Cataract Induction by 1,2-Naphthoquinone
II. Mechanism of Hydrogenperoxide Formation and Inhibition by Iodide

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Naphthalene cataract is probably due to peroxide production through naphthoquinone (NQ) redox cycling and/or glutathione conjugation. Both mechanisms yield losses of essential SH-groups in cristallins and are thus probably involved in protein modification finally visible as lens opacity. 1,2-Naphthoquinone produces H_2O_2 in the presence of either ascorbate, glutathione, NADH or – to a lesser extend – by homogenates of lens protein preparations. In the presence of 1,2-naphthoquinone and the above reductive additions, both, oxygen uptake and H_2O_2 formation can be observed. Reductive oxygen activation in these systems are diminuated by iodide in a concentration-dependent manner. Since maleimide-treated proteins are less capable to activate oxygen by 1,2-naphthoquinone, a direct oxygen activation by the interactions of 1,2-naphthoquinone with protein-SH is indicated. Catalysis of "diaphorase"-type (dia) enzymes *via* NADH – dia – 1,2-NQ – O_2 seems not to operate in hydrogenperoxide production during 1,2-naphthoquinone lens toxicity.

Introduction

Several results of basic research during the last decades clearly indicate that senile lens turbidity is mediated by so-called co- and syncataractogenic processes. During syncataractogenesis oxidative damage due to different mediators has been indicated by different research groups [1–5]. It has been proposed that in 70 to 80% of all types of cataract oxidative procedures are involved yielding decreased content of SH-groups, increase of H_2O_2 in aqueous humour, fluorescence of cristallins and activity losses of lens enzymes. During all these processes covalent bonds are newly introduced in lens proteins resulting in aggregation and thus higher molecular weights of the cristallins [3, 5, 6]. Since it seems impossible to reverse these processes by topically applied drugs, the aim of topical cataract treatment can only be seen in retardation or slowering of the "turbidity-producing" process. Thus, it is also understandable that the activity of corresponding drugs can hardly be proven by clinical tests. Therefore, animal models have been used where artificially induced cataract formation is thought to be closly related to *in vivo* conditions simulating senile cataracts. In this context, naphthalene cataract in animals is one very common model [7–9]. The dibenzene configuration of naphthalene is metabolized in the liver and in the iris by cooperation of monooxygenases and phenol-oxidases producing 1,2-naphthoquinone which in turn seems to be one of the final cataract inducing chemicals.

According to Rossa and Pau [9] naphthalene cataract may be used as a model for subcapsular cataract which is one of the most abundant senile cataract forms. Metabolic naphthalene transformation in mammals may operate *via* P_{450} catalysis where an intermediary 1,2-epoxide is formed which is converted into the diole by epoxide hydrolases [10].

The next step, catalyzed by phenoloxidases, may transform the 1,2-dioles into the *o*-quinones which in turn can react with SH-groups of glutathione to form conjugates [11]. The addition of glutathione onto 1,2-naphthoquinones is essentially coupled with the loss of vital SH-groups of proteinacous and non-proteinacous nature and seems to cooperate with H_2O_2 formation. 1,2-Naphthoquinones seem to be mainly formed in the pigmented iris [7] indicating that this metabolite is first appearing in the aqueous humour from where it may finally penetrate anterior lens tissues. Therefore it also seems to be very likely that interactions of naphthoquinones with contents of the aqueous humour such as ascorbic acid and glutathione are of great

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importance. In addition to these activities, reactions with oxidoreductases (diaphorases) and phenol oxidases in the lens epithelia or in the cortex might be operating. We recently described model reactions indicating that iodide can actively interfere with different types of oxygen activation in the aqueous humour as well as in the lens [12–15]. For this purpose isolated rabbit, pig and bovine lenses have been used for describing the basic mechanisms of these interactions. In this context, isolated eye bulbs from rabbits have been used for testing penetration rates of iodide into the aqueous humour and different parts of the lens [16, 17]. Our models clearly show that iodide penetrates the corneal tissues and functions as an antioxidant preventing different types of photodynamic damage [12, 13, 16, 17]. In the present communication we describe possible mechanisms of naphthoquinone toxicity and the function of iodide preventing oxidative damage due to naphthoquinone-protein-SH interactions where hydrogen peroxide may operate as one of the toxic products.

Materials and Methods

Oxygen uptake has been determined potentiometrically in 2 ml vessels with an oxygen electrode (Rank Brothers, England) in combination with a two-channel recorder. The temperatures of the reaction mixtures were 22, 25 and 37 °C, containing different oxygen saturations of 532, 506 and 414 nmol O_2/2 ml, respectively.

