Novel Role of Protein Kinase C Delta Tyr^{311} Phosphorylation in Vascular Smooth Muscle Cell Hypertrophy by Angiotensin II

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Short title: PKC delta in Angiotensin II-induced Hypertrophy

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Supplemental Methods and Figures

Supplemental Methods

Reagents and Antibodies

AngII was purchased from Sigma Chemical Co. (St. Louis, MO). PP2, PP3, AG1478 and staurosporine were purchased from Calbiochem (La Jolla, CA). RNH6270 and YM-254890 were gifts from Sankyo Pharmaceutical Co Ltd. (Tokyo, Japan) and Astellas Pharma Inc. (Tokyo, Japan), respectively.

Sources and dilutions of the primary antibodies used are as follows. Phospho-specific antibodies for Tyr^{311}-phosphorylated PKCδ (1:10,000) and Tyr^{1068}-phosphorylated EGF receptor (1:6,000) were purchased from Biosource (Camarillo, CA). Phosphospecific antibody for Ser^{473}-phosphorylated Akt (1:4,000), total Akt antibody (1:6,000) and cleaved caspase-3 antibody (1:10,000) were purchased from Cell Signaling (Beverly, MA). Phosphospecific antibody for Tyr^{204}-phosphorylated ERK1/2 (1:20,000) and antibodies against PKCδ (1:10,000), PKCα (1:10,000) and Src (1:10,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for GAPDH (1:10,000) was purchased from Chemicon International (Temecula, CA). Antibody for GFP (1:10,000) was purchased from BD Biosciences (Palo Alto, CA).

Cell culture

Rat aortic VSMCs were obtained and maintained as described previously. Subcultured cells from passages 3-12 were used in the experiments and showed more than 99% positive immunostaining of smooth muscle α-actin antibody [1]. For both short-term (2-10 min) and long-term (3 days) experiments, cells were cultured to ~80% confluency and then made quiescent by incubating in serum-free media for 2-3 days. Average confluency before AngII stimulation is a little more than 80% but still less than 90% since VSMCs do not proliferate significantly after the serum starvation without a mitogen.

Statistical Analysis

Unless otherwise stated, the data presented in this study are representative of a minimum of three independent experiments yielding similar results. The data were analyzed using one-way ANOVA followed by a post-hoc modified t-test (Figure 1, 2A and 2B) or a two-way ANOVA (Figure 3B, 4 and 5).

Reference

1. Eguchi S, Matsumoto T, Motley ED, Utsunomiya H, Inagami T. Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. Possible requirement of Gq-mediated p21ras activation coupled to a Ca2+/calmodulin-sensitive tyrosine kinase. ***J Biol Chem***. 1996; 271:14169-14175.

