Preventive and therapeutic effects of caffeic acid against inflammatory injury in striatum of MPTP-treated mice

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Abstract

Preventive or therapeutic effects of caffeic acid in brain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mice against inflammatory injury were examined. Caffeic acid at 0.5, 1 or 2% was supplied either pre-intake or post-intake for 4 weeks. Brain caffeic acid content was increased by caffeic acid pre-intake at 1 and 2%, and post-intake at 2% ($P<0.05$). MPTP treatment enhanced the release of interleukin (IL)-1beta, IL-6, tumor necrosis factor (TNF)-alpha, IL-4 and IL-10 ($P<0.05$). Pre-intake of caffeic acid decreased the production of test cytokines ($P<0.05$); however, post-intake only at 2% reduced the level of IL-1beta, IL-6 and TNF-alpha ($P<0.05$). MPTP treatment up-regulated mRNA expression of inducible nitric oxide synthase (iNOS), neuronal NOS, cyclooxygenase (COX)-2, glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1, and increased production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) ($P<0.05$). Caffeic acid pre-intake at test doses and post-intake at 2% declined the expression of iNOS, COX-2 and GFAP; and lowered the production of NO and PGE_2 ($P<0.05$). MPTP treatment suppressed mRNA expression of brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and tyrosine hydroxylase (TH), and lowered dopamine level ($P<0.05$). Caffeic acid pre-intake retained the expression of these factors, maintained TH activity and protein production, and dopamine synthesis ($P<0.05$). These results suggest that caffeic acid is a potent neuroprotective agent against the development of Parkinson's disease.

Keywords: Caffeic acid; Parkinson's disease; Cytokine; NOS activity; Neurotrophin

1. Introduction

Neuro-inflammatory process has been considered as an important mechanism responsible for Parkinson's disease progression (Hirsch and Hunot, 2009). Proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-6, and nitric oxide (NO) are increased in cerebrospinal fluid of patients with Parkinson's disease (Mogi et al., 1994; Ferger et al., 2004). Postmortem examination in patients with Parkinson's disease reveals a loss of dopaminergic neurons in the substantia nigra associated with a massive astrogliosis and excessive microglial activation (Hirsch et al., 2003). Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba-1) are markers of astrogliosis and microglial activation, respectively. The over-production of these markers are highly associated with the generation of inflammation associated neurotoxic molecules such as IL-1beta, TNF-alpha and NO (Little and O'Callagha, 2001; Sugama et al., 2009). In addition, the elevated activity and expression of inducible nitric oxide synthase (iNOS) and neuronal NOS (nNOS) enhance NO formation and deteriorate Parkinson's disease (Okuno et al., 2005; Hancock et al., 2008). Thus, there is an increasing interest to examine the use of appropriate agent(s) to reduce the production of these markers in order to prevent or improve inflammatory damage in Parkinson's disease.

Caffeic acid is a phenolic acid naturally occurring in many plant foods such as carrot, tomato, strawberry and blueberry (Sun et al., 2009; Sochor et al., 2010). Several *in vivo* studies have indicated that this compound possesses anti-inflammatory activities (Yamada et al., 2006; Chao et al., 2009). Li et al. (2008) observed that caffeic acid, as a 5-lipooxygenase inhibitor, exhibited dopaminergic neuroprotective property. The study of Vauzour et al. (2008) revealed that caffeic acid could provide protection against 5-S-cysteinyl-dopamine-induced neurotoxicity in mouse cortical neurons. Those previous

studies suggested that caffeic acid was a potent dopamine-restorative agent; however, it remains unknown that caffeic acid could protect brain against Parkinson's disease associated inflammatory progression.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease has been widely used as a model for investigating effects of anti-Parkinson's disease agents (Bezard et al., 2001; Tsai et al., 2010). In this present study, MPTP was used to induce Parkinson's disease-like neurotoxicity in mice. Pre-intake and post-intake effects of caffeic acid at various doses against inflammatory injury in striatum of MPTP-treated mice were examined. The impact of this agent on neurotrophins such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) was determined. Furthermore, the effects of this agent on striatal level of dopamine metabolites such as 3,4-dihydroxyphenylacetic acid (DOPAC) were also evaluated.

2. Materials and methods

2.1. Animals

Three- to four-week-old male C57BL/6 mice were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12-h light-12-h dark schedule, and fed with water and mouse standard diet for one week acclimation. Use of the mice was reviewed and approved by China Medical University animal care committee. Mice with body weight at 22.3±1.4 g were used in all experiments.

2.2. Experimental design

Caffeic acid (99%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and at 0.5, 1 or 2 g was mixed with 99.5, 99 or 98 g powder diet (PMI Nutrition International

LLC, Brentwood, MO, USA). In preventive study, caffeic acid at three doses was supplied to mice for 4 weeks, mice then were treated by daily subcutaneous injection of vehicle saline or MPTP (24 mg/kg body weight) for 6 consecutive days. In therapeutic study, mice were treated by MPTP first, and followed by caffeic acid supplementation for 4 weeks. After sacrificed by decapitation, brain was quickly removed and the striatum was collected. The striatum at 0.1 g was homogenized on ice in 2 ml of phosphate saline buffer (PBS, pH 7.2) and the filtrate was collected. Protein concentration of striatal filtrate was determined by a commercial assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin as a standard. In all experiments, the sample was diluted to a final concentration of 1 mg protein/ml.

2.3. Caffeic acid content in brain tissue

An HPLC method described in Yamada et al. (2006) was used to analyze the brain content of intact form of caffeic acid, in which an octadecylsilica column (4.6 x 250 mm, Wakopak, Wako Pure Chemical Industry, Tokyo, Japan), and a mobile phase consisting of 95.6% H_2O, 4.1% ethyl acetate and 0.3% acetic acid were used at 30°C with a flow rate of 0.8 ml/min.

2.4. Measurement of dopamine, DOPAC and homovanillic Acid (HVA)

The levels of dopamine, DOPAC and HVA were determined by HPLC methods (Richardson et al., 2006). Briefly, the striatum was homogenized in 0.1 mol/l perchloric acid solution containing 0.1 mM ethylene-diaminetetraacetic acid (EDTA). After centrifuging at 12,000 xg for 60 min at 4°C, the supernatant was collected for analysis. HPLC equipped with a coulometric electrode array detector was used to quantify.

