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The Neuroprotective Antioxidant α-lipoic Acid Induces Detoxication Enzymes in Cultured Astroglial Cells

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α-Lipoic acid (LA), an antioxidant with broad neuroprotective capacity, is thought to act by scavenging reactive oxygen species and stimulation of glutathione synthesis. LA shows structural resemblance to dithiolethiones, like anethole dithiolethione (ADT). ADT protects against oxidative damage, primarily by induction of phase II detoxication enzymes, in particular NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-*S*-transferase (GST). Therefore, we investigated whether LA, like ADT, is capable also of inducing these protective enzymes.

Our data show that LA, like ADT, induces a highly significant, time- and concentration dependent, increase in the activity of NQO1 and GST in C6 astroglial cells. The LA or ADT mediated induction of NQO1 was further confirmed by quantitative PCR and western blot analysis.

This work for the first time unequivocally demonstrates LA mediated upregulation of phase II detoxication enzymes, which may highly contribute to the compounds' neuroprotective potential. Moreover, the data support the notion of a common mechanism of action of LA and ADT.

Keywords: Parkinson's disease; Glutathione-*S*-transferase; NAD(P)H:quinone oxidoreductase; Neuroprotection; Astrocyte; Antioxidant

Abbreviations: ADT, anetholedithiolethione; ARE, antioxidant response element; CDNB, 1-chloro-2,4-dinitrobenzene; DA, dopamine; DMSO, dimethylsulfoxide; GSSG, glutathione disulfide; GST, glutathione-*S*-transferase; LA, α-lipoic acid; NQO1, NAD(P)H:quinone oxidoreductase; Nrf, NF-E2-related factor; PD, Parkinson's disease; ROS, reactive oxygen species; SN, substantia nigra

INTRODUCTION

Oxidative stress is well accepted as a pathogenic factor in neurodegenerative syndromes, in particular Parkinson's disease (PD). PD is characterized at the cellular level by loss of dopamine-producing neurons in a brain area known as the substantia nigra (SN). One of the leading hypotheses concerning the pathogenesis of PD is that oxidative stress in the SN, caused by the production of reactive oxygen species (ROS) exceeding cellular antioxidant capacity, results in lipid peroxidation, loss of protein structure, and DNA damage which culminates in the death of susceptible dopaminergic neurons.[1] Due to their strong propensity for redox cycling, dopamine (DA) quinones formed during oxidative DA metabolism in the SN are thought to contribute substantially to oxidative stress mediated neurodegeneration in PD.[2]

NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-*S*-transferase (GST), two enzymes which according to insights into the functional consequences of their action can both be classified as phase II detoxication enzymes,[3–5] play a major role in cellular protection against the toxic effects of electrophiles like DA-quinones. Thus, as outlined recently, compounds capable of co-ordinately stimulating the activity of these enzymes may prove to be active as neuroprotective drugs in PD.[2]

Induction of phase II detoxication enzymes in general, and NQO1 and GST in particular, is evoked by an extraordinary variety of chemical agents, including a group of cyclic, sulphur-containing compounds known as dithiolethiones.[4,5] The phase II detoxication enzyme-inducing capacity of dithiolethiones has been linked to the reactivity of

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these compounds with sulfhydryl groups and subsequent activation of redox-sensitive gene transcription.[6] Interestingly, dithiolethiones show a distinct structural similarity to α-lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid),[2] a natural dithiol antioxidant with broad-spectrum neuroprotective efficacy.[7] Not surprisingly, most of the current interest in LA is centered around the free radical scavenging properties of exogenously supplied LA, its role in the regeneration of vitamin E and vitamin C, as well as the ability of LA to enhance intracellular levels of the endogenous antioxidant glutathione, presumably by increasing the availability of the precursor cysteine.[8,9] In addition, however, considering its structural characteristics it is feasible that LA, alike dithiolethiones, is also capable of inducing phase II detoxication enzymes, a feature which would contribute considerably to the compound's neuroprotective capacity. Hence, to address this issue and in line with our long-standing interest in astrocytes as target for neuroprotective treatment in PD, in the present study, we used our recently established C6 astroglial cell-line model[10] to compare the effect(s) of LA on NQO1 and GST activity with those of anethole dithiolethione (ADT, 5-(4-methoxyphenyl)-3*H*-1,2-dithiole-3-thione), a dithiolethione previously shown by us to protect astrocytes against ROS-mediated damage.[11]

MATERIALS AND METHODS

Cell Culture and Treatment of Cells

Rat C6 astroglioma cells were cultured as described previously.[10] Two days after seeding the cells in 12-well culture plates at a density of 4×10^4 cells/well, culture medium was replaced by freshly prepared medium containing either vehicle (DMSO) or experimental drugs, i.e. DL-LA (ICN Biomedicals Zoetermeer, The Netherlands) or ADT (Solvay Pharma). Following incubation for 24 and/or 48 h, cultures were evaluated for the catalytic activity of GST (EC 2.5.1.18) and NQO1 (EC 1.6.99.2), respectively, using the assays described below. In addition, the expression of NQO1 was also monitored at the mRNA and protein level.

