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Molecules and Cells

Protective Effect of Urocortin on 1-Methyl-4-Phenylpyridinium-Induced Dopaminergic Neuronal Death

Yonjung Kim, Myoung Kyu Park, and Sungkwon Chung*

Recent studies have indicated that the corticotropin releasing hormone (CRF)-related peptide, urocortin, restores key indicators of damage in animal models for Parkinson's disease (PD). However, the molecular mechanism for the neuroprotective effect of urocortin is unknown. 1-Methy-4-phenylpyridinium (MPP^+) induces dopaminergic neuronal death. In the present study, MPP^+-induced neuroblastoma SH-SY5Y cell death was significantly attenuated by urocortin in a concentration-dependent manner. The protective effect of urocortin involved the activation of CRF receptor type 1, resulting in the increase of cyclic AMP (cAMP) levels. Various cAMP-enhancing reagents mimicked the effect of urocortin, while inhibitors for protein kinase A (PKA) blocked the effect of urocortin, strongly implicating the involvement of cAMP-PKA pathway in the neuroprotective effect of urocortin on MPP^+-induced cell death. As the downstream of this signal pathway, urocortin promoted phosphorylation of both glycogen synthase kinase 3β and extracellular signal-regulated kinases, which are known to promote cell survival. These neuroprotective signaling pathways of urocortin may serve as potential therapeutic targets for PD.

INTRODUCTION

In Parkinson's disease (PD), dopaminergic neurons in the substantia nigra pars compacta (SNc) progressively degenerate, resulting in dopaminergic neuronal cell loss and deficiency of dopamine in the striatum (Nicotra and Parvez, 2002). Death of dopaminergic neurons probably occurs as a result of apoptotic, excitotoxic, free-radical mediated events, and neuroinflammation (Blum et al., 2001; Gandhi and Wood, 2005; Vaux and Korsmeyer, 1999; Whitton, 2007). Toxicological factors, such as lipopolysaccharide, 6-hydroxydopamine, and 1-methy-4-phenyl- pyridinium (MPP^+), have been used to mimic the etiology of PD (Gandhi and Wood, 2005; Vaux and Korsmeyer, 1999). MPP^+, a toxin metabolite converted from 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, induces pathological changes resembling PD (Jacquy et al., 1993).

Urocortin, a corticotrophin releasing factor (CRF)-related peptide, may be neuroprotective since it promotes the survival of rat hippocampal neurons against oxidative and excitotoxic cell death (Pedersen et al., 2002). Urocortin appears to protect cultured hippocampal neurons, cortical neurons, cerebellar granule cells, and dopaminergic neurons from neurotoxin-induced cell death (Abuirmeileh et al., 2007; 2009; Bayatti et al., 2003; Facci et al., 2003; Pedersen et al., 2002). Urocortin also protects from spontaneous cell death related to aging (Huang et al., 2009). Urocortin mediates its effects through at least two high affinity membrane receptors, CRF receptor type 1 (CRFR1) and CRF receptor type 2 (CRF2R), which are expressed not only in SNc dopaminergic neurons but also in various neurons and glial cells (Huang et al., 2009; Kim et al., 2009; Pedersen et al., 2002; Stevens et al., 2003). Therefore, elucidating the signaling pathway for the neuroprotective effect of urocortin may confer potential therapeutic implications in PD. However, the molecular mechanisms are unknown for the neuroprotective effect of urocortin.

CRF receptors are coupled to multiple G-proteins that activate intracellular signaling pathways (Grammatopoulos et al., 2001; Perrin and Vale, 1999). These include mitogen-activated protein kinase (MAPK) pathway, in particular, extracellular signal-regulated kinases (ERKs). $ERK_{1/2}$ constitute a widely conserved family of serine/threonine protein kinases involved in many cellular programs such as cell proliferation, cell differentiation, cell movement, and cell survival (Courchesne, 1989). Activation of $ERK_{1/2}$ by CRF ligands results in neuroprotection (Brar et al., 2000; Pederson et al., 2002) and cell differentiation (Cibelli et al., 2001). Also, glycogen synthase kinase 3 (GSK3) participates in the regulation of protein synthesis, cell proliferation, cell differentiation, and apoptosis (Grimes and Jope, 2001), contributing to neuronal cell death induced by neurotoxic insults (Chen et al., 2004; Kelly et al., 2004; Koh et al., 2008; Xu et al., 2003). GSK3β plays a critical role in the promotion of apoptosis in neurons (Chin et al., 2005; Mora et al., 2001).

The present study was undertaken to resolve the molecular mechanism for the protective effect of urocortin on MPP^+-induced apoptotic death in human neuroblastoma SH-SY5Y cells. The results demonstrate that the activation of CRF1R results in increased levels of cyclic AMP (cAMP), which in turn activate protein kinase A (PKA). As well, it is demonstrated that

Department of Physiology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea
*Correspondence: schung@med.skku.ac.kr

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urocortin promotes phosphorylation of both GSKβ and $ERK_{1/2}$, which are known to promote cell survival.

MATERIALS AND METHODS

Materials

All chemicals used in this study were purchased from Sigma-Aldrich, unless otherwise indicated. H-89 and Rp-8-br-cAMP were obtained from Calbiochem. Anti-GSK3b, anti-phospho GSK3β, anti-$ERK_{1/2}$ and anti-phospho $ERK_{1/2}$ antibodies were purchased from Cell Signaling Technology. Tissue culture media and reagents were obtained from Invitrogen.

Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection. Cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics in a CO_2 humidified incubator in an atmosphere of 95% air and 5% CO_2.