All reactions contained 100 mM phosphate buffer, pH = 7.8.

Chemicals were from Sigma GmbH, Merck, Darmstadt or Boehringer, Mannheim, F.R.G.

Lens proteins were prepared from bovine lenses by stepwise ammoniumsulphate precipitation (35–75% saturation) with following 16 h dialysis against 50 mM phosphate buffer, pH = 7.8. The *post mortem* time of the bulbi between slaughtering and lens protein precipitation was approximately 4 h. Precipitated and dialyzed protein extracts were stored in small portions at −16 °C. The protein content was determined with the Biorad protein assay according to Bradford [18] yielding 2.54 mg/ml protein. Determination of SH groups was done according to Ellman [19, 20] with 5,5′-dithiobis-nitrobenzoic acid (DTNB) and spectrophotometric quantification at 412 nm. The protein extract with 2.54 mg/ml protein was determined as 3.6 mM in SH groups, according to a SH density of 1.42 μmol SH/mg protein.

The 1,2-naphthoquinone-4-sulfonic acid (sodium salt) contained traces of other reducing substances which could not be separated after several recristallizations. Further details to the experiments are given in the corresponding tables and figures, experiments have been done at least twice or as often as indicated.

Results and Discussion

Oxygen uptake in the presence of 1,2-NQ

1,2-NQ-sulfonate in the presence of lens protein, NADH, ascorbate or glutathione catalyzes oxygen reduction shown as oxygen uptake from an aqueous oxygen solution. The slow oxygen consumption by 1,2-NQ solutions (basal rate) can be explained with traces of reducing substances as impurities.

Rapid oxygen uptake is observed in the presence of 0.25 mM NADH or ascorbic acid where the strongest oxygen uptake is observed during the first minute of reaction and decreasing oxygen reduction in the second and third minute. Constant rates of oxygen reduction are observed in the presence of glutathione or lens proteins where the rate in the presence of lens proteins (0.25 mg protein/2 ml) is approximately half of the rate observed in the presence of 1 mM glutathione (Table I, Fig. 1). After addition of catalase after 6 min of reaction

Table I. Stimulation of the oxygen consumption [nmol O_2/min].

Naphtho-quinone	Addition of		
3.1	0.1 ml LCE (lens cortex extract)	8.25	(n = 4)
4.25	NADH	106.4	(1. min)
		66.5	(2. min)
		37.2	(3. min)
4.0	ascorbic acid	58.5	(1. min)
		37.2	(2. min)
		26.2	(3. min)
2.1	glutathione	16.0	(constant)

Reaction conditions:
NQ-sulfonic acid 1 mM
NADH 0.25 mM
Ascorbic acid 0.1 mM
Glutathione 1 mM
T = 22 °C

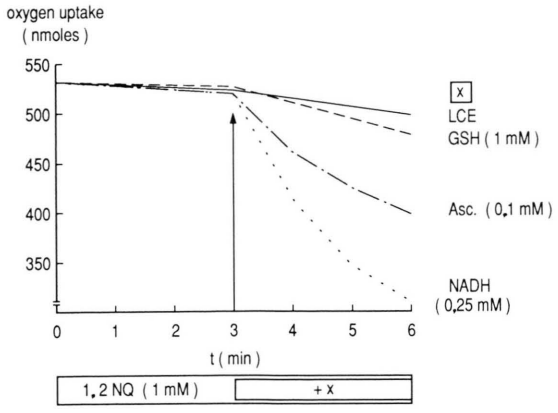

Fig. 1. Oxygen consumption by glutathione, ascorbate and NADH in the presence of 1,2-naphthoquinone. Reaction conditions as indicated in Table I.

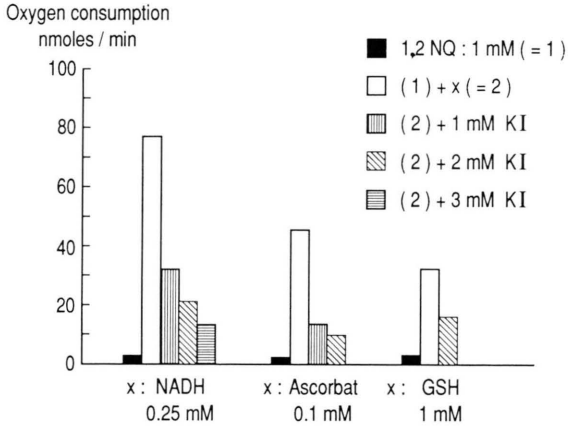

Fig. 2. Inhibition of O_2 uptake in the presence of 1,2-NQ and different electron donors by iodide.

