

Berberine Reverses Nitroglycerin Tolerance Through Suppressing PKC α Activity in Vascular Smooth Muscle Cells

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Abstract

Purpose

The aim of the present study was to evaluate the effects of berberine on nitroglycerin (NTG) tolerance and explore the underlying mechanism involved.

Methods

NTG tolerance was induced by pre-exposure of Sprague-Dawley rat aortas to NTG *in vitro* or by pretreating Sprague-Dawley rats with NTG patch *in vivo*. The aortas were pre-treated with berberine or PKC inhibitors for different durations of time before induction of NTG tolerance. NTG-induced vasorelaxations was measured on wire myograph. Primary vascular smooth cells (VSMCs) were used to dissect the underlying mechanism of berberine-induced inhibition of NTG tolerance.

Results

NTG tolerance induced by either prior exposure of rat aortas to NTG *in vitro* or pretreatment with NTG patch *in vivo* was reversed by co-treatment with berberine, as well as the inhibitors of PKC and PKC α . The mechanistic study revealed that PKC α participated in the development of NTG tolerance as NTG increased the activity of PKC α with enriched PKC α membrane localization and elevated phosphorylation of PKC α in VSMCs, which was reversed by berberine or PKC α inhibitors.

Conclusion

The present study is probably the first demonstration that berberine reverses NTG tolerance through inhibiting PKC α activity in VSMCs and PKC α is an important contributor to the development of NTG tolerance. These new findings suggest that berberine could become a promising drug for prevention of NTG tolerance and that targeting PKC α in VSMCs is likely to be a potential therapeutic target for reversal of NTG tolerance in blood vessels.

Introduction

Nitroglycerin (NTG), a nitric oxide (NO)-releasing vasodilator, is widely used in the clinical treatment of angina pectoris, congestive heart failure, and myocardial infarction [1]. However, long-term therapy with NTG is frequently associated with a progressive reduction of hemodynamic and antiaggregatory effects (a phenomenon termed nitrate tolerance) with a common manifestation of attenuated anti-hypertensive effects of nitrates [2, 3]. This limits the clinical efficacy on nitrate therapy for patients with stable angina pectoris, congestive heart failure, and acute myocardial infarction [4]. Although a large volume of studies described possible cellular mechanisms underlying nitrate tolerance and intervention, our understanding

of molecular process involved in nitrate tolerance is still limited. Multiple contributing factors are reported and they include sulfhydryl depletion [5], mtALDH inactivation [6], vascular oxidative stress [7, 8], cGMP-cGMP-dependent protein kinase (PKG) inhibition [9], and phosphodiesterase 1A1 up-regulation [10]. Endothelin-1-induced PKC activation was reported to involve NTG tolerance [11] and pharmacological inhibition of the PKC activity with N-benzoyl-staurosporine inhibited the development of NTG tolerance in rat arteries *in vitro* [12]. PKC is a family of kinases with at least eleven isoforms that can phosphorylate actin filament-associated proteins to regulate vascular tone [13]. Inactive form of cytoplasmic PKC can be activated after translocation to the cell membrane. However, the previous study did not show the isoform(s) of PKC responsible for the development of NTG tolerance.

Berberine-containing medicinal plants have been used for over 2000 years in China. As a major alkaloid extracted from the roots and bark of many plants, such as Cortex phellodendri (Huangbai) and Rhizoma coptidis (Huanglian), berberine possesses strong anti-inflammatory [14] and antimicrobial activities [15]. Growing evidence suggests the beneficial effect of berberine against cardiovascular and metabolic diseases. Berberine lowers blood glucose in type 2 diabetes patients through increasing insulin receptor expression [16], ameliorates diabetic neuropathy by blocking PKC/TRPV1 activation [17], attenuates atherogenesis through inhibiting LDLR expression [18], alleviates pressure overload-induced cardiac hypertrophy and dysfunction through enhancing autophagy [19], retards vascular remodeling and inflammation [20], reduces aortic stiffness *via* suppression of transient receptor potential vanilloid 4 channel [21] and protects cardiac myocytes against ischemia-reperfusion injury [22]. Our previous study showed that berberine reduced proliferation of VSMCs and produced vasorelaxations through both endothelium-dependent and -independent mechanisms [23]. These vaso protective actions of berberine prompted us to wonder whether berberine is effective to inhibit NTG tolerance. Against this background, we investigate the effect of berberine on the induction of NTG tolerance in rat arteries and the underlying mechanisms involving PKC pathway in vascular smooth muscle cells.

Materials And Methods

Animals

All animal experiments were approved by CUHK Animal Experimentation Ethics Committee, Capital Medical University Animal Experimentation Ethics Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley (SD) rats (200–250 g) were purchased from the Laboratory Animal Center of Chinese University of Hong Kong (CUHK) and Beijing SPF Biotechnology Co., Ltd. Rats were kept under constant temperature and humidity in a 12-hour controlled dark/light cycle. Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed on a ventilator. The rats were randomized into three groups: 6 with sham control, 6 with NTG patch and 6 with NTG patch plus berberine treatment. For the rats with NTG patch, a region either on the dorsal surface of the thorax or the area between the scapulae was shaved and a NTG patch (glyceryl trinitrate transdermal patch releasing 5 mg of glyceryl trinitrate in 24 hours, Schwarz Pharma AG, Monheim am Rhien, Germany) was applied to the shaved skin. Rats in the sham group received a similar

procedure without NTG patch. The rat with NTG patch plus berberine were treated with berberine (30 mg/kg/d, i.p.) one day before application of NTG patch and the co-treatment with NTG patch lasted three days.

Adenovirus construction tail injection

To knock down PKC α expression, oligonucleotides: cccgGTCCTTCACGTTCAAATTAAGCTC GAGTTTAATTTGAACGTGAAGGACTTTTTC (forward) and tcgagaaaaGTCCTTCACGTTCAAA TTAAACTCGAGTTTAATTTGAACGTGAAGGAC (reverse) against the rat PKC α were annealed and cloned into pAdTrack-U6, and this clone was named as Ad-PKC α shRNA. After sequencing, pAdtrack-U6 vectors with scramble sequence or with above inserted sequences were recombined with pAdeasy-1 in BJ5183 *E. coli* to generate the recombined adenoviral vectors. After *PacI* (New England Biolabs, MA, USA) digestion, adenoviral plasmids were transfected into HEK293 cells to generate infectious adenovirus particles. The adenoviruses were purified by CsCl gradient ultracentrifugation. Amplification was conducted thrice to obtain sufficient active adenovirus (10^8 pfu) to infect rat aortas (24 hours) or to perform the tail vein injection twice with an interval of five days. pAdtrack-U6-Scramble (named as Ad-Scramble) adenoviruses were used as control.