Pharmacological treatments and cell viability test

Cell viability was measured using a conventional 3-(4,5-dimethyl thiazoly)-2,5-diphenyl-tetrazolium bromide (MTT)-based assay. Briefly, 10 μl of an MTT solution was added to each well with 80% confluent SH-SY5Y cells and incubated at 37°C for 2 h. Optical density was measured at 450 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader. The MPP^+-induced cell death was measured after treating SH-SY5Y cells with various concentrations of MPP^+ between 3.12 μM and 400 μM for 24 h. MPP^+ was dissolved in DMSO. At concentrations used in the experiment, DMSO itself was without effect on cell death. To test the protective effect of urocortin on MPP^+-induced cell death, cells were pretreated with various concentrations of urocortin (0.1-1000 nM) for 1 h, followed by the addition of 400 μM MPP^+ in the continued presence of urocortin. Cell viability was measured after 24 h. Similarly, CRF receptor antagonists were pretreated along with 100 nM urocortin for 1 h, which was followed by the addition of 400 μM MPP^+ in the continued presence of urocortin and antagonists. Cell viability was measured after 24 h.

Intracellular cAMP measurement

SH-SY5Y cells were plated on six-well plates (10^6 cells/well) and treated with various concentrations of urocortin for 24 h. Cells were detached and resuspended in 50 mM sodium acetate assay buffer (pH 5.8). cAMP level was analyzed with a cAMP ELISA kit according to the manufacturer's instructions (cAMP Biotrak EIA system; Amersham Pharmacia Biotech).

Western blotting

Cells were treated for 24 h with either 400 μM MPP^+ or 100 nM urocortin alone or with both. In some experiments, cells were treated either with 100 nM antagonist for CRF receptor or with 5 μM H-89 for 1 h prior to the treatment with 400 μM MPP^+ and 100 nM urocortin for an additional 24 h. Then, cells were rinsed twice with ice-chilled phosphate buffered saline (PBS), harvested, and lysed in a solution containing 1% Triton ×100, 0.5% sodium dodecyl sulfate (SDS), 0.75% deoxycholate, 75 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl (pH 7.4), 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), 2 mM sodium orthovanadate, 4.2 mg/ml NaF, 17.8 mg/ml sodium pyrophosphate, and 1% protease/phosphatase inhibitors for 1 h. Lysates were centrifuged for 30 min (10,000 × g). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to a nitrocellulose membrane (Potran). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at 22-25°C, and incubated with the primary antibody at 4°C overnight at 22-25°C. The Western blot was developed using an enhanced chemiluminescence Western detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Urocortin protects SH-SY5Y neuroblastoma cells from MPP^+-induced neuronal cell death

In the rat mesencephalic and striatal neurons, MPP^+ induces apoptotic cell death (Mochizuki et al., 1994). MPP^+ also induces apoptosis in neuroblastoma SH-SY5Y cells (Itano and Nomura, 1995), which serve as a model for human dopaminergic neurons (Ross and Biedler, 1985). When we treated SH-SY5Y cells with various concentrations of MPP^+ between 3.12 μM and 400 μM for 24 h, MPP^+ induced cell death in a concentration-dependent manner (Fig. 1A). Cell viability was quantified by using an established MTT assay. The cell viability at 400 μM MPP^+ was 60.0 ± 2.1% of the control level (n = 20). Then, we tested whether urocortin protects SH-SY5Y cells from MPP^+-induced cell death. Urocortin itself did not affect cell viability at concentrations up to 1000 nM (Fig. 1B, n = 21). Cells were pretreated with different concentration of urocortin for 1 h, which was followed by treatment with 400 μM MPP^+ for an additional 24 h in the continued presence of urocortin. Urocortin attenuated MPP^+-induced cell death in a concentration-dependent manner (Fig. 1C, n = 11). Cell viability was increased to 78.5 ± 2.1% of the control level by 100 nM urocortin, even in the presence of 400 μM MPP^+.

In SH-SY5Y cells, the mRNA level of CRF1R is higher than that of CRF2R (Schoeffter et al., 1999). When Western blots were done using antibodies specific to each receptor type, endogenous protein level of CRF1R was detected, while CRF2R expression was not (data not shown). To confirm that the protective effect of urocortin on MPP^+-induced cell death involved the activation of the urocortin receptor, the effects of CRF receptor antagonists were tested. Cells were pretreated with 100 nM urocortin (URO) and 100 nM astressin, a CRFR antagonist (Brar et al., 2004), for 1 h prior to the addition of 400 μM MPP^+. When the viability of cells was measured 24 h later, the presence of astressin was observed to significantly reduce the cell viability to 64.8 ± 3.8% of the control level (Fig. 1D, n = 11). Next, the effect of 100 nM antalarmin, a specific antagonist for CRF1R (Brar et al., 2004), was ascertained. The presence of antalarmin also reduced the cell viability to 67.6 ± 2.4% of the control level (Fig. 1D, n = 16). Cell viability was not affected by the presence of astressin or antalarmin itself for 24 h (data not shown). Together, these results suggest that the protective effect of urocortin on MPP^+-induced neuronal cell death was via the activation of CRF1R.

