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Possible Role of Interleukin-6 in PC12 Cell Death Induced by MPP⁺ and Tetrahydroisoquinoline

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Abstract. Interleukin (IL)-6 has been shown to protect neuronal cells from cell death induced by various stimulants. Although neuronal cells including PC12 cells were shown to produce IL-6, little is known about the effects of dopaminergic neurotoxins, 1,2,3,4-tetrahydroisoquinoline (TIQ) and 1-methyl-4-phenylpyridinium ion (MPP⁺), on IL-6 expression in PC12 cells. In the present study, we investigated the role of IL-6 in the TIQ- and MPP⁺-induced cell death in PC12 cells. Treatment with 3.2 mM TIQ for 24 h caused a delayed cell death (lactate dehydrogenase (LDH) leakage and nuclear DNA fragmentation) markedly 72 h after the addition. Addition of 0.4 mM MPP⁺ caused LDH leakage and nuclear DNA fragmentation 24 h after the addition. The cell death induced by MPP⁺ was inhibited by an inhibitor of caspases, z-Val-Ala-Asp(OMe)-fluoromethylketone. The cell death induced by TIQ or MPP⁺ was inhibited by nerve growth factor and 10% serum and significantly enhanced by the treatment with anti-IL-6 antibody. Both neurotoxins decreased the IL-6 mRNA level in PC12 cells without changing the other tested mRNA levels (IL-1 α , β -actin, etc.). These findings suggest that dopaminergic neurotoxins cause cell death in PC12 cells at least partially by changing IL-6 expression.

Keywords: 1,2,3,4-tetrahydroisoquinoline, 1-methyl-4-phenylpyridinium ion, apoptosis, interleukin-6, PC12 cell

Introduction

A series of naturally occurring neurotoxic 1,2,3,4-tetrahydroisoquinoline (TIQ) derivatives such as salsolinols exist in the mammalian brain, and TIQ derivatives are candidates for endogenous parkinsonogenic compounds (1, 2). The cell death caused by the TIQ derivatives in dopaminergic human neuroblastoma SH-SY5Y cells proved to be apoptotic (2). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been considered as a potential agent of Parkinson's disease. MPTP is converted to 1-methyl-4-phenylpyridinium ion (MPP⁺), an active metabolite of MPTP, in the brain. PC12 cells, a clonal rat pheochromocytoma cell line, have been

used as a model cell line to investigate the mechanism of action of dopaminergic neurotoxins since PC12 cells produce dopamine and possess the dopamine transporters. Both TIQ and MPP⁺ are taken up into dopaminergic neurons including PC12 cells by the dopamine transporters (3, 4). Previously, we reported that MPP⁺ induced cell death and/or apoptosis in PC12 cells (5) and GH3 cells (a cell line from rat anterior pituitary, Ref. 6). Although TIQ and MPP⁺ have been shown to cause apoptosis in various neuronal cells including PC12 cells (7–9), several reports showed that these neurotoxins induced non-apoptotic cell death and/or necrosis in neuronal cells (10–12). In addition, the precise mechanism(s) of the toxicity has not been well revealed.

Recent evidence suggests that cytokines such as inter-

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leukin (IL)-6 play an important role in neuronal functions including differentiation and survival in the brain (13). Wu and Bradshaw (14) reported that IL-6 enhanced the nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells. In addition, IL-6 was capable of protecting fetal rat dopaminergic neurons from the neurotoxicity of MPP⁺ (15). The addition of IL-6 protected PC12 cells from serum deprivation- and various stimulants-induced toxicity (16–19). Although neuronal cells including PC12 cells were shown to produce IL-6 in themselves (20–22), it has not been well established that IL-6 synthesized in neurons can be effective to protect themselves from cell death. In addition, little is known about the effects of neurotoxins on IL-6 mRNA levels in neuronal cells. In the present study, we show that treatment with dopaminergic neurotoxins (TIQ and MPP⁺) decreased IL-6 mRNA levels and the treatment with the anti-IL-6 antibody enhanced TIQ- and MPP⁺-induced cell death in PC12 cells. The neurotoxins-induced cell death may be modulated by the deprivation of IL-6 in PC12 cells.

Materials and Methods

Materials

TIQ and MPP⁺ were purchased from Sigma-Aldrich (Steinheim, Germany) and Sigma (St. Louis, MO, USA), respectively. The cytotoxicity detection kit for measurement of lactate dehydrogenase (LDH) activity was from Roche (Mannheim, Germany). z-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD-fmk) was purchased from Biomol (Plymouth Meeting, PA, USA). NGF (2.5S) and the Apoptosis Screening Kit were from Wako (Osaka). Recombinant human IL-6 was purchased from PeproTech (Rocky Hill, NJ, USA). Anti-rat IL-6 antibody and an assay kit for rat IL-6 were purchased from Genzyme (Cambridge, MA, USA).