Rat aortic rings and vascular functional assay

Thoracic aortas of NTG patch-treated SD rats or sham rats were dissected in Krebs solution (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl $_2$, 1 mM MgCl $_2$, 25 mM NaHCO $_3$, 1.2 mM KH $_2$ PO $_4$, and 11 mM D-glucose) and cut into ring segments. Each ring was suspended in organ bath (Danish Myo Technology, Aarhus N, Denmark) and the changes in isometric force were recorded as described[24]. Rings were first contracted with 60 mmol/L KCl to test the viability and then rinsed twice in Krebs solution; For acute NTG treatment, aortic rings were incubated in NTG (30 μ M) for 90 min, and washed 4 times in Krebs solution in an interval of 15 min. The rings were contracted by phenylephrine (1 μ M) to obtain a steady tension before cumulative addition of NTG (3 nM – 50 μ M). Berberine (3 μ M), Go6976 (2 μ M, PKC α / β inhibitor), or GF109203X (2 μ M, PKC general inhibitor) was individually added to bathing solution 30 min before 90-min treatment with NTG (30 μ M) or 30 min before addition of NTG in aortas of NTG patch-treated rats. Phenylephrine, NTG and Go6976 were dissolved in water while berberine was prepared in DMSO.

Isolation and culture of rat aortic primary vascular smooth muscle cells (VSMCs)

VSMCs were isolated from the aorta of male SD rats (200–250 g) by enzymatic digestion [25]. Briefly, the thoracic aortas were removed aseptically from SD rats and cleaned gently of perivascular connective tissue in sterile Hanks' solution. The isolated aorta was incubated for 30 min in Hanks' solution containing 1 mg/mL collagenase (type II, Worthington Biochemical Corp., Freehold, NJ) and 0.5 mg/mL elastase (type I, Sigma-Aldrich, USA) at 37°C. The adventitia was carefully removed, and the luminal surface was scraped with forceps to remove endothelial cells. After dissection, the aorta was placed in fresh enzyme solution, minced into 1-mm pieces, and incubated (37°C, 95% O $_2$, 5% CO $_2$) for additional 2

hours. The resulting cell-tissue suspension was filtered through an 85- μ m stainless steel mesh. The filtrate was collected and 20% fetal calf serum (FCS, Gibco) was added to inactivate enzymes. The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells of passages 3 to 5 at around 80% confluence were used in the experiments.

Separating the fraction of cell membrane and cell cytoplasm

The VSMCs treated for 1 min with NTG (30 μ M) or phorbol myristate acetate (PMA, Sigma-Aldrich, 100 nM) with or without berberine co-treatment (3 μ M, 1 min) was homogenized in cold lysis buffer (RIPA, 50 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 1 mM PMSF, 10 g/ml leupeptin, 1 mM Na₃V₀4, 5 mM NaF, 1mM DTT), then centrifuged for 10 min at 500 g at 4 °C to remove debris and nuclei. Subsequently, the supernatant was centrifuged for 45 min at 120,000 g at 4 °C. The supernatant was the cytosol fraction and the pellets were re-suspended in 1% Triton X-100 in a lysis buffer and centrifuged for 10 min at 10,000 g at 4 °C to remove insoluble debris and thus obtain the cell membrane fraction in supernatant.

Western blotting

VSMCs and rat aorta proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). After a complete homogenization on ice, the samples were centrifuged and the supernatants were fractionated by 10% SDS-PAGE and electro-transferred onto a PVDF membrane. After blocking with Tris-buffered saline (TBS) containing 5% non-fat milk, the membranes were probed with primary antibodies against PKC α , p-PKC α , PKC θ , PKC δ , PKC ξ , PKC ζ , PKC μ (1:1000, Cell signaling Technology, USA); GAPDH (Anti-GAPDH (1:5000, Ambion, Austin, TX, USA) and Caveolin 1 (1:1000, Abcam, Cambridge, UK) overnight at 4°C. A horseradish peroxidase-conjugated secondary antibody was used for the enhanced chemiluminescence (ECL) detection. Immunoreactive materials on the membrane were then exposed to X-ray film. The band intensity on the film was analyzed by densitometry.

Immunofluorescence staining

VSMCs were fixed, blocked and incubated with a mouse monoclonal anti- α -SMA antibody (Sigma-Aldrich, USA), a mouse monoclonal anti-Caveolin 1 (Abcam, Cambridge, UK) and a rabbit monoclonal anti-PKC α antibody (1:100 dilution) at 4°C overnight. Fluorescein-conjugated secondary antibodies (anti-mouse FITC, green; anti-rabbit TRITC, red; 1:100 dilution) were then added to the samples, followed by 1-hour incubation. The slides were washed and covered with mounting medium. Confocal microscopic images were captured on Olympus Fluoview 1000 (FV1000, Olympus, Tokyo, Japan) and analyzed with Olympus Fluoview Version 1.5.

Drugs and chemicals

NTG was purchased from Hameln Pharmaceuticals Ltd (Hameln, Germany). Nitroglycerin patch was purchased from Schwarz Pharma AG (Schwarz Pharma AG, Monheim am Rhien, Germany). Berberine, PMA, Go6976, GF109203X, acetylcholine and phenylephrine were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA).

Statistical analysis

Results represent mean \pm standard deviation (SD) of *n* separate experiments. Concentration-response curves were analyzed using GraphPad Prism software (Version 4.0). Statistical significance was determined by Student's *t*-test (two-tailed) or one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test when more than two treatments were compared. $P < 0.05$ indicates statistical difference between groups.

Results

In vivo and in vitro treatment with berberine attenuates or prevents the development of NTG tolerance

Pre-incubation (90 min) of SD rat aortas with NTG (30 μ M) resulted in markedly attenuated relaxant responses to NTG (3 nM – 50 μ M) (Fig. 1a-b) without affecting acetylcholine-induced NO-mediated relaxations (Fig. 1c), while 30-min treatment with berberine (3 μ M) prior to addition of NTG (30 μ M) reversed the induction of NTG tolerance (Fig. 1b). Likewise, *in vivo* 3-day treatment of the rats with NTG patch caused a significant rightward shift of the concentration–response curve to NTG without affecting maximum relaxation (Fig. 1d and Table 1). The effect of NTG patch on NTG tolerance was reversed by either 30-min acute incubation of the aortas from NTG patch-treated rats with berberine (Fig. 1e) or intraperitoneal (IP) injection of berberine (30 mg/kg/d for 1 day followed by 3-day co-treatment with NTG patch) (Fig. 2a, b and Table 2). The maximum relaxation in response to NTG was similar in all treatment groups.

Table 1
NTG-induced relaxations in rat aortas.

