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Upregulation of guanylyl cyclase expression and activity in striatum of MPTP-induced parkinsonism in mice

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Abstract

The aim of our study was to investigate the expression and the activity of soluble guanylyl cyclase (GC) and phosphodiesterase (PDE) activities that regulate cGMP level in the striatum, hippocampus, and brain cortex in an animal model of PD, induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We observed the increase of total activity and protein level of GC in striatum after MPTP injection. It was accompanied by an enhancement of both mRNA expression and protein level of GCβ1 subunit. MPTP induces mRNA expression and elevates protein concentration of GCβ1 in striatum up to 14 days after its injection, which in turn causes a marked enhancement of cGMP formation. Furthermore, the activation of GC occurs through change of maximal enzyme activity (V_{max}). Simultaneously, no change in PDE activity has been detected in all investigated regions of the brain after MPTP. MPTP injection caused the elevation of GCβ1 protein level in both the membrane and cytosol fractions being significantly higher in cytosol. Western blot analysis demonstrated about 45–67% decrease of tyrosine hydroxylase protein content in striatum. These data suggest that NO/cGMP signaling pathway may at least partially contribute to dopaminergic fiber degeneration in the striatum, the damage attributed to PD.

Keywords: Guanylyl cyclase; cGMP; MPTP; Parkinson's disease; Phosphodiesterase; Tyrosine hydroxylase

Parkinson's disease (PD) is attributed to a profound reduction of dopamine concentration in the striatum due to a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1–3].

Recent studies suggest that free radicals may play a role in the pathogenesis of PD and nitric oxide (NO) may be one of the major reactive radicals involved in MPTP-induced toxicity [4–7]. NO is synthesized by three isoforms of nitric oxide synthase (NOS): constitutive neuronal (nNOS, NOS-1), endothelial (eNOS, NOS-3), and inducible (iNOS, NOS-2). Neuronal and inducible NOS expressions and activities are elevated in PD, leading to abnormalities and death of dopaminergic neurons in SNpc [5,8,9]. In agreement with above-mentioned changes are data which indicated that nNOS inhibitors, e.g., 7-nitroindazole (7-NI) and *S*-methylthiocitrulline or iNOS inhibitor (*S*-methylisothiourea), protected mice and baboons from MPTP-induced nigral cell loss [10–13]. A similar effect has been observed on mice that lack the gene for nNOS or iNOS, however, in nNOS-deficient and iNOS-deficient mice protection against MPTP neurotoxicity was only partial [5,8]. Taken together, animal experiments have generally documented an important role of NO in PD but the mechanism of its influence still remains unknown.

More details in favor of regulating role of NOS isoforms in PD pathogenesis have been achieved experimentally by using their inhibitors. In mice, inhibition of nNOS and iNOS activities attenuates MPTP-induced

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decrease in striatal dopamine levels [5,7,8,14,15]. Recognized as selective nNOS blocker 7-NI inhibits MPTP-induced 3-nitrotyrosine formation [16] and/or acts via inhibition of monoamine oxidase B [17] whereas iNOS, *S*-methylisothiourea, leads to reduction of liposaccharide-induced cell death in substantia nigra [12]. On the contrary, under conditions of oxidative stress, NO reacts with superoxide anion (O^{2-}) to form peroxynitrite ($ONOO^{-}$), which in turn leads to the production of highly toxic hydroxyl radicals [18,19].

Recently the evidence was obtained, suggesting that the meaningful mechanism through which NO acts in the central nervous system (CNS) is an activation of soluble guanylyl cyclase (sGC) [20–22]. NO binds to the prosthetic heme group of the sGC which in turn leads to about 200-fold increase in sGC activity [23,24]. This enzyme catalyzes the conversion of GTP to cGMP. The increase of second messenger, cGMP, modulates the activity of cGMP-dependent protein kinase (PKG), phosphodiesterase (PDE), and ion channels, well-known processes which lead to alterations in cellular function [25,26].

NO-sensitive GC is a heterodimeric enzyme composed of four subunits (α1, α2, β1, and β2) that have been identified so far [27]. Coexpression of the α1, α2 subunits with the β1 subunit results in a catalytically active GC. In contrast to β1 subunit, the β2 subunit does not express a catalytically active enzyme with any other subunits [28–30]. Both α subunits share a highly conserved C-terminal catalytic part of enzyme whereas the regulatory N-terminal domains differ considerably with only 30% identical amino acids. The N-terminal domains of the α1 and β1 subunits have been required for proper coordination of the prosthetic heme group [31,32]. The differences in primary structure, investigated by extensive analysis of purified isoforms α1β1 and α2β1, did not reveal any regulatory or kinetic property differences [27]. The α1β1 subunit is found in the cytosolic fraction, whereas the α2β1 is bound with PDZ domains and is recruited to the membrane fraction [33]. The highest amounts of α1β1 and α2β1 isoforms have been predominantly found in equal amounts in striatum, brain cortex, hippocampus, and cerebellum [34–36].

