4-Methylcatechol-induced heme oxygenase-1 exerts a protective effect against oxidative stress in cultured neural stem/progenitor cells via PI3 kinase/Akt pathway

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ABSTRACT

4-Methylcatechol (4MC), a stimulator of the synthesis of neurotrophin family members in various cells, was able to up-regulate the expression of heme oxygenase (HO)-1, a redox-sensitive inducible stress protein, in neural stem/progenitor cells (NS/PCs). RT-PCR analysis showed that 4MC induced HO-1 mRNA expression in a dose- and a time-dependent manner. The increase in HO-1 mRNA was followed by an increase in HO-1 protein content, which was confirmed by ELISA and Western blotting analysis. When NS/PCs were pretreated with 4MC before exposure to hydrogen peroxide (H₂O₂), most of the cells were rescued from the H₂O₂-induced death. 4MC enhanced the phosphorylation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) and Akt in a time-dependent manner. Pretreatment of cultures with a selective inhibitor of PI3 kinase (PI3K)/Akt, but not with one of MAPK/ERK, inhibited both the 4MC-induced HO-1 expression and neuroprotective effect, demonstrating that PI3K/Akt signaling pathway played a significant role in 4MC-induced HO-1 induction and neuroprotection. Taken together, our results suggest that 4MC activates the expression of HO-1 through the PI3K/Akt signaling pathway and that the HO-1 protein inhibits the death of NS/PCs induced by oxidative stress.

Neurotrophic factors have been thought to be key molecules to regulate neuronal degeneration/regeneration processes of some neurological diseases. We have particularly focused on neurotrophins, a family of well-studied representative neurotrophic factors, and found 4-methylcatechol (4MC), a low-molecular-weight compound having a stimulatory effect on the synthesis of neurotrophins including brain-derived neurotrophic factor (BDNF) in cultured neurons, cultured astrocytes, and embryonic brain in vivo (6, 21). We also found that 4MC itself has a neurotrophin-like signal transducing activity, that is to say, 4MC can activate (i.e. phosphorylate) mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinases (ERK)1/2 (28). MAPK/ERK1/2 signaling pathway is known to activate the cAMP-response element binding protein (CREB) (30), which plays important roles in both neurogenesis and neuronal survival (19).

During the last decade, considerable interest in research on neural stem cells (NSCs), the most immature progenitor cells in the nervous system, has emerged, because these cells have been revealed to be present not only in the embryonic brain but also in the adult brain (7, 27, 31). Numerous studies are being directed at examining whether embryonic NSC-transplantation or endogenous NSC-activation

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is effective for the treatment of neurological diseases (12) or CNS injury (20). In particular, a growing body of evidence has accumulated to indicate the involvement of hippocampal neurogenesis in depressive disorders. For example, neuroplastic phenomena such as significant atrophy within the hippocampus are involved in the cognitive deficits observed during depressive episodes, and the hippocampal neurogenesis is stimulated by most antidepressant drugs (23). The recent report (5) that the cell loss observed in depression is considered to be the result from the decreased level of BDNF and BDNF-regulated signaling pathway prompted us to investigate the effects of 4MC on NSCs in the present study.

In order to examine the effects of 4MC, we used neurosphere-forming cells cultured from the embryonic rat telencephalon. Such neurospheres are composed of plural populations of cells such as neural (neuronal or glial) progenitor cells (PCs) in addition to the NSCs (26). Therefore, we refer to them as “neural stem/progenitor cells (NS/PCs)” hereinafter.

MATERIALS AND METHODS

Primary cultures. NS/PCs were isolated from the telencephalon of Wistar rats (Nippon SLC, Shizuoka, Japan) at embryonic day 15, and cultured as described before (22). To cause proliferation of the cells in non-adherent culture dishes or adherent culture dishes, we maintained the cells in proliferation medium daily supplemented with fibroblast growth factor (FGF)-2 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 10 ng/mL. For preparation of adherent cultures for experiments, cells were plated at a cell density of 1 × 10^5 cells/cm^2 onto poly-L-ornithine-coated plates or dishes and cultured for 3 days in the proliferation medium containing FGF-2. For studies to examine the effects of any agents, cells were washed twice with the proliferation medium before the subsequent procedure. 4MC was purchased from Tokyo Kasei (Tokyo, Japan). U0126 and LY294002 were obtained from Wako (Osaka, Japan).