Treatment	EC ₅₀ (μM)	E _{max} (%)	n
Control ¹	0.030 ± 0.012	99.26 ± 0.58	6
NTG pre-exposure ¹	8.089 ± 3.793*	88.02 ± 3.64	6
Berberine (1 μM) + NTG pre-exposure ¹	4.704 ± 2.311	90.79 ± 1.92	6
Berberine (3 μM) + NTG pre-exposure ¹	0.052 ± 0.013 [#]	99.54 ± 0.46	6
Control ²	0.033 ± 0.006	99.56 ± 0.64	6
NTG pre-exposure ²	7.736 ± 1.804*	84.23 ± 3.45	6
GF109203X + NTG pre-exposure ²	0.142 ± 0.078 [#]	97.95 ± 0.93	6
Control ³	0.025 ± 0.006	99.01 ± 0.98	6
NTG pre-exposure ³	9.501 ± 3.962*	87.15 ± 3.24	6
Go6976 + NTG pre-exposure ³	0.113 ± 0.017 [#]	92.39 ± 2.40	6
Control (Ad Scramble) ⁴	0.030 ± 0.013	98.26 ± 0.48	6
Ad Scramble + NTG pre-exposure ⁴	6.204 ± 1.566*	86.44 ± 4.41	6
Control (Ad PKCa shRNA) ⁴	0.077 ± 0.020	95.23 ± 0.89	6
Ad PKCa shRNA + NTG pre-exposure ⁴	0.124 ± 0.015 [#]	89.05 ± 1.57	5

EC₅₀, concentration for 50% of maximal effect. E_{max}, maximal effect. 1, data points to Fig. 1b; 2, data points to Fig. 3a; 3, data points to Fig. 6a; 4, data point to Fig. 6c. **P* < 0.05 vs. Control with the same number; [#]*P* < 0.05 vs. NTG pre-exposure with the same number.

Table 2
NTG-induced relaxations in aortas of NTG patch-treated rats.

Treatment	EC ₅₀ (μM)	E _{max} (%)	n
Sham ⁵	0.073 ± 0.034	99.11 ± 0.61	6
NTG patch ⁵	8.921 ± 2.355*	93.33 ± 2.17	6
Berberine (ex vivo) + NTG patch ^{5.1}	0.071 ± 0.050 [#]	98.46 ± 1.54	6
Berberine (in vivo) + NTG patch ⁵	0.173 ± 0.089 [#]	100.00 ± 0.00	6
NTG patch ⁶	8.829 ± 3.377	94.01 ± 1.88	6
GF109203X (ex vivo) + NTG patch ⁶	0.227 ± 0.170 [#]	96.73 ± 2.08	6
Sham ⁷	0.106 ± 0.029	98.10 ± 0.51	6
NTG patch ⁷	9.002 ± 3.332*	93.09 ± 2.03	6
Go6976 (ex vivo) + NTG patch ⁷	0.158 ± 0.086 [#]	96.16 ± 1.46	6

EC₅₀, concentration for 50% of maximal effect. E_{max}, maximal effect. 5, data points to Fig. 2b; 5.1, data points to Fig. 1e; 6, data points to Fig. 3a; 7, data points to Fig. 6b. **P* < 0.05 vs. Sham with the same number; [#]*P* < 0.05 vs. NTG patch with the same number.

PKC inhibitor reverses NTG tolerance

PKC signaling plays important roles in regulating vascular tone [13]. To examine the mechanisms underlying the induction of NTG tolerance in rat aortas and the beneficial effect of berberine against NTG tolerance, we used PKC inhibitors to treat rat aortas and showed that 30-min treatment with a non-selective PKC inhibitor (GF109203X, 2 μM) substantially inhibited NTG tolerance (Fig. 3a) in both NTG pretreated (90 min)-aortas and in aortas from NTG patch-treated rats (Fig. 3b). These results clearly suggest a significant contributing role of PKC activation in the *in vitro* and *in vivo* induction of NTG tolerance. Indeed, 90-min incubation with the PKC activator, phorbol myristate acetate (PMA) attenuated NTG-induced relaxation in rat aortas and this effect was also reversed by the co-treatment with either GF109203X (2 μM) or berberine (3 μM) (Fig. 3c).

Enhanced membrane translocation of PKCα and augmented intracellular Ca²⁺ concentration in the induction of NTG tolerance

Since non-selective PKC inhibitor GF109203X either inhibited or reversed the induction of NTG tolerance we next examined which PKC isoform can be inhibited by berberine to reverse NTG tolerance. Primary rat aortic VSMCs were cultured and exposed to NTG (30 μM) or PMA (100 nM) for 1 min (PKC isoform membrane translocation occurs within 1–2 min according to a previous publication [26], concomitantly with or without berberine (3 μM, 1 min) or GF109203X (2 μM, 1 min). Western blotting results showed that

treatment with either NTG or PMA induced the enrichment of PKC α in the membrane fraction with concurrent reduction of its content in the cytosolic fraction; this effect of NTG or PMA on PKC α membrane translocation was reversed by prior treatment with berberine or GF109203X (Fig. 4a). Expect for PKC α , NTG treatment also increased the level of PKC θ albeit to a lesser degree without affecting the amount of the membrane fraction of other PKC isoforms including PKC δ , PKC ξ , PKC ζ , PKC μ . However, the NTG-induced small elevation of PKC θ on the membrane fraction was not reversed by the co-treatment of berberine or GF109203X. Of note, PMA-induced increases of PKC θ and PKC μ on the membrane fraction were inhibited by berberine and GF109203X (Fig. 4a and Supplemental Fig. 1.). Active PKC isoforms always translocate to the cell membrane in their phosphorylated form. We measured phosphorylated PKC α in VSMCs after treatment with GF109203X (2 μ M, 1 min) or PKC α/β inhibitor Go6976 (2 μ M, 1 min) and observed that GF109203X and Go6976 indeed blocked the PMA-induced elevation of PKC phosphorylation (Supplemental Fig. 2.). Consistent with the immunoblotting results, immunofluorescence also showed that treatment with NTG or PMA increased the transfer of PKC α from the cytoplasm to the cell membrane, which was inhibited by berberine (Fig. 4b). As the PKC α activity is Ca²⁺-dependent, we next measured the intracellular Ca²⁺ concentration [Ca²⁺]_i in primary rat aortic VSMCs using Fluo-4 fluorescence and found that NTG concentration-dependently decreased phenylephrine-induced elevation of [Ca²⁺]_i and this effect was attenuated by prior exposure to NTG. The attenuated effect of NTG (30 μ M) pretreatment on [Ca²⁺]_i was reversed by co-treatment with either berberine (3 μ M) or the selective PKC α inhibitor, Go6976 (2 μ M) (Fig. 4c, d).

