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The Plant Phenolic Diterpene Carnosol Suppresses Sodium Nitroprusside-Induced Toxicity in C6 Glial Cells

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Carnosol, a naturally occurring bioactive phenolic diterpene originating from rosemary and sage, has been shown to exert antioxidant and anti-inflammatory effects. This study examined possible protective effects of carnosol on sodium nitroprusside (SNP)-induced cytotoxicity in C6 glial cells. Carnosol (1−10 μM) dose-dependently attenuated SNP (100 μM)-induced cell death and NO production. SNP-induced apoptotic characteristics, including DNA fragmentation, caspase-3 activation, and c-jun N-terminal protein kinase (JNK) phosphorylation, were significantly suppressed by carnosol (10 μM). In addition, carnosol pretreatment restored the level of reduced glutathione (GSH), which was diminished by SNP treatment. Although both SNP (100 μM) and carnosol (10 μM) stimulated the HO-1 expression time-dependently, SNP caused a temporal increase in HO-1 in early time periods (3−6 h) before cell death occurred. In contrast, carnosol induced the sustained expression of HO-1 until a late time point (24 h). The addition of 1 μM zinc protoporphyrin IX (ZnPP), a specific HO inhibitor, with SNP or carnosol further reduced cell viability. Also, the addition of ZnPP inhibited the protective effect of carnosol against SNP-induced cytotoxicity in C6 cells. These results suggest that carnosol possesses abilities to inhibit SNP-mediated glial cell death through modulation of apoptotic events and induction of HO-1 expression.

KEYWORDS: Carnosol; SNP; glial cell; apoptosis; GSH; heme oxygenase-1

INTRODUCTION

Astroglial cells (astrocytes) play an essential role in maintaining the functions of neurons in the brain. Astrocytes act as a structural support within the central nervous system (CNS); their processes contact various cell types in CNS such as neurons, other glial cells, endothelial cells, and blood vessels. It has been suggested that astrocytes and microglia can be activated and produce toxic mediators to neurons during the pathogenesis of various neurodegenerative diseases (*1*). Although reactive astrocytes produce cytokines and other neurotoxic products, they can also secrete neuroprotective molecules. Several studies have demonstrated that astrocytes assist outgrowth and survival of neurons by secreting a number of neurotrophic factors including glial cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (*2*, *3*).

Recent papers suggest that astrocytes particularly exert neuroprotective effects in Parkinson's disease (PD) via up-regulating antioxidant enzyme-related molecules, such as protease-activated receptor-1 (PAR-1) and NF-E2-related factor (Nrf2) (*4*, *5*). Moreover, recent evidence suggests that astrocytes appear not to attack a pathological target, as do microglia (*6*). For these reasons, astrocytes are regarded as the first line of defense against neuropathological conditions. Because dysfunction or loss of astrocytes leads to cell death and dysfunction of the surrounding neurons, much interest has focused on the protection of astrocytes, which may be a promising way to treat neurodegenerative diseases.

Accumulating evidence from epidemiological and dietary intervention studies has implicated that naturally occurring polyphenols may play a useful role in preventing neurodegeneration (*7*, *8*). Carnosol and carnosic acid are major polyphenolic diterpenes found in Lamiaceae herbs, rosemary and sage (*9*). Carnosic acid is easily converted into carnosol by oxidation. Carnosol has multiple biological activities including antioxidant, anti-inflammatory, antimicrobial, antitumor, and neuromodulatory effects (*9*, *10*). We have previously shown that carnosol has protective effects against rotenone-induced neurotoxicity in nigral dopaminergic neuronal cells (*11*). Also, carnosic acid and carnosol protected cortical neurons by activating the Keap1/Nrf2 pathway and protected the HT22 neuronal cells against oxidative glutamate toxicity via activation of transcriptional antioxidant-responsive element (*12*).

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Although neuronal cell survival by carnosol has been reported, the effect of carnosol against glial cell death has not been studied. Therefore, we examined the protective effects of carnosol against sodium nitroprusside (SNP)-induced C6 glial cell damage. C6 cells have been well characterized and applied as a useful astroglial model because these cells express specific characteristic astrocyte markers, such as glutamine synthetase (GS) and glial fibillary acidic protein (GFAP). In this study, we found for the first time that carnosol-mediated protection in C6 glial cells was involved in the attenuation of apoptotic cell death and modulation of antioxidative molecules.

MATERIALS AND METHODS

Materials. Carnosol was obtained from Cayman Chemical (Ann Arbor, MI). The specific JNK inhibitor SP600125, the protein kinase C (PKC) inhibitor Gö6983, cell-permeable PKC inhibitor Gö6976, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, the specific protein kinase A (PKA) inhibitor PKI, and the specific Akt/PKB inhibitor triciribine were purchased from Calbiochem (San Diego, CA). SNP and other chemicals were purchased from Sigma (St. Louis, MO). Antibodies against HO-1, Nrf2, Sp1, and actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against caspase-3 and phospho-JNK were from Cell Signaling Technology Inc. (Beverly, MA).