In comparison to other parts of the brain, the striatum contains the highest activity and expression of GC and cGMP levels [37]. Recent studies suggest that striatal NO/cGMP pathway is involved in synaptic plasticity, neurotransmitter release, and excitation of cholinergic interneurones [38–40]. There is controversy about the role of cGMP in neurodegeneration neurons and cell death. Cyclic GMP is involved in glutathione depletion [41] and apoptosis [42]. Changes in sGC gene expression have not yet been demonstrated in response to PD.

Our results indicate that both the expression and the activity of GC were elevated in the striatum at 3, 7, and 14 days after MPTP injection. In addition, these changes were accompanied by an increased cGMP production. However, no alteration in PDE activity was found. Thus, our results suggest that sGC/cGMP signaling pathway may be involved in the regulation of PD process.

Materials and methods

Materials. Rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody was from Biocom International (USA), anti-GCαβ antibody was from Alexis Biochemicals (Germany), and anti-GCβ1 antibody and anti-rabbit IgG conjugated with horseradish peroxidase (AR-HRP) were purchased from Sigma (St. Louis, MO, USA). Nitrocellulose membrane was obtained from Bio-Rad (Vienna, Austria). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), TRI-reagent, 1-isobuthyl-1-methylxantine (IBMX), and all other reagents were purchased from Sigma (St. Louis, MO, USA). RT kit was obtained from Promega (Madison, WI, USA) and Master Mix was obtained from Quigen (Hilden, Germany). Cyclic GMP kit and ECL kit were obtained from Amersham–Pharmacia Biotech, (Piscataway, NJ, USA). Protease inhibitors were from Boehringer–Mannheim (Mannheim, Germany).

Animals and treatment with MPTP. Eight-week-old C57/BL mice (20–25 g) were used from Animals Farm, Lomna, Poland. All experimental procedures were approved by a I Local Ethic Committee for Animals Experiments, Nencki Institute, Warsaw, Poland.

Mice were housed in a temperature-controlled room under a 12-h light/12 dark cycle with free access to food and water. On the day of experiment, mice C57/BL received three injections of MPTP in saline at 2 h intervals at a total dose of 40 mg/kg. Control mice obtained only saline. Mice were killed after 3, 7, and 14 days. Different parts of the brain: striatum, hippocampus, cerebellum, and brain cortex were quickly isolated on ice-cold glass Petri dish. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analyses. Cyclic GMP, mRNA, protein level and activity of GC, TH protein level, and PDE activity were determined in striatum, brain cortex, and hippocampus at 3, 7, and 14 days after MPTP injection.

Preparation of sub-cellular fraction. The striatum was homogenized in 10 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, and protease inhibitors (one tablet/10 ml) in a Dounce homogenizer by 14 strokes. Then the homogenate was centrifuged for 10 min at 10,000*g* to obtain membrane fraction and the supernatant, crude cytosolic fraction. The protein content of homogenate and each sub-cellular fraction were determined by the method of Lowry et al. [43].

Determination of cGMP level. Cyclic GMP level was determined using a commercially available ELISA kit (Amersham–Pharmacia Biotech, Piscataway, NJ) according to the procedure supplied by manufacturer. This method allows measuring cGMP in the range of 2–512 pmol/well, and it is based on competition between cGMP present in the sample with peroxidase-labeled cGMP for binding to specific anti-cGMP antibody. Briefly, different parts of the brain were homogenized with 20% TCA and centrifuged at 2200*g* for 10 min. The supernatant was washed four times with water–saturated diethyl ether and neutralized with 1 N NaOH to pH 7.4 before determination of cGMP. Then samples were acetylated. Acetylated samples were incubated with anti-cGMP antibody for 2 h at 4 °C and with cGMP conjugated to horseradish peroxidase for 60 min at 4 °C. 3,3′5′5′-Tetramethylbenzidine was used as a substrate for horseradish peroxidase. To quantify the cGMP level, a standard curve was performed using a standard solution of cGMP.

Determination of guanylyl cyclase activity. Guanylyl cyclase activity was assayed as described by Bonkale et al. [44]. The striatum, hippocampus, and brain cortex were homogenized in ice-cold 10 mM Tris–HCl buffer, pH 7.4, with a glass-Teflon homogenizer. For each group, enzyme activities were determined in the presence of 1 mM sodium

nitroprusside (SNP) used to generate NO. The reaction medium contained 4 mM of 1-isobuthyl-1-methylxantine (IBMX), 1 mM SNP, and various concentrations of $MgCl_2$-GTP (0.25–4 mM). The reaction was performed in a final volume of 300 μl, and was initiated by addition of 300 μg protein, and incubated for 15 min at 37 °C. Then it was terminated with 200 μl of 20% trichloroacetic acid (TCA). Samples were mixed vigorously and centrifuged at 2200*g* for 10 min at 4 °C. The supernatant was washed four times with water–saturated diethyl ether and then neutralized with 1 M NaOH to pH 7.4 before determination of cGMP. Fifty microliter aliquots of the supernatant were assayed for cGMP content using a commercial cGMP enzyme immunoassay kit (Amersham, TNP 226). Values for the maximal enzyme activity (V_{max}) and affinity (K_m) were determined from Hanes plot as (S) versus [S/V] of data that were fitted by linear regression.

