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# Protein kinase Cδ contributes to phenylephrine-mediated contraction in the aortae of high fat diet-induced obese mice

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ABSTRACT

The down-regulation of α-adrenoceptor-mediated signaling casacade has been implicated in obesity but the underlying mechanism remains largely unknown. The present study investigated whether inositol 1,4,5-trisphosphate (IP3) receptor and protein kinase C (PKC) were involved in the reduction of $\alpha_1$-adrenoceptor agonist phenylephrine-evoked contraction in aortae of high fat diet-induced obese (DIO) mice. C57BL/6 mice were fed with a rodent diet containing 45 kcal% fat for 16 weeks to induce obesity. Isolated mouse aortae were suspended in myograph for isometric force measurement. Protein phosphorylations and expressions were determined by Western blotting. In C57BL/6 mouse aortae, phenylephrine-induced contraction was partially inhibited by either IP3 receptor antagonist heparin or PKC inhibitor GFX, and the combined treatment with heparin and GFX abolished the contraction. Phenylephrine-induced contraction was significantly less in the aortae of DIO mice than those of control mice; only GFX but not heparin attenuated the contraction, indicating a diminishing role of IP3 receptor in DIO mice. Western blotting showed the reduced expression and phosphorylation of IP3 receptor and the down-regulated expression of PKC, PKCβ, PKCδ, and PKCζ in DIO mouse aortae. Importantly, PKCδ was more likely to maintain phenylephrine-mediated contraction in DIO mouse aortae because that (1) PKCδ inhibitor rottlerin but not PKCα and PKCβ inhibitor Gö6976, PKCβ inhibitor hispidin, or PKCζ pseudosubstrate inhibitor attenuated the contraction; and (2) PKCδ phosphorylation was increased but phosphorylations of PKCα, PKCβ, and PKCζ were reduced in DIO mouse aortae. The present study thus provides additional insights into the cellular mechanisms responsible for vascular dysfunction in obesity.



## 1. Introduction

Obesity is a situation with excess fat deposit in the body because of an imbalance of energy intake and expenditure [1]. Obesity is an important risk factor for the development of type 2 diabetes, atherosclerosis, and hypertension [2–5], leading to increased cardiovascular morbidity and mortality [6–8]. Both animal and clinical studies indicate that obesity is closely associated with vascular dysfunction [9–12].

In 1993, Ratz et al. reported that α-adrenoceptor-mediated vascular response is selectively down-regulated in renal arteries of obese hypertensive dog [13]. Subsequent studies demonstrate that the phenylephrine ($\alpha_1$-adrenoceptor agonist)-mediated contractions are reduced in mesenteric arteries from ob/ob mouse [14], old obese Zucker rats [9], obese spontaneously hypertensive rat [15], and in aortae from renovascular hypertensive rats [16] in comparison with arteries from respective control animals.

$\alpha_1$-Adrenoceptor activation results in a rapid breakdown of phosphatidylinositol 4,5-bisphosphate, leading to the formation of the two intermediate intracellular second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 stimulates IP3 receptor on the sarcoplasmic reticulum to trigger $Ca^{2+}$ release into cytosol while diacylglycerol activates protein kinase C (PKC), thus jointly increasing the force and sensitivity of vascular smooth muscle contraction [17–19]. The present study therefore investigated whether alterations of expression and activity of IP3 receptor or PKC contributed to the reduced contraction induced by phenylephrine in the aortae of high fat diet-induced obese (DIO) mice.

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## 2. Materials and methods

### 2.1. Chemicals

Anti-phospho-PKCα (Thr638), anti-phospho-PKCδ (Thr505), anti-phospho-PKCζ (Thr410), anti-PKCα, anti-PKCβ, anti-PKCδ, anti-PKCζ, and anti-PKC antibodies were obtained from Bioword Technology (Louis Park, MN, USA). Anti-phospho-IP3 Receptor (Ser1756) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IP3 Receptor antibody was purchased from Abcam (Cambridge, MA). Anti-GAPDH antibody was obtained from Ambion (Austin, TX). HRP-conjugated swine anti-rabbit and anti-mouse IgG were from DakoCytomation (Carpinteria, CA, USA). Immobilon-P polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA, USA) and chemiluminescence (ECL reagents) was obtained from Amersham Pharmacia (GE Healthcare Life Sciences, Buckinghamshire, UK). Phenylephrine, heparin, and Gö6976 were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). GF 109203X (GFX), and rottlerin were from Tocris Bioscience (Bristol, UK). Hispidin, PKCζ pseudosubstrate inhibitor, and anti-phospho-PKCβI (Thr642) antibody were from Calbiochem (Merck Millipore International, USA). Phenylephrine and heparin were dissolved in distilled water. Other drugs were dissolved in DMSO. DMSO (0.1% v/v) did not modify phenylephrine-induced contraction.

