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ORIGINAL RESEARCH COMMUNICATION

Role of Reactive Oxygen Species-Related Enzymes in Neuropeptide Y and Proopiomelanocortin-Mediated Appetite Control: A Study Using Atypical Protein Kinase C Knockdown

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Abstract

Aims: Studies have reported that redox signaling in the hypothalamus participates in nutrient sensing. The current study aimed to determine if the activation of reactive oxygen species-related enzymes (ROS-RE) in the hypothalamus participates in regulating neuropeptide Y (NPY)-mediated eating. Moreover, possible roles of proopiomelanocortin (POMC) and atypical protein kinase C (aPKC) were also investigated. Rats were treated daily with phenylpropanolamine (PPA) for 4 days. Changes in the expression levels of ROS-RE, POMC, NPY, and aPKC were assessed and compared. ***Results:*** Results showed that ROS-RE, POMC, and aPKC increased, with a maximal response on Day 2 (anorectic effect) and with a restoration to the normal level on Day 4 (tolerant effect). By contrast, NPY expression decreased, and the expression pattern of NPY proved opposite those of ROS-RE and POMC. Central inhibition of ROS production by ICV infusion of ROS scavenger attenuated PPA anorexia, revealing a crucial role of ROS in regulating eating. Cerebral aPKC knockdown by ICV infusion of antisense aPKC modulated the expression of ROS-RE, POMC, and NPY. ***Conclusion:*** Results suggest that ROS-RE/POMC- and NPY-containing neurons function reciprocally in regulating both the anorectic and tolerant effects of PPA, while aPKC is upstream of these regulators. ***Innovation:*** These results may further the understanding of ROS-RE and aPKC in the control of PPA anorexia. *Antioxid. Redox Signal.* 15, 2147–2159.

Introduction

Recent evidence indicates that differential metabolic signals in the hypothalamus have responsibility for the fuel utilization and for the distinct responses of neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons (26, 52). For example, POMC neurons utilize glucose as the primary fuel source in a metabolic state of satiety, while NPY neurons utilize free fatty acids as the primary fuel source in a metabolic state of hunger (1, 23). The generation of reactive oxygen species (ROS) during fuel utilization plays a crucial role in regulating neuronal responses in a substrate-dependent manner, in addition to serving as a byproduct of substrate oxidation (2). For example, during positive energy balance, glucose-utilizing POMC neurons fire, which leads to ROS accumulation in these cells (23). By contrast, during negative energy balance, fatty acid-utilizing NPY neurons are activated, but ROS levels are not increased in these cells (1). This study aims mainly to examine the essential role of ROS-related enzymes (ROS-RE) in the regulation of POMC- and NPY-mediated appetite suppression evoked by phenylpropanolamine (PPA) treatment.

The mechanism underlying the appetite-suppressing effect of PPA, a sympathomimetic agent, relates to the release of catecholamine, which acts on NPY- (6, 21, 55) and POMC-containing (27, 28) neurons to suppress appetite. NPY, a

Innovation

Current research reveals that ROS-RE/POMC- and NPY-containing neurons in hypothalamus function reciprocally in regulating PPA-induced anorexia and that PKC activation is isotype specific during PPA treatment, because only one isotype (α, δ, or λ) from among cPKC, nPKC, and aPKC is involved in PPA anorexia. This result favors the therapeutic research of some anti-obesity drugs.

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member of the central orexigenic system, plays an essential role in the regulation of daily eating and energy homeostasis (34, 55, 58). POMC, a member of the anorexigenic system, participates in the regulation of appetite suppression and energy expenditure (3, 64). Although PPA-containing (or catecholamines-stimulating) compounds relate to an increase in oxidative stress (31), catecholamine can reduce the amount of ROS present by increasing superoxide dismutase (SOD) activity in the nervous system (47). Thus, the authors speculated that the activation of ROS-RE might have a hand in the regulation of NPY- and POMC-mediated appetite suppression during PPA treatment.

Both SOD and glutathione S-transferase (GST) belong to the ROS-RE family of proteins. We know that SOD-1 and SOD-2 catalyze the dismutation of superoxide, which results in the production of hydrogen peroxide (H_2O_2). To prevent damage, H_2O_2 must be quickly decomposed to water and oxygen by catalase, one of the antioxidant defense systems that can be activated during PPA treatment (28). Glutathione-related enzymes, such as glutathione peroxidase and GST, represent antioxidative enzymes that may function in the detoxification process. GST-α, expressed in the brain, plays an essential role in the neuroprotection against lithium-induced oxidative stress (48). Thus, endogenous antioxidants, including SOD-1 and GST-α, might play a role in the regulation of NPY-mediated PPA anorexia.

The three subfamilies of PKC isotypes include conventional PKC (cPKCs, including the α, βI, βII, and γ isotypes), which are Ca^{2+} and diacylglycerol (DAG) dependent during activation; novel PKC (nPKCs, including the δ, ϵ, η, and θ isotypes), which are Ca^{2+} independent but DAG dependent; and atypical PKC (aPKCs, including the ζ and τ/λ isotypes), which are both Ca^{2+} and DAG independent (37, 59). PKC is a redox-sensitive molecule, which can be activated by redox action (63). Moreover, PKCs are cell-signaling proteins that are particularly sensitive to redox stress because the modification of their redox-sensitive regions interferes with their activities and, thus, with their biological effects (13). The suggestion that aPKCs are involved in Alzheimer's and Parkinson's diseases may provide support for the hypothesis that these kinases participate in neuronal plasticity or neuroprotection in the brain (9, 41, 46). Reports have indicated the involvement of signals of cPKCα and nPKCδ in the regulation of the appetite-suppressing effect of PPA (27, 28). During PPA treatment, aPKCλ was activated and expressed in a manner similar to that of cPKCα and nPKCδ (27); thus, we speculated that aPKCλ might participate in the control of NPY/POMC-mediated feeding and ROS-RE expression during PPA treatment.

Although PPA can induce several physiological effects, including nasal decongestion (60), exacerbation of neuropsychiatric symptoms (14), and appetite suppression (22, 28), as a neurotoxin, PPA can increase the frequency of oxidative stress-related brain disturbances (31, 61). Despite its toxic effect, PPA has an anti-addictive effect (43) and can be clinically used to improve male stress urinary incontinence (54) and to prevent weight gain after smoking cessation in patients (40). Thus, the complex and uncertain effects of PPA in the brain need further investigation. This study also intended to expand the authors' previous examinations of the molecular mechanisms underlying the effect of PPA-evoked feeding behavior. Comparing to our recent report (28), we will provide new evidence that (a) PKCλ is located on the site of arcuate (ARC) neurons in the hypothalamus, (b) only one isotype (α, δ, or λ) from among cPKC, nPKC, and aPKC was involved in PPA anorexia, and (c) ICV injection of ROS scavenger can modulate PPA-mediated eating.