Determination of PDE activity. The homogenate (300 μg protein) was incubated with 4 mM non-specific inhibitor of cGMP-dependent PDE, IBMX, for 15 min at 37 °C in 10 mM Tris–HCl buffer, pH 7.4, containing 2 mM Ca^{2+}. It was terminated with 200 μl of 20% TCA. Samples were mixed vigorously and then centrifuged at 2200*g* for 10 min at 4 °C. The supernatant was washed four times with water–saturated diethyl ether and then neutralized with 1 M NaOH to pH 7.4 before determination of cGMP.

Gel electrophoresis and Western blotting for GCβ1 subunit and tyrosine hydroxylase (TH). The homogenate or subcellular fraction (40 μg protein) was mixed with an equal volume of sample buffer (62.5 mM Tris–HCl, 2% SDS, 100 mM DTT, 0.2 mM 2-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue, pH 6.8 [45]). The samples were heated for 5 min at 95 °C. The proteins were analyzed by electrophoresis on 10% polyacrylamide gel [45]. Then proteins were electrophoretically transferred from the SDS–polyacrylamide gel to nitrocellulose membranes (Bio-Rad). The membrane was blocked in 5% milk powder (non-fat dried milk) in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at 37 °C. Then the blot was incubated with rabbit anti-GCβ1 (Sigma, USA) or rabbit anti-GCαβ (Alexis, Germany) (diluted 1:10000 in TBS-T containing 5% (w/v) non-fat milk) or rabbit anti-TH antibody (Biocom, USA) (diluted 1:1000 in PBS-T containing 2% non-fat milk) overnight at 4 °C temperature. According to the manufacturer, applied antibody (anti-GCαβ, Alexis, Germany) recognizes predominantly the β1 subunit of human, bovine, and rat soluble guanylate cyclase. The GCβ1 antibody complex was identified with goat-anti rabbit IgG conjugated with horseradish peroxidase (AR-HRP, diluted 1:8000 in TBS-T or PBS-T containing 5% (w/v) non-fat milk), and incubated for 1 h at RT. The GCβ1-AR-HRP and TH-AR-HRP were visualized with ECL kit (Amersham–Pharmacia Biotech, Piscataway, NJ, USA) and Hyperfilm-Kodak (Sigma, MO, USA). The optical densities of the sGCβ1 and TH bands on the immunoblot were quantified using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Determination of GCβ1 subunit mRNA-RT-PCR method. RNA isolation was performed using Sigma TRI-reagent according to manufacturer's manual. Reverse transcription of 5 μg of total RNA was performed in a final volume of 20 μl using 1.500 U AMV reverse transcriptase, 0.5 μg oligo$(dT)_5$ as a primer, and 1 mM each dNTP in one cycle: 42 °C for 1 h and 99 °C for 5 min with subsequent cooling to 4 °C. Polymerase chain reaction of 5 μl (cDNA) RT product was carried out according to the manufacturer's manual in a total volume of 50 μl using *Taq* PCR Master Mix and 20 pmol of each primer (GCβ1 sense: 5′-GCC GTA CTC TTG CCT GGA AG-3′; antisense: 5′-GAC CAT AAT TGC GGA TCA TCA CCA-3′. GAPDH sense: 5′-TG AAGGTCGGAGTCAACGGATTTGGT-3′; antisense: 5′-CATG TGGGCCATGAGGTCCACCAC-3′ in 30 cycles: 1 min 94 °C, 2 min 60 °C, and 3 min 72 °C, with pre-denaturation 94 °C for 5 min. Then the samples were cooled to 4 °C. Fifteen microliters of PCR product was loaded onto one lane with 3 μl of sample buffer and electrophoresed at 100 V through 2% agarose gel containing 200 μg/l ethidium bromide. The quantity of GAPDH mRNA (positive control) and GCβ1 mRNA was estimated by densitometric analysis of the gel in UV light using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Statistic analysis. The results are expressed as means ± SEM. Differences among means were analyzed using one-way or two-way ANOVA. When two-way ANOVA was appropriate, the different fraction and days after MPTP treatment were used as independent factors. When ANOVA showed significant differences, for each end point, the group means were compared using Newman–Keuls post hoc test. In all, null hypothesis was rejected at the 0.05 level.

The dependence between cGMP concentration and TH protein content was calculated using Pearson's linear correlation test. All statistical analyses were performed by using STATISTIC version 4.0. (Sofware, Kraków, Poland).

Results

Total GC protein level was determined in striatum after MPTP injection using rabbit polyclonal anti-GCαβ antibody. Western blot analysis has shown that MPTP induced the increased GC protein level at 3, 7, and 14 days after treatment. GC protein level increased about

