner. These results suggest that β-cry predominantly undergoes chemical modification by 1,2-NQ, causing its aggregation, which is suppressed by the chaperone-like protein, α-cry. This β-cry aggregation may be, at least in part, involved in the induction of cataract caused by 1,2-NQ.

Key words: 1,2-Naphthoquinone, Crystalline, Covalent modification, Aggregation, Chaperone

INTRODUCTION

Naphthalene is an important industrial compound, which is ubiquitous in the environment. The most important sources of naphthalene that the general public is exposed to are emissions from fuel combustion (Fraser et al., 1998) and cigarette smoke (Lu and Zhu, 2007). The epidemiological relationship between smoking and cataract has been well studied and there is evidence of a dose-response relationship between the cumulative effects of smoking and the risk of cataract development (Nirmalan et al., 2004; Kelly et al., 2005). Naphthalene has been shown to be a cataractogenic agent in rabbits (Van Heyningen and Pirie, 1967; Van Heyningen, 1979) and murine rodents (Xu et al., 1992a). On the basis of the development and morphology of naphthalene-induced cataract, it was considered a potential model for human subcapsular senile cataract (Lee and Chung, 1998). Naphthalene is initially metabolized by cytochrome P-450 isozymes, yielding naphthalene-1,2-oxide in the liver (Wilson et al., 1996). Naphthalene-1,2-oxide and its metabolites are converted into 1,2-dihydroxynaphthalene by aldose reductase isozymes in the lens (Van Heyningen, 1979; Xu et al., 1992b; Lee and Chung, 1998). This catechol metabolite transforms into 1,2-naphthoquinone (1,2-NQ) by auto-oxidation (Smithgall et al., 1988). 1,2-NQ has two chemical characteristics: 1) generation of reactive oxygen species (ROS) through redox cycling (Kleber et al., 1991; Kröner et al., 1991; Sun et al., 2006) and 2) covalent modification of proteins through nucleophilic amino acid residues such as cysteine, histidine and lysine (Fig. 1) (Smithgall et al., 1988; Iwamoto et al., 2007; Kumagai et al., 2012). Although previous studies have reported that naphthalene-induced cataract is due to metabolic activation that produces 1,2-NQ (Rees and Pirie, 1967; Rao et al., 1992), the participation of chemical modifications by 1,2-NQ in the cataract formation remains to be elucidated.

The ocular lens is a transparent organ containing thiol-rich proteins. Crystallins, which are structural proteins in the lens, account for approximately 90% of the total soluble proteins, consisting of α-crystallins (α-cry), β-cry...
and γ-cry. α-Cry is a key member of the small heat shock protein family (Narberhaus, 2002) and acts as a molecular chaperone by preventing the aggregation of β-cry and γ-cry possessing many cysteine residues in the interior of the protein under stress conditions (Horwitz, 1992). We have developed an immunoblot analysis to detect proteins modified by 1,2-NQ with specific antibodies against 1,2-NQ (Miura and Kumagai, 2010). With this procedure we found that approximately 40% of naphthalene molecules were metabolized to 1,2-NQ by 9,000 g of mouse liver supernatant in the presence of NAD(P)H, suggesting that 1,2-NQ, causing protein S-arylation, is a major reactive metabolite of naphthalene. In the present study, we examined the aggregation of β-cry by 1,2-NQ through covalent modifications. We also explored whether α-cry affects β-cry aggregation mediated by 1,2-NQ, as α-cry possesses chaperone activity.

MATERIALS AND METHODS

Materials

1,2-NQ was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). α-Cry and β-cry from bovine eye lens were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The polyclonal antibody against 1,2-NQ was prepared as reported previously (Miura and Kumagai, 2010). Anti-β-cry was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were of the highest purity available.

Preparation of bovine crystallins

To regenerate oxidized thiol groups on bovine α- and β-cry, each cry (20 mg) was incubated with 10 mM dithiothreitol (DTT) in 50 mM potassium phosphate buffer (pH 7.4) for 1 hr on ice. The reaction mixture was dialyzed against 1,000 mL of 50 mM potassium phosphate buffer (pH 7.4) and 0.1 mM EDTA overnight (twice). Protein content was determined by the bicinchoninic acid protein assay (Smith et al., 1985) with bovine serum albumin as a standard. Reduced cry were stored at -80°C before use.

