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Involvement of heme oxygenase-1 induction via Nrf2/ARE activation in protection against H_2O_2-induced PC12 cell death by a metabolite of sesamin contained in sesame seeds

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ABSTRACT

Induction of phase II antioxidant enzymes by activation of Nrf2/ARE (antioxidant response element) signaling has been considered as a promising strategy to combat with oxidative stress-related diseases. In the present study, we tested for potential effects of sesamin, a major lignan contained in sesame seeds, its stereoisomer episesamin, and their metabolites on Nrf2/ARE activation in rat pheochromocytoma PC12 cells. Luciferase reporter assays showed that primary metabolites of sesamin and episesamin, SC-1 and EC-1 were the most potent ARE activators among all tested compounds. SC-1 {(1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane} enhanced nuclear translocation of Nrf2 and up-regulated expression of phase II antioxidant enzymes including heme oxygenase-1 (HO-1). Treatment with SC-1 resulted in increased phosphorylation of p38 MAP kinase and transient increase in intracellular ROS levels. *N*-acetylcysteine (NAC) treatment abolished p38 phosphorylation as well as HO-1 induction caused by SC-1, indicating that ROS are upstream signals of p38 in Nrf2/ARE activation by SC-1. Furthermore, preconditioning with SC-1 attenuated H_2O_2-induced cell death in a dose-dependent manner. Finally, treatment with a HO-1 inhibitor, Zn-protoporphyrin (ZnPP), and overexpression of a dominant-negative mutant of Nrf2 diminished SC-1-mediated neuroprotection. Our results demonstrate that SC-1 is capable of protecting against oxidative stress-induced neuronal cell death in part through induction of HO-1 via Nrf2/ARE activation, suggesting its potential to reduce oxidative stress and ameliorate oxidative stress-related neurodegenerative diseases.

1. Introduction

Oxidative stress is implicated in numerous diseases including neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD).[1] Reactive oxygen species (ROS) are generated not only in mitochondria during electron transport and oxidative phosphorylation but also in plasma membranes by NADPH oxidase (NOX). Excess amounts of ROS damage high molecular weight molecules such as DNA, protein and lipid, resulting in cellular dysfunction and apoptosis. At lower levels, however, ROS play an important role as signaling molecules in diverse physiological processes. Enhancement of the antioxidant capacity by pharmacological or nutritional intervention could reduce oxidative stress and ameliorate oxidative stress-related diseases. The capacity can be increased by induction of phase II antioxidant enzymes as well as superoxide dismutase (SOD) and catalase. The phase II antioxidant enzymes include heme oxygenase-1 (HO-1), γ-glutamylcysteine synthase (γ-GCS), glutathione synthase (GS) and glutathione peroxidase (GPX). HO-1 catalyzes heme to biliverdin, which is converted to bilirubin that functions as a potent antioxidant. The cellular antioxidant, glutathione, is synthesized by the sequential action of γ-GCS and GS. GPX removes hydrogen peroxide by glutathione oxidation.

The expression of phase II enzymes is regulated by transcription factor Nrf2 (nuclear factor E2-related factor 2). Nrf2 binds to specific DNA enhancer sequences termed antioxidant response element (ARE) present within the promoter region of genes encoding for phase II enzymes, resulting in up-regulation of their transcription.[2–4] Under unstressed conditions, Nrf2 binds to

Abbreviations: ARE, antioxidant response element; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; Keap1, kelch-like ECH-associated protein 1; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; ZnPP, Zn-protoporphyrin.

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Keap1 (kelch-like ECH-associated protein 1) in the cytoplasm, which is an adapter protein for the ubiquitin ligase Cullin 3 (Cul3), and undergoes constitutive proteasome-dependent degradation. Once covalent or oxidative modification of cysteine sulfhydryl groups takes place by electrophiles or ROS, Keap1 changes its conformation, resulting in Nrf2 release and translocation into the nucleus. Naturally-derived compounds such as curcumin and sulforaphane are known to interact with Keap1 and activate the Nrf2/ARE signaling.[5,6] It has been also demonstrated that phosphorylation by protein kinases enhances nuclear translocation of Nrf2.[7]

In Asia, sesame seeds and oil have been widely consumed for thousands of years and linked to health benefits. Sesamin and its stereoisomer, episesamin, were identified as major lignans in refined sesame oil.[8] Dietary intake of sesamin and/or episesamin has been reported to show various beneficial effects on health including cholesterol- and blood pressure-lowering property,[9–13] and protective capacity against alcohol-induced liver injury and rotenone-induced dopaminergic neuronal loss.[14,15] In the body, sesamin and episesamin are metabolized by cytochrome P-450 and catechol-O-methyltransferase (COMT)[16] (Supplementary Fig. 1). Sesamin and episesamin are initially metabolized by P-450 to SC-1 {(1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane} and EC-1 {(1*R*,2*S*,5*R*,6*R*)- and (1*R*,2*R*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane}, which are then metabolized to SC-2 and EC-2, respectively. The resultant compounds, SC-1 and SC-2, and EC-1 and EC-2 are further metabolized by COMT to SC-1m and SC-2m, and EC-1m and EC-2m, respectively. Some metabolites have been shown to possess strong antioxidant activity in vitro.[17] We have recently reported that SC-1 induces neuronal differentiation of PC12 cells, a model of neuronal cells, through activation of ERK1/2 signaling pathway that is essential for NGF (nerve growth factor)-mediated neurite outgrowth.[18]

In the present study, we first investigated effects of sesamin, episesamin and their metabolites on Nrf2/ARE signaling by luciferase reporter assays in PC12 cells and found that SC-1 and EC-1 were the most potent ARE enhancer activators. We then investigated and elucidated the molecular mechanisms underlying Nrf2/ARE activation by SC-1 and its neuroprotective effects on oxidative stress-induced neuronal cell death.