### 2.2. Animal protocols

The present study was approved by the Animal Research Ethics Committee of Chinese University of Hong Kong and the Animal Care and Use Review Committees of Peking University Health Science Center. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male C57BL/6 mice were fed with a rodent diet containing 45 kcal% fat (Research Diets Inc., New Brunswick, NJ, USA) for 16 weeks starting from 6 weeks old to generate obesity (DIO).

### 2.3. Isometric force measurement

Mice were sacrificed by $CO_2$ suffocation. Aortae from mice were removed and placed in ice–cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, and 11 D-glucose. Aortae were cleaned of adhering tissue and cut into ring segments of 2 mm in length and suspended in myograph (Danish Myo Technology, Aarhus, Denmark) for recording of changes in isometric tension. To visualize the contractions, rings were exposed for 30 min to heparin (IP3 receptor antagonist, 10 mg/ml), GFX (PKC inhibitor, 2 μmol/L), Gö6976 (inhibitor of PKCα and PKCβ, 1 μmol/L), hispidin (PKCβ inhibitor, 10 μmol/L), rottlerin (PKCδ inhibitor, 10 μmol/L), and PKCζ pseudosubstrate inhibitor (PS-PKCζ, 10 μmol/L) before the application of phenylephrine (0.003–10 μmol/L).

### 2.4. Western blot analysis

Isolated aortae were homogenized in RIPA lysis buffer containing 1 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium fluoride, and 2 μg/mL β-glycerolphosphate. The homogenated were centrifuged at 20,000*g* for 20 min at 4 °C. Protein lysates (15 μg) were separated by electrophoresis and transferred onto PVDF membrane. Blots were blocked with 1% bovine serum albumin or 5% non-fat milk for 1 h and incubated overnight at 4 °C with primary antibodies. After washing, blots were incubated with HRP-conjugated swine anti-rabbit or anti-mouse IgG.

