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SEA0400, a specific inhibitor of the Na^+–Ca^{2+} exchanger, attenuates sodium nitroprusside-induced apoptosis in cultured rat microglia

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1 Using SEA0400, a potent and selective inhibitor of the Na^+–Ca^{2+} exchanger (NCX), we examined whether NCX is involved in nitric oxide (NO)-induced disturbance of endoplasmic reticulum (ER) Ca^{2+} homeostasis followed by apoptosis in cultured rat microglia.

2 Sodium nitroprusside (SNP), an NO donor, decreased cell viability in a dose- and time-dependent manner with apoptotic cell death in cultured microglia.

3 Treatment with SNP decreased the ER Ca^{2+} levels as evaluated by measuring the increase in cytosolic Ca^{2+} level induced by exposing cells to thapsigargin, an irreversible inhibitor of ER Ca^{2+}-ATPase.

4 The treatment with SNP also increased mRNA expression of CHOP and GPR78, makers of ER stress.

5 SEA0400 at 0.3–1.0 μM protected microglia against SNP-induced apoptosis.

6 SEA0400 blocked not only the SNP-induced decrease in ER Ca^{2+} levels but also SNP-induced increase in CHOP and GRP78 mRNAs.

7 SEA0400 did not affect capacitative Ca^{2+} entry in the presence and absence of SNP.

8 SNP increased Na^+-dependent $^{45}Ca^{2+}$ uptake and this increase was blocked by SEA0400.

9 These results suggest that SNP induces apoptosis *via* the ER stress pathway and SEA0400 attenuates SNP-induced apoptosis *via* suppression of the ER stress in cultured microglia. Our findings imply that NCX plays a role in ER Ca^{2+} depletion under pathological conditions.
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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ER, endoplasmic reticulum; HBSS, Hanks' balanced saline solution; MTT, 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; NCX, Na^+–Ca^{2+} exchanger; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SR, sarcoplasmic reticulum; Z-VAD-FMK, carbobenzoxy-L-valyl-L-alanyl-β-methyl-L-aspart-1-yl-fluoromethane

Introduction

Microglia are the most plastic cell population of the central nervous system. In response to pathological conditions, microglia undergo a stereotypic activation process composed of proliferation, migration, and morphological and functional changes in the central nervous system (Kreutzberg, 1996; Gonzalez-Scarano & Baltuch, 1999; Streit *et al*., 1999). The activated microglia secretes diverse inflammatory and cytotoxic factors such as nitric oxide (NO) and tumor necrosis factor-α (Banati *et al*., 1993). These factors may be involved in the pathogenesis of various neurodegenerative diseases (Minghetti & Levi, 1998; Gonzalez-Scarano & Baltuch, 1999). Lee *et al*. (2001b) demonstrated that mouse microglial cells undergo apoptosis upon inflammatory activation and that NO is a major autocrine mediator in the process.

The Na^+–Ca^{2+} exchanger (NCX) plays a critical role in the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Hryshko & Philipson, 1997; Matsuda *et al*., 1997; Blaustein & Lederer, 1999; Shigekawa & Iwamoto, 2001). Previous studies have shown that NCX activity is stimulated by NO in vascular smooth muscle cells (Furukawa *et al*., 1991), astrocytes (Asano *et al*., 1995) and C6 glioma cells (Amoroso *et al*., 2000). From these observations, it is possible that NCX activity may be involved in the effects of NO. NCX is largely distributed close to the sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) Ca^{2+} stores in smooth muscle (Lederer *et al*., 1990; Moore *et al*., 1993) and astrocytes (Juhaszova *et al*., 1996). In addition, Golovina *et al*. (1996) reported that Ca^{2+} entry across plasmalemma *via* NCX increases the Ca^{2+}

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level of ER stores in astrocytes, and Chernaya *et al*. (1996) reported using transfected Chinese hamster ovary cells expressing NCX that Ca^{2+} release from intracellular stores induces regulatory activation of NCX activity. These findings suggest that NCX plays a role in regulation of the intracellular Ca^{2+} stores in the SR/ER. The ER is the major intracellular Ca^{2+} storage site: the Ca^{2+} concentration in the lumen of the ER is 3–4 orders of magnitude greater than that of the cytosol, a gradient that is maintained by ER Ca^{2+}-ATPase (Mendolesi & Pozzan, 1998). Under conditions of oxidative or chemical stress, the ER undergoes a stress response termed the unfolded protein response (Kozutsumi *et al*., 1988; Wooden *et al*., 1991). Recent studies show that NO-induced apoptosis is mediated by the ER stress pathway in pancreatic β cells, p53-deficient microglia and RAW 264.7 macrophages (Kawahara *et al*., 2001; Oyadomari *et al*., 2001; Gotoh *et al*., 2002). However, it is not known whether NCX is involved in ER stress-mediated apoptosis. We have recently shown that the novel compound SEA0400 is a potent and selective inhibitor of NCX (Matsuda *et al*., 2001). This inhibitor certainly contributes to studies on the role of NCX. The present study first demonstrates that sodium nitroprusside (SNP) causes apoptosis *via* the ER stress pathway in cultured microglia. Moreover, we examine the effect of SEA0400 on SNP-induced apoptosis to clarify the role of NCX in the ER stress-mediated apoptosis in cultured microglia.

Methods

Materials

The following drugs were used: fetal bovine serum, isolectin B_4, ouabain, monensin, QuantiPro BCA assay kit, 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), SNP, thapsigargin, *S*-nitroso-*N*-acetylpenicillamine (SNAP), $K_3Fe(CN_6)$ and deferoxamine (Sigma-Aldrich Inc., St Louis, MO, U.S.A.); Eagle's MEM (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan); L-glutamine, EGTA, sodium lauryl sulfate (SDS), *N,N*-dimethylformamide (DMF) and Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan); and RNase A (Wako Chemical Industries, Ltd, Osaka, Japan); EDTA (Dojindo Laboratories, Kumamoto, Japan); proteinase K, M-MLV reverse transcriptase and *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA, U.S.A.); Vistra Green (Amersham Pharmacia Biotech U.K. Ltd, Buckinghamshire, England); Hoechst 33258, fura-2/AM and LIVE/DEAD® Viability/Cytotoxicity assay kit (Molecular Probe Inc., Eugene, OR, U.S.A.); carbobenzoxy-L-valyl-L-alanyl-β-methyl-L-aspart-1-yl-fluoromethane (Z-VAD-FMK) (Peptide Institute Inc., Osaka, Japan); ionomycin (Calbiochem-Novabiochem Co., Ltd, La Jolla, CA, U.S.A.). $^{45}Ca^{2+}$ was purchased from Amersham Biosciences K.K. (Tokyo, Japan). SEA0400 was synthesized by Taisho Pharmaceutical Co., Ltd (Saitama, Japan).

Preparation of microglia

Microglia were obtained from the cerebral cortices of 1-day-old Wistar rats (Japan SLC, Shizuoka, Japan) essentially as previously reported (Nagano *et al*., 2004). Briefly, a mixed glial cell culture plated in 75-cm^2 flasks was grown in Eagle's minimum essential medium containing 10% fetal bovine serum and 2 mM of L-glutamine in 5% CO_2 atmosphere at 37°C for 14 days. The medium was changed twice a week. This was then agitated on an orbital shaker at 140 r.p.m. at 37°C for 2 h. The supernatant medium was collected and centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in fresh medium and seeded in plastic plates at a density of 10^3 cells mm^{-2} for most experiments or in glass plates at a density of 250 cells mm^{-2} for measurement of $[Ca^{2+}]_i$. The medium was then changed 20 min after seeding. All experiments were performed after secondary culture for 1 day. Reverse transcription–polymerase chain reaction (RT–PCR) analysis showed that ionized calcium-binding adapter molecule 1 mRNA for a maker of microglia was observed in this microglial preparation, while microtubule-associated protein-2, glial fibrillary acidic protein and proteolipid protein mRNAs (markers for neuron, astrocyte and oligodendrocyte) were not.

