Regulation of NAD(P)H quinone oxireductase 1 (NQO1) in SHSY-5Y neuroblastoma cells by chemopreventive agents

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Abstract
Introduction: High level of polyunsaturated fatty acids of lipid bilayer membrane coupled with increased oxygen consumption exposed brain cells to oxidative stress. Oxidative stress has been implicated as the etiological factor of many neurodegenerative diseases. Chemopreventive agents both synthetic and natural have the potential of inducing endogenous antioxidants with the capability of attenuating oxidant induced damage. This work examined the ability of tert-butylated hydroxyl quinone (tBHQ), ethoxyquin, coumarin and selenium to induce NAD(P)H quinine oxireductase 1 (NQO1) in neuroblastoma cell line.

Method: SHSY-5SY neuroblastoma cells were cultured in medium containing 100 μM of the individual chemopreventive agents or in combination with 50 nM sodium selenite. The cells were harvested after 24 h exposure with the chemopreventive agents. Protein levels were determined by immunoblotting, while enzyme assay was done using dichloro-phenol-indo-phenol as a substrate in the presence of NADH and FAD to determine reductase activity, while dicumarol was used as an inhibitor of NQO1.

Results: Coumarin, ethoxyquin and tBHQ-induced NQO1 marginally (1.2-1.5-fold). Treatment of cells with selenium as sodium selenite together with tBHQ-induced NQO1 by about 3-fold compared with control. The enzyme activity was significantly increased by all chemopreventive agents (p<0.05).

Conclusion: Inducers of endogenous antioxidants that can pass blood–brain barrier provide hope of delaying and attenuating neurodegenerative diseases associated with either increased free radical production or genetic predisposition.

1. Introduction

Brain consumes 20% of body oxygen despite constituting only 2% of body weight.¹ Polynsaturated fatty acids which are associated with the generation of reactive oxygen species constitute the greater part of nervous tissue cell membrane lipid bilayer.¹ Lower antioxidant concentration in addition to compartmentalization of antioxidant enzymes in different locations, such as catalase in neurons, while glutathione peroxidase 1 in astrocytes, exposed brain cells to injurious effects of oxidative stress.² Generation of oxidative stress in the brain is further exacerbated by high level of iron.² Metabolism of excitatory amino acids, influx of calcium into cytosol and metabolism of neurotransmitters such as adrenaline in neural cells are also potential sources of free radicals.²

Though the etiological factors of neurodegenerative diseases such as motor degenerative disease (MND), Alzheimer’s disease (AD) and Parkinson’s disease (PD) may be diverse, but all converge in the involvement of reactive oxygen species in their pathogenesis.²

Reduction of quinines and related compounds by NQO1 involves two-electron transfer from NADH or NAPDH.³,⁴ The flavoenzyme reduced the formation of ROS through decreasing one electron reduction in the associated redox cycling.⁴,⁵ It acts as an antioxidant enzyme through reduction of the oxidized form of vitamin E to a product with antioxidant properties.⁵ Furthermore, NQO1 is also a phase II detoxifying enzyme believed to be involved in cancer prevention.⁶ It also activates enzyme for some anticancer drugs,⁶ that play an important role in regulating the activity of these agents that are used in tumor targeting.

Diverse xenobiotics and oxidants such as tert-butylhydroquinone are known to induce the expression of NQO1.⁷ Higher organisms have evolved elaborate systems of protection against neurotoxic effects of electrophilic metabolites. Induction of phase II enzymes such as glutathione-S Transferases, NAD-(P):quinone reductase and increased intracellular reduced glutathione provide a robust protection against oxidative stress and the effect of toxic xenobiotics. Low concentrations of diverse chemical agents are known to activate...
transcriptional induction of phase II enzymes with the attending consequence of blocking chemical carcinogenesis. The induction by these chemical agents is mediated via antioxidant electrophile response element.8

The synergistic effects of expansive set of antioxidant/detoxification genes regulated by Cap ‘n’ Collar (CNC) transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) provide a powerful means of removing ROS/RNS species by binding to ARE/EpRE consensus sequence.9,10 tertbutylhydroquinone (tBHQ) an inducer of Nrf2 genes has been demonstrated to induce NQO1 via Nrf2 transcriptional factor in brain thereby reducing cortical damage and sensorimotor deficit after ischemia-reperfusion in rats.10

This study was an attempt to evaluate the potential of some chemopreventive agents property in the induction of NAD[P]H:[quinone acceptor]oxidoreductase (NQO1 EC 1.6.99.2), a phase two enzyme with a variety of antioxidant functions in the brain.