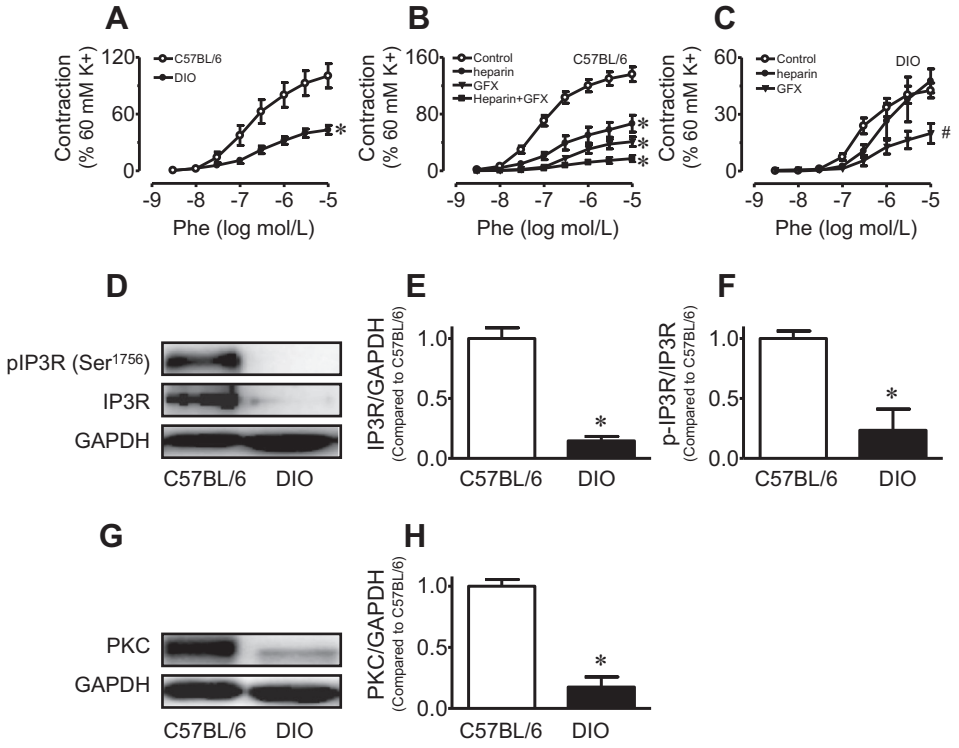


**Fig. 1.** IP3 receptor and PKC signaling were impaired in DIO mouse aortae. (A) Phenylephrine-induced aortic contraction was reduced in DIO mice compared to C57BL/6 mice. Effects of IP3 receptor antagonist heparin (10 mg/ml, 30 min) and PKC inhibitor GF 109203X (GFX, 2 μmol/L, 30 min) on phenylephrine-induced contraction in aortae from C57BL/6 (B) and DIO mice (C). (D–F) The phosphorylation and expression of IP3 receptor (IP3R) in mouse aortae. (G, H) PKC expression in mouse aortae. $^{*}P<0.05$ vs C57BL/6 control; $^{\#}P<0.05$ vs DIO control. Results are means ± SEM ($n = 4–8$).

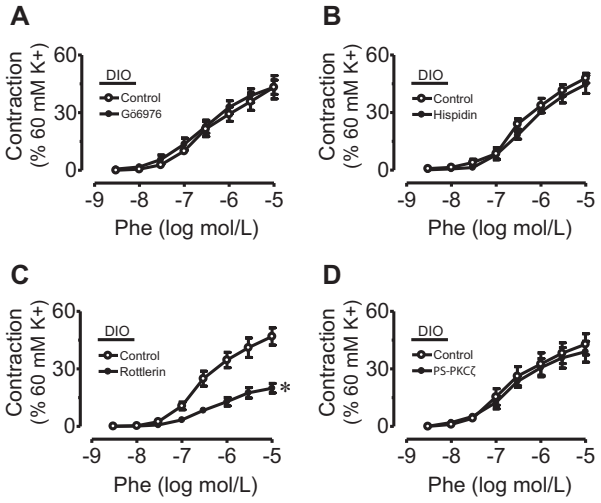


**Fig. 2.** Effects of PKC inhibitors on phenylephrine-induced contraction in DIO mouse aortae. Effects of PKCα and PKCβ inhibitor Gö6976 (1 μmol/L, 30 min) (A), PKCβ inhibitor hispidin (10 μmol/L, 30 min) (B), PKCδ inhibitor rottlerin (10 μmol/L, 30 min) (C), and PKCζ pseudosubstrate inhibitor (PS-PKCζ, 10 μmol/L, 30 min) (D) on phenylephrine-induced contraction in DIO mouse aortae. $^{*}P < 0.05$ vs control. Results are means ± SEM ($n = 4$).

Immunoreactive bands were visualized by chemiluminescence and exposed to Kodak Image Station 440 for densitometric analysis.

### 2.5. Statistical analysis

The contraction was expressed as percentage of 60 mmol/L KCl-induced tension. Results are means ± SEM and n represents aortae from different mice. Statistical significance was determined by two-tailed Student's *t*-test and nonparametric test. $P < 0.05$ was considered significantly different.

## 3. Results

### 3.1. IP3 receptor signaling was impaired in aortae of diet-induced obese mice

Phenylephrine induced significantly less contractions in aortae from DIO mice than those from C57BL/6 control mice (Fig. 1A). Since phenylephrine-induced contraction is mediated by $Ca^{2+}$ release from IP3-stimulated sarcoplasmic reticulum $Ca^{2+}$ stores [18] and PKC activation in vascular smooth muscle [19], we examined the effects of IP3 receptor antagonist heparin and PKC inhibitor GFX on the contraction. As shown in Fig. 1B, phenylephrine-induced contraction was markedly inhibited by either heparin or GFX but nearly abolished by a combined treatment of the two inhibitors in C57BL/6 mouse aortae. However, phenylephrine-induced contraction in DIO mouse aortae was only inhibited by GFX but not by heparin (Fig. 1C). The expressions of IP3 receptor (Fig. 1D and E) and PKC (Fig. 1G and H) were down-regulated in aortae from DIO mice compared to those from C57BL/6 mice. In addition, the phosphorylation of IP3 receptor was also reduced in