Supplemental Figure Legends

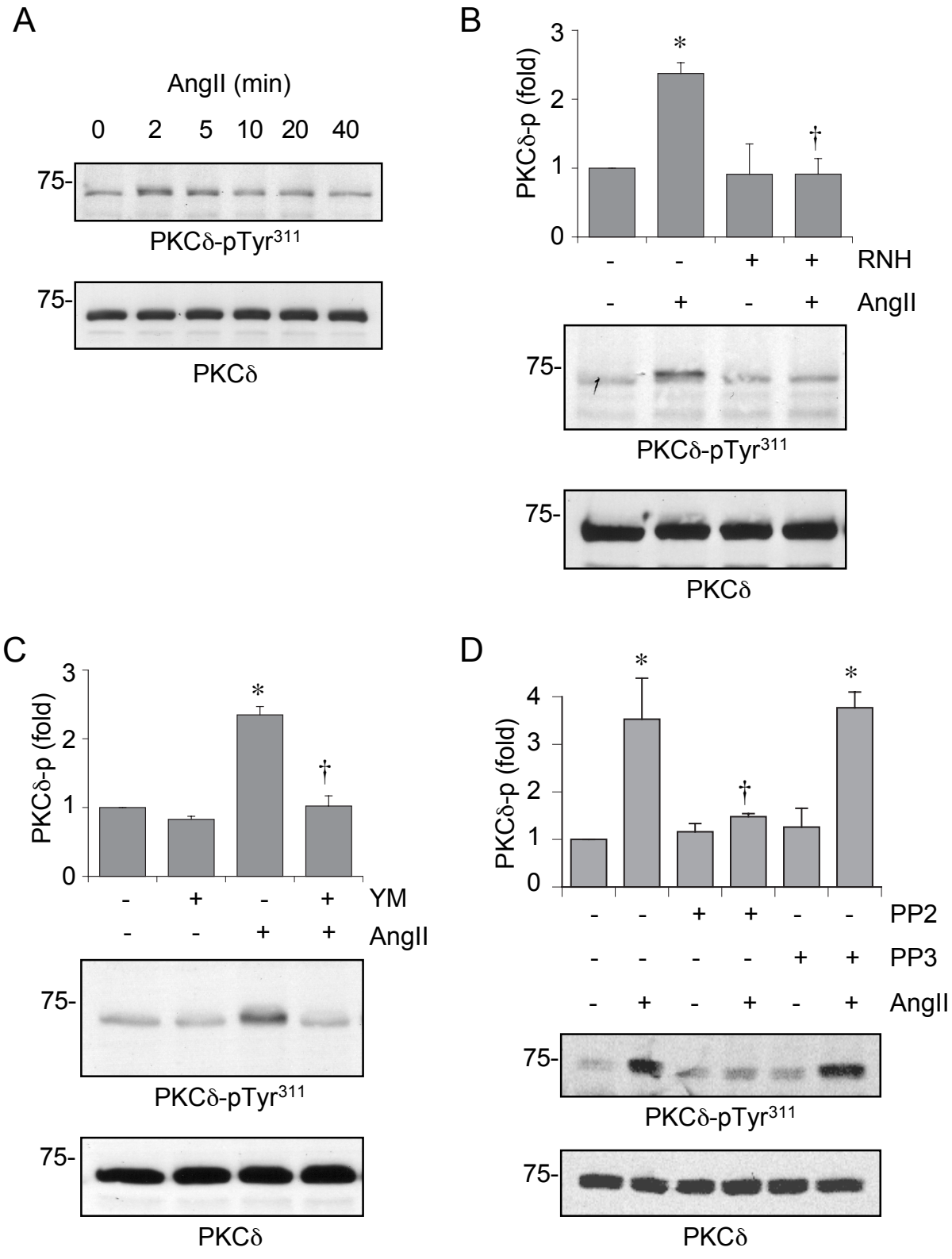
Figure S1: A. VSMCs were stimulated with 100 nmol/L AngII for the indicated time periods. **B** and **C**. VSMCs were pretreated with (A) AT_1 receptor blocker, RNH6270 (10 μmol/L) or (B) a selective G_q inhibitor, YM-254890 (1 μmol/L) for 30 min and stimulated with AngII (100 nmol/L) for 2 min. **D**. VSMCs were pretreated with a Src family kinase inhibitor, PP2 (5 μmol/L) or its negative control, PP3 (5 μmol/L) for 30 min and stimulated with 100 nmol/L AngII for 2 min. The cell lysates were immunoblotted with phospho-selective antibody which detects PKCδ Tyr^{311} phosphorylation and anti-PKCδ antibody. The PKCδ phosphorylation at Tyr^{311} was measured by densitometry and shown as mean ± SEM for three independent experiments. $*P < 0.05$ compared to the basal control. $\dagger P < 0.05$ compared to the stimulated control.