Thiol content

Reduction in thiol groups in α- and β-cry (600 µg each) following incubation with 100 µM 1,2-NQ (pH 7.4) for 1 hr was determined as reported previously (Kumagai et al., 2002). Briefly, a 0.1-mL aliquot of the incubation mixture was mixed with 0.3 mL of 0.2 M Tris-HCl (pH 8.2)/20 mM EDTA, followed by 20 µL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 0.15 mL of 5% SDS. Each resulting mixture was measured at 412 nm against a blank to determine the content of the thiol groups. Quantitation of protein thiol content was measured using an extinction coefficient of 13.6 mM⁻¹•cm⁻¹.

Identification of modification sites in proteins

Bovine β-cry (4 µM) was reacted with 100 µM 1,2-NQ in 50 mM KPi (pH 7.4) at 25°C for 30 min. The native or 1,2-NQ-modified bovine β-cry was then incubated with 2 mM Tris(2-carboxyethyl) phosphine at 25°C for 10 min, then the mixture was alkylated by adding 5 mM 2-iodoacetamide in 50 mM ammonium bicarbonate buffer at 25°C in the dark for 20 min. The resulting protein was digested using mass spectrometry grade modified trypsin (100 ng; Promega, Madison, WI, USA) at 37°C overnight. Aliquots of the resulting peptides were analyzed using a nanoAcquity ultrahigh performance liquid chromatography system (nanoUPLC; Waters, Milford, MA, USA) equipped with a BEH130 nanoAcquity C18 column (100 mm long, 75 µm i.d., 1.7 µm particle diameter), which was kept at 35°C. Mobile phase A [water containing 0.1% (v/v) formic acid] and mobile phase B [acetonitrile containing 0.1% (v/v) formic acid] were mixed in a linear fashion using a gradient program. The instrument was calibrated immediately before each series of experiments. The flow rate was 0.3 µL/min, and the mobile phase composition started at 3% B for 1 min, then linearly increased over 74 min to 40% B, which was maintained for 4 min, then linearly increased over 1 min to 95% B, which was maintained for 5 min, and then linearly decreased over 1 min to 3% B. The total runtime, including conditioning the column to the initial conditions, was 100 min. The eluted peptides were transferred to the nano-electrospray source of a Synapt high definition Q-TOF mass spectrometer (Waters) through a Teflon capillary union and a precut PicoTip (Waters). The system was controlled, and the mass spectral data were analyzed using MassLynx version 4.1 software (Waters). The mass spectrometer used electrospray ionization with a capillary voltage of 3.5 kV and a sampling cone voltage of 40 V. A low collision energy (6 eV) was used to generate intact peptide precursor ions, and an elevated collision energy (stepped from 15 to 30 eV) was used to generate...
peptide product ions. The source temperature was 100°C, and the detector was operated in positive ion mode. Data were collected in centroid mode, and the m/z range was 50-1990. All analyses were acquired using an independent reference. Glu-1-fibrinopeptide B (m/z 785.8426), used as an external mass calibrant, was infused through the nanoLockSpray ion source and sampled every 10 sec. Biopharmlynx version 1.2 software (Waters) was used to perform baseline subtraction, smoothing and deisotopying, to identify de novo peptide sequences and to perform database searches.

Protein aggregation assay

The aggregation of cry was determined as previously described (Horwitz, 1992). Briefly, under normal conditions, crystallins were incubated with dimethyl sulfoxide (DMSO) or 1,2-NQ in 50 mM potassium phosphate buffer (pH 7.4) at 25°C for 1 hr. For measurement of thermal aggregation, β-cry was reacted with DMSO or 1,2-NQ in the presence or absence of α-cry or DTT in 50 mM potassium phosphate buffer (pH 7.4) at 25°C for 15 min, followed by incubation at 55°C for 1 hr. The protein aggregation was determined by measuring the apparent absorption due to scattering at 360 nm using a Shimadzu UV-1800 double-beam spectrometer (Shimadzu Co., Kyoto, Japan) equipped with a temperature controller.

Immunoblot analysis

The above-described reaction was performed, and then terminated by addition of SDS-PAGE loading buffer. Samples were adjusted to a protein content of 1 μg, and then mixed with half the volume of SDS-PAGE loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 24% glycerol, 6% SDS, 15 mM 2-mercaptoethanol and 0.015% bromophenol blue. The mixtures were heated at 95°C for 5 min, and then loaded on 15% SDS-polyacrylamide gels and subjected to electrophoresis (Laemmli, 1970) and western blot analysis (Kyhse-Andersen, 1984). The membrane was blocked with blocking solution at 4°C for 1 hr, and then probed with anti-1,2-NQ (0.5 μg/mL) for 1 hr. Next, the membrane was incubated with secondary anti-rabbit IgG antibody coupled to horseradish peroxidase. Finally, the proteins were visualized by an enhanced chemiluminescence system (Chemi-Lumi One; Nacalai Tesque, Inc., Kyoto, Japan) and recorded on X-ray films (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Statistical analysis

Significance was assessed with a t test, and P < 0.05 was considered significant.

