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The sigma-1 receptor protects against cellular oxidative stress and activates antioxidant response elements

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ABSTRACT

Sigma-1 receptors are associated with Alzheimer's disease, major depressive disorders, and schizophrenia. These receptors show progrowth/antiapoptotic properties via their chaperoning functions to counteract ER (endoplasmic reticulum) stress, to block neurodegeneration, and to regulate neuritogenesis. The sigma-1 receptor knock out mouse offered an opportunity to assess possible mechanisms by which the sigma-1 receptor modulates cellular oxidative stress. Nuclear magnetic resonance (NMR) metabolomic screening of the WT (wild type) and sigma-1 KO (knockout) livers was performed to investigate major changes in metabolites that are linked to oxidative stress. Significant changes in protein levels were also identified by two-dimensional (2D) gel electrophoresis and mass spectrometry. Increased levels of the antioxidant protein peroxiredoxin 6 (Prdx6), and the ER chaperone BiP (GRP78) compared to WT littermates were detected. Oxidative stress was measured in WT and sigma-1 KO mouse liver homogenates, in primary hepatocytes and in lung homogenates. Furthermore, sigma-1 receptor mediated activation of the antioxidant response element (ARE) to upregulate NAD(P)H quinone oxidoreductase 1 (NQO1) and superoxide dismutase 1 (SOD1) mRNA expression in COS cells was shown by RT PCR. These novel functions of the sigma-1 receptor were sensitive to well-known sigma ligands via their antagonist/agonist properties.

1. Introduction

Sigma receptors are unique nonopioid binding sites that are distinct from other known neurotransmitters or hormone receptors and ubiquitously expressed in different tissues (Hayashi and Su, 2008; Su et al., 2010). They were initially proposed as opioid receptors based on the binding of N-allyl-normetazocine (SKF-10047) (Martin et al., 1976). This mischaracterization, however, was later corrected when the sigma receptors were found to be insensitive to a common opioid receptor antagonist, naloxone (Iwamoto, 1981; Su, 1982). The sigma-1 receptor subtype has been cloned (Hanner et al., 1996) and shares about 90% identity and 95% similarity across mammalian species. The guinea pig sigma-1 receptor has been expressed in *E. coli* and purified to homogeneity (Ramachandran et al., 2007). The sigma-2 receptor has yet to be cloned. Pharmacological studies have indicated that the sigma-1 receptor is able to bind a wide range of compounds (Hayashi and Su, 2004; Su et al., 2010) and is able to mediate various cellular events in the nervous system (Monnet, 2005; Hayashi and Su, 2008). Steroids such as progesterone (Hayashi and Su, 2004) and DHEA, amines such as dimethyltryptamine (DMT) (Fontanilla et al., 2009), and lipids such as sphingosine (Ramachandran et al., 2009) have been identified as endogenous ligands for the sigma-1 receptor. A partial classification of some of the primary molecules that have been assessed as sigma-1 receptor agonists and antagonists has been reviewed (Su et al., 2010) (for structures of the agonist and antagonists used in this study, see Supplemental Figure 1).

Sigma-1 receptors have been reported to modulate intracellular calcium (Novakova et al., 1998; Wu and Bowen, 2008) via a direct interaction with L-type voltage gated calcium channels (Tchedre et al., 2008), increased synthesis of inositol triphosphate (IP3) (Novakova et al., 1998), and by interaction through the C-terminal region (amino acids 102–223) with ankyrin-B 220 to disrupt ankyrin-B 220 and IP3 receptor-3 interactions (Wu and Bowen, 2008). Both production of IP3 (Robison et al., 1995) and release of calcium from ER (endoplasmic reticulum) stores (Roveri et al., 1992) have been suggested to occur on exposure of cells to oxidative stress. Recently, sigma-1 receptors have been reported to function as novel ligand-operated chaperones that form a regulated chaperone machinery complex with another ER chaperone, BiP, to counteract ER stress (Hayashi and Su, 2007). Accumulation of misfolded proteins due to ER stress

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can release calcium from the ER lumen and activate the ER chaperone machinery (Zhang and Kaufman, 2008). This process, simultaneously, leads to generation and accumulation of intracellular reactive oxygen species since correct protein folding is an energy-consuming process that requires oxidizing conditions to form intramolecular and intermolecular disulphide bonds. Reducing equivalents are transferred from thiol groups from protein-folding substrates to molecular oxygen resulting in generation of reactive oxygen species (Cuozzo and Kaiser, 1999). Additional oxidative stress can also result from the depletion of reduced glutathione, which is consumed in reactions that reduce unstable and improperly formed disulphide bonds (Cuozzo and Kaiser, 1999). In addition, the calcium released from the ER is concentrated in the matrix of the mitochondria and causes depolarization of the inner mitochondrial membrane, disrupts electron transport and further increases reactive oxygen species production (Gorlach et al., 2006). Mitochondrial reactive oxygen species can additionally increase calcium release from the ER by sensitizing ER calcium-release channels and increase protein misfolding (Zhang and Kaufman, 2008).

Beneficial effects of reactive oxygen species occur at low/moderate concentrations and involve physiological roles in cellular responses in defense against environmental pathogens (Keisari et al., 1983), in the functioning of cellular signaling pathways (Droge, 2002), and the induction of a mitogenic response (Valko et al., 2007). However, overproduction of reactive oxygen species leads to oxidative stress, a deleterious process that can be an important mediator of damage to cell structure and has been implicated in various pathological conditions involving cardiovascular diseases, cancer, neurological disorders, diabetes, ischemia/reperfusion and aging (Butterfield et al., 2002; Hildeman et al., 2003; Valko et al., 2007; Paravicini and Touyz, 2008).

In this paper, we report that sigma-1 receptors function against cellular oxidative stress as evidenced by metabolic and proteomic examination of the sigma-1 KO (knockout) and WT (wild type) littermates. Furthermore through activation of antioxidant response element (ARE) genes sigma-1 receptors may provide additional layers of protection during ER stress in addition to its chaperoning activities as reported earlier (Hayashi and Su, 2007).

