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Dual Neuroprotective Pathways of a Pro-Electrophilic Compound via HSF-1-activated Heat Shock Proteins and Nrf2-activated Phase 2 Antioxidant Response Enzymes

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Abstract

Activation of the Keap1/Nrf2 pathway and consequent induction of phase 2 antioxidant enzymes is known to afford neuroprotection. Here, we present a series of novel electrophilic compounds that protect neurons via this pathway. Natural products, such as carnosic acid (CA), are present in high amounts in the herbs rosemary and sage as *ortho*-dihydroquinones, and have attracted particular attention because they are converted by oxidative stress to their active form (*ortho*-quinone species) that stimulate the Keap1/Nrf2 transcriptional pathway. Once activated, this pathway leads to the production of a series of antioxidant phase 2 enzymes. Thus, such dihydroquinones function as redox-activated “pro-electrophiles.” Here, we explored the concept that related *para*-dihydroquinones represent even more effective bioactive pro-electrophiles for the induction of phase 2 enzymes without producing toxic side effects. We synthesized several novel *para*-hydroquinone-type pro-electrophilic compounds (designated D1 and D2) in order to analyze their protective mechanism. DNA microarray, PCR, and Western blot analyses showed that compound D1 induced expression of heat-shock proteins (HSPs), including HSP70, HSP27 and DnaJ, in addition to phase 2 enzymes such as hemeoxygenase-1 (HO-1), NADP(H) quinine-oxidoreductase1, and the Na⁺-independent cystine/glutamate exchanger. Treatment with D1 resulted in activation of Nrf2 and HSF-1 transcriptional elements, thus inducing phase 2 enzymes and HSPs, respectively. In this manner, D1 protected neuronal cells from both oxidative and endoplasmic reticulum (ER)-related stress. Additionally, D1 suppressed induction of GRP78, an ER chaperone protein, and inhibited hyperoxidation of peroxiredoxin 2 (PRX2), a molecule that in

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it reduced state can protect from oxidative stress. These results suggest that D1 is a novel pro-electrophilic compound that activates both the Nrf2 and HSF-1 pathways, and may thus offer protection from oxidative and ER stress.

Introduction

Activation of the Nrf2/antioxidant response element (ARE) pathway

Electrophilic molecular species can induce the expression of a set of antioxidant enzymes, called 'phase 2 enzymes,' which include hemeoxygenase-1 (HO-1), NADPH quinone oxidoreductase 1 (NQO1), sulfiredoxin, glutamyl cysteine ligase modifier subunit (GCLM), and the Na⁺-independent-cystine/glutamate exchanger (xCT), all of which provide efficient cytoprotection by regulating the intracellular redox state (Talalay 2000; Itoh K *et al.* 2004; Sato and Lipton 2007; Soriano *et al.* 2008; Sato *et al.* 2000, 2001, 2003, 2006). A key cascade in activating the transcription of genes encoding phase 2 enzymes involves the Keap1/Nrf2 pathway, which is comprised of Keap1, a regulator protein, and Nrf2, a transcription factor that when released from Keap1 in the cytoplasm, enters the nucleus and binds to the antioxidant response element (AREs) (Talalay 2000; Itoh K *et al.* 2004; Padmanabhan *et al.* 2006; Sato *et al.* 2006; Wang *et al.* 2010). Mechanistically, when electrophiles react with critical cysteine residues on Keap1 protein to form a covalent adduct, they perturb this system, thereby releasing Nrf2 and allowing it to be translocated from the cytoplasm into the nucleus, where it binds to AREs and stimulates transcription of phase 2 enzyme genes (Itoh K *et al.* 2004; Padmanabhan *et al.* 2006; Sato *et al.* 2009b). Thus, Nrf2 has been considered a potential therapeutic target for the treatment of neurodegenerative diseases (Mattson and Cheng 2006; Sato and Lipton 2007; Vargas and Johnson 2009).