RT-PCR. Total RNA from the cells in 6-well plates was prepared by use of Isogen (Nippon Gene, Tokyo, Japan), basically composed of guanidine isothiocyanate, and transcribed into cDNA by using a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). The synthesized cDNA was amplified by PCR using each pair of primers for HO-1, BDNF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR conditions were as follow: HO-1, 23 cycles at 60°C; BDNF, 27 cycles at 55°C; GAPDH, 27 cycles at 55°C, respectively. The following primer pairs were used: HO-1, 5’-accccc gaggtcaagcacag-3’ and 5’-ttttctecgggctctetg-3’; BDNF, 5’-ggaattcgagtgatgaccatccttttccttac-3’ and 5’-cggatccctatcttcccctttataagtg-3’; GAPDH, 5’-cggagtcaacggatttggtcgtat-3’ and 5’-agccttctccagtgggtggaagac-3’.

Microarray experiments. Total RNA prepared from the cells in 10 cm-dishes were cleaned up by using an RNeasy Mini Kit (Qiagen, GmbH, Germany). Two micrograms of total RNA was then amplified/labeled by using an Amino Allyl MessageAmp™ aRNA Kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. The labeled antisense RNA (referred to as aRNA) was fragmented and then applied to a DNA microarray plate (Sigma, St Louis, MO, USA) containing approximately 7,800 probe sets corresponding to 65-mer-length rat cDNAs. The arrays were scanned in a microarray scanner CR BIO Ile (Hitachi, Tokyo, Japan), and analyzed by using a DNASIS Array (Hitachi).

HO-1 ELISA. Cells in 6-well plates were incubated with test drugs for various times. Cell extracts were prepared with 0.1 mL of Extraction Buffer according to the protocol of the Rat HO-1 ELISA Kit (Assay Designs, Ann Arbor, MI, USA).

Western immunoblot analysis. Cells in 6-well plates were incubated with test drugs for various times, and the cell extracts were prepared as previously described (28). The antibodies and their sources were the following: rabbit IgG antibody against HO-1, from Sigma; rabbit antibody against MAPK 1/2 (Erk1/2-CT) which recognizes the C-terminal 35 amino acids of the rat 44 kDa MAPK1/ERK1 and 42 kDa MAPK2/ERK2, from Upstate (Lake Placid, NY, USA); rabbit antibodies against phospho-p44/42 MAPK (Thr202/Tyr204) which recognizes the phosphorylated ERK1/2 (pERK1/2), Akt, and phospho-Akt (Ser473), from Cell Signaling (Danvers, MA, USA); and alkaline phosphatase-conjugated anti-rabbit IgG (H+L), from Promega (Madison, WI, USA).

Immunocytochemistry. Cells were plated on micro slide/cover glasses placed in 24-well plates (4 × 10^4 cells/well). Immunocytochemical analysis was per-
induction in NS/PCs. We previously showed that BDNF facilitates neuronal differentiation of NSCs by inducing the mRNA expression of basic helix-loop-helix transcription factors Mash 1 and Math 1 (14). We then examined the effect of 4MC on the cell differentiation of NS/PCs. After the cells had been cultured for 5 h in FGF-2-free medium containing 10 μM 4MC and then cultured for 5 days in FGF-2/4MC-free medium, we detected nestin-positive (immature neural stem cells, not shown), Tuj-1-positive (neurons), GFAP-positive (astrocytes), and CNPase-positive (oligodendrocytes) cells by using an immunocytochemical technique (Fig. 1A). At culture day 0, the cultures contained over 95% nestin-positive cells (data not shown). After 5 days, the percentage of nestin-positive cells decreased apparently; and Tuj-1-positive (24.5 ± 0.7%), GFAP-positive (26.3 ± 1.3%), and CNPase-positive (3.6 ± 0.4%) cells emerged (open bars in Fig. 1B). The 4MC-treated cultures contained slightly greater numbers of Tuj-1-positive (26.5 ± 0.8%), GFAP-positive (27.1 ± 1.1%), and CNPase-positive (4.8 ± 0.4%) cells (closed bars in Fig. 1B); but there was no difference in the percentages of these cell populations between