2. Materials and methods

2.1. Reagents

Primers for quantitative real-time RT-PCR were synthesized by Integrated DNA Technologies, Coralville, IA. TransIT-LT1 transfection reagent was purchased from Mirus Bio, Madison, Wisconsin. Radioactive Na[^{125}I] for the preparation of [^{125}I] IAF was from Perkin-Elmer Life Sciences, Wellesley, MA. Luciferase assay kit (cat no E1550) and RNAsin (cat no N2511) were purchased from Promega, Madison, WI. All other chemicals were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise mentioned.

2.2. Preparation of tissue homogenates from wild type and the sigma-1 receptor KO mice

All animals were handled in accordance with the "Animal Care and Use Guidelines" of the University of Wisconsin-Madison. The sigma-1 receptor KO mice were obtained from the "Mutant Mouse Regional Resource Centers" (MMRRC) at University of California, Davis. Liver and lung from WT and the sigma-1 receptor KO littermates (3 months old) (n = 6–10) were minced and homogenized by four bursts of 10 s each using a Brinkman polytron on setting 6 (10 ml buffer gm^{-1} of wet tissue). Homogenization was performed in an ice-cold sodium phosphate buffer (10 mM, pH 7.4) containing 0.32 M sucrose and a cocktail of protease inhibitors [20 mg/ml leupeptin, 5 mg/ml soybean trypsin inhibitor, 100 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM benzamidine, and 1 mM EDTA]. The homogenates were snap frozen in liquid N_2, and were stored at −80 °C with a final protein concentration of approximately 10 mg/ml.

2.3. Isolation of primary hepatocytes and cell culture

Primary hepatocytes were isolated from adult WT and sigma-1 KO mice (C57BL/6J) as described by Bumgardner et al. (1998) with slight modifications. Briefly, the livers were sequentially perfused *in situ* with 2.5 mM EGTA in calcium-free Dulbecco's phosphate buffer (3–4 ml min^{-1} for 5 min at 37 °C) and with 0.05% collagenase type IV in a 1% albumin and balanced salt solution under the same conditions for 15 min for digestion. The livers were transferred to a Petri dish, where the liver tissue was gently minced and filtered (40 μM) to remove large aggregates. Liver cells were washed three times in DMEM with 10% fetal bovine serum (FBS) and centrifuged at 35×*g* for 5 min between washes. Hepatocytes were purified on a discontinuous 60% Percoll gradient (Pharmacia, Uppsala, Sweden) in a 50-ml conical tube and centrifuged at 140×*g* for 15 min. The hepatocyte pellet was resuspended in DMEM containing 10% FBS. Both COS-7 cells and primary hepatocyte culture were maintained with DMEM supplemented with 10% FBS containing penicillin and streptomycin.

2.4. Metabolomic screening

The WT and KO mouse livers were frozen in liquid N_2, ground, and extracted with water to assess metabolites. Two 2-D Heteronuclear Single Quantum Coherence (HSQC) spectra were collected on a Bruker DMX 500 MHz along with the metabolite standards at 2 mM, 5 mM, and 10 mM. Raw data were processed by the NMRPIPE program, and the processed data were analyzed by the SPARKY program. Three hundred milligrams of dried liver yielded 20 mg of dried extract which was dissolved in 0.3 ml of 5 mM HEPES, 0.5 mM DSS, and 0.5 mM sodium azide. (NMR experiments were performed at the NMRFAM, Dept. of Biochemistry, UW-Madison).

2.5. Two dimensional gel electrophoresis and mass spectrometry

The liver homogenates of both WT and the sigma-1 receptor KO mice were centrifuged at 100,000×*g* to separate membrane and cytosolic fractions. Two dimensional gel electrophoresis (performed by Kendrick Labs, Madison, WI) on 200 μg of the cytosolic and membrane fractions was separated initially using a 17 cm pH 3.5–10 linear IPG strip in duplicate followed by conventional 12% SDS-PAGE. The gels were stained with Coomassie blue, dried and unique spots were used for identification of proteins using MALDI-TOF-TOF-MS at the Biotechnology Center, University of Wisconsin-Madison.

2.6. Measurement of oxidative stress

Oxidative stress levels were measured using the methods reported by Bejma and Ji (1999) with slight modifications. Known concentrations of tissue homogenates, primary hepatocytes or COS-7 cell lysates were incubated with 50 mM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in DMEM for 30 min at 37 °C in the dark and the fluorescence of 2′,7′-dichlorofluorescin (DCF) was measured at 485/530 nm (exi/emi). The fluorescence of the DCFH-DA solution without any samples was taken as the blank.

2.7. Measurement of ARE activation

The ARE-luciferase and GC-ARE (mutant) constructs of the human NQO1 gene were a kind gift from Dr Jeff Johnson, University of Wisconsin-Madison and reported earlier (Lee et al., 2001). Both the sigma-1 receptor and the luciferase reporter construct were co-transfected into COS-7 cells (approximately 1×10^6 cells) using TransIT-LT1 transfection reagent. After 48 h of transfection, cells were

treated with different sigma ligands for 24 h with a final concentration of 10 μM and ARE activation was measured using a luciferase assay kit (Promega, Madison) following the manufacturer's protocol. COS-7 cells, transfected with ARE-Luciferase or mutant GC-ARE, were treated with well-known ARE activator tertiary butyl hydroquinone (t-BHQ) at a concentration of 100 μM for 4 h after 2 days of transfection, for the positive control and negative control, respectively. Expression of the sigma-1 receptor was confirmed using western blot analysis and further utilized for transfection efficiency.

