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PYK2/PDZ-RhoGEF Links Ca²⁺ Signaling to RhoA

Z. Ying, F. R.C. Giachini, R. C. Tostes and R. C. Webb

Arterioscler Thromb Vasc Biol, October 1, 2009; 29 (10): 1657-1663.

[Abstract] [Full Text] [PDF]

Salicylates dilate blood vessels through inhibiting PYK2-mediated RhoA/Rho-kinase activation

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Cardiovasc Res, July 1, 2009; 83 (1): 155-162.

[Abstract] [Full Text] [PDF]

Role of Focal Adhesion Kinase in Flow-Induced Dilation of Coronary Arterioles

R. Koshida, P. Rocic, S. Saito, T. Kiyooka, C. Zhang and W. M. Chilian

Arterioscler Thromb Vasc Biol, December 1, 2005; 25 (12): 2548-2553.

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Altered PYK2 phosphorylation by ANG II in hypertensive vascular smooth muscle

PETRA ROCIC,¹ TINA M. GRIFFIN,² CHASTITY N. McRAE,¹ AND PAMELA A. LUCCHESI¹

¹Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294; and ²Cardiovascular Institute, Stritch School of Medicine, Loyola University, Maywood, Illinois 60153

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Rocic, Petra, Tina M. Griffin, Chastity N. McRae, and Pamela A. Lucchesi. Altered PYK2 phosphorylation by ANG II in hypertensive vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 282: H457–H465, 2002; 10.1152/ajpheart.00546.2001.—Vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) exhibit increased cell growth compared with normotensive Wistar-Kyoto rats (WKY). ANG II stimulates growth via G_q-protein-coupled signaling that involves changes in cytosolic intracellular Ca²⁺ concentration ([Ca²⁺]_i) and activation of protein kinase C (PKC) and mitogen-activated protein kinases. This study examines the role of the proline-rich tyrosine kinase 2 (PYK2) in hypertensive VSMC. Basal PYK2 phosphorylation in SHR VSMC was increased compared with WKY (0.44 ± 0.02 vs. 0.20 ± 0.02-fold). ANG II-induced activation of PYK2 in SHR VSMC was of greater magnitude (2.2 ± 0.2-fold in SHR; 1.4 ± 0.1-fold in WKY) and occurred more rapidly (peak activation at 2 min in SHR vs. 5 min in WKY). This effect was blocked by pretreatment with the [Ca²⁺]_i chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid or the PKC inhibitor chelerythrine. Basal and ANG II-stimulated c-Fos expression was increased in SHR versus WKY VSMC. PYK2 downregulation with antisense oligonucleotides blocked ANG II-induced c-Fos expression. Increased PYK2 activation may be altered signaling cascades that regulate cell growth in hypertensive VSMC.

mitogen-activated protein kinase; spontaneously hypertensive rats; protein kinase C; calcium; c-Fos

ABNORMAL VASCULAR SMOOTH MUSCLE CELL (VSMC) function is a key feature of hypertension. Chronic changes that occur in hypertensive blood vessels include aberrant VSMC growth, alterations in extracellular matrix production, and remodeling, as well as adventitial and endothelial dysfunction. Altered VSMC growth leads to medial thickening and progressive luminal narrowing (19, 23). The mechanisms responsible for altered VSMC phenotype and function in hypertension remain unknown.

A commonly used model for studying genetic hypertension is the spontaneously hypertensive rat (SHR). VSMC isolated from SHR exhibit a distinct phenotype compared with those isolated from the normotensive

Wistar-Kyoto rat (WKY), characterized by an increase in cell growth, altered protein kinase C (PKC) activity, enhanced Na⁺/H⁺ exchanger activity, increased Ca²⁺-dependent activation of extracellularly regulated mitogen-activated protein (ERK1/2 MAP) kinases (2, 16), and increased expression of transcription factors responsible for the regulation of genes involved in cell growth, including c-Fos (12). c-Fos is one of the early response genes that has been shown to be activated in response to ANG II in VSMC (27). Touyz et al. (29) recently reported an increase in ANG II-induced c-Fos mRNA expression in SHR VSMC that required ERK1/2.