Berberine reverses NTG-induced phosphorylation of PKC α in VSMCs

Primary VSMCs were cultured and treated with NTG (30 μ M) for different durations of time. The Western blotting results showed that 1-min exposure of VSMCs to NTG increased PKC α phosphorylation and this effect declined gradually and returned to the baseline at 15 min (Fig. 5a). Likewise, the increased phosphorylated PKC α level was also observed in PMA (100 nM, 1 min)-treated VSMCs (Fig. 5b). Co-treatment with either berberine (3 μ M) or Go6976 (2 μ M) reversed the increase of PKC α phosphorylation in response to NTG or PMA (Fig. 5B). In rat aortas, 10-min treatment with NTG also elevated PKC α phosphorylation, which was reversed by co-treatment of berberine or Go6976 (Fig. 5c). Likewise, the increased phosphorylated PKC α was also detected in the aortas of NTG patch-treated rats, which was reversed by berberine (30 mg/kg/d, i.p.) administered 1 day before NTG patch application (Fig. 5d).

Knockdown of PKC α restores NTG tolerance

To further determine the critical role of PKC α in the induction of NTG tolerance, Go6976 (2 μ M) was used to treat the aortas from NTG patch-treated rats or the rat aortas with prior 90-min exposure to 30 μ M NTG. The results showed that Go6976 pre-treatment (30 min) inhibited NTG tolerance (Fig. 6a, b). Furthermore, Ad-PKC α shRNA (10⁸ pfu) and ad-scramble were administered to SD rats *via* tail vein injection. Ten days after injection, the expression of PKC α was analyzed in rat aortas and NTG-induced relaxations were measured. Ad-PKC α shRNA reduced the expression of PKC α in rat aortas and improved NTG-induced

relaxations in prior 90-min NTG (30 μ M)-treated aortas (Fig. 6c), further supporting the significant role of PKC α activation in the induction of NTG tolerance in rat arteries.

Discussion

The major findings of the present study include (i) NTG-induced increase of PKC α activity is critically involved in the induction of NTG tolerance in rat aortas and (ii) both *in vivo* and *in vitro* treatment with berberine reverses NTG tolerance through inhibiting the PKC α activity in VSMCs.

Nitrates are the NO donors widely used to treat patients with angina pectoris, congestive heart failure, and myocardial infarction. However, chronic nitrate administration results in nitrate tolerance in patients while the mechanisms underlying this deleterious effect are not fully understood [27]. Although it has been always challenging to present a uniform hypothesis for the development of NTG tolerance, the most causative studies on NTG tolerance focused on the role of endothelial cells and endothelial function [28], while less was directed to the role of VSMCs despite the fact that NTG and all other NO donors are the endothelium-independent vasodilators. The NTG-induced overproduction of superoxide anions was used to explain eNOS uncoupling and endothelial dysfunction in nitrate tolerance [8, 7], while other studies argued that superoxide anion is probably not the primary mediator of nitrate tolerance because neither tiron nor dimethylsulfoxide, the intracellular ROS scavengers, affects nitrate tolerance [29, 30]. Furthermore, NTG tolerance still developed in NADPH oxidase subunit knockout mice receiving chronic administration of NTG [31]. In addition, some reports indicate that eNOS may not be critically involved in nitrate tolerance as eNOS deficient mice also developed NTG tolerance [32]. Taken together, these studies suggest that other more important mechanisms are yet to be revealed during the development of NTG tolerance in blood vessels. In the present study, acetylcholine-induced endothelium-dependent relaxations were not affected in aortas from rats with 3-day NTG-patch treatment, suggesting that NTG tolerance was more likely to occur in VSMCs rather than in endothelial cells. Therefore, in this study we chose VSMCs to investigate the mechanisms underlying NTG tolerance.

PKC signaling is known to play important roles in regulating vascular tone through phosphorylating the actin filament-associated proteins, calponin and caldesmon in VSMCs [13]. Previous studies suggested that PKC signal might be implicated in NTG tolerance [11, 12]. However, these studies did not reveal the isoform(s) of PKC involved in NTG tolerance. The present study demonstrates that the activity of PKC α was induced by NTG treatment as evidenced by the elevated levels of both phosphorylation and membrane bound PKC α in NTG-treated VSMCs, which can be reversed by PKC inhibitor GF109203X or by selective PKC α inhibitor Go6976. More importantly, we found that genetic knockdown or the pharmacological inhibition of PKC α inhibited or even reversed NTG tolerance in rat aortas both *in vivo* and *in vitro*, thus supporting a critical role of PKC α activation during the development of NTG tolerance in rat arteries and suggesting that PKC α is likely a molecular target for intervention to inhibit NTG tolerance.

Berberine, an isoquinoline alkaloid possesses a wide spectrum of pharmacological properties, including anti-inflammation, anti-oxidative stress and anti-apoptosis [14, 33]. In addition, berberine also exerts

cardiovascular protective action to inhibit atherosclerosis [18], cardiac hypertrophy [19], and aortic stiffness [21]. The present demonstrates for the first time that both *in vitro* and *in vivo* treatment with berberine is effective to reverse the development of NTG tolerance in rat aortas through normalizing the increased membrane bound PKC α and PKC α phosphorylation in NTG-treated VSMCs or in the aortas from NTG patch-treated rats. These new results clearly indicate that PKC α inhibition is most likely to mediate the beneficial effect of berberine to prevent the development of NTG tolerance. Consistent with our results, other studies also showed a suppressive effect of berberine on PKC or PKC α signaling in non-vascular tissues such as lumbar spinal cords and breast cancer cells [34, 17], although one study described an stimulatory effect of berberine on PKC in hepatocytes [35], indicating the effect of berberine on the PKC activity may be tissue- or disease-dependent.

Due to the multiplicity of NTG tolerance-inducing mechanisms, a growing list of compounds appear to be effective in preventing the development of NTG tolerance and they include vitamin C [36], vitamin E [37], thiols [38], hydralazine [39], L-arginine [40], adrenergic receptor blockers[41], phosphodiesterase inhibitor[42], angiotensin-converting enzyme inhibitor [43], and diuretics [44]. However, most of these drugs were only tested in basic experiments or were administered to healthy volunteers and they are yet to be tested clinically in patients who develop NTG tolerance. In addition, NTG and other drugs such as hydralazine, adrenergic blockers, angiotensin-converting enzyme inhibitors or diuretics share the similar effect to dilate blood vessels and lower blood pressure through different mechanisms of actions. These drugs are commonly use as monotherapy or in combination to decrease cardiac afterload and/or preload and thus to improve cardiac performance, and they have rarely been used to ameliorate NTG tolerance. Our new findings strongly suggest that berberine is a promising drug to inhibit NTG tolerance acting through mechanisms that are distinct from other agents reported in literature and that berberine could be potentially developed into an adjunct agent to prevent NTG tolerance for patients receiving nitrates therapy. Nevertheless, the therapeutic value of berberine needs to be confirmed in future clinical studies.