2.8. Quantitative real-time RT-PCR

Total RNA was purified from COS-7 cells (approximately 2×10^6 cells) with RNeasy Mini Kit (Qiagen) using the manufacturer's protocol. Complementary-DNA sequences were prepared by annealing RNA (1 μg) with 250 ng of a 5:1 mixture of random and oligo(dT) primers heated at 68 °C for 10 min. This was followed by incubation with Moloney murine leukemia virus (MMLV) reverse transcriptase (50 units) (GIBCO/BRL) combined with 10 mM DTT, RNAsin, and 0.5 mM dNTPs at 42 °C for 1 h. Reactions were diluted to a final volume of 150 μl and heat inactivated at 98 °C for 5 min. Reactions (25 μl) contained 2.5 μl of cDNA, 12.5 μl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 200 nM of appropriate primers. Product accumulation was monitored by SYBR Green fluorescence. Control reactions lacking reverse transcriptase (RT) yielded very low signals. Relative expression levels were determined from a standard curve of serial dilutions of nontransfected COS-7 cells in cDNA samples and were normalized to the expression of *RpII* 215.

Real-time RT-PCR primers

RpII 215

Forward, 5′-CGAATCCGCATCATGAACAG-3′
Reverse, 5′-TGCATCGCAGGAAGACATCA-3′

HMOX-1

Forward, 5′-CCACCAAGTTCAAGCAGCTCTA-3′
Reverse, 5′-GCTCCTGCAACTCCTCAAAGAG-3′

NQO1

Forward, 5′-GAACTTCAATCCCATCATTTCCAG-3′
Reverse, 5′-CAGCTTCTTTTGTTCAGCCACAAT-3′

SOD1

Forward, 5′-AGGTGTCTTTCGAAGATTCTGTGATC-3′
Reverse, 5′-TTTCTTCATTTCCACCTTTGCC-3′

CAT

Forward, 5′-GAGCAGCCCTGACAAAATGC-3′
Reverse, 5′-GGTAGGGACAGTTCACAGGTATCTG-3′

2.9. Statistical analysis

P values were calculated using the software "GraphPad Prism" version 4.0c (GraphPad Software Inc, San Diego, CA).

3. Results

3.1. Sigma-1 receptor KO mouse showed higher oxidative stress

To explore the possible role of the sigma-1 receptor during oxidative stress, we studied primarily the sigma-1 KO mouse liver. We have performed both metabolomic screening and 2D gel electrophoresis of liver homogenates of both the WT and sigma-1 receptor KO mice to identify major changes in the metabolites and proteins (Figs. 1 and 2). Interestingly, metabolomic screening of the liver samples (n = 6) indicated that the sigma-1 receptor KO mouse livers had significant increases in the levels of metabolites that are well-known signatures of cellular oxidative stress such as oxidized glutathione (GSSG) and glutamate, and those that have protective roles against oxidative stress such as alanine, glutamine, lactic acid, AMP, myo-inositol, and betaine (Fig. 1). An increase in threonine levels and a decrease in valine levels were also observed in the sigma-1 receptor KO livers compared to the WT liver (Fig. 1). Two dimensional electrophoresis followed by mass spectrometric analysis revealed that the antioxidant protein Prdx6 (Fatma