Reaction conditions:

1,2-NQ-sulfonic acid	1 mM
Potassium iodide	1, 2, 3 mM
NADH	0.5 µmol
Ascorbic acid	0.1 mM
Glutathione	1 mM

$T = 22$ °C

time, approximately 50% of the amount of oxygen taken up is released as molecular oxygen. According to the reaction $H_2O_2 \xrightarrow{\text{CATALASE}} H_2O + \frac{1}{2} O_2$ an oxygen release of 50% during catalase treatment is indicating hydrogen peroxide as final reaction product of oxygen reduction (data not shown).

Since oxygen reduction is achieved merely in the presence of 1,2-NQ and NADH, GSH or ascorbate, a purely chemical reduction of oxygen *via* 1,2-NQ is indicated. A stimulation of oxygen uptake by lens protein preparations is probably due to the conjugation of SH groups to 1,2-NQ *i.e.* by reduction of the quinone. Formation of hydrogenperoxide occurs apparently without a lag phase where concentration-dependent ascorbic acid or NADH show approximately equal rates. The observed oxygen uptake is inhibited by iodide in a concentration-dependent manner (Fig. 2).

Inhibition of H_2O_2 formation by iodide

As shown in Fig. 2 increasing concentrations of iodide inhibit oxygen uptake by 1,2-NQ in the presence of either NADH, ascorbic acid or GSH. 1 mM of KI inhibit between 50% (GSH) and approximately 70% (ascorbate) of the basic rate. From the above results one might conclude that iodide and the above electron donors (NADH, GSH, ascorbate) interfere in the 1,2-NQ-oxygen redox cycle.

Lens proteins as electron donors: dependence of oxygen consumption on temperature and transition metals

Oxygen uptake in the presence of lens proteins and 1,2-NQ is stimulated at elevated temperatures: 37 °C instead of 22 °C increase oxygen reduction by approximately 40%. Chelex treatment of lens protein results in partial loss of oxygen reducing activities of the lens protein (Table II). Likewise, KI

Table II. Influence of protein on oxygen consumption [nmol/min].

T	NQ	Addition of LCE (0.1 ml)
22 °C	3.1 ± 1.8 (n = 7)	8.25 ± 0.7 (n = 4)
37 °C	5.0 ± 1.1 (n = 8)	10.8 ± 0.2 (n = 3)
37 °C (Chelex-treated buffer)	2.9 ± 1.0 (n = 13)	9.9 ± 0.2 (n = 3)

inhibits oxygen reduction by lens protein in the presence of 1,2-NQ (Table III). The results in Table III documents that oxygen reduction and thus H_2O_2 formation is partially due to the catalysis by transition metals (*cf.* Table II).

There is no accelerated reduction of 1,2-naphthoquinone by oxidoreductases. The stimulating effect of the lens cortex extract may be correlated with its content in SH groups.

Another enzymatic effect, the presence of peroxidases which may destroy hydrogenperoxide at the expense of iodide, is not feasible in this context, since iodide inhibits the first step (oxygen uptake), and is not involved in the following decrease of H_2O_2 by a peroxidase (data not shown).

Influence of sulfhydryl groups in 1,2-NQ-mediated oxygen reduction

To get information about the reactivity of SH groups with 1,2-naphthoquinone concerning the oxygen reducing capacity of the protein, we compared a glutathione solution, SH equimolar to the lens protein extract (3.6 mM SH in LCE). The content in sulfhydryl groups was determined according to Ellman as described in Materials and Methods.

The results in Table IV indicate that glutathione-SH show a higher rate in oxygen reduction than the protein-SH groups. The sulfhydryl groups in the small tripeptide seem to be better accessible for the naphthoquinone/oxygen redox cycle as compared to protein-SH.

Treatment with maleimide (1 and 10 mM) for 4 h and subsequent dialysis for 17 h results in partial or full loss in their specific activity as compared to a control of lens proteins incubated in buffer and dialyzed for the same time in the absence of maleimide. Maleimide (MI) *per se* has no influence on the determination of SH groups (Table V, Fig. 3).

As shown in Fig. 3, 1,2-NQ-dependent oxygen reduction yielding hydrogenperoxide is due to the availability of SH groups in the protein.

Table III. Influence of iodide on oxygen consumption [nmol/min].