Glutathione-*S*-transferase Assay

GST activity was assayed spectrophotometrically (340 nm) by monitoring the GST-catalyzed conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB, Sigma, St Louis, MO, USA) at 30°C.[12] After correction for protein content, specific enzyme activity was calculated using $\varepsilon_{\mathrm{CDNB}} = 9600\,\mathrm{l\,Mol^{-1}\,cm^{-1}}$.

NQO1 Enzyme Activity, Protein and mRNA Detection

NQO1 activity was determined spectrophotometrically (550 nm) by measuring NADPH-dependent-, menadiol-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) as described previously.[10] After correction for protein content, specific enzyme activity was calculated using $\varepsilon_{\mathrm{reducedMTT}} = 11,600\,\mathrm{l\,Mol^{-1}\,cm^{-1}}$.

Western blot analysis was performed using a mouse monoclonal antibody recognizing rat/human NQO1 protein (B771) in combination with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Denmark).[10] Protein bands were detected with ECL chemiluminescence (NEN Life Science Products, Boston, MA, USA), and quantified using an Imagestation (NEN Life Science Products).

To determine *NQO1* gene expression relative to β-actin, quantitative real-time PCR was performed using a combination of a 96-well spectrofluorometric thermal cycler (ABI Prism® 7700 sequence detection system and software; PE Biosystems, Foster City, CA, USA) and SYBR Green I dye chemistry.[13] In brief, upon isolation of total RNA using the RNeasy procedure (Qiagen, Chatsworth, CA, USA), reverse transcription of oligo (d15)T primed total RNA (1 μg) was performed for 1 h at 42°C as described previously.[10] PCR on cDNA was carried out in MicroAmp® optical 96-well reaction plates with 0.5 U AmpliTaq Gold® DNA polymerase using the recommended dNTP concentrations and buffer as supplied by the manufacturer. Primer pairs used: rat *NQO1* forward 5′-AACGTCATTCTCTGGCCA-ATTC-3′ /reverse 5′-GCCAATGCTGTACACCAG-TTGA-3′ (Genebank accession No J02640) and rat β-actin forward 5′-GCTCCTCCTGAGCGCAAG-3′/reverse 5′-CATCTGCTGGAAGGTGGACA-3′ (Genebank accession No J00691) (Isogen Bioscience, Maarssen, The Netherlands). The *NQO1* relative gene expression was calculated using the comparative threshold cycle (C_T) method. In brief, while the ΔC_T value for each sample is calculated by subtracting the C_T values for *NQO1* and β-actin, respectively, and the $\Delta\Delta C_T$ value by subtracting the ΔC_T value by the mean ΔC_T value of the vehicle treated control, the amount of target, i.e. normalized to an endogenous reference and relative to control, is given by $2^{-\Delta\Delta C_T}$.[13]

Statistical Analysis

Unless indicated otherwise, the values given represent the mean ± SD obtained from three independent experiments performed in triplicate. Statistical comparisons between groups were made