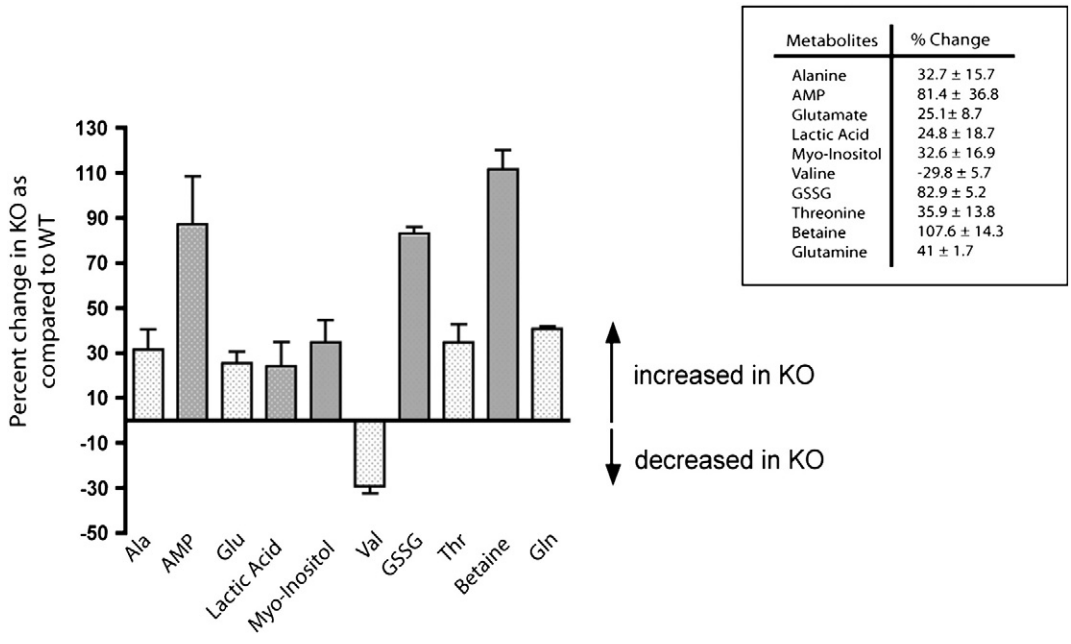


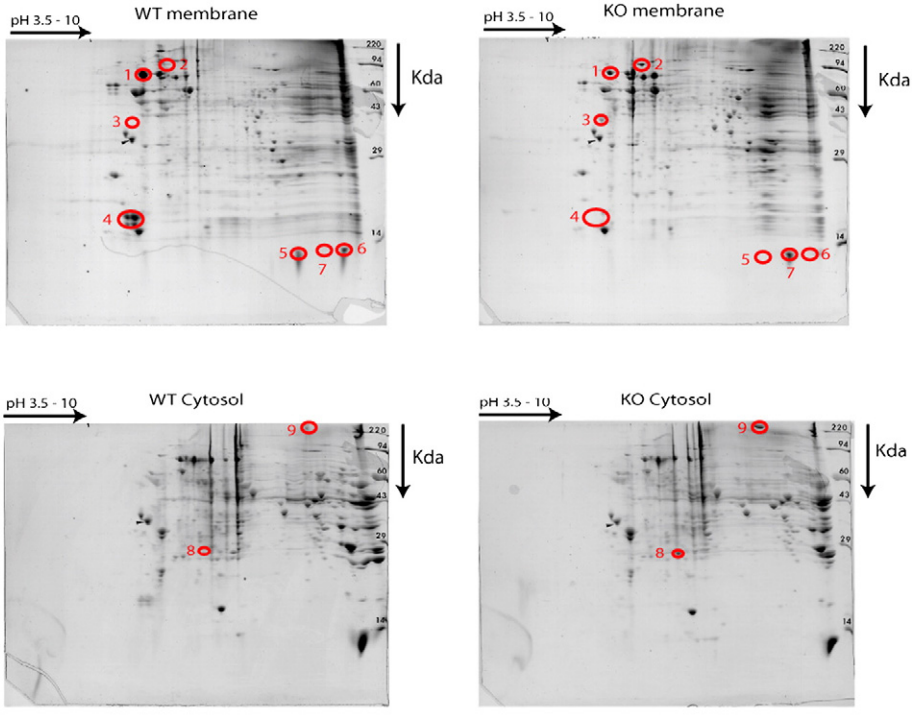
Fig. 1. Changes in metabolites in the WT and sigma-1 receptor KO mouse livers. Higher levels of oxidized glutathione (GSSG) and glutamate in the KO livers indicate that the KO mice were under oxidative stress. Error bars represent mean ± S.E.M. from different experiments (n = 6).

et al., 2005; Power et al., 2008), ER chaperone BiP (Schroder and Kaufman, 2005), 40S ribosomal protein SA, and carbamoyl phosphate synthetase 1 were upregulated in the sigma-1 receptor KO livers compared to the WT livers (Fig. 2). In addition, major urinary proteins and HSPA5 were downregulated in KO livers (Fig. 2). These results support the general conclusion that the sigma-1 receptor KO mouse has higher levels of oxidative stress than the WT mouse.

To confirm this view, we also measured the oxidative stress levels in the liver and lung tissues from both the WT (n = 8) and sigma-1 receptor KO mice (n = 9) using the oxidative conversion of DCFH-DA to the highly fluorescent DCF (LeBel et al., 1992) by endogenous reactive oxygen species. The oxidative stress levels in both tissue homogenates of the sigma-1 KO mice were significantly increased compared to that of the WT (Fig. 3A). For further confirmation, we measured the oxidative stress levels in the isolated primary hepatocytes from both animals and found a similar pattern; that is, the sigma-1 KO hepatocytes had nearly a twofold higher oxidative stress level compared to the WT hepatocytes (Fig. 3B).

3.2. Decrease in oxidative stress levels by over-expression of the guinea pig sigma-1 receptor in COS-7 cells

To further validate the functions of the sigma-1 receptor in protection against oxidative stress, we performed a gain-of-function analysis using COS-7 cells as a model cell line [in which the sigma-1 receptor cannot be detected by photoaffinity labeling, Supplemental Figure 2A, or by western blot (data not shown)] and the guinea pig sigma-1 receptor as the model receptor (the sigma-1 receptor shares approximately 90% identity and 95% similarity across various species). Also no significant effects of sigma-1 receptor ligands on reactive oxygen species levels in COS cells alone could be detected (Supplemental Figure 2B). Transient expression of the guinea pig sigma-1 receptor in COS-7 cells showed nearly 50% decrease in the measured levels of conversion of DCFH-DA to DCF (n = 4) compared to vehicle transfected COS-7 cells (Fig. 4). These results indicated that the over-expressed guinea pig sigma-1 receptor in COS-7 cells is functionally protective against oxidative stress either alone or via



Spot	Protein Identified	Up / Down Regulated	MW (kDa)	pI	Sequence Covered
1	Heat Shock Protein 5 (HSP 5)	Down	72.4	5.01	60%
2	**BiP (GRP78)**	Up	72.3	5.07	40%
3	40S Ribosomal Protein SA (34/67 kDa laminin receptor)	Up	24.8	6.53	59%
4	Major Urinary Protein (18.7 kDa)	Down	17.4 - 18.6	4.78 - 4.82	79 - 100%
5	Chain B, Chimeric Human Mouse Carbomonoxy Hemoglobin	Down	15.6	7.27	47%
6	Hemoglobin a, adult chain 2	Down	15.1	7.96	54%
7	b-Globin	Up	16.2	7.4	68%
8	**Peroxiredoxin 6 (Prdx6)**	Up	24.9	5.71	78%
9	Carbamoyl Phosphate Synthetase 1	Up	164.5	6.48	48%

Fig. 2. Two dimensional gel electrophoresis of 200 μg of membrane (100,000 ×*g* pellet) and cytosolic fractions of the liver homogenates of the WT and sigma-1 receptor KO mice as described in the Materials and methods section. The gels were stained with Coomassie blue and marked spots showed the presence and absence of the unique proteins (shown by circled numbered spots), which were selected for sequence identification by mass spectrometry (shown in the lower panel). Upregulation of the antioxidant protein Prdx6 and the ER chaperone BiP was observed in the KO livers.