2. Results

2.1. Identification of sesamin and episesamin derivatives that activate the ARE enhancer

In order to determine the ARE enhancer activity of sesamin, episesamin and their derivatives (Supplementary Fig. 1), we performed luciferase reporter assays. Although sesamin and episesamin showed no ARE enhancer activity, their metabolites generated by P450 enzymes, SC-1 and EC-1, increased the enhancer activity by more than twofold (Fig. 1). SC-2, a metabolite of SC-1 generated by P450, retained the ARE enhancer activity, but SC-1m, another metabolite of SC-1 generated by COMT, lost the enhancer activity. In contrast, EC-2 and EC-1m both of which were yielded from EC-1 did not activate the ARE enhancer. These results indicate that SC-1 and EC-1 converted by P450 from sesamin and episesamin are capable of activating Nrf2/ARE signaling. Hereafter, we focused on SC-1 as a representative of these two active compounds.

2.2. Induction by SC-1 of phase II antioxidant/detoxification enzymes

Activation of Nrf2/ARE signaling results in up-regulation of expression of phase II antioxidant/detoxification enzymes such as HO-1, γ-GCS catalytic subunit (γ-GCSc) and NAD(P)H dehydrogenase, quinone 1 (NQO-1). γ-GCSc associates with γ-GCS modifier subunit (γ-GCSm) to form γ-GCS. NQO-1 catalyzes the two-electron reduction of quinone compounds. As shown in Figure 2A, HO-1 protein induction was seen at 6 h after treatment with SC-1 (10 μM), peaked at 12 h, and then decreased at 24 h. SC-1 increased HO-1 expression in a dose-dependent manner (1, 5, 10 μM). SC-1 at 10 μM up-regulated HO-1 mRNA expression by more than sixfold at 6 h after treatment (Fig. 2B). Furthermore, SC-1 induced expression of other phase II enzymes including γ-GCSc and NQO-1 (Supplementary Fig. 2). These results suggest that SC-1 up-regulates expression of phase II enzymes as a result of Nrf2/ARE activation.

2.3. Translocation of Nrf2 into the nucleus by SC-1

Upon Nrf2/ARE activation, Nrf2 is released from Keap1 and translocated into the nucleus, where it activates the ARE enhancer

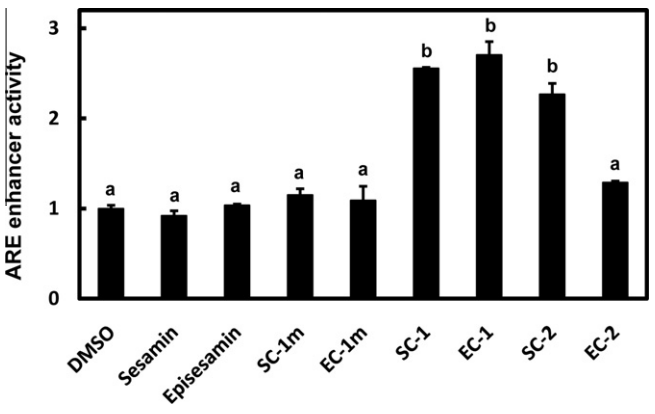

Figure 1. Effects of sesamin, episesamin and their derivatives on Nrf2/ARE activation in PC12 cells. PC12 cells transfected with the ARE firefly luciferase reporter vector and Renilla luciferase control vector were treated with sesamin, episesamin or their derivatives at 10 μM for 24 h, followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to the DMSO control. Data are expressed as mean ± SD (n = 3). Statistical significance was determined by one way ANOVA and Fisher's-multiple range test (p <0.01).

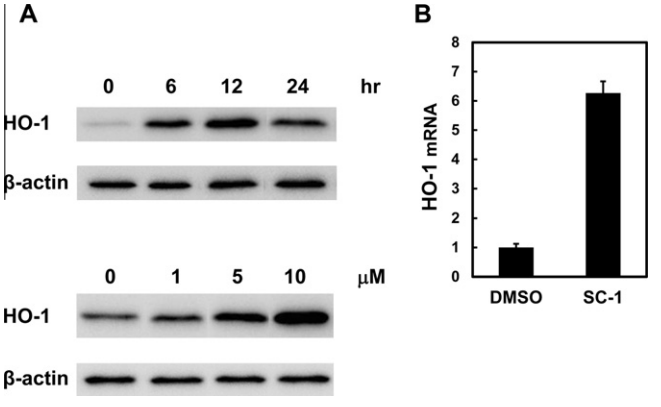

Figure 2. Effects of SC-1 on HO-1 expression in PC12 cells. (A) PC12 cells were treated with SC-1 (10 μM) for indicated times (upper column). PC12 cells were treated for 24 h with increasing concentrations of SC-1 (lower column). After treatments, cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown. (B) PC12 cells were treated with SC-1 (10 μM) for 6 h. Then, total RNA was harvested and subjected to quantitative real-time RT-PCR for HO-1 and 18S ribosomal RNA. The relative expression level of HO-1 mRNA was expressed as a ratio to 18S ribosomal RNA mRNA. Data are expressed as mean ± SD (n = 3).