Hyperoxidation of peroxiredoxin (PRX)

Aside from the glutathione system, the thioredoxin (TRX)-PRX pathway is arguably the most important antioxidant system that limits accumulation of intracellular peroxides via redox reactions at critical cysteine residues (Holmgren and Lu 2010; Winyard *et al.* 2005; Immenshuh and Baumgart-Vogt 2005). Expression of TRX-PRX is controlled by the Nrf2/ARE system (Soriano *et al.* 2008). In particular, TRX1-PRX2 is extremely abundant in the brain. The TRX1-PRX2 system detoxifies peroxides by transferring reducing equivalents from NADPH to peroxides via TRX reductase, TRX1, and finally PRX2 (Winyard *et al.* 2005; Immenshuh and Baumgart-Vogt 2005). The TRX1-PRX2 system can react with at least two types of oxidants, nitric oxide (NO) and peroxide (Fang *et al.* 2007). Through reaction with NO, PRX2 is S-nitrosylated at redox-active cysteine residues Cys-51 and Cys-172 to form PRX2-SNO, thus inhibiting the neuroprotective effect of PRX2 against oxidative stress (Fang *et al.* 2007). S-nitrosylation of PRX2, resulting in PRX2 dysfunction, therefore mechanistically links nitrosative and oxidative stress to neurodegeneration (Fang *et al.* 2007). In the absence of NO, PRX2 forms PRX2-SOH via reaction with peroxide, and can be further oxidized to a sulfinic (-SO₂H) or sulfonic (-SO₃H) acid derivative. This hyperoxidation of PRX2 causes inactivation of its peroxidase/neuroprotective activity (Rhee *et al.* 2007). Although PRX2-SO₂/3H cannot be reduced by TRX, it can be reduced back to

the catalytically active free thiol form in eukaryotic cells by the ATP-dependent reductase, sulfiredoxin 1 (SRXN1; Rhee *et al.* 2007). The activity of SRXN1 restores inactive PRX2-SO₂/3H back to the TRX cycle and prevents permanent oxidative inactivation of PRX2 by strong oxidative insults (Soriano *et al.* 2008). The induction of SRXN may represent a critical event for the neuroprotective effect of thiol-containing electrophilic compounds (Soriano *et al.* 2008).

HSF-1 system

Oxidative stress can also lead to endoplasmic reticulum (ER) stress, which may play a key role in chronic neurodegeneration; in this case, accumulation of misfolded proteins in the ER induces ER dysfunction (Bukau *et al.* 2006; Bredesen 2008; Morimoto 2008; Kim *et al.* 2008; Nakamura and Lipton 2009). To limit such stress, a powerful endogenous protective mechanism is represented by the induction of molecular chaperones, including heat-shock proteins (HSPs) such as HSP70 and DnaJ, as well as heat-shock factor-binding protein (HSBP) and heat-shock 105-kDa/110-kDa protein 1 (HSPH1). These molecular chaperones are known to suppress protein misfolding (Bukau *et al.* 2006; Morimoto 2008; Kim *et al.* 2008; Nakamura and Lipton 2009). The expression of molecular chaperones after exposure to various types of cell stress is known to be regulated by heat-shock transcription factor 1 (HSF-1). Under unstressed conditions, HSF-1 is localized in the cytosol and is inactivated in a protein complex including HSP90. Upon exposure to stress-inducing compounds, HSF-1 dissociates from the HSP90 protein complex, translocates into the nucleus, and binds to the HSE response element (HSE) in the promoter region of various molecular chaperone genes to induce their expression. For this reason, HSF-1 has been considered to be a potential target for the treatment of neurodegenerative diseases (Fujimoto *et al.* 2005; Morimoto 2008; Trott *et al.* 2008; Fujikake *et al.* 2008). In fact, several HSF-1 activators reportedly protect neurons and the brain *in vivo* (Sano 2001; Lu *et al.* 2004; Trott *et al.* 2008; Fujikake *et al.* 2008).