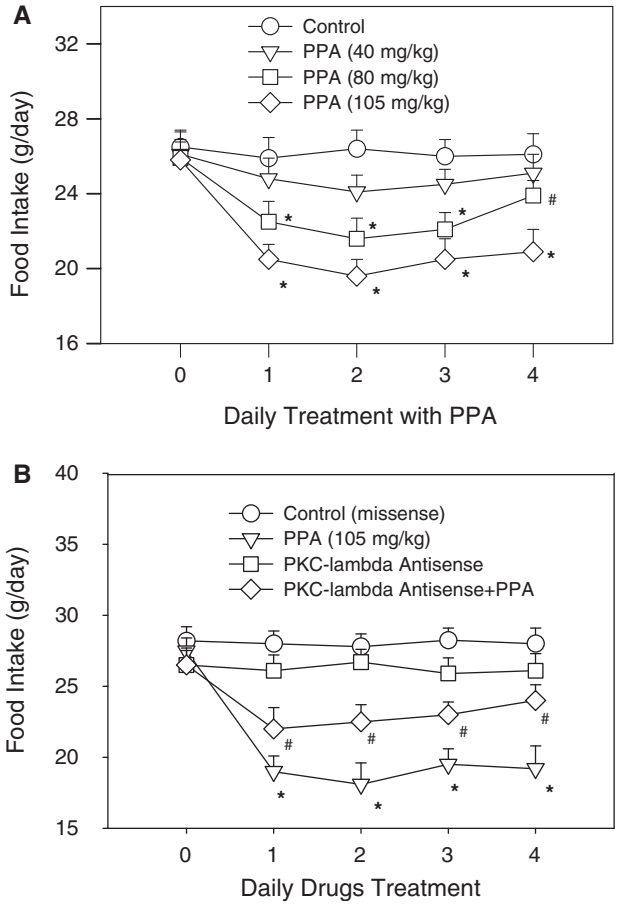

FIG. 1. The effect of daily phenylpropanolamine (PPA) on food intake over a 4-day period (A) and the effect of PKCλ antisense (or missense) pretreatment on daily PPA-mediated food intake over a 4-day period (B). (A) PPA at dose of 40, 80, or 105 mg/kg was daily administered to rats at 6:00 PM. The first injection of PPA was conducted at the end of Day 0. Each point represents the mean ± SEM of 6–8 rats. *Bars* are means ± SEM; $n = 5$–6 per group; $^{*}p < 0.05$ vs. control group of each treatment day' $^{\#}p < 0.05$ vs. the Day 1 group. **(B)** Daily missense or antisense treatment (20 μg/10 μl/day, ICV) was administered 1 h before daily PPA treatment; $^{*}p < 0.05$ vs. the missense groups of each treatment day; $^{\#}p < 0.05$ vs. the PPA-treated groups of each treatment day. *Bars* are mean ± SEM; $n = 6$–8 per group.

Results

Effects of daily PPA on food intake

The change of food intake following PPA treatment is shown in the upper panel of Figure 1. Statistical analysis by two-way ANOVA revealed significant dose-dependent [$F(3, 28) = 20.22$, $p < 0.05$] and time-dependent effects [$F(4, 35) = 5.68$, $p < 0.05$]. Followed by Dunnett's test, it revealed that daily PPA (80 mg/kg) produced marked decreases in food intake from Day1 to Day 3 (anorectic effect) and a return to normal intake (tolerant effect) on Day 4, but daily PPA (105 mg/kg) produced a continuous anorectic response during a 4-day period of time.

Based on these findings, PPA at a dose of 80 mg/kg was employed for most of the subsequent studies, since after 2 days there was a drug tolerance, while PPA at a dose of 105 mg/kg was used for studies of GSH-EE administration, aPKC antisense, and immunohistochemistry (IHC) since it could exert a larger anorectic response.

Effect of ICV injections of aPKCλ antisense on PPA anorexia

As shown in the lower panel of Figure 1, aPKCλ antisense can partially block PPA-induced anorexia, indicating the involvement of aPKCλ gene during PPA treatment. Using two-way ANOVA to measure the effect of aPKCλ antisense, significant drug-dependent [$F(3, 27) = 5.86$, $p < 0.05$] and time-dependent effects [$F(4, 34) = 6.43$, $p < 0.05$] were revealed. Comparing the food intake between antisense/PPA-treated and PPA-treated rats, it revealed significant effects from Day 1 to Day 4. Furthermore, it also revealed significant effects from Day 1 to Day 4 if comparing between antisense/PPA-treated and missense-treated (control) rats. The feeding response in missense-treated rats was similar to that in saline-treated rats. Moreover, the anorectic response in missense/PPA-treated rats was not significantly changed when compared to that in PPA-treated rats. These results revealed the noninterference of missense treatment in this study and also revealed that PKCλ knockdown could modify the feeding responses in PPA-treated rats.

Effects of PPA on NPY and PKCλ gene expression

As described in our previous report (20), hypothalamic NPY contents were dose-dependently decreased on Day 1 and Day 2 but with a gradual return to normal levels on the following days. This change of NPY was expressed in a manner consistent with the alteration of NPY mRNA levels shown in the present study. Results shown in Figure 2 revealed that daily PPA (80 mg/kg) resulted in a decrease in NPY but an increase in aPKCλ expression. Statistical analysis with one-way ANOVA indicated a decrease of NPY mRNA contents [$F(4, 25) = 5.85$, $p < 0.05$] from Day 1 to Day 3 with a maximum decrease of about 40% on Day 2, but an increase of aPKCλ mRNA [$F(4, 25) = 4.78$, $p < 0.05$] from Day 1 to Day 3, with a maximum increase of about 200% on Day 2 as compared with the control group. Moreover, it revealed a significant increase of aPKCλ [$F(4, 25) = 4.12$, $p < 0.05$] from Day 1 to Day 3 with a maximum increase of about 200% on Day 2 as compared with the control group. These results revealed that the NPY gene was downregulated and expressed in a manner reciprocal to that of the aPKCλ gene, which was upregulated during PPA treatment.

Effects of aPKCλ antisense on NPY and aPKCλ mRNA levels

Results shown in Figure 3 revealed that aPKCλ antisense pretreatment could attenuate a PPA (80 mg/kg)-induced decrease in NPY mRNA levels and increase in aPKCλ mRNA levels. Using GAPDH as the internal standard, the ratio of NPY (or aPKCλ) mRNA over GAPDH mRNA in each group was calculated and compared. By one-way ANOVA followed by Dunnett's test ($p < 0.05$), it was revealed that NPY mRNA content decreased in both PPA-treated and antisense/PPA-treated rats compared with the control (missense-treated) group [$F(2, 18) = 3.72$, $p < 0.05$]. Moreover, there was a significant reverse of NPY mRNA content in antisense/PPA-treated rats compared with the PPA-treated group. These results revealed that PKCλ signaling was involved in the regulation of NPY gene expression in PPA-treated rats.

Statistical analysis revealed that aPKCλ mRNA content was increased in PPA-treated group but decreased in aPKCλ