Cell viability

MTT reduction activity was measured using a colorimetric assay (Matsuda *et al*., 1996; 1998). The cells were incubated at 37°C for 4 h after addition of 0.5 mg ml^{-1} MTT. Then solubilizing solution (20% SDS, 50% DMF, 2% acetic acid, 2.5% 1 N HCl, pH 4.7) was added to extract the dark-blue crystals. After complete extraction, the absorbance (at wavelength 570 nm) was measured on the BioRad Model 3550 EIA plate reader. MTT reduction activity was expressed as a percentage of the control. The incubation time and the cell number used for the reaction were optimized for quantitation of MTT reduction. In some experiments, cell viability was determined cytochemically using the LIVE/DEAD® Viability/Cytotoxicity assay kit. The cells were incubated with 1 μM calcein AM and 4 μM ethidium homodimer at room temperature for 30 min and then rinsed three times to remove excess dye. A fluorescence microscope ECLIPSE TE300 (Nikon Corp., Tokyo, Japan) was used to visualize individual cells. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence. Ethidium homodimer enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. Ethidium homodimer is excluded by the intact plasma membrane of live cells.

Analysis of DNA ladder

The cells were lysed in cell lysing buffer (10 mM Tris, 10 mM EDTA, 0.5%. Triton X-100; pH 8.0) and the lysate was centrifuged at 13,000 g for 20 min to separate intact from fragmented chromatin. The supernatant, containing fragmented DNA, was incubated with 0.5 mg ml^{-1} RNase A for 1 h and then incubated with 0.2 mg ml^{-1} proteinase K at 37°C for 1 h. Isopropanol (50%) and NaCl (0.5 M) were added and the mixture was kept overnight at −20°C. The precipitate was collected by centrifugation at 13,000 g for 20 min. The pellet was dissolved in DNA-solubilizing buffer (10 mM Tris, 1 mM EDTA, 0.5% SDS; pH 8.0). Equal amounts of DNA samples were subjected to 1.8% agarose gel electrophoresis. DNA in the gel was stained with Vistra Green and detected with FluorImager 595 (Amersham Pharmacia Biotech U.K. Ltd).

Hoechst 33258 staining

The cells were fixed with 4% paraformaldehyde for 15 min at 4°C and stained with 1 μg ml^{-1} Hoechst 33258 for 15 min at room temperature. A fluorescence microscope ECLIPSE TE300 was used to visualize individual nuclei. The cells showing nuclear condensation are expressed as apoptotic cells.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined as reported previously (Takuma *et al*., 1994; Matsuda *et al*., 1998). Briefly, the cells, plated on a glass coverslip, were incubated for 30 min in Hanks' balanced saline solution (HBSS) containing 5 μM fura-2/AM and then rinsed three times to remove excess dye. After perfusion with HBSS for 15 min, $[Ca^{2+}]_i$ was measured by the ratio-imaging of fura-2 fluorescence (510 nm emission excited by 340 and 380 nm illumination) using an AQUACOSMOS image processor (Hamamatsu Photonics K.K., Shizuoka, Japan). The fluorescence ratios were calculated.

Capacitative Ca^{2+} entry was evaluated by measuring the increase in $[Ca^{2+}]_i$ as reported previously (Williams *et al*., 2000; Thyagarajan *et al*., 2002). The cells were preincubated with Ca^{2+}-free HBSS for 20 min. They were then stimulated with 100 nM thapsigargin and 1 mM $CaCl_2$ was added to induce Ca^{2+} entry. The resultant increase in $[Ca^{2+}]_i$ was indicative of capacitative Ca^{2+} entry. The maximum $[Ca^{2+}]_i$ after addition of $CaCl_2$ was defined as MAX, while the minimum $[Ca^{2+}]_i$ before addition of $CaCl_2$ was defined as MIN. The MIN was $[Ca^{2+}]_i$ measured in the last period without addition of $CaCl_2$.

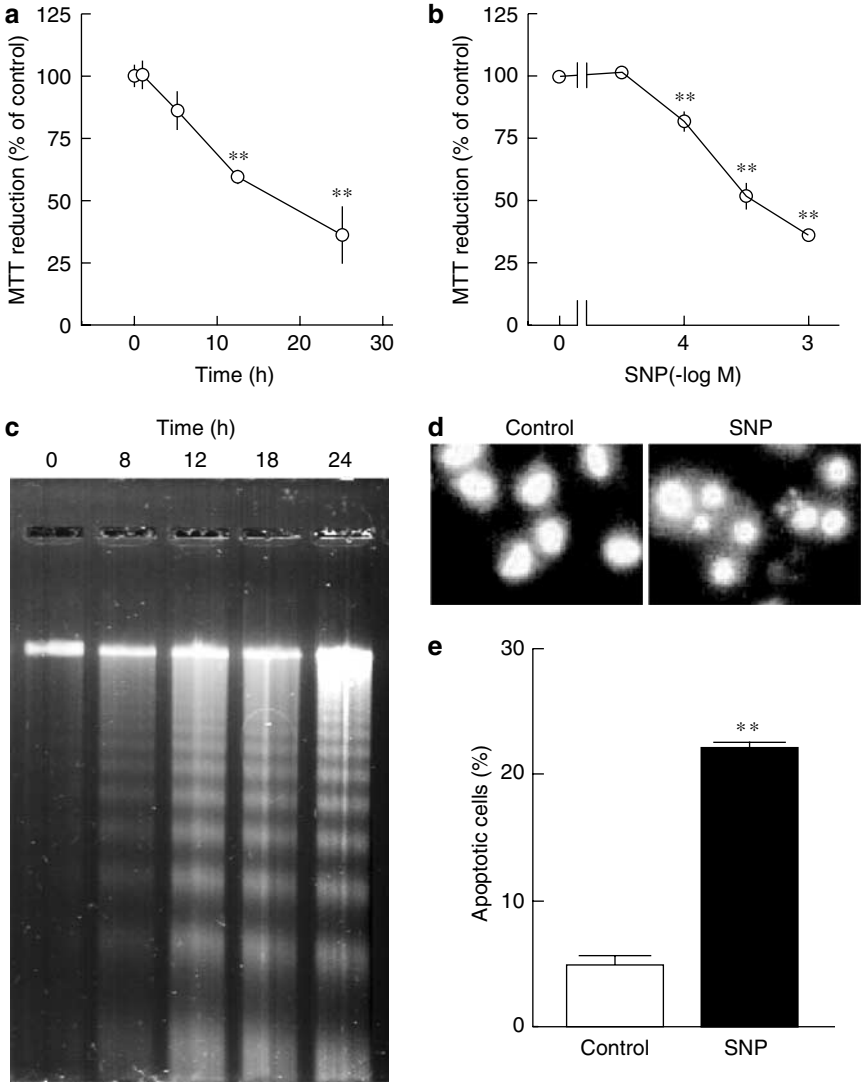

Figure 1 Effect of SNP on cell injury in cultured microglia. (a) and (b) show MTT reduction activity. The cells were treated with SNP at 1 mM for the indicated time (a) or at the indicated concentrations (b) for 12 h. Results are means ± s.e.m. of six determinations. $^{**}P<0.01$, significantly different from control (Dunnett's test). (c) DNA ladder formation is shown. The cells were treated with SNP at 1 mM for the indicated time, and DNA samples were separated on 1.8% agarose gel electrophoresis and stained with Vistra Green. Results of agarose gel electrophoresis are representative of four independent experiments. (d) and (e) show nuclear condensation. The cells were treated with SNP at 1 mM for 12 h, and stained with Hoechst 33258. Results are representative (d) and means ± s.e.m. of four determinations (e). $^{**}P<0.01$, significantly different from control (*t*-test).

