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Amphetamine-evoked changes of oxidative stress and neuropeptide Y gene expression in hypothalamus: Regulation by the protein kinase C-δ signaling

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ABSTRACT

Amphetamine (AMPH), a psychostimulant, is an appetite suppressant and may be regarded as a neurotoxin. It was reported that superoxide dismutase (SOD) and neuropeptide Y (NPY) participated in AMPH-mediated behavior response. However, molecular mechanisms underlying this action are not well known. Using feeding behavior as an indicator, this study investigated if protein kinase C (PKC)-δ signaling was involved. Rats were given daily with AMPH for 4 days. Changes in hypothalamic NPY, PKCδ and SOD mRNA contents were measured and compared. Results showed that the up-regulations of PKCδ and SOD mRNA levels following AMPH treatment were concomitant with the down-regulation of NPY mRNA level and the decrease of feeding. To further determine if PKCδ was involved, intracerebroventricular infusions of PKCδ antisense oligonucleotide were performed at 1 h before daily AMPH treatment in freely moving rats, and results showed that PKCδ knock-down could block the anorectic response and restore partially both NPY and SOD mRNA levels in AMPH-treated rats. It is suggested that central PKCδ signaling may play a functional role in the regulation of AMPH-mediated appetite suppression via a modification of hypothalamic NPY gene expression. Moreover, the increase of SOD during AMPH treatment may favor this modification.

1. Introduction

Amphetamine (AMPH) is a well-known appetite suppressant. After the approval of the treatment of obesity in the 1940s and 1950s, AMPH is served presently as a prototype for the development of subsequent anorectic drugs [1,2]. AMPH is neurotoxic to brain partly due to the increase of oxidative stress during drug treatment [3,4]. The drug 3,4-methylenedioxymethamphetamine (MDMA, Adam), an AMPH derivatives, had emerged over the last two decades as a common "club drug" due to their hallucinogenic effect with relatively low toxicity [5]. In human, AMPH can be employed to treat attention-deficit/hyperactivity disorder due to its psychomotor effects such as increased attention, restlessness, and feelings of confidence [6]. Basing on these effects of AMPH, mechanisms behind the anorectic, psychomotor and neurotoxic effects of AMPH have been investigated extensively.

The anorectic effect of AMPH is implicated in the release of biogenic amine in the brain, which may lead to an inhibitory action on hypothalamic neuropeptide Y (NPY) [7–9]. NPY is a powerful appetite stimulant in the brain and may play critical roles in periodic eating behavior and body weight maintenance [10]. Central administration of NPY can induce hyperphagia even under conditions of satiation, resulting in an increase of fat deposition, a decrease of energy expenditure and a promotion of obesity [11,12]. Although previous studies showed that some intracellular signal transductions, including protein kinase C (PKC)-α [13], *c-fos*/*c-jun* [14] and protein kinase A [15] signaling, were involved in NPY gene expression during AMPH treatment, however, possible role of PKCδ signaling is still unknown. Thus, the present study was aimed to investigate if PKCδ signaling was involved in NPY-mediated appetite suppression in AMPH-treated rats.

PKC isotypes have been divided into three subfamilies: conventional (cPKCs, including α, βI, βII, and γ), novel (nPKCs, including δ, ε, η, and θ), and atypical (aPKCs, including ζ and λ) [16,17]. PKCδ isotype is the first nPKC isoform identified and is expressed ubiquitously among cells and tissues, suggesting that PKCδ has universal rather than cell-type specific role in mammals [18]. PKCδ can be found in rat brain [20] and may play a role in the stress-stimulated signal pathway [21]. For example, PKCδ can be activated by diacylglycerol through tyrosine phosphorylation and protein complex formation in stress-stimulated cells [18,19]. Numerous evidence reveal that PKCδ is an oxidative stress-sensitive kinase involv-

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ing in MPP^+-induced oxidative stress [22], dopaminergic neuronal degeneration [23,24] and apoptotic cell death in animal model of Parkinson's disease [25].

The enzyme superoxide dismutase (SOD), including CuZn–SOD (SOD-1) and Mn–SOD (SOD-2), is essential in destroying oxygen-based radicals. Increasing evidence reveal that SOD is activated and involved in antioxidant defense mechanism in AMPH-treated rats [4,26]. Moreover, superoxide radicals seem to participate in AMPH-induced neurodegeneration because its toxicity is attenuated in an animal model of SOD-1 transgenic mice [27]. Thus, we suspected that hypothalamic SOD-1 gene might be activated during AMPH treatment, which could decrease the oxidative stress and favor the normalization of feeding behavior and NPY gene expression. Thus, another purpose of this study was to evaluate if PKCδ signaling participated in SOD-1 gene expression during AMPH treatment.

Recently, approaches by modulating isotype-selective inhibitors or activators had been applied in the improvement of nervous diseases [28,29]; therefore, our results might be helpful for the therapeutic research of AMPH-like anti-obesity drugs. In addition, results might also be helpful to understand the signaling mechanism of some nervous diseases induced by repeated administrations of AMPH, such as AMPH-induced psychological dependence [65] and behavioral sensitization [66].

2. Materials and methods

2.1. Animal treatments

Male Wistar rats (200–300 g, Animal Center of National Cheng Kung University Medical College) were housed individually in a cage, maintained at 22 ± 2 °C according to a 12 h light:12 h dark cycle (light on at 6:00 am), and habituated to frequent handling. Drug administration and food intake assessment (LabDiet, PMI Nutrition International, Brentwood, MO, USA) were performed daily at the beginning of dark phase (6:00 pm). This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. This study has been approved and reviewed by National Science Council in Taiwan, ROC.

To examine the effect of AMPH (D-amphetamine, Sigma–Aldrich, MO, USA) on feeding behavior, rats (n = 6–8 for each group) were injected intraperitoneally (IP) with the drug at doses of 0, 2 or 4 mg/(kg-day) for 4 days. The first injection of AMPH was conducted at the end of Day 0 (i.e., at 6:00 pm), which was regarded as the beginning of Day 1. The intake data were calculated as the total amount of food during the previous day. The body weight data were calculated as daily body weight change when compared with the previous day.