2.5. Cytokine measurements

Striatum was homogenized in 10 mM Tris-HCl buffered solution (pH 7.4) containing 2

M NaCl, 1 mM EDTA, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 9000 xg for 30 min at 4°C. The resultant supernatant was used for cytokine determination. The levels of IL-1beta, IL-6, TNF-alpha, IL-4 and IL-10 were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA, USA). Samples were assayed in duplicates according to manufacturer's instructions. The sensitivity of the assay, i.e., the lower limit of detection, was 5 nmol/l for IL-1beta, IL-6, IL-4, IL-10 and 10 nmol/l for TNF-alpha.

2.6. Determination of nitrite and prostaglandin E (PGE)$_2$

The production of NO was determined by measuring the formation of nitrite. Briefly, 100 µl of supernatant was treated with nitrate reductase, NADPH and FAD, and incubated for 1 h at 37 ºC in the dark. After centrifuging at 6,000 xg, the supernatant was mixed with Griess reagent for color development. The absorbance at 540 nm was measured and compared with a sodium nitrite standard curve. The production of PGE_2 was determined using a PGE_2 EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.7. BDNF and GDNF quantification

BDNF or GDNF level was determined using anti-BDNF or anti-GDNF monoclonal antibody. Briefly, striatum was dissected and homogenized at 4°C in 300 µl lysis buffer containing 137 mM NaCl, 20 mM Tris, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonylflouride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 0.5 mM sodium orthovanadate. After centrifuged at 10,000 xg for 10 min, the supernatant was used, and BDNF or GDNF level was determined by commercially available kits (Promega, Madison, WI, USA) according to the manufacture's instructions. Absorbance was measured at 450

nm using a plate reader. Data were expressed as pg/mg protein.

2.8. Activity of total NOS, cyclooxygenase-2 (COX-2) and tyrosine hydroxylase (TH)

The method described in Sutherland et al. (2005) was used to measure total NOS activity. Briefly, total NOS activity was determined via incubating 30 μl of homogenate with 10 mM β-nicotinamide adenine dinucleotide phosphate, 10 mM L-valine, 3000 U/ml calmodulin, 5 mM tetrahydrobiopterin, 10 mM $CaCl_2$, and a mixture of 100 μM L-arginine containing L-[^{3}H]arginine. COX-2 activity was assayed by a commercial assay kit (Cayman Chemical Co., Ann Arbor, MI, USA), and colorimetrically monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine at 590 nm. TH activity was assayed by the method described in Neff et al. (2006). Brain was homogenized in 10 mM Tris acetate buffer. After centrifuged, 30 μl supernatant was added to a mixture containing 40 mM sodium acetate, 200 μM 6-methyl-5,6,7,8-tetrahydropteridine, 10 μg/100 μl catalase, 1 mM ferrous ammonium sulfate and 200 μM L-tyrosine with 1 μCi of [^{3}H]-L-tyrosine. The reaction was terminated by adding charcoal in 0.01 M HCl, and radioactivity in the supernatant was counted in a scintillation counter.

2.9. Real-time polymerase chain reaction for mRNA expression

Total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). One μg RNA was used to generate cDNA, which was amplified using Taq DNA polymerase. PCR was carried out in 50 μl of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U Taq DNA polymerase. The specific oligonucleotide primers of targets are shown in Table 1. The cDNA was amplified under the following reaction conditions: 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min. 28 cycles were

performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) and 35 cycles were performed for others. Generated fluorescence from each cycle was quantitatively analyzed by using the Taqman system based on real-time sequence detection system (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA, USA). In this study, mRNA level was calculated as percentage of the control group.

2.10. Western blot analysis of TH

Brain tissue was homogenized in buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Alsrich Chemical Co., St. Louis, MO, USA). This homogenate was further mixed with buffer (60 mM Tris-HCl, 2% SDS, and 2% β-mercaptoethanol, pH 7.2), and boiled for 5 min. Sample at 40 μg protein was applied to 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) for 1 h. After blocking with a solution containing 5% nonfat milk for 1 h to prevent non-specific binding of antibody, membrane was incubated with mouse anti-TH monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 4^{o}C overnight, and followed by reacting with horseradish peroxidase-conjugated antibody 3.5 h at room temperature. The detected bands were quantified by Scion Image analysis software (Scion Corp., Frederick, MD, USA), and GAPDH was used as a loading control.

2.11. Statistical analysis

The effect of each treatment was analyzed from 10 mice (n = 10) in each group. All data were expressed as mean ± standard deviation (S.D.). Statistical analysis was done using one-way analysis of variance (ANOVA), and post-hoc comparisons were carried out using Dunnet's t-test. P values <0.05 were considered as significant.

3. Results

As shown in Table 2, all treatments did not affect daily water intake, feed intake, final body weight and brain weight (P>0.05). Brain caffeic acid content was significantly increased in mice treated by caffeic acid alone at 2%, caffeic acid pre-intake at 1 and 2%, and caffeic acid post-intake at 2% (P<0.05). MPTP treatment significantly increased the brain level of IL-1beta, IL-6, TNF-alpha, IL-4 and IL-10 (Table 3, P<0.05). Pre-intake of caffeic acid at test doses significantly decreased the release of five cytokines (P<0.05); however, post-intake only at 2% significantly lowered MPTP-caused increase of IL-1beta, IL-6 and TNF-alpha (P<0.05). As shown in Fig. 1, MPTP treatment significantly enhanced mRNA expression of IL-1beta, TNF-alpha and IL-10 (P<0.05). Pre-intake of caffeic acid significantly reduced the expression of three test cytokines (P<0.05); however, post-intake of this compound only at 2% significantly declined expression of IL-1beta and TNF-alpha (P<0.05).