RESULTS AND DISCUSSION

The thiol contents of commercial α-cry and β-cry after reduction with DTT under the conditions described in the Materials and Methods were 34.0 ± 0.3 and 75.0 ± 5.2 nmol/mg of protein, respectively (n = 3). However, incubation of α-cry or β-cry (600 μg) with 100 μM 1,2-NQ at pH 7.4 and 25°C for 1 hr caused a reduction in thiol groups (α-cry, 2.2 nmol/mg of protein; β-cry, 42.6 nmol/mg of protein), suggesting that β-cry more readily underwent covalent modification through presumably thiol groups by 1,2-NQ compared with α-cry. To confirm this possibility, we examined the β-cry modification sites by 1,2-NQ and found that 1,2-NQ modified β-cry at least at Cys117 and Lys125 (Fig. 2, Table 1 and Table 2). Unexpectedly, western blot analysis with specific antibodies against 1,2-NQ showed that the level of its covalent binding to β-cry was almost identical to its binding to α-cry (Fig. 3A) when using a higher concentration of 1,2-NQ (100 μM). This suggests that in α-cry, nucleophilic basic amino acid residues (e.g., lysine and histidine) rather than cysteine residues were predominantly modified by 1,2-NQ. Covalent binding of 1,2-NQ to proteins has also been seen with protein tyrosine phosphatase 1B in A431 cells (Iwamoto et al., 2007), peroxiredoxin 6 in A549 cells (Takayama et al., 2011), and thioredoxin (Shinkai et al., 2012).

Under these conditions, 1,2-NQ induced β-cry aggregation in a concentration-dependent manner, whereas this electrophile did not affect α-cry (Fig. 3B). We also found that 1,2-NQ facilitated dimerization of β-cry through presumably a bridge formation of 1,2-NQ as evaluated by western blot analysis with anti-1,2-NQ and anti-β-cry antibodies (data not shown). As shown in Fig. 4, 1,2-NQ-mediated aggregation of β-cry was markedly elevated by increasing the temperature to 55°C from 25°C (see Fig. 3), whereas naphthalene lacking electrophilicity had no effect on the aggregation, indicating that this concentration-dependent aggregation of β-cry caused by 1,2-NQ was dependent on covalent binding of this quinone.

As α-cry has been reported to be a small heat shock protein (Narberhaus, 2002), we thought that a lens protein exhibiting chaperone activity may affect the aggregation of β-cry during exposure to 1,2-NQ. To address this hypothesis, we incubated β-cry (200 μg) and 8 μM 1,2-NQ with or without different amounts of α-cry (Fig. 5). Pretreatment with a large amount of thiol-reducing compound such as DTT blocked covalent binding of 1,2-NQ to β-cry, and thus β-cry aggregation, further supporting the idea that covalent binding of 1,2-NQ is involved in the aggregation of β-cry.
Table 1. 1,2-NQ-modified peptides in bovine β-cry identified by nanoUPLC-MS.

| Position | Peptide sequence                  | Calculated mass | Observed mass | Site   |
|----------|-----------------------------------|-----------------|---------------|--------|
| 110-122  | LMSFRPICSANHK + 1,2-NQ            | 1658.77         | 1658.80       | Cys117 |
| 123-131  | ESKITIFEK + 1,2-NQ               | 1249.62         | 1249.63       | Lys125 |

Bovine β-cry (4 µM) was reacted with 100 µM 1,2-NQ for 30 min. The native or 1,2-NQ-treated β-cry was digested with trypsin and analyzed by nanoUPLC-MS as described in the Materials and Methods. A mass number of 156.0 was used to calculate the effect of the modification of nucleophilic amino acid residues by 1,2-NQ. The MS² data are shown in Table 2.
even 8 µg of α-cry markedly decreased β-cry aggregation, whereas heated α-cry (100°C, 1 hr) lost this inhibitory action (data not shown). This protective effect of α-cry on β-cry aggregation was concentration-dependent. At 200 µg of α-cry, almost complete blockage of 1,2-NQ-mediated β-cry aggregation was observed (Fig. 5B) without suppression of covalent binding of 1,2-NQ to β-cry (Fig. 5A). This suggests that the α-cry inhibition of β-cry aggregation induced by 1,2-NQ through covalent binding was not due to decreased binding of β-cry to 1,2-NQ. Rather it seems likely that α-cry works as a chaperone of the modified β-cry.