RT–PCR

Total RNAs were isolated using the acid guanidium thiocyanate–phenol–chloroform method and converted into cDNA by reverse transcriptase (Hosoi *et al*., 1997). The oligonucleotides designed as PCR primers and PCR using *Taq* DNA polymerase are shown in Table 1. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with Vistra Green. The fluorescence was measured with FluorImager 595. The numbers of amplifications and the amounts of cDNAs used for the reaction were optimized for quantitation of RNAs. The β-actin housekeeping gene was simultaneously reverse transcribed and amplified as the internal reference standard to control for variations in product abundances. The signal intensities were quantified using ImageQuant 1.11 software (Amersham Pharmacia Biotech U.K. Ltd).

Na^+–Ca^{2+} exchange activity

Na^+–Ca^{2+} exchange activity was determined by assaying Na^+-dependent $^{45}Ca^{2+}$ uptake, which was performed by increasing the intracellular Na^+ concentration, using 1 mM ouabain and 20 μM monensin. Ouabain was added 5 min before the addition of $^{45}Ca^{2+}$, and monensin was added simultaneously with the isotope. $^{45}Ca^{2+}$ uptake was determined in HBSS as reported previously (Takuma *et al*., 1994, Matsuda *et al*., 1998).

Statistical analysis

Results were expressed as the means ± s.e.m. The results were examined by one-way ANOVA, and then individual group means were compared with the Tukey–Kramer test or Dunnett's test, using software package Statview 5.0J for Apple Macintosh (SAS Institute Inc., Cary, NC, U.S.A.). The results between two groups were analyzed by Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

Results

Treatment with SNP decreased cell viability in a dose-dependent manner with apoptotic change in cultured rat microglia (Figure 1). The effect of 1 mM SNP was dependent on treatment time (Figure 1a), and SNP at concentrations higher than 0.3 mM decreased MTT reduction activity

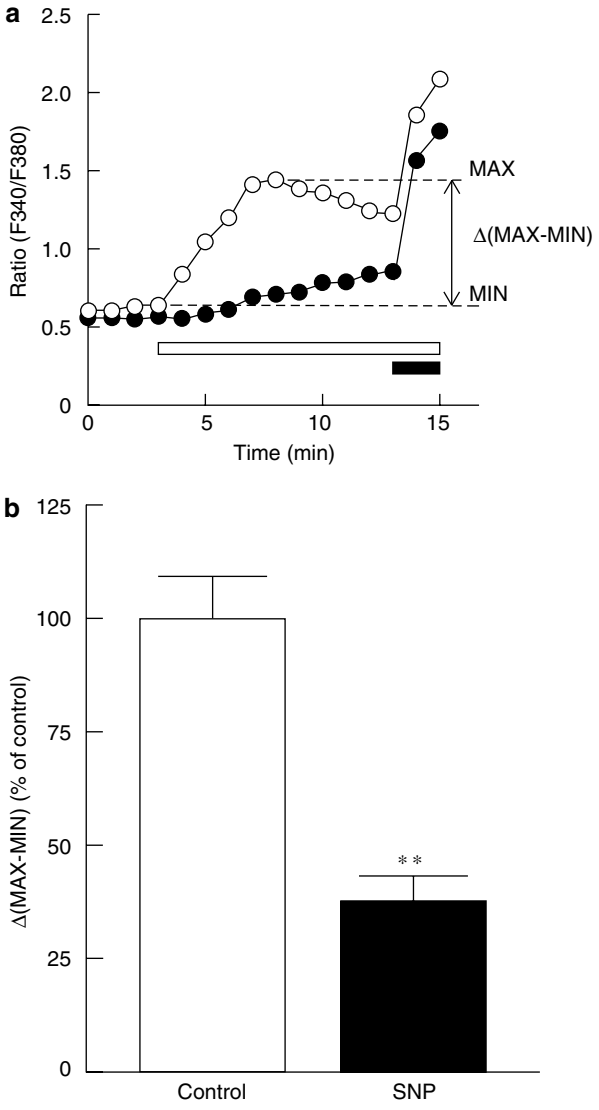

Figure 2 Effect of SNP on thapsigargin-evoked $[Ca^{2+}]_i$ response in cultured microglia. (a) Results are means of four independent experiments. The cells were pretreated in the absence (open circles, control) or presence (closed circles) of SNP at 1 mM for 1 h, and then stimulated by 1 μM thapsigargin (open bar), and 1 μM ionomycin (closed bar) was used as a positive control. (b) Quantitative results of thapsigargin-evoked $[Ca^{2+}]_i$ response are shown. Results are means ± s.e.m. of 84 cells. $^{**}P<0.01$, significantly different from control (*t*-test).

Table 1 Oligonucleotide primers, reaction cycles and temperatures and times for the amplification of β-actin, CHOP and GRP78 cDNAs by PCR analysis

	Left primer (5′–3′) *Right primer (5′–3′)*	*Reaction cycles* *Reaction temperatures (°C)/times (s)*
β-Actin	GATGGTGGGTATGGGTCAGAAGGA GCTCATTGCCGATAGTGATGACCT	16 94→60→72/30→30→60
CHOP	GCAGCTGAGTCTCTGCCTTT GCTCGTTCTCTTCAGCAAGC	22 94→60→72/30→30→60
GRP78	GACATTTGCCCCAGAAGAAA TCAAATTTGGCCCGAGTAAG	22 94→60→72/30→30→60

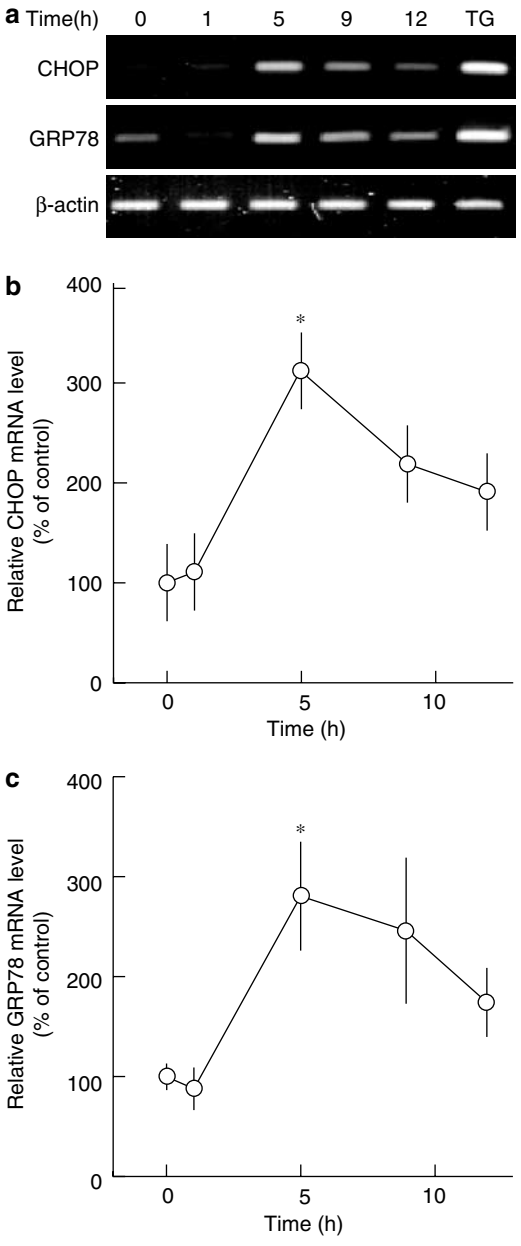

Figure 3 Effects of SNP on CHOP and GRP78 mRNAs in cultured microglia. (a) RT–PCR products are shown. The cells were treated with SNP at 1 mM for the indicated time and 1 μM thapsigargin for 5 h (TG, as a positive control), and the mRNAs were determined by RT–PCR. Results of agarose gel electrophoresis are representative of four independent experiments. (b) and (c) show quantitative results of CHOP and GRP78 mRNAs, respectively. Results are means$\pm$s.e.m. of four determinations. $^*P<0.05$, significantly different from control (Dunnett's test).