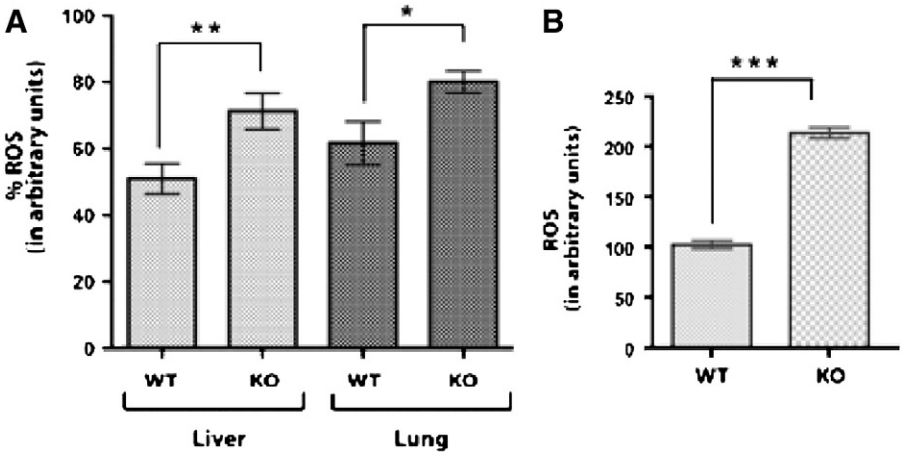


Fig. 3. Sigma-1 receptor KO mice have higher oxidative stress. A. Reactive oxygen species (ROS) levels in liver and lung tissue homogenates (50 μg) from WT and sigma-1 receptor KO mice. Both liver and lung tissues from KO mouse showed higher levels of reactive oxygen species compared to that of WT tissues. $^{**}P<0.001$, $^{*}P<0.01$ by unpaired Student t test, mean ± S.E.M. of triplicate measurements from different mice (for WT n = 8 and for KO n = 9). B. Reactive oxygen species levels in primary hepatocytes isolated from the WT and sigma-1 receptor KO mice. Approximately 2.5×10^6 cells were used in each condition. Cells were lysed using 500 μl of lysis buffer (25 mM Tris–HCl pH 7.8 supplemented with 2 mM DTT, 10% glycerol, 1% Triton X-100) and the protein concentrations in cell lysates were measured. 150 μg of total lysate was used for measurement of reactive oxygen species. The sigma-1 receptor KO hepatocytes showed higher levels of reactive oxygen species compared to that of WT hepatocytes and indicated that sigma-1 receptors are protective against oxidative stress. $^{***}P<0.0001$ by unpaired Student t test, mean ± S.E.M. of three separate experiments (n = 3).

endogenous agonist(s). Administration of the sigma-1 receptor antagonists (Hayashi and Su, 2008) haloperidol, BD 1047, and BD 1063 further increased the oxidative stress levels in sigma-1 receptor transfected COS-7 cells compared to control (no drug treatment) whereas (+)-pentazocine, a sigma-1 receptor agonist (Hayashi and Su, 2008), showed a decrease in oxidative stress levels (Fig. 4). To check whether this function is cell specific or not, we also measured reactive oxygen species levels in RAW 264.7 cells after over-expressing sigma-1 receptor and found that over-expression of sigma-1 receptor in RAW 264.7 cells reduced the reactive oxygen species levels (nearly 30%) consistently (data not shown).

3.3. Sigma-1 receptors activate the antioxidant response elements (ARE)

We further investigated whether the sigma-1 receptor might be involved in signaling pathways which activate the 'antioxidant response element' (ARE), a *cis*-acting regulatory enhancer found in the 5′ flanking region of many phase II detoxification enzymes and antioxidant proteins such as NAD(P)H:quinone oxidoreductase (NQO), γ-glutamyl cysteine-synthase (γ-GCS), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and heme oxygenase-1 (HMOX-1) (Nguyen et al., 2003). Therefore, we investigated ARE activation in COS-7 cells transfected with sigma-1 receptors using an ARE-luciferase reporter construct with a minimal promoter region of the NQO1 gene which was reported previously (Lee et al., 2001). Interestingly, we found approximately an 8–10 fold increase in ARE activation in the presence of transfected sigma-1 receptors compared to controls. We also tested the effects of sigma-1 receptor agonists and antagonists and found that the sigma-1 receptor antagonists haloperidol, BD1047, and BD 1063 reduced ARE activation compared to control (no drug treatment) whereas the sigma-1 receptor agonists such as (+)-pentazocine showed increased ARE activation over the control (Fig. 5A). The reduction by haloperidol on ARE activation was completely reversed by (+)-pentazocine cotreatment in a dose dependent manner (Fig. 5B). In addition, we performed quantitative PCR of the NQO1, HMOX-1, SOD1 and CAT, the gene products under the control of the ARE enhancer (Nguyen et al., 2003). Transfection of the sigma-1 receptor itself increased the NQO1 and SOD1 mRNA levels almost twice over the mock transfected COS-7 cells (Fig. 5C) while the mRNA levels of HMOX-1 and CAT remain unchanged (data not shown). Addition of the sigma-1 agonist (+)-pentazocine further increased the NQO1 and SOD1 mRNA levels significantly over the sigma-1 receptor transfection alone condition (with no ligands) whereas the antagonist haloperidol lowered the NQO1 and SOD1 mRNA levels nearly to that of sigma-1 receptor transfection alone (Fig. 5C). No change in the HMOX-1 and CAT mRNAs was observed (data not shown), indicating that the sigma-1 receptor selectively activated the ARE of SOD1 and NQO1 genes.

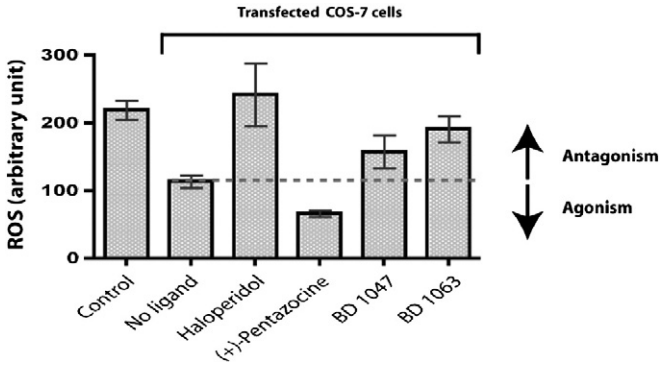


Fig. 4. Sigma-1 receptor protects COS-7 cells from oxidative stress. Reactive oxygen species (ROS) levels in COS-7 cells after transfection with the guinea pig sigma-1 receptors. Approximately 3×10^6 cells were used in each condition and cell lysates containing 150 μg of total proteins were used to measure reactive oxygen species in each condition. The sigma-1 receptor transfected COS-7 cells showed nearly a 50% reduction in reactive oxygen species levels compared to vehicle transfected (Mock) COS-7 cells. For determination of agonist/antagonist effects of the various sigma-1 receptor ligands, guinea pig sigma-1 receptor transfected COS-7 cells were treated with sigma-1 ligands for 24 h with a final concentration of 10 μM before reactive oxygen species level measurements. The sigma-1 receptor antagonists haloperidol, BD 1047, and BD 1063 increased reactive oxygen species levels whereas the agonist (+)-pentazocine lowered the reactive oxygen species levels in the sigma-1 receptor transfected COS-7 cells. Error bars represent mean ± S.E.M. from four separate experiments (n = 4).