To assess the effect of daily AMPH on hypothalamic NPY, PKCδ and SOD-1 protein and mRNA levels, rats (n = 5–6 each group) were injected daily with AMPH (0 or 2 mg/kg) for 1, 2, 3 or 4 days depending on the group of rats. Rats received AMPH 40 min before being anesthetized (pentobarbital, 30 mg/kg, IP) and decapitated. Their hypothalamus was removed from the brain immediately and subjected to determinations of protein or mRNA levels or stored at −80 °C until the day to use.

To determine the effect of PKCδ antisense on anorectic response of AMPH, intracerebroventricular (ICV) injections with missense or antisense (20 μg in a 10-μl vehicle) were conducted daily at 1 h before AMPH treatment for 4 days. Before AMPH (4 mg/kg; IP) treatment, rats were injected (ICV) with similar dose of missense or antisense daily for 2–3 days until the response of feeding behavior was reduced slightly in antisense group. This is due to the fact that either continuous or repeated ICV injections of antisense may be necessary to maximize behavioral effect and especially to block the synthesis of constitutively active gene product [30,31].

Another experiment was designed to determine the effects of pretreatment of PKCδ antisense or missense on NPY and SOD-1 mRNA levels in AMPH-treated rats. Rats (n = 6–8 for each group) were injected every day with antisense or missense (20 μg in a 10 μl vehicle; ICV) at 1 h before AMPH treatment for 4 days. Before AMPH (4 mg/kg; IP) treatment, rats were injected (ICV) with similar dose of antisense or missense daily for 2–3 days until the response of feeding behavior was reduced slightly in antisense group. At 40 min after AMPH treatment, rats were anesthetized and the hypothalamus of each rat was removed from the brain and its NPY and SOD-1 mRNA contents were determined by RT-PCR.

To determine the effect of PKCδ antisense (or missense) alone on PKCδ protein and mRNA levels, rats (n = 4–6 for each group) were injected daily with antisense or missense (20 μg in a 10 μl vehicle; ICV) for 2–3 days until the response of feeding behavior was reduced slightly in antisense group. At 40 min after the last treatment of antisense or missense, the hypothalamus was removed from the brain and its PKCδ protein and mRNA contents were determined by Western blot and RT-PCR, respectively.

2.2. RNA extraction

Hypothalamic NPY, PKCδ and SOD-1 mRNA levels were measured in a block of mediobasal hypothalamic tissue as described previously [32]. The block of mediobasal hypothalamic tissue was dissected rostral-caudally from the optic chiasma to the mammillary body, and extended laterally from the midline of hypothalamus to the perihypothalamic nucleus and superiorly to the anterior commissure. Total RNA was isolated from tissues using a modified guanidinium thiocyanate–phenol–chloroform method [33]. Each hypothalamic block was homogenized in 1 ml of TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, Connecticut, USA). After an incubation at 22 °C for 5 min, each sample was added with 0.2 ml of chloroform, shaken vigorously for 15 s, incubated at 22 °C for 3 min, and then centrifuged at 12,000 × g for 15 min under 4 °C. After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at 22 °C for 10 min and centrifuged at 12,000 × g for 15 min under 4 °C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7500 × g for 5 min under 4 °C. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at −80 °C. The RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Japan).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany), RNA was transcribed reversely into single-stranded cDNA. For each sample, 8 μl of sterile DEPC water containing 2 μg of RNA were added to oligo-p(dT)15 primer (0.8 μg/μl) followed by being denatured at 65 °C for 15 min, cooled at 25 °C for 10 min, then added to a reaction mixture consisting of 10× reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), $MgCl_2$ (25 mM), RNase inhibitor (40 unit/μl), and AMV reverse transcriptase (25 unit/μl). Reaction mixtures were incubated at 42 °C for 2 h and then heated to 95 °C for 5 min to terminate the reaction followed by soaking at 16 °C. PCR was carried out subsequently by mixing 3 μl of cDNA product with mastermix solution consisting of DEPC water, 10× reaction buffer, $MgCl_2$ (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 μg/μl each), and Taq polymerase (5 unit/μl). With GAPDH being used as an internal standard calibrator, PCR reactions for NPY were carried out on a PCR thermocycler

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Table 1
The sequences of primers used in RT-PCR.

	Primer	Sequence 5′ → 3′	Size of product (base pairs)
NPY	Forward	GGGCTGTGTGGACTGACC	264
	Reverse	GGAAGGGTCTTCAAGCCT	
PKC δ	Forward	CACCATCTTCCAGAAAGAACG	352
	Reverse	CTTGCCATAGGTCCCGTTGTTG	
SOD-1	Forward	GAG CAT GGGTTCCATGTCCAT	279
	Reverse	ACTTTCTTCATT TCCACCTTTGCC	
GAPDH	Forward	TCCCTCAAGATTGTCAGCAA	309
	Reverse	AGATCCACAACGGATACATT	

NPY, neuropeptide Y; PKC, protein kinase C; SOD, superoxide dismutase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

(GeneAmp PCR System 2700, CA, USA) with the following steps: 91 °C for 1 min (denaturing), 60 °C for 1 min (annealing), and 72 °C for 30 s (extension) for 28 cycles followed by a final elongation step at 72 °C for 7 min, and finally the PCR products were soaked at 16 °C. PCR reactions for other molecules analyzed were carried out in steps similar to those described above except the changes of two steps (annealing and cycles) that were described as follows: PKCδ (55 °C, 25), SOD-1 (60 °C, 25) and GAPDH (52 °C, 25). All PCR products were measured during the exponential phase of the DNA amplification in the present study. The sequences of primers used in RT-PCR were shown in Table 1.