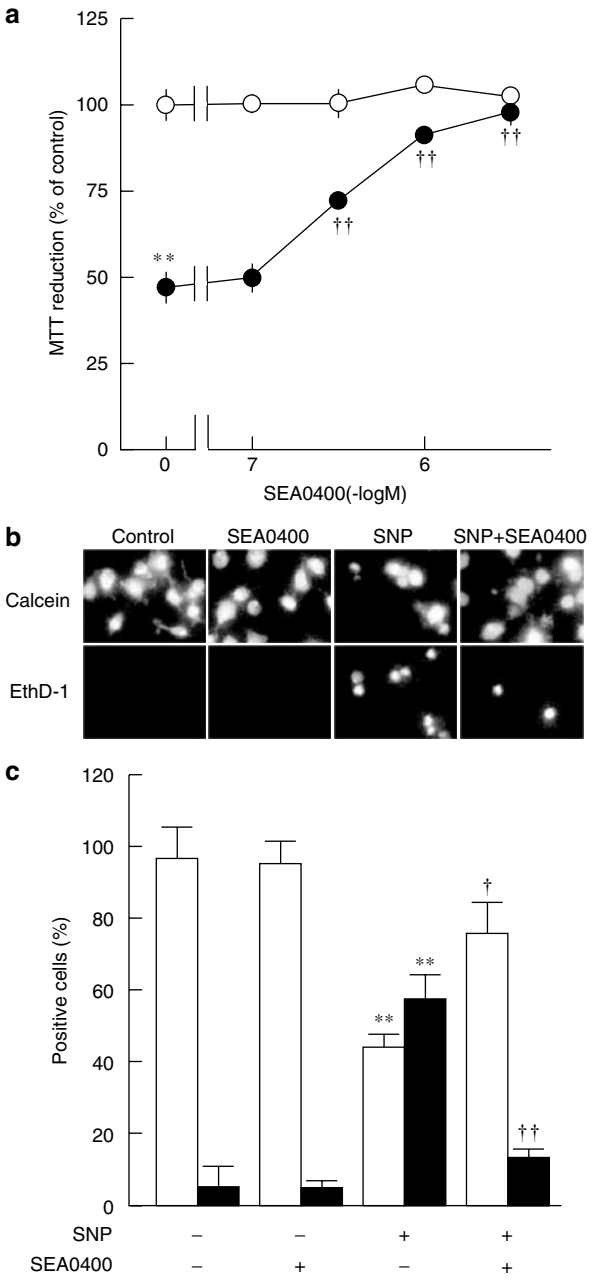

Figure 4 Effect of SEA0400 on cell injury induced by SNP in cultured microglia. (a) The cells were treated with SNP at 1 mM for 12 h and then MTT reduction was measured. SEA0400 at the indicated doses was added 30 min before SNP treatment. Open and closed symbols indicate control and SNP treatment, respectively. Results are means$\pm$s.e.m. of five determinations. $^{**}P<0.01$, significantly different from control. $^{\dagger\dagger}P<0.01$, significantly different from the values without SEA0400 (Tukey–Kramer test). (b) and (c) show LIVE/DEAD® Viability/Cytotoxicity assay. The cells were treated with SNP at 1 mM for 12 h in the presence or absence of 1 μM SEA0400, and then stained with calcein-AM (live cells) and ethidium homodimer (EthD-1) (dead cells). SEA0400 was added 30 min before SNP treatment. (b) Results are representative of three independent experiments. (c) Quantitative results are shown. Open and closed columns indicate calcein-AM and EthD-1 staining, respectively. Results are means$\pm$s.e.m. of five determinations. $^{**}P<0.01$, significantly different from control. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, significantly different from the values without SEA0400 (Tukey–Kramer test).

(Figure 1b). The effects of SNP on biochemical markers of apoptosis are shown in Figure 1c–e. SNP treatment for 8–24 h caused DNA ladder formation (Figure 1c). Furthermore, it caused nuclear condensation: the control cells had oval and unequally stained nuclei, while the treated cells had numerous fragmented and pyknotic nuclei (Figure 1d). The effect of SNP on nuclear condensation was statistically significant (Figure 1e). SNP-induced decrease in cell viability was blocked by the pan-caspase inhibitor Z-VAD-FMK (10 μM): MTT reduction activities (%, means$\pm$s.e.m. of four determinations)

were 100±1 (control without Z-VAD-FMK), 48±6 (SNP treatment without the inhibitor), 100±2 (control with the inhibitor) and 90±4 (SNP treatment with the inhibitor). Furthermore, the treatment with SNP resulted in dose- and time-dependent decreases in mitochondrial membrane potential, which plays a role in apoptotic process (data not shown). It is likely that the effect of SNP is due to NO, because of the following observations. $K_3Fe(CN)_6$ did not affect MTT reduction activity, and the effect of SNP on MTT reduction activity was not affected by the iron ion chelator deferoxamine (data not shown). Furthermore, treatment with SNAP, another NO donor, at 3 mM for 12 h also decreased MTT reduction activity (data not shown).

Figure 2 shows the effect of SNP treatment on the level of ER Ca^{2+} stores in cultured rat microglia. The level of ER Ca^{2+} stores was evaluated by measuring the increase in $[Ca^{2+}]_i$ induced by exposing cells to thapsigargin, an irreversible inhibitor of ER Ca^{2+}-ATPase (Doutheil *et al*., 2000). Thapsigargin at 1 μM increased $[Ca^{2+}]_i$ in control cells, and this increase in $[Ca^{2+}]_i$ was significantly suppressed by pretreatment of cells with SNP at 1 mM for 60 min. In the control and SNP-pretreated cells, ionomycin-induced Ca^{2+} signals were similar, suggesting that there is no difference in Fura-2/AM loading between cells. Figure 3 shows the effect of SNP treatment on mRNA expression of CHOP and GRP78, which are induced in response to ER stress (Sidrauski *et al*., 1998; Kaufman, 1999; Mori, 2000). Treatment with SNP or thapsigargin for 5 h significantly increased the mRNA levels of CHOP and GRP78.