MPTP treatment increased brain NO and PGE_2 levels, as well as elevated total NOS and COX-2 activities (Table 4, P<0.05). Caffeic acid pre-intake at three doses and post-intake at 2 % significantly lowered the production of NO and PGE_2, and activity of total NOS and COX-2 (P<0.05). MPTP also up-regulated mRNA expression of iNOS, nNOS and COX-2 (Fig. 2, P<0.05). Pre-intake of caffeic acid at 1 and 2% declined the expression of iNOS, nNOS and COX-2 (P<0.05). Post-intake of this compound at 2% significantly suppressed the expression of iNOS and COX-2 (P<0.05). MPTP significantly increased GFAP and Iba-1 expression (Fig. 3, P<0.05). Caffeic acid pre-intake at three doses and post-intake at 2 % repressed GFAP expression (P<0.05), and neither pre-intake nor post-intake of this compound affected Iba-1 expression (P>0.05).

As shown in Fig. 4, Fig. 5 and Table 5, MPTP treatment significantly decreased mRNA expression of DAT, BDNF, GDNF and TH, lowered BDNF and GDNF content, and reduced

TH protein level and activity (P<0.05). Caffeic acid treatments failed to affect DAT expression (P>0.05), but its pre-intake retained both expression and production of BDNF, GDNF and TH, as well as TH activity (P<0.05). Caffeic acid post-intake only at 2 % restored TH activity, expression and protein generation (P<0.05). As shown in Table 6, MPTP treatment significantly decreased the striatal content of dopamine, DOPAC and HVA (P<0.05). The pre-intake of caffeic acid dose-dependently attenuated MPTP-induced dopamine loss; but only at 1 and 2 % significantly retained DOPAC and HVA content (P<0.05). Post-intake of this compound at 2 % significantly restored dopamine, DOPAC and HVA levels (P<0.05).

4. Discussion

In our present study, caffeic acid pre-intake markedly increased caffeic acid content in brain and attenuated MPTP-caused inflammatory stress by lowering the production of inflammatory cytokines, NO and PGE_2, as well as declining activity and mRNA expression of cytokines, nNOS, COX-2 and GFAP in striatum. Furthermore, we found pre-intake of this compound retained expression, production and activity of BDNF, GDNF and TH. These findings support that this compound is an effective preventive agent against the development of neurodegenerative diseases such as Parkinson's disease. Since the mRNA expression of NOS, COX-2 and BDNF was mediated by caffeic acid, this agent might be able to penetrate blood brain barrier and exert its functions at the level of transcription. On the other hand, post-intake of caffeic acid only at high dose (2%) increased its deposit in brain, and mildly improved MPTP-induced inflammatory injury. Thus, further study is recommended to examine the neuro-restorative effects of this compound at higher doses and/or longer period of supplement.

Suppressing TNF-alpha response has been considered as a promising target for inflammatory treatment in Parkinson's disease (Madrigal et al., 2002; Tansey et al., 2008). Our present study found that caffeic acid pre-intake effectively decreased subsequent MPTP-induced over-production and over-expression of TNF-alpha and IL-6, which consequently mitigated inflammatory stress and in turn spared the formation of anti-inflammatory cytokines, IL-4 and IL-10. These findings indicated that caffeic acid exhibited preventive effects against inflammatory reactions via suppressing inflammatory cytokines production. In addition, we notified that post-intake of caffeic acid only at high dose declined production and expression of IL-1beta and TNF-alpha. These findings suggested that this compound was a mild therapeutic agent to attenuate inflammatory damage in already existed Parkinson's disease condition. iNOS and nNOS, two isoforms of NOS, are involved in pathological progression of Parkinson's disease (Levecque et al., 2003; Silverman, 2009). Elevated NO production resulted from enhanced activity and over-expression of these NOS is an important neurotoxic effector responsible for the loss of dopaminergic neurons and the expression of proinflammatory cytokines (Eberhardt et al., 2000; Choi et al., 2002). Our present study found that caffeic acid pre-intake effectively down-regulated iNOS and nNOS expression, and diminished total NOS activity, which consequently lowered production of NO and proinflammatory cytokines. These results once again supported that this compound could provide anti-inflammatory activities against Parkinson's disease, and partially explained the action modes of this compound. However, post-intake of caffeic acid only at high dose declined iNOS expression and total NOS activity. These findings implied that caffeic acid might provide mild therapeutic effect via regulating NO pathway.

COX-2 is the rate-limiting enzyme for synthesis of PGE_2, a pro-inflammatory mediator.

Enhanced COX-2 expression promoted microglial activation of substantia nigra pars compacta (Liu and Hong, 2003; Teismann et al., 2003), which accelerated the loss of dopaminergic neurons and favored the release of inflammatory cytokines, including IL-1beta and TNF-alpha (Vijitruth et al., 2006). In our present study, elevated expression and activity of COX-2 in brain from MPTP-treated mice indicated that COX-2 was involved in MPTP-induced brain inflammatory injury, and responsible for increased production of PGE_2 and inflammatory cytokines. Furthermore, we found that caffeic acid pre-intake at three doses and post-intake at high dose reduced COX-2 expression, mitigated COX-2 activity, lowered PGE_2 generation and rescued dopamine. These findings suggest that the preventive and therapeutic effects of caffeic acid against inflammation and dopamine loss in brain were partially due to this compound repress COX-2 and PGE_2. In addition, the observed up-regulated expression of GFAP and Iba-1 agreed that both astrogliosis and microglial activation has been enhanced in those MPTP-treated mice. Woiciechowsky et al. (2004) reported that astrogliosis induced by cytokines such as IL-1beta and IL-6 could accelerate GFAP release, which favored neuroinflammatory response and neuronal loss. We found that pre-intake of caffeic acid effectively suppressed GFAP expression, which suggested that this compound might be able to directly interrupt astrogliosis. The other possibility was that caffeic acid pre-intake already reduced the production of inflammatory cytokines, which in turn attenuated astrogliosis. On the other hand, we also notified that post-intake of caffeic acid at high dose diminished GFAP expression, which indicated that astrogliosis has been mitigated, and this might be partially ascribed to caffeic acid already lowered IL-6 and TNF-alpha. These findings implied that this compound at high dose could exhibit therapeutic effects against astrogliosis.