Novel electrophiles

Hydroquinone-type compounds, such as carnosic acid (CA) and *tert*-butyl hydroquinone (TBHQ), are not themselves electrophilic but become electrophilic via oxidative conversion to the *quinone*, which occurs when they encounter free radicals generated by cellular oxidative stress (Sato *et al.* 2006, 2008a, 2008b, 2009a; Lipton 2007; Tamaki *et al.* 2010; Lee *et al.* 2003; Kraft *et al.* 2004; Shih *et al.* 2005). Thus, these *hydroquinone-type* compounds act as pro-electrophilic drugs, which require conversion from *hydroquinone* to *quinone* in order to exert their neuroprotective effect (Sato *et al.* 2008a and 2009a). In the present study, we synthesize new parahydroquinone-type molecular compounds in order to generate improved neuroprotective electrophiles. We prepared two novel pro-electrophilic molecules (D1 and D2, Fig. 1C) for this purpose and investigated their protective actions against oxidative and ER stress-induced neuronal cell death. We found that the HSF-1/HSE pathway may be activated along with the Nrf2/ARE pathway in the neuroprotective action of such electrophilic drugs.

Materials and methods

Materials

Antibodies and their sources were as follows: anti-Nrf2 and anti-78 kDa glucose-regulated protein (GRP78) polyclonal rabbit IgGs (H300 and H129, respectively, Santa Cruz Biotech, Santa Cruz, CA), anti-HSF-1 polyclonal rabbit IgG (#4356, Cell Signaling Tech, Danvers, MA), anti-HO-1 polyclonal rabbit IgG (OSA-150, Assay design, Plymouth Meeting, PA), anti-HSP70 monoclonal mouse IgG (200-301-A27, Rockland Immunological Inc., Gilbertsville, PA), anti-actin monoclonal mouse IgM (#MAB1501, Millipore, Billerica, MA), anti-microtubule-associated protein2 (MAP2) monoclonal mouse IgG (HM2, Sigma-Aldrich, St. Louis, MO), anti-NeuN monoclonal IgG (A60, Millipore, Billerica, MA), anti-PRX2 rabbit polyclonal IgG (LF-PA0007, Lab Frontier, Clear Lake, IA), anti-PRX2-SO₃H rabbit polyclonal antibody (ab16830, Abcam, Cambridge, MA), peroxidase-conjugated anti-mouse IgM (Calbiochem, Darmstadt, Germany), peroxidase-conjugated anti-rabbit IgG, FITC-conjugated anti-rabbit IgG, and rhodamine-conjugated anti-mouse IgG, (Jackson Immuno Research Laboratories, West Grove, PA). Other chemicals including dimethylsulfoxide (DMSO), sodium glutamate, fluorescein diacetate (FDA), hydrogen peroxide (H₂O₂) and Hoechst 33,258 were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibody against human Srxn1 is courtesy of Dr. Sue Goo Rhee, Ewha Womans University, Seoul, South Korea (Chang *et al.* 2004).

Culture of human retinal pigmented epithelial cells 19 (ARPE-19) cells

The outermost layer of human retina is the retinal pigment epithelium (RPE). This RPE represents a primary target of injury in age-related macular degeneration (AMD), which is thought to be induced in part by oxidative stress. ARPE-19 cells are a primary human cell line derived from human RPE. To study the biochemistry and molecular biology of oxidative stress in ARPE-19 cells, several investigators have used these cells for in vitro culture. For example, exposure of ARPE-19 cells to H₂O₂ is known to induce apoptosis and has thus been used as an *in vitro* model of retinal degeneration triggered by oxidative stress. ARPE-19 cells were obtained from ATCC and maintained in 10-cm dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Zareba *et al.* 2006). The cells were introduced into wells of a 24-well plate at a density of 10⁵ cells/cm² and incubated for 24 h. The medium was exchanged for serum-free medium containing the compounds to be tested (D1 or D2; 20 μ M), and the cultures were incubated for 24 h. Then, H₂O₂ or tunicamycin (TM) was added and the cells incubated for an additional 4 h or 24 h, respectively. Finally, the cells were stained with FDA (1 μ M) and Hoechst dye 33,258 (5 μ g/ml) and then observed under epifluorescence microscopy.

Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD). An analysis of variance was performed for multiple comparisons and a Student's *t*-test for single comparisons.

Other Methods are mentioned in the Supporting Document.