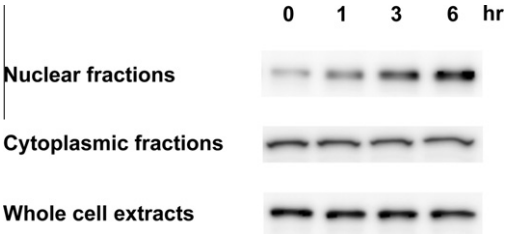

Figure 3. Effects of SC-1 on nuclear translocation of Nrf2 in PC12 cells. At indicated times after treatment with SC-1 (10 μM), nuclear and cytoplasmic fractions as well as whole cell extracts were prepared from PC12 cells and subjected to Western blot analysis for Nrf2. A representative blot from three independent experiments is shown.

present in the promoter region of genes encoding for phase II enzymes. Increase in nuclear Nrf2 was first detected at 1 h after treatment with SC-1, which was further enhanced up to 6 h, whereas the levels of Nrf2 in the cytoplasmic fraction were unaffected or slightly decreased (Fig. 3).

2.4. Activation of signal transduction by SC-1

Nuclear translocation of Nrf2 is potentiated by its phosphorylation, to which various signal transduction pathways have been linked. Treatment with SC-1 resulted in phosphorylation of p38 and JNK at 30 min after treatment, which gradually increased until 120 min (Fig. 4A). Phosphorylated ERK1/2 was also seen at 30 min after SC-1 treatment, but its level remained unchanged thereafter. In order to determine which signal transduction pathways are involved in Nrf2/ARE activation, we pretreated cells with their specific-inhibitors prior to SC-1 treatment. Pretreatment for 1 h with a p38 inhibitor, SB203580 (10 μM), attenuated HO-1 protein induction 5 h after treatment with SC-1, whereas a JNK-specific inhibitor, SP600125, and MEK1/2 inhibitors, PD98059 and U0126, showed little or no effect on HO-1 induction (Fig. 4B). Taken together, it was suggested that activation of the p38 MAPK pathway is in part involved in Nrf2/ARE activation and HO-1 induction by SC-1 presumably through Nrf2 phosphorylation.

2.5. Transient production of ROS by SC-1

Modification of the cysteine sulfhydryl groups of Keap1 by ROS and electrophiles changes its conformation, resulting in Nrf2 release and translocation into the nucleus. Here, we investigated if SC-1 could generate ROS. The intracellular ROS level was increased 0.5 h after treatment with SC-1, which remained elevated at 1 h and then gradually decreased to the basal level at 6 h (Fig. 5A). Pretreatment for 1 h with *N*-acetylcysteine (NAC) at 2.5 mM, which

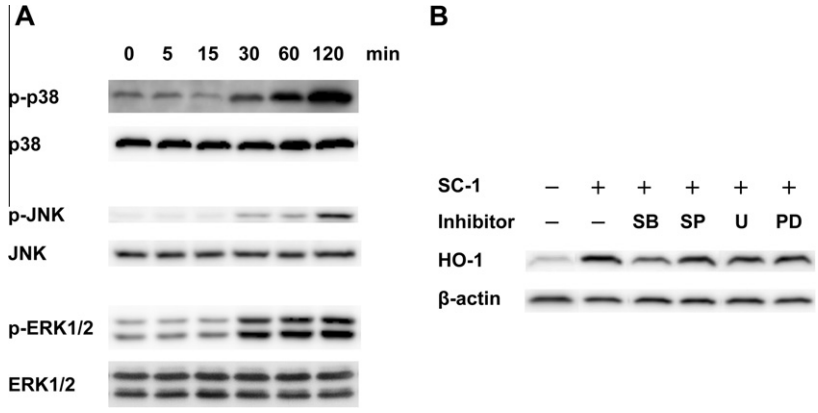

Figure 4. Effects of SC-1 on signal transduction in PC12 cells. (A) At indicated times after treatment with SC-1 (10 μM), cell lysates were prepared from PC12 cells and subjected to Western blot analysis for phosphorylated and total p38, JNK and ERK1/2 proteins. A representative blot from three independent experiments is shown. (B) PC12 cells were pretreated for 1 h with either SB203580 (10 μM), SP600125 (10 μM), U0126, (5 μM) or PD98059 (10 μM), and then treated for 5 h with SC-1 (10 μM). Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. A representative blot from three independent experiments is shown.

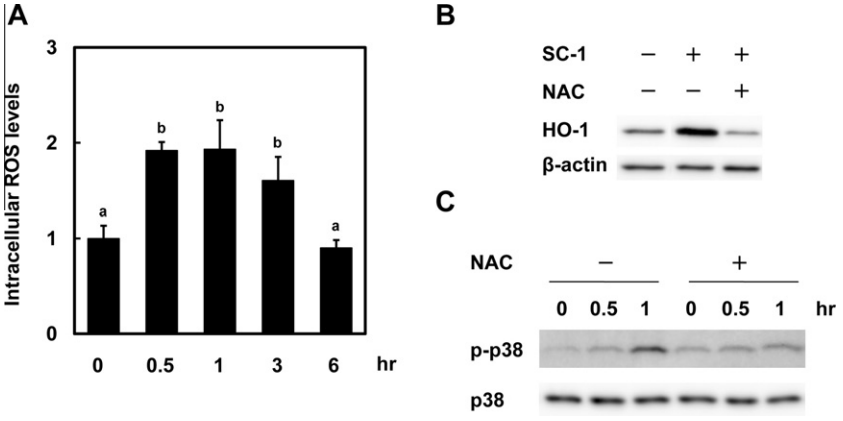

Figure 5. Effects of SC-1 on the intracellular ROS level in PC12 cells. (A) At indicated times after treatment with SC-1 (10 μM), PC12 cells were subjected to measurement for intracellular ROS levels using the fluorescent probe CM-H_2DCFDA. The ROS levels were expressed as fluorescence per mg of protein. Data are expressed as mean ± SD (n = 3). Statistical significance was determined by one way ANOVA and Fisher's-multiple range test (p <0.05). (B) PC12 cells were pretreated for 1 h with or without NAC (2.5 mM) and then treated for 24 h with SC-1 (10 μM). Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. A representative blot from three independent experiments is shown. (C) PC12 cells were pretreated for 1 h with or without NAC (2.5 mM) and then treated for indicated times with SC-1 (10 μM). Cell lysates were prepared and subjected to Western blot analysis for phosphorylated and total p38. A representative blot from three independent experiments is shown.