DAT is involved in dopamine homeostasis and sensitivity to dopaminergic

neurotoxicants (Kurosaki et al., 2003). The results of our present study revealed the pre-intake of caffeic acid substantially retarded MPTP-induced dopamine depletion in the striatum without alleviating MPTP-induced DAT depletion. Apparently, the increased dopamine level from this compound was not associated with DAT expression. Both BDNF and GDNF are neurotrophic and potent survival factors for dopaminergic neurons (Rosenbald et al., 2000; Ghitza et al., 2010). TH is the rate-limiting enzyme for dopamine synthesis and also a key molecule in dopaminergic functions because it converts tyrosine to L-DOPA, which is then converted to dopamine (Nakashima et al., 2009). In our present study, MPTP treatment decreased the production, expression and/or activity of BDNF, GDNF and TH, which partially explained the MPTP-caused dopamine loss. Furthermore, we found that caffeic acid pre-intake markedly counteracted neuro-toxic effects of MPTP and reserved the expression, activity and generation of BDNF, GDNF and TH, which benefited dopamine synthesis in brain. These findings implied that caffeic acid could prevent Parkinson's disease progression through regulating neurotrophic factors, stabilizing TH and protecting dopaminergic neurons. On the other hand, we notified that caffeic acid post-intake at high dose restored TH activity, mRNA expression and protein production, which consequently favored dopamine formation. Our data of dopamine, DOPAC and HVA also agreed that post-intake of this compound at that dose improved synthesis of dopamine and its metabolites. These findings suggested that this agent at high dose might provide therapeutic effect via restoring dopamine level.

In conclusion, caffeic acid pre-intake at three doses and post-intake at high dose effectively elevated brain caffeic acid content, and alleviated MPTP-caused inflammatory damage and dopamine loss. This agent exhibited anti-inflammatory activities by decreasing inflammatory cytokines levels, suppressing NO, PGE_2 and GFAP production, reserving

neurotrophic factors levels, as well as regulating mRNA expression of NOS, COX-2 and TH in striatum, which consequently retained neurotransmitters such as dopamine, DOPAC and HVA. These results suggest that caffeic acid is a potent neuro-protective agent against the development of Parkinson's disease.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Figure legend.

Fig. 1. mRNA expression of IL-1beta, TNF-alpha and IL-10 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. #*P*<0.05 vs. MPTP group.

Fig. 2. mRNA expression of iNOS, nNOS, COX-2 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. #*P*<0.05 vs. MPTP group.

Fig. 3. mRNA expression of GFAP and Iba-1 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. #*P*<0.05 vs. MPTP group.

Fig. 4. mRNA expression of DAT, BDNF, GDNF and TH in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. #*P*<0.05 vs. MPTP group.

Fig. 5. Protein level of TH, determined by western blot analysis, in striatum from mice. Bands from left to right are control, 2% caffeic acid, MPTP, 0.5% caffeic acid+MPTP, 1% caffeic acid+MPTP, 2% caffeic acid+MPTP, MPTP+0.5% caffeic acid, MPTP+1% caffeic acid, and MPTP+2% caffeic acid.

Table 1

Forward and reverse primers for real time PCR analysis.

Target	Forward	reverse
IL-1beta	5’-TCA TGG GAT GAT GAT GAT AAC CTG CT-3’	5’-CCC ATA CTT TAG GAA GAC ACG GAT-3’
TNF-alpha	5’-GGC AGG TCT ACT TTG GAG TCA TTG-3’	5’-ACA TTC GAG GCT CCA GTG AAT TCG-3’
IL-10	5'-GGC CCT TTG CTA TGG TGT CC-3’	5'- AAG CGG CTG GGG GAT GAC-3’
iNOS	5′-GAC GAG ACG GAT AGG CAG AG-3’	5′-CTT CAA GCA CCT CCA GGA AC-3’
nNOS	5′-TAT GTG GCA GAA GCT CCA GA-3’	5′-CGG CTG GAT TTA GGA CTT TG-3’
COX-2	5’-CCA GCA GGC TCA TAC TGA TAG GA-3’	5’-GCA GGT CTG GGT CGA ACT TG-3’
GDNF	5′-AAG GTC ACC AGA TAA ACA AGC GG-3′	5′-TCA CAG GAG CCG CTG CAATATC-3′
BDNF	5’-ATC CAA ATA TGG CAC AGC AA-3’	5’-TTC TGC CTG AGT TTT GAT GC-3’
DAT	5’-ATC AAC CCA CCG CAG ACA CCA GT-3’	5’-GGC ATC CCG GCA ATA ACC AT-3’
GFAP	5’ATT GCT GGA GGG CGA AGA A-3’	5’-CGG ATC TGG AGG TTG GAG AA-3’
Iba-1	5’-CTT GAA GCG AAT GCT GGA GAA-3’	5’-GGA GCC ACT GGA CAC CTC TCT-3’
TH	5’-CCC CAC CTG GAG TAC TTT GTG-3’	5’-CTT GTC CTC TCT GGC ACT GC-3’
GAPDH	5’-TGA TGA CAT CAA GAA GGT GGT GAA G-3’	5’-CCT TGG AGG CCA TGT AGG CCA T-3’

Table 2

Water intake (WI), feed intake (FI), body weight, brain weight and brain caffeic acid content of mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10.

	WI ml/mouse/d	FI g/mouse/d	Body weight g	Brain weight g	Caffeic acid mg/100 g wet tissue
Control	2.3 ± 0.4[a]	2.4 ± 0.5[a]	27.1 ±1.0[a]	0.48±0.05[a]	-*
caffeic acid, 2	2.5 ± 0.6[a]	2.1 ± 0.6[a]	28.3 ± 0.9[a]	0.45±0.08[a]	12.4±1.8[c]
MPTP	2.1 ± 0.5[a]	2.7 ± 0.7[a]	27.3 ± 1.1[a]	0.42±0.04[a]	-
caffeic acid, 0.5 + MPTP	2.0 ± 0.7[a]	3.0 ± 0.8[a]	29.0 ± 1.2[a]	0.50±0.07[a]	-
caffeic acid, 1 + MPTP	2.3 ± 0.4[a]	2.2 ± 0.6[a]	27.2 ± 0.8[a]	0.51±0.06[a]	1.7±0.8[a]
caffeic acid, 2 + MPTP	2.4 ± 0.5[a]	2.3 ± 0.9[a]	28.9 ± 1.3[a]	0.49±0.08[a]	4.1±1.0[b]
MPTP + caffeic acid, 0.5	2.2 ± 0.6[a]	2.5 ± 0.7[a]	28.5 ± 0.6[a]	0.46±0.05[a]	-
MPTP + caffeic acid, 1	2.4 ± 0.7[a]	2.1 ± 0.6[a]	27.8 ± 0.8[a]	0.50±0.06[a]	-
MPTP + caffeic acid, 2	2.1 ± 0.3[a]	2.6 ± 0.4[a]	27.7 ± 0.9[a]	0.44±0.06[a]	1.5±0.6[a]

*Means too low to be detected.