The protective effect of urocortin is mediated via cAMP-PKA pathway

CRF receptors mainly couple to G_s proteins, leading to the activation of adenylyl cyclase and the synthesis of cAMP (Dautzenberg and Wille, 2004). cAMP is an archetypal second messenger that functions as an intracellular mediator for neurotransmitters and hormones (Sutherland, 1972). The elevation of cAMP levels promotes the survival of many different types of neurons including sympathetic, sensory, cholinergic, and dopaminergic neurons (Goldberg and Barres, 2000). In particular, several studies have shown that cAMP-elevating agents

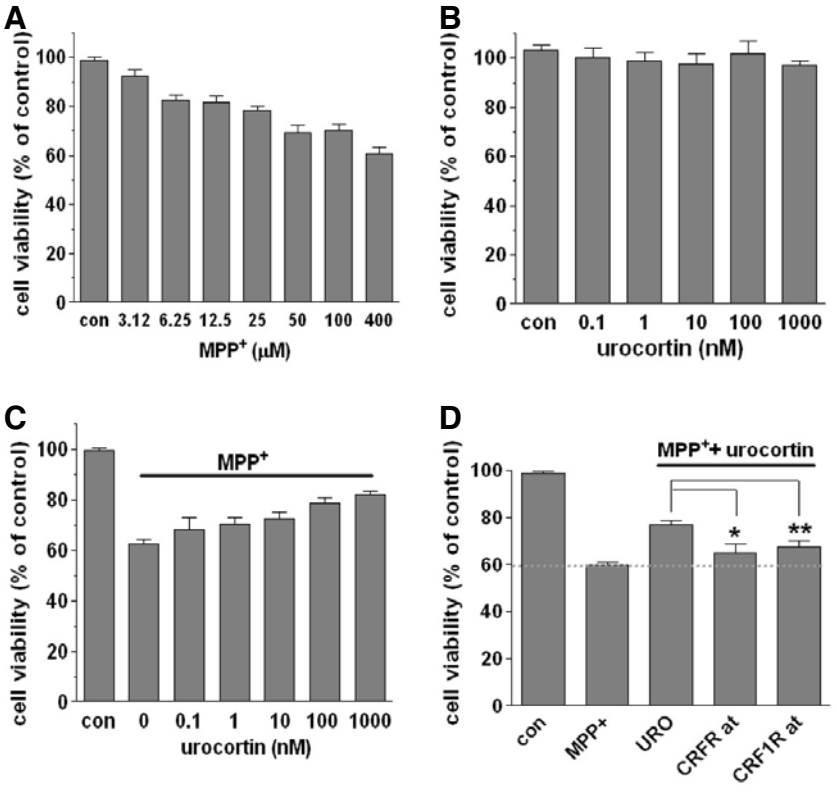

Fig. 1. Urocortin protectes SH-SY5Y cells from MPP^+-induced cell death. (A) MPP^+ induces cell death in a dose-dependent manner. SH-SY5Y cells were treated with indicated concentrations of MPP^+ for 24 h, and the cell viability was measured by using MTT assay (n = 20). (B) The effect of urocortin on cell death. Cells were treated with indicated concentrations of urocortin for 24 h (n = 21). (C) MPP^+-induced cell death is rescued by urocortin in a dose-dependent manner. Cells were pretreated with indicated concentrations of urocortin for 1 h, followed by treatment with 400 μM MPP^+ for an additional 24 h. Then, cell viability was measured (n = 11). (D) The protective effect of urocortin on MPP^+-induced cell death is via the activation of CRF1R. The presence of 100 nM urocortin (URO) protected cells from 400 μM MPP^+-induced cell death as expected. Cells were treated with urocortin together either with CRF receptor antagonist (CRFR at), 100 nM astressin, or with the CRF1R specific antagonist (CRF1R at), 100 nM antalarmin, for 1 h prior to the addition of 400 μM MPP^+. When the viability of cells was measured after 24 h (n = 16), the presence of these antagonists significantly reduced the cell viability. **represents $p < 0.01$ and * represents $p < 0.05$ from paired *t*-tests.

can prevent the spontaneous death of dopaminergic neurons in mesencephalic cultures (Hartikka et al., 1992; Mena et al., 1995; Michel and Agid, 1996; Mourlevat et al., 2003). To examine whether the protective effect of urocortin on MPP^+-induced cell death was due to the elevation of cAMP level, the intracellular cAMP content was measured after incubating cells with urocortin for 24 h. We chose 24 h incubation time before measuring the intracellular cAMP content, since the protective effect of urocortin was measured in the same incubation condition. Urocortin increased the intracellular cAMP levels in a dose-dependent manner as shown in Fig. 2A (n = 5). The presence of 100 nM urocortin increased the cAMP level by 52 ± 9% compared to the control level (Fig. 2B, n = 5).

Various cAMP-enhancing reagents were used to determine whether the increased cAMP level mimicked the effect of urocortin on MPP^+-induced cell death. For this purpose, cells were pretreated with the membrane permeable cAMP analogue dibutyryl cyclic adenosine monophosphate (db-cAMP; 1 mM) or 8-bromo-cyclic adenosine monophosphate (8-br-cAMP; 100 μM). The concentrations of these cAMP analogues were used previously for protecting neuronal cells from death (Facci et al., 2003; Troadec et al., 2002). Even in the presence of 400 μM MPP^+, cell viability was increased to 74.2 ± 2.6% (n=13) or 73.1 ± 2.5% (n = 13) of the control level when we included db-cAMP or 8-br-cAMP, respectively (Fig. 2C). In addition, when cells were pretreated with 10 μM of the adenylyl cyclase activator forskolin (Facci et al., 2003; Troadec et al., 2002), cell viability increased to 80.2 ± 3.6% of the control level (n = 13), which was similar to the level obtained with 100 nM urocortin. Together, these results suggest that increased cAMP level protects cells from MPP^+-induced cell death.

The increased cellular cAMP level would induce the activation of PKA. To elucidate the role of PKA on urocortin action, cells were pretreated with 50 μM Rp-8-bromo-cyclic adenosine monophosphate (Rp-8-br-cAMP), a specific inhibitor of PKA (Facci et al., 2003). The presence of Rp-8-br-cAMP significantly inhibited the protective effect of urocortin, decreasing cell viability to 62.8 ± 2.7% of the control level (Fig. 2D, n = 10). Also, pretreating cells with 10 μM H-89, a general PKA inhibitor (Facci et al., 2003), reduced cell viability to 58.8 ± 4.1% of the control level (n = 13). Taken together, these results indicate that cAMP-PKA pathway plays a critical role for the neuroprotective effect of urocortin on MPP^+-induced cell death.