Although we provide new evidence demonstrating that berberine inhibits NGT tolerance through suppressing PKC activity in VSMCs and rat aortas, we are also aware of several limitations in the present study. First, both *in vitro* and *in vivo* results in rodents cannot be confirmed by the effect of berberine in patients. Second, although we confirmed that berberine reversed NTG tolerance both *in vivo* and *in vitro* through PKC α inhibition, we still cannot discount other PKC α inhibition-independent mechanism, albeit in a lesser degree mediating the effect of berberine against NTG tolerance. Third, we only used male SD rats in the present study and do not know whether there is any gender difference in the pharmacological effect of berberine in inhibiting NTG tolerance.

In summary, the present study provides new mechanistic insights into the important role of PKC α activation in the development of NTG tolerance. Berberine is potent and effective to prevent the development of NTG tolerance through inhibiting the PKC α activity in VSMCs. The present study not only deepens the understanding of VSMC-associated mechanisms underlying NTG tolerance by revealing the critical role of PKC α , but also highlights the therapeutic potential of berberine as an adjuvant agent to enhance clinical efficacy of NO donors in the treatment of coronary heart disease.

Declarations

Author contributions

Huina Zhang and Jinghui Dong carried out most of the experiments and designed some experiments and wrote the manuscript. Chi-Wai Lau performed animal experiments. Yu Huang designed the experiments, edited the manuscript and financed the study.

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Compliance with Ethical Standards

Conflict of interest

The authors declare no competing financial interests.

Ethical Approval

All animal experiments were approved by CUHK Animal Experimentation Ethics Committee, Capital Medical University Animal Experimentation Ethics Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Availability of Data and Material

Additional data and material information are available upon request.

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Figures

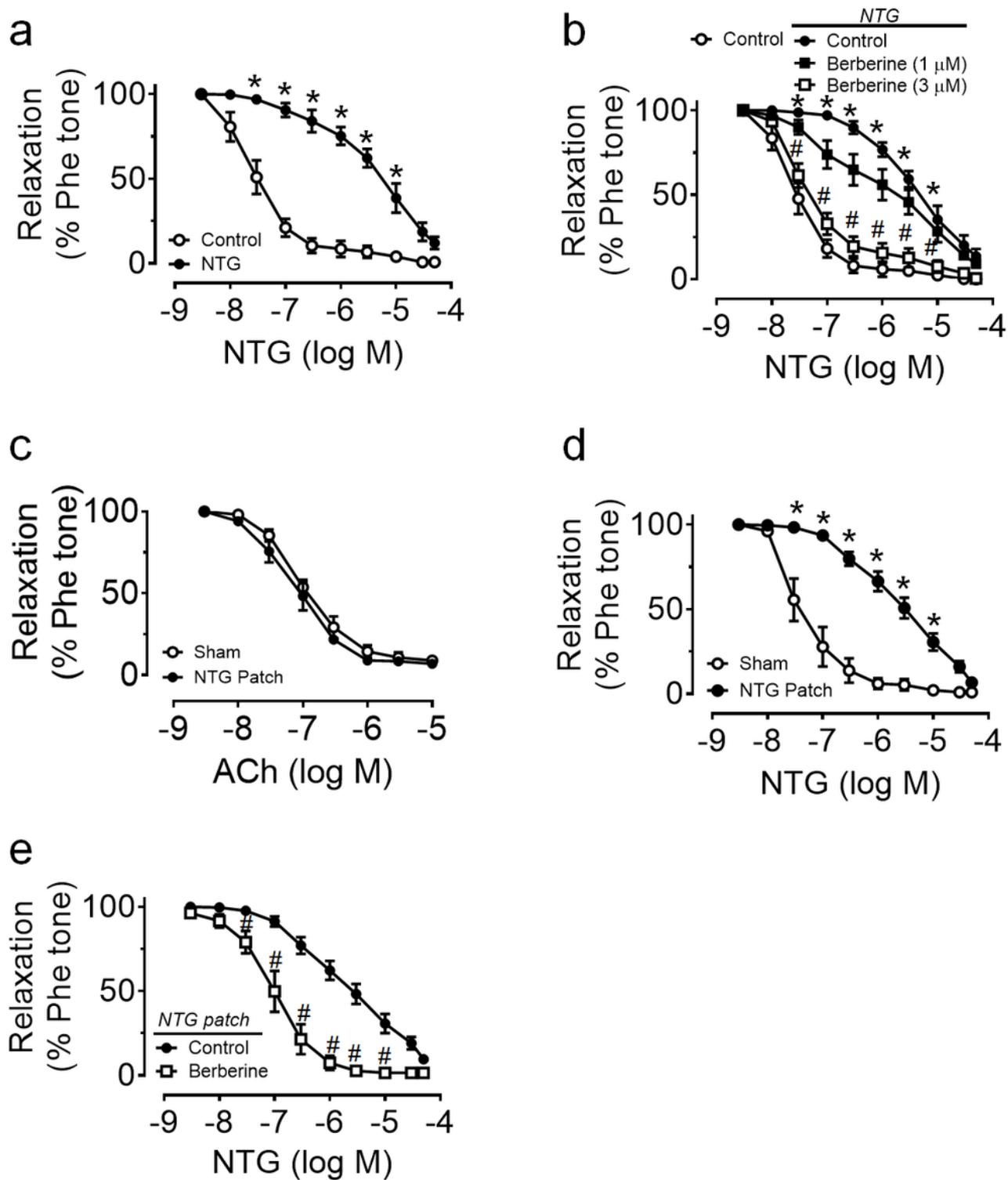


Figure 1

Berberine reverses NTG tolerance in vitro. (a) NTG pretreatment (30 μ M, 90 min) attenuated dilator responses to NTG in rat aortas. (b) Pretreatment of rat aortas with berberine (3 μ M, 30 min) in vitro restores NTG-induced aortic relaxations. (c) NTG pretreatment did not influence acetylcholine-induced relaxation. (d) In aortas from rats receiving 3-day treatment with NTG patch exhibited markedly blunted relaxations in response to NTG. (e) NTG patch-induced attenuated aortic relaxations was reversed by 30-

min berberine pre-treatment before addition of NTG. Results are mean \pm SD (n=6). *P < 0.05 vs. Control (a and b) or Sham (d). # P < 0.05 vs. NTG or NTG patch (b and e).