Figure S2: A. VSMCs were infected with retrovirus encoding control vector, wild type PKCδ tagged with GFP, or PKCδ Y311F mutant tagged with GFP. VSMCs were then stimulated with 100 nmol/L AngII for 3 days. Cell volume was analyzed with a Coulter counter. **B.** VSMCs were infected with retrovirus encoding control vector, wild type PKCδ, or PKCδ Y311F mutant. The cells were stimulated with 100 nmol/L AngII for 3 days. Afterwards, cell proliferation was measured by a CellTiter 96 Aqueous cell proliferation/viability assay kit. The data was presented as fold basal mean ± SEM for three independent experiments. **C**. VSMCs were infected with adenovirus encoding control vector or wild type PKCδ. The cells were stimulated with 100 nmol/L AngII for 24 hours. Afterwards, DNA synthesis was assessed by a BrdU incorporation kit. The data was presented as mean ± SEM (n=5).

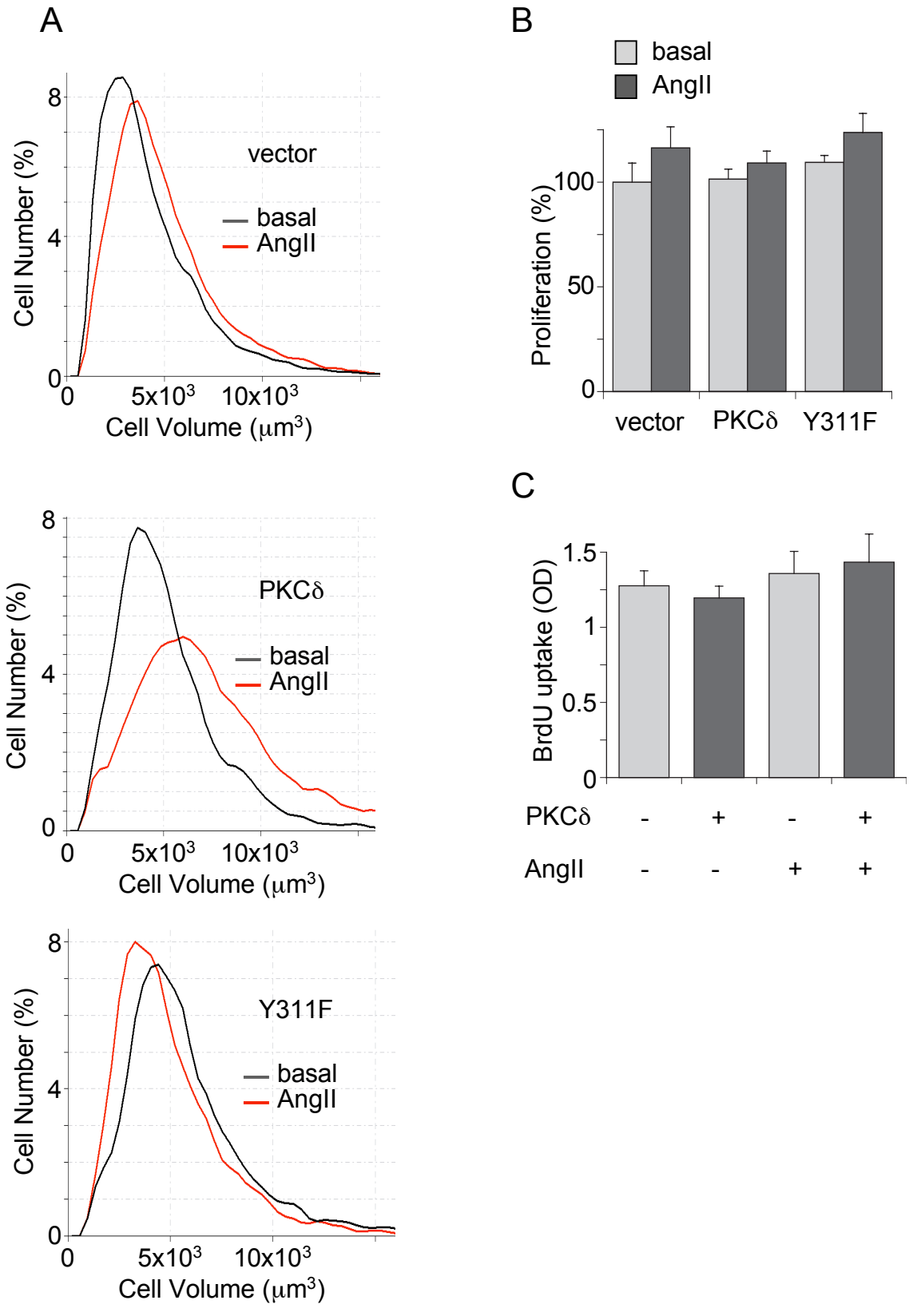
Figure S3: A. VSMCs were infected with adenovirus encoding wild type PKCδ or control empty vector, and stimulated with 100 nmol/L AngII for 4 hours. **B.** VSMCs were stimulated with 100 nmol/L AngII or 1 mmol/L staurosporine (Staur) for 4 hours. The cell lysates were immunoblotted with cleaved caspase-3 selective antibody, anti-GAPDH antibody and anti-PKCδ antibody, as indicated. Note that an apoptosis inducer, staurosporine, markedly stimulated cleavage of caspase 3. Data shown are representative from three independent experiments.