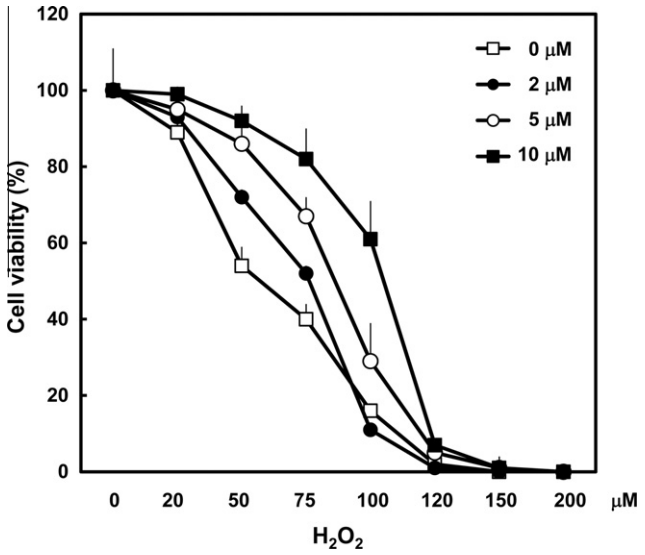

Figure 6. Neuroprotective effects of SC-1 in PC12 cells. PC12 cells were pretreated for 6 h with or without increasing concentrations of SC-1 (2, 5, 10 μM) and then treated for 20 h with or without indicted concentrations of H_2O_2. The number of living cells was determined by measuring ATP levels in the cells. Cell viability was calculated as a ratio to control without treatments. Data are expressed as mean ± SD (n = 3).

elevates intracellular glutathione levels, eliminated HO-1 induction by SC-1 at 24 h after treatment (Fig. 5B), suggesting that transient ROS production is involved in Nrf2/ARE activation by SC-1. Furthermore, we examined whether ROS that were generated transiently by SC-1 participated in activation of p38. As shown in Figure 5C, NAC treatment abolished SC-1-induced phosphorylation of p38, indicating that ROS are upstream signals of p38 in Nrf2/ARE activation by SC-1.

2.6. Protection against H_2O_2-induced cell death by SC-1

Since various compounds have been shown to protect against cell death by enhancing the antioxidant capacity via Nrf2/ARE activation, we tested if SC-1 could ameliorate oxidative stress-induced neuronal cell death. We pretreated PC12 cells with SC-1 for 6 h prior to treatment with different concentrations of H_2O_2. SC-1 significantly attenuated H_2O_2-induced cell death in a dose-dependent manner (Fig. 6). For instance, pretreatment with SC-1 (2, 5, 10 μM) increased cell viability after treatment with 75 μM of H_2O_2 from 40% to 52%, 67% and 82%, respectively. These results show that preconditioning by SC-1 protects against oxidative stress-induced PC12 cell death.

2.7. Attenuation of SC-1-mediated neuroprotection by inhibition of HO-1 and Nrf2/ARE activation

In order to provide direct evidence for the involvement of HO-1 induction and Nrf2/ARE activation in SC-1-mediated neuroprotection, we inhibited their activities by using a HO-1 inhibitor, Zn-protoporphyrin (ZnPP) and Nrf2 dominant-negative mutant expression vector, respectively. Treatment with ZnPP alone (5 and 10 μM) deceased viable cells after treatment with 75 μM of H_2O_2 from 40% to 21% and 18%, respectively (Fig. 7A). In the presence of ZnPP, however, neuroprotective effects mediated by SC-1 (10 μM) were significantly attenuated. On the other hand, overexpression of a dominant-negative mutant of Nrf2 diminished SC-1-mediated neuroprotection (Fig. 7B). Taken together, these results suggest that SC-1 protects against H_2O_2-induced cell death through HO-1 induction via Nrf2/ARE activation.

3. Discussion

In the present study, we tested if sesamin, episesamin and their metabolites (Supplementary Fig. 1) could activate Nrf2/ARE signaling in PC12 cells and found that SC-1 and EC-1, which were generated by oxidative demethylenation of one of two methylenedioxyphenyl moieties of sesamin and episesamin, were the most potent ARE activators (Fig. 1). Further oxidative demethylenation of another methylenedioxyphenyl moiety of SC-1 did not affect the ARE enhancer activity (SC-2), but that of EC-1 totally abolished it (EC-2). On the other hand, methylation of a catechol hydroxyl