Cell Culture and Treatments. Rat C6 glioma cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)−streptomycin (100 μg/mL) at 37 °C in 5% CO_2. Because C6 cells in later passages have shown many properties of astrocytes, we used the cells in later passages in our experiments. C6 cells were cultured at a seeding density of 2.5×10^5 cells/mL. Usually, 1 day before any treatments, the culture medium was changed to DMEM medium with 0.5% FBS to reduce the serum effect. When indicated, cells were treated with 100 μM SNP for 24 h or pretreated with carnosol for 1 h prior to addition of SNP. To prevent direct interaction between carnosol and SNP in the culture medium, at the end of the carnosol pretreatment, the medium was changed to fresh low-serum culture medium. To estimate cell viability, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction assay was used in combination with total cell counting, using trypan blue dye exclusion, as previously described (*13*). In a single experiment, each treatment was performed in triplicate.

Measurement of Nitric Oxide. Nitrite (NO^-_2) in culture medium was measured as an indicator of NO production. One hundred microliters of culture supernatant was mixed with an equal volume of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylendiamine dihydrochloride in water) and incubated under reduced light at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate reader. Sodium nitrite ($NaNO_2$) was used as standard to calculate nitrite concentration.

Detection of Apoptosis. Apoptotic cell death was analyzed by detection of oligonucleosomal fragmentation of DNA and nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI). For DNA fragmentation analysis, nuclear DNA was isolated as described before (*13*), electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with DAPI as described before (*13*). Percentage of apoptotic cells, which coincided with morphological criteria of apoptosis such as nuclear condensation and segmentation, was counted as a ratio of apoptotic nuclei over the total number of nuclei.

Measurement of the Activation of Caspase-3. Caspase-3 activity was determined according to a previously described method (*14*). Briefly, cells were scraped from the plate with cold lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), incubated for 5 min on ice, and clarified by centrifuging at 10000*g* at 4 °C for 10 min. The caspase-3 substrate, 200 μM AC-DEVD-pNA (Biomol, Plymouth Meeting, PA), was added to 10 μg protein samples. Formation of *p*-nitroaniline (*p*NA) from the reaction was measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA) with 405 nm wavelength over a 60 min period. Activity was expressed as picomoles of substrate hydrolyzed per minute.

Measurement of Cellular GSH Content. The intracellular reduced glutathione (GSH) was measured according to a previously described method (*15*). Briefly, cells were homogenized in 0.1 M phosphate buffer (pH 7.4) and further resuspended with an equal volume of 10% trichloroacetic acid. After 10 min of vigorous shaking, samples were centrifuged at 21000*g* for 10 min at 4 °C. Thirty microliter aliquots of the resulting supernatant were mixed with 0.01 M phosphate buffer (pH 7.5, 570 μL), NADPH (4 mM, 50 μL), and glutathione reductase (6 U/mL, 100 μL). After 5 min of incubation at 37 °C, 5,5′-dithiobis(2-nitrobenzoic acid) (10 mM, 50 μL) was added just before the absorbance was read. The formation of 2-nitro-5-thiobenzoic acid was measured at 412 nm for 6 min. Total GSH was determined from a standard curve constructed using known amounts of GSH. Values were expressed as a relative content of control cells.

Immunoblot. For immunoblot analysis, total cell proteins were prepared from the C6 cells grown under various experimental conditions. Cells were washed twice with 1× phosphate-buffered solution (PBS) and then lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 mg/mL phenylmethanesulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate in PBS). After 30 min of incubation on ice, cell lysates were centrifuged at 14000*g* for 10 min at 4 °C. The supernatants were used as the total cell lysates. In some experiments, nuclear extracts were prepared from C6 cells using a Nuclear Extraction kit (Cayman Chemical). Protein concentration was determined by using the BSA protein assay kit (Bio-Rad, Hercules, CA). Each 20 μg of protein samples was separated on 10−15% SDS-PAGE and blotted onto PVDF membrane. The membrane was treated with primary antibody for caspase-3 (1:1000 dilution), HO-1 (1:4000 dilution), p-JNK (1:1000 dilution), Nrf2 (1:1000 dilution), Sp1 (1:1000 dilution), or actin (1:4000 dilution) and then reacted with HRP-conjugated secondary antibody. Immunoreactive bands were detected by ECL chemiluminescence kit (GE Healthcare, Piscataway, NJ).

Statistical Analysis. The data were expressed as the mean ± SEM. Data were first analyzed using one-way factorial analysis of variance (ANOVA). Tukey's multiple-comparison test or Student's *t* test was then performed to compare treated samples, and $p < 0.05$ was considered to be significant.