We studied the effect of the selective NCX inhibitor SEA0400 on SNP-induced cell injury in cultured rat microglia (Figure 4). SNP at 1 mM for 12 h decreased MTT reduction activity to nearly 50% of the control and SEA0400 attenuated the effect of SNP on MTT reduction activity in a dose-dependent manner, although SEA0400 on its own did not affect MTT reduction activity (Figure 4a). The cytochemical study using LIVE/DEAD® Viability/Cytotoxicity assay also showed that SEA0400 protected microglia against SNP-induced cell death (Figure 4b and c). Figure 5 shows the effect of SEA0400 on DNA ladder formation and nuclear condensation in cultured rat microglia. Treatment with SNP at 1 mM for 12 h caused DNA ladder formation, and the effect was inhibited by SEA0400 in a dose-dependent manner (Figure 5a). SEA0400 on its own did not cause DNA ladder formation (data not shown). A fluorescence microscopic image using Hoechst 33258 showed that SNP caused nuclear condensation and this effect was significantly blocked by

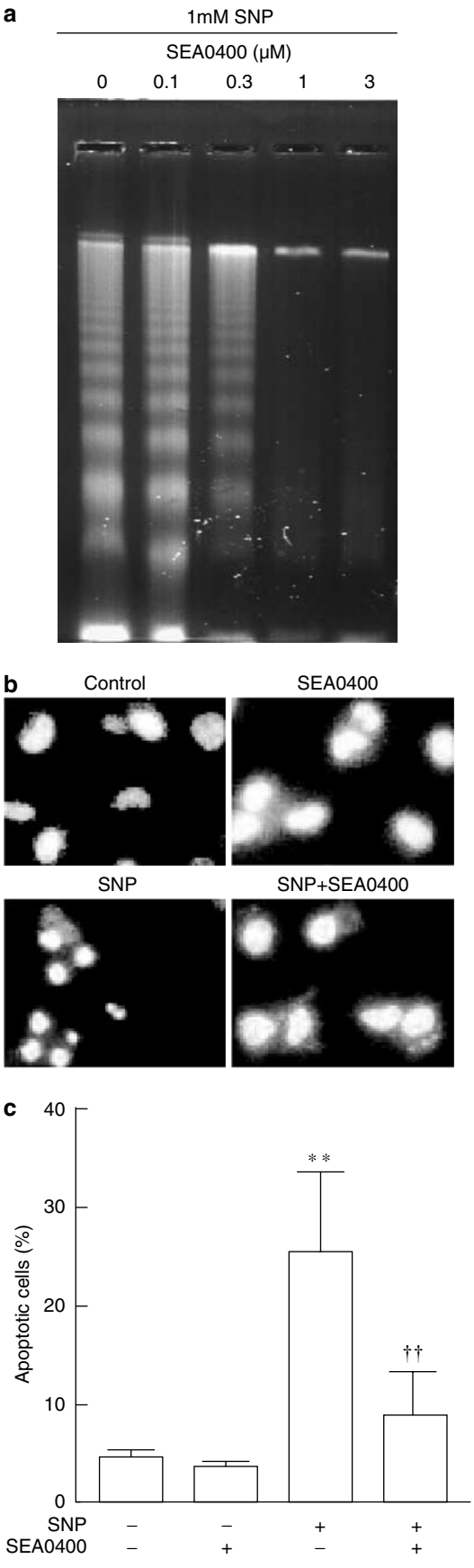

Figure 5 Effect of SEA0400 on DNA ladder formation and nuclear condensation in cultured microglia. (a) DNA ladder formation is shown. The cells were treated with SNP at 1 mM for 12 h in the presence of SEA0400 at the indicated doses, and then DNA samples were separated on 1.8% agarose gel electrophoresis and stained with Vistra Green. SEA0400 was added 30 min before SNP treatment. Results of agarose gel electrophoresis are representative of three independent experiments. (b) and (c) show nuclear condensation. The cells were treated with 1 mM SNP for 12 h in the presence or absence of 1 μM SEA0400, and then stained with Hoechst 33258. SEA0400 was added 30 min before SNP treatment. (b) Results are representative of three independent experiments. (c) Quantitative results are shown. Results are means±s.e.m. of three determinations. $^{**}P<0.01$, significantly different from control. $^{\dagger\dagger}P<0.01$, significantly different from the values without SEA0400 (Tukey–Kramer test).

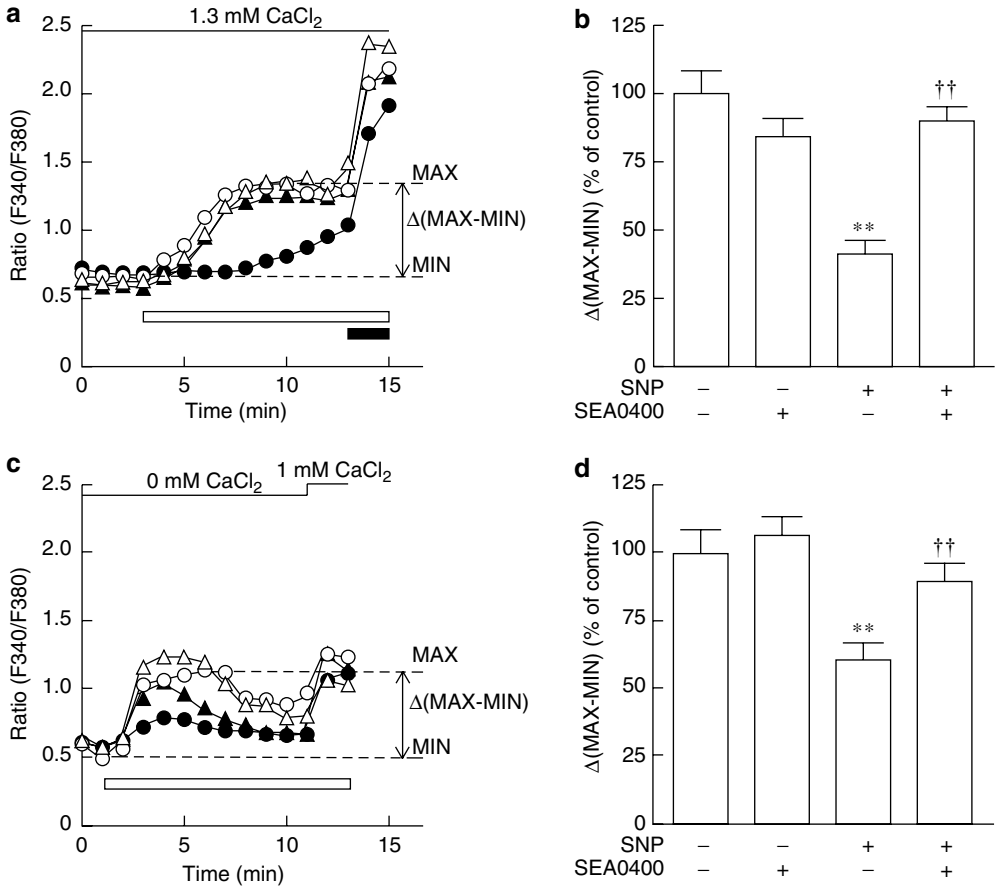

Figure 6 Effects of SEA0400 on thapsigargin-evoked $[Ca^{2+}]_i$ response modified by SNP in cultured microglia. The experiments were carried out in Ca^{2+}-containing (a, b) and Ca^{2+}-free (c, d) HBSS. (a, c) Results (means of 21 cells) are representative of 2–3 independent experiments. The cells were pretreated with (closed circles or triangles) and without (open circles or triangles) SNP at 1 mM for 1 h in the presence (open or closed triangles) or absence (open or closed circles) of 1 μM SEA0400 and then stimulated with 1 μM thapsigargin (open bar). SEA0400 was added 15 min before SNP treatment. The closed bar (a) indicates 1 μM ionomycin, which is used as a positive control. (b, d) Quantitative results of thapsigargin-evoked $[Ca^{2+}]_i$ response are shown. $^{**}P<0.01$, significantly different from control; $^{\dagger\dagger}P<0.01$, significantly different from the values without SEA0400 (Tukey–Kramer test).