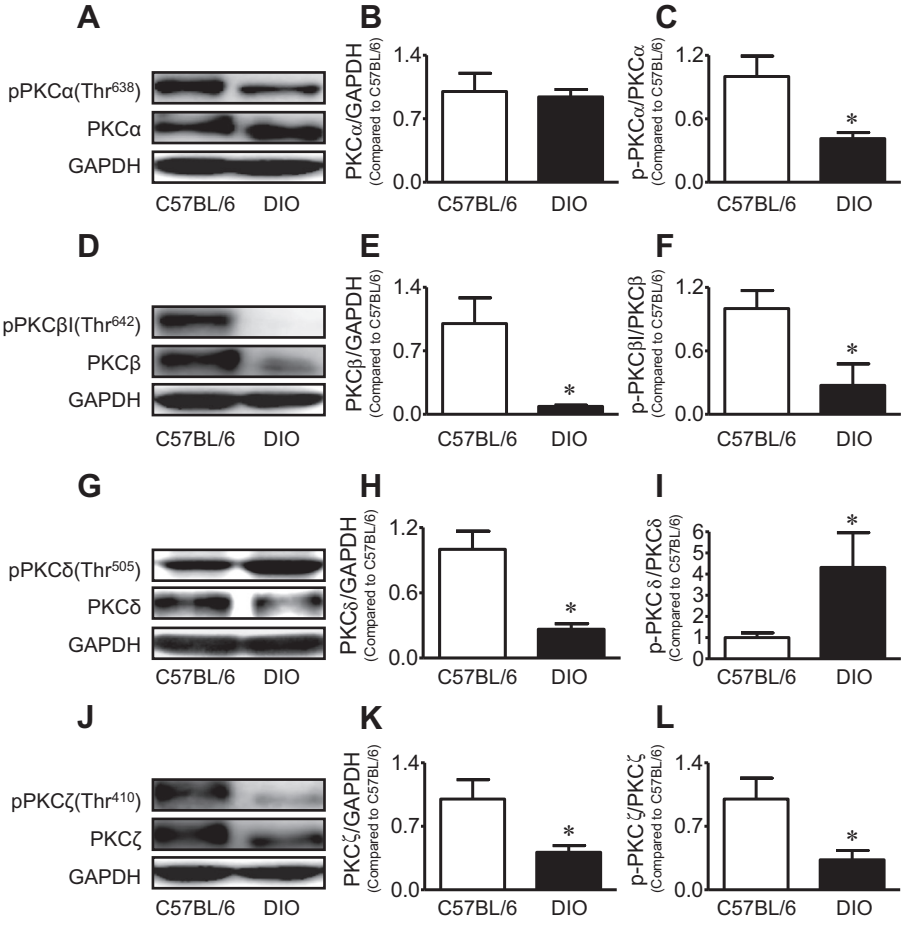


**Fig. 3.** The phosphorylations and expressions of PKC isoforms in mouse aortae. Phosphorylations and expressions of PKCα (A–C), PKCβ (D–F), PKCδ (G–I), and PKCζ (J–L) in the aortae from C57BL/6 and DIO mice. $^{*}P < 0.05$ vs C57BL/6. Results are means ± SEM ($n = 4$).

DIO mouse aortae (Fig. 1D and F). Taken together, these results suggest that IP3 receptor and PKC signaling were likely impaired and PKC might play a greater role in phenylephrine-induced contraction in aortae of DIO mice.

### 3.2. Phenylephrine-induced contraction was most likely mediated by PKCδ in DIO mouse aortae

PKC increases vascular smooth muscle $Ca^{2+}$ sensitivity, thus enhancing muscle contraction [18,20]. PKCα and PKCθ participate in phenylephrine-induced smooth muscle contraction of the rat cavernosum [21]. Since phenylephrine-induced contraction was mostly mediated through PKC activation in DIO mouse aortae (Fig. 1C), we next used various pharmacological inhibitors of PKC isoforms to examine which isoforms were the significant contributors. We found that phenylephrine-induced contraction was unaffected by PKCα and PKCβ inhibitor Gö6976 (1 μmol/L, Fig. 2A), PKCβ inhibitor hispidin (10 μmol/L, Fig. 2B), or PKCζ pseudosubstrate inhibitor (PS-PKCζ, 10 μmol/L, Fig. 2D) in DIO mouse aortae. By contrast, PKCδ inhibitor rottlerin (10 μmol/L, Fig. 2C) significantly attenuated the contraction. The present data indicate that PKCδ is likely to be the major isoform to mediate phenylephrine-induced aortic contraction in DIO mice.