[a-c]Means in a column without a common letter differ, $P<0.05$.

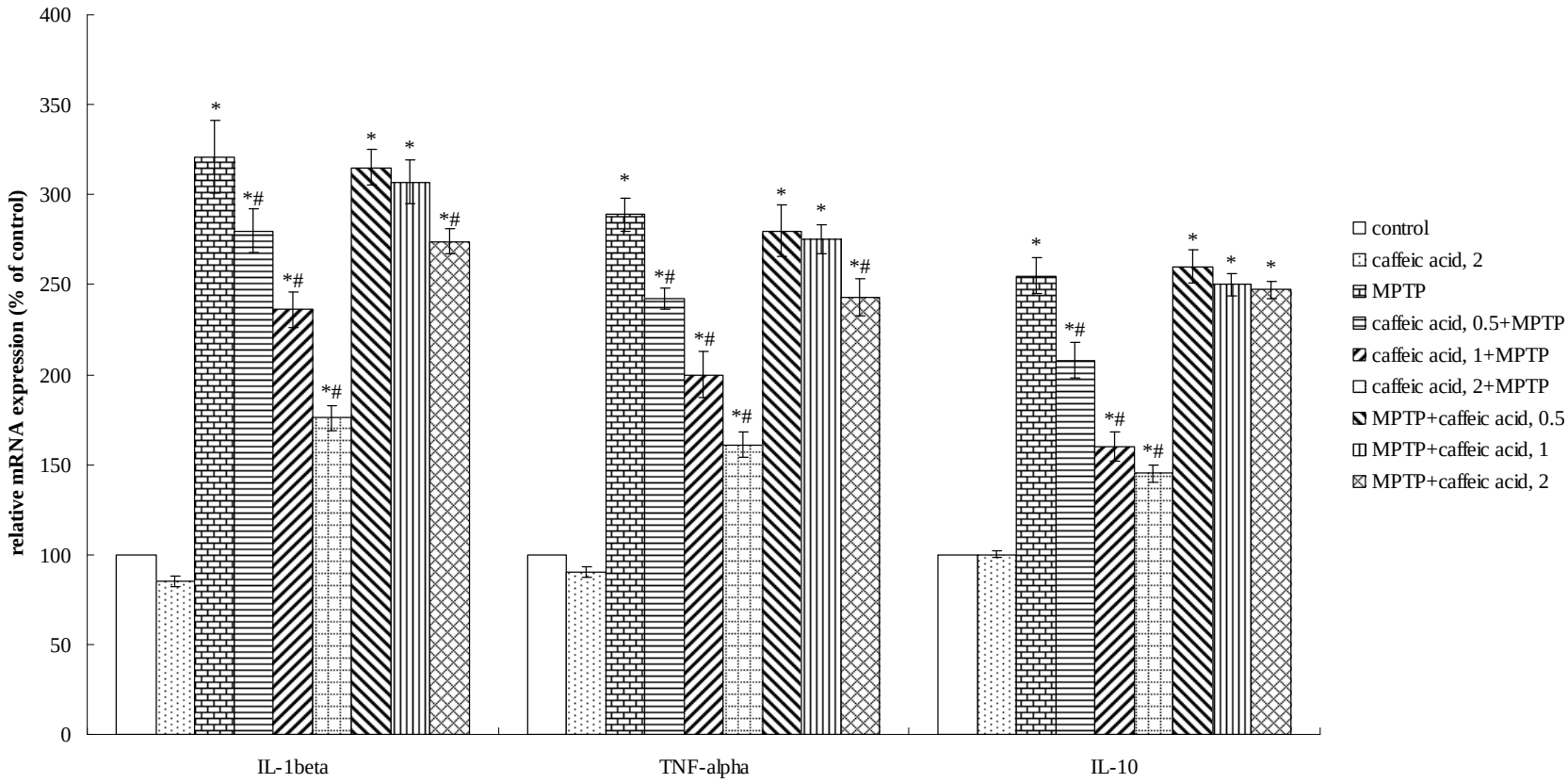
Table 3

Level (pg/ml) of IL-1beta, IL-6, TNF-alpha, IL-4 and IL-10 in the striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10.

	IL-1beta	IL-6	TNF-alpha	IL-4	IL-10
Control	19.3±2.4[a]	18.0±1.7[a]	15.8±1.0[a]	13.5±0.8[a]	15.3±1.0[a]
caffeic acid, 2	18.1±1.7[a]	17.4±1.3[a]	13.6±1.2[a]	14.0±0.6[a]	14.7±1.1[a]
MPTP	153.5±10.2[e]	134.4±12.1[d]	142.9±11.7[e]	110.5±6.1[c]	116.7±6.5[c]
caffeic acid, 0.5 + MPTP	90.4±5.0[c]	85.1±6.3[c]	90.3±7.3[c]	53.4±3.7[b]	68.7±4.3[b]
caffeic acid, 1 + MPTP	86.7±6.4[c]	60.7±4.2[b]	64.7±5.9[b]	50.1±3.1[b]	63.5±3.5[b]
caffeic acid, 2 + MPTP	64.0±3.3[b]	56.3±3.5[b]	61.2±4.5[b]	46.8±3.3[b]	60.1±4.0[b]
MPTP + caffeic acid, 0.5	149.5±9.0[e]	127.9±10.6[d]	139.8±9.5[e]	109.2±7.9[c]	113.8±8.8[c]
MPTP + caffeic acid, 1	141.8±8.2[e]	121.0±8.3[d]	133.4±7.5[e]	103.5±6.6[c]	101.4±5.7[c]
MPTP + caffeic acid, 2	110.9±6.1[d]	91.4±7.8[c]	108.1±8.0[d]	99.4±3.7[c]	102.1±4.2[c]

[a-e]Means in a column without a common letter differ, $P<0.05$.