Several lines of evidence strongly indicate that biotransformation of naphthalene to electrophilic metabolites such as 1,2-NQ is involved in cataract formation (Kleber et al., 1991; Kröner et al., 1991). While 1,2-NQ has been found to be associated with induction of cataract during oxidative stress through a redox cycling and

Table 2. MS<sup>+</sup> data for the 1,2-NQ-modified peptides produced from bovine β-cry.

| Position | Assignment | Calculated mass | Observed mass | Modifier |
|----------|------------|-----------------|---------------|----------|
| 110-122  | b1         | 114.092         | 114.091       | + 1,2-NQ (Cys) |
|          | b9*        | 1191.532        | 1191.552      | + 1,2-NQ (Cys) |
|          | y5         | 556.284         | 556.289       | + 1,2-NQ (Lys) |
|          | a3         | 304.170         | 304.172       | + 1,2-NQ (Lys) |
|          | y13-NH<sub>3</sub>* | 1642.750       | 1642.764      | + 1,2-NQ (Cys) |
| 123-131  | y3         | 423.224         | 423.226       | + 1,2-NQ (Lys) |
|          | y4         | 536.308         | 536.309       | + 1,2-NQ (Lys) |
|          | y5         | 637.356         | 637.355       | + 1,2-NQ (Lys) |
|          | y7*        | 1034.555        | 1034.557      | + 1,2-NQ (Lys) |
|          | y9-H<sub>2</sub>O* | 1232.619       | 1232.621      | + 1,2-NQ (Lys) |
|          | y9*        | 1250.630        | 1250.630      | + 1,2-NQ (Lys) |

Fig. 3. Covalent binding of 1,2-NQ to cry and their aggregation. Bovine α-cry or β-cry (200 µg) was reacted with 1,2-NQ (8 or 100 µM) at 25°C for 1 hr in 50 mM potassium buffer (pH 7.4). (A) Covalent binding of 1,2-NQ to cry was analyzed by western blotting with anti-1,2-NQ antibodies. (B) The aggregation of α-cry and β-cry was monitored by light scattering at 360 nm. CBB staining was used as a loading control. Data are expressed as the mean ± SD (n = 3). *p < 0.05, **p < 0.01.

Fig. 4. Thermal enhancement of β-cry aggregation by 1,2-NQ modification. Bovine β-cry (200 µg) was reacted with DMSO, 1,2-NQ (0.05-8 µM) or naphthalene (8 µM) at 25°C for 15 min in 50 mM potassium phosphate buffer (pH 7.4), followed by incubation at 55°C for 1 hr. Covalent binding of 1,2-NQ to β-cry was analyzed by western blotting with anti-1,2-NQ antibodies (A) and β-cry aggregation was monitored by light scattering at 360 nm (B). CBB staining was used as a loading control. Data are expressed as the mean ± SD (n = 3). **p < 0.01.
covalent attachment of cellular proteins, the present study indicated that α-cry and β-cry are molecular targets of 1,2-NQ via covalent binding and that β-cry, but not α-cry, is sensitive to 1,2-NQ, leading to its aggregation presumably through S-arylation. Interestingly, 1,2-NQ extensively bound to α-cry as evaluated by western blotting (Fig. 3A), but it had little effect on α-cry aggregation (Fig. 3B) and function as a chaperone (Fig. 5B). Although further research is required, we speculate that the chaperone-like protein, α-cry, plays a role in repression of 1,2-NQ-mediated β-cry aggregation during metabolic activation of naphthalene in the lens.

A previous study has reported that exposure of mice to 1,2-NQ induced increased intracellular calcium concentration and subsequent activation of calpains (Qian and Shichi, 2001), which are capable of truncating β-cry and α-cry, thereby causing structural perturbation and diminished chaperone activity, respectively (Biswas et al., 2005). These phenomena are potentially implicated in the cataract formation induced by 1,2-NQ. The present study thus suggests that 1,2-NQ-mediated covalent modification of β-cry may also be involved in aggregation of this structural lens protein during 1,2-NQ-mediated suppression of α-cry activity.

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**Conflict of interest**---- The authors declare that there is no conflict of interest.

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