4. Discussion

Reactive oxygen species induce oxidative damage to macromolecular structures such as membranes and DNA and contribute to cellular damage as well as cell death through apoptosis and necrosis (Cave et al., 2005). Increased ligand binding activity to sigma-1 receptors in retinal Muller cells has been observed *in vitro* when the cells were treated with NO and reactive oxygen species donors (Jiang et al., 2006) and protection of human umbilical vein endothelial cells (HUVEC) from oxidative stress by the antioxidant methyl gallate,

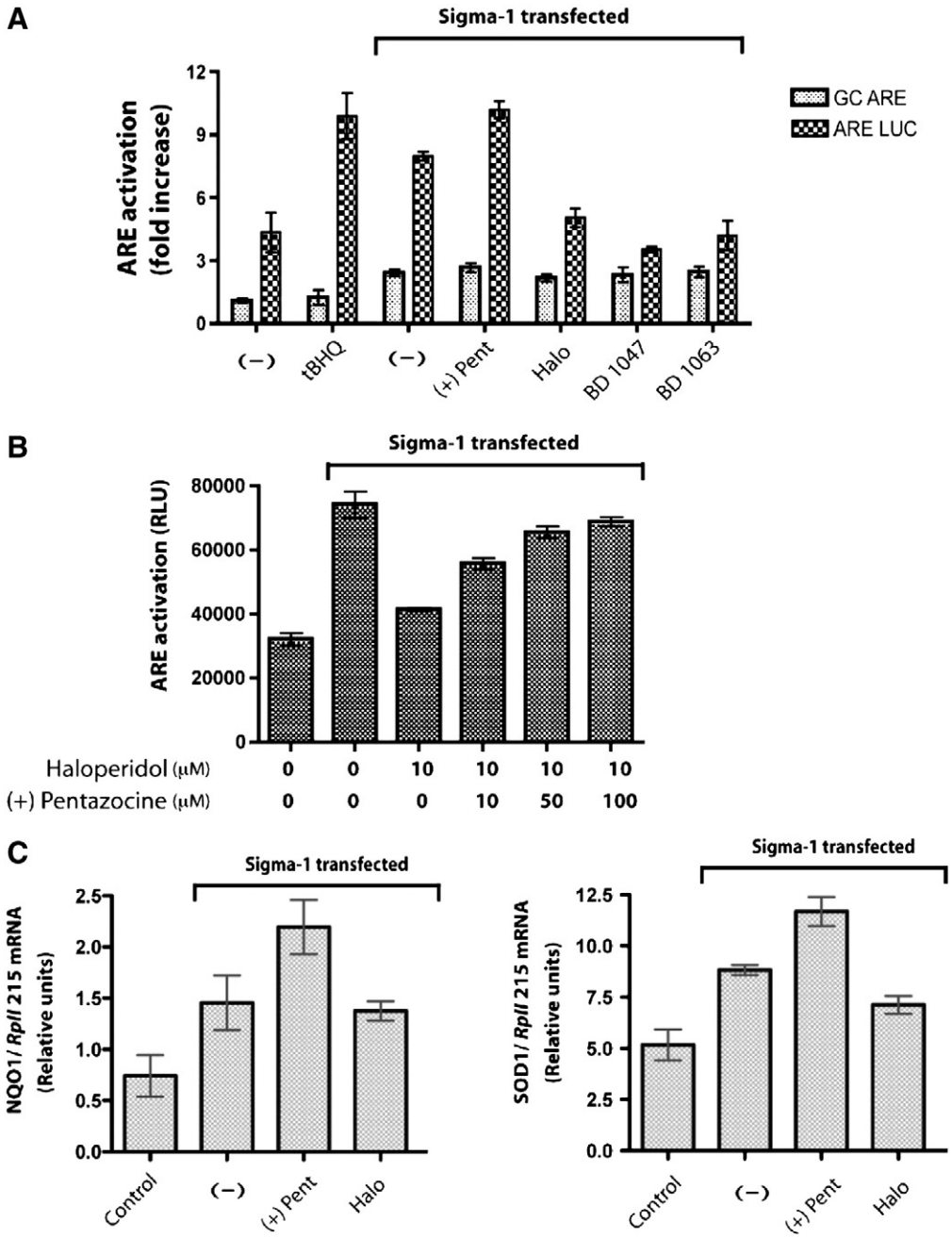


Fig. 5. Sigma-1 receptor activates 'antioxidant response elements' (ARE). A. ARE activation by the sigma-1 receptors and its ligands in COS-7 cells (approximately 1×10^6 cells) using a luciferase reporter gene assay. Specificity of ARE activation was determined by using mutant GC-ARE luciferase construct, which is not activated by the known ARE activator, tertiary butyl hydroquinone (t-BHQ). Transfections of sigma-1 receptor in COS-7 cells showed almost a twofold specific ARE activation compared to that of the vehicle transfected COS-7 cells. The sigma-1 receptor agonist (+)-pentazocine (10 μM) showed a further increase in the specific ARE activation compared to that no drug treatment [(−) condition], whereas the antagonists haloperidol, BD 1047, and BD 1063 lowered the specific ARE activation. Error bars represent mean ± S.E.M. from three separate experiments (n = 3). B. Reversal of haloperidol-mediated antagonism of ARE activation (n = 3). COS-7 cells transfected with guinea pig sigma-1 receptor (approximately 1×10^6 cells) were treated with 10 μM of haloperidol for 24 h 2 days after transfection and lowered ARE activation was observed. Co-treatment of 50–100 μM (+)-pentazocine, a sigma-1 receptor agonist, reversed the antagonism shown by 10 μM of haloperidol. Error bars represent mean ± S.E.M. from different experiments (n = 3). C. Real-time RT-PCR analysis of NQO1 and SOD1 mRNAs — the gene products which are under control of the ARE enhancer, in COS-7 cells. Transfection of sigma-1 receptor alone increased the SOD1 and NQO1 mRNA levels almost twofold compared to that of vehicle transfected condition (control) (n = 4). Treatment with the sigma-1 receptor agonist (+)-pentazocine (10 μM) showed a further increase in the NQO1 and SOD1 mRNA levels compared to the no drug treatment condition whereas the treatment of the antagonist haloperidol (10 μM) showed reverse effects. Error bars represent mean ± S.E.M. from four separate experiments (n = 4).