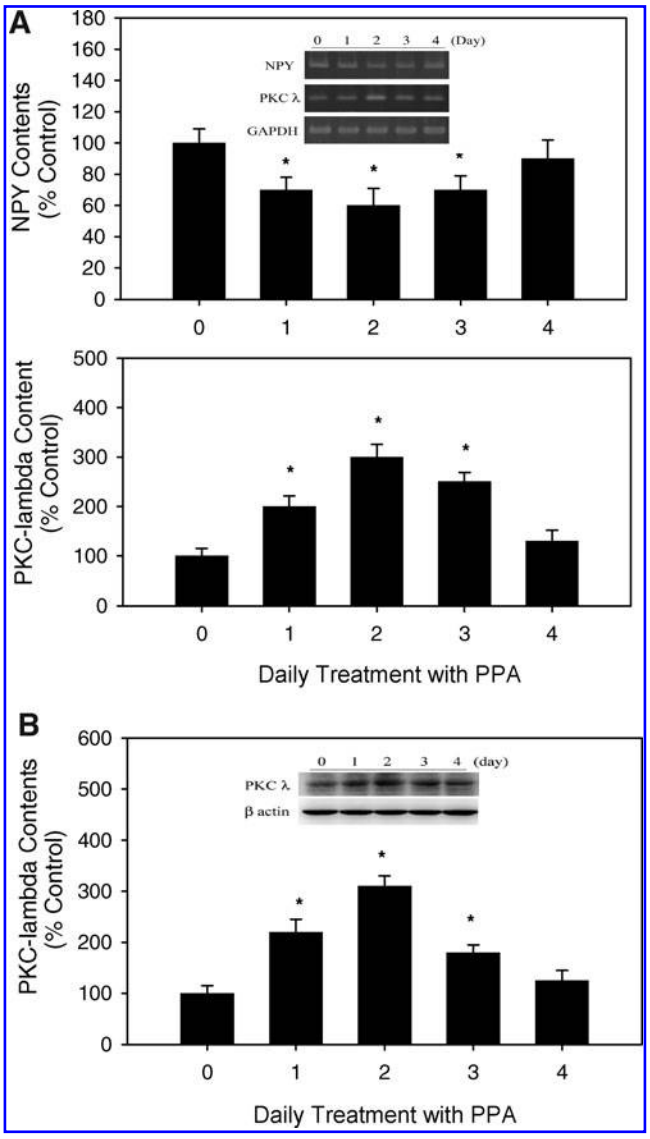

FIG. 2. Effects of daily PPA on hypothalamic NPY and aPKCλ mRNA levels over a 4-day period. (A) Results show relative densitometric values for RT-PCR products in PPA-treated and control groups. Contents of NPY and aPKCλ mRNA in PPA-treated group are indicated as the percentage of control (Day 0). Inserted gel: the result of RT-PCR analyzing mRNA levels of NPY and aPKCλ in stained ethidium bromide gels. **(B)** Results show relative densitometric values for Western blot of aPKCλ and NPY in control and PPA-treated groups. Contents of aPKCλ and NPY in PPA-treated groups are indicated as the percentage of the control group. *Bars* are mean ± SEM; $n = 6$–8 each group; *$p < 0.05$ vs. control. Inserted gel: the results of Western blot analyzing the contents of aPKCλ and NPY. *Bars* are mean ± SEM; $n = 6$–8 each group; *$p < 0.05$ vs. control. NPY, neuropeptide Y; PKC, protein kinase C.

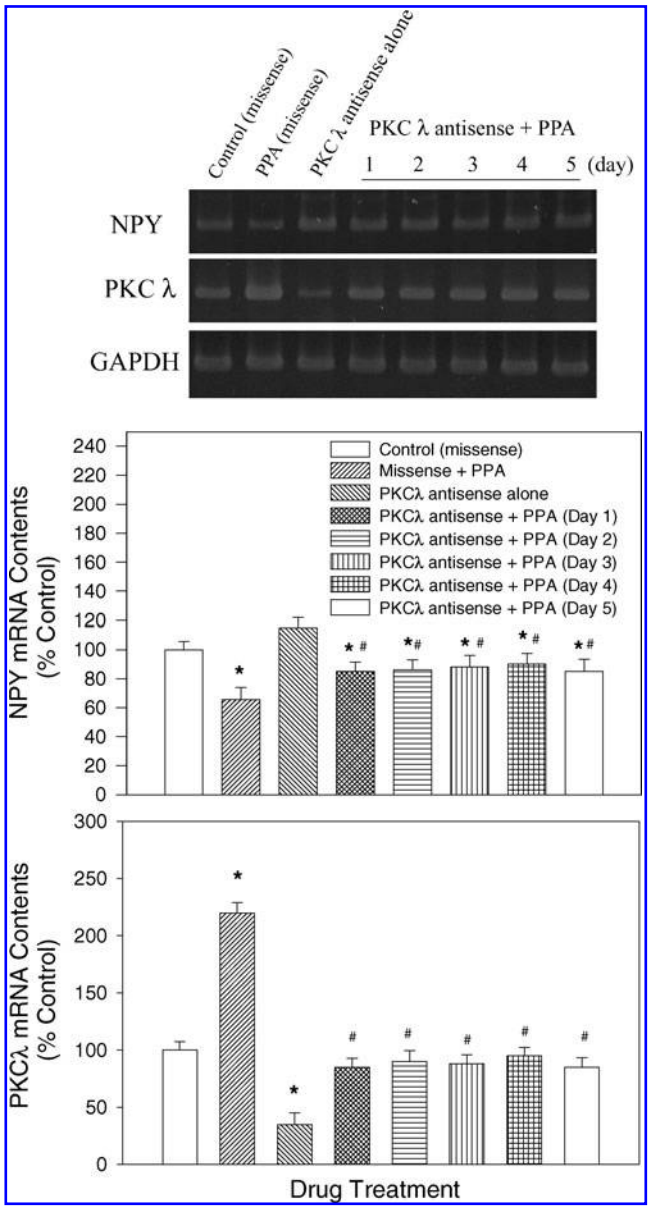

FIG. 3. The effect of aPKCλ antisense (or missense) pretreatment on daily PPA-induced changes of NPY and aPKCλ mRNA levels over a 5-day period. Daily missense or antisense treatments (20 μg/10 μl/day, ICV) were administered 1 h before daily PPA treatment; $^{*}p<0.05$ vs. the missense groups of each treatment day; $^{\#}p<0.05$ vs. the PPA-treated groups of each treatment day. *Bars* are mean ± SEM; $n=6$–8 per group.

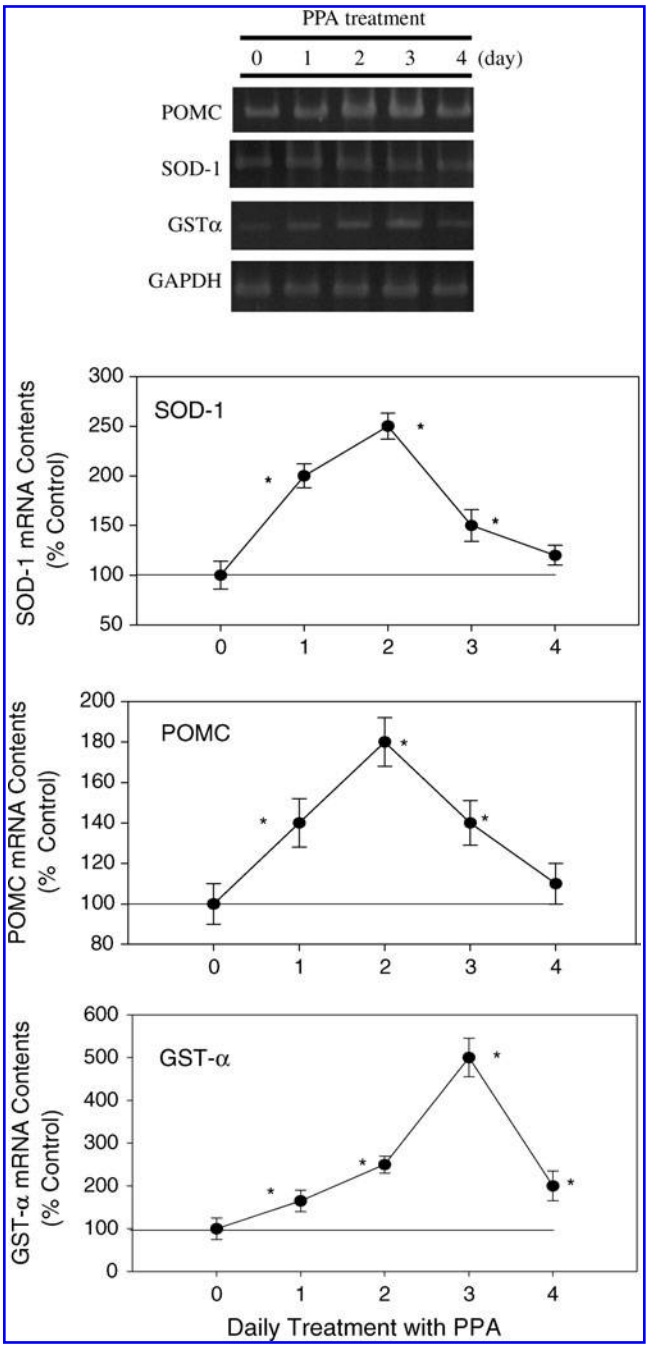

FIG. 4. Effects of daily PPA on hypothalamic POMC, SOD-1, and GST-α mRNA levels over a 4-day period. *Upper panel*: RT-PCR analysis of mRNA levels of NPY and aPKCλ in stained ethidium bromide gels. *Lower panels:* relative densitometric values for RT-PCR products in PPA-treated and control groups. Contents of POMC, SOD-1, and GST-α mRNA levels in PPA-treated group are indicated as the percentage of control (Day 0). *Bars* are mean ± SEM; $n=6$–8 each group; $^{*}p<0.05$ vs. control.

antisense-treated group as compared with the control group [$F(2, 18)=3.12$, $p<0.05$]. Moreover, a significant effect on aPKCλ mRNA content was also observed in antisense/PPA-treated rats as compared with the PPA-treated group. These results revealed that aPKCλ antisense could effectively inhibit PKCλ expression during PPA treatment.