Urocortin increases phosphorylation of GSK3β and $ERK_{1/2}$

Several mechanisms have been proposed to explain the survival promoting effect of cAMP in neurons. Specifically, it has been suggested that cAMP induces the phosphorylation of GSK3β (Li et al., 2000) or stimulates the MAPK/$ERK_{1/2}$ signaling pathway (Troadec et al., 2002; Villalba et al., 1997). To investigate the cellular mechanism for the protective effect of urocortin on MPP^+-induced cell death, GSK3β and $ERK_{1/2}$ levels were measured, as were the levels of their phosphorylated products, pGSK3β and p$ERK_{1/2}$. For this purpose, SH-SY5Y cells were treated with either 400 μM MPP^+ or 100 nM urocortin alone, and with both.

Figure 3A shows a typical Western blot result, and Figs. 3B and 3C show the densitometric analysis of the results. MPP^+ decreased the level of pGSK3β (Fig. 3A). In contrast, urocortin significantly increased the level of pGSK3β. When cells were treated with both MPP^+ and urocortin, the level of pGSK3β was comparable with that of control cells. This is more clearly demonstrated in Fig. 3B where the ratio of pGSK3β/GSK3β was maintained at higher level by urocortin even in the presence of MPP^+ (n = 14). Similar to pGSK3β, the level of p$ERK_{1/2}$ was decreased by MPP^+, while it was increased by urocortin (Figs.

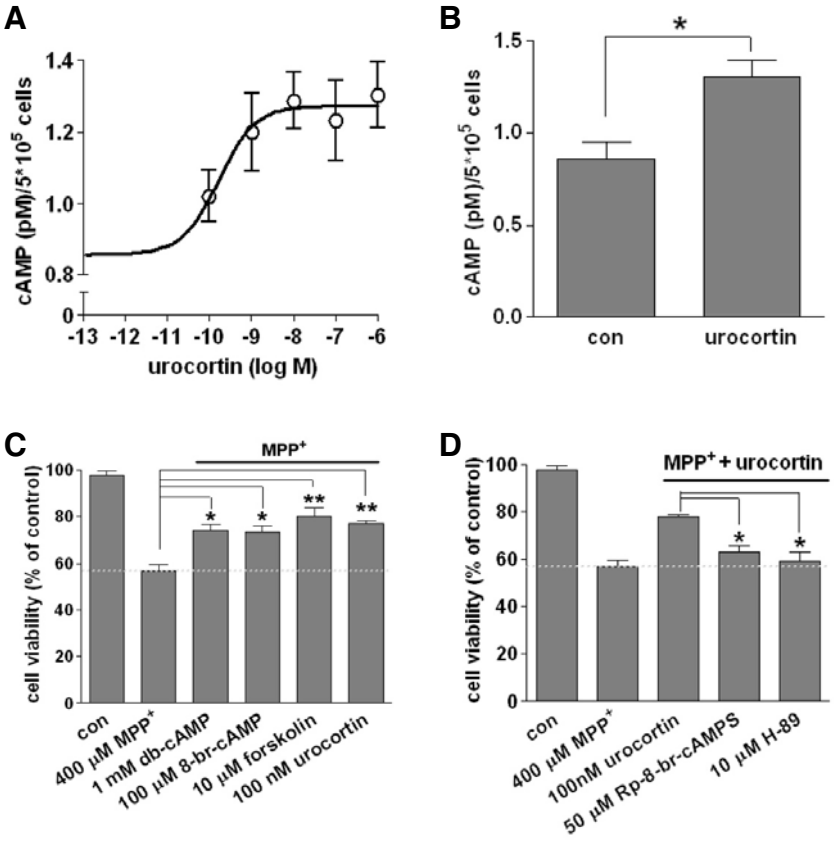

Fig. 2. The protective effect of urocortin is mediated by cAMP-PKA pathway. (A) Intracellular cAMP content was measured in SH-SY5Y cells after incubating cells with indicated concentrations of urocortin for 24 h. Data are the mean ± SEM of five independent experiments. (B) Summarized result showing that 100 nM urocortin increases intracellular cAMP level (n = 5). * represents $p < 0.05$ from paired *t*-tests. (C) Effects of various cAMP-enhancing reagents on MPP^+-induced cell death. Cells were treated with dibutyryl cyclic adenosine monophosphate (db-cAMP, 1 mM), 8-bromo-cyclic adenosine monophosphate (8-br-cAMP, 100 μM), or adenyl cyclase activator (forskolin, 10 μM) together with 400 μM MPP^+ for 24 h (n = 13). Cell viability level obtained with 400 μM MPP^+ is indicated by a broken line in the figure. (D) The presence of PKA inhibitors, Rp-8-bromo-cyclic adenosine monophosphate (Rp-8-br-cAMP, 50 μM) or H-89 (10 μM) significantly attenuates the protective effect of urocortin on MPP^+-induced cell death (n = 13). ** represents $p < 0.01$ and * represents $p < 0.05$ from paired *t*-tests.