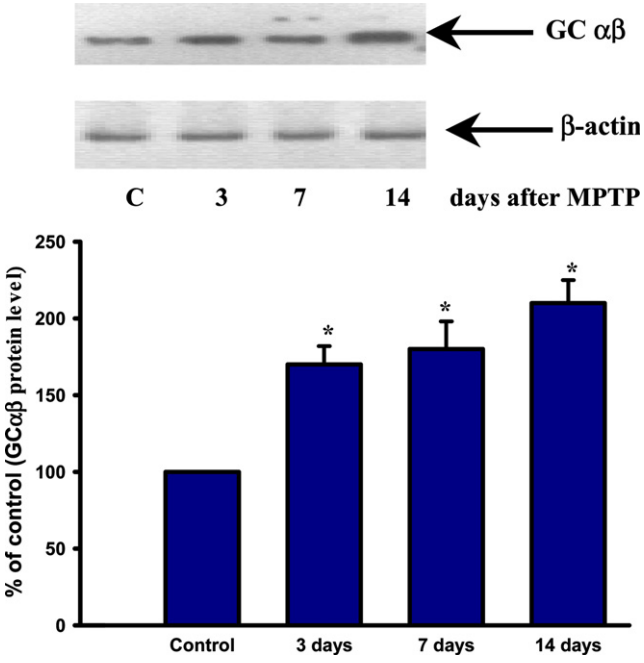

Fig. 1. Western blot for the detection of GC protein level in striatum after MPTP injection. Forty micrograms of protein was subjected to 10% polyacrylamide gel SDS–PAGE and analyzed for GC protein by immunoblotting using rabbit polyclonal anti-GCαβ antibody (1:1000 diluted in T-TBS containing 5% milk). GC antibody complex was identified with the goat anti-rabbit IgG horseradish peroxidase conjugate (AR-HP) and visualized by using ECL kit (Amersham, UK). Results represent a typical immunoblot from three separate experiments. Western blot for GC protein is shown at the top and actin protein at the bottom: lane C—control, 3, 7, and 14 days after injection of MPTP. Analysis of densitometry: results are plotted as means ± SEM from four separate experiments and expressed as percentage relative to control value. The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. *$p < 0.01$ versus the control values.

Table 1
Kinetic parameters of guanylyl cyclase activity in different parts of brain control mice and after MPTP treatment

	V_{max} (pmol/mg protein/min)	K_m (mM GTP-$MgCl_2$)
Striatum		
Control	178 ± 12	0.999 ± 0.003
3 days	$435 \pm 10^*$	0.990 ± 0.005
7 days	$555 \pm 15^*$	0.985 ± 0.002
14 days	$667 \pm 11^*$	0.993 ± 0.001
Hippocampus		
Control	56 ± 5	1.005 ± 0.002
3 days	57 ± 7	0.949 ± 0.001
7 days	49 ± 5	1.001 ± 0.004
14 days	56 ± 4	1.004 ± 0.003
Brain cortex		
Control	38 ± 5	1.003 ± 0.005
3 days	38 ± 3	1.006 ± 0.003
7 days	34 ± 4	1.004 ± 0.001
14 days	33 ± 7	0.999 ± 0.003

Values for the maximal enzyme activity (V_{max}) and affinity (K_m) were determined from Hanes plot as (S) versus [S/V] of data that were fitted by linear regression. Results are expressed as means ± SEM of data from four separate experiments performed in duplicate. The statistical analysis performed by one-way ANOVA, using the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant.

* $p < 0.05$ versus the control value.

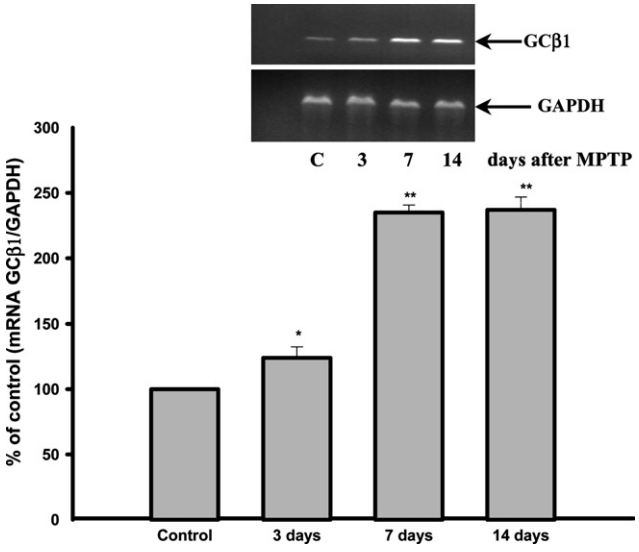

Fig. 2. RT-PCR analysis of GCβ1 mRNA after MPTP injection in striatum. RT-PCR was carried out as described in Materials and methods. RT-PCR products were electrophoresed on 2% agarose gel. The quantitative assessment of GCβ1 and GAPDH mRNA expression was done by using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech. GCβ1 mRNA PCR products were normalized by the intensity of GAPDH expression. A typical RT-PCR products is representative of four separate experiments. Results are plotted as means ± SEM from four separate experiments and expressed as percentage relative to control value. The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. *$p < 0.001$ and **$p < 0.0001$ versus the control values.

1.7–2.0 times up to 14 days after MPTP injection in comparison to control value (Fig. 1).