Results

Induction of phase 2 enzymes and HSPs by the novel pro-electrophilic drug, D1

We hypothesized that the biological activity of D1 and D2 should be closely related to their transcriptional activation of the Nrf2/ARE pathway. In order to examine this possibility, we initially performed a microarray analysis using ARPE-19 cells exposed to these compounds vs. control diluent (Table S1). The top 20 genes induced by 20 μ M D1 or D2 are listed in Table S1. D1 induced several phase 2 enzymes such as HO-1 and xCT, as well as HSPs. In contrast, induction of these genes by D2 was very small or completely negative. In order to confirm that D1 indeed induced the genes encoding phase 2 enzymes and HSPs, we performed an RT-PCR analysis using primers for HO-1, xCT, GCLM, and NQO-1 (phase 2 enzymes) as well as for HSP70, DnaJ, HSBP, and HSPH1 (HSPs) (Fig. 1A). All of these were significantly induced by D1, although the magnitude of induction varied from gene to gene. In contrast, these same genes were only very weakly induced by D2 (Fig 1A). HO-1, xCT, and HSP70 were strongly induced by D1, whereas GCLM, NQO1, DNAJ, HSBP, and HSPH1 manifested weaker, yet significant induction. Next, we confirmed the induction of HO-1 and HSP70 at the protein level by performing Western blot analysis (Fig 1B). D1 induced HO-1 and HSP70 proteins in a dose-dependent manner. Taken together, these data suggested that D1 potently induced both phase 2 enzymes and HSPs.

Translocation of Nrf2 after treatment with D1

Induction of phase 2 enzymes and HSPs is largely regulated by the transcription factors Nrf2 and HSF-1, respectively. For transcriptional activation, both factors must be in the nucleus. Thus, we determined the distribution of Nrf2 and HSF-1 in cells in the presence and absence of D1 (20 μ M). In an analogous fashion to NF- κ B and I κ B, Nrf2 translocates from the cytoplasm into the nucleus after dissociation from Keap1. In the absence of D1, we observed that Nrf2 was predominantly localized in the cytoplasm (Fig. 2A). When D1 was added, we found a dramatic translocation of Nrf2 into the nucleus (Fig. 2A). In contrast, in some but not all cases, HSF-1 has been found in the nucleus even under basal conditions (Nakai *et al.* 1995; Mercier *et al.* 1999). Here, we found that HSF-1 was present in the nucleus under control conditions even in the absence of D1 (Fig. 2B).

Activation of ARE and HSE after treatment with D1

Next, we investigated whether D1 would activate both Nrf2 and HSF-1. To test this notion, we performed reporter gene assays on ARPE-19 cells transfected with the cDNAs under the transcriptional control of the ARE (Fig. 3A) and HSE (Fig. 3B). We found that the response to D2 was completely negative, whereas D1 significantly activated both transcriptional elements, indicating that D1 activated both the Nrf2/ARE and HSF-1/HSE systems.

Reduction of GRP78 by D1

We next studied whether D1 could ameliorate ER stress through induction of HSPs. In particular, HSP70 is engaged in a number of folding events, including folding newly synthesized proteins, transporting proteins across membranes, refolding of misfolded or aggregated proteins, and controlling the activity of regulatory proteins (Morimoto 2008). To

monitor the effect of D1 on ER stress, we performed Western blotting using anti-GRP78, a marker of ER stress (Fig. 4). GRP78 is rapidly induced during ER stress in the typical unfolded protein response (UPR) (Bukau *et al.* 2006). Thus, if D1 could ameliorate ER stress by induction of HSPs, we would expect that the level of GRP78 would be decreased. We found that D1 markedly increased the levels of HO-1 and HSP70 both in the absence and presence of 1 μ M TM (Fig. 4B), suggesting that ER stress did not affect the induction of these proteins. However, the addition of 1 μ M TM markedly increased the level of GRP78 protein, indicating that TM induced ER stress, but this increase was markedly attenuated by D1 (Fig. 4A). These results suggest that D1 could relieve ER stress, possibly through induction of HSPs.