RESULTS

C6 glial cells are widely used to study astrocytic pathogenesis, because this cell line expresses some representative astrocytic markers, such as glial fibrillary acidic protein (GFAP) and S-100 protein (*16*). Therefore, in this study, C6 cells were adopted as a model system to investigate the role of carnosol against SNP-mediated astrocytic glial cell death. In the present study, the effect of carnosol on SNP-induced C6 cell viability loss was assessed by XTT assay and confirmed with trypan blue exclusion test. Previous studies showed that various cell lines were treated with SNP at concentrations ranging from 10 μM to 5 mM according to the initial seeding density and treatment periods (*17*, *18*). In our experimental conditions, the effect of SNP on C6 cell death was detectable at concentrations of 25−100 μM after 24 h of treatment, and 100 μM SNP induced approximately 50% cell loss (**Figure 1B**). Thus, we did subsequent experiments using 100 μM SNP. When exposed to various concentrations of carnosol (1−10 μM) for 24 h, those concentrations of carnosol did not show any cytotoxicity in C6 cells (**Figure 1B**). To investigate the effect of carnosol on SNP-induced glial cell death, C6 cells were pretreated with 1−10 μM carnosol for 1 h, followed by 100 μM SNP treatment for 24 h. As shown in **Figure 1C**, pretreatment of carnosol attenuated SNP-induced cytotoxicity dose dependently. In addition, carnosol dose-dependently inhibited SNP-induced NO production (**Figure 1D**). Although the addition of a high concentration of carnosol (10 μM) to cells produced almost complete blocking of SNP-induced cell death (95.4% viability), the same concentration of carnosol attenuated NO production only 50%. This suggests that the protective effect of carnosol is not simply due to the attenuation of SNP-mediated NO

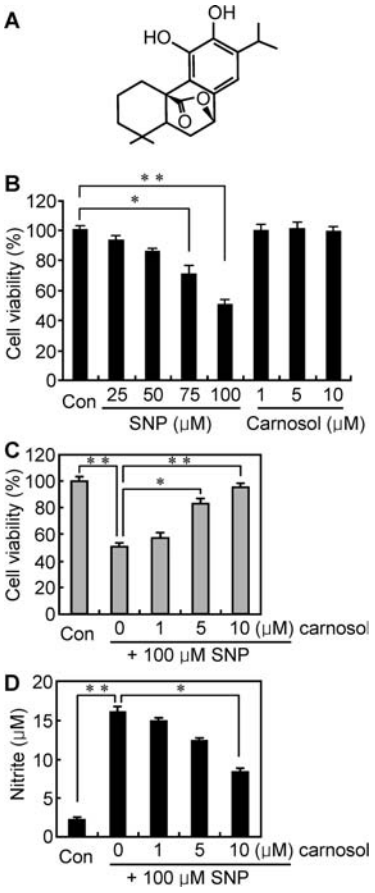

Figure 1. Effects of carnosol (**A**) on SNP-induced cytotoxicity and NO production in C6 glial cells. (**B**) Indicated concentrations of SNP or carnosol were added to the C6 glial cell cultures for 24 h. Cell viability was assessed by XTT reduction assay as described under Materials and Methods. (**C**) Cells were pretreated with various concentrations of carnosol (1, 5, and 10 μM) for 1 h and incubated for 24 h with SNP (100 μM). Cell viability was expressed a percentage of the untreated control (Con). (**D**) Under conditions described for panel **C**, the amount of NO released from cells was evaluated by the Griess reaction. The amount of NO production was quantitatively assessed using $NaNO_2$ as a standard. Data are the mean $\pm$ SEM from three independent experiments in triplicate. Statistically significant differences among groups were determined by one-way ANOVA, followed by Tukey's post hoc test. $*$, $p < 0.05$; $**$, $p < 0.01$.

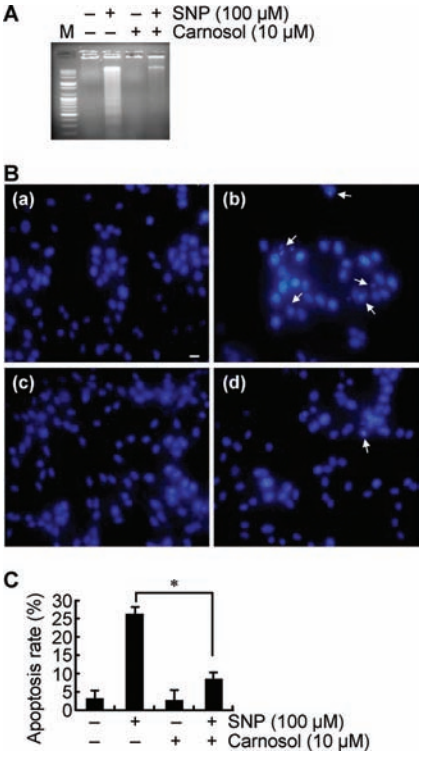

Figure 2. Carnosol prevents apoptotic nuclear changes induced by SNP in C6 glial cells. (**A**) Carnosol inhibited DNA fragmentation induced by SNP in C6 cells. Cells were treated with carnosol (10 μM) for 1 h followed by SNP (100 μM) treatment for a further 24 h, and the integrity of nuclear chromosomal DNA was determined by 1.5% agarose gel electrophoresis as described under Materials and Methods. Lane M, 100 bp plus DNA ladders (Bioneer, Seoul, Korea). (**B**) Representative DAPI-stained fluorescence photomicrographs show the nuclei morphology of C6 cells: (**a**) untreated control cells, (**b**) SNP (100 μM; 24 h), (**c**) treated with carnosol (10 μM; 24 h) alone, (**d**) carnosol (10 μM; 1 h) followed by SNP (100 μM; 24 h). Arrows indicate condensed chromatin, reduced nuclei, and fragmented nuclei in apoptotic cells. Scale bar indicates 10 μm. (**C**) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. Results are expressed as mean $\pm$ SEM of three independent experiments. $*$, $p < 0.05$ compared with SNP treatment alone (Student's t test).

generation. Thus, the next experiments sought to determine molecular mechanisms of carnosol-mediated protective effect against SNP-induced C6 cell death.