The increase of GC protein level after MPTP treatment in striatum was accompanied by an enhancement of enzyme activity. Non-treated and MPTP treated mice expressed a higher activity of guanylyl cyclase in striatum than in other parts of the brain. Two kinetics parameters, i.e., maximal enzyme activity (V_{max}) and affinity for substrate (K_m), were determined in different parts of the brain in control and in animals injected with MPTP (Table 1). The maximal enzyme activity (V_{max}) increased significantly at 3, 7, 14 days after MPTP injection in striatum when the results were compared to values detected in control (non-treated) mice (Table 1). The V_{max} values changed from control mice (178 ± 12 pmol/mg protein/min) to 435 ± 10–667 ± 11 pmol/mg protein/min in striatum, taken from MPTP treated mice. The enzyme's affinity for substrate (K_m) did not alter after MPTP injection in the striatum. MPTP did not change V_{max} and K_m in other parts of the brain studied, i.e., in hippocampus and brain cortex (Table 1).

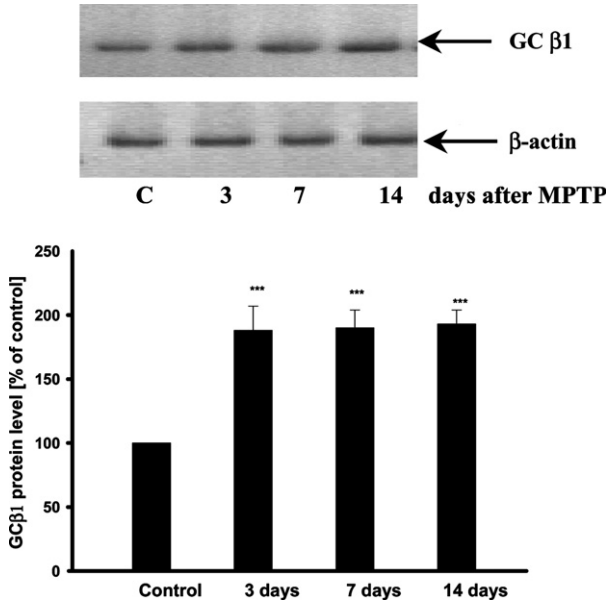

Fig. 3. Western blot analysis for GCβ1 subunit following MPTP treatment in striatum. Forty micrograms of protein was subjected to 10% polyacrylamide gel SDS–PAGE and analyzed for GCβ1 by immunoblotting using polyclonal rabbit anti-GCβ1 antibody (1:1000 diluted in T-TBS containing 5% milk). GC antibody complex was identified with the anti-rabbit IgG horseradish peroxidase conjugate (AR-HP) and visualized by using ECL kit (Amersham, UK). Results represent a typical immunoblot from four separate experiments. Western blot: lane, MW—molecular marker of protein (Amersham, UK) C—control, 3, 7, and 14 days after injection MPTP. Analysis of densitometry: results are plotted as means ± SEM from four separate experiments and expressed as percentage relative to control value. The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. ***$p < 0.0001$ versus the control values.

The expression of mRNA and the protein level of GCβ1 subunit, which is responsible for the activity expressed by enzyme, were measured in the present study. mRNA GCβ1 was normalized by the intensity of GAPDH (housekeeping gene) expression. RT-PCR analysis indicates that MPTP treatment induced the increase of mRNA GCβ1 at 3, 7, and 14 days after injection in the striatum (Fig. 2). mRNA GCβ1 was elevated at all investigated days after MPTP administration. Moreover, Western blot analysis using anti-GCβ1 antibody shows that MPTP injection caused about a 200% increase of the GCβ1 protein level in striatum at 3, 7, and 14 days after its administration in comparison to control samples (Figs. 3A and B).

GCβ1 subunit was found in cytosol and membrane fractions of the striatum (Fig. 4). Densitometric analysis demonstrated the same amount of GCβ1 protein level in cytosolic and membrane fractions of the striatum in control group of animals. MPTP enhanced the protein level of GCβ1 subunit at 3, 7, and 14 days after injection in both subcellular fractions compared to appropriate control. We observed a significantly higher GCβ1 subunit protein level in cytosolic fraction than in membrane fraction (Figs. 4A and B).

An increase of CG V_{max} value produced an increase of cGMP concentration (Fig. 5). MPTP induced 1.74-, 1.9-, and 1.75-fold increase of cGMP level in striatum at 3, 7, and 14 days after injection (Fig. 5). Basal level of cGMP was 10 times higher in striatum in comparison to other parts of the brain: hippocampus, brain cortex, midbrain, and cerebellum (data not shown). Basal level of cGMP in striatum was 232 ± 10 pmol/mg protein. MPTP did not alter cGMP concentration in all other investigated parts of the brain (data not shown).

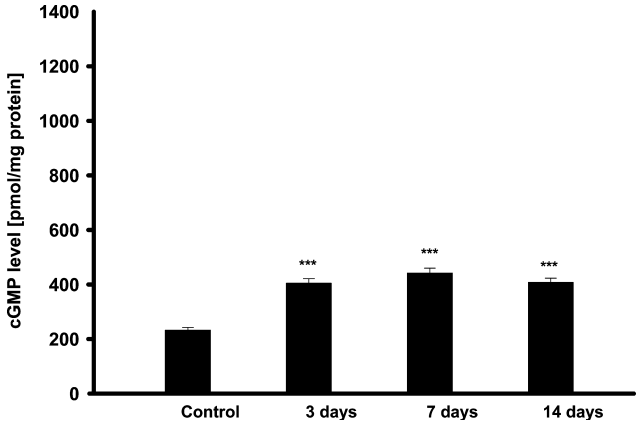

Fig. 5. Basal level of cGMP in striatum with MPTP-induced parkinsonism. The tissue was homogenized in 20% TCA, then cGMP level was determined using immunoassay ELISA kit as described in Materials and methods. Results are expressed as means ± SEM of data from five separate experiments performed in triplicate. The statistical analysis performed by one-way ANOVA followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. ***$p < 0.001$ versus the control value.