Culture of PC12 cells and the treatment with TIQ and MPP⁺

PC12 cells were grown in collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; ThermoTrace, Melbourne, Australia). For experiments, subconfluent PC12 cells were cultured with DMEM containing 1% FBS in the presence of TIQ or MPP⁺ for the indicated period. For TIQ treatment, PC12 cells were incubated with TIQ for 24 h, and the cells were cultured with the TIQ-free medium for an additional 24 h and 48 h. To verify the effects of NGF and FBS, the cells were cultured for 24 h with DMEM containing 50 ng/ml NGF and 10% FBS before treatment with neurotoxins for 24 h. The same concentrations of NGF and FBS

were added again in the TIQ-free medium. To verify the effect of caspase inhibitor, the cells were preincubated for 2 h with the medium containing 30 μ M ZVAD-fmk. The neurotoxins and ZVAD-fmk were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide in the medium was 0.5%. The vehicle containing 0.5% dimethyl sulfoxide alone did not show the toxicity for 24 and 48 h.

LDH leakage assay

Cell death was estimated by the LDH leakage method as described previously (6). LDH leakage (%) was defined as the ratio of LDH activity in the culture medium and total activity [% = (extracellular activity) / (extracellular activity and remaining cellular activity) per well].

Measurement of nuclear DNA fragmentation and condensation

DNA fragmentation induced by TIQ was measured by the Apoptosis Screening Kit (Wako) using TdT-mediated dUTP nick end labeling (TUNEL) method. The procedures were in accordance to the manufacturer's protocols. For fluorescence microscopy, cells were stained with the chromatin dye Hoechst 33258 (10 μ M, Wako), as described previously (23). Nuclei of apoptotic cells were observed as fragmented.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of mRNA levels in PC12 cells

Total RNA was isolated with TRIzol reagent (Sigma), and total RNA (2.5 μ g) was used for reverse transcription (RT) to generate cDNA. RT cDNA (0.12 μ g) was then used for the polymerase chain reaction (PCR) amplification. The primers for IL-6 were sense 5'-CAAGAGACTTCCAGCCAGTTGC-3' and antisense 5'-TTGCCGAGGTAGACCTCATAGTGACC-3' (expected product 614 bp); for IL-1 α , they were sense 5'-CTAAGAACTACTTCACATCCGCAGC-3' and antisense 5'-CTGGAATAAAACCCACTGAGGTAGG-3' (623 bp); for β -actin, they were sense 5'-TGCCCCA TCTATGAGGGTTACG-3' and antisense 5'-TAGAA GCATTTGCGGTGCACG-3' (650 bp); for bax, they were sense 5'-TGGCGATGAACTGGACAACAAC-3' and antisense 5'-CCC GAAGTAGGAAAGGAGGC-3' (301 bp); for tyrosine hydroxylase, they were sense 5'-GCTTCAGAAGGGCCGTCTCAG-3' and antisense 5'-CTTGTACTGGAAGGCAATCTC-3' (608 bp). The amplification cycles were within the linear range. Verification of the specific gene was established by their predicted size and restriction enzyme treated-fragment. Computer imaging analysis (NIH Image 1.57) was used to quantify the density of each band and to obtain semi-

quantitative information on the relative changes in mRNA levels. Since the level of β -actin mRNA did not change by TIQ or MPP⁺ treatment, the mRNA of this housekeeping gene was used as an internal control and the ratios of RT-PCR products of IL-6 to β -actin were determined.

Statistics

Values are means $\pm$ S.E.M. of more than 3 independent experiments. In the case of multiple comparisons, the significance of difference was determined using one-way analysis of variance followed by the Bonferroni test. *P* values <0.05 were considered significant.