Fig. 1. mRNA expression of IL-1beta, TNF-alpha and IL-10 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. $^{\#}$*P*<0.05 vs. MPTP group.


relative mRNA expression (% of control)
400
350
300
250
200
150
100
50
0
IL-1beta
TNF-alpha
IL-10
control
caffeic acid, 2
MPTP
caffeic acid, 0.5+MPTP
caffeic acid, 1+MPTP
caffeic acid, 2+MPTP
MPTP+caffeic acid, 0.5
MPTP+caffeic acid, 1
MPTP+caffeic acid, 2

1

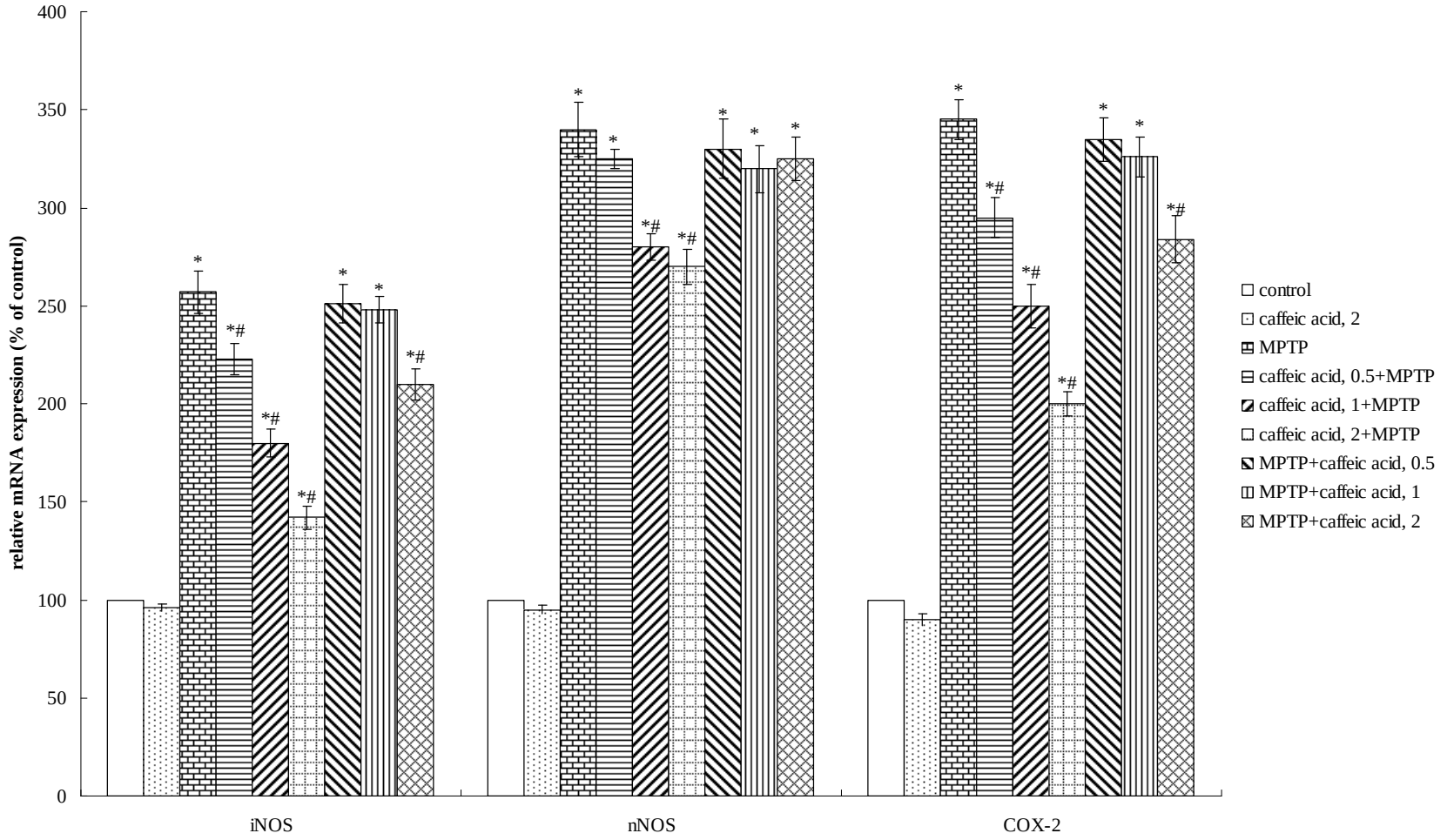
Table 4

Level of nitrite and PGE_2, activity of total NOS and COX-2 in the striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10.

	Nitrite μM/mg protein	PGE_2 pg/g protein	Total NOS pmol/min/mg protein	COX-2 U/mg protein
Control	9.8±1.0[a]	1120±104[a]	18.4±0.8[a]	0.28±0.07[a]
caffeic acid, 2	10.1±1.2[a]	1075±93[a]	19.0±1.0[a]	0.31±0.05[a]
MPTP	37.5±2.3[e]	2514±178[e]	54.7±4.6[e]	2.57±0.14[e]
caffeic acid, 0.5 + MPTP	29.1±1.5[d]	2170±191[d]	47.2±3.7[d]	2.20±0.09[d]
caffeic acid, 1 + MPTP	24.3±1.4[c]	1731±128[c]	40.5±2.2[c]	1.80±0.11[c]
caffeic acid, 2 + MPTP	18.6±1.8[b]	1403±115[b]	28.1±1.8[b]	1.32±0.07[b]
MPTP + caffeic acid, 0.5	35.9±2.5[e]	2490±165[e]	54.0±5.0[e]	2.60±0.15[e]
MPTP + caffeic acid, 1	34.6±1.9[e]	2342±133[e]	53.8±4.2[e]	2.51±0.12[e]
MPTP + caffeic acid, 2	26.0±0.8[c]	2081±98[d]	46.3±2.9[d]	2.13±0.09[d]