SEA0400 at 1 μM (Figure 5b and c). SEA0400 also attenuated the SNP-induced decrease in mitochondrial membrane potential in a dose-dependent manner (data not shown). Furthermore, SEA0400 protected cultured microglia against SNAP (3 mM for 12 h)-induced decrease in cell viability (data not shown).

Figure 6 shows the effects of SEA0400 and SNP on thapsigargin-induced increase in $[Ca^{2+}]_i$ in cultured rat microglia. The thapsigargin-induced Ca^{2+} response was examined under the conditions in the presence (Figure 6a and b) and absence (Figure 6c and d) of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, all cells in this experiment showed similar Ca^{2+} responses to ionomycin. SNP treatment significantly inhibited the thapsigargin-induced increase in $[Ca^{2+}]_i$. SEA0400 attenuated the SNP-induced decrease in Ca^{2+} response to thapsigargin, although SEA0400 on its own affected neither basal Ca^{2+} levels nor the thapsigargin-induced Ca^{2+} response in control cells. The similar effects of SNP and SEA0400 on thapsigargin-induced Ca^{2+} response were observed in the absence of extracellular Ca^{2+}, although the thapsigargin-induced increase in Ca^{2+} levels was less in the absence of extracellular Ca^{2+} than in the presence. Figure 7 shows the effects of SEA0400 and SNP on the expression of ER stress-associated genes CHOP and GRP78 in cultured rat microglia. SNP at 1 mM for 5 h caused increases in CHOP and GRP78 mRNA levels, and SEA0400 attenuated the increases in these mRNA levels in a dose-dependent manner. SEA0400 on its own did not affect the mRNA levels of CHOP and GRP78 (data not shown). β-Actin mRNA expression was unaffected by SNP or SEA0400.

Tunicamycin causes ER stress in Ca^{2+}-independent mechanisms, and staurosporine causes apoptosis in an ER stress-independent mechanism. SEA0400 did not affect tunicamycin- or staurosporine-induced decrease in MTT reduction activity (Figure 8). In agreement with the previous report (Thyagarajan *et al*., 2002), SNP inhibited capacitative Ca^{2+} entry (Figure 9) and increased Na^+-dependent $^{45}Ca^{2+}$ uptake (Figure 10) in microglia. SEA0400 did not affect capacitative Ca^{2+} entry in the presence or absence of SNP (Figure 9), while it attenuated SNP-induced increase in NCX activity (Figure 10).

Discussion

Lee *et al*. (2001a) reported that overactivation of microglia by LPS/interferon-γ induced apoptosis *via* two apoptotic signaling pathways involving the production of NO. In

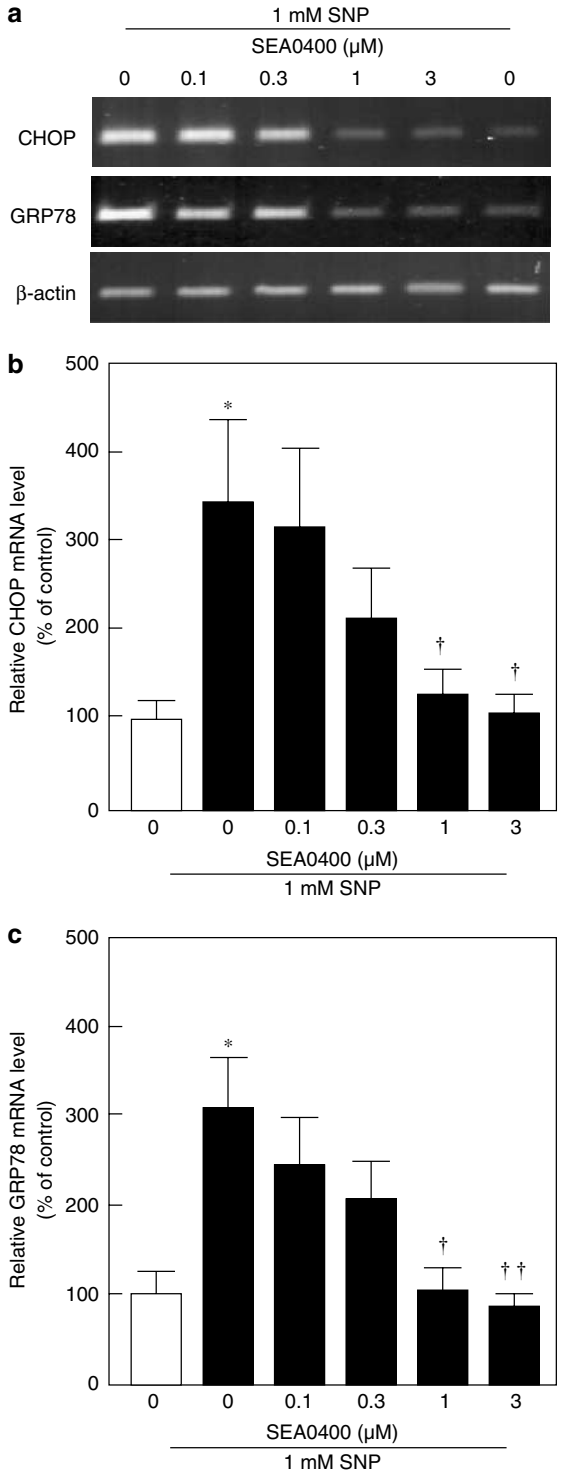

Figure 7 Effects of SEA0400 on SNP-induced expression of CHOP and GRP78 mRNAs in cultured microglia. (a) RT–PCR products are shown. The cells were treated with SNP at 1 mM for 12 h in the presence of SEA0400 at the indicated doses, and the RNAs were determined by RT–PCR. SEA0400 was added 30 min before SNP treatment. Results of agarose gel electrophoresis are representative of four independent experiments. (b) and (c) show quantitative results of CHOP and GRP78 mRNAs, respectively. Results are means ± s.e.m. of four determinations. $^*P<0.05$, significantly different from control. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, significantly different from the values without SEA0400 (Tukey–Kramer test).