We next investigated the effect of carnosol on SNP-induced apoptotic characteristics, such as DNA laddering, nuclear morphology changes, and caspase-3 activation in C6 glial cells. A significant proportion of SNP-mediated cell death was apoptotic, based on DNA fragmentation (**Figure 2A**) as well as DAPI-stained nuclear changes in morphology (**Figure 2B**). However, carnosol pretreatment effectively inhibited those apoptotic events (**Figure 2A**,**B**). As quantified in **Figure 2C**, although SNP increased the apoptotic rate to 26.0 $\pm$ 0.9%, carnosol pretreatment prior to exposure to SNP caused a statistically significant reduced percentage of apoptotic rate (8.2 $\pm$ 1.1%). Furthermore, we examined the involvement of caspase-3 activation in our experimental conditions (**Figure 3**). As shown in **Figure 3A**, Western blotting showed that SNP treatment induced the cleavage of caspase-3 from its native 35 kDa form to an active 17 kDa fragment, but this caspase-3 activation was blocked by carnosol pretreatment. In addition, a time-dependent increase in the activation of caspase-3 was detected in SNP-treated C6 cells using specific colorimetric peptidyl substrate, Ac-DEVD-pNA (**Figure 3B**). However, carnosol (10 μM) pretreatment significantly attenuated the effects of SNP on caspase-3 activity. These results suggest that the protective effect of carnosol is mediated by anti-apoptotic pathway.

Because activation of JNKs has been known in apoptosis induction before caspase-3 activation, we investigated whether the protective effect of carnosol against cell death induced by SNP occurs via blocking JNK activation. As shown in **Figure 4**, the expression of phosphorylated JNK proteins was potently induced in 2−4 h after SNP treatment, and this JNK activation was blocked by carnosol pretreatment in C6 cells. Next, we confirmed whether JNK activation is critical for the effects of SNP on cell death. As shown in **Figure 4C**, pretreatment with the specific JNK inhibitor SP600125 (2 μM) partially blocked SNP-induced cell death. Because carnosol pretreatment did not significantly change the level of ERKs and p38 activation (data not shown), our results suggest that only JNKs among MAP kinase family members are required for SNP-mediated C6 glial cell death.

GSH has been known to play an important role in protecting against oxidative pathophysiology related to neurodegenerative

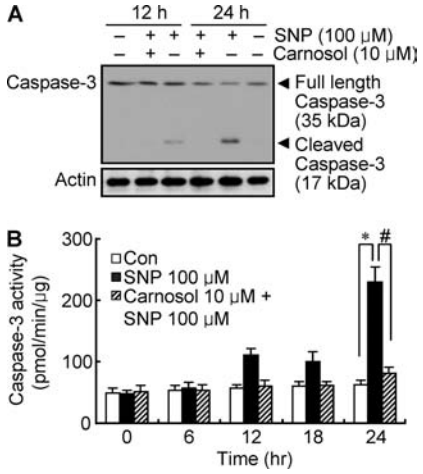

Figure 3. Effect of carnosol on the activation of caspase-3 in C6 cells. (**A**) The cells were treated with SNP (100 μM) for 12 or 24 h, in the absence or presence of 10 μM carnosol. Carnosol was added 1 h before SNP treatment. The expressions of native full-length caspase-3 (35 kDa) and activated fragment of caspase-3 (17 kDa) were detected by Western blotting using specific antibody (top panel). Anti-actin was used for normalization (bottom panel). (**B**) Caspase-3 enzyme activity was measured by using specific peptidyl substrate, Ac-DEVD-*p*NA, at various time points. Activity was calculated as picomoles of substrate hydrolyzed per minute per microgram of protein. All values represent mean ± SEM of three independent experiments. ∗, $p < 0.05$ (Student's *t* test), indicates the statistical significance as compared with untreated control. #, $p < 0.05$ (Student's *t* test), indicates statistically significant differences from treatment with SNP alone.

disorders (*19*). Therefore, we investigated if the protective effect of carnosol against SNP-mediated C6 cell death may also be related to modulation of GSH. As shown in **Figure 5**, incubation of C6 cells with 100 μM SNP for 12 and 24 h significantly decreased GSH contents to 83 and 60%, respectively. Carnosol (10 μM) treatment caused an elevation of GSH level at 12 and 24 h, although a tendency to decrease effect was observed (127% at 12 h and 121% at 24 h). In addition, pretreatment with carnosol markedly attenuated SNP-induced GSH depletion in C6 cells. The amount of GSH in cultures pretreated with carnosol (10 μM) before the SNP (100 μM) treatment increased by 20.5% (after 12 h) and 29.0% (after 24 h), respectively. These results suggest that the protective effect of carnosol is mediated via, at least in part, modulation of intracellular GSH generation.