ANG II regulates VSMC function through activation of the ANG type 1 (AT₁) receptor (23). Signaling cascades initiated by AT₁ receptor activation have been shown to regulate a variety of cellular processes that control the observed phenotypic changes in SHR VSMC, including gene transcription and p70 ribosomal phosphorylation. These cellular responses to AT₁ receptor activation are initiated by a myriad of signaling cascades, including PKC, ERK1/2 MAP kinase, and phosphatidylinositol 3-kinase (PI3 kinase) (5, 7, 25).

However, the proximal signaling intermediates that transduce signals from the AT₁ receptor to the intracellular signaling cascades remain to be identified. We (16) have previously demonstrated that ANG II-induced, Ca²⁺-dependent ERK1/2 activation was increased in SHR VSMC compared with WKY VSMC despite no observable difference in ANG II-induced Ca²⁺ transients or total ERK1/2 expression. The results suggest that the expression or activation of a Ca²⁺-dependent regulator of the ERK1/2 pathway is increased in SHR compared with WKY VSMC.

An attractive candidate is the Ca²⁺-dependent, non-receptor, proline-rich tyrosine kinase 2 (PYK2). Sabri et al. (24) showed that ANG II activates PYK2 in a Ca²⁺- and PKC-dependent manner in VSMC. This is compatible with the observed Ca²⁺- and PKC-dependent activation of ERK1/2 MAP kinases. PYK2 has been shown to interact with the upstream regulators of the ERK1/2 pathway, Src, Shc, Grb2, and Ras (26). Therefore, we hypothesized that, like ERK1/2, PYK2

Address for reprint requests and other correspondence: P. A. Lucchesi, Dept. of Physiology and Biophysics, MCLM-986, 1530 Third Ave. S., Birmingham, AL 35294-0005 (E-mail: lucchesi@physiology.uab.edu).

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regulation by vasoactive agonists may be altered. Rocic et al. (20) have also shown that PYK2 interacts with both the ERK1/2 and the PI3-kinase signaling pathways in VSMC and that the inhibitors of these pathways prevent ANG II-induced protein synthesis (8).

In this study, we show increased basal PYK2 phosphorylation in SHR compared with WKY VSMC. ANG II caused a more rapid and potent PYK2 activation in SHR than in WKY VSMC in a Ca^{2+} - and PKC-dependent manner. We then show a greater dependence of PYK2 activation on Ca^{2+} in SHR VSMC and determine that typical, novel, and atypical PKC isoforms contribute to the greater activation of PYK2 in SHR VSMC. Finally, we show that PYK2 forms a signaling complex with the upstream regulators of the ERK1/2 MAP kinase pathway and that the formation of this complex is more rapid and of a greater magnitude in hypertensive VSMC. Therefore, PYK2 may represent a key signaling molecule that is differentially regulated in hypertensive VSMC (21).

METHODS

Materials and antibodies. ANG II was purchased from Sigma. 1,2-Bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA), chelerythrine, and phorbol dibutyrate (PDBU) were from Calbiochem. Monoclonal PYK2, polyclonal pTyr, and isoform-specific PKC antibodies were from Pharmagen. Monoclonal pTyr antibodies were from Upstate Biotechnology. Polyclonal c-Fos antibodies were from Santa Cruz Biotechnology. PYK2 antisense oligonucleotides were custom designed by Biognostik (Göttingen, Germany). Lipofectamine Plus was purchased from GIBCO-BRL.

Cell culture. VSMC from 10- to 12-wk-old SHR and WKY were isolated from the thoracic aorta by collagenase digestion and cultured as described (16). Passages 3–5 were used for all experiments. The animal protocol was approved by the University of Alabama-Birmingham Animal Care and Use Committee.