2.4. Gel electrophoresis

After RT-PCR, 8 μl of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel (Difco, Detroit, MC, USA). Gels stained by ethidium bromide (0.5 μg/ml, Sigma–Aldrich Co., MO, USA) were visualized under UV light, photographed, and then scanned densitometrically (Hoefer, San Francisco, CA, USA). Ratios of NPY and GAPDH mRNA for each treatment day were calculated to determine relative NPY mRNA levels. Contents of NPY mRNA in AMPH-treated group were indicated as the percentage of control group. Similar steps were used to determine hypothalamic PKCδ and SOD-1 mRNA levels.

2.5. Lateral ventricular cannulation

A surgery of rat was performed under anesthesia with pentobarbital (30 mg/kg, IP) using stereotaxic apparatus (Kopf Model 900, Tujunga, CA, USA). The target of cannulation was close to the junction between the right lateral ventricle and the third ventricle (coordinates: 0.8 mm posterior to Bregma, 1.5 mm from the midline, and 3.5–4.0 mm below the dura) [34]. A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless-steel screws and dental cement. A correct placement was confirmed by observing a transient and rapid inflow of vehicle in PE tube connected with a 28 g injector cannula. The cannula was then occluded with a 28 g stylet. For ICV infusion of antisense, the stylet was replaced with a 28 g injector cannula extending 0.5 mm below the tip of guide cannula. Behavioral testing began at 1 week after the surgery. For all experiments verification of cannula placement was done by the administration of angiotensin II (100 ng/rat; Sigma–Aldrich Co., MO, USA). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the ventricles [35]. Only data from rats drinking more than 10 ml within 30 min were included in this study.

2.6. ICV injection of antisense ODN

A 16-mer antisense oligodeoxynucleotide was complementary to the translation initiation region of mRNA specific for mouse PKCδ [36]. The sequences of the PKCδ antisense and missense ODN were 5′-AGGGTGCCATGATGGA-3′, and 5′-TCGATCATGGCACCCT-3′, respectively. We used ODNs that were phosphorothioate-modified (S-ODN) only on the three terminal bases of both the 5′ and 3′ ends (Proligo Pty Ltd., Singapore), because these S-ODNs had been shown to produce sequence-specific effects without detectable toxicity in brain region and was regarded as a well-established agent in several vertebrate systems [31,37]. Both antisense and missense S-ODN were dissolved in artificial cerebrospinal fluid containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.4.

2.7. Western blotting

Hypothalamic protein was extracted by the additions of 0.1 ml cell-lysis buffer and protease inhibitor to the tissue, and then subjected to a centrifugation of 12,000 × *g* for 10 min at 4 °C. Supernatant was used for the quantification of protein by a method of Western Blot described previously [67]. Protein (50 μg/lane) was detected by electrophoreses processing on a 12.5% SDS polyacrylamide gel (100 V, 2.5 h) with running buffer (containing glycine 3 g, Tris 14.7 g, and 10% SDS 5 ml in 1 l) and then transferred onto a nitrocellulose membrane by a method of electroblotting (90 mA, 1 h) performed by using a transfer electroblot unit (Bio-Rad Laboratories, Hercules, CA) in a transfer buffer (containing Tris 1.5 g, glycine 7.15 g and methanol 200 ml in a total volume of 1 l). The blot was blocked by blocking buffer containing 5% non-fat milk in Tris-buffered saline (0.1% Tween-20), and the membrane was incubated overnight with a specific antibody against one of the three proteins: PKCδ (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), SOD-1 (1:500 dilution) and β-actin (1:2500 dilution) (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA). After washing twice (with washing buffer containing Tris 1.33 g, Nacl 9 g and 0.5 ml Tween-20 in 1 l) and incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed by 4-chloro-1-napthol/3,3′-diaminobenzidine, 0.9% (w/v) NaCl in Tris–HCl buffer (Sigma Chemical Co., St. Louis, MO, USA). Relative photographic density was quantified by scanning the photographic negative film on a Gel Documentation and Analysis System (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

2.8. Statistical analysis

Data were presented as mean ± SEM. Two-way or one-way ANOVA followed by Dunnett's test was used to detect significances among groups. $P < 0.05$ was considered to be statistically significant.

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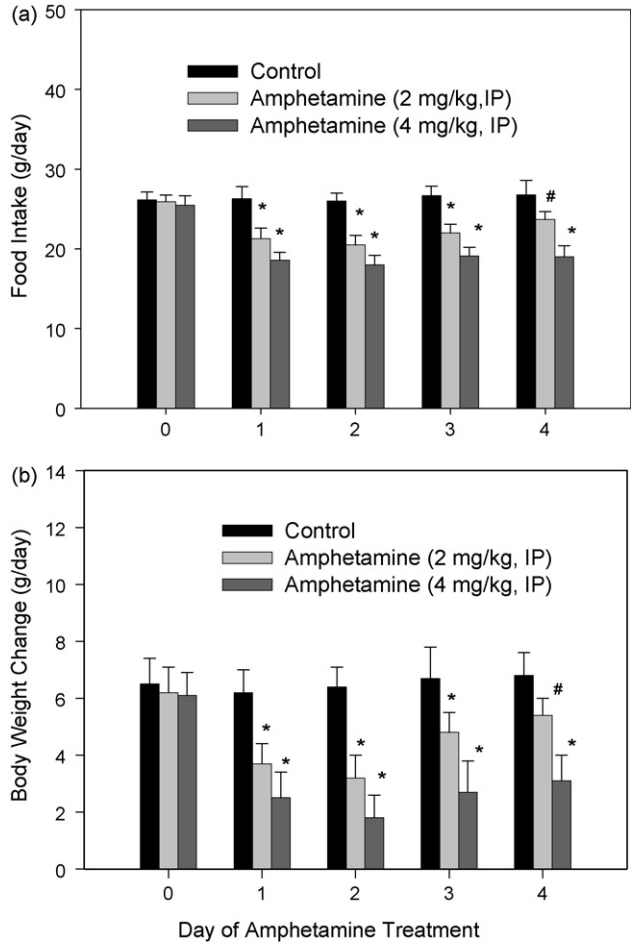

Fig. 1. Effects of daily treatment with amphetamine (AMPH) on daily food intake (upper panel) and body weight change (lower panel) over a 4-day period. Various doses of AMPH (0, 2 or 4 mg/(kg-day), IP) were administered to rats once a day (at 6:00 pm of each day) for 4 days. The first injection of AMPH was conducted at the end of Day 0. Each point represents the mean ± SEM of 6–8 rats. $^{*}P < 0.05$ vs. the control group of each treatment day. $^{\#}P < 0.05$ vs. the treatment group on Day 2. IP; intraperitoneally.