Several studies have suggested that HO-1 expression plays a critical role in mediating antioxidant and anti-apoptotic defense mechanisms (*20*, *21*). As shown in **Figure 6A**, significant induction of HO-1 protein was observed in C6 cells treated with SNP. HO-1 induction by SNP (100 μM) peaked at 3−6 h after treatment and rapidly decreased after 12 h of exposure (**Figure 6A**,**B**). Of interest, carnosol alone also increased HO-1 protein expression in C6 cells. The increase of the HO-1 protein by carnosol was time-dependent and reached a maximum level at 12 h, which was sustained until 24 h with moderate decline (**Figure 6A**,**B**). Additionally, we analyzed the HO-1 expression in the presence of both SNP and carnosol (**Figure 6C**). Of interest, the combined treatment with SNP and carnosol induced a boosted expression of HO-1 over those obtained with each drug separately. Although the cell viability was maintained until HO-1 was expressed, the increased cell death was observed after the HO-1 level decreased (data not shown). These results suggest that the HO-1 expression is correlated with cell viability in C6 cells. To confirm the exact role of HO-1 expression, C6 cells were treated with ZnPP, a

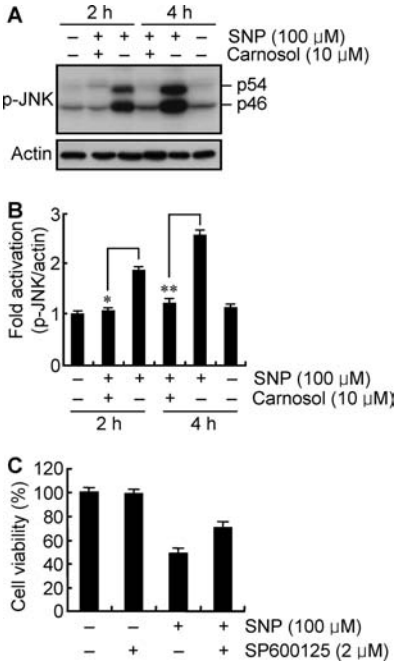

Figure 4. Effect of carnosol on SNP-induced JNK activation in C6 glial cells. Activation of JNKs in response to SNP was attenuated by carnosol in C6 cells. (**A**) Carnosol (10 μM) was added to the cultures for 1 h prior to the addition of SNP (100 μM) for different time periods (2, 4 h), and the expressions of phosphorylated JNKs were analyzed by Western blotting using specific antibody. Anti-actin was used for normalization. (**B**) The activation of JNKs was estimated by densitometric analysis of each protein band and assessed as the ratio against the value of untreated control. (**C**) Effect of JNK inhibitor on cell viability was determined. C6 cells were treated with a specific JNK inhibitor SP600125 (2 μM) 1 h prior to the addition of SNP (100 μM). Cell viability was assessed by XTT reduction assay after 24 h. All values represent mean ± SEM of three independent experiments. ∗, $p < 0.05$, ∗∗, $p < 0.01$ (Student's *t* test), as compared with the SNP treatment alone.

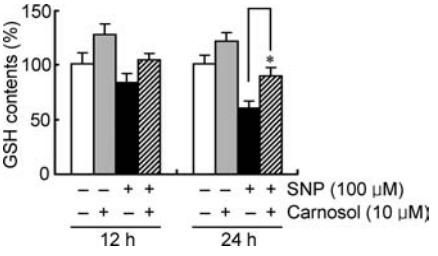

Figure 5. Effect of carnosol on GSH level in C6 glial cells. Cells were incubated in the absence or presence of carnosol (10 μM; 1 h) and then treated with SNP (100 μM) for 12 or 24 h. The intracellular GSH contents were determined by the change in absorbance induced by the addition of NADPH and glutathione reductase as described under Materials and Methods. The basal contents of GSH in untreated control cells were taken as 100%. All values represent mean ± SEM of three independent experiments in triplicate. ∗, $p < 0.05$ (Student's *t* test), compared with SNP treatment alone.

well-known inhibitor of HO-1. As shown in **Figure 6D**, the protective effect of carnosol on SNP-induced cell death was reversed by the addition of ZnPP. Similarly, when the C6 cells were treated with carnosol plus ZnPP and with SNP plus ZnPP, those cells became more sensitive and more cell death was triggered. These results suggest that the induced HO-1 can delay SNP-induced cell death and that induction of HO-1 expression is essential for carnosol to attenuate SNP-induced cytotoxicity in C6 cells.