**RESULTS AND DISCUSSION**

**Effects of 4MC on BDNF mRNA expression and differentiation of NS/PCs**

At first, we determined by RT-PCR whether 4MC had the ability to induce BDNF in NS/PCs. When GAPDH mRNA was used as an internal control, the amount of RT-PCR product representing BDNF mRNA in NS/PCs treated with 10 and 25 μM 4MC for 6 h was 1.4 ± 0.078 and 1.6 ± 0.12 times, respectively, greater than that of the control culture (P < 0.01, data not shown). These results demonstrate that 4MC had the ability to induce significantly
the control group and 4MC-treated groups. Taken together, the above findings show that a BDNF-mimicking effect of 4MC on NS/PCs did not occur, probably because the increased level of BDNF protein was too small to affect the differentiation of the NS/PCs.

Identification of the genes induced by 4MC-treatment
To evaluate the biological effect of 4MC on NS/PCs, we performed an oligo hybridization-based DNA array analysis with total RNA isolated from non-treated cells or cells treated for 6 h with 4MC (10 or 25 μM). This analysis revealed that 4MC-treatment up-regulated the transcription of several genes, prominently that of the following 3 genes: HO-1, galectin (Gal)-3, and adrenomedullin (ADM), which are known to encode proteins playing significant roles in the nervous system (2, 4, 8, 33). The fold increase in induction/soft score was 4.0 (10 μM 4MC) and 2.5 (25 μM 4MC) for HO-1; 3.4 (10 μM 4MC) and 2.3 (25 μM 4MC) for Gal-3; and 1.8 (10 μM 4MC) and 2.8 (25 μM 4MC) for ADM. The data from the DNA array analysis were confirmed by an RT-PCR experiment. The fold increase was 7 (10 μM 4MC) and 13.7 (25 μM 4MC) for HO-1 mRNA; 4 (10 μM 4MC) and 6.5 (25 μM 4MC) for Gal-3 mRNA; and 2.2 (10 μM 4MC) and 5.8 (25 μM 4MC) for ADM mRNA, as compared with the non-treated cells. Among these 3 genes, we focused on HO-1, a heat-shock protein (Hsp) known as Hsp32, for the following reasons: 1) the fold increase in gene induction was the highest for it among these 3 genes, and 2) NS/PCs have been reported to be very sensitive to an increased level of reactive oxygen species (ROS) (17), and oxidative stress-induced apoptosis has often been observed during NSC therapy (16) and therapeutic irradiation of brains (13).

Induction of HO-1 expression by 4MC
We then examined the induction of the HO-1 gene by conducting an RT-PCR experiment, in which the cells were cultured in medium containing 10 μM 4MC for various times (0, 0.5, 1, 2, 4, 7, and 10 h). GAPDH mRNA was used as an internal control, as its level was constant during the experiment (Fig. 2A). The level of HO-1 mRNA, which was very low in the control cells, started to increase at 1 h in the 4MC-treated cells; and its maximal increase was achieved by 4-h treatment (7.1 ± 0.6-fold). When the cells were incubated with various concentrations (0, 1, 2, 5, 10, and 20 μM) of 4MC for 4 h or 7 h, the amount of HO-1 mRNA increased in a dose-dependent manner (Fig. 2B).

Next, using ELISA, we examined the effect of 10 μM 4MC-treatment on the expression of HO-1 protein. This protein was at a very low level in control cells (6.71 ± 0.69 ng/mL); but the induction was evident as early as 4 h, causing the level to become 19.6 ± 1.27-fold greater than that of the control after 7 h (Fig. 3A). Western blotting analysis (Fig. 3B) also showed that 18 h-treatment with 4MC significantly induced the expression of HO-1 protein (14.4 ± 1.23-fold versus control cells).