3. Results

3.1. The effect of AMPH treatment on feeding behavior and body weight

Changes of food intake in rats receiving AMPH were shown in upper panel of Fig. 1. Using two-way ANOVA to measure the effect of AMPH, a significant dose effect [$F(2, 21) = 10.8$, $P < 0.05$] and time effect [$F(4, 35) = 3.6$, $P < 0.05$] was revealed, but the interaction effect was not significant. Dunnett's test ($P < 0.05$) revealed that AMPH (2 mg/(kg-day)) reduced the food intake from Day 1 to Day 3, and that AMPH (4 mg/(kg-day)) reduced food intake from Day 1 to Day 4 when compared to controls. Moreover, AMPH (2 mg/(kg-day)) on Day 4 showed significant effect when compared to that on Day 2. This result revealed that 2 mg/(kg-day) AMPH could produce a marked anorectic response on Day 2 and induced a gradual tolerant effect on the following days. However, with a dose of 4 mg/(kg-day) AMPH, it could produce a continuous anorectic response during a 4-day period.

Similar results were obtained in body weight change shown in lower panel of Fig. 1. Statistical results by two-way ANOVA showed significant dose-dependent [$F(2, 21) = 4.87$, $P < 0.05$] and time-dependent effects [$F(4, 35) = 3.36$, $P < 0.05$]. It revealed that daily AMPH (2 mg/kg) produced a marked body weight change from Day 1 to Day 3 and a return to normal intake on the following days, but daily AMPH (4 mg/kg) produced a continuous body weight change during a 4-day period.

Therefore, AMPH at dose of 2 mg/(kg-day) was employed for most of the subsequent measures, except antisense studies, since there was a tolerance. While AMPH at dose of 4 mg/(kg-day) was used for behavioral studies (antisense studies) since it can exert a more significant effect than 2 mg/(kg-day) AMPH on the decrease of NPY expression and feeding response.

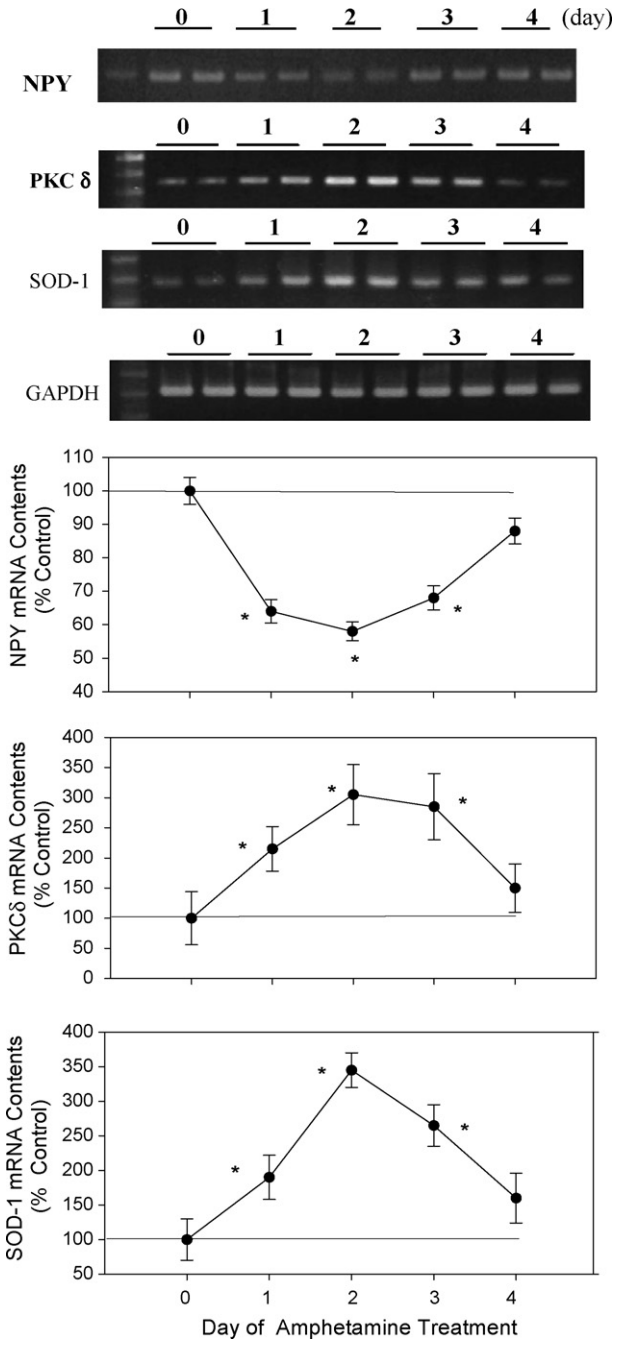

Fig. 2. Effects of daily AMPH on hypothalamic NPY, PKCδ and SOD-1 mRNA levels over a 4-day period. Upper panel: the results of RT-PCR analyzing mRNA levels of NPY, PKCδ and SOD-1 in stained ethidium bromide gels. Lower panel: relative densitometric values for RT-PCR products in AMPH- and saline-treated groups. Content of NPY, PKCδ and SOD-1 mRNA in AMPH-treated group was indicated as the percentage of control (Day 0). Bars were mean ± SEM. $N = 4$–6 each group. $^{*}P < 0.05$ vs. control.