2. Materials and methods

2.1. Cell-line propagation

Human neuroblastoma cells were grown in Dulbecco’s modified medium (Gibco-BRL) supplemented with 15% (v/v) heat activated fetal calf serum, 500 i.u./ml penicillin and 500 mg/ml streptomycin at 37 °C in a humidified air/O2 (19:1) atmosphere. The cells were plated at a density of 2.5 × 10^6 cells in 400 cm² flask and grown to confluency. SHSY-5Y cells were split and seeded at a density of 10^6 cells in 400 cm² and grown to 80% confluency in media containing the following chemicals:

- a) 0.1% Dimethyl sulfoxide DMSO (control).
- b) 0.1% DMSO, 50 nM sodium selenite.
- c) 0.1% DMSO and 100 μM tBHQ.
- d) 0.1% DMSO, 50 nM sodium selenite and 100 μM tBHQ.
- e) 0.1% DMSO and 100 μM coumarin.
- f) 0.1% DMSO, 50 nM sodium selenite and 100 μM coumarin.
- g) 0.1% DMSO and 100 μM ethoxyquin.
- h) 0.1% DMSO, 50 nM sodium selenite and 100 μM ethoxyquin.

Stock solutions of Coumarin, ethoxyquin and tBHQ were prepared in DMSO. Final working concentration of the chemicals gave a concentration of 0.1% DMSO in the media.

The cells were then incubated for 18 h under the same conditions. The experiments were performed in triplicate.

Cells were then harvested and washed twice with phosphate buffered saline (PBS) and lysed in 10 mM phosphate pH 7.2 containing 0.1 M MgCl₂, 1 mM EDTA by sonication (Soniprep 150 MSE), by being given 3 pulses (amplitude 15) for 10 s each, with 10 s cooling interval between each pulse.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

2.2.1. Resolution of proteins by SDS-PAGE

Proteins were analyzed by SDS-PAGE according to Laemmli11 (1970) using a Bio-Rad mini-Protean vertical electrophoresis kit. Typically, 10-μg proteins from mouse cytosol were resolved in a 12% (w/v) polyacrylamide gel with an applied electromotive force (EMF) of 200 V.

2.2.2. Immunoblotting

Western blotting was carried out using a modified method of Towbin et al12(1979). Proteins that had been resolved by SDS-PAGE were electrotransferred to nitro-cellulose membrane (Millipore, Watford, Herts, UK). Even loading was determined by staining the nitrocellulose with Ponceau S prior to blocking with defatted milk. The blots were then probed with the human NAD[P]H quinone oxoreductase 1 antibody raised in rabbit. Band intensities were determined using a molecular dynamics model 300A computing densitometer.

2.2.3. Protein estimation

Estimation of protein concentration was performed by the method of Bradford13 (1976) adapted for the use on Cobas Fara centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, Herts, U.K.) (as adopted by Golloway et al14 (1999)).

2.3. NAD(P)H quinone oxoreductase 1 (NQO1) assay

Quinone reductase (QR EC 1.6.99.2) activity was determined as described by Benson et al15(1980) and adapted on the Cobas Fara centrifugal analyzer. 5 μl of tissue cytosol was added to a solution containing 100 μM NADH and 50 μM FAD. The enzyme reaction was initiated by adding 10 μl of 0.32 mM dichloro-phenol-indo-phenol (DCPIP). Quinone reductase-mediated reduction of DCPIP was monitored at 660 nm. The reaction again was monitored by mixing 5 μl of cytosol to a solution containing 100 μM NADH, 50 μM FAD and 10.67 μM dicumarol.

The specific activity was the difference between the slope with and without dicoumarol (vDCPIP 21,000 L mol⁻¹ cm⁻¹).

2.4. Antibodies

Human NADP(H) oxoreductase 1 antibody was a gift from Prof J.D. Hayes Biomedical Research Center, Faculty of Medicine, Ninewells Hospital University of Dundee. SHS-5SY cells were kind gift of Dr Kuren of Neuroscience department, Ninewells Hospital University of Dundee.