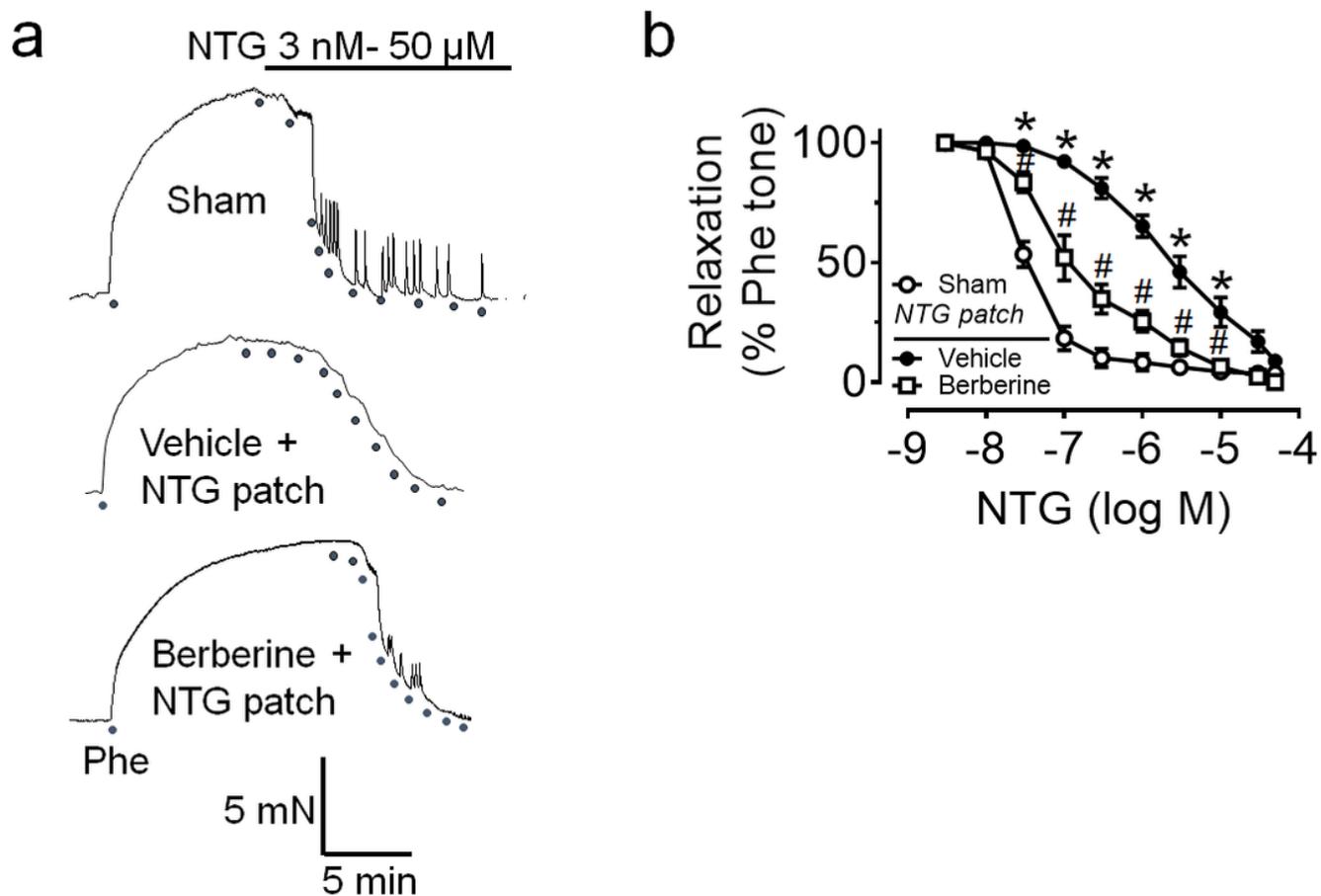


Figure 2

Berberine inhibits NTG tolerance in vivo. (a) Representative traces showing NTG-induced aortic relaxations in sham control (upper panel), following 3-day NTG patch treatment without (middle panel) or with (lower panel) 1-day berberine pretreatment. (b) The effect of in vivo berberine treatment (30 mg/kg/d, 1 day before NTG patch treatment) on NTG-induced aortic relaxations. Results are mean \pm SD (n=6). *P < 0.05 vs. Sham control. # P < 0.05 between NTG patch plus Vehicle and NTG patch plus berberine.

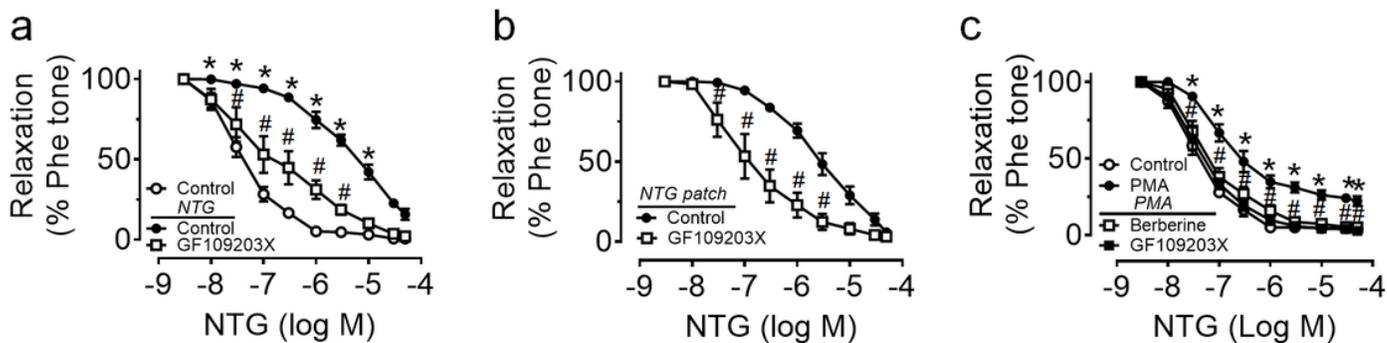


Figure 3

PKC inhibitors attenuate NTG tolerance. (a-b) The acute inhibitory effect of a PKC general inhibitor (GF109203X, 2 μ M, 30 min) inhibited NTG tolerance in aortas with prior 90-min exposure to 30 μ M NTG (A) or in rats receiving 3-day NTG patch treatment (b). (c) Treatment with PMA (100 nM, 90 min) attenuated NTG-induced aortic relaxations and this effect was reversed by pre-treatment of berberine (3 μ M, 30 min) or GF109203X (2 μ M, 30 min). Results are mean \pm SD (n=6). *P < 0.05 vs. Control. #P < 0.05 vs. NTG (a) or NTG patch (b) or PMA (c).