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3.2. The effect of AMPH treatment on NPY, PKCδ and SOD-1 mRNA levels

Results shown in Fig. 2 revealed that a daily treatment of AMPH (2 mg/kg) for 4 days resulted in a significant decrease in NPY mRNA levels, but significant increases in PKCδ and SOD-1 mRNA levels. Analysis with one-way ANOVA followed by Dunnett's test ($P < 0.05$) indicated a significant decrease of NPY mRNA contents [$F(4, 25) = 4.7$, $P < 0.05$] on Day 1, Day 2 and Day 3 as compared with the control group. Moreover, the changes of NPY mRNA levels were consistent with the changes of feeding behavior during 4-day repeated treatments of AMPH, revealing the involvement of NPY gene in AMPH anorexia. Statistical results also indicated a significant increase of PKCδ mRNA [$F(4, 25) = 2.8$, $P < 0.05$] from Day 1 to Day 3 and a significant increase of SOD-1 mRNA [$F(4, 25) = 3.1$, $P < 0.05$] from Day 1 to Day 3 as compared with the control group.

3.3. Effects of AMPH treatment on NPY, PKCδ and SOD-1 protein contents

AMPH could decrease hypothalamic NPY contents that were decreased markedly on Day 1 with a gradual return to normal level

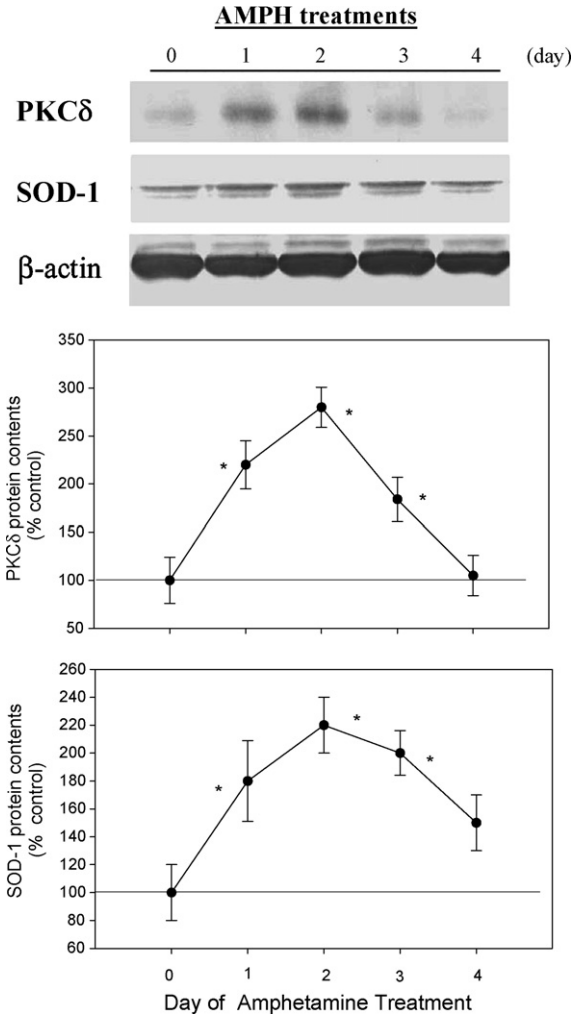

Fig. 3. Effects of AMPH (2 mg/(kg-day)) treatment on hypothalamic PKCδ and SOD-1 protein contents over a 4-day period. Upper panel: the results of Western Blot analyzing the contents of PKCδ and SOD-1. Lower panel: relative densitometric values for Western Blot of PKCδ and SOD-1 in saline- and AMPH-treated groups. Contents of PKCδ and SOD-1 in AMPH-treated groups were indicated as the percentage of the control group. Bars were mean ± SEM. $N = 4$–6 each group. $^{*}P < 0.05$ vs. control.

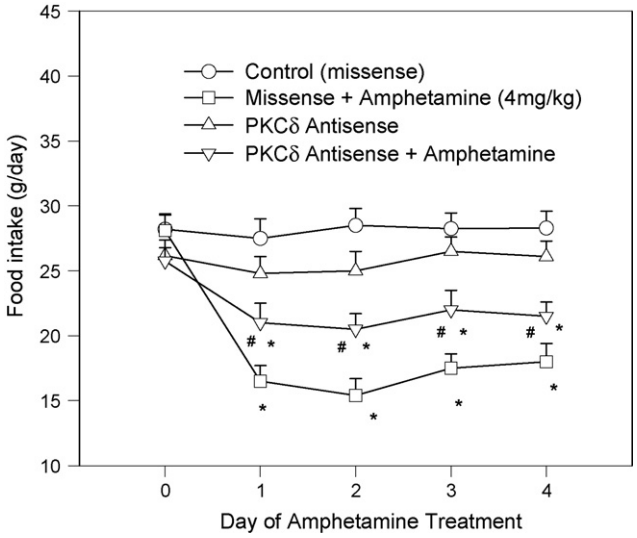

Fig. 4. The effect of PKCδ antisense (or missense) pretreatment on AMPH (4 mg/(kg-day))-induced feeding behavior over a 4-day period. Daily missense or antisense treatment (20 μg/10 μl/day, ICV) was administered 1 h before daily AMPH treatment. $^{*}P < 0.05$ vs. the missense groups of each treatment day. $^{\#}P < 0.05$ vs. the AMPH-treated groups of each treatment day. Bars are mean ± SEM. $N = 6$–8 per group.

on the followings during a 4-day period in AMPH-treated rats as described in our previous report [38]. This change of NPY contents was expressed in a manner consistent with the alteration of NPY mRNA levels shown in the present study. Results shown in Fig. 3 reveal that daily AMPH increase PKCδ and SOD-1 protein contents during a 4-day period. Using β-actin as the internal standard, the ratio of PKCδ (or SOD-1) over β-actin in each group was calculated and compared. Analysis with one-way ANOVA revealed an increase of PKCδ content [$F(4, 25) = 2.5$, $P < 0.05$] from Day 1 to Day 3 and an increase of SOD-1 content [$F(4, 25) = 2.8$, $P < 0.05$] from Day 1 and Day 3 as compared with the control. These results revealed that both PKCδ and SOD-1 were activated for 3 days during AMPH treatment, which were parallel with expression of PKCδ and SOD-1 mRNA levels.