Effects of PPA on POMC, GST, and SOD-1 mRNA levels

Results shown in Figure 4 revealed that daily PPA (80 mg/kg) resulted in significant increases in POMC, SOD-1, and GST-α mRNA levels. Using GAPDH as the internal standard, the ratio of POMC (or SOD-1 and GST-α) mRNA over GAPDH mRNA in each group was calculated and compared. Statistical analysis indicated significant increases of POMC mRNA [$F(4, 25)=5.85$, $p<0.05$], GST-α mRNA [$F(4, 25)=3.88$, $p<0.05$], and SOD-1 mRNA [$F(4, 25)=4.16$, $p<0.05$] from Day

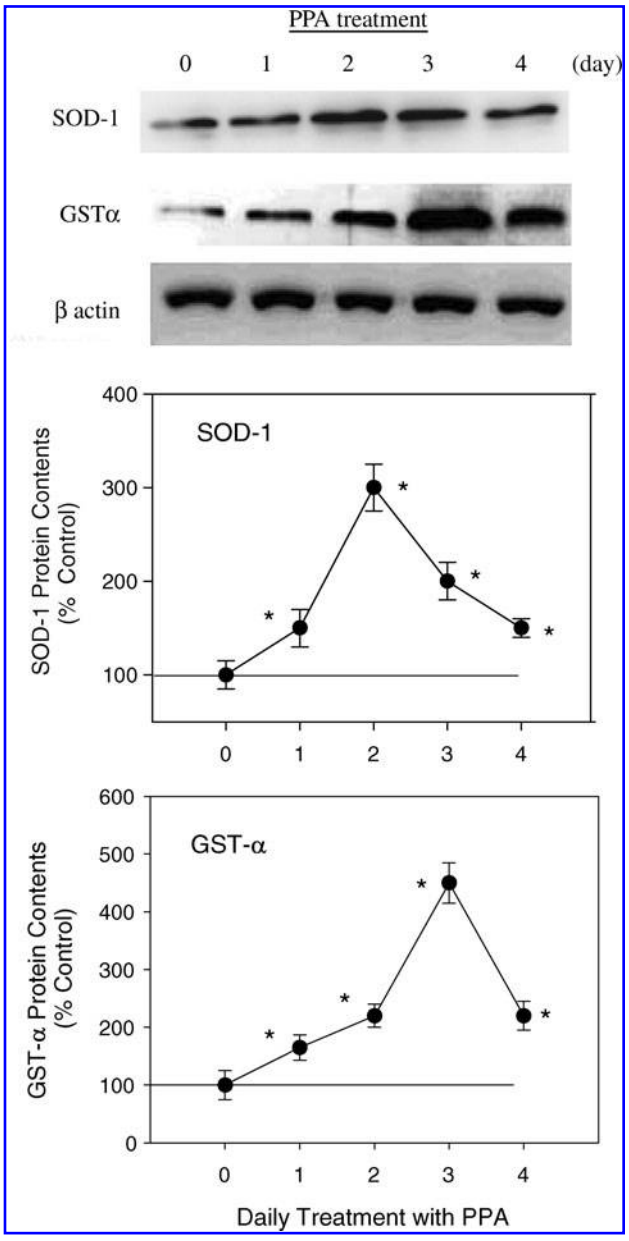

FIG. 5. Effects of daily PPA on hypothalamic SOD-1 and GST-α protein contents over a 4-day period. *Upper panel:* Western blot analysis of the contents of SOD-1 and GST-α. *Lower panels:* relative densitometric values for Western blot of protein contents in control and PPA-treated groups. Contents of protein in PPA-treated groups are indicated as the percentage of the control group. *Bars* are mean ± SEM; $n = 6$–8 each group; *$p < 0.05$ vs. control.

1 to Day 4 compared with the control. These results revealed that POMC, SOD-1, and GST-α mRNA levels were increased for 4 days with maximum increases of about 200% ~ 450% on Day 2 or Day 3, compared with the controls during PPA treatment.

Effects of PPA on SOD-1 and GST-α protein contents

As shown in Figure 5, daily PPA could increase SOD-1 and GST-α with a maximum increase of about 200% ~ 450% on Day 2 or Day 3 during a 4-day period of PPA treatment. Using β-actin as the internal standard, the ratio of SOD-1 (or GST-α) over β-actin in each group was calculated and compared. Analysis with one-way ANOVA revealed that all protein contents were increased from Day 1 to Day 4, comparing with the control group during PPA treatment. The statistical values were SOD-1 [$F(4, 25) = 3.75$, $p < 0.05$] and GST-α [$F(4, 25) = 4.16$, $p < 0.05$]. These results revealed that SOD-1 and GST-α were increased and expressed in a way similar to those of their own mRNA levels during PPA treatment.

Effects of aPKCλ antisense/PPA co-administration on POMC, SOD, and GST mRNA levels

Results shown in Figure 6 revealed that aPKCλ antisense/PPA co-administration could partially attenuate the levels of PPA-elevated POMC, SOD-1, and GST-α mRNA. Using GAPDH as the internal standard, the ratio of POMC (or SOD-1, GST-α) mRNA over GAPDH mRNA in each group was calculated and compared. By one-way ANOVA, it revealed that mRNA contents of POMC [$F(6, 35) = 3.82$, $p < 0.05$], SOD-1 [$F(6, 35) = 4.25$, $p < 0.05$], and GST-α [$F(6, 35) = 3.89$, $p < 0.05$] were increased in the PPA-treated and the antisense/PPA-treated groups as compared with the control group. Moreover, a partial reverse of POMC, SOD-1, and GST-α mRNA contents was observed in antisense/PPA-treated rats compared with the PPA-treated group. Results revealed that aPKCλ was involved in regulating POMC, SOD-1, and GST-α expressions in PPA-treated rats.

Effect of PPA on hypothalamic aPKCλ expression

Results of IHC staining shown in Figure 7 indicate a significant influence of PPA on the expression of aPKCλ. The expression of aPKCλ-immunoreactive particles (yellow-brown color) appeared to be increased in the region of ARC in rats receiving 105 mg/kg PPA (*t*-test, $p < 0.05$). In ARC, the number of aPKCλ-immunoreactive particles was digitally detected by the software (NIS- Elements BR 3.0) through the instrument of Digital Sigh DS-Fi1 system (Nikon Corporation, Japan). Results showed that aPKCλ immunoreactivity was increased about 350% in PPA-treated rat.

Effect of the pretreatment with GSH-EE on PPA-induced feeding

Results shown in Figure 8A reveal that pretreatment with GSH-EE before daily PPA (105 mg/kg) could attenuate a PPA-induced anorectic response. Statistical analysis with two-way ANOVA revealed significant dose-dependent [$F(3, 28) = 19.21$, $p < 0.05$] and time-dependent effects [$F(4, 35) = 5.76$, $p < 0.05$]. Moreover, it revealed that co-administration with GSH-EE/PPA attenuated the decreases of food intake from Day1 to Day 4 compared with the PPA-treated group. The expression of feeding in vehicle-treated rats was similar to that in saline-treated rats. Moreover, the expression of feeding in GSH-EE-treated rats remained unchanged compared to that in vehicle-treated rats, revealing the noninterference of vehicle in this study.