Figure S4: A. VSMCs were infected with adenovirus encoding a kinase-inactive PKCδ mutant (K376A) or control empty vector, and stimulated with 100 nmol/L AngII for 3 days. Cell volume was analyzed with a Coulter counter. **B.** VSMCs were infected with adenovirus encoding K376A or control vector. The cells were stimulated with 100 nmol/L AngII for 3 days. Afterwards, cell proliferation was measured by a CellTiter 96 Aqueous cell proliferation/viability assay kit. The data was presented as fold basal mean ± SEM for three independent experiments.

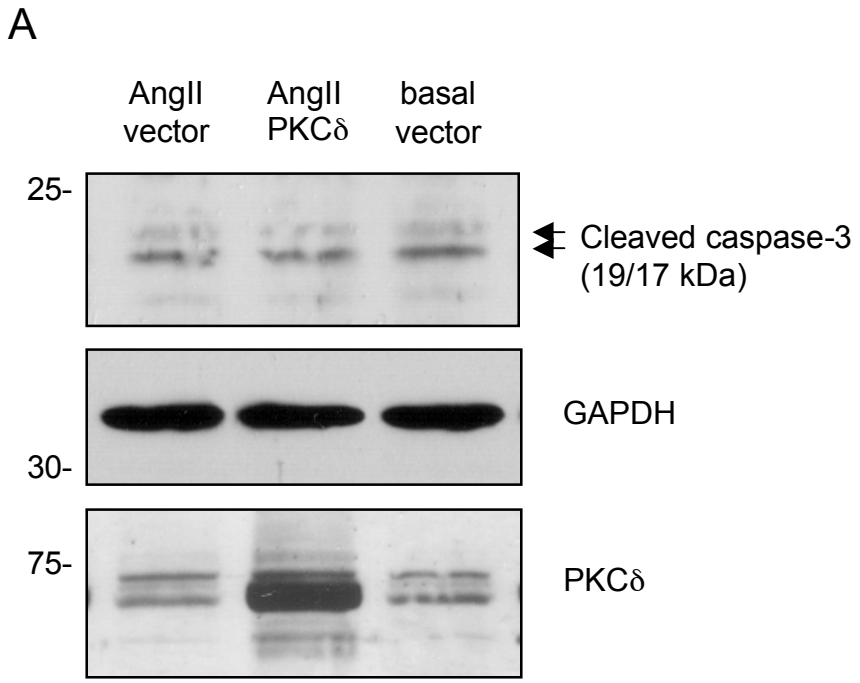
Supplement Figure S1



Supplement Figure S2



Supplement Figure S3



Supplement Figure S4