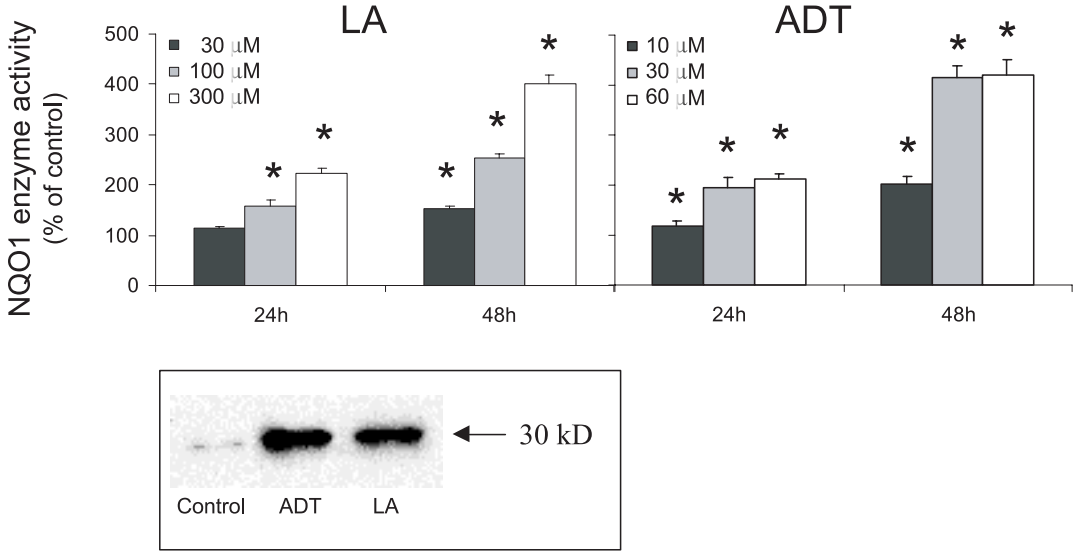

FIGURE 1 Upper panel: Time and concentration dependent effects of LA and ADT on NQO1 enzyme activity in cultures of C6 astroglioma cells. Specific NQO1 enzyme activity (nmol/min/mg protein) of vehicle-treated controls was: 1182 ± 105 (24 h) and 676 ± 97 (48 h). *Significantly different from control. Lower panel: Effect of 24 h treatment with ADT (60 μM, lane 2), LA (300 μM, lane 3) or vehicle control (lane 1) on NQO1 protein levels in C6 astroglioma cultures as determined by Western blot analysis. Results shown are from a representative experiment and demonstrate a 3.6 and 2.7 fold increase in NQO1 protein levels compared to control after treatment with ADT or LA, respectively. Similar data were obtained in two other independent experiments.

using a two-way analysis of variance (ANOVA) followed by a Newman–Keuls *post-hoc* test. p values <0.01 were considered significant.

RESULTS

Recently, we demonstrated that *NQO1* expression is increased upon exposure of C6 astroglial cells to the anti-Parkinsonian drug L-Dopa.[10] In the current study, the same cell line was used to monitor the effects of LA and ADT on NQO1 enzyme activity. In these experiments, both LA and ADT were used in concentrations that were previously shown to confer cytoprotection in various *in vitro* models.[9,11] As shown in Fig. 1, upper panel, exposure of C6 astroglial cells to LA (30–300 μM) or ADT (10–60 μM) for up to 48 h resulted in a highly significant, time- and concentration dependent increase in NQO1 activity. Since NQO1 activity is known to be determined primarily by the amount of NQO1 protein, which in turn is regulated at the level of *NQO1* gene transcription,[14] in addition NQO1 mRNA and protein levels were measured to establish a common basis for the LA and ADT induced upregulation of NQO1 activity. As shown in Table I, treatment of C6 astroglial cells with LA (300 μM) or ADT (60 μM) for 24 h indeed resulted in a marked and comparable increase in NQO1 relative gene expression. Similar results were obtained by western blot analysis of NQO1 protein levels in LA or ADT treated C6 astroglial cells, (Fig. 1, lower panel).

To further confirm the capacity of LA to act as a general inducer of phase II detoxication enzymes, GST enzyme activity in C6 astroglial cells was measured following exposure to LA (100 or 300 μM) or ADT (30 or 60 μM) for up to 48 h. As shown in Fig. 2, in line with the results on NQO1, treatment with LA or ADT resulted in a highly significant and sustained increase in the catalytic activity of GST in C6 astroglial.

DISCUSSION

Thus far, the neuroprotective efficacy of LA has been attributed almost exclusively to its ability to directly

TABLE I Relative *NQO1* gene expression in C6 astroglioma cells after treatment with LA or ADT

Drug	Concentration (μM)	NQO1 relative gene expression (normalized to control)
–		1.0 (0.9–1.2)
LA	300	6.3 (5.7–6.9)*
ADT	60	5.9 (5.6–6.2)*

C6 astroglioma cultures were exposed for 24 h to vehicle or drugs at the indicated concentrations. Values represent the mean and range (as calculated by evaluating the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the SD of the respective $\Delta\Delta C_T$ value) obtained from six culture wells, determined in two independent experiments. *Significantly different from control.