Results shown in Figure 8B indicate the picture and statistical result of reverse transcription-polymerase chain reaction (RT-PCR). By one-way ANOVA, it revealed that NPY mRNA contents [$F(6, 35) = 3.13$, $p < 0.05$] decreased in the

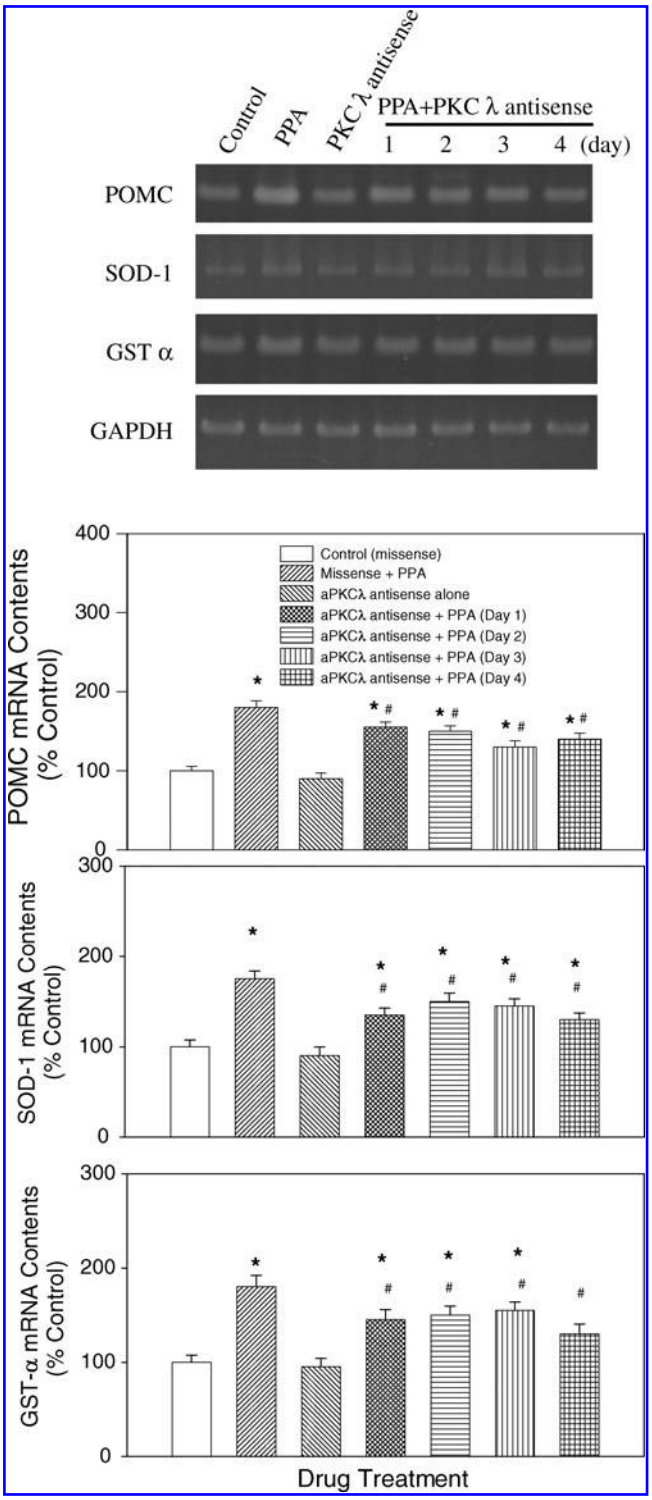

FIG. 6. Effects of daily aPKCλ antisense pretreatment on hypothalamic POMC, SOD-1, and GST-α mRNA levels in PPA-treated rats over a 4-day period. POMC, SOD-1, and GST-α mRNA levels were measured by RT-PCR. Contents of mRNA in drugs-treated rats are indicated as the percentage of control. *Bars* are mean ± SEM; $n = 6–8$ per group; $^{*}p < 0.05$ vs. control (missense alone); $^{\#}p < 0.05$ vs. missense/PPA-treated group.

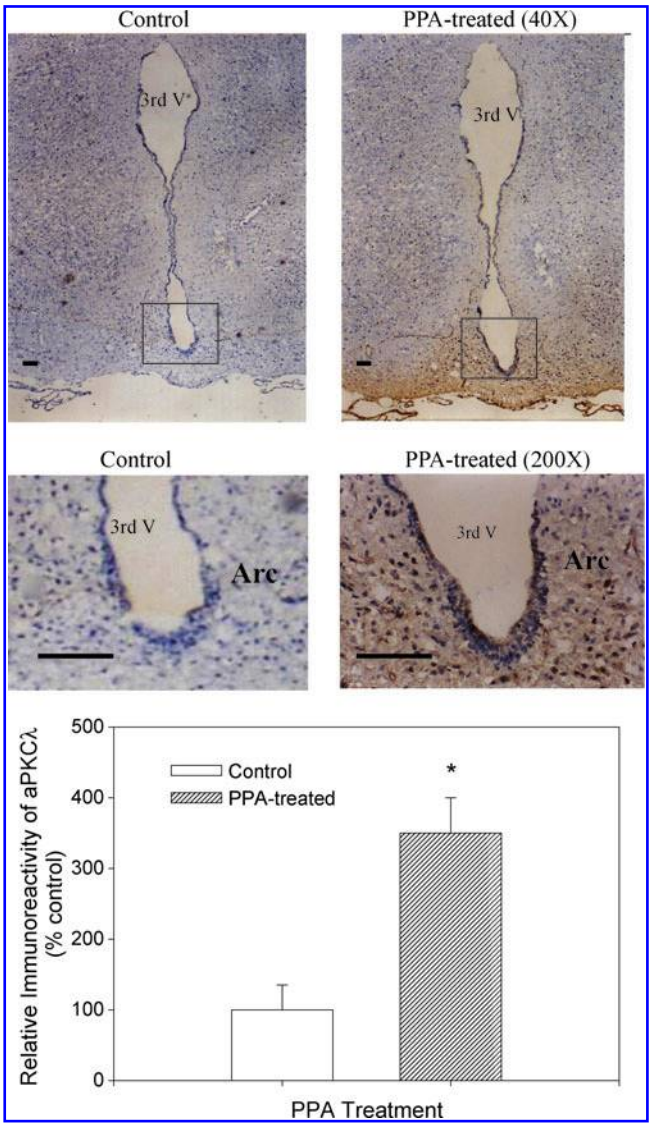

FIG. 7. Immunohistochimical characterization of aPKCλ particles in hypothalamus in rats receiving PPA (105 mg/kg) treatment. *Upper panels:* photomicrographs showing aPKCλ immunoreactivity (*yellow-brown color*) in rats receiving saline and PPA. *Middle panels*: the magnification (X5) of ARC observed in upper panels. *Lower panel*: relative densitometric values for aPKCλ-immunoreactive particles in arcuate nucleus (ARC) in PPA-treated rat comparing with the control group. Frontal sections through the ARC level were obtained. 3rd V: the third ventricle. *Scale bars* = 50 μm.

PPA-treated group but restored to normal in the GSH-EE/PPA-treated group, while SOD-1 mRNA contents [$F(6, 35) = 3.29$, $p < 0.05$] increased in the PPA-treated group but restored to normal in the GSH-EE/PPA-treated group. These results revealed that GSH-EE could restore the PPA-induced increase of SOD-1 and the decrease of NPY mRNA levels toward normal level.