2.5. Statistical analysis

The mean value for each experiment was the average of triplicate tests in the same plate. Data are reported as the mean ± the standard error of the mean. Differences between treated and untreated were analyzed by one-way or multiple factor analysis of variance and the Student's unpaired t-test. Statistical significance was considered when p was less than 0.05 using Statview package (UK).

3. Results

3.1. NADPH quinone reductase 1 (NQO1)

NADPH quinone reductase 1 (NQO1) activity was significantly increased (p < 0.05) by tBHQ or ethoxyquin (approx 100-fold) and to a lesser extent by coumarin (approx. 14-fold) compared with control. Furthermore the addition of selenium potentiated the effects of tBHQ on NQO1 activity to 179 and 290-fold increase compared with selenium treated and control respectively (Table 1). Addition of selenium as sodium selenite also increased the enzyme activity by 1.7-fold compared with control. Co-administration of selenite and ethoxyquin has reduced the activity of the enzyme to 43 and 70-fold compared with selenite treated and control respectively (Table 1). However, co-administration Coumarin and selenite increased enzyme activity by 7 and 18-fold compared with selenite and control respectively.

Immunoblotting analysis in Fig. 1 shows both ethoxyquin and coumarin in either the presence or absence of selenium caused a marginal induction of NQO1 (1.2-fold). Treatment with tBHQ...
caused also a small induction of NQO1 in the absence of sodium selenite (1.5-fold). However, addition of sodium selenite to tBHQ induced NQO1 by 3-fold, which is commensurate with the observed increase in NQO1 activity in Table 1. Addition of selenite to tBHQ increased the activity of NQO1 from 100-fold in absence of selenite to 290-fold in the presence of selenite compared with control.

4. Discussion

Treatment of the neuroblastoma cell line SHSY-5Y with tBHQ and selenite caused a substantial induction of NQO1 (Fig. 1). This increase was accompanied by further increase in NQO1 activity. However, it is puzzling that co-treatment with ethoxyquin and sodium selenite attenuates an ethoxyquin-mediated increase in NQO1 activity. Such attenuation is likely to have been cause by selenite, as treatment with ethoxyquin alone caused an increase in NQO1 activity.

Though NQO1 is not a selenoprotein, but has been observed that selenium induces the expression of NQO1.16 In this study NQO1 activity was increased by 1.7-fold (p < 0.05) compared with control without significant increase in protein level (Table 1 and Fig. 1). Selenium as integral part of selenoproteins participating in antioxidant enzymes was examined by western blotting.20 Addition of selenite to SHSY-5Y cells which had been treated as follows: Lane (1) no treatment, (2) 50 nM selenium, (3) 100 nM tBHQ, (4) 100 nM tBHQ + 50 nM selenium, (5) 100 nM ethoxyquin, (6) 100 nM ethoxyquin + 50 nM selenium, (7) 100 µM coumarin, (8) 100 µM coumarin + 50 nM selenium, (9) standard.

<table>
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<tr>
<th>Treatment</th>
<th>nmol/min/mg protein</th>
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<tr>
<td>Control</td>
<td>34.21 ± 2.01</td>
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<tr>
<td>Selenium</td>
<td>55.23 ± 15.13</td>
</tr>
<tr>
<td>t-BHQ</td>
<td>3320.87±30.34*</td>
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<tr>
<td>t-BHQ + selenium</td>
<td>9860.52 ± 201.23**</td>
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<tr>
<td>Coumarin</td>
<td>485.25 ± 22.50</td>
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<tr>
<td>Coumarin + selenium</td>
<td>595.52 ± 55.24**</td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>3565.62 ± 155.32*</td>
</tr>
<tr>
<td>Ethoxyquin + selenium</td>
<td>2365.25 ± 173.98*</td>
</tr>
</tbody>
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NQO1 NAD(P)H quinone oxidoreductase.
**Statistical difference of p < 0.05 with respect to control with selenium.

All enzymes activities are expressed as nmol/min/mg protein and are average value from three separate cell lysate ± SEM. Statistical analyses were performed using unpaired Student’s t-test.

References