Results

Cell death induced by TIQ or MPP⁺ in PC12 cells

In PC12 cells, serum-deprivation alone caused a marked cell toxicity (16, 18). Similarly, we reported that the basal (vehicle-induced) LDH leakage in the period of 24 h was over 20% in serum-free DMEM (5). In the present study, PC12 cells were treated with TIQ and MPP⁺ in the DMEM containing 1% FBS in order to avoid the effect of serum-deprivation. The addition of 3.2 mM TIQ did not stimulate LDH leakage in the period of 24 h after the addition; the LDH leakages were $4.5 \pm 1.2\%$ ($n = 4$) and $5.3 \pm 0.9\%$ ($n = 5$) in the control and the TIQ-treated cells, respectively. Next, we investigated the effect of TIQ at 48 and 72 h after the TIQ addition. In these experiments, PC12 cells were cultured with 3.2 mM TIQ for 24 h, and then the cells were cultured in the TIQ-free medium for an additional 24 and 48 h; the total times after the TIQ addition were 48 and 72 h, respectively. The treatment with TIQ significantly stimulated LDH leakage at 72 h after the addition (Fig. 1A and Table 1). The effects induced by 0.8 and 1.6 mM TIQ on LDH leakage were limited and/or marginal, and the effect of 3.2 mM TIQ was not significant at 48 h after the addition. The culture of PC12 cells with 50 ng/ml of NGF or 10% FBS decreased both the basal and the 3.2 mM TIQ-induced LDH leakage, although the TIQ toxicity was still observed in the treated cells. The addition of 3.2 mM TIQ significantly caused DNA fragmentation assayed by the TUNEL method 48 h after the addition, and the effect of TIQ 72 h after the addition was remarkable (Fig. 1B). Treatment with 30 μ M ZVAD-fmk (a pan-caspase inhibitor) did not inhibit the TIQ-induced LDH leakage (data not shown), probably because of degradation of ZVAD-fmk during the long culture period (72 h).

Addition of 0.4 mM MPP⁺ slightly but significantly induced LDH leakage from PC12 cells in the period of 24 h after the addition (Fig. 2A), as described previously

Fig. 1. TIQ-induced cell death and DNA fragmentation in PC12 cells. PC12 cells were cultured with vehicle or 50 ng/ml of NGF in the DMEM containing 1% FBS for 24 h. In some cases, PC12 cells were cultured in the DMEM containing 10% serum for 24 h. The cells were further cultured with and without 3.2 mM TIQ for 24 h, and then the cells were further cultured with the TIQ-free fresh medium containing 1% FBS for an additional 48 h. An identical concentration of NGF was added again to the NGF-treated cells 24 h after the TIQ addition. In Panel A, LDH leakage was measured as described in Materials and Methods. Data are presented as percentages of LDH leakage from the control PC12 cells. The absolute values of LDH leakage were similar to those in Table 1. In Panel B, DNA fragmentation in the control cells (open column) and the 3.2 mM TIQ-treated cells (hatched column) were determined by TUNEL methods. Each experiment was done in triplicate, and values are means $\pm$ S.E.M. for 3–4 independent experiments. ^a*P*<0.01, significantly different from the control values without TIQ. ^b*P*<0.01, significantly different from the value with TIQ.

(5, 24). The addition of 0.2 mM MPP⁺ slightly but not significantly induced LDH leakage. The LDH leakage induced by 0.4 mM MPP⁺ was almost completely inhibited in the PC12 cells cultured with 50 ng/ml of NGF. In addition, the LDH leakage induced by MPP⁺ in the presence of 10% FBS was $7.4 \pm 0.9\%$ ($n = 4$), which was similar to that in the control cells (Table 1). Treatment with 30 μ M ZVAD-fmk inhibited MPP⁺-induced LDH leakage almost completely. The

Table 1. Effects of anti-IL-6 antibody on MPP⁺- and TIQ-induced LDH leakage in PC12 cells

Addition	Control	Anti-IL-6 antibody
	LDH Leakage (%)	
Experiment I (72 h)		
None	12.7 ± 2.5	11.2 ± 0.5
TIQ	33.6 ± 0.7 ^a	48.9 ± 3.0 ^{a,b}
Experiment II (24 h)		
None	7.6 ± 0.7	7.2 ± 0.4
MPP ⁺	13.5 ± 1.0 ^a	19.2 ± 1.1 ^{a,b}

PC12 cells were cultured with vehicle or 1 μ g/ml of anti-IL-6 antibody for 2 h and then cultured with vehicle or 3.2 mM TIQ for an additional 72 h (Experiment I) or with 0.4 mM MPP⁺ for an additional 24 h (Experiment II). In experiment I, 1 μ g/ml of anti-IL-6 antibody was further added to the antibody-treated cells 24 h after the TIQ addition. Values are means $\pm$ S.E.M. for 3 independent experiments. ^a P <0.01, compared with the control values without the neurotoxins. ^b P <0.05, compared with the value without anti-IL-6 antibody.