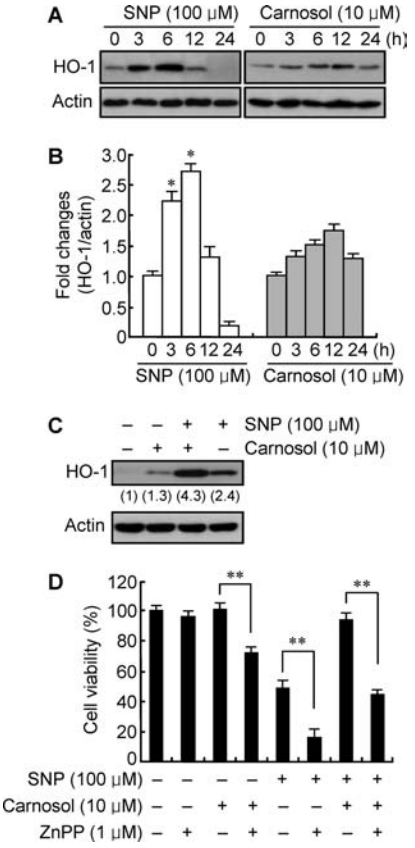

Figure 6. Induction of HO-1 expression involves C6 cell survival. (**A**) Differential time-dependent induction of HO-1 by SNP and carnosol. C6 cells were incubated with the indicated concentrations of SNP or carnosol. HO-1 expression was analyzed by Western blotting. At the same conditions, actin expression was determined for normalization. (**B**) The intensity of each band was estimated by densitometric analysis, and the fold changes of HO-1 induction were plotted as a graph. (**C**) Carnosol boosted SNP-induced HO-1 expression. C6 cells were pretreated with 10 μM carnosol 1 h before the addition of SNP (100 μM), and then the cells were incubated for 3 h. The expression of HO-1 was determined by Western blot analysis. After estimation of densitometric analysis of each protein band, fold changes of protein levels are indicated in parentheses under the HO-1 image. (**D**) HO-1 inhibitor, ZnPP, reversed the protective effect of carnosol and enhanced SNP-induced cell death. C6 cells were pretreated with carnosol (10 μM) for 6 h and further incubated with ZnPP (1 μM) for 30 min. After this pretreatment, the medium was replaced and then cells were treated with SNP (100 μM) for 24 h. Cell viability was assessed by XTT reduction assay. Results are expressed as the mean $\pm$ SEM from three independent experiments in triplicate. $*$, $p < 0.05$ (Student's t test), compared with untreated control cells. $**$, $p < 0.05$ (Student's t test), compared with carnosol- and/or SNP-treated cells.

Next, we analyzed the signaling pathways used by carnosol to induce HO-1 expression. C6 cells were preincubated for 30 min with pharmacological inhibitors, such as PKI (PKA inhibitor), LY294002 (PI3K inhibitor), Gö6976 (cell-permeable PKC-α,β inhibitor), Gö6983 (PKC-$\alpha,\beta,\gamma,\delta,\zeta$ inhibitor), or triciribine (specific Akt/PKB inhibitor) and then stimulated with 10 μM carnosol for 12 h. As shown in **Figure 7A**, inhibition of PKA and PKC pathways had little or no effect on HO-1 expression, suggesting that these kinases are not related to HO-1 up-regulation by carnosol. However, PI3K and Akt/PKB inhibition significantly reduced the stimulated HO-1 expression by carnosol. These results suggest that the PI3K-Akt/PKB pathway is required for the induction of HO-1 by carnosol in C6 glial cells.

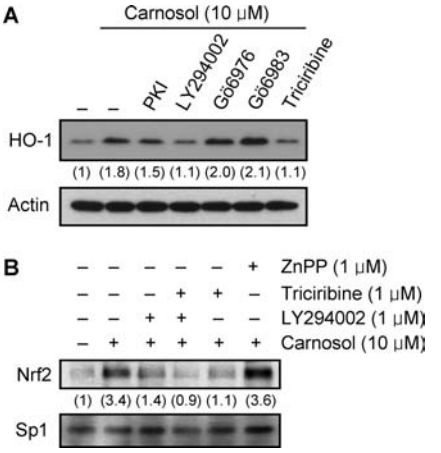

Figure 7. Induction of HO-1 and translocation of Nrf2 by carnosol via PI3 kinase and Akt/PKB. (**A**) Blocking PI3K and Akt/PKB prevents HO-1 up-regulation by carnosol. C6 cells were preincubated with either 0.5 μM PKI (PKA inhibitor), 1 μM LY294002 (PI3K inhibitor), 1 μM Gö6976 (cell-permeable PKC inhibitor), 1 μM Gö6983 (PKC inhibitor), or 1 μM triciribine (specific Akt/PKB inhibitor) for 30 min and then incubated with 10 μM carnosol for 12 h. Whole cell protein extracts were prepared and subjected to Western blotting with specific antibody for HO-1. Actin expression was used to show equal sample loading. (**B**) Carnosol-induced Nrf2 nuclear translocation was reduced by PI3K and Akt/PKB inhibition. C6 cells were preincubated with triciribine (1 μM) or LY294002 (1 μM) for 30 min and then incubated with 10 μM carnosol for 3 h. Nuclear extracts were analyzed by Western blotting with anti-Nrf2 antibody. The specific nuclear protein Sp1 expression was determined for normalization. The fold changes of HO-1 or Nrf2 protein levels are shown in parentheses. Results shown are representative of three separate experiments.