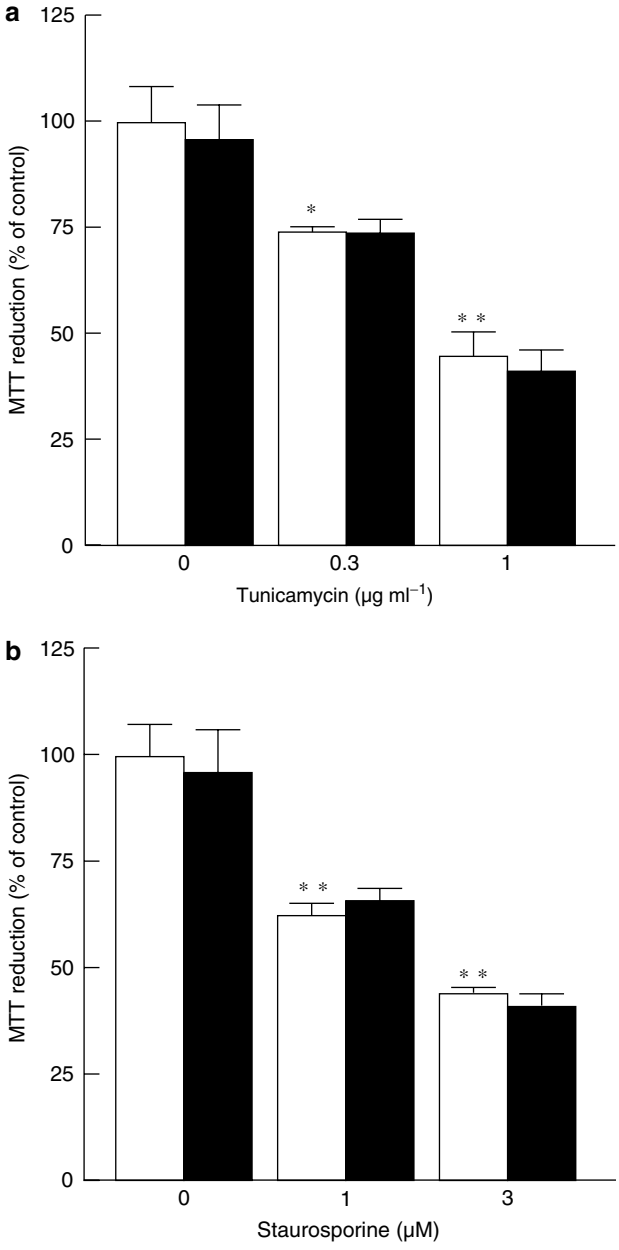

Figure 8 Effect of SEA0400 on cell injury induced by tunicamycin and staurosporine in cultured microglia. The cells were treated with tunicamycin (a) or staurosporine (b) at the indicated doses in the presence (closed columns) and absence (open columns) of SEA0400 at 1 μM for 12 h and then MTT reduction activity was measured. SEA0400 was added 30 min before tunicamycin or staurosporine. Results are means ± s.e.m. of five determinations. $^*P<0.05$, significantly different from control (Tukey–Kramer test).

NO-induced apoptosis, attention has recently been focused on ER stress pathway. NO depletes ER Ca^{2+} levels and causes ER stress, resulting in apoptosis in primary neuronal cells (Doutheil *et al*., 2000), p53-deficient microglial cells (Kawahara *et al*., 2001) and pancreatic β cells (Oyadomari *et al*., 2001). Furthermore, Gotoh *et al*. (2002) have demonstrated that the ER stress pathway involving ATF6 and CHOP plays a key role in NO-mediated apoptosis in macrophages. The present study demonstrates for the first time that SNP induces apoptosis *via* the ER stress pathway in cultured rat microglia.

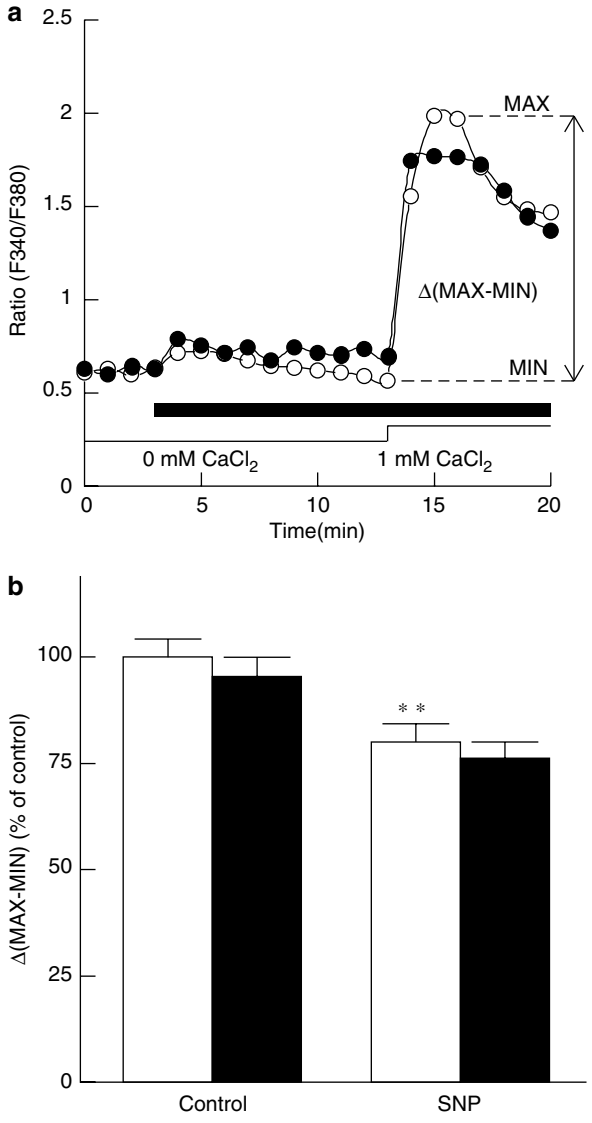

Figure 9 Effects of SNP and SEA0400 on capacitative Ca^{2+} entry in cultured microglia. Results (means of 20 cells) are representative of four independent experiments. The cells were pretreated in the absence (open circles, control) and presence (closed circles) of SNP at 1 mM for 1 h, and preincubated in Ca^{2+}-free HBSS for 20 min. They were stimulated with 100 nM thapsigargin (TG, closed bar), and 1 mM $CaCl_2$ was added to induce capacitative Ca^{2+} entry. (b) Quantitative results are shown. The cells were treated with SNP at 1 mM in the presence (closed columns) and absence (open columns) of SEA0400 at 1 μM for 1 h. SEA0400 was added 30 min before SNP treatment. Results are means of 75–84 cells of four independent experiments. $^{**}P<0.01$, significantly different from control (Tukey–Kramer test).

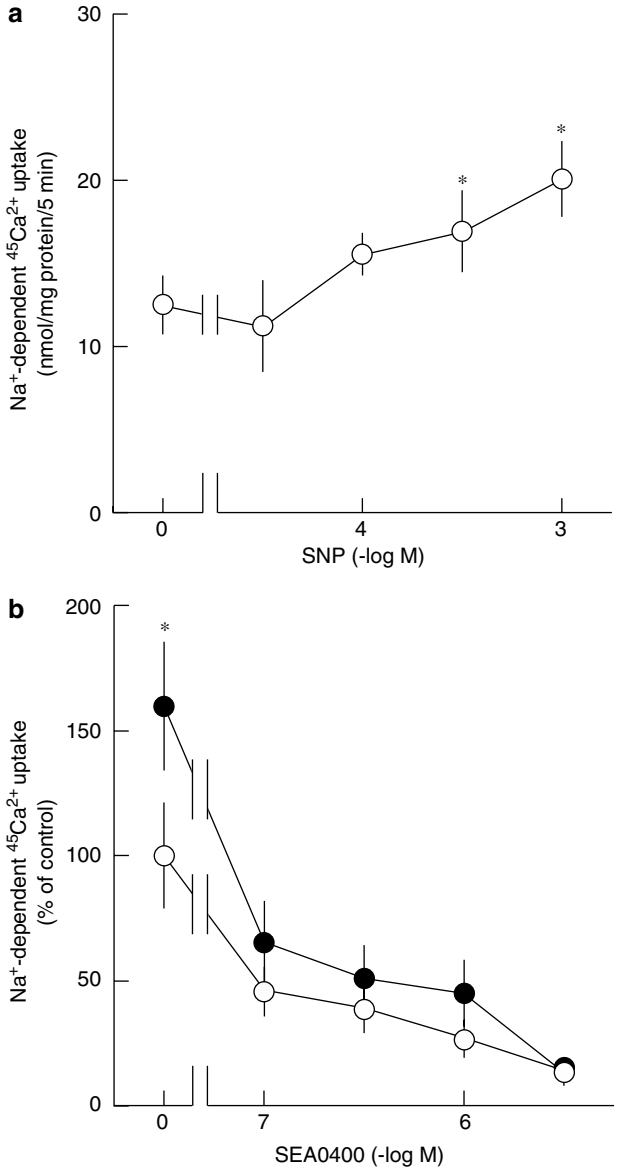

Figure 10 Effects of SNP and SEA0400 on Na^+-dependent $^{45}Ca^{2+}$ uptake in cultured microglia. (a) The cells were treated with SNP at the indicated doses for 10 min, and then Na^+-dependent $^{45}Ca^{2+}$ uptake was determined. The results are means ± s.e.m. of six determinations. $^*P<0.05$, significantly different from control (Dunnett's test). (b) The cells were treated with SNP at 1 mM in the presence (closed circles) and absence (open circles) of SEA0400 at the indicated doses for 10 min, and then Na^+-dependent $^{45}Ca^{2+}$ uptake was determined. SEA0400 was added 5 min before SNP and was present during treatment. The results are means ± s.e.m. of six determinations. $^*P<0.05$, significantly different from control (Tukey–Kramer test).