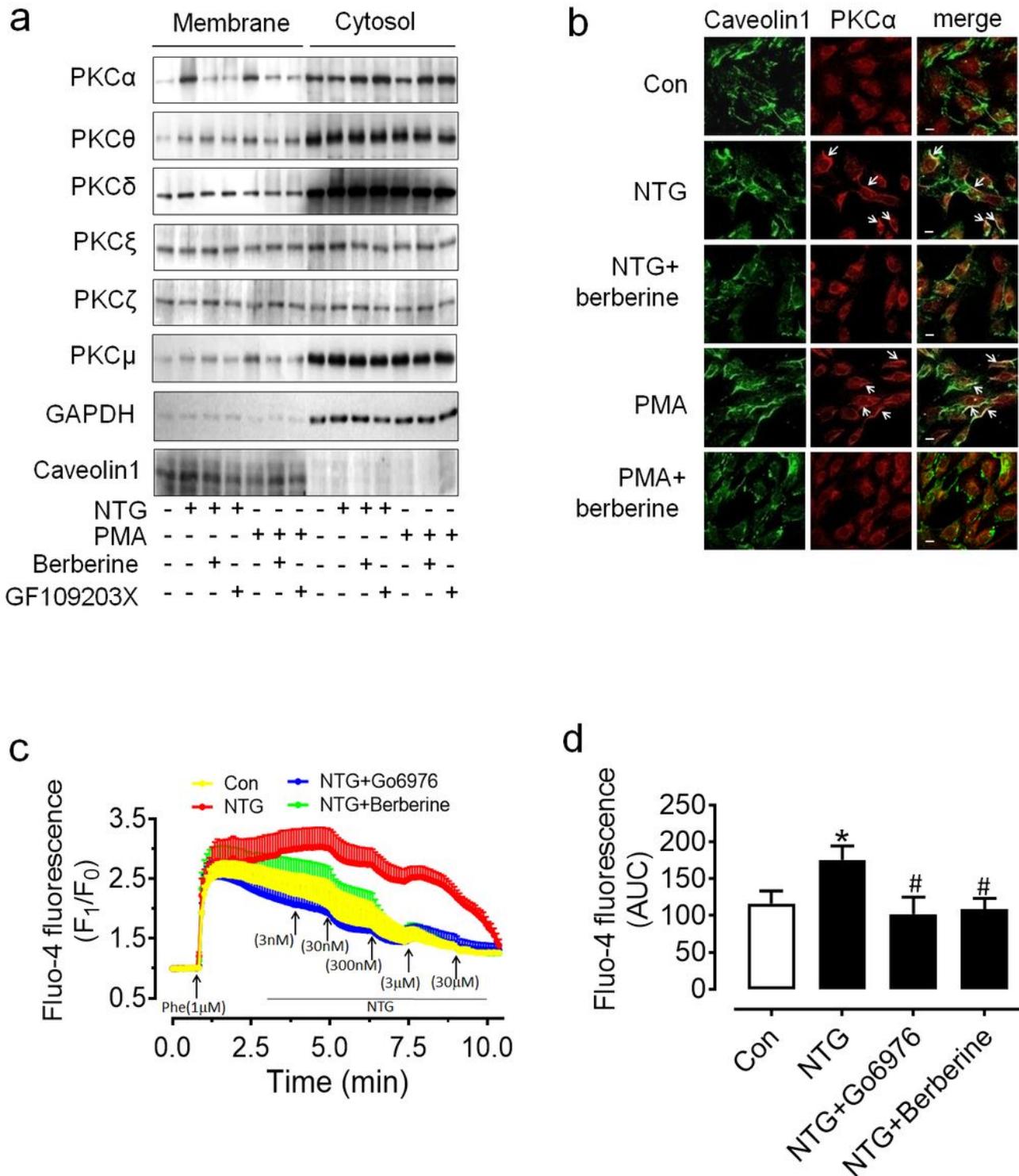


Figure 4

Berberine inhibits membrane location of PKC α induced by NTG in primary rat vascular smooth muscle cells. (a) The expression of membrane or cytosol PKC isoforms (including PKC α , PKC θ , PKC δ , PKC ξ , PKC ζ , PKC μ) was detected by Western blotting (upper panel) after separating the fractions in VSMCs treated with NTG (30 μ M, 1 min) or PMA (100 nM, 1 min) concomitantly with berberine (3 μ M, 1 min) or GF109203X (2 μ M \times 1 min). GAPDH was used as cytosol internal control while caveolin 1 as membrane internal control. (b) Immunofluorescence of intracellular location of PKC α in VSMCs after treatment with NTG or PMA with or without berberine pretreatment. Green, Caveolin 1 signal showing the cell membrane. Red, PKC α signal, white arrows showing the membrane binding of PKC α , which merged with the green signal of Caveolin 1 appearing yellow color. (c-d) Fluo-4 fluorescence measurement of intracellular free calcium (c) and summarized results (d) showing that NTG concentration-dependently decreased phenylephrine-induced intracellular Ca $^{2+}$ concentration in VSMCs and this effect was attenuated in NTG-pretreated VSMCs (30 μ M, 90 min). The latter was reversed by co-treatment of berberine (3 μ M) or Go6976 (2 μ M, protein kinase C α/β inhibitor). Results are mean \pm SD (n=5). *P < 0.05 vs. Control. #P < 0.05 vs. NTG.