Discussion

Several observations strongly support the involvement of ROS signaling in nutrient sensing in the hypothalamus (1, 2, 23). The present study found that daily PPA treatment

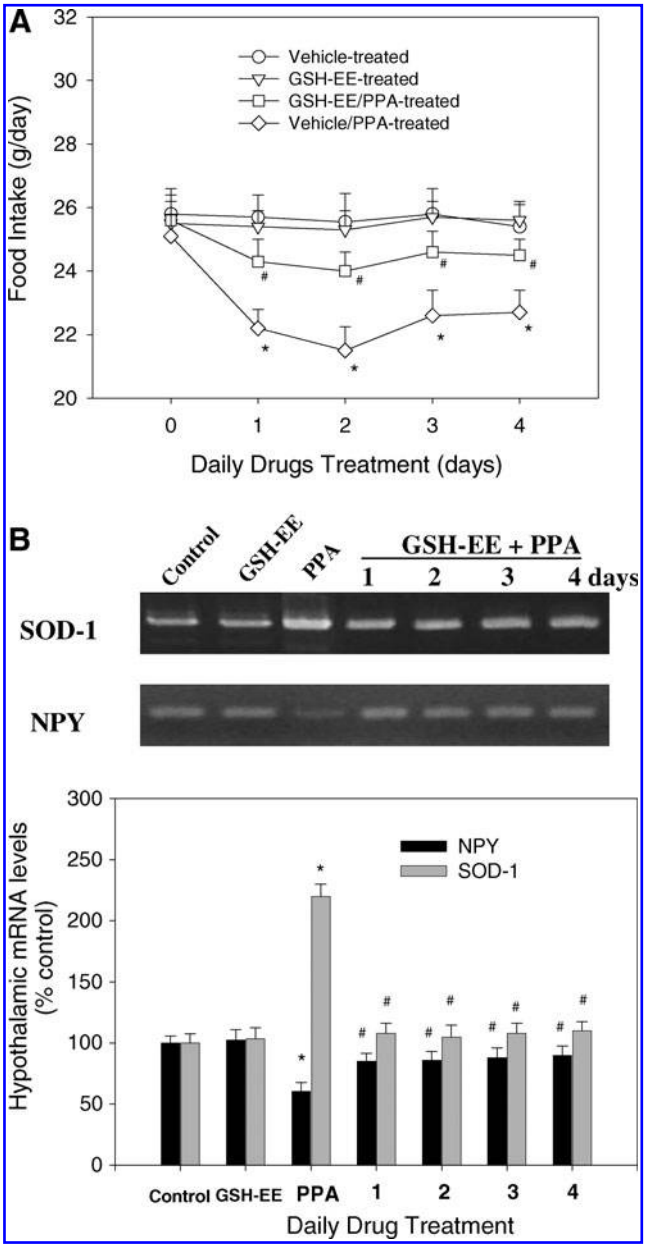

FIG. 8. (A) The effect of daily pretreatment with GSH-EE on PPA-induced feeding behavior and (B) results of RT-PCR showing the GSH-EE/PPA-induced changes of hypothalamic NPY and SOD-1 mRNA levels. GSH-EE, an ROS scavenger, was ICV administered at 40 min before PPA (105 mg/kg; IP) treatment; $^{*}p < 0.05$ vs. the control (vehicle-treated) groups; $n = 6$–8 per group; $^{\#}p < 0.05$ vs. the vehicle/PPA-treated group. GSH-EE, glutathione ethyl-ester, which is dissolved in ACSF solution.

promoted subtle ROS-RE elevation within the hypothalamus, which related to an increase of POMC activity and a decrease of NPY activity. Moreover, central inhibition of ROS production modulated the anorectic response of PPA and the expression levels of POMC and NPY mRNA. These results provide evidence to support the hypothesis that ROS signaling has an essential role in the regulation of POMC/NPY expression and PPA-induced appetite suppression.

Current results show that daily PPA administration could markedly reduce food intake and NPY expression during the initial 2 days (anorectic effect) and, in turn, reverse this effect gradually on the subsequent days, with food intake and NPY expression returning to normal (tolerant effect). Thus, NPY is involved in both the anorectic response of PPA, which was related to a decrease in NPY expression, and in the tolerant response of PPA, which was related to the restoration of NPY gene expression (21). Moreover, expression levels of ROS-RE, POMC, and aPKCλ during a 4-day period of PPA treatment were upregulated, with the maximum increase observed on Day 2 or Day 3. The manner of these expressions was almost opposite to that of the feeding response and NPY expression, which had the maximum decrease on Day 2. These results imply that ROS-RE, POMC, and aPKCλ might function in a manner opposite that of NPY during the regulation of PPA-induced feeding behavior.

The present findings reveal that ROS-RE participates in the regulation of PPA-induced feeding. Increasing evidence points to the significance of ROS activation during fuel utilization in the regulation of energy homeostasis in the hypothalamus (19, 24). In the ARC, mitochondrial uncoupling protein 2 may modulate the efficiency of metabolic processes, such as mediate ghrelin's action on NPY neurons by lowering free radicals (1, 19). Therefore, some nutrients targeting the inhibition of mitochondrial decay can prevent oxidative damage by increasing the endogenous antioxidant content (32). The present results revealed that ROS-RE, including GST-α and SOD-1, were activated and expressed in a manner concomitant with the expression of POMC but in a manner converse to that of NPY during PPA treatment. These results imply that ROS-RE/POMC- and NPY-containing neurons are expressed in a reciprocal manner to regulate appetite during PPA treatment. Studies have reported that POMC neurons can increase firing during the metabolic state of positive energy balance (or in satiety), while NPY neurons can increase firing during negative energy balance (or in hunger) (11). In the present study, rats treated with PPA reached a metabolic state similar to those in satiety during the first 2 days of drug treatment (*i.e.*, during the period of anorectic response) and, thus, could reduce food intake. On the subsequent days (*i.e.*, during the period of the tolerant response), the rats reached a metabolic state similar to those in hunger and, thus, could gradually reverse the amounts of food intake. Our current results revealed that the POMC and ROS-RE expression levels increased during the first 2 days of treatment but reversed gradually to normal during the subsequent days of PPA treatment. By contrast, NPY expression decreased during the first 2 days but gradually reversed toward normal during the subsequent days of PPA treatment. These results suggest that the induction of anorectic response of PPA related to the increases in the POMC and ROS-RE expression levels as well as to the decrease in NPY expression during the first 2 days of PPA treatment, while the gradual restorations of NPY, POMC, and ROS-RE expressions during the subsequent days were related to the induction of the tolerant response of PPA treatment. Further examination revealed that an ICV infusion of GSH-EE, which aimed to inhibit ROS formation, efficiently attenuated the anorectic response of PPA and restored the expression levels of SOD-1 and NPY to normal. This suggested that the activation of ROS-sensitive mechanisms sufficiently promotes satiety via the modulation of NPY gene expression.

The increased ROS-RE and POMC contents during the first 2 days of PPA treatment might relate to the decreased inhibitory action of NPY on POMC neurons. Some previous evidence suggests that NPY can inhibit POMC-containing neurons by releasing GABA (8) via a unidirectional input from NPY to POMC (17). Thus, the catecholamine released during PPA treatment might at first exert its inhibitory action on NPY neurons, which in turn increase POMC activity by a mechanism of disinhibition, finally resulting in a reduced food intake. Moreover, aPKCλ knockdown could modulate the response of feeding and the expression levels of ROS-RE, POMC, and NPY, suggesting the involvement of aPKCλ in the regulation of ROS-RE-, POMC-, and NPY-mediated PPA anorexia.

These findings suggest that the elevated aPKCλ level in the hypothalamus might be located in the area containing catecholamine-releasing neurons that were upstream of NPY- and POMC-containing neurons. In accordance with this view, a previous report indicated that aPKCs appear to mediate the dopaminergic enhancement of spike firing in the nucleus accumbens shell and may therefore play a critical role in the dopamine-dependent goal-directed behaviors (18). By contrast, this result contradicts some *in vivo* or *in vitro* data indicating that PKC activity localizing in NPY-like neurons mediates the intoxicating effect of ethanol in flies (5) and that PKC participates in the inhibition of dopamine receptor-mediated NPY release in PC12 cells (4). However, these experiments did not identify the PKC isotype, thus, making it difficult to correlate the results of these experiments with the present results. Ethanol can enhance ROS formation, which relates to alcohol-induced oxidative damage, neurodegeneration (16), and possibly brain injury (30).