LDH leakage at 48 h after the addition of 0.4 mM MPP⁺ was greater than 50%, and the severe effect of MPP⁺ was hardly inhibited by the NGF and ZVAD-fmk treatment (data not shown). Next, we performed nuclear staining with the chromatin dye Hoechst 33258 (Fig. 2B). The fragmentation of chromatin was observed in PC12 cells cultured with 0.4 mM MPP⁺, but not in the untreated cells. The addition of 0.4 mM MPP⁺ caused DNA fragmentation assayed by the TUNEL method 24 h after the addition, although the value showed wide variation (data not shown). These findings suggest that both TIQ and MPP⁺ caused apoptosis at

least partially in PC12 cells.

Effects of IL-6 and anti-IL-6 antibody on TIQ- and MPP⁺-induced cell death

It is reported that the addition of 20 ng/ml of IL-6 protected PC12 cells from serum deprivation-induced cell death (16, 18). The addition of 10 or 20 ng/ml of IL-6 showed a protective effect against 3.2 mM TIQ- or 0.4 mM MPP⁺-induced LDH leakage in some experiments; about 50% inhibition of the LDH leakages induced by the neurotoxins ($n=3$). However, the protective effect of IL-6 was not constant; the effect of IL-6 was not observed in some cases ($n=3$). The severe LDH leakages (greater than 50%) induced by 6.4 mM TIQ and by 0.8 mM MPP⁺ were not inhibited by IL-6 (data not shown). It is reported that neutralization of supernatant IL-6 by the addition of anti-IL-6 antibody inhibited the neuronal differentiation in PC12 cells (25). Next, we investigated the effect of 1 μ g/ml of anti-IL-6 antibody on LDH leakages by the neurotoxins (Table 1). The antibody treatment, which alone showed no effect on cell death, significantly enhanced 3.2 mM TIQ- and 0.4 mM MPP⁺-induced LDH leakages. The antibody treatment did not affect the appearance of toxicity induced by 3.2 mM TIQ; the LDH leakages at 48 h after the TIQ addition were $10.4 \pm 1.2\%$ in the control group, $11.2 \pm 2.1\%$ in the TIQ-treated group, and $13.3 \pm 2.5\%$ in the TIQ plus anti-IL-6 antibody-treated group, respectively ($n=3$). Similarly, the toxicity of 0.4 mM MPP⁺ at 12 h after the addition was not affected by the antibody treatment. The severe LDH leakage induced by the treatment with 0.4 mM

Fig. 2. MPP⁺-induced cell death and DNA fragmentation. PC12 cells were cultured in DMEM containing 1% FBS with vehicle (Control) or 50 ng/ml of NGF for 24 h. Then the cells were further cultured with 0.4 mM MPP⁺ for 24 h. Data are each the mean $\pm$ S.E.M. for 3–5 independent experiments. ^a P <0.05, significantly different from the value in the control cells. ^b P <0.05, significantly different from the value with MPP⁺. In Panel B, nuclear DNA fragmentation was observed by the staining with Hoechst 33258. Data are representative of 3 independent experiments.

MPP⁺ for 48 h was not enhanced by the antibody treatment; the values in the absence and presence of the antibody were $55 \pm 14\%$ and $59 \pm 11\%$ ($n = 3$), respectively.

Decrease of IL-6, not IL-1 α , mRNA levels induced by TIQ and MPP⁺

The constitutive expression of IL-1 α and IL-6 mRNAs is shown in PC12 cells (21, 22). In our experiment, the constitutive expressions of IL-1 α and IL-6

mRNAs in PC12 cells were demonstrated (Fig. 3). In PC12 cells treated with 3.2 mM TIQ for 72 h or 0.4 mM MPP⁺ for 24 h, the IL-6 mRNA level markedly decreased compared with that in the control cells. A slight decrease of IL-6 mRNA level was observed at 48 h after the TIQ addition (data not shown). The detectable changes in the IL-6 mRNA levels were not observed at early time points (4 and 12 h after the MPP⁺ addition and 24 h after the TIQ addition). The decreases of IL-6 mRNA level induced by TIQ and MPP⁺ were not due to non-specific effects because the mRNA level of IL-1 α was not decreased by the neurotoxins. In addition, mRNA levels of other proteins such as β -actin and tyrosine hydroxylase remained constant with and without neurotoxin treatment. Although oxidative stress is reported to increase the mRNA level of bax (a pro-apoptotic protein) in PC12 cells (26), the mRNA level of bax was not changed by TIQ and MPP⁺ in the present study. We could not examine the effects of the neurotoxins on the expression of IL-6 in GH3 cells since the constitutive expression of IL-6 mRNA was very low (data not shown).