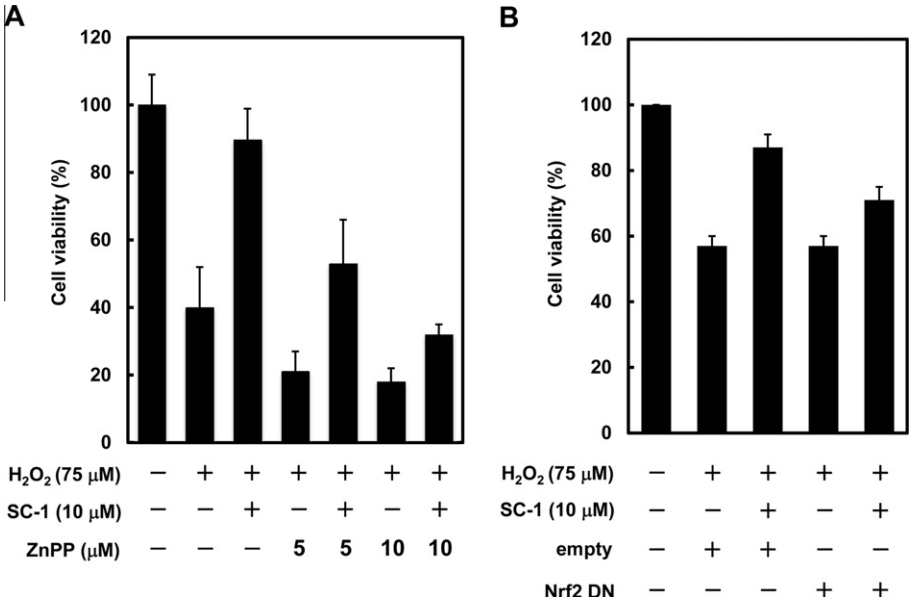

Figure 7. Effects of inhibition of HO-1 and Nrf2/ARE activation on SC-1-mediated neuroprotecion in PC12 cells. (A) PC12 cells on 96-well plates were pretreated for 6 h with or without SC-1 (10 μM) and then treated for 20 h with or without 75 μM H_2O_2 in the absence or presence of ZnPP (5 or 10 μM). (B) The ARE firefly luciferase reporter vector (2 μg) and the Renilla luciferase control vector (40 ng) were transfected into PC12 cells on 6-well plates with either empty vector or Nrf2 dominant-negative mutant expression vector (250 ng). 24 h after transfection, cells were replated onto 96-well plates. Then, cells were pretreated for 6 h with or without SC-1 (10 μM) and treated for 20 h with or without 75 μM H_2O_2. The number of living cells was determined by measuring ATP levels in the cells. Cell viability was calculated as a ratio to control without treatments and transfection. Data are expressed as mean ± SD (n = 3).

group of SC-1 and EC-1 eliminated the enhancer activity (SC-1m and EC-1m, respectively). These structure and function relationship studies indicate that primary metabolites of sesamin and episesamin, SC-1 and EC-1, are capable of activating Nrf2/ARE signaling in PC12 cells.

SC-1 treatment resulted in increased expression of HO-1 mRNA and protein (Fig. 2), which was preceded by nuclear translocation of Nrf2 (Fig. 3). We provided evidence suggesting that two distinct mechanisms are involved in Nrf2/ARE activation by SC-1. First, SC-1 increased phosphorylation of p38 MAP kinase and pretreatment with a p38 inhibitor, SB203580, diminished HO-1 induction caused by SC-1 (Fig. 4). Since it has been reported that Nrf2 phosphorylation by protein kinases facilitates its nuclear translocation, these results suggest that Nrf2 phosphorylation by p38 may participate in Nrf2/ARE activation by SC-1. Second, SC-1 treatment transiently increased the intracellular ROS level and glutathione replenishment by NAC abolished SC-1-induced HO-1 expression (Fig. 5). Oxidative modification of cysteine sulfhydryl groups of Keap1 by ROS has been shown to change its conformation, resulting in Nrf2 release. It is therefore likely that ROS transiently generated by SC-1 modify sulfhydryl groups of Keap1, thereby activating Nrf2/ARE signaling. Intriguingly, pretreatment with NAC also abolished p38 phosphorylation induced by SC-1, suggesting that ROS are upstream signals of p38 activation in Nrf2/ARE activation by SC-1 (Fig. 8). Nevertheless, the precise mechanisms by which SC-1 induces a transient increase in ROS, despite its potent radical scavenging activity in vitro, remain to be elucidated. In addition to these two mechanisms, covalent modification of the sulfhydryl groups of Keap1 has been proposed as another mechanism of Nrf2/ARE activation. Naturally-derived compounds such as sulforaphane as well as curcumin, caffeic acid phenetyl ester, zerumbone, sesquiterpene lactones and kavalactone have been proposed to interact with Keap1 as electrophiles or Michael reaction acceptors and activate Nrf2/ARE signaling.[5,6,19–22] However, it is unlikely that SC-1 directly interacts with Keap1, since SC-1 does not contain any α,β-unsaturated carbonyl group and also HO-1 induction by SC-1 was completely eliminated by pretreatment with NAC.

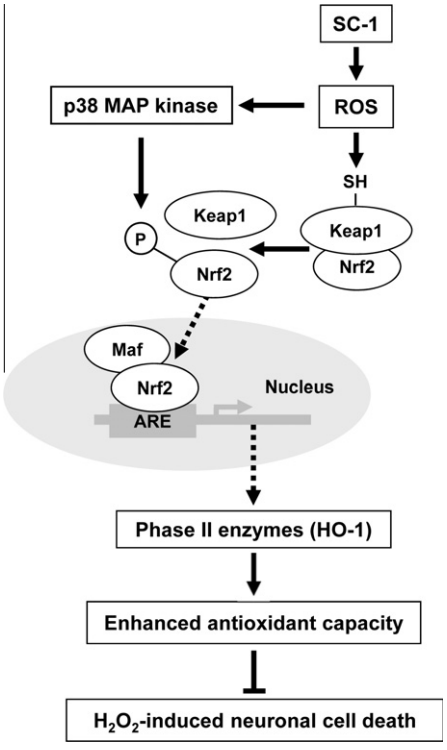

Figure 8. Schematic representation of mechanisms underlying neuroprotection by SC-1 through Nrf2/ARE activation. SC-1 transiently increases the intracellular level of ROS, which not only directly interact with sulfhydryl groups of Keap1 but also activate p38 and possibly phosphorylate Nrf2. The resultant Nrf2/ARE activation induces expression of phase II enzymes such as HO-1 and enhances the antioxidant capacity in the cells, resulting in protection against H_2O_2-induced neuronal cell death.