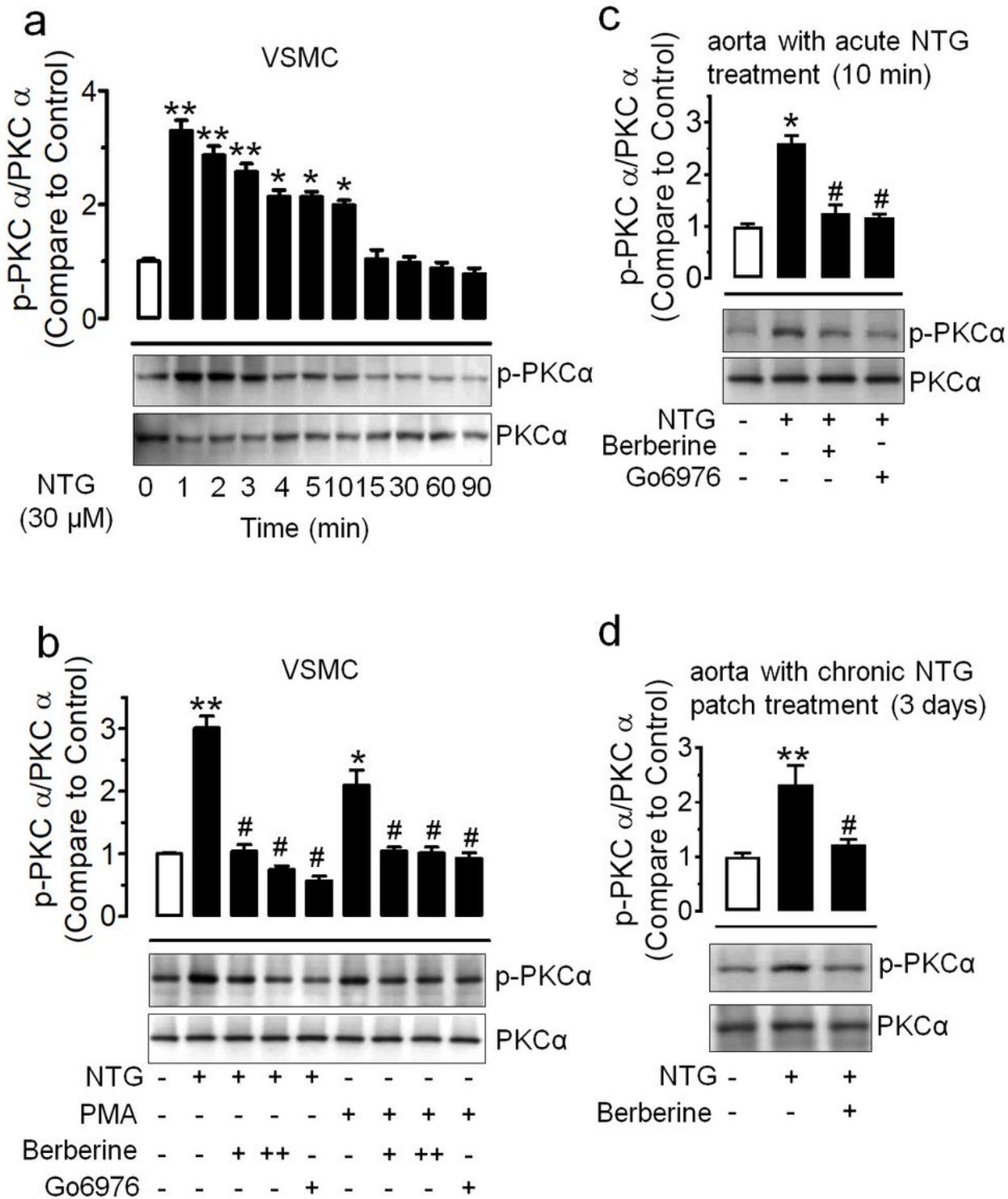


Figure 5

Berberine reverses NTG-induced PKC α phosphorylation in primary vascular smooth muscle cells. (a) PKC α phosphorylation was measured by Western blotting in VSMCs treated with NTG (30 μ M) for different durations of time. (b) PKC α phosphorylation was measured by Western blotting in VSMCs treated with NTG (30 μ M, 1 min) or PMA (100 nM, 1 min) with or without the co-treatment of berberine (3 μ M) or Go6976 (2 μ M). (c-d) Western blotting results showing the level of PKC α phosphorylation in rat

aortas incubated with NTG with or without co-treatment of berberine or Go6976 (c) and in aortas from NTG patch-treated rats with or without berberine in vivo pretreatment (d). Results are mean \pm SD (n=5-6). *P < 0.05 vs. non-reagent treatment. #P < 0.05 vs. NTG

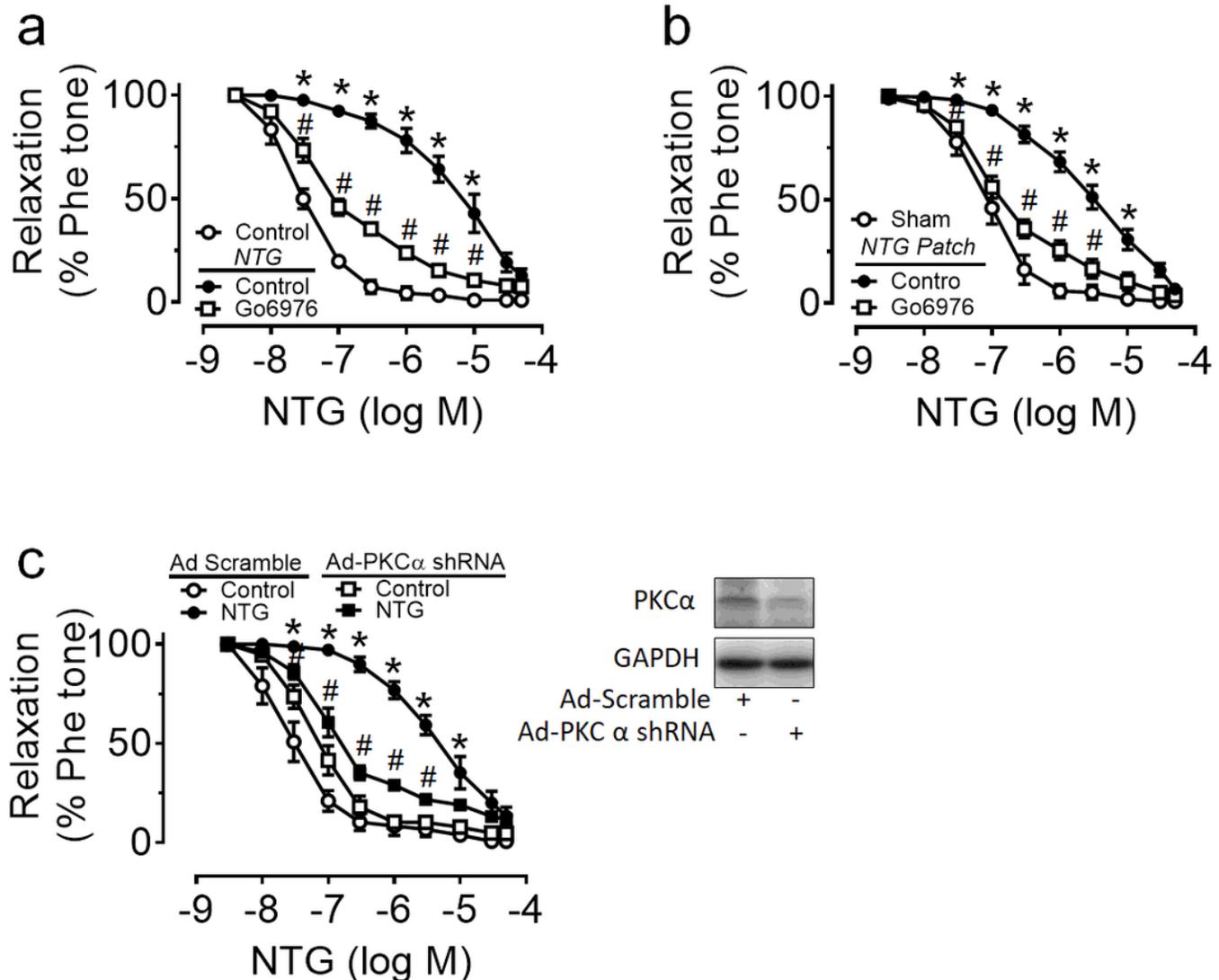


Figure 6

PKC α shRNA reverses NTG tolerance in vivo. (a-b) Pre-treatment of PKC specific inhibitor (Go6976, 2 μ M, 30 min) largely inhibited NTG tolerance in rat aortas acutely pre-exposed to NTG (a) and in aortas of NTG patch-treated rats (b). (c) NTG tolerance was reversed by knockdown of PKC α in aortas from rats 7 days after ad-PKC α shRNA tail vein injection. Results are mean \pm SD (n=5-6). *P < 0.05 vs. Control (a) or Sham (b) or Ad Scramble Control. #P < 0.05 vs. NTG (a) or NTG patch Vehicle (b) or Ad Scramble NTG (c).

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