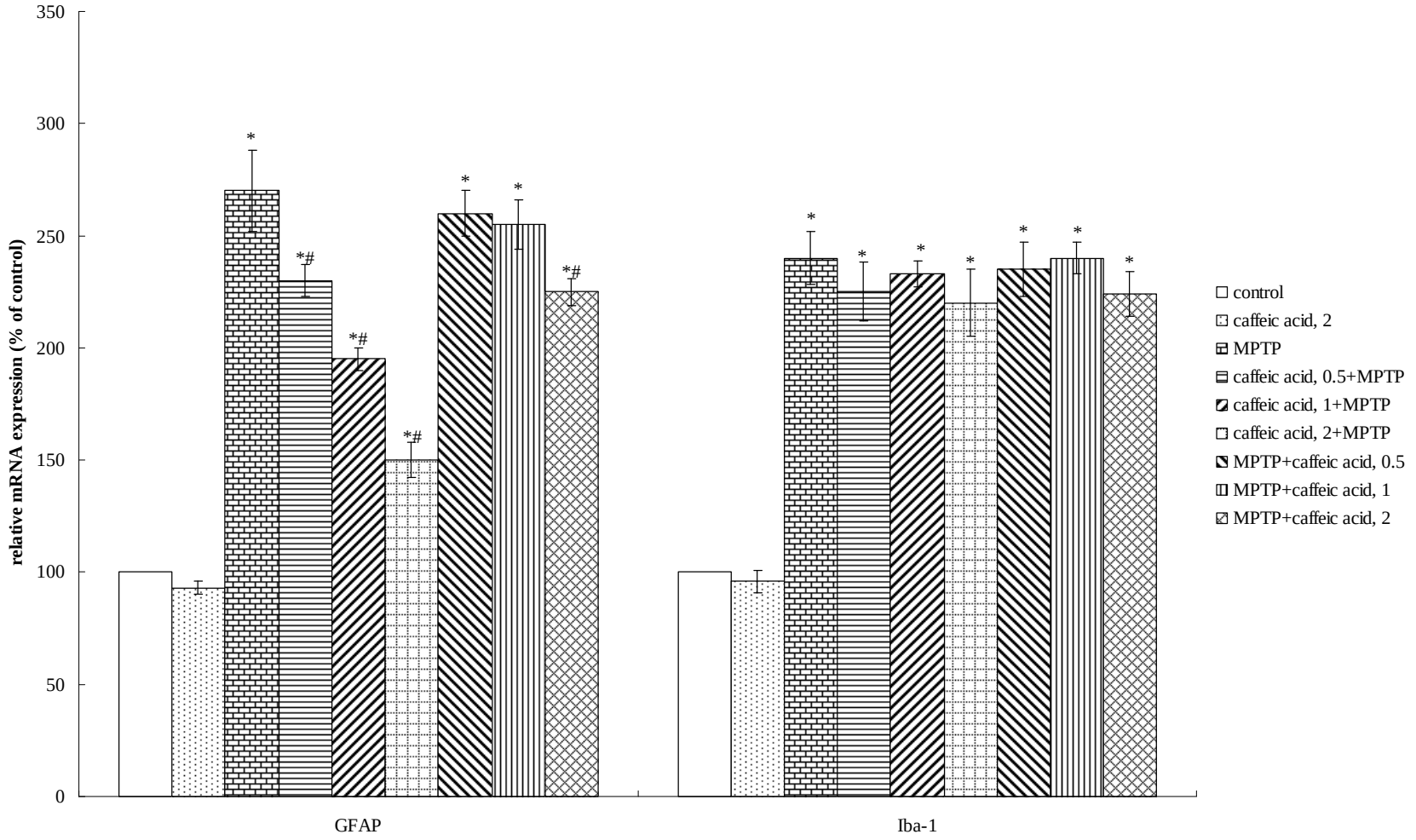
[a-e]Means in a column without a common letter differ, $P<0.05$.

Fig. 2. mRNA expression of iNOS, nNOS, COX-2 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. $^{\#}$*P*<0.05 vs. MPTP group.


relative mRNA expression (% of control)
400
350
300
250
200
150
100
50
0
iNOS
nNOS
COX-2
control
caffeic acid, 2
MPTP
caffeic acid, 0.5+MPTP
caffeic acid, 1+MPTP
caffeic acid, 2+MPTP
MPTP+caffeic acid, 0.5
MPTP+caffeic acid, 1
MPTP+caffeic acid, 2