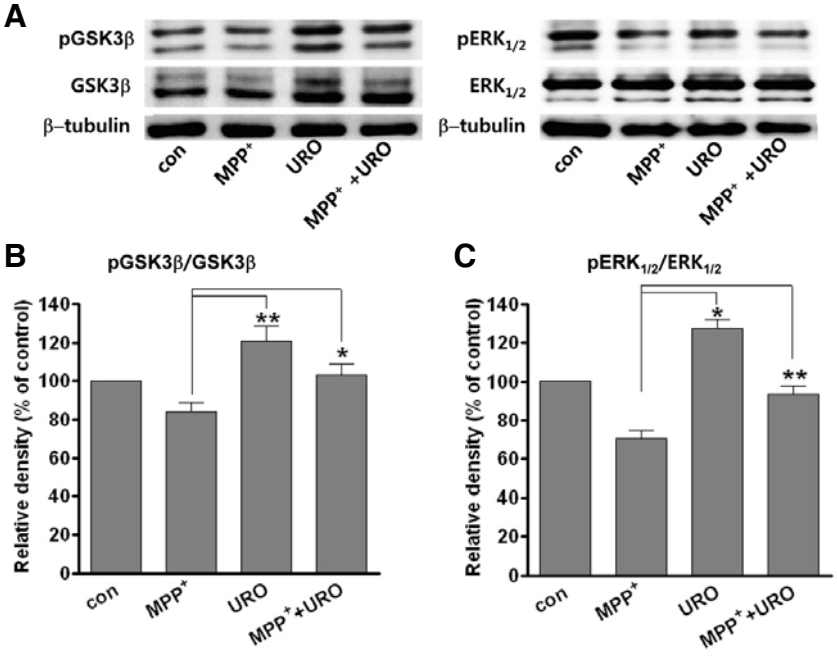

Fig. 3. Urocortin prevents the effects of MPP^+ on the levels of both phosphorylated GSK3β and phosphorylated $ERK_{1/2}$. (A) A representative Western blot showing the levels of GSK3β and $ERK_{1/2}$ from SH-SY5Y cells. Also shown are the levels of their phosphorylated forms (pGSK3β and $pERK_{1/2}$). Cells were treated for 24 h with either 400 μM MPP^+ or 100 nM urocortin alone, or with both. (B, C) Summarized densitometric analysis data for the ratio of pGSK3β/GSK3β (B) and $pERK_{1/2}$/$ERK_{1/2}$ (C). Treating cells with MPP^+ decreased the levels of pGSK3β and $pERK_{1/2}$, while urocortin increased those levels. Urocortin maintained the levels of pGSK3β (n = 14) and $pERK_{1/2}$ (n = 13) at higher level comparable with that of control cells even in the presence of MPP^+. ** represents $p < 0.01$ and * represents $p < 0.05$ from paired *t*-tests.

3A and 3C, n = 13). The level of $pERK_{1/2}$ was also maintained at higher level by urocortin, even in the presence of MPP^+. These results indicate that urocortin can promote phosphorylation of both GSK3β and $ERK_{1/2}$, which are known to promote cell survival. However, the increase of phosphorylation of both GSK3β and $ERK_{1/2}$ by urocortin was minimal (about 20%). This weak response may be due to our measurement at 24 h post-treatment with urocortin. Since we tested the effects of urocortin on MPP^+-induced cell death and on the level of cAMP after 24 h incubation time, we used the same incubation condition for measuring phosphorylation of both GSK3β and $ERK_{1/2}$.

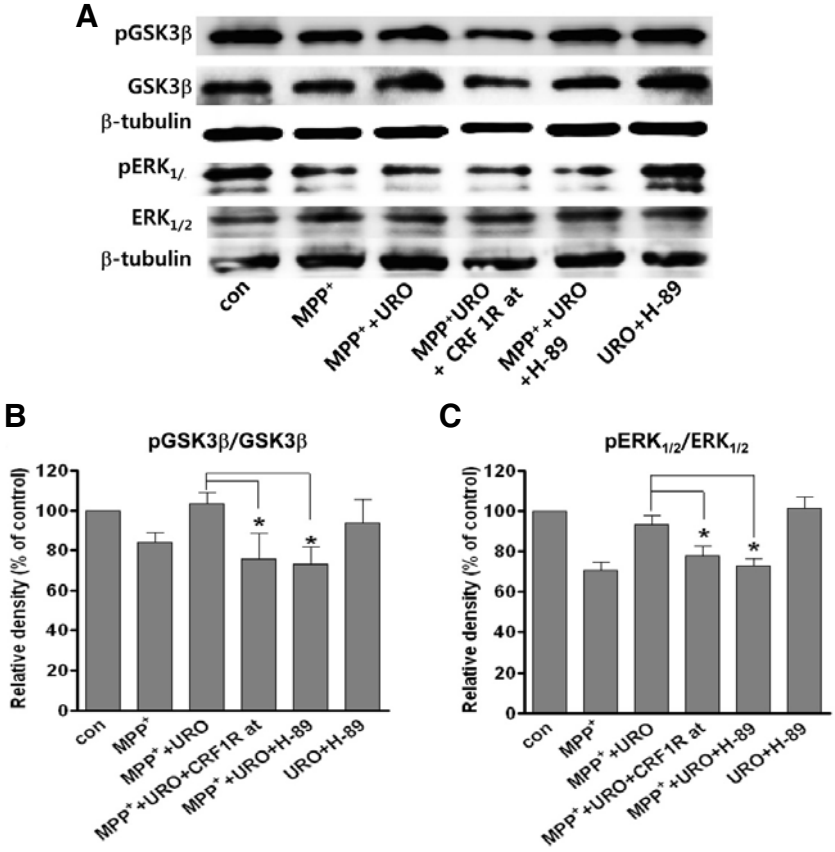

Fig. 4. cAMP-PKA pathway mediates urocortin-induced phosphorylation of GSK3β and $ERK_{1/2}$. (A) A representative Western blot showing the levels of GSK3β and $ERK_{1/2}$ from SH-SY5Y cells. Also shown are the phosphorylated forms (pGSK3β and $pERK_{1/2}$). Cells were treated either with 100 nM antalarmin, a specific antagonist for CRF1R (CRF1R at), or with 5 μM H-89 for 1 h prior to the treatment with 400 μM MPP^+ and 100 nM urocortin for an additional 24 h. (B, C) Summarized densitometric analysis data for the ratio of pGSK3β/GSK3β (B) and $pERK_{1/2}$/$ERK_{1/2}$ (C). The levels of pGSK3β (n = 5) and $pERK_{1/2}$ (n = 7) were maintained at lower level comparable with that of MPP^+-treated cells by antalarmin or H-89 even in the presence of urocortin (n = 6).