Inhibition of PRX2 hyperoxidation by D1

One of the most effective protective mechanisms against peroxide is the TRX1-PRX2 antioxidant system in the brain (Winyard *et al.* 2005; Immenschuh and Baumgart-Vogt 2005). When there is a peroxide overload, thiol groups on PRX2 are oxidized to -SOH (sulfenic acid), which is reversible by TRX1, but intense insult results in hyperoxidation to -SO₂H (sulfinic acid) or -SO₃H (sulfonic acid) that is not enzymatically reversed by TRX1. However, SRXN1 can reduce -SO_{2/3}H to -SH in the presence of TRX, ATP, and NADPH (Rhee *et al.* 2007). Thus, we hypothesized that this defense system might play a role in the protective effect afforded by D1 against oxidative stress. To test this idea, we examined the ratio of PRX2-SO_{2/3}H/PRX2 at the protein level in ARPE-19 cells exposed to 1 mM H₂O₂. The ratio was very low under control conditions but increased after exposure to H₂O₂ (Fig. 5A). Pretreatment with D1 significantly inhibited the ratio, suggesting that D1 led to chemical reduction of PRX2-SO_{2/3}H to PRX2-SH. Since the Nrf2/ARE pathway mediates transcriptional activation of SRXN1 (Soriano *et al.* 2008) and we had shown that D1 activates the ARE, we monitored SRXN1 expression by RT-PCR (Fig. 5B) and western blot (Fig. 5C) after treatment with D1. Indeed, we found that D1 increased SRXN1 message and protein, consistent with transcriptional induction (Fig. 5B and C).

Protective effect of D1 against oxidative and ER stress in ARPE-19 cells and cortical neurons

Importantly, our previous work had shown that pro-electrophilic drugs afford protection against oxidative stress (Sato *et al.* 2006, 2008a, 2008b, 2009a, 2009b; Sato and Lipton 2007); thus, we tested D1 in this manner. Initially, we examined whether D1 could protect ARPE-19 cells from H₂O₂. This retinal-derived cell lines is often used as an experimental model of oxidative stress in age-related macular degeneration (Wada *et al.* 2001; Kim *et al.* 2003; Zareba *et al.* 2006; Mandel *et al.* 2009). We found that exposure to 1 mM H₂O₂ for 4 h in serum-free medium induced extensive cell death (Fig. 6), but D1 significantly protected the cells. Similarly, D1 also protected primary cortical neurons in culture from H₂O₂ (Fig. S2).

Next, we considered the possible effect of D1 on ER stress because many reports have suggested that induction of HSPs can increase cell resistance to ER stress, and we had found that D1 increased HSPs. Indeed, we observed that D1 decreased susceptibility to ER stress elicited by TM in both ARPE-19 cells and primary cortical neurons (Figs. 6 and S2).

Protective effect of D1 against oxidative glutamate toxicity in HT22 cells

Finally, we examined the protective effects of D1 against oxidative glutamate toxicity in mouse hippocampal HT22 cells, representing another type of oxidative stress. In these cells, a high concentration of glutamate induces cell death through depletion of glutathione, which is induced by inhibition of the glutamate-cystine antiporter (Tan *et al.* 1998; Sagara *et al.* 2000). We found that the addition of D1 significantly protected the cells against oxidative glutamate toxicity ($IC_{50} = 8.6 \mu M$), whereas D2 was ineffective (Fig. S3). The protective effect of D1 required preincubation prior to the addition of the glutamate. In fact, when D1 was added simultaneously with glutamate, the cells were not protected (data not shown). This finding is consistent with the mechanism shown above that transcriptional events are essential for the protective effects of D1.