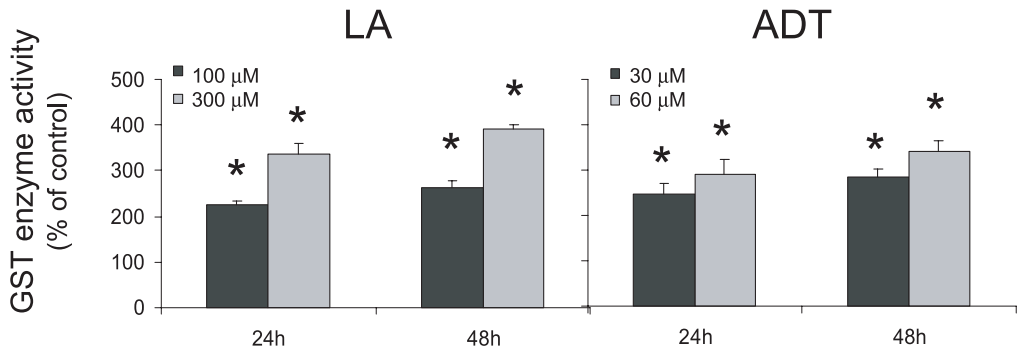

FIGURE 2 Time and concentration dependent effects of LA and ADT on GST enzyme activity in cultures of C6 astroglioma cells. Specific GST enzyme activity (nmol/min/mg protein) of vehicle-treated controls was: 24 ± 1.5 (24 h) and 31 ± 3 (48 h). *Significantly different from control.

scavenge a broad spectrum of ROS in combination with its stimulatory effect on glutathione synthesis.[7] The major finding of the present study is that, in addition, LA is a potent inducer of the phase II detoxication enzymes NQO1 and GST in astroglial cells. NQO1 and GST, which are known to be expressed by brain astrocytes *in vivo*,[15,16] play a crucial role in the detoxication of a wide range of highly neurotoxic agents that have been implicated in oxidative stress-mediated neurodegeneration. These include DA-quinones and aldehydes like the lipid peroxidation product 4-hydroxynonenal.[2,17] Thus, the simultaneous upregulation of NQO1 and GST activity in astroglial cells induced by LA may contribute substantially to the compound's neuroprotective capacity. It should be noted however, that because of our use of a racemic mixture of LA, at the present time no definite conclusion can be drawn about the contribution of either of the two stereoisomers.

Using NQO1 mRNA and protein levels as parameters, our data moreover suggest, that LA, alike ADT, mediates its stimulatory effect on the activity of these phase II detoxication enzymes through an increase in gene expression leading to enhanced protein levels. In fact, although definite information on the underlying mechanism is lacking for LA, dithiolethiones such as ADT have been shown to induce gene expression of NQO1 and various GST subtypes through activation of a common element present in the promotor region of the respective genes, which is known as the antioxidant response element (ARE).[5] Activation of the ARE by dithiolethiones is thought to follow modulation of cysteine moieties present in cytosolic signaling proteins and subsequent nuclear translocation of transcription factors such as Nrf1 and Nrf2, that bind and activate the ARE with high affinity.[5] Besides disulfides like the dithiolethiones, also vicinal dithioles, such as 1,2-ethanedithiol and 2,3-dimercapto-1-propanol, activate the ARE resulting in upregulation of phase II enzyme activity.[6] This suggests that reduction of the disulfide bond present in both LA and ADT might be a prerequisite in the molecular events culminating in the transcriptional activation of ARE-containing phase II genes by these compounds. In this context, it is of interest that LA readily undergoes intracellular reduction into dihydrolipoic acid through the action of a number of flavo-enzymes, in particular lipoamide dehydrogenase and glutathione disulfide (GSSG) reductase.[9,18] As described very recently, the redox couple formed in this manner, which consist of LA and its vicinal dithiol form, optimally fits the requirements for induction of a protective "phase II response".[19] Whether activation of the ARE indeed underlies the LA-induced upregulation of NQO1 and GST activity in C6 astroglial cells, as observed in the current study, remains to be investigated.

In addition to phase II detoxication enzymes, ADT and related dithiolethiones are also known to upregulate the expression of a broad spectrum of other protective enzymes and proteins. Besides antioxidant enzymes such as heme-oxygenase I, manganese-dependent superoxide dismutase and catalase, these include γ-glutamylcysteine synthetase, i.e. the enzyme catalyzing the rate-limiting step in glutathione synthesis, and the iron-binding protein ferritin.[20,21] Moreover, dithiolethiones have been shown to stimulate the activity of enzymes responsible for maintenance of the cellular redox-state, in particular GSSG reductase and glucose 6-phosphate dehydrogenase.[22] In light of the current results, it is tempting to suggest that a similar mode of action may underlie the remarkable range of (neuro)protective effects documented for LA both *in vitro* and *in vivo*. Experiments designed to test this hypothesis are underway in our laboratory.

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