3.4. Effects of ICV injection of PKCδ antisense on AMPH anorexia

As shown in Fig. 4, a pretreatment of PKCδ antisense in AMPH-treated rats could block partially the anorectic response of AMPH, indicating the involvement of δ gene in AMPH anorexia. Using two-way ANOVA to analyze the effect of PKCδ antisense pretreatment on AMPH anorexia from Day 1 to Day 4, a significant dose-dependent [$F(3, 21) = 4.1$, $P < 0.05$] and time-dependent effects [$F(4, 34) = 3.6$, $P < 0.05$] were revealed, however, the interaction effect is not significant. Comparing the food intake between antisense/AMPH-treated and missense/AMPH-treated rats in every day, significant effect was seen from Day 1 to Day 4. Furthermore, significant effect was also seen from Day 1 to Day 4 by comparing between antisense/AMPH-treated and control (missense alone) rats. The feeding response in missense alone-treated rats was similar to that in saline-treated rats (shown in Fig. 1) during a 4-day period of treatment. Moreover, the anorectic response in missense/AMPH-treated rats was not changed significantly when compared to that in AMPH alone-treated rats during a 4-day period of treatment. These results revealed the non-interference of missense treatment in this study. This result indicated that PKCδ knock-down could modify the feeding responses of repeated AMPH treatments.

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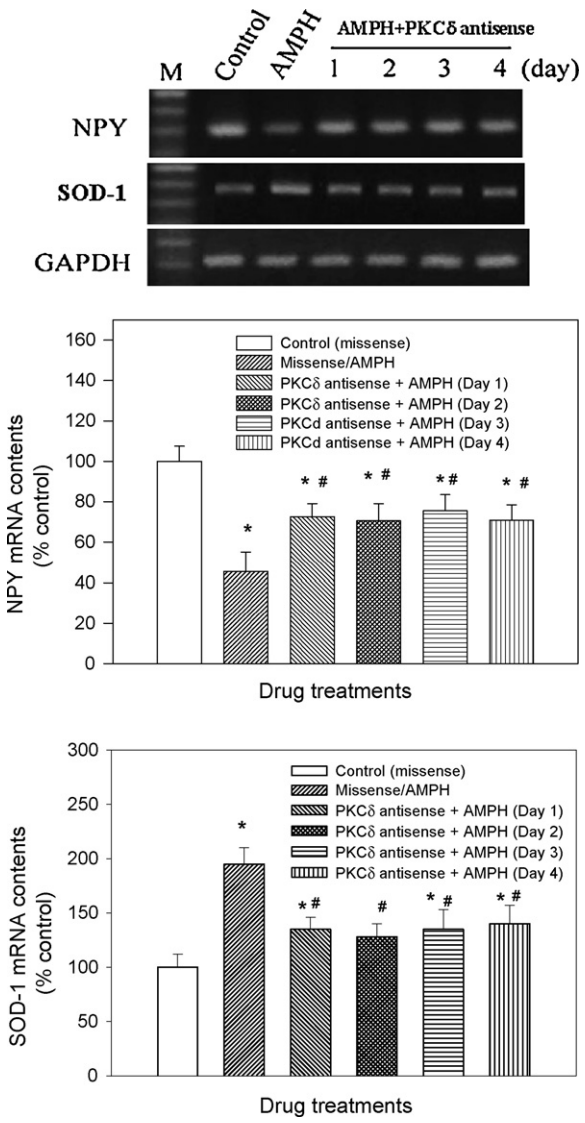

Fig. 5. Effects of PKCδ antisense pretreatment on NPY and SOD-1 mRNA level in missense/AMPH (4 mg/(kg-day))-treated rats over a 4-day period. NPY and SOD-1 mRNA levels were measured by RT-PCR. Content of NPY and SOD-1 mRNA in drugs-treated rats was indicated as the percentage of control. Bars are mean ± SEM. $N = 4–6$ per group. $^{*}P < 0.05$ vs. control (missense alone), $^{\#}P < 0.05$ vs. missense/AMPH-treated group.

3.5. Effects of PKCδ antisense pretreatment on NPY and SOD-1 mRNA levels

Results shown in Fig. 5 revealed that pretreatment with PKCδ antisense in AMPH-treated rats resulted in a partial restoration of hypothalamic NPY and SOD-1 mRNA contents toward normal level. Using GAPDH as the internal standard, the ratio of NPY (or SOD-1) mRNA over GAPDH mRNA in each group was calculated and compared. By one-way ANOVA followed by Dunnett's test ($P < 0.05$), it revealed that NPY mRNA content [$F(2, 18) = 3.5, P < 0.05$] decreased in both AMPH-treated and antisense/AMPH-treated rats as compared with the control (missense-treated) group. Moreover, significant decrease was also observed in antisense/AMPH-treated rats as compared with the AMPH-treated group.

Similarly, statistical analysis revealed that PKCδ was also involved in the regulation of SOD-1 gene expression in AMPH-treated rats. SOD-1 mRNA content increased in both AMPH-treated and antisense/AMPH-treated rats as compared with the control (missense-treated) group [$F(2, 18) = 4.25$, $P < 0.05$]. Moreover, significant decrease was also observed in antisense/AMPH-treated rats as compared with the AMPH-treated group.

3.6. Effects of PKCδ antisense on hypothalamic PKCδ mRNA and protein levels

Results shown in the left panel of Fig. 6 revealed that pretreatment of PKCδ antisense in vehicle-treated rats resulted in a significant decrease of PKCδ mRNA level. Using GAPDH as the internal standard, the ratio of PKCδ mRNA over GAPDH mRNA in each group was calculated and compared. Statistical analysis by t-test ($P < 0.05$) revealed that PKCδ mRNA content decreased in antisense-treated rats as compared with the control (missense-treated) group. This result revealed that ICV injection of PKCδ antisense was effective to reduce the hypothalamic PKCδ mRNA level in rats.