Discussion

Involvement of both the HSF/HSE and Nrf2/ARE pathways in protection by pro-electrophilic drugs

An important point of the present study is the discovery that the HSF-1/HSE pathway is a contributing neuroprotective mechanism that is activated by the novel electrophile, D1. In previous studies, we and others had found that activation of the Nrf2/ARE pathway is an important neuroprotective mechanism for electrophilic compounds (Satoh and Lipton 2007; Vargas and Johnson 2009). This pathway represents an endogenous protective mechanism against oxidative stress because it stimulates transcription of several phase 2 enzymes that are involved in redox regulation. Additionally, it has been previously reported that activation of Nrf2 in turn stimulates HSP70 production (Renaldi et al. 2011). Accordingly in the present study, we show that D1 also activates the HSF-1 transcriptional pathway to decrease the level of hyperoxidized PRX2. By microarray analysis and RT-PCR, we show that D1 induces the phase 2 enzymes α CT and GCLM, which are involved in the regulation of glutathione metabolism. This finding is particularly important for the potential clinical tolerability for D1 since electrophiles that have proven to be toxic actually react with GSH to lower the cell content of reduced glutathione (Satoh and Lipton 2007). Additionally, we found that D1 induces SRXN1, which subsequently reduces hyperoxidized PRX2 to native PRX2 (Fig. 5).

Here, we show that the HSF-1/HSE pathway also contributes to protection by at least some types of electrophilic compounds, including D1. The HSF-1/HSE pathway has been considered an endogenous protective mechanism, primarily from ER stress (Morimoto 2008). We show that our novel compound D1 can not only protect from oxidative stress by activating the Nrf2/ARE pathway, but also attenuates ER stress by activating the HSF-1/HSE pathway (Fig. 7).

The HSF-1/HSE system

The ER is a reticulated organelle in which proteins are synthesized and modified for proper folding. Approximately 30% of newly synthesized proteins in normal cells are misfolded, and some of these proteins are refolded to achieve their correct structures. This process of refolding is facilitated by ER chaperones. However, other proteins remain misfolded,

accumulate in the ER, and induce ER stress (Bukau *et al.* 2006; Morimoto 2008). Transcriptional induction of molecular chaperones is governed by the stress-inducible heat-shock transcription factor known as HSF1, which plays a key regulatory role in the response to environmental stress (Bukau *et al.* 2006; Morimoto 2008). HSF-1-modulating compounds such as HSP90 inhibitors have protective effects against various types of stress including ER stress (Fujimoto *et al.* 2005; Fujikake *et al.* 2008; Trott *et al.* 2008). Interestingly, radicicol and geldanamycin, HSF-1 activators, protect neurons against oxidative stress (Sano 2001; Xiao *et al.* 2002). Thus, this HSF-1/HSE system may be protective against oxidative stress in addition to ER stress. In this background, we found in the present study that D1-mediated induction of HSPs plays a role in neuroprotection against ER stress as well as against oxidative stress. While electrophiles have been shown to affect HSPs (Groeger and Freeman 2010), to our knowledge, this is the first report of a pro-electrophilic drug inducing transcription via the HSF-HSE pathway in addition to the Nrf2/ARE pathway.