Preconditioning is defined as resistance to insults in response to previous short episodes.[23] Ischemic preconditioning enhances resistance to cerebral and myocardial infarction.[24,25] In cell culture models, it has been demonstrated that cells acquire resistance to an otherwise lethal heat shock insult after repeated exposures to non-lethal heat shock.[26] Pretreatment with 4-hydroxynonenal (4-HNE), a toxic by-product of lipid peroxidation, activates Nrf2/ARE signaling and protects against 6-hydroxydopamine-induced PC12 cell death.[27] We have previously reported that preconditioning by a sesquiterpene lactone enhances Nrf2/ARE activation caused by oxidative stress in PC12 cells.[20] We have also demonstrated that a kavalactone derivative protects against H_2O_2-induced PC12 cell death via a preconditioning effect on Nrf2/ARE activation.[22] In the present study, pretreatment with SC-1 attenuated H_2O_2-induced cell death in a dose-dependent manner (Fig. 6). Furthermore, treatment with a HO-1 inhibitor, Zn-protoporphyrin (ZnPP), and overexpression of a dominant-negative mutant of Nrf2 diminished SC-1-mediated neuroprotection (Fig. 7), providing direct evidence for the involvement of HO-1 induction and Nrf2/ARE activation in SC-1-mediated neuroprotection. Furthermore, in addition to antioxidant enzymes, Nrf2/ARE activation has been shown to induce expression of anti-inflammatory proteins.[28] Thus, preconditioning by Nrf2/ARE activators could be a promising strategy to combat with oxidative stress-related diseases including neurodegenerative diseases.

Accumulating evidence has suggested beneficial effects of sesamin on health and diseases.[9–14] In an animal model of Parkinson's disease that is associated with oxidative stress, dietary intake of sesamin showed cytoprotective effects against rotenone-induced loss of dopaminergic neurons.[15] It has been also reported that sesamin and sesamolin, another lignan in sesame oil, attenuates PC12 cell death induced by hypoxia and hydrogen peroxide through suppression of ROS generation.[29] In vitro, SC-1 and EC-1 exhibit a strong scavenging activity against superoxide anion and hydroxyl radical, although their parental compounds, sesamin and episesamin, lack this property.[16,17] It is therefore possible that neuroprotective effects caused by SC-1 could be explained in part by direct ROS scavenging. However, our results have clearly indicated that SC-1 protects against H_2O_2-induced cell death through activation of Nrf2/ARE signaling and induction of phase II antioxidant enzymes such as HO-1, providing a novel mechanism by which sesamin derivatives exert protective effects against oxidative stress-induced neuronal cell death.

We have recently reported that SC-1 targets NGF signaling pathway and induces neuronal differentiation in PC12 cells.[18] These findings and our present results suggest that regular dietary intake of sesamin might reduce oxidative stress and enhance NGF function in the brain, thereby contributing to brain health. Similarly to SC-1, carnosic acid extracted from rosemary has been shown to activate Nrf2/ARE signaling through interaction with Keap1 and thus decrease the infarct size in a rodent model of brain infarction.[30] The authors also reported that carnosic acid can induce neuronal differentiation in PC12 cells.[31] Since cholinergic neurons are more vulnerable than other cells in AD,[32–34] and mature cholinergic neurons in the basal forebrain are highly dependent on the NGF activity,[35,36] NGF has been proposed as a potential therapeutics for AD with an expectation that enhancement of NGF function could prevent and compensate neuronal and synaptic loss.[37] On the other hand, it has been widely accepted that oxidative stress is involved in the pathogenesis of neurodegenerative diseases such as AD, PD and amyotrophic lateral sclerosis. It would be thus of great

interesting to identify bi-functional molecules that are capable of activating Nrf2/ARE signaling as well as enhancing NGF function like SC-1 and carnosic acid. It is also noteworthy that SC-1 promoted neuronal differentiation through activating the ERK1/2-MAPK signaling pathway that is located downstream from the NGF tyrosine kinase receptor, TrkA, while it activated Nrf2/ARE signaling through p38 phosphorylation. These findings suggest that SC-1 modulates distinct signaling pathways and thereby exerts diverse physiological effects.

When rats were fed with diet containing sesamin, SC-1 predominates in the plasma, which is presumably due to high enzymatic activity of P450 in the liver.[38] In contrast, in the brain, the concentrations of sesamin and episesamin were much higher than those of SC-1 and EC-1 when both compounds were orally administered (Tomimori et al., unpublished data). These findings suggest that SC-1 and EC-1 may not be able to effectively path through the blood brain barrier (BBB) because of their less lipophilic nature compared with sesamin and episesamin. Alternatively, SC-1 and EC-1 generated by P450 from sesamin and episesamin may be readily metabolized by COMT in the brain. Nevertheless, sesamin and episesamin that reached the brain are eventually metabolized. SC-1 and EC-1 transiently generated during the process of metabolizing sesamin and episesamin may activate Nrf2/ARE signaling.

4. Conclusion

We have demonstrated that SC-1 protects against H_2O_2-induced neuronal cell death through induction of HO-1 via Nrf2/ARE activation. Thus, SC-1 has a potential to reduce oxidative stress and ameliorate oxidative stress-related neurodegenerative diseases.