### 3.3. The altered phosphorylation and expression of PKC isoforms in mouse aortae

To further confirm the primary role of PKCδ, we determined the levels of both phosphorylation and expression of various PKC isoforms in mouse aortae. First, the PKCα expression was similar in aortae between C57BL/6 and DIO mice (Fig. 3A and B), but expressions of PKCβ (Fig. 3D and E), PKCδ (Fig. 3G and H), and PKCζ (Fig. 3J and K) were all significantly less in DIO mouse aortae compared to C57BL/6 mouse aortae. Second, the phosphorylations of PKCα (Fig. 3A and C), PKCβI (Fig. 3D and F), and PKCζ (Fig. 3J and L) were decreased in aortae of DIO mice compared to those of C57BL/6 mice. By contrast, the PKCδ phosphorylation was increased in DIO mouse aortae (Fig. 3G and I). These results indicate that PKCδ is most likely to mediate phenylephrine-induced contraction in DIO mouse aortae.

## 4. Discussion

The present study provide new evidence that (1) the impaired contractile response to $\alpha_1$-adrenoceptor agonist is probably associated with the reduced expression and phosphorylation of IP3 receptor and PKC in DIO mouse aortae; and (2) PKCδ isoform is most likely to mediate phenylephrine-induced contraction while the IP3 receptor loses its role in DIO mouse aortae.

Upon stimulation of Gq protein-coupled receptors, the IP3 receptor participate in $Ca^{2+}$-dependent contraction in response to several vasoconstrictors [22,23]. Loss of the IP3 receptor function in neuropeptide-secreting neurons leads to obesity in adult *Drosophila* [24]. The present results show a significant reduction in phenylephrine-induced contraction in aortae of DIO mice compared to those of normal C57BL/6 mice. The IP3 receptor antagonist heparin inhibited the contraction only in C57BL/6 mouse aortae but not in DIO mouse aortae. Moreover, both expression and phosphorylation of the IP3 receptor were decreased in DIO mouse aortae. These results suggest that obesity may have impaired the IP3 receptor signaling in blood vessels.

Phosphatidylinositol-bisphosphate is hydrolyzed by phospholipase C to form IP3 and diacylglycerol; the latter is the endogenous activator of PKC [23,25]. PKC is known to raise vascular smooth muscle $Ca^{2+}$ sensitivity and thus augments muscle contraction [18,20]. The present results show that PKC inhibitor GFX attenuated phenylephrine-induced contraction in C57BL/6 mouse aortae but it also inhibited the contraction in DIO mouse aortae, suggesting an important role of PKC in maintaining phenylephrine-induced contraction in obesity. In addition, the present study demonstrates that the expression level of PKC was reduced in DIO mouse aortae. There are several PKC isoforms including conventional PKCs (PKCα, β1, β2, and γ), novel PKCs (PKCδ, ε, θ, and η), and atypical PKCs (PKCζ and τ/λ) with a variety of biological functions [26,27]. Further experiments revealed that PKCδ inhibitor rottlerin significantly inhibited the contraction while inhibitors of PKCα, PKCβ, or PKCζ were without effect. In addition, the expressions of PKCβ, PKCδ, and PKCζ were significantly decreased in DIO mouse aortae compared to C57BL/6 mouse aortae but the PKCα expression was not different in aortae between DIO and control mice. Moreover, PKCα, PKCβ, and PKCζ phosphorylations were also reduced in DIO mice. By contrast, PKCδ phosphorylation was elevated in DIO mouse aortae. Previous reports described an increased PKCδ activity in the liver of obese Zucker rats [28] or in response to phenylephrine preconditioning in isolated rat ventricular myocytes [29], thus indicating the significant role of PKCδ in obesity or phenylephrine-induced cellular responses. Taken together, the present study indicates that PKCδ is the most likely PKC isoform to mediate the phenylephrine-induced contraction in DIO mouse aortae.

In summary, the present study demonstrates the loss of IP3 receptor expression and activity and the impairment of PKC signalings may account for the attenuated contraction triggered by $\alpha_1$-adrenoceptor agonist in the aortae of obese mice. Furthermore, PKCδ is most likely required for maintaining the aortic contraction in obese mice. The present findings thus provide additional insight into the cellular mechanisms underlying vascular dysfunction in obesity.

## Author disclosure statement

No competing financial interests exist.

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