The Nrf2/ARE system

Several groups, including our own, have reported that the Nrf2/ARE system is the primary target of electrophilic neuroprotective compounds such as NEPP11, CA, TBHQ, celastrol, plumbagin, ceftriaxone, and 3H-1,-dithiole-3-thione (Kraft *et al.* 2004; Sato *et al.* 2006; Sato and Lipton 2007; Soriano *et al.* 2008; Trott *et al.* 2008; Lewerenz *et al.* 2009; Vargas and Johnson 2009; Son *et al.* 2010; Wang *et al.* 2010). Para-hydroquinone type electrophiles, such as TBHQ, can produce oxidative stress themselves via conversion to a quinone, which in turn may be responsible for activation of the Keap1/Nrf2 pathway (Imhoff and Hansen 2010). Because D1 is also a para-hydroquinone-type electrophile, it is possible that D1 might activate the Nrf2 pathway by this mechanism. Prior microarray analyses using CA and TBHQ have suggested that many of the genes induced by electrophiles are phase 2 enzymes (Takahashi *et al.* 2009; Tamaki *et al.* 2009; Lee *et al.* 2003). One of the enzymatic pathways so induced results in an increase in glutathione synthesis (Sato *et al.* 2008a, 2008b; Shih *et al.* 2005). Accordingly, in the present study we show that D1 induced GCLM and xCT, both of which are involved in the regulation of glutathione metabolism. Several groups, including our own, have proposed that prolonged activation of this pathway by electrophilic compounds may be an effective pharmacological approach to help combat neurodegenerative diseases. This proposal was based on the hypothesis that oxidative stress mediates, at least in part, the neuronal damage caused by various types of stress (Sato and Lipton 2007; Hara and Snyder 2007; Nakamura and Lipton 2007). Several reports, however, have suggested that oxidative stress is not the initial event leading to neurodegeneration. Instead, ER stress, precipitated by the accumulation of specific misfolded proteins, such as β -amyloid, α -synuclein or polyQ-containing peptides, is often considered to be the inciting event (Hara and Snyder 2007; Kim *et al.* 2008; Bredesen 2008; Nakamura and Lipton 2009). Thus, in order for an electrophilic compound to be maximally effective for neurodegenerative diseases, mechanistic action against ER stress in addition to oxidative stress would be most helpful. Our present discovery with the novel pro-electrophilic drug D1 appears to meet this requirement, and thus further investigation of this and related compounds is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ARE	antioxidant response element
ARPE	apical retinal pigment epithelium
CA	carnosic acid
DIV	days <i>in vitro</i>
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
E17	embryonic day 17
ER	endoplasmic reticulum
FCS	fetal calf serum
FDA	fluorescein diacetate
GCLM	glutamyl cysteine ligase modifier subunit
GRP78	78 kDa glucose-regulated protein
H₂O₂	hydrogen peroxide
HO-1	heme oxygenase-1
HSE	heat-shock factor response element
HSF-1	heat-shock factor-1
HSP	heat-shock protein
HSPH1	heat-shock 105kDa/110kDa protein 1
HSBP	heat-shock factor-binding protein
HSE	heat-responsive element
MAP2	microtubule-associated protein 2
NQO1	NADPH quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NEPP	NEurite outgrowth-Promoting Prostaglandin
NO	nitric oxide
PBS (-)	Ca ²⁺ , Mg ²⁺ (-)-phosphate-buffered saline
PRX2	peroxiredoxin2
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SRXN1	sulfiredoxin1
TBHQ	<i>tert-butyl</i> hydroquinone
TRX	thioredoxin
TM	tunicamycin
UPR	unfolded protein response
xCT	Na ⁺ -independent cystine-glutamate exchanger

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Fig. 1. Induction of phase-2 enzymes and HSPs by D1

(A) PCR analysis of phase 2 and HSP genes induced by 20 μM D1. Total RNAs were extracted from ARPE cells after treatment with 20 μM D1 or D2 for 24 h in serum-free medium. RT-PCR was performed with the specific primers listed in the Materials and Methods. (B) Western blot analysis to show dose-dependent induction of HO-1 and HSP70 proteins by D1. Lysates were prepared from cells after treatment with various concentrations of D1; 10 μg protein samples were loaded per lane and subjected to SDS-PAGE prior to immunoblotting. The experiments were repeated three times with similar results. *Lower panel:* Cell lysates (10 μg protein/lane) were subjected to SDS-PAGE and immunoblotted for HO-1 and HSP70. *Upper panel:* Analysis of HO-1 and HSP70 normalized to β-actin by taking the ratio of their densitometric values on immunoblots. * $p < 0.05$ from control cells.

Fig. 2. Nuclear translocation of Nrf2 and HSF-1 after treatment with D1

ARPE-19 cells were plated at 10^5 cells/cm² and incubated for 24 h. The medium was then exchanged to serum-free medium containing vehicle (DMSO) or D1 (20 μ M), and the cells were incubated for an additional 24 h. Cells were fixed and stained (green) with anti-Nrf2 (**A**) or anti-HSF-1 antibody (**B**) as well as with Hoechst dye 33,258 (5 μ M, blue). Scale bar, 50 μ m. Double-positive cells are marked by arrows, indicating nuclear localization. Each experiment was repeated at least twice.