5. Experimental

5.1. Cell culture and treatments

Rat PC12 pheochromocytoma cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal bovine serum. Sesamin, episesamin and their metabolites were isolated and purified, and their chemical structures were confirmed as previously described.[16,39] In the present study, the following eight compounds were used (Supplementary Fig. 1); (1*R*,2*S*,5*R*,6*S*)-2,6-bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (sesamin), (1*R*,2*S*,5*R*,6*R*)-2,6-bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (episesamin), (1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0] octane (SC-1), (1*R*,2*S*,5*R*,6*R*)- and (1*R*,2*R*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0] octane (EC-1), (1*R*,2*S*,5*R*,6*S*)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (SC-2), (1*R*,2*S*,5*R*,6*R*)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (EC-2), (1*R*,2*S*,5*R*,6*S*)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (SC-1m), (1*R*,2*S*,5*R*,6*R*)- and (1*R*,2*R*,5*R*,6*S*)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (EC-1m). p38 inhibitor (SB203580), JNK inhibitor (SP600125) and MEK1/2 inhibitors (PD98059 and U0126) were purchased from Calbiochem (La Jolla, CA, USA). *N*-acetylcysteine (NAC) and Zn-protoporphyrin (ZnPP) were from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA), respectively.

5.2. Transfection and luciferase assays

The ARE reporter gene containing two copies of the ARE enhancer sequence found in the promoter of glutathione S-transferase Ya subunit gene[40] was previously described (pGL3-ARE-TK-luc).[20] The Nrf2 dominant-negative mutant expression vector (pEF-dominant-negative Nrf2) and its empty vector (pEF) are kind gifts from Dr. Jeffrey A. Johnson, University of Wisconsin–Madison.[41] Plasmids were transfected into cells by using TransIT-LT1 reagent (Mirus, Madison, WI, USA). Luciferase activities were measured using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA) and Pikkagene Dual luciferase assay system (TOYO B-Net, Tokyo, Japan).

5.3. Western blot analysis

Anti-HO-1 (SPA-896) and -β-actin (A5441) antibodies were from Stressgen Bioreagents (Ann Arbor, MI, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Antibodies against Nrf2 (sc-722), γ-GCSc (sc-22755) and NQO-1 (sc-16464) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies recognizing p-p38 (#9211), p-JNK (#9251), p-ERK1/2 (#9101), p38 (#9212), JNK (#9252) and ERK1/2 (#9102) were obtained from Cell Signaling Technology (Beverly, MA, USA). Whole cell extracts were prepared using the RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing protease inhibitor cocktails and phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich). Nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Rockford, IL, USA). Extracts were separated by SDS–PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk for 1 h, membranes were incubated overnight at 4 °C with a primary antibody and then reacted with a HRP-conjugated secondary antibody for 1 h. Immunoreactive proteins were visualized using the ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) and the LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

5.4. Total RNA extraction and reverse transcriptase-PCR for mRNA detection

Total RNA was extracted using the SV Total RNA Isolation System (Promega). RNA was reverse-transcribed to cDNA using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed on the Thermal Cycler Dice Real Time System (Takara, Ohtsu, Japan) using SYBR Premix Ex Taq (Takara). Primer sequences for HO-1 and 18S ribosomal RNA were described previously.[20] The relative expression level of HO-1 mRNA was expressed as a ratio to 18S ribosomal RNA mRNA.

5.5. Measurement of intracellular ROS levels

Intracellular ROS levels were determined using a fluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) obtained from Molecular Probes (Eugene, OR, USA). After incubation with 10 μM CM-H_2DCFDA for 30 min at 37 °C, cell lysates were prepared and subjected to fluorescence measurement at the excitation wavelength of 490 nm and the emission wavelength of 530 nm using the fluorescence microplate reader, MTP-600F (Corona Electric, Ibaragi, Japan).

5.6. Measurement of cell viability

Cell viability was determined by the CellTiter-Glo Luminescent Cell Viability Assay Kit according to the manufacturer's instruction (Promega).

5.7. Statistical analysis

Statistical significance was determined by one way ANOVA and Fisher's-multiple range test.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.059.