Immunoprecipitation. Growth-arrested VSMC were treated with 100 nM ANG II \pm inhibitors for the indicated times. Lysates were prepared as previously described (24). Protein concentrations were measured using a bicinchoninic acid assay (Pierce). PYK2 phosphorylation was monitored by two methods. First, cell lysates were immunoprecipitated with a monoclonal PYK2 antibody and phosphorylation was assessed by Western blot analysis with monoclonal phospho-Tyr antibodies. Second, cell lysates were immunoprecipitated with a polyclonal anti-phospho-Tyr (pTyr) antibody and phosphorylated PYK2 was detected by Western blot analysis with monoclonal anti-PYK2 antibodies. Both techniques produced nearly identical results. For immunoprecipitation experiments, equal amounts of protein (500 μ g) were immunoprecipitated with monoclonal anti-PYK2 antibodies overnight at 4°C. Immune complexes were collected by incubation with protein G-agarose or protein A-sepharose beads for 2 h at 4°C. The beads were centrifuged, washed twice in lysis buffer, and resuspended in 3 \times Laemmli sample buffer. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis.

Western blotting. VSMC lysates (20–30 μ g of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed as described (20) using anti-PYK2 (1:1,000) and anti-PKC isoform-

specific antibodies, monoclonal anti-pTyr antibodies (1:1,000, Upstate Biotechnology), or anti-c-Fos antibodies and horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by enhanced chemiluminescence and quantified by laser densitometry.

PYK2 antisense oligonucleotide incorporation. WKY and SHR VSMC were grown in 10% fetal calf serum-Dulbecco's modified Eagle's medium (DMEM) to 60% confluency. Cells were washed three times in Opti minimal essential medium 1 h before antisense treatment. VSMC were treated with PYK2 antisense oligonucleotides (0.75 μ M) for 8 h using Lipofectamine Plus (10 μ g/ml) as a transfection reagent. After 8 h, the medium was replaced with serum-free DMEM and left overnight. The next day, the medium was replaced with fresh serum-free DMEM for at least 1 h before treatment with ANG II.

Data analysis. All experiments use passage-matched sets of SHR and WKY VSMC. Data are expressed as means \pm SD for at least $n = 3$ experiments. To compare basal PYK2 activation between SHR and WKY VSMC, unstimulated cells from both sets were compared with positive controls (PC12 cell lysate) and were corrected for any differences in lane loading as detected by immunoblotting with anti-total PYK2 antibodies. c-Fos and PKC expression in SHR VSMC was compared with WKY. Statistical analyses of these data were performed with the use of paired Student's *t*-tests (InStat Software). To determine the extent of PYK2 phosphorylation by ANG II, time points were then compared with SHR and WKY controls (set = 1). Statistical analysis was performed using InStat software by one-way analysis of variance, followed by Bonferroni test as appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

Basal phosphorylation of PYK2 in SHR and WKY VSMC. We have previously shown that phosphorylation of PYK2 correlates well with PYK2 kinase activity (24). Basal PYK2 phosphorylation, detected by immunoprecipitation with anti-phosphotyrosine antibodies, followed by Western blot analysis with anti-PYK2 antibodies, was significantly (twofold) higher in SHR VSMC (0.44 ± 0.02 vs. total) compared with WKY VSMC (0.2 ± 0.02 vs. total PYK2) (Fig. 1).

ANG II induces differential PYK2 phosphorylation in SHR vs. WKY VSMC. We then examined the effects of ANG II on PYK2 phosphorylation in SHR and WKY VSMC. Passage-matched sets of SHR and WKY VSMC were growth arrested and then treated with 100 nM ANG II for 0–60 min. Cell lysates were immunoprecipitated with anti-PYK2 antibodies, and phosphorylated PYK2 was measured by Western blot analysis with monoclonal anti-pTyr antibodies. ANG II caused significant phosphorylation of PYK2 as early as 0.5 min. Peak ANG II-induced PYK2 phosphorylation was of a greater magnitude in SHR compared with WKY VSMC (2.2 ± 0.2 -fold at 2 min in SHR; 1.4 ± 0.1 -fold at 5 min in WKY) (Fig. 2A). The kinetics of PYK2 phosphorylation were quite different in the two cell types. Maximum ANG II-induced PYK2 phosphorylation was observed at 2 min in SHR compared with maximum activation at 5 min in WKY VSMC (Fig. 2A). These blots were then stripped and re probed with anti-PYK2 antibodies to confirm equal lane loading. Analogous