Fig. 3. Transcriptional activation of ARE and HSE after treatment with D1

ARPE cells were plated at 10^5 cells/cm², incubated for 24 h, and then transfected with cDNA reporter constructs (ARE- or HSE-luciferase). After 5 h in serum-containing medium, the medium was exchanged for serum-free medium containing vehicle (DMSO) or D1 (20 μ M). Cell lysates were obtained after 24 h later and subjected to reporter gene assays. Values are mean $\pm$ SD; * p < 0.01. Results represent two independent experiments.

Fig. 4. Reduction in GRP78 levels after treatment with D1

ARPE-19 cells were plated at 10^5 cells/cm² and incubated for 24 h. Then, the medium was exchanged for serum-free medium containing vehicle (DMSO) or D1 (10 or 20 μ M), and incubated for an additional 24 h; following this, TM 1 (μ M) or vehicle was added to the cells and incubated for 8 h. *Lower panel*: Cell lysates (10 μ g protein/lane) were subjected to SDS-PAGE and immunoblotted for GRP78 (A), or HO-1 and HSP70 (B). *Upper panel*: Analysis of GRP78 (A), or HO-1 and HSP70 (B), normalized to β -actin by taking the ratio of their densitometric values on immunoblots. * $p < 0.05$. Each experiment was replicated at least twice.

Fig. 5. Effect of D1 on PRX2 hyperoxidation and SRXN1 Induction

(A) Inhibition of PRX2 hyperoxidation. ARPE-19 cells were plated at 10^5 cells/cm² and incubated for 24 h. Then, the medium was exchanged for serum-free medium containing vehicle (DMSO) or D1 (20 μ M), and the cells were incubated for an additional 24 h, after which H₂O₂ (1 mM) or vehicle was added to the cells for 4 h. *Lower panel:* Cell lysates (10 μ g protein/lane) were subjected to SDS-PAGE and immunoblotted for PRX2-SO_{2/3}H. *Upper panel:* Analysis of PRX2-SO_{2/3}H normalized to total PRX2 by taking the ratio of their densitometric values on the immunoblots. * $p < 0.05$. Each experiment was repeated at least twice. Samples loaded onto the gel in each experiment were from duplicate wells of a

6-well plate. Membranes were probed with β -actin to insure equal loading. **(B) Induction of SRXN1.** For mRNA levels, total RNA was extracted from ARPE-19 cells after treatment for 24 h with vehicle or D1 (20 μ M) in serum-free medium and subjected to RT-PCR for SRXN1 and β -actin. For protein levels, lysates were prepared from cells after treatment with vehicle or D1 (20 μ M); 10 μ g protein samples were loaded per lane and subjected to SDS-PAGE prior to immunoblotting. The experiments were repeated two times with similar results. Analysis of SRXN1 normalized to total β -actin by taking the ratio of their densitometric values on the immunoblots. * $p < 0.05$.

Fig. 6. (A) Protective effects of D1 on ARPE-19 cells

ARPE-19 cells were plated at 10^5 cells/cm² and incubated for 24 h. Then, the medium was exchanged for serum-free medium containing vehicle (DMSO) or D1 20 μM, and the cells were incubated for an additional 24 h. Thereafter, the cells were incubated with 1 mM H₂O₂, 3 μM TM, or vehicle for 4 h and then stained with FDA (green) and Hoechst 33,258 (blue). Note that cells labeled only with blue represent dead cells while blue/green cells are living. Scale bar, 100 μm. **(B) Statistical analysis of protective effects of D1.** Number of surviving cells is scored on the abscissa. * $p < 0.01$. Each experiment was performed at least three times. Cells labeled only with blue represent dead cells, while blue/green cells were scored as alive.

Fig. 7. Proposed protective mechanism of D1

D1 transcriptionally activates both the Nrf2/ARE system and the HSF-1/HSE system, inducing phase 2 enzymes and HSPs, respectively, thus protecting neurons against oxidative stress and ER stress. Some of the phase 2 enzymes induced by D1 are capable of reversing the hyperoxidation of PRX2, thus affording neurons significant resistance to oxidative stress. By also inducing HSPs, D1 attenuates protein misfolding, thus ameliorating ER stress.