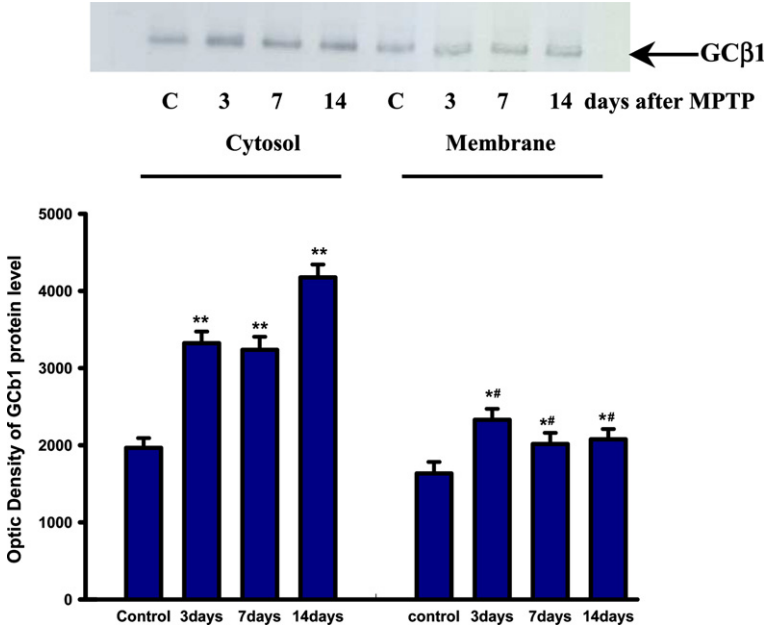

Fig. 4. Western blot for the detection of GCβ1 subunit protein level following MPTP treatment in cytosol and membrane fractions of striatum. Cytosol and membrane fractions (40 μg of protein) were subjected to 10% polyacrylamide gel SDS–PAGE and analyzed for GCβ1 by immunoblotting using polyclonal rabbit anti-GCβ1 antibody (1:1000 diluted in T-TBS containing 5% milk). GC antibody complex was identified with the anti-rabbit IgG horseradish peroxidase conjugate (AR-HP) and visualized by using ECL kit (Amersham, UK). Results represent a typical immunoblot from three experiments. Analysis of densitometry is plotted as means ± SEM from four separate experiments and expressed as optic densitometry units. The statistical analysis performed by two-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. *$p < 0.001$ and **$p < 0.0001$ versus the appropriate control values, $^{\#}p < 0.01$ versus the appropriate groups of cytosol fraction.

Cyclic GMP level was elevated about 0.5-fold after 15 min of incubation at 37 °C in striatum, hippocampus, and brain cortex of non-treated, treated with MPTP mice, compared to basal level of cGMP (data not shown). Non-specific inhibitor of PDE, IBMX, enhanced 2-fold cGMP concentration in striatum compared to the value without IBMX (Fig. 6). MPTP did not change PDE activity in striatum as compared to the basal cGMP level (Fig. 7). There was no difference in PDE activity in other parts of the brain (data not shown).

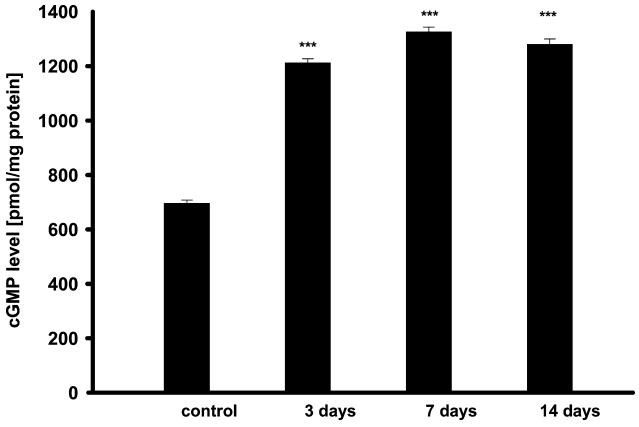

Fig. 6. Effect of PDE inhibitor, 1-isobuthyl-1-methylxantine (IBMX), on cGMP concentration in striatum after MPTP injection. Homogenate was incubated with 4 mM IBMX for 15 min at 37 °C. Then the reaction was stopped by 200 μl of 20% TCA and cGMP level was determined using immunoassay ELISA kit as described in Materials and methods. Results are expressed as means ± SEM of data from five separate experiments performed in triplicate. The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. ***$p < 0.001$ versus the control value.