In the present study, antisense aPKCλ partially interrupted the effects of PPA anorexia and NPY expression, suggesting that a component of an aPKCλ-independent pathway mediated the effect of PPA anorexia. nPKCδ and cPKCα signals also play roles in this effect in conscious rats (27, 28); therefore, co-activation of cPKCα, nPKCδ, and aPKCλ signals in the hypothalamus, probably including other protein kinase-mediated signaling, might have a synergistic role in modulating NPY gene expression in PPA-treated rats. This synergistic effect revealed an essential role and a complex modulation of NPY gene expression in the hypothalamus in conscious rats.

The activation of aPKCλ signaling might relate to the function of antioxidative stress mediated by ROS-RE. Current results revealed that SOD-1 and GST-α genes were upregulated, with maximum increases of approximately 250% and 450%, respectively, on Day 2 or Day 3 during PPA treatment. This pattern of ROS-RE gene expression resembled that of aPKCλ during PPA treatment. Moreover, aPKCλ knockdown during PPA treatment could downregulate SOD-1 and GST-α expression but upregulate NPY gene expression in the present study. Previous evidence revealed the enhanced activities of SOD and GST in the brain of curcumin-treated rats, which show protective effects against aluminum-induced toxicity (49). Curcumin, a yellow pigment extracted from the rhizome of the plant, can exert a protective effect by upregulating the activity of antioxidant enzyme (49). Moreover, the aPKCλ isotype is involved in the oxidative stress-mediated nuclear translocation of Nrf2 (38). Thus, aPKCλ-mediated activation of ROS-RE may have possibly involved in the reduction of oxidative stress, which would favor the restoration of NPY gene expression and feeding behavior.

As signals of cPKCα, nPKCδ, and aPKCλ are all involved in the NPY-mediated anorectic action of PPA, Ca^{2+} and DAG might be necessary for the induction of NPY-mediated PPA anorexia. Furthermore, the activation of PKC was isotype-specific during PPA treatment because only one isotype (α, δ, or λ) from among cPKC, nPKC, and aPKC was involved in PPA anorexia, while other isotypes (such as β, γ, ϵ, etc.) were expressed differently (27). This result agrees with a previous report indicating that cPKC, nPKC, and aPKC could all respond to ethanol stimulation in vascular smooth muscle cell during the initial phase of activation of oxidative stress-related enzymes (25).

The expression of aPKCλ immunoreactivity in the area of the ARC increased following PPA treatment. This result might support our view on the involvement of aPKCλ signaling in the regulation of NPY- and POMC-mediated appetite suppression because both NPY- and POMC-ergic pathways originate in the ARC and project into other areas in the hypothalamus (55). Thus, ICV injections of antisense aPKCλ could effectively interrupt the actions of aPKCλ, NPY, and POMC in the present study. Consistent with the present result, the literature has reported the expression of aPKCλ in the hypothalamic ARC and paraventricular nuclei based on immunohistochemical staining and that the hypothalamic aPKCλ signaling plays a key role in food intake inhibition induced by lipopolysaccharide, but not leptin and insulin; therefore, ICV administration with aPKCλ inhibitor can abolish this effect (53). Redox stress may play some roles in leptin and insulin signaling. Leptin can reduce appetite by inhibiting the antioxidant system in the mouse brain (29), while it can protect neurons against cell death by inducing the production of SOD-2 and lessening mitochondrial oxidative stress (15). The anorexigenic effect of insulin requires an increase of hypothalamic ROS (24).

PKCs are sensitive to redox stress because the modification of their redox-sensitive regions interferes with their biological effects; therefore, PKCs are logical candidates for redox modification by oxidants and antioxidants. Moreover, the intracellular redox state can control the selectivity of PKC isoform activation (13). The present study found that the change of ROS-RE was consistent with that of aPKCλ during PPA treatment, and both ROS-RE and aPKCλ participated in the appetite-suppressing effect of PPA. Moreover, aPKCλ knockdown could interfere with the activation of ROS-RE, which could modulate the appetite-suppressing effect of PPA. Furthermore, the inhibition of ROS production could modulate NPY expression, which was related to the change of aPKCλ during PPA treatment. These results revealed the essential role of ROS in the modulation of aPKCλ signaling during the regulation of appetite-suppressing effect of PPA.

A recent report suggested that the routine preclinical toxicology studies should initially precede current strategies in the development of anti-obesity drugs and their safety concerns (10). Our results provide the preclinical toxicology evidence that hypothalamic ROS-RE, including SOD-1 and GST-α, participates in the anorectic effect of PPA. Although the molecular mechanisms are more evident, PPA may not prove an ideal anti-obesity drug due to the side effects of this compound. PPA, now withdrawn from the market, had the risks of myocardial injury and hemorrhagic stroke (33, 44). The risks are possibly related to

the increase of ROS in injured tissues. The present result that the inhibition of ROS production can modulate the behavioral response of PPA implies possible modulation of the ROS-sensitive pathway under certain physiological conditions. Thus, this finding might prove helpful in furthering the understanding and development of amphetamine-like anti-obesity drugs.

Conclusions

The present results suggest that ROS signaling plays an essential role in the induction of the appetite-suppressing effect of PPA, which may be reciprocally regulated by POMC- and NPY-containing neurons, while cerebral aPKCλ signaling participates in this effect by stimulating POMC/ROS-RE activity and inhibiting NPY neurotransmission.

Materials and Methods

Animal treatments

Male rats of the Wistar strain, with a weight of 200 $\sim$300 g, were obtained from the National Laboratory Animal Center. They were housed individually in cages, maintained at a temperature of 22 $\pm$ 2°C, in a room with a 12-hour light-dark cycle (light on at 6:00 AM), and habituated to frequent handling. The administration of drugs and the checking of food intake were performed every day at the beginning of the dark phase (6:00 PM). All procedures were 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 the National Science Council, Taiwan, ROC.

To investigate the effect of daily treatment of PPA ($\pm$-phenylpropanolamine) on feeding behavior, rats (n = 6–8 for each group) were injected intraperitoneally (IP) with the drug at doses of 0, 40, 80, or 105 mg/kg daily for 4 days. The first time PPA was injected was at the end of Day 0 (*i.e.,* at the beginning of Day 1) and the intake data was calculated with respect to the food amount of the previous day.

To determine the effect of daily PPA (0 or 80 mg/kg; i.p.) on hypothalamic NPY, POMC, aPKCλ, SOD-1, and GST-α mRNA levels, rats were injected with the drug for 1, 2, 3, or 4 days, depending on the group. Rats were divided into five groups (n = 6–8 for each group) according to the day they were to be sacrificed. On the day of sacrifice, rats received a treatment of 80 mg/kg PPA to enhance the effect of the drug. Following the decapitation, the hypothalamus was removed to determine mRNA levels.

Studies to assess the effects of ICV injections of aPKCλ antisense on PPA (105 mg/kg)-induced anorexia and on the changes of mRNA levels of NPY, POMC, aPKCλ, SOD-1, and GST-α were described in the appropriate sections.

To examine the effect of PPA on the expression of aPKCλ immunoreactivity, the rats were given PPA (0 or 105 mg/kg; n = 6–8) once a day for 2 days. Forty minutes after the second day of PPA treatment, the rats were sacrificed under ether anesthesia by transaortic perfusion with a fixative solution as described above.

To determine the effects of glutathione ethyl-ester (GSH-EE) on the anorectic response of PPA and on the changes of NPY and SOD-1 mRNA levels, rats (n = 6–8 for each group) were infused daily with GSH-EE (20 μl in concentration of 1 μmole/l; ICV; the infusion rate was 4 μl/min) 40 min before the daily PPA treatment (105 mg/kg; IP) for 4 days. GSH-EE is a potent ROS scavenger that is particularly effective in restoring the mitochondrial glutathione redox state to a reduced state (GSH) (2).