Results shown in the right panel of Fig. 6 revealed that pretreatment of PKCδ antisense in vehicle-treated rats resulted in a significant decrease of PKCδ protein level. Using β-actin as the internal standard, the ratio of PKCδ over β-actin in each group was calculated and compared. Statistical analysis by t-test ($P < 0.05$) revealed that PKCδ content decreased in antisense-treated rats as compared with the control (missense-treated) group. This result revealed that ICV injection of PKCδ antisense was effective to reduce the hypothalamic PKCδ protein level in rats.

4. Discussion

Hypothalamic NPY has been reported to participate in the anorectic effect of AMPH. However, until recently, little was known about the role of PKCδ signaling in this effect. In this study, we found that PKCδ signaling in hypothalamus was activated during AMPH treatment, which was relevant to the inhibitions of NPY gene expression and the induction of AMPH-induced anorectic response.

Results shown in Fig. 1 revealed that daily AMPH could induce markedly the anorectic responses on Day 1 and Day 2, which were followed by a gradual tolerant response on the subsequent days. This alteration of feeding response was consistent with the change of NPY gene expression. This result confirmed that NPY gene was involved in the anorectic response of AMPH, which was related to the down-regulation of NPY gene, and in the induction of AMPH tolerance, which was relevant to the restoration of NPY gene expression [15]. Similar results could be observed in the alteration of body weight change during AMPH treatment, revealing that the weight-reducing effect of AMPH might reflect a combined effect of drug on food intake and on thermogenesis of brown adipose tissue [39,40].

The PKCδ gene was also activated during AMPH treatment. However, instead of being inhibited as observed in NPY gene expression, PKCδ gene was activated following daily AMPH treatment. Moreover, the PKCδ mRNA levels after drug treatment were increased from Day 1 to Day 2 but returned gradually to normal level on the subsequent days. This alteration of PKCδ mRNA levels was expressed in a manner almost reciprocal to the change of NPY mRNA levels, implying that PKCδ might play an inhibitory role in the reduction of NPY gene expression during AMPH treatment. To test this possibility, we examined the effects of ICV injection of PKCδ antisense on NPY gene expression in AMPH-treated rats. Results revealed that PKCδ knock-down could attenuate the anorectic response of AMPH and restore partially the NPY mRNA level toward normal in AMPH-treated rat. Thus, it is suggested that the activation of central PKCδ signaling is involved in the regulation of AMPH anorexia by an inhibition on NPY gene expression. Although a recent report [41] indicated that PKC signaling could be inhibited via dopamine D2 receptor to decrease the NPY release in PC12 cells, however, it was an *in vitro* mechanism and the mediated PKC iso-

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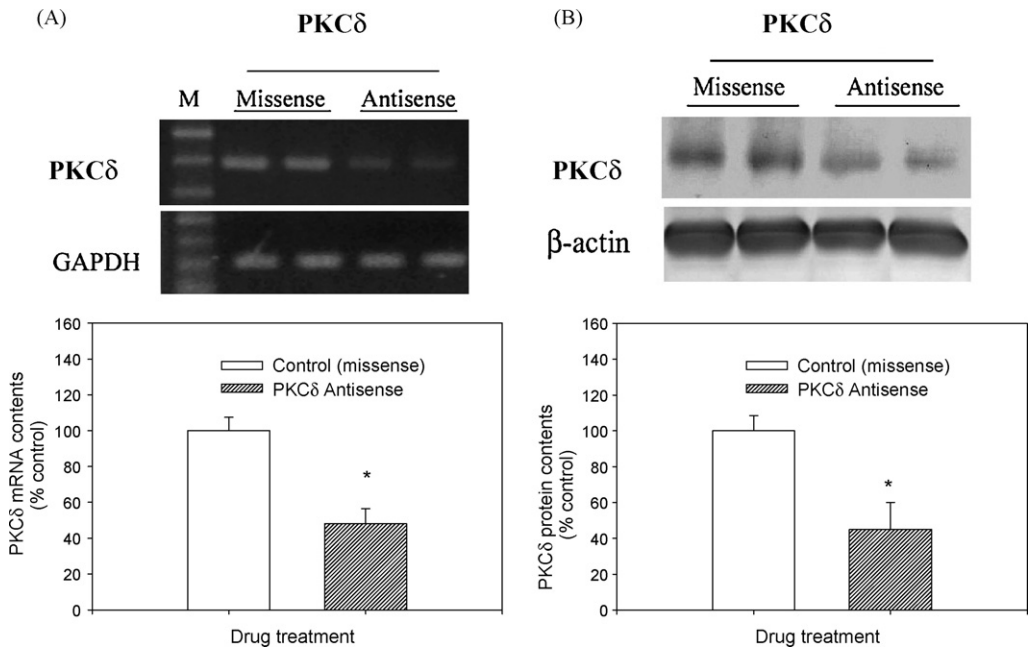

Fig. 6. Effects of PKCδ antisense (or missense) treatment on hypothalamic PKCδ mRNA and protein levels. (A) Upper panel: the results of RT-PCR analyzing mRNA levels of PKCδ in stained ethidium bromide gels. Lower panel: relative densitometric values for RT-PCR products of PKCδ mRNA in missense- and antisense-treated groups. Content of PKCδ mRNA in antisense-treated group was indicated as the percentage of control (missense-treated). (B) Upper panel: the results of Western blot analyzing the contents of PKCδ protein. Lower panel: relative densitometric values for PKCδ protein in missense- and antisense-treated groups. Content of PKCδ protein in antisense-treated group was indicated as the percentage of control. Bars were mean ± SEM. $N = 4–6$ each group. $^{*}P < 0.05$ vs. control.

type was a calcium-dependent one which was different from the calcium-independent PKCδ isotype in the present study.