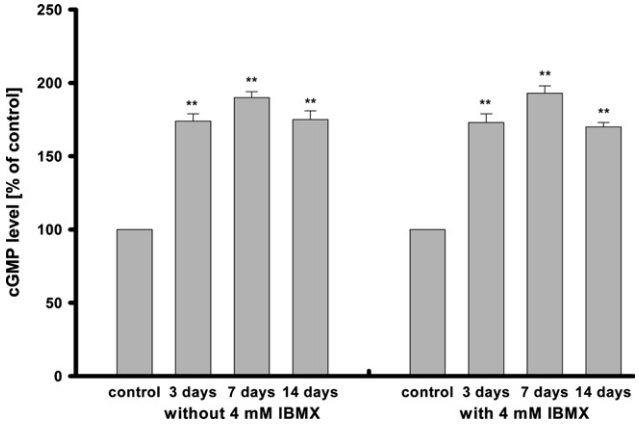

Fig. 7. Phosphodiesterase activity in striatum with MPTP-induced parkinsonism. Homogenate was incubated without and with 4 mM IBMX for 15 min at 37 °C. Then the reactions were stopped by 200 μl of 20% TCA and cGMP level was determined using immunoassay ELISA kit as described in Materials and methods. Cyclic GMP concentrations are 348 ± 9 and 695 ± 13 pmol/mg protein in control group without and with IBMX, respectively. Data are expressed as means ± SEM from five separate experiments performed in triplicate and expressed as percentage of appropriate control (without IBMX, with IBMX), The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. **$p < 0.01$ versus the control value.

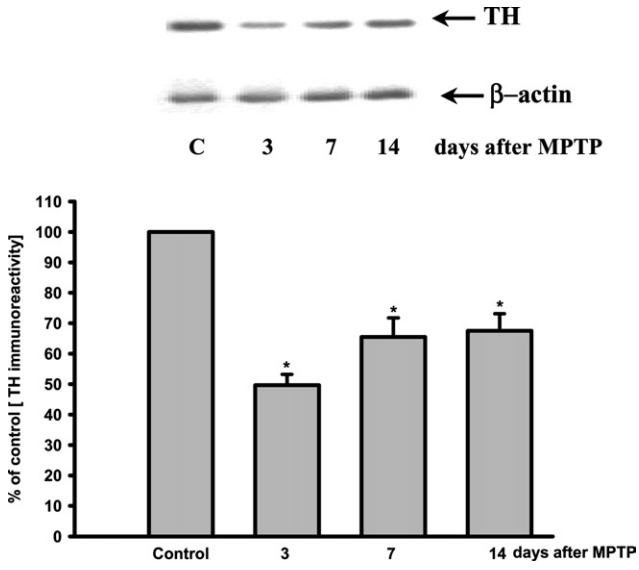

Fig. 8. Western blot analysis of tyrosine hydroxylase (TH) protein level in striatum with MPTP-induced parkinsonism. Forty micrograms of protein was subjected to 10% polyacrylamide gel SDS–PAGE and analyzed for TH by immunoblotting using polyclonal rabbit anti-TH antibody (1:1000 diluted in T-TBS containing 5% milk). GC antibody complex was identified with the anti-rabbit IgG horseradish peroxidase conjugate (AR-HP) and visualized by using ECL kit (Amersham, UK). Results represent a typical immunoblot from three experiments and analysis of densitometry data from four separate experiments expressed as percentage of control value. The statistical analysis performed by one-way ANOVA, followed by the Newman–Keuls post hoc test. Statistical probability of $p < 0.05$ was considered significant. *$p < 0.05$ versus the control value.

To identify the damage of dopaminergic neurons, specific antibodies were raised against tyrosine hydroxylase (TH). Western blot analysis has shown that the injection of MPTP decreased the TH protein level in striatum by 45%, 65%, and 67% at 3, 7, and 14 days of posttreatment (Fig. 8). Moreover, the correlation between cGMP concentration and TH protein content was calculated. We have obtained a strong inverse linear correlation ($r = -0.858$, $n = 16$, $p < 0.001$).

Discussion

There is a growing body of evidence to suggest that neurotoxicity of MPTP may be mediated by NO [2,5,8]. In the present study, we extended these findings, showing that MPTP induces mRNA expression and elevates protein concentration of GCβ1 in striatum up to 14 days after its injection, which in turn causes a marked enhancement of cGMP formation. Furthermore, the

activation of GC occurs through a change of maximal enzyme activity (V_{max}). Simultaneously, no differences in PDE activity have been detected. Of importance is the finding that MPTP injection caused an elevation of GCβ1 protein level either in membrane or in cytosolic fraction, being significantly higher in cytosol (Fig. 4). These data suggest that NO/cGMP mechanism may at least partially contribute to degeneration of dopaminergic fibers in the striatum, the damage attributed to PD.

Kinetic properties of GC detected in our study have revealed that its affinity for substrate (K_m) is identical among investigated groups whereas its effectiveness estimated as V_{max} is elevated solely in MPTP treated striatum samples (Table 1). As expected, an increase of GC V_{max} value produced an increase of cGMP concentration (Fig. 5). Since we have not found any changes in PDE activity in the same samples, these data point to the fact that the alteration in GC V_{max} was probably the main causative factor elevating the rate of cGMP formation.