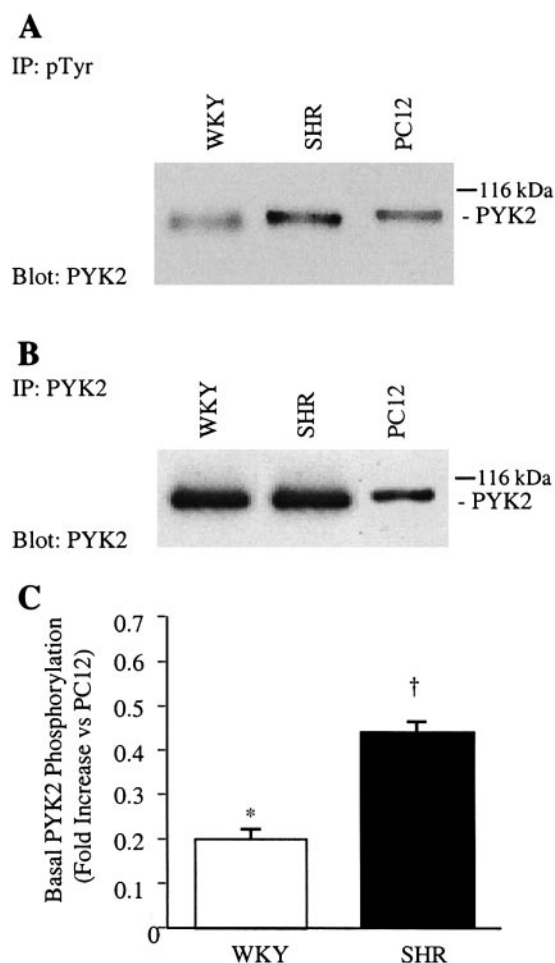


Fig. 1. Proline-rich tyrosine kinase 2 (PYK2) basal phosphorylation in spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY) vascular smooth muscle cells (VSMC). **A:** lysates from unstimulated VSMC were immunoprecipitated (IP) with anti-phospho-Tyr (pTyr) antibodies, and Western blot analysis was performed with anti-PYK2 antibodies. **B:** unstimulated VSMC lysates were immunoprecipitated with anti-PYK2 antibodies, and Western blot analysis was performed with anti-PYK2 antibodies. **C:** cumulative data from $n = 12$ experiments. PYK2 expression and basal phosphorylation were normalized to PC12 lysates. * $P < 0.05$ vs. PC12; † $P < 0.01$, SHR vs. WKY VSMC.

results were obtained when pTyr immunoprecipitates were blotted with anti-PYK2 antibodies (Fig. 2B).

ANG II-induced PYK2 phosphorylation requires an increase in cytosolic Ca^{2+} . To determine the ability of increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to stimulate PYK2 phosphorylation, we first treated VSMC with 1 μ M ionomycin, a Ca^{2+} ionophore, for 5 min. Treatment with ionomycin resulted in PYK2 tyrosine phosphorylation in both cell types. However, the magnitude of this phosphorylation was 1.7-fold higher in SHR (4.9 ± 0.5 -fold vs. control) compared with WKY (3.0 ± 0.2 -fold vs. control) (Fig. 3A). To assess the requirement for cytosolic $[Ca^{2+}]_i$ in ANG II-induced PYK2 activation, we examined the effect of the Ca^{2+} chelator BAPTA on PYK2 phosphorylation. Pretreatment with 50 μ M BAPTA resulted in a significant reduction in ANG II-depend-

ent PYK2 phosphorylation at all time points examined (Fig. 3B). In SHR VSMC, maximal PYK2 phosphorylation was inhibited $\sim 80\%$ by Ca^{2+} chelation (from 10.3 ± 1.1 -fold to 2.5 ± 0.5 -fold vs. control). In WKY VSMC, BAPTA pretreatment resulted in a $\sim 70\%$ inhibition of ANG II-induced PYK2 phosphorylation (from 6.5 ± 1.1 -fold to 2.0 ± 0.4 -fold vs. control). In fact, there was no significant difference in ANG II-induced PYK2 phosphorylation between SHR and WKY VSMC after $[Ca^{2+}]_i$ chelation (Fig. 3B). These results indicate that ANG II-induced PYK2 activation is dependent on the increase in cytosolic Ca^{2+} and that the Ca^{2+} dependence of PYK2 activation is greater in SHR compared with WKY VSMC.