Nrf2, a member of the cap'n'collar family of transcription factors, regulates the expression of phase 2 genes encoding detoxifying and antioxidant enzymes including HO-1 (*5*). Therefore, we sought to determine whether carnosol induced nuclear translocation of Nrf2 in C6 cells. Although Nrf2 was weakly found in untreated control, carnosol treatment increased Nrf2 accumulation in the nucleus within 3 h (**Figure 7B**). Interestingly, inhibition of Akt/PKB and/or PI3K attenuated carnosol-induced Nrf2 nuclear translocation. However, when cells were pretreated with the HO-1 inhibitor ZnPP, Nrf2 nuclear accumulation by carnosol was not hampered, suggesting that Nrf2 nuclear translocation precedes HO-1 expression. Altogether these results suggest that the PI3K-Akt/PKB signaling pathway mediates the induction of HO-1 through the nuclear translocation of Nrf2 in C6 glial cells.

DISCUSSION

The present study revealed that carnosol can inhibit SNP-induced apoptosis in C6 glial cells. Carnosol, a naturally occurring phytopolyphenol found in rosemary, has been reported to exhibit several beneficial biological activities including potent antioxidant and anti-inflammatory effects (*10*). Therefore, carnosol has been investigated as a therapeutic potential agent against various diseases.

Resent studies have suggested that astrocytes play dual roles in Parkinson's disease (PD). Astrocytes secrete protective molecules to the peripheral surround while at the same time producing NO and pro-inflammatory molecules, leading to damaging surrounding neurons (*2*, *6*). Therefore, blocking the dysfunction of astrocytes can offer the initial strategy for treatment of PD and related diseases.

NO has been thoroughly investigated for its multipotent ability to act as a neurotransmitter or as a neurotoxic agent. Some recent studies have suggested that NO induces apoptosis in various cell types including astrocytes (*22*, *23*). In this study, SNP was used as an NO generator. Although SNP produces NO, it also generates cyanide and free iron (*24*). To distinguish the role of NO, cyanide, and free iron in the SNP-induced glial cell death, C6 cells were treated with ferric ammonium citrate (1 mM) or potassium ferricyanide (1 mM). However, treatment with ferric ammonium citrate or potassium ferricyanide did not change the cell viability in our experimental conditions (data not shown). Thus, we can speculate that NO may be a cytotoxic mediator involved in SNP-induced apoptosis of C6 glial cells. In addition, we found that the exogenous NO donor, SNP, activated C6 astrocytes and induced their own production of NO (**Figure 1D**).

In our results, carnosol suppressed the NO production (**Figure 1D**) in C6 glial cells, which was in agreement with previous results that demonstrated carnosol could attenuate the NO production in macrophages (*25*). From these results, it is clear that carnosol attenuates NO generation in SNP-activated C6 glial cells, but it is at present unclear whether carnosol, as an antioxidant, scavenges intracellular NO direct. A previous paper suggested that SNP could cause long-lasting oxidative stress by inducing hydroxyl radical generation (*26*). Carnosol and related antioxidant compounds have been known to scavenge hydroxyl radicals and other oxidants (*27*). Our results showed that carnosol (10 μM) pretreatment completely prevented the C6 glial cell death induced by SNP (**Figure 1C**). However, when our results were analyzed in detail, carnosol partially (around 50%) attenuated NO production. This means that carnosol may scavenge SNP-induced reactive hydroxyl radicals while at the same time suppressing SNP-mediated NO generation.

Previous studies using SNP showed the activation of apoptosis molecules during cell death in both rat primary cultured astrocytes and C6 glial cells (*28*, *29*). In accordance with the previous findings, our results revealed that SNP caused the typical apoptotic changes, such as nuclear condensation, DNA fragmentation, caspase-3 activation, and JNK phosphorylation in C6 glial cells (**Figures 2**−**4**). Of interest, carnosol pretreatment effectively suppressed essential morphologic forms of apoptotic nuclei and dramatically attenuated JNK and caspase-3 activation. These results strongly suggest that carnosol protects against the apoptotic cell death by SNP in glial cells. According to our knowledge, this is the first time evidence identified anti-apoptotic effects of carnosol in glial cells. However, when our results (**Figure 2**) were examined in detail, 100 μM SNP treatment for up to 24 h decreased cell viability to 50%, although only a 26% apoptosis rate was observed under the same condition. Therefore, it is likely that there is another type of cell death, such as necrosis induced by SNP. It has been proposed that NO can trigger mitochondrial depolarization, oxidative stress, and, subsequently, either necrotic or apoptotic cell death (*29*, *30*). Because carnosol (10 μM) almost completely blocked death of SNP-activated C6 glial cells (**Figure 1**), we can speculate that carnosol may inhibit both apoptotic and necrotic processes. Even though necrosis is involved in glial cell death, apoptosis is the major event in glial cell death and associated with neuropathological conditions (*22*, *28*). Recent data show that ROS can stimulate autophagy (also known as type II programmed cell death) as well as induce apoptosis (type I programmed cell death) (*31*). Several signaling pathways, such as p53, Bcl-2, and PI3K/Akt pathways, may regulate both autophagy and apoptosis processes (*31*). Therefore, it has been suggested that there are some connections between autophagy and apoptosis. When cells are exposed to stress signals, autophagy is induced early at the pre-apoptotic lag phase to suppress apoptosis by eliminating damaged organelles and cytoplasmic components. However, extensive autophagy can trigger either apoptosis or necrotic cell death. Although it has been suggested that there are some connections between different cell death mechanisms, there is no clear-cut mechanistic interaction between regulators of apoptosis, autophagy, and necrosis. This study was focused on the apoptotic cell death induced by SNP. However, we could not exclude whether autophagy and necrosis may play an important role or not during SNP-mediated glial cell death. In the future, we need more study to understand the mechanisms of interactions and alterations between apoptosis and autophagy and necrosis.