The rationale for selective investigation of β1 subunit in the present study is based on the knowledge, indicating that only two isoforms of GC (α1β1 and α2β1) possess catalytic activity against substrate [27–30]. Furthermore, recent studies did not reveal any regulatory and kinetic differences between α1β1 and α2β1 isoforms [27]. Additionally, the striatum also contains a high sGC activity [46] and expresses abundantly mRNA for both α1 and β1 subunits of the enzyme [37,47]. The extent of changes in GC β1 subunit protein level was comparable to those observed in total enzyme activity (Figs. 1 and 3). It therefore makes sense to postulate that GCβ1 subunit may account for enhancement of cGMP in striatum of MPTP injected mice. Moreover, the measurement of GCβ1 subunit in cytosolic and membrane fractions has shown that the former is to a greater extent engaged in cGMP formation. This is because the same stimulus, e.g., MPTP, caused a significantly higher total protein level of GCβ1 subunit in the cytosol fraction in comparison to the membrane one (Fig. 4). Recent study has demonstrated the major level of α1β1 and α2β1 enzyme heterodimers in the brain, both occurring there in a comparable amount [36]. Russwurm et al. [33] detected that after interaction with PDZ domain, α2β1 heterodimer is recruited to the membrane fraction. Keeping in mind the above mentioned data and the fact that GCβ1 expression was higher in cytosol than in membrane fraction (Fig. 4) allow one to assume that first of all changes of GCα1β1 activity were the subject, leading to an elevation of the cGMP level in striatum after MPTP injection.

Many experimental and postmortem studies provided evidence supporting the involvement of oxidative stress in the pathogenesis of PD [5–7,48]. There are data, indicating that NO may be a reactive radical involved in cell death producing highly toxic hydroxyl radicals [18,19]. In central and peripheral nervous system many actions of NO are exerted through the stimulation of sGC. The latter causes an increase of cGMP level in a concentration-dependent manner [49] and the activation of many physiological and pathological processes [25]. In line with a view suggesting a possible role of NO/cGMP pathway in regulation of brain function are the findings based on immunochemistry and immunohistology showing a coexistence of GC with nNOS in some brain regions [50].

In our study cGMP was markedly elevated in striatum of MPTP treated mice in comparison to sedentary control animals (Fig. 5). Thus, it is tempting to speculate that the increase of cGMP can mediate neurodegeneration of dopaminergic neurons in striatum. This effect may be induced by cGMP-dependent regulation of $[Ca^{2+}]_i$ concentration, phosphorylation of PLA2, PLC, GAP-32, depletion of GSH, and stimulation of apoptosis [41,51]. Ca^{2+} ion is known to be involved in a programmed cell death [52]. Recently obtained evidences have shown that cGMP analogue (CTP-cGMP) elevates intracellular calcium through the activation of cGMP-modulated calcium channels [41]. Moreover, it was also seen in the same experiment that the direct modulation of calcium channel properties by cGMP and in turn the accumulation of intracellular Ca^{2+} proceeds to death of hippocampal nerve cell line [41].

The reduced glutathione (GSH), recognized as the predominate form of glutathione within the cell, is known to protect cells from oxidative stress (damage) [9,51]. In recent years, a beneficial contribution of GHS in neurotoxicity has been indicated in fetal midbrain cultures by their pretreatment with GSH synthesis inhibitor, L-buthionine-(*S*,*R*)-sulfoximine (BSO) [51]. On the contrary, reduction in medium GSH level to 50% of baseline concentration caused cell death in response to neurotrophic doses of NO [51]. Data obtained by Li et al. [41] documented a requirement for sGC/cGMP in nerve cell death caused by GSH depletion.

The striatum expresses one of the highest PDE activities among brain structures [46]. Thus, an enhancement of cGMP detected in our study in mice striatum after MPTP treatment could have been caused by decreasing PDE activity. However, we did not find any detectable changes in this variable (Fig. 6). In other words, we demonstrated that PDE is not playing a role in the regulation of cGMP concentration in striatum of mice with experimentally induced parkinsonism. It should be noted that this regulatory pathway may play an important role in keeping cGMP concentration in some parts of the brain [53].

The fact that MPTP injection can effectively induce PD in mice was shown in the present study by measurement of tyrosine hydroxylase (TH) protein level (Fig. 8). Western blot analyses demonstrated about 45–67% decrease of TH protein content in striatum. These values are within the range established by others as an ade-

quate indicator of PD [5,8,54]. To further corroborate the view that cGMP may be involved in degenerative process of striatum dopaminergic fibers we calculated the correlation between cGMP concentration and TH protein content. A strong inverse linear correlation obtained in this study additionally confirms a possible role of cGMP in the development of PD.

In summary, the results presented herein indicate that MPTP injection can increase the expression and the activity of GC that can cause an increase of cGMP formation in mice striatum. We have also found that an increased protein level of cytosolic GCβ1 fraction was probably the relevant factor responsible for elevated cGMP formation. Our data suggest that NO/cGMP signaling pathway can be considered as a possible regulatory mechanism involved in neurodegeneration of dopaminergic fibers in the striatum.

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