Discussion

A series of TIQ derivatives such as salsolinols, which exist in the mammalian brain and are structurally related to MPP⁺, are potent inhibitors of NADH ubiquinone reductase (complex I) in mitochondria and candidates of endogenous neurotoxins (1, 2). Both TIQ and MPP⁺ are reported to cause apoptosis in PC12 cells (7, 8) and dopaminergic neurons (2, 6, 8, 27). However, several investigators suggest that MPP⁺ elicits non-apoptotic cell death in PC12 cells (11, 28). In the present study, we confirmed that both TIQ- and MPP⁺-caused cell death are accompanied with DNA fragmentation at least partially, and we showed that the TIQ toxicity was inhibited by NGF and FBS.

In the present study, treatment with anti-IL-6 antibody significantly enhanced the neurotoxins-induced cell death (Table 1). Functional IL-6 receptors are expressed both in the native and the differentiated PC12 cells (20). IL-6 protected PC12 cells from serum deprivation (16, 18)-, hydrogen peroxide (17)-, anticancer agents (18)-, or 6-hydroxydopamine- and 4-hydroxynonenal (19)-induced toxicity. IL-6 protected against MPP⁺ neurotoxicity in cultures of fetal rat dopaminergic neurons (15). Our results and these findings suggest that IL-6 protects PC12 cells against various neurotoxins including TIQ and MPP⁺. It is reported that IL-6 protected PC12 cells from serum deprivation through the Akt pathway (18) and that constitutive activation of Akt protected PC12 cells from the MPP⁺ toxicity

Fig. 3. Decrease in IL-6 mRNA level in TIQ- and MPP⁺-treated PC12 cells. Panel A: PC12 cells were cultured for 24 h with vehicle (lane 1) or 0.4 mM MPP⁺ (lane 2) and for 72 h with vehicle (lane 3) or 3.2 mM TIQ (lane 4). Total RNA was isolated from the respective cells and used for RT-PCR analysis using specific primers. Quantitative analysis of the ratio of RT-PCR products of IL-6 to β -actin is shown in Panel B. Values are means $\pm$ S.E.M. for 3 separate experiments. ^a $P < 0.01$, compared with the control.

(29). It should be determined whether the protective effect of IL-6 on the neurotoxins-induced cell death is mediated by the Akt pathway in the future. The real effect of exogenously added IL-6 on the neurotoxins-induced cell death has not yet been revealed because of the endogenous existence of IL-6 in the culture medium of PC12 cells. It is reported that the cellular levels of IL-6 varied depending on the conditions such as serum and the activities of Ca^{2+} -dependent kinases in PC12 cells (22, 30). Otherwise, cell-derived proteases may modulate the IL-6 response by the proteolytic pathway of IL-6 and IL-6 receptors (31).

It is reported that expression of IL-6 mRNA in PC12 cells increased not only by physiological stimulations such as depolarization (22), but also by chemical hypoxia (21). In neuronal cells including PC12 cells, the dopaminergic neurotoxins such as MPP^+ , oxidized dopamine and 6-hydroxydopamine regulate various gene expressions (9, 24, 32–34). In the present study, we found that both TIQ and MPP^+ markedly down-regulated IL-6, not IL-1 α , mRNA levels in PC12 cells at 72 h and 24 h after the addition, respectively (Fig. 3). However, the decrease of IL-6 mRNA (and probably IL-6 protein) induced by TIQ and MPP^+ does not appear to be an initial and sufficient pathway for cell death in PC12 cells. The reasons are as follows: 1) the decrease of IL-6 mRNA level was not ahead of the DNA fragmentation and/or cell death induced by the neurotoxins, 2) treatment with anti-IL-6 antibody alone did not cause cell death, and 3) the treatment with anti-IL-6 antibody did not accelerate the appearance of TIQ and MPP^+ toxicity. Since the treatment with anti-IL-6 antibody enhanced a rather moderate, but not severe, cell death induced by the neurotoxins, the decrease of IL-6 by the neurotoxins is likely to enhance the cell death in a cooperative manner.

All the findings taken together, TIQ and MPP^+ may decrease the IL-6 level and regulate cell death in PC12 cells. To our knowledge, this is the first study demonstrating that TIQ and MPP^+ , dopaminergic neurotoxins, regulate IL-6 mRNA level in neuronal cells. Although we tried to examine the effects of the neurotoxins on IL-6 protein level, we could not make a definitive conclusion because of wide variations. The effects of TIQ and MPP^+ on IL-6 mRNA and the protein levels in other dopaminergic neurons should be examined. Further studies are needed to clarify the role of cytokines such as IL-6 on the neurotoxins-induced cell death.

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