A common denominator of postulated pathogenic mechanisms in neurodegenerative diseases is a vicious cycle of oxidative stress. Because aerobic organisms are exposed to harmful oxidative stress, they have a complex antioxidant defense system. Of the various natural antioxidants in the nervous system, the glutathione (GSH) redox cycle is the primary defense mechanism (*19*). GSH, a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) ubiquitously existing in most cells, exerts critical protective effects as a representative natural antioxidant. Under normal conditions, the cellular level of GSH is properly maintained, but a vicious cycle of oxidative stress decreases the glutathione level in neurodegenerative diseases (*32*). Recent data suggest that SNP caused depletion of cellular GSH in cholinergic neurons (*17*). Adversely, carnosol has been shown to increase the level of GSH in neuronal cells and preadipocyte fibroblast (*12*, *33*). However, the inducibility of GSH by carnosol in glial cells has not been reported until now. Our results clearly show GSH elevation by carnosol and reduction by SNP in C6 glial cells (**Figure 5**). Furthermore, the reduction of cellular GSH by SNP was compensated to normal level by carnosol pretreatment. Thus, we propose that carnosol exerts a beneficial effect by enhancing the GSH-mediated defensive potential of glial cells.

As illustrated in **Figure 1A**, carnosol contains a catechol ring, which can convert into electrophilic quinone upon oxidation. From this structural consideration, carnosol potentially makes adduct formation with biological enzymes or proteins. Previous study suggested that carnosol activates the Nrf2 by forming an adduct with Keap1 protein, an inhibiting regulator of Nrf2 (*33*). Therefore, carnosol potently activates the Keap1/Nrf2 pathway, which results in the activation of the antioxidant-response element (ARE) and subsequent induction of phase 2 enzymes including HO-1 and γ-glutamyl cystein ligase (*9*, *34*). HO-1 is known to exert an antioxidant defense activity in various cell types including astrocytes (*20*, *21*). HO-1 has been characterized as a prominent marker for oxidative stress because it is induced by stimuli producing oxidative stress. It has been suggested that the rapid up-regulation of HO-1 against cytotoxic stimuli may provide partially protective effect and delay the cell death (*35*). Previous studies demonstrated that several natural dietary components, such as quercetin and baicalein, protect glial cells against oxidative stress through the induction of HO-1 expression (*36*,*37*). Also, carnosol induced the up-regulation of HO-1 expression via activation of the PI3K-Akt survival pathway in PC12 cells (*34*). However, the role of HO-1 expression in carnosol's protection of glial cells was not studied. In this study, we have proved that carnosol induced moderate HO-1 up-regulation for a long time in C6 glial cells (**Figure 6A**,**B**). Although SNP also induced the up-regulation of HO-1 expression in our experimental conditions, the pattern of HO-1 expression was quite different from that of carnosol mediation. The increase of the HO-1 protein peaked as early as 6 h after exposure with SNP, and then it declined rapidly (**Figure 6A**,**B**). The expression of HO-1 by carnosol was slightly increased, peaking at 12 h and still moderately elevated even at

24 h after treatment. In addition, the combination of carnosol and SNP treatment resulted in an additive effect in HO-1 expression (**Figure 6C**). Thus, these results suggest that brief up-regulation of HO-1 expression by SNP is associated with adaptive resistance to toxic nitrosative stress. When the increased HO-1 expression in the C6 glial cells was inhibited by HO-1 inhibitor ZnPP, the sensitivity of C6 cells to the SNP-induced cytotoxicity was significantly increased and the protective effects induced by carnosol were markedly decreased (**Figure 6D**). These results supported the essential role of HO-1 in providing a resistant function against SNP-mediated injury and delaying glial cell death.

It has been proposed that activation of intracellular signaling pathways and regulation of transcription factor Nrf2 are principally involved in HO-1 induction (*34*). Depending on the cell types and various stimuli, regulatory roles of different kinases including ERK1/2, JNK, p38 MAP kinase, PKA, or PKC for HO-1 induction were observed (*21*, *35*). As shown in **Figure 7**, we found that carnosol increased the level of Nrf2 in the nucleus and subsequent HO-1 expression via PI3K and Akt/PKB pathways. The present study has demonstrated for the first time that carnosol can protect glial cells. Carnosol significantly suppressed SNP-induced apoptotic nuclei changes, accompanied by reducing caspase-3 enzyme activity, JNK activation, and NO generation in C6 cells. In addition, we have found that carnosol maintained the cellular level of GSH to prevent the effects of SNP and induced constitutive but moderate overexpression of HO-1 for prevention of glial cell damage. This study may open up a novel strategy using carnosol against neurodegenerative diseases that correlate with astrocyte damage.

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