Characterization of PKC isoforms involved in ANG II-induced PYK2 phosphorylation. To determine the role of PKC isoforms in PYK2 activation, we pretreated VSMC with the non-isoform-specific PKC inhibitor chelerythrine. Pretreatment with 5 μ M chelerythrine chloride for 45 min decreased the ANG II-dependent PYK2 phosphorylation to basal levels at all time points. Moreover, ANG II-induced PYK2 phosphorylation was not different between SHR and WKY VSMC in the presence of the PKC inhibitor (Fig. 4A).

To gain insight as to which PKC isoforms may be involved in PYK2 regulation in SHR and WKY VSMC, we pretreated VSMC for 24 h with PDBU to downregulate phorbol ester-sensitive PKC isoforms. In SHR, PKC downregulation by PDBU led to a $\sim 70\%$ decrease in ANG II-induced PYK2 phosphorylation (6.9 ± 0.8 -fold to 2.3 ± 0.6 -fold vs. control). In WKY VSMC, PKC inhibition with PDBU led to a $\sim 60\%$ decrease in PYK2 activation (4.4 ± 0.2 -fold to 2.9 ± 0.48 -fold vs. control) (Fig. 4B).

Twenty-four hours of PDBU pretreatment caused complete downregulation of PKC- α , - δ , and - ϵ , a partial downregulation of PKC- β and - γ , but had no effect on the atypical PKC- ζ isoform (Fig. 4B). Interestingly, the expression of PKC- δ , - ϵ , and - ζ , and to a lesser degree PKC- β appears to be elevated in SHR compared with WKY VSMC (Fig. 5).

PYK2 antisense oligonucleotides block increased c-Fos expression in SHR VSMC. We (22) have previously shown that PYK2 downregulation inhibits ANG II-induced protein synthesis in VSMC. To determine the functional consequences of increased PYK2 activation in SHR VSMC, we assessed the effects of PYK2 downregulation on c-Fos expression. PYK2 antisense oligonucleotides significantly decreased PYK2 total protein levels to the same extent in both WKY and SHR VSMC (data not shown). PYK2 antisense treatment had no effect on the expression of the closely related focal adhesion kinase. Basal c-Fos protein expression was increased in SHR versus WKY VSMC (5.5 ± 0.1 -fold). Treatment with ANG II for 24 h resulted in an increase in c-Fos expression that was greater in SHR than WKY (4.4 ± 0.08 -fold in SHR vs. 2.7 ± 0.01 -fold in WKY). PYK2 downregulation by antisense oligonucleotides reduced basal c-Fos expression and completely blocked ANG II-induced c-Fos expression in both cell types (Fig. 6).