has been linked to up-regulation of the sigma-1 receptor gene (Whang et al., 2005). It has been also reported that sigma-1 receptor ligands can protect retinal cells against oxidative stress (Smith et al., 2008) and provide protection by increased transcription of antiapoptotic bcl-2 mRNA to preserve a favorable *bcl-2*/*bax* ratio in injured neurons (Yang et al., 2007) as well as in CHO cells via NFkappaB pathway (Meunier and Hayashi, 2010). An inverse correlation between the levels of Bcl2 family proteins and reactive oxygen species has already been established (Hildeman et al., 2003).

The sigma-1 receptor knockout mouse has been reported previously to be viable and fertile showing no overt constitutive phenotype (Langa et al., 2003). Our study provides an explanation for the functional redundancy of the sigma-1 receptor KO mouse. From our study as well as other published reports (Whang et al., 2005; Hayashi and Su, 2007;

Yang et al., 2007; Smith et al., 2008), it is clear that the sigma-1 receptor has both antioxidative as well as chaperoning activities, which are sensitive to sigma-1 receptor ligands. Thus, it is expected that the sigma-1 KO mouse would have higher levels of proteins and/or metabolites, which have either antioxidative or chaperoning activities in the cells. In fact, metabolomic studies revealed that the KO livers had higher oxidized glutathione (GSSG) and glutamate levels (Fig. 1). Glutathione has previously been well characterized as the major cellular "redox buffer" in conjugation with thioredoxins for maintaining intracellular "redox homeostasis" and higher amounts of oxidized glutathione are an indicator of oxidative stress (Valko et al., 2007). Glutamate has also been well documented as an inducer of oxidative stress-related endothelial death by apoptosis (Parfenova et al., 2006). Thus, the increase in GSSG and glutamate indicated the presence of oxidative stress in the KO livers (Fig. 1). On the other hand, alanine has been shown to possess cytoprotective effects against free radical-induced injury in various tissues (Grosser et al., 2004) and glutamine, as a precursor of glutathione, is required to maintain high levels of glutathione and to avoid oxidative stress damage (Amores-Sanchez and Medina, 1999). Thus, increases in glutamine and alanine provide protection against oxidative stress (Fig. 1). Moreover, organic osmolytes, such as betaine and myo-inositol, which are also upregulated in the KO livers, are involved in cell volume homeostasis as well as in cell protection against oxidative stress (Warskulat et al., 2004) and may act as "chemical chaperones" to stabilize native protein structure and protein function (Welch and Brown, 1996). Increases in lactate and AMP (Fig. 1) indicated a metabolic stress which favors anaerobic consumption of glucose to generate ATP in a less effective manner than through the TCA cycle and oxidative phosphorylation, a reactive oxygen species generating process. Identification of changes in protein levels also supports the hypothesis for functional redundancy in KO mice (Fig. 2). Two dimensional gel electrophoresis followed by mass spectrometric identification showed dramatic upregulation of the ER chaperon BiP and antioxidant protein peroxiredoxin 6 (Prdx6) in the sigma-1 receptor KO mouse livers (Fig. 2) which most likely occurs to counteract the deficiencies in chaperoning as well as to provide antioxidative functions (Manevich and Fisher, 2005) due to lack of the sigma-1 receptors. A similar increase in Prdx6 has been reported in sigma-1 receptor knockdown experiments, using siRNA in primary cultures of mouse hippocampal neurons (Tsai et al., 2012).

In order to evaluate functionally the sigma-1 receptors further, we investigated the possible role of the sigma-1 receptor in oxidative stress and found that this receptor is protective against cellular oxidative stress (Figs. 3A, B, and 4) without possessing direct reactive oxygen species scavenging activity (data not shown). The sigma-1 KO mice showed higher levels of oxidative stress (Fig. 3A and B) and transfection of the sigma-1 receptor into COS-7 cells showed reduced oxidative stress (Fig. 4). Transfection of the sigma-1 receptor in COS-7 cells resulted in activation of the ARE in a manner that was enhanced by the sigma-1 receptor agonist, (+)-pentazocine and reduced by the sigma-1 receptor antagonists, haloperidol, BD1047 and BD1063 (Fig. 5A). The inhibition of ARE activity by haloperidol was reversed by increasing concentrations of (+)-pentazocine (Fig. 5B). Moreover the sigma-1 receptor agonist (+)-pentazocine was able to increase the mRNA levels of the housekeeping antioxidant proteins NQO1 and SOD1 whereas the sigma-1 receptor antagonist, haloperidol, was without effect (Fig. 5C). SODs convert superoxide to hydrogen peroxide (Storz, 2007), which is further neutralized by catalase. NQO1 genes encode cytosolic flavoenzymes that catalyze the beneficial two-electron reduction of quinones to hydroquinones and reduce the formation of reactive oxygen species by preventing the unwanted one-electron reduction of quinones in the presence of molecular oxygen (Vasiliou et al., 2006). ARE promoters are under transcriptional control of the transcription factor NF-E2-related factor 2 (Nrf2) (Johnson et al., 2008) which indicates that the sigma-1 receptor is capable of signaling through this transcriptional pathway in an as yet unknown mechanism. The sigma-1 receptor has been previously linked to the regulation of transcription factor events that are associated with oxidative stress (Meunier and Hayashi, 2010).