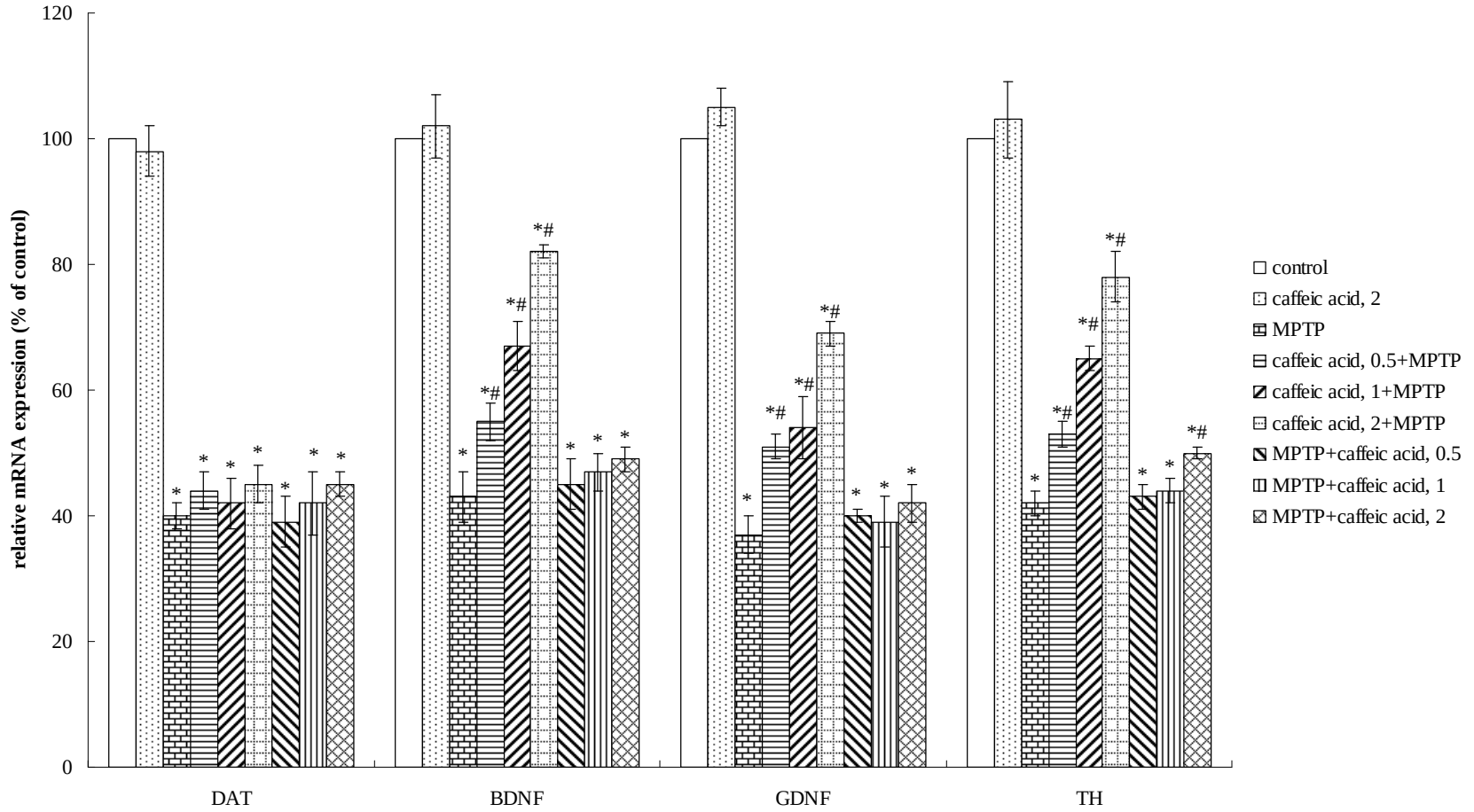
1

Fig. 3. mRNA expression of GFAP and Iba-1 in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. $^{\#}$*P*<0.05 vs. MPTP group.


350
300
250
200
150
100
50
0
relative mRNA expression (% of control)
GFAP
Iba-1
control
caffeic acid, 2
MPTP
caffeic acid, 0.5+MPTP
caffeic acid, 1+MPTP
caffeic acid, 2+MPTP
MPTP+caffeic acid, 0.5
MPTP+caffeic acid, 1
MPTP+caffeic acid, 2

1

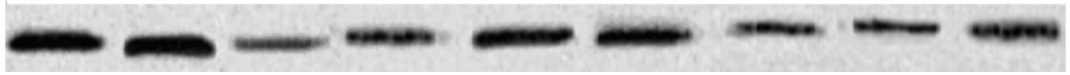
Fig. 4. mRNA expression of DAT, BDNF, GDNF and TH in striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10. **P*<0.05 vs. control group. $^{\#}$*P*<0.05 vs. MPTP group.


relative mRNA expression (% of control)
120
100
80
60
40
20
0
DAT
BDNF
GDNF
TH
control
caffeic acid, 2
MPTP
caffeic acid, 0.5+MPTP
caffeic acid, 1+MPTP
caffeic acid, 2+MPTP
MPTP+caffeic acid, 0.5
MPTP+caffeic acid, 1
MPTP+caffeic acid, 2

1

Fig. 5. Protein level of TH, determined by western blot analysis, in striatum from mice. Bands from left to right are control, 2% caffeic acid, MPTP, 0.5% caffeic acid+MPTP, 1% caffeic acid+MPTP, 2% caffeic acid+MPTP, MPTP+0.5% caffeic acid, MPTP+1% caffeic acid, and MPTP+2% caffeic acid.

TH



GAPDH

Table 5

Content of BDNF and GDNF, and TH activity in the striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10.

	BDNF pg/mg protein	GDNF pg/mg protein	TH nmol/mg protein
Control	28±3[d]	65±4[d]	1.83±0.25[e]
caffeic acid, 2	32±4[d]	71±6[d]	1.94±0.18[e]
MPTP	8±2[a]	20±3[a]	0.35±0.09[a]
caffeic acid, 0.5 + MPTP	15±3[b]	29±5[b]	0.79±0.12[b]
caffeic acid, 1 + MPTP	16±2[b]	34±4[b]	1.06±0.14[c]
caffeic acid, 2 + MPTP	21±3[c]	42±3[c]	1.42±0.08[d]
MPTP + caffeic acid, 0.5	7±1[a]	19±2[a]	0.37±0.05[a]
MPTP + caffeic acid, 1	10±2[a]	21±2[a]	0.41±0.07[a]
MPTP + caffeic acid, 2	10±3[a]	22±4[a]	0.70±0.10[b]

[a-e]Means in a column without a common letter differ, $P<0.05$.

Table 6

Content (ng/mg protein) of dopamine, DOPAC and HVA in the striatum from mice treated with 2% caffeic acid alone, MPTP alone, caffeic acid followed by MPTP, or MPTP followed by caffeic acid. Values are mean ± S.D., n=10.

	dopamine	DOPAC	HVA
control	9.60±1.25[e]	0.84±0.07[d]	0.92±0.10[d]
caffeic acid, 2	9.53±1.02[e]	0.87±0.05[d]	0.95±0.12[d]
MPTP	1.37±0.07[a]	0.42±0.06[a]	0.40±0.04[a]
caffeic acid, 0.5 + MPTP	2.25±0.13[b]	0.46±0.03[a]	0.43±0.04[a]
caffeic acid, 1 + MPTP	4.08±0.34[c]	0.67±0.06[c]	0.61±0.07[b]
caffeic acid, 2 + MPTP	6.32±0.70[d]	0.70±0.08[c]	0.75±0.06[c]
MPTP + caffeic acid, 0.5	1.47±0.11[a]	0.43±0.05[a]	0.42±0.03[a]
MPTP + caffeic acid, 1	1.55±0.15[a]	0.45±0.06[a]	0.45±0.07[a]
MPTP + caffeic acid, 2	2.10±0.24[b]	0.57±0.08[b]	0.59±0.09[b]

[a-e]Means in a column without a common letter differ, $P<0.05$.

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