HO-1 has been observed to be present in various neuronal cells such as astrocytes (1, 32), neurons (3, 11), PC12 cells (18) and neuro 2A cells (10); but little is known about this enzyme in NS/PCs. To our
knowledge the present results are the first to show that HO-1 is expressed in cultured NS/PCs.

**Neuroprotective effect of 4MC against oxidative stress**

HO-1 activity may have either protective effect or toxic effect against oxidative stress in various cells depending upon the cell population and the type of injury. Many reports have demonstrated that HO-1 has potent antioxidant and anti-apoptotic activities (2); whereas some others have indicated that the reactive iron, a HO-1 product, has a potent cytotoxic effect (29). In order to investigate the effect of 4MC on the viability of NS/PCs, we treated the cells with various concentrations of 4MC for 5 h, and then incubated them for an additional 18 h in the proliferation medium without 4MC. The MTT assay showed that 4MC itself did not affect the viability of the cells (left columns of Fig. 4A), suggesting that 4MC-induced HO-1 likely acted not as an inducer of cell death.

We then investigated whether 4MC could prevent the cell death of NS/PCs due to oxidative stress. As ROS, we exposed the cells to hydrogen peroxide (H$_2$O$_2$), which was previously shown to induce acute apoptosis in cultured NS/PCs in both time- and dose-dependent manners (17). Cells were pretreated with 10 μM 4MC or not for 5 h, and then were exposed to H$_2$O$_2$ at various concentrations (0, 0.1, 0.2, 0.3, 0.5 or 1.0 mM) for 30 min. After the cells had been incubated for an additional 18 h without 4MC/H$_2$O$_2$, and then they were subjected to the MTT assay. The ratios of the value for the H$_2$O$_2$-treated cells to that value for the control cells were calculated, and are shown on the ordinate. Values are presented as the mean ± SEM (n = 6). Significant differences in values between the H$_2$O$_2$-treated and non-treated cells (*$P < 0.05$, ***$P < 0.001$; Student’s t test) and in those indicated by the brackets are shown (**$P < 0.001$; Student’s t test).
duced the cells from the death induced by H$_2$O$_2$ (Fig. 4B). When pretreated with various concentrations of 4MC (5 ~ 100 μM) for 5 h before the exposure to 1.0 mM H$_2$O$_2$, the neuroprotective effect of 4MC was dose dependent (right columns of Fig. 4A). These results suggest that 4MC has the ability to prevent ROS-mediated cell loss of NS/PCs.

Effect of 4MC on the phosphorylation of MAPK/ERK and Akt

We then characterized the related cell signaling pathway of 4MC-induced HO-1 expression and its cytoprotective effect against oxidative stress in NS/PCs. It has been well documented that the MAPK signal transduction pathway participates in the regulation of HO-1 expression. For example, the results of a previous study using PC12 cells (18) showed that NGF induces HO-1 via the MAPK pathway and that HO-1 protects cells against apoptosis by serum deprivation-induced oxidative stress. Furthermore, another study also using PC12 cells (9) showed that kaempferol, a natural flavonoid, and rhamnocitrin, a compound found in various plants, can augment cellular antioxidant defense capacity, at least in part, through regulation of HO-1 expression and MAPK signal transduction. We thus examined at first the pERK1/2 levels in NS/PCs by Western blotting when the cells were cultured for various times (0, 15, 30, 45, and 60 min) in medium containing 10 μM 4MC. 4MC-treatment induced the phosphorylation of ERK1/2 in NS/PCs in a time-dependent manner (Fig. 5A), as observed in neurons (28). We then pre-