In cells, ER calcium levels are regulated by intake through the ER-calcium ATPase and release through the ryanodine and IP3 receptors. Upon depletion of ER calcium due to ER stress or via agonist stimulation, the sigma-1 receptors dissociate from BiP leading to prolonged Ca^{2+} signaling from ER into mitochondria by chaperoning the IP3 type3 receptors at the ER mitochondrial interface (mitochondrion-associated ER membrane or MAM) (Hayashi and Su, 2007). Increase in the mitochondrial calcium generates more ATP by the TCA cycle and oxidative phosphorylation (ox-phos) via allosteric activation of several enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, and alpha-ketoglutarate dehydrogenase, as well as stimulation of ATP synthase (complex V), alpha-glycerophosphate dehydrogenase, and adenine nucleotide translocase (Brookes et al., 2004). As a result, the effect of elevated mitochondrial Ca^{2+} concentration is the coordinated upregulation of the entire ox-phos machinery, resulting in faster respiratory chain activity and higher ATP output to meet the cellular ATP demand (Brookes et al., 2004). However, stimulation of the TCA cycle and ox-phos by Ca^{2+} will also enhance oxidative stress (reactive oxygen species) by forcing the mitochondria to function faster and consume more O_2 (Brookes et al., 2004). Indeed, mitochondrial reactive oxygen species generation correlates well with metabolic rate where faster metabolism simply results in more respiratory chain electron leakage (Brookes et al., 2004). Thus, the reactive oxygen species generated in the mitochondrial inner membrane (mainly superoxide) is released into the mitochondrial matrix and the cytosol (Storz, 2007). In our study, we found that the NQO1 and SOD1 mRNAs were upregulated in the sigma-1 receptor transfected COS-7 cells via the activation of ARE and the sigma receptor agonists and antagonists further modulated this function (Fig. 5). This novel sigma-1 receptor function, in addition to its chaperoning activity and channeling ER calcium to mitochondria via IP3 type 3 receptors, mediates protective effects during ER stress. Although transcription of the ARE gene battery has also been reported previously during ER stress (Cullinan and Diehl, 2004), our results indicate a novel route of ARE activation to increase NQO1 and SOD1 mRNA levels via the sigma-1 receptor to protect the cells against ER stress generated reactive oxygen species as shown schematically in Fig. 6.

5. Conclusion

In summary, the sigma-1 receptors protect the cell from oxidative stress as observed from the modulation of reactive oxygen species levels in the primary hepatocytes from the sigma-1 receptor knockout animal as well as in COS-7 cells when transfected. By reducing levels of reactive oxygen related oxidative stress, sigma-1 receptors are likely to provide additional layers of protection during ER stress in addition to its chaperoning activities (Hayashi and Su, 2007; Tsai et al., 2009). Application of sigma-1 receptor agonists is likely to be a useful therapeutic strategy in the treatment of several diseases that involve oxidative stress (Maurice and Su, 2009) such as cardiovascular diseases, cancer, neurodegenerative disorders, diabetes, ischemia/reperfusion, Alzheimer's (Villard et al., 2009; Su et al., 2010; Villard et al., 2010) and CNS inflammatory conditions associated with cocaine and HIV (Su et al., 2010; Yao et al., 2010).

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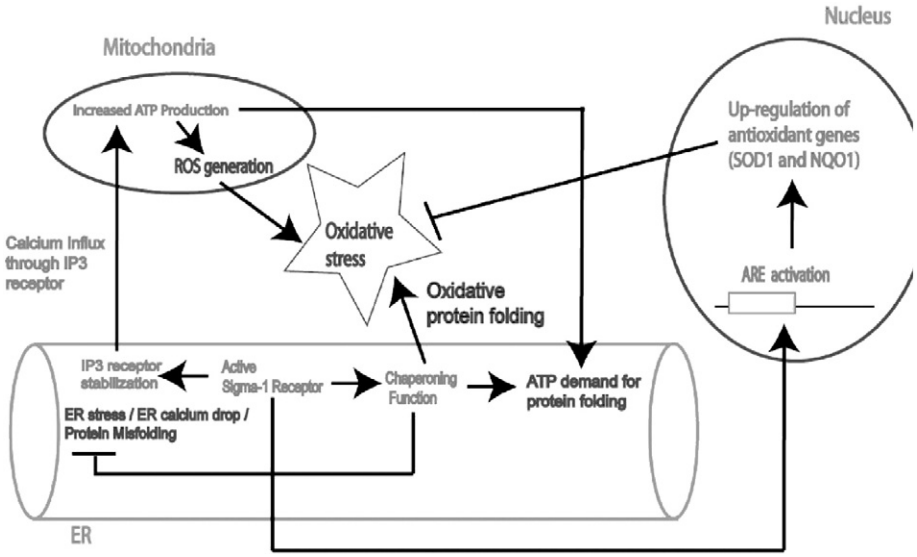

Fig. 6. Putative model for the mechanism of sigma-1 receptor mediated protection against cellular oxidative stress. ER stress or ER calcium drop leads to accumulation of unfolded proteins. Sigma-1 receptor functions as an ER chaperone to protect against ER stress and increase calcium mobilization from ER to mitochondria by stabilizing IP3 receptor (Hayashi and Su, 2007). Calcium influx into the mitochondria leads to increase ATP generation (Brookes et al., 2004) to counteract ATP demand for chaperoning function. Simultaneously, mitochondria also produce more reactive oxygen species (ROS) due to more respiratory chain electron leakage (Brookes et al., 2004). In addition, correct protein folding is an oxidative process, which also increases cellular oxidative stress (Cuozzo and Kaiser, 1999). Sigma-1 receptors counteract cellular oxidative stress by upregulation of antioxidant genes such as SOD1 and NQO1, through the activation of antioxidant response elements (ARE).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2012.01.030.

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