cAMP-PKA pathway mediates urocortin-induced phosphorylation of GSK3β and $ERK_{1/2}$

Since it was presently demonstrated that the neuroprotective effect of urocortin was via the activation of CRF1R, and that urocortin promoted the phosphorylation of GSK3β and $ERK_{1/2}$, it was appropriate to test the effect of a specific antagonist for CRF1R on the levels of pGSK3β and $pERK_{1/2}$. As expected, the level of pGSK3β was decreased by MPP^+, while it was maintained at higher level by urocortin (Fig. 4A). However, 100 nM antalarmin, a specific antagonist for CRF1R, abolished the effect of urocortin on the level of pGSK3β. Antalarmin decreased the ratio of pGSK3β/GSK3β to a level comparable with that of MPP^+-treated cells, even in the presence of urocortin (Fig. 4B, n = 5). Similarly, in the presence of antalarmin, the effect of urocortin on the level of $pERK_{1/2}$ was abolished (Fig. 4A), and the ratio of $pERK_{1/2}$/$ERK_{1/2}$ was decreased to a lower level (Fig. 4C, n = 7). The presence of antalarmin itself did not affect the levels of pGSK3β and $pERK_{1/2}$ (data not shown).

The neuroprotective effects of CRF and urocortin are attributed to the increase of cAMP level and the phosphorylation of GSK3β in rat primary cerebellar granule neurons (Facci et al., 2003). Also CRF triggers strong ERK phosphorylation, which is induced by a cAMP-dependent mechanism in AtT20 cells (Van Kolen et al., 2010). Since it has been presently demonstrated that the protective effect of urocortin on MPP^+-induced cell death was via the activation of cAMP-PKA pathway, it was appropriate to ascertain the effects of PKA inhibitor on the levels of pGSK3β and $pERK_{1/2}$. Even in the presence of urocortin, inhibition of PKA by H-89 decreased the pGSK3β level significantly to a level that was comparable with that of MPP^+-treated cells (Figs. 4A and 4B). This result indicates that the effect of urocortin on the level of pGSK3β was prevented by inhibiting PKA. Also, H-89 attenuated urocortin-induced phosphorylation of $ERK_{1/2}$ (Figs. 4A and 4C). The presence of H-89 itself did not affect the levels of pGSK3β and $pERK_{1/2}$. These results are consistent with the suggestion that urocortin activates the cAMP-PKA signal pathway, inducing phosphorylation of GSK3β and $ERK_{1/2}$, which may be responsible for the protective effect of urocortin on MPP^+-induced cell death.

The present study has demonstrated that urocortin increases the survival of dopaminergic SH-SY5Y cells from MPP^+-induced cell death via the cAMP-PKA pathway. It is also shown that the activation of this signal pathway is necessary for urocortin-induced phosphorylation of GSK3β and $ERK_{1/2}$, which is known to be protective. Thus, the results identify the molecular mechanism of the action of urocortin on MPP^+-induced cell death. Since MPP^+ is frequently used for *in vitro* as well as *in vivo* model for PD, activation of the urocortin signaling pathway may have therapeutic possibilities.

Urocortin increases intracellular cAMP levels followed by phospholyation of GSK3β that contributes to urocortin-mediated protection in spontaneous cell death related to aging (Huang et al., 2009). We confirmed the role of GSK3β for the protective effect of urocrotin on MPP^+-induced cell death as shown in the schematic model for the action of urocortin in Fig. 5. We also identified the phosphorylation of GSK3β through cAMP-PKA pathway by urocortin. However, it is possible that other pathways, which may work independently of cAMP activation, are involved in the protection. Other recent studies also showed that GSK3β may be a downstream target of the PI-3 kinase-Akt antiapoptotic signal pathway (Pap and Cooper, 1998). Elevation of intracellular cAMP levels in rat cerebellar granule neu-

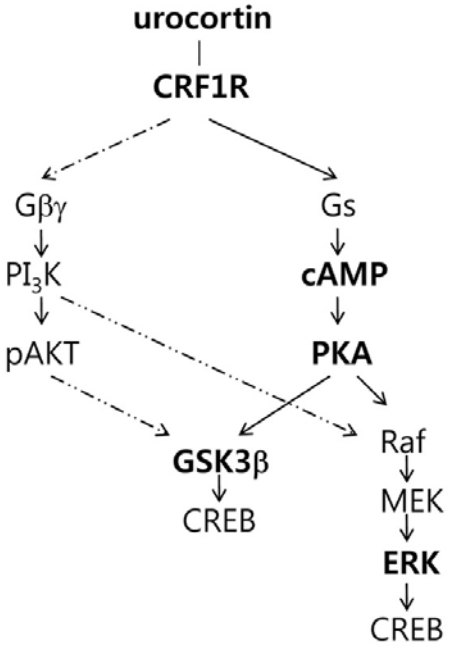

Fig. 5. Signal transduction pathways for the neuroprotective effect of urocortin. Based on the data from this study, urocortin activates cAMP-PKA pathway. Then, the phosphorylated forms of both $ERK_{1/2}$ and GSK3β increase, which are known to play important roles for the cell survival. Thus, urocortin protects cells from MPP^+-induced cell death. A dotted arrow indicates the pathway not determined in this study, and the signaling molecules tested in this study were presented as bold letters.

rons leads to phosphorylation of GSK3β in a process that is independent of Akt activation and activates PKA directly, which may phosphorylate GSK3β and inhibit apoptotic activity in neurons (Li et al., 2000).