References and notes

1. Sies, H. *Am. J. Med.* **1991**, *91*, 31S.
2. Itoh, K.; Chiba, T.; Takahashi, S.; Ishii, T.; Igarashi, K.; Katoh, Y.; Oyake, T.; Hayashi, N.; Satoh, K.; Hatayama, I.; Yamamoto, M.; Nabeshima, Y. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 313.
3. Nguyen, T.; Yang, C. S.; Pickett, C. B. *Free Radical Biol. Med.* **2004**, *37*, 433.
4. Na, H. K.; Surh, Y. J. *Mol. Carcinog.* **2006**, *45*, 368.
5. Balogun, E.; Hoque, M.; Gong, P.; Killeen, E.; Green, C. J.; Foresti, R.; Alam, J.; Motterlini, R. *Biochem. J.* **2003**, *371*, 887.
6. Dinkova-Kostova, A. T.; Holtzclaw, W. D.; Cole, R. N.; Itoh, K.; Wakabayashi, N.; Katoh, Y.; Yamamoto, M.; Talalay, P. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11908.
7. Hayes, J. D.; McMahon, M. *Cancer Lett.* **2001**, *174*, 103.
8. Fukuda, N.; Zhang, L.; Kodama, M.; Sakono, M.; Ide, T.; Yamamoto, K.; Sugano, M. *J. Nutr. Sci. Vitaminol. (Tokyo)* **1999**, *45*, 437.
9. Hirose, N.; Inoue, T.; Nishihara, K.; Sugano, M.; Akimoto, K.; Shimizu, S.; Yamada, H. *J. Lipid Res.* **1991**, *32*, 629.
10. Penalvo, J. L.; Hopia, A.; Adlercreutz, H. *Eur. J. Nutr.* **2006**, *45*, 439.
11. Kita, S.; Matsumura, Y.; Morimoto, S.; Akimoto, K.; Furuya, M.; Oka, N.; Tanaka, T. *Biol. Pharm. Bull.* **1995**, *18*, 1283.
12. Matsumura, Y.; Kita, S.; Tanida, Y.; Taguchi, Y.; Morimoto, S.; Akimoto, K.; Tanaka, T. *Biol. Pharm. Bull.* **1998**, *21*, 469.
13. Matsumura, Y.; Kita, S.; Morimoto, S.; Akimoto, K.; Furuya, M.; Oka, N.; Tanaka, T. *Biol. Pharm. Bull.* **1995**, *18*, 1016.
14. Akimoto, K.; Kitagawa, Y.; Akamatsu, T.; Hirose, N.; Sugano, M.; Shimizu, S.; Yamada, H. *Ann. Nutr. Metab.* **1993**, *37*, 218.
15. Fujikawa, T.; Kanada, N.; Shimada, A.; Ogata, M.; Suzuki, I.; Hayashi, I.; Nakashima, K. *Biol. Pharm. Bull.* **2005**, *28*, 169.
16. Nakai, M.; Harada, M.; Nakahara, K.; Akimoto, K.; Shibata, H.; Miki, W.; Kiso, Y. *J. Agric. Food Chem.* **2003**, *51*, 1666.
17. Kiso, Y. *Biofactors* **2004**, *21*, 191.
18. Hamada, N.; Fujita, Y.; Tanaka, A.; Naoi, M.; Nozawa, Y.; Ono, Y.; Kitagawa, Y.; Tomimori, N.; Kiso, Y.; Ito, M. *J. Neural Transm.* **2009**, *116*, 841.
19. Nakamura, Y. Y. C.; Murakami, A.; Ohigashi, H.; Osawa, T.; Uchida, K. *FEBS Lett.* **2004**, *572*, 245.
20. Umemura, K.; Itoh, T.; Hamada, N.; Fujita, Y.; Akao, Y.; Nozawa, Y.; Matsuura, N.; Iinuma, M.; Ito, M. *Biochem. Biophys. Res. Commun.* **2008**, *368*, 948.
21. Wruck, C. J.; Gotz, M. E.; Herdegen, T.; Varoga, D.; Brandenburg, L. O.; Pufe, T. *Mol. Pharmacol.* **2008**, *73*, 1785.
22. Tanaka, A.; Hamada, N.; Fujita, Y.; Itoh, T.; Nozawa, Y.; Iinuma, M.; Ito, M. *Bioorg. Med. Chem.* **2010**, *18*, 3133.
23. Calabrese, E. J. *Toxicol. Appl. Pharmacol.* **2004**, *197*, 125.
24. Kirino, T. *J. Cereb. Blood Flow Metab.* **2002**, *22*, 1283.
25. Raphael, J. *Semin. Cardiothorac. Vasc. Anesth.* **2010**, *14*, 54.
26. Rattan, S. I. *Biochem. Mol. Biol. Int.* **1998**, *45*, 753.
27. Chen, Z. H.; Saito, Y.; Yoshida, Y.; Sekine, A.; Noguchi, N.; Niki, E. *J. Biol. Chem.* **2005**, *280*, 41921.
28. van Muiswinkel, F. L.; Kuiperij, H. B. *Curr. Drug Targets CNS Neurol. Disord.* **2005**, *4*, 267.
29. Hou, R. C.; Huang, H. M.; Tzen, J. T.; Jeng, K. C. *J. Neurosci. Res.* **2003**, *74*, 123.
30. Satoh, T.; Kosaka, K.; Itoh, K.; Kobayashi, A.; Yamamoto, M.; Shimojo, Y.; Kitajima, C.; Cui, J.; Kamins, J.; Okamoto, S.; Izumi, M.; Shirasawa, T.; Lipton, S. A. *J. Neurochem.* **2008**, *104*, 1116.
31. Kosaka, K.; Mimura, J.; Itoh, K.; Satoh, T.; Shimojo, Y.; Kitajima, C.; Maruyama, A.; Yamamoto, M.; Shirasawa, T. *J. Biochem.* **2010**, *147*, 73.
32. Davies, P.; Maloney, A. J. *Lancet* **1976**, *2*, 1403.
33. Bartus, R. T.; Dean, R. L., 3rd; Beer, B.; Lippa, A. S. *Science* **1982**, *217*, 408.
34. Whitehouse, P. J.; Price, D. L.; Struble, R. G.; Clark, A. W.; Coyle, J. T.; Delon, M. R. *Science* **1982**, *215*, 1237.
35. Hefti, F.; Weiner, W. J. *Ann. Neurol.* **1986**, *20*, 275.
36. Fischer, W.; Wictorin, K.; Bjorklund, A.; Williams, L. R.; Varon, S.; Gage, F. H. *Nature* **1987**, *329*, 65.
37. Cattaneo, A.; Capsoni, S.; Paoletti, F. *J. Alzheimers Dis.* **2008**, *15*, 255.
38. Nakano, D.; Kwak, C. J.; Fujii, K.; Ikemura, K.; Satake, A.; Ohkita, M.; Takaoka, M.; Ono, Y.; Nakai, M.; Tomimori, N.; Kiso, Y.; Matsumura, Y. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 328.
39. Perez, C.; Almonacid, L. N.; Trujillo, J. M.; Gonzalez, A. G.; Alonso, S. J.; Navarro, E. *Phytochemistry* **1995**, *40*, 1511.
40. Morimitsu, Y.; Nakagawa, Y.; Hayashi, K.; Fujii, H.; Kumagai, T.; Nakamura, Y.; Osawa, T.; Horio, F.; Itoh, K.; Iida, K.; Yamamoto, M.; Uchida, K. *J. Biol. Chem.* **2002**, *277*, 3456.
41. Lee, J. M.; Calkins, M. J.; Chan, K.; Kan, Y. W.; Johnson, J. A. *J. Biol. Chem.* **2003**, *278*, 12029.