![Fig. 5 Effect of 4MC on the phosphorylation of ERK1/2 (A) and Akt (B) of NS/PCs and effects of a MEK/ERK inhibitor and a PI3K/Akt inhibitor on 4MC-induced HO-1 expression (C) and 4MC-induced neuroprotective effect against oxidative stress (D) of NS/PCs. (A and B) Cells were cultured in the presence of 10 μM 4MC for various times (0, 15, 30, 45, and 60 min). Cell lysates were then prepared and subjected to Western blotting analysis. (C) Cells were pretreated for 30 min with 10 μM LY294002 or 10 μM U0126, subsequently incubated for 5 h in the presence of 10 μM 4MC, and then for 18 h in the absence of inhibitor/4MC. Cell lysates were then prepared and subjected to Western blotting analysis. (D) Cells were pretreated for 30 min with 10 μM LY294002 or 10 μM U0126, sequentially incubated for 5 h with 10 μM 4MC, for 30 min with 0.3 mM H$_2$O$_2$, and then for an additional 18 h in the medium lacking H$_2$O$_2$; and then they were applied to the MTT assay. Significant differences in values between the treated and non-treated cells (*P<0.05, **P<0.01, ***P<0.001; Student’s t test) and in those indicated by the brackets are shown (*P<0.05, **P<0.01, ***P<0.001; Student’s t test).
treated the cells with U0126, an inhibitor of the ERK1/2 upstream kinase MEK1/2, 30 min before incubation in the presence of 4MC. As shown in Fig. 5C, pretreatment of NS/PCs for 30 min with LY294002, a specific PI3K inhibitor that blocks the activation of Akt, abolished the 4MC-induced increase in HO-1 expression by about half. Fig. 5D shows that pretreatment with U0126 did not block 4MC-mediated attenuation of H₂O₂-induced cell death. U0126 itself had no effect on the basal level of HO-1 expression or neuroprotective activity (data not shown). These results negate the possibility that the MAPK/ERK signaling pathway contributed to the 4MC-induced HO-1 induction and neuroprotection in NS/PCs.

We next examined the phosphorylated Akt (pAkt) levels in NS/PCs after 4MC-treatment, because several studies have independently demonstrated that HO-1 up-regulation is mediated by PI3K activity; for example, the results of an earlier study using C6 glioma cells showed that prostaglandin E₂ induces HO-1 via the PI3K pathway as well as via the PKA pathway and that HO-1 is responsible for anti-apoptotic effect of the prostaglandin against oxidant stress (24). Another study using PC12 cells showed that peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of Nrf-E2-related factor-2 (15). We thus examined the pAkt levels in NS/PCs by Western blotting, when the cells were cultured for various times (0, 15, 30 and 60 min) in medium containing 10 μM 4MC. As shown in Fig. 5B, Akt was time-dependently phosphorylated in response to 4MC. As shown in Fig. 5C, pretreatment of NS/PCs for 30 min with LY294002, a specific PI3K inhibitor that blocks the phosphorylation of Akt, abolished the 4MC-induced increase in HO-1 expression by about half. As shown in Fig. 5D, pretreatment of NS/PCs with LY294002 decreased the 4MC-induced anti-apoptosis effect on NS/PCs by about half; whereas LY294002 itself had no effect on the basal level of HO-1 expression or neuroprotective activity (data not shown). These results suggest that 4MC caused the induction of HO-1 at least in part via the PI3K/Akt pathway in NS/PCs and that HO-1 played an important role in cellular protection against ROS. The detailed molecular signaling responsible for 4MC-induced-HO-1 expression remained to be clarified, and we will investigate the contribution of other signal pathways such as the c-Jun NH₂-terminal kinase (JNK)-p38 pathway.

In conclusion, the present results suggest that 4MC activates the expression of HO-1 through the PI3K/Akt signaling pathway and that the HO-1 protein inhibits the death of NS/PCs induced by oxidative stress. We observed that 4MC had the ability to induce HO-1 in cultured neurons and to exert neuroprotective activity when the neurons were exposed to H₂O₂ (data not shown). 4MC is likely not to play an important role(s) in the neurogenesis via BDNF-induction, but rather in the neuroprotection via HO-1-induction. Recent data suggest that oxidative stress participates in NSC therapy (16), therapeutic irradiation of brains (13), normal aging, neurodegeneration (25) and neurotoxicity of some drugs such as methamphetamine (11). In future studies we will investigate whether 4MC may be a useful drug to induce the cytoprotective protein HO-1 in vivo as well as it does in vitro.

REFERENCES