Drugs, chemicals, and reagents

Chow (LabDiet) was purchased from PMI Nutrition International (Brentwood, MO). PPA, Tris-HCl solution, angiotensin II, ethidium bromide, and GSH-EE were purchased from Sigma-Aldrich (St. Louis, MO); antibodies against aPKCα, β-actin, and SOD-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while GST-α was purchased from Gibco BRL, Life Technologies, Inc., (Rockville, MD). RT-PCR was processed using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany). TRIZOL reagent (Life Technologies, Inc., Carlsbad, CA) was used in tissue homogenization. Agarose gel was purchased from Difco (Detroit, MC). Antisense and DNA primer were synthesized by Proligo Pty Ltd (Singapore).

RNA extraction

Hypothalamic NPY, POMC, aPKCα, SOD-1, and GST-α mRNA levels were measured in a block of mediobasal hypothalamic tissue as described previously (35). Total RNA was isolated from the hypothalamus using a modified guanidinium thiocyanate-phenol-chloroform method (7). Each hypothalamic block was homogenized in 1 ml of TRIZOL reagent using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, CT). Other steps were referred to in our previous report (28).

Semiquantification of RT-PCR

Using the 1st Strand cDNA Synthesis Kit, RNA was reversely transcribed into single-stranded cDNA. Other steps were referred to in our previous report (28). PCR was subsequently carried out by mixing 3 μl of cDNA with the mastermix solution consisting of DEPC water, 10x reaction buffer, $MgCl_2$ (25 m*M*), deoxynucleotide mix (10 m*M* 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 (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA) with the following steps: 91°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 30 sec (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 for changes in two steps (annealing and cycles) that were described as follows: aPKCλ, 60°C, 28), SOD-1 (60°C, 25), POMC (55°C, 25), GST-α (57°C, 27), and GAPDH (52°C, 25). Sequences of primers used in this experiment are shown in Table 1.

Gel electrophoresis

After RT-PCR, 8 μl of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 μg/ml) were visualized under UV light, photographed, and then scanned densitometrically (Hoefer, San Francisco, CA). Ratios of NPY and GAPDH mRNA for each treatment day were calculated to

Table 1. Summary of Primer Sequences used in RT-PCR

Genes	*Primer*	*Sequence 5′→3′*	*Size of product (base pairs)*
NPY	Forward Reverse	GGGCTGTGTGGACTGACC GGAAGGGTCTTCAAGCCT	264
aPKCλ	Forward Reverse	ATACCGCGCTCCCTGTCTGTGAAA ATGCTAACTGTGACCGGGCTAACG	408
SOD-1	Forward Reverse	GAG CAT GGGTTCCATGTCCAT ACTTTCTTCATT TCCACCTTTGCC	279
GST-α	Forward Reverse	CAGGAGTGGAGT TTGAAGAGA AGAGGGAAAGAGGTCAGAAG	443
POMC	Forward Reverse	GGCCTTCCCCCTGGAGTTCA TTGATGATGGCGTTCTTGAA	742
GAPDH	Forward Reverse	TCCCTCAAGATTGTCAGCAA AGATCCACAACGGATACATT	309

determine relative NPY mRNA levels. Contents of NPY mRNA in PPA-treated group were indicated as the percentage of the control group. Similar steps were used to determine the contents of SOD-1, GST-α, and aPKCλ mRNA of each isotype.

Lateral ventricular cannulation

Stereotaxic surgery (Kopf Model 900, Tujunga, CA) for each rat was performed under anesthesia with pentobarbital (30 mg/kg, IP). 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 $\sim$ 4.0 mm below the dura) (42). Other steps were referred to in our previous report (28). For all experiments, verification of cannula placement was done by histochemistry or by the administration of angiotensin II (100 ng/rat). Angiotensin II reliably induces water drinking in nondeprived rats when administered into the ventricles (44). Only data from rats drinking more than 10 ml within 30 min were included in this study.

ICV injection of antisense ODN

To determine the effect of aPKCλ antisense on the anorectic response of PPA, rats (n = 6–8 for each group) were injected daily with missense or antisense (20 μg in a 10-μl vehicle; ICV) at 1 h before the daily treatment of PPA (105 mg/kg; IP) for 4 days. Before PPA treatment, rats were ICV injected with similar dose of missense or antisense daily for 2–3 days until the response of feeding behavior was slightly reduced in the antisense group. This is due to the fact that either continuous or repeated ICV injections of antisense may be necessary to maximize the behavioral effect and especially to block the synthesis of the constitutively active gene product (39, 62). A 20-mer antisense oligodeoxynucleotide complementary to the translation initiation region of mRNA specific for PKCι/λ (36) was designed. The sequences were: antisense, 5′-CAA AAGAGGATTGATATACT-3′, and missense, 5′-AGTATA TCAATCCTCTTTTG-3′. 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 the brain region and was regarded as a well-established agent in several vertebrate systems (12, 39, 57). Both antisense and missense S-ODN were dissolved in artificial cerebrospinal fluid (ACSF) containing 140 m*M* NaCl, 3.35 m*M* KCl, 1.15 m*M* $MgCl_2$, 1.26 m*M* $CaCl_2$, 1.2 m*M* Na_2HPO_4, 0.3 m*M* NaH_2PO_4, pH 7.4.

Another experiment was designed to determine the effect of pretreatment with aPKCλ antisense (or missense) on the expression of NPY, POMC, SOD-1, GST, and aPKCλ mRNA levels in PPA-treated rats. Rats (n = 6–8) were treated daily with antisense or missense 1 h before the daily PPA treatment (105 mg/kg; IP) for 4 days. Forty minutes after PPA treatment, the hypothalamus was removed from the brain and its NPY, POMC, SOD-1, GST-α, and aPKCλ mRNA contents were determined by RT-PCR.

Western blot analysis

To determine the effect of PPA on the expression of NPY, SOD-1, GST-α, and aPKCλ, rats were treated daily with PPA (80 mg/kg; n = 6–8 each group) for 4 days. At 40 min after PPA treatment, the hypothalamus was removed to determine the protein contents by Western blotting. Protein samples extracted from the hypothalamus tissues were separated in a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then incubated separately with specific antibodies of aPKCλ, GST-α, β-actin, and SOD-1. Other steps were referred to in our previous report (28).

IHC

The rats were sacrificed under ether anesthesia by transaortic perfusion with 50 ml heparinized saline, and then treated with 250 ml of fixative (4% paraformaldehyde in 0.1 *M* phosphate buffer; pH 7.4). Brains were removed and immersed immediately in the fixative for 90 min, washed in 0.1 *M* phosphate buffer saline (PBS), and then placed in 0.1 *M* PBS containing 30% sucrose at 4°C for 48 h. Coronal sections (10 μm thick) prepared by cryostat were collected in gelatin-coated slides and stored at −80°C until further use. Brain sections were processed for IHC using the indirect immunoperoxidase procedure as described previously (50). Sections were washed in 0.01% PBS, incubated with 0.2% hydrogen peroxidase (20 min), washed in 0.01% PBS, and then incubated with blocking solutions (Vector Laboratories, Inc., Burlingame, CA) at a 1:100 dilution for 30 min. Sections were incubated with goat aPKCλ antibody (1:500; Santa Cruz Bio-

technologies) at 4°C for 24 h. Other steps were referred to in our previous report (20).

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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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ACSF = artificial corticospinal fluid
ANOVA = analysis of variance
ARC = arcuate nucleus
DAG = diacylglycerol
DEPC = diethyl pyrocarbonate
GAPDH = glyceraldehydes-3-phosphate dehydrogenase
GSH-EE = glutathione ethyl-ester
GST = glutathione S-transferase
ICV = intracerebroventricle
IHC = immunohistochemistry
IP = intraperitoneally
NPY = neuropeptide Y
ODN = oligodeoxynucleotides
PKC = protein kinase C
POMC = pro-opiomelanocortin
PPA = phenylpropanolamine
ROS-RE = reactive oxygen species-related enzymes
RT-PCR = reverse transcription-polymerase chain reaction
SOD = superoxide dismutase
S-ODN = phosphorothioate oligodeoxynucleotides

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