NQ	Addition of	
2.8	LCE 0.1 ml	9.7
	+KI	1.1
3.3	glutathione	7.0
	+extract 0.1 ml	11.6
	+KI	3.3
4.1	ascorbic acid	6.2
	+LCE 0.1 ml	8.7
	+KI	1.4

Reaction conditions:
NQ-sulfonic acid 1 mM
Ascorbic acid 0.01 mM
Glutathione 0.1 mM
Potassium iodide 1 mM
$T = 37$ °C

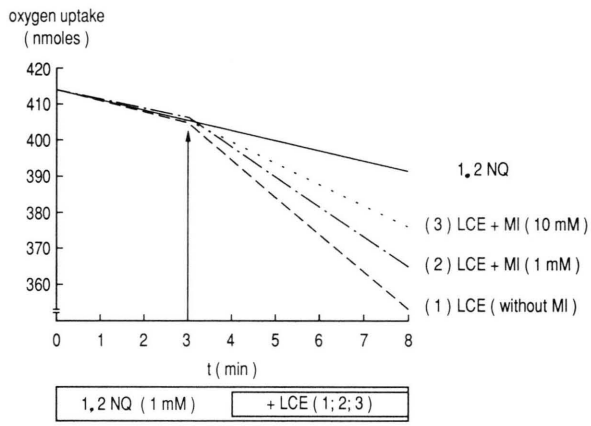

Fig. 3. Dependence on free SH groups of oxygen consumption [nmol/min] by lens cortex extract.

Table IV. Oxygen reduction by 1,2-NQ and glutathione or LCE [nmol/min].

1,2-NQ	+ GSH	+ LCE
	100 µl 5.33 ± 0.8 ($n = 3$)	100 µl 6.74 ± 0.25 ($n = 3$)
	200 µl 15.0 ± 1.3	200 µl 11.8 ± 0
	500 µl 29.2 ± 2.9	500 µl 18.4 ± 0.7

Reaction conditions:
1,2-NQ 0.5 mM
Glutathione 3.6 mM
LCE

Table V. SH content of LCE after maleimide treatment.

LCE	"Fresh" control 1	"Aged" control 2	Incubated with maleimide 1 mM	10 mM
mol SH/g LFW	7.9 ± 0.1	4.6 ± 0.6	2.2 ± 0.3	0.05
Reaction conditions:				
DTNB	0.1 mM			
T =	15 min			
V =	3 ml			

Influence of lens proteins on oxygen uptake by 1,2-NQ in the presence of NADH

In order to examine whether diaphorase activity could catalyze NADH-dependent oxygen uptake *via* naphthoquinone reduction, NADH and LCE were incubated in an oxygen electrode. As shown in Table VI, NADH alone strongly stimulates oxygen uptake. The addition of extract does not further stimulate this rate. The effect of 1 or 10 mM maleimide on the lens protein-mediated oxygen uptake clearly shows that no increased oxygen uptake is measured by the simultaneous presence of NADH and lens protein. This result indicates that the NADH – 1,2-NQ – redox reaction is not catalyzed by a flavoprotein (diaphorase)-like activity in the LCE.

Table VI. Influence of LCE/NADH on the oxygen consumption by 1,2-NQ [nmol/min].

NQ	Addition of	
3.1	NADH	26.9 (1. min)
		8.3 (2. u. 3. min)
	+0.1 ml LCE	13.7
2.9	NADH	22.8 (1. min)
		10.3 (3. min)
		6.2 (3.min)
	+0.1 ml LCE (1 mM maleimide)	9.9
2.1	NADH	26.9 (1. min)
		6.2 (2. u. 3. min)
	+0.1 ml LCE (10 mM maleimide)	5.4

Conclusions

The presented results indicate that

1. 1,2-naphthoquinones are chemically reduced by biological reducing agents (antioxidants) such as NADH, ascorbic acid or glutathione.
2. SH groups in the lens proteins can partially substitute for these reducing agents whereby oxygen uptake through 1,2-NQ is observed. The addition of catalase demonstrates that H_2O_2 is the product of these redox reactions. Maleimide treatment of lens proteins indicates that free SH groups in the proteins act as electron donating groups thus allowing a purely chemical interaction between proteins, NADH, glutathione and ascorbate with certain toxic quinones. We therefore can conclude that cataractogenic activity of 1,2-naphthoquinone is due to

a. SH, NADH and ascorbate depletion in aqueous humour and lens cortex and
b. formation of toxic H_2O_2 through chemical interactions.

Acknowledgements

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