SNP decreased MTT reduction activity, and caused DNA ladder formation and nuclear condensation in cultured microglia. The decrease in cell viability was also observed by LIVE/DEAD® Viability/Cytotoxicity assay based on intracellular esterase activity and plasma membrane integrity. These findings suggest that SNP treatment causes apoptotic cell death in cultured microglia. This is also supported by the findings that a caspase inhibitor blocks SNP-induced cell injury. The SNP molecule contains Fe and ferricyanide, but the iron ion chelator deferoxamine did not reverse the effect of SNP and $Fe_3K(CN)_6$ did not affect cell viability. Furthermore, another NO donor, SNAP, decreased MTT reduction activity in cultured microglia. It is thus likely that the cytotoxic effect of SNP is due to NO in cultured microglia. With respect to ER stress, CHOP and GRP78 are increased by ER stress (Sidrauski *et al*., 1998; Kaufman, 1999; Mori, 2000). SNP attenuated thapsigargin-induced Ca^{2+} signal, suggesting a decrease in ER Ca^{2+} levels, and the NO donor increased the expression of CHOP and GRP78 mRNAs in cultured microglia. In this study, the change in ER Ca^{2+} level and

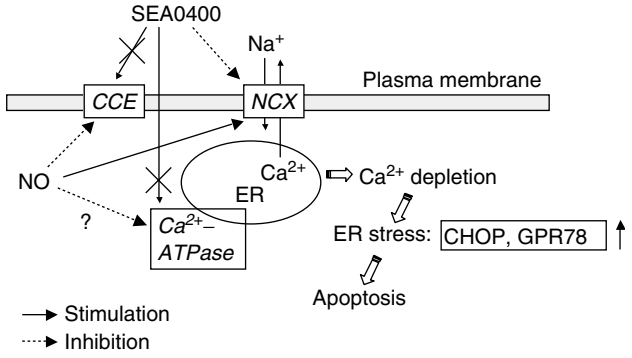

Figure 11 Model of NO-induced cell injury in cultured microglia. NO stimulates NCX activity and inhibits capacitative Ca^{2+} entry (CCE) and Ca^{2+}-ATPase. These effects may lead to Ca^{2+} depletion in ER, resulting in ER stress-mediated apoptosis. SEA0400 attenuates NO-induced decrease in ER Ca^{2+} levels and apoptosis. The effect may be mediated by NCX, since SEA0400 is a highly selective inhibitor of NCX.

the induction of the stress protein mRNAs were observed 1 and 5 h after treatment, respectively. That is, SNP-induced disturbance of ER Ca^{2+} homeostasis is followed by the expression of stress proteins. These results suggest that SNP induces apoptosis *via* the ER stress pathway in microglia.

Using the selective NCX inhibitor SEA0400, the present study examined whether NCX is involved in NO-induced apoptosis. SEA0400 at 0.3–1.0 μM attenuated the decrease in cell viability induced by SNP and attenuated SNP-induced DNA ladder formation and nuclear condensation. Similar protection by SEA0400 was observed in SNAP-induced cell injury (data not shown). These observations suggest that SEA0400 protects microglia against NO-induced apoptosis. We previously reported that Ca^{2+} reperfusion-induced apoptosis is mediated by excess Ca^{2+} influx *via* NCX in the reverse mode in cultured astrocytes (Matsuda *et al*., 1996). In contrast to Ca^{2+} reperfusion experiments in astrocytes, we did not detect any effect of SNP on $[Ca^{2+}]_i$ in cultured microglia (data not shown). Therefore, the present study focused on the effect of SEA0400 on NO-induced ER stress. We observed that SEA0400 blocked the SNP-induced decrease in ER Ca^{2+} levels. ER Ca^{2+} levels are closely coupled with capacitative Ca^{2+} entry (influx of extracellular Ca^{2+}). The effect of SEA0400 on the SNP-induced decrease in ER Ca^{2+} levels was also observed in the absence of extracellular Ca^{2+}. This result suggests that SNP and SEA0400 affect preferentially the process of ER Ca^{2+} depletion rather than the process of Ca^{2+} influx. In this line, we also observed that SEA0400 blocked the SNP-induced increase in the expression of CHOP and GRP78 mRNAs in microglia. These effects of SEA0400 were observed at doses similar to those required to protect the cells against SNP-induced decrease in cell viability. Taken together, the present study suggests that the protective effect of SEA0400 against NO-induced apoptosis is mediated by attenuation of the ER stress.

Little is known of the mechanism underlying NO-induced ER stress. It has been proposed that NO induces ER stress by disturbing ER Ca^{2+} homeostasis in cells, since NO inhibits SR Ca^{2+}-ATPase activity (Ishii *et al*., 1998; Viner *et al*., 1999). In addition, Thyagarajan *et al*. (2002) reported that NO induces apoptosis *via* an inhibition of capacitative Ca^{2+} entry. The inhibitory effect of NO on capacitative Ca^{2+} entry may lead to a decrease in ER Ca^{2+} levels. However, SEA0400 affected neither Ca^{2+} response to thapsigargin, a typical inhibitor of Ca^{2+}-ATPase, in cultured microglia (data not shown), nor capacitative Ca^{2+} entry in the presence or absence of SNP in cultured microglia. On the other hand, previous studies show that NO stimulates NCX activity in vascular smooth muscle cells (Furukawa *et al*., 1991), astrocytes (Asano *et al*., 1995) and C6 glioma cells (Amoroso *et al*., 2000). The present study showed that SNP stimulated NCX activity in cultured microglia and this effect was blocked by SEA0400. The finding suggests that NCX is involved in NO-induced cell injury in microglia. How is NCX involved in NO-induced cell injury? In cardiac cells, it is widely accepted that NCX transports a portion of the Ca^{2+} released from the SR out of the cells. NCX is expressed on the plasma membrane close to regions of SR/ER in smooth muscle cells (Moore *et al*., 1993) and astrocytes (Juhaszova *et al*., 1996). Considering the close relationship between NCX and intracellular Ca^{2+} stores, it is likely that NO-induced decrease in ER Ca^{2+} is mediated by Ca^{2+} efflux *via* activation of NCX in the forward mode. It should be noted that the role of NCX might be specific for Ca^{2+}-dependent ER stress, since SEA0400 did not protect against tunicamycin- or staurosporine-induced cell injury.

In conclusion, the present study demonstrates that NCX is involved in NO-induced ER stress, resulting in apoptosis in cultured microglia (Figure 11). NO decreases ER Ca^{2+} levels, resulting in ER stress, which induces apoptosis in microglia. This effect may be mediated by the forward mode of NCX, since the effect is inhibited by SEA0400. The present finding provides a new insight into the role of NCX: the exchanger contributes to extrusion of ER Ca^{2+} out of the cells *via* a possible interaction with ER under pathological conditions. In this connection, Ma *et al*. (2000) have recently reported that physical interaction between ER and plasma membrane is necessary for activation of plasma membrane store-operated Ca^{2+} channels, and they suggested that ER moves dynamically in the cells.

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