In addition to GSK3β the activation of $ERK_{1/2}$ by urocortin has been reported from various cell types (Brar et al., 2004). Activation of $ERK_{1/2}$ by CRF results in cytoprotection and cell differentiation (Brar et al., 2000; Cibelli et al., 2001; Pederson et al., 2002). Both stimulatory and inhibitory effects via cAMP have been observed by cAMP-mediated alterations of ERK signaling (Stork and Schmitt, 2002). Considering all of these results together, it can be concluded that the phosphorylated forms of both $ERK_{1/2}$ and GSK3β may play important roles for the cell survival.

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REFERENCES

Abuirmeileh, A., Lever, R., Kingsbury, A.E., Lees, A.J., Locke, I.C., Knight, R.A., Chowdrey, H.S., Biggs, C.S., and Whitton, P.S. (2007). The corticotrophin-releasing factor-like peptide urocortin reverses key deficits in two rodent models of Parkinson's disease. Eur. J. Neurosci. *26*, 417-423.

Abuirmeileh, A., Harkavyi, A., Kingsbury, A., Lever, R., and Whitton, P.S. (2009). The CRF-like peptide urocortin greatly attenuates loss of extracellular striatal dopamine in rat models of Parkinson's disease by activating CRF(1) receptors. Eur. J. Pharmacol. *604*, 45-50.

Bayatti, N., Zschocke, J., and Behl, C. (2003). Brain region-specific neuroprotective action and signaling of corticotropin-releasing hormone in primary neurons. Endocrinology *144*, 4051-4060.

Blum, D., Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R., and Verna, J.M. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog. Neurobiol. *65*, 135-172.

Brar, B.K., Jonassen, A.K., Stephanou, A., Santilli, G., Railson, J., Knight, R.A., Yellon, D.M., and Latchman, D.S. (2000). Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. J. Biol. Chem. *275*, 8508-8514.

Brar, B.K., Chen, A., Perrin, M.H., and Vale, W. (2004). Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides. Endocrinology *145*, 1718-1729.

Chen, G., Bower, K.A., Ma, C., Fang, S., Thiele, C.J., and Luo, J. (2004). Glycogen synthase kinase 3beta (GSK3beta) mediates 6-hydroxydopamine-induced neuronal death. FASEB J. *18*, 1162-1164.

Chin, P.C., Majdzadeh, N., and D'Mello, S.R. (2005). Inhibition of GSK3beta is a common event in neuroprotection by different survival factors. Brain Res. Mol. Brain Res. *137*, 193-201.

Cibelli, G., Corsi, P., Diana, G., Vitiello, F., and Thiel, G. (2001). Corticotropin-releasing factor triggers neurite outgrowth of a catecholaminergic immortalized neuron via cAMP and MAP kinase signalling pathways. Eur. J. Neurosci. *13*, 1339-1348.

Courchesne, W.E., Kunisawa, R., and Thorner, J. (1989). A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. Cell *58*, 1107-1119.

Dautzenberg, F.M., and Wille, S. (2004). Binding differences of human and amphibian corticotropin-releasing factor type 1 (CRF(1)) receptors: identification of amino acids mediating high-affinity astressin binding and functional antagonism. Regul. Pept. *118*, 165-173.

Facci, L., Stevens, D.A., Pangallo, M., Franceschini, D., Skaper, S.D., and Strijbos, P.J. (2003). Corticotropin-releasing factor (CRF) and related peptides confer neuroprotection via type 1 CRF receptors. Neuropharmacology *45*, 623-636.

Gandhi, S., and Wood, N.W. (2005). Molecular pathogenesis of Parkinson's disease. Hum. Mol. Genet. *14*, 2749-2755.

Goldberg, J.L., and Barres, B.A. (2000). The relationship between neuronal survival and regeneration. Annu. Rev. Neurosci. *23*, 579-612.

Grammatopoulos, D.K., Randeva, H.S., Levine, M.A., Kanellopoulou, K.A., and Hillhouse, E.W. (2001) Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. J. Neurochem. *76*, 509-519.

Grimes, C.A., and Jope, R.S. (2001). The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog. Neurobiol. *65*, 391-426.

Hartikka, J., Staufenbiel, M., and Lubbert, H. (1992). Cyclic AMP, but not basic FGF, increases the *in vitro* survival of mesencephalic dopaminergic neurons and protects them from $MPP(^+)$-induced degeneration. J. Neurosci. Res. *32*, 190-201.

Huang, H.Y., Lin, S.Z., Chen, W.F., Li, K.W., Kuo, J.S., and Wang, M.J. (2009). Urocortin modulates dopaminergic neuronal survival via inhibition of glycogen synthase kinase-3beta and histone deacetylase. Neurobiol. Aging [in press].

Itano, Y., and Nomura, Y. (1995). 1-methyl-4-phenyl-pyridinium ion (MPP^+) causes DNA fragmentation and increases the Bcl-2 expression in human neuroblastoma, SH-SY5Y cells, through different mechanisms. Brain Res. *704*, 240-245.

Jacquy, J., Mettens, P., Blum, S., Jacqmotte, N., and Vanderheyden, J.E. (1993). Long-latency auditory evoked responses in Parkinson disease and in parkinsonism induced by neuroleptics. Acta Neurol. Belg. *93*, 119-129.