Based on these findings, it is possible that AMPH may at first activate the PKCδ signaling in a distinct population of hypothalamic neurons, such as anorexic dopamine neurons, and then inhibits the NPY-producing neurons. It is because that PKC can modulate dopamine release in tissues of AMPH treated-synaptoneurosomes [42,43] and that PKCδ isotype can modulate dopamine synthesis in dopaminergic neurons [44]. Similarly, AMPH may activate at first the PKCδ signaling in anorexic proopiomelanocortin (POMC)-producing neurons [45] and then inhibits NPY neurons to decrease appetite. It was reported that both NPY- and α-MSH-immunoreactive neurons were embedded in dense, intermingling networks of NPY- and α-MSH axons and their neuronal wiring in hypothalamus possessed a different role in feeding behavior [46,47]. Moreover, it was also reported that the activities of two populations of appetite-related neurons (NPY/AgRP- and POMC-containing neurons) in hypothalamus were regulated reciprocally by a number of peripheral and central neuromodulatory systems that influenced energy balance [48,49].

In addition to PKCδ gene, the present results also showed that SOD-1 mRNA levels were increased following AMPH treatment. The changes of SOD-1 mRNA levels during AMPH treatments were consistent with the alterations of PKCδ mRNA levels since both of them showed the most significant levels (about threefolds) on Day 2. This result revealed that PKCδ might play a protective role to prevent AMPH-induced neurotoxicity via the activation of SOD-1 gene, which could help to normalize NPY gene expression. This possibility was confirmed in the present study by a further experiment showing that PKCδ knock-down could restore partially both SOD-1 and NPY mRNA levels. Current concepts of the molecular mechanisms underlying the long-lasting neurodegenerative effects of AMPH have been centered on the formation of oxidative stress [50–52]. Moreover, PKCδ was associated with the activation of oxidative stress in high-fat diet-induced obese and insulin-resistant mice [53] and was responsible for the increase of oxidative stress relevant to the advanced glycation end product (AGE)-induced neuroblastoma cell death [54]. Thus, it is rational that the activation of PKCδ may participate in the antioxidative stress mechanism that favors the normalizations of NPY gene expression and feeding behavior in AMPH-treated rats.

The present results revealed that PKCδ antisense could block partially the decreasing effects of feeding behavior and NPY gene expression in AMPH-treated rats, suggesting a component of PKCδ-independent pathway mediating the appetite-suppressing effect of AMPH. Interestingly, except PKCδ isotype, it was reported that the PKCα was also elevated and expressed in a manner similar to PKCδ isotype and that PKCα could modulate NPY gene expression during a 4-day period of AMPH treatment [13]. This result revealed the possibility that there was a certain cross-regulation between PKCα and PKCδ signaling [55,56]. Several reports indicated that both isoforms could promote a number of biological effects and could regulate mutually the expression and activity of other isoforms [57,58]. Thus, the co-activation of PKCα and PKCδ during AMPH treatment might cooperate with each other in the modulation of NPY gene expression.

Recently, PKCδ and PKCα isotypes were emerging as an oxidative stress-sensitive kinase [25,59–61]. AMPH has been regarded as a neurotoxin since it could increase the oxidative state of animals and produce an oxidative damage to dopaminergic neurons [62,63]. In addition to PKCδ and PKCα signaling, the PKA signaling was also activated and participated in an antioxidative stress mechanism during AMPH [15] or phenylpropanolamine treatment [64]. Thus, it is possible that PKCα, PKCδ and PKA signaling in hypothalamus are co-activated because of the increased oxidative stress induced by AMPH and they are implicated altogether in the activation of SOD gene and the normalization of NPY gene expression.

Recently, PKCδ inhibitor was used clinically to prevent the apoptotic cell death in animal model of Parkinson's disease, suggesting that pharmacological modulation of PKCδ might offer a novel therapeutic strategy for the treatment of Parkinson's disease [25]. Moreover, PKCδ inhibitor was able to prevent AGE-induced

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neuronal death, suggesting a therapeutic route to improve several neurodegenerative diseases in the brain [54]. The present data provided a molecular basis for the anorectic effect of AMPH and implied that manipulations at the level of PKC might allow the development of therapeutic agents to improve the undesirable properties of AMPH.

In summary, the present results showed that hypothalamic PKCδ signaling was involved in AMPH-induced anorexia via the modulation of NPY gene expression and that the increased SOD during AMPH treatment might be helpful for this modulation.

Conflict of interest

None.

Acknowledgements

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出席國際會議心得報告

2009-09-01

撰寫人：郭東益

此次是我第一次由國科會補助出席國際會議，會議名稱爲：第36屆國際生理科學研討會(36TH International Congress of Physiological Sciences)，於二零零九年七月二十七至八月一日，共六天，在日本京都舉行。

由大會所提供的資料顯示參與者有2000-3000人，可說是大型研討會。議題區分九大類，各類在不同時間及不同會議室研討。我分配在第四類: Neural Cell Signaling: Appetite, hypertension, breathing. 標號: P2AM-18-1，主題爲：Activation of c-fos/c-jun signaling are involved in neuropeptide Y gene expression in amphetamine-mediated appetite suppression. (p110 of Program Book)

此次榮幸親眼看到日本皇太子恭臨盛會，並致詞祝賀大會成功。並聽到不少生理屆大師級學者演講，收獲不少。並且認識不少國外專家，得知各實驗室研究趨勢，也獲知良多，希望以後能有更多機會做此接觸。

出席國際會議心得報告

2009-09-01

撰寫人：郭東益

此次是我第一次由國科會補助出席國際會議，會議名稱爲：第36屆國際生理科學研討會(36TH International Congress of Physiological Sciences)，於二零零九年七月二十七至八月一日，共六天，在日本京都舉行。

由大會所提供的資料顯示參與者有2000-3000人，可說是大型研討會。議題區分九大類，各類在不同時間及不同會議室研討。我分配在第四類: Neural Cell Signaling: Appetite, hypertension, breathing. 標號: P2AM-18-1，主題爲：Activation of c-fos/c-jun signaling are involved in neuropeptide Y gene expression in amphetamine-mediated appetite suppression. (p110 of Program Book)

此次榮幸親眼看到日本皇太子恭臨盛會，並致詞祝賀大會成功。並聽到不少生理屆大師級學者演講，收獲不少。並且認識不少國外專家，得知各實驗室研究趨勢，也獲知良多，希望以後能有更多機會做此接觸。