Kelly, S., Zhao, H., Hua Sun G., Cheng, D., Qiao, Y., Luo, J., Martin, K., Steinberg, G.K., Harrison, S.D., and Yenari, M.A. (2004). Glycogen synthase kinase 3beta inhibitor Chir025 reduces neuronal death resulting from oxygen-glucose deprivation, glutamate excitotoxicity, and cerebral ischemia. Exp. Neurol. *188*, 378-386.

Kim, Y., Park, M.K., and Chung, S. (2009). Regulation of somatodendritic dopamine release by corticotropin-releasing factor via the inhibition of voltage-operated Ca^{2+} channels. Neurosci. Lett. *465*, 31-35.

Koh, S.H., Noh, M.Y., and Kim, S.H. (2008). Amyloid-beta-induced neurotoxicity is reduced by inhibition of glycogen synthase kinase-3. Brain Res. *1188*, 254-262.

Li, M., Wang, X., Meintzer, M.K., Laessig, T., Birnbaum, M.J., and Heidenreich, K.A. (2000). Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. Mol. Cell Biol. *20*, 9356-9363.

Mena, M.A., Casarejos, M.J., Bonin, A., Ramos, J.A., and García Yébenes, J. (1995). Effects of dibutyryl cyclic AMP and retinoic acid on the differentiation of dopamine neurons: prevention of cell death by dibutyryl cyclic AMP. J. Neurochem. *65*, 2612-2620.

Michel, P.P., and Agid, Y. (1996). Chronic activation of the cyclic AMP signaling pathway promotes development and long-term survival of mesencephalic dopaminergic neurons. J. Neurochem. *67*, 1633-1642.

Mochizuki, H., Nakamura, N., Nishi, K., and Mizuno, Y. (1994). Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rat. Neurosci. Lett. *170*, 191-194.

Mora, A., Sabio, G., Gonzalez-Polo, R.A., Cuenda, A., Alessi, D.R., Alonso, J.C., Fuentes J.M., Soler, G., and Centeno, F. (2001). Lithium inhibits caspase 3 activation and dephosphorylation of PKB and GSK3 induced by K^+ deprivation in cerebellar granule cells. J. Neurochem. *78*, 199-206.

Mourlevat, S., Troadec, J.D., Ruberg, M., and Michel, P.P. (2003). Prevention of dopaminergic neuronal death by cyclic AMP in mixed neuronal/glial mesencephalic cultures requires the repression of presumptive astrocytes. Mol. Pharmacol. *64*, 578-586.

Nicotra, A., and Parvez, S. (2002). Apoptotic molecules and MPTP-induced cell death. Neurotoxicol. Teratol. *24*, 599-605.

Pap, M., and Cooper, G.M. (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J. Biol. Chem. *273*, 19929-19932.

Pedersen, W.A., Wan, R., Zhang, P., and Mattson, M.P. (2002). Urocortin, but not urocortin II, protects cultured hippocampal neurons from oxidative and excitotoxic cell death via corticotropin-releasing hormone receptor type I. J. Neurosci. *22*, 404-412.

Perrin, M.H., and Vale, W.W. (1999). Corticotropin releasing factor receptors and their ligand family. Ann. N.Y. Acad. Sci. *885*, 312-328.

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A., and Ginty, D.D. (1999). Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science *286*, 2358-2361.

Ross, R.A., and Biedler, J.L. (1985). Presence and regulation of tyrosinase activity in human neuroblastoma cell variants *in vitro*. Cancer Res. *45*, 1628-1632.

Schoeffter, P., Feuerbach, D., Bobirnac, I., Gazi, L., and Longato, R. (1999). Functional, endogenously expressed corticotropin-releasing factor receptor type 1 (CRF1) and CRF1 receptor mRNA expression in human neuroblastoma SH-SY5Y cells. Fundam. Clin. Pharmacol. *13*, 484-489.

Stevens, S.L., Shaw, T.E., Dykhuizen, E., Lessov, N.S., Hill, J.K., Wurst, W., and Stenzel-Poore, M.P. (2003). Reduced cerebral injury in CRH-R1 deficient mice after focal ischemia: a potential link to microglia and atrocytes that express CRH-R1. J. Cereb. Blood Flow Metab. *23*, 1151-1159.

Stork, P.J., and Schmitt, J.M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. *12*, 258-266.

Sutherland, E.W. (1972). Studies on the mechanism of hormone action. Science *177*, 401-408.

Troadec, J.D., Marien, M., Mourlevat, S., Debeir, T., Ruberg, M., Colpaert, F., and Michel, P.P. (2002). Activation of the mitogen-activated protein kinase ($ERK_{1/2}$) signaling pathway by cyclic AMP potentiates the neuroprotective effect of the neurotransmitter noradrenaline on dopaminergic neurons. Mol. Pharmacol. *62*, 1043-1052.

Van Kolen, K., Verstraeten, K., Royaux, I., De Hoogt, R., Gutknecht, E., and Peeters, P.J. (2010). Corticotropin releasing factor-induced ERK phosphorylation in AtT20 cells occurs via a cAMP-dependent mechanism requiring EPAC2. Neuropharmacology *58*, 135-144.

Vaux, D.L., and Korsmeyer, S.J. (1999). Cell death in development. Cell *96*, 245-254.

Villalba, M., Bockaert, J., and Journot, L. (1997). Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. J. Neurosci. *17*, 83-90

Whitton, P.S. (2007). Inflammation as a causative factor in the aetiology of Parkinson's disease. Br. J. Pharmacol. *150*, 963-976.

Xu, J., Culman, J., Blume, A., Brecht, S., and Gohlke, P. (2003). Chronic treatment with a low dose of lithium protects the brain against ischemic injury by reducing apoptotic death. Stroke *34*, 1287-1292.