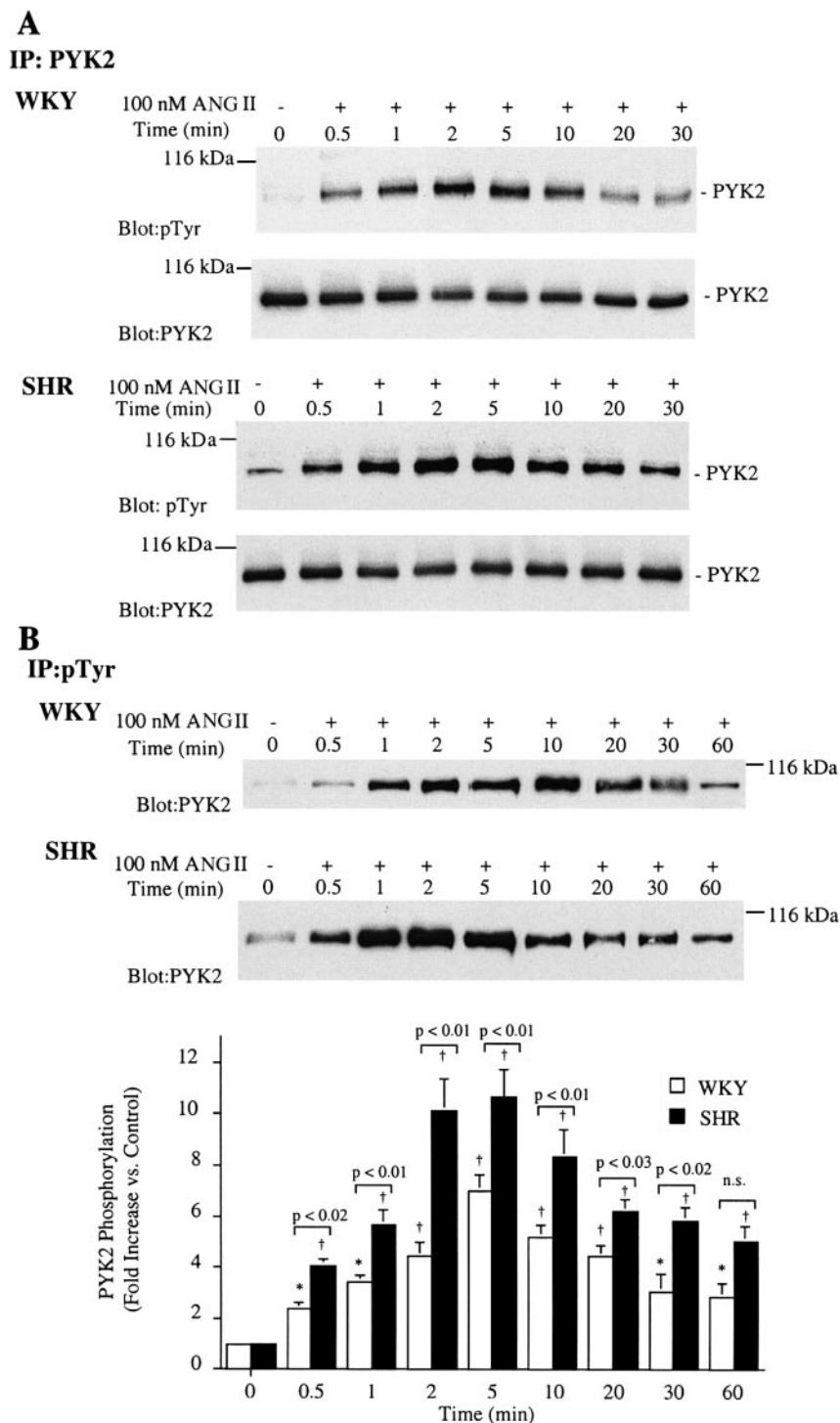


Fig. 2. ANG II-induced PYK2 phosphorylation in SHR and WKY VSMC. *A*: immunoprecipitation with anti-PYK2 antibodies. VSMC were treated with 100 nM ANG II for 0–30 min. Cell lysates were immunoprecipitated using anti-PYK2 antibodies. Immunoblot analysis was performed using monoclonal anti-pTyr antibodies. Blots were then stripped and reprobed using anti-PYK2 antibodies. *B*: immunoprecipitation with anti-pTyr antibodies. VSMC were treated with 100 nM ANG II for 0–60 min. Cell lysates were immunoprecipitated using anti-pTyr antibodies. Immunoblot analysis was performed using anti-PYK2 antibodies. *Top*: representative immunoblots. *Bottom*: cumulative results from $n = 12$ experiments. * $P < 0.05$ vs. control; † $P < 0.01$ vs. control. NS, not significant

DISCUSSION

The present findings suggest that the Ca^{2+} -sensitive tyrosine kinase PYK2 may be an important signaling molecule whose regulation is significantly altered in hypertensive VSMC. In Fig. 1, we show that basal PYK2 phosphorylation is significantly increased in hypertensive VSMC. Because phosphorylation correlates well with PYK2 activation, these results suggest that basal PYK2 activity is increased in SHR VSMC. Our

data show significant differences in the magnitude and kinetics of PYK2 activation by ANG II between SHR and WKY VSMC. Compared with WKY, ANG II-induced PYK2 phosphorylation occurred more rapidly (2 compared with 5 min) and was ~2- to 3-fold greater in magnitude (Fig. 2). These findings are compatible with previous results showing increased and more rapid ERK1/2 MAP kinase activation in SHR VSMC (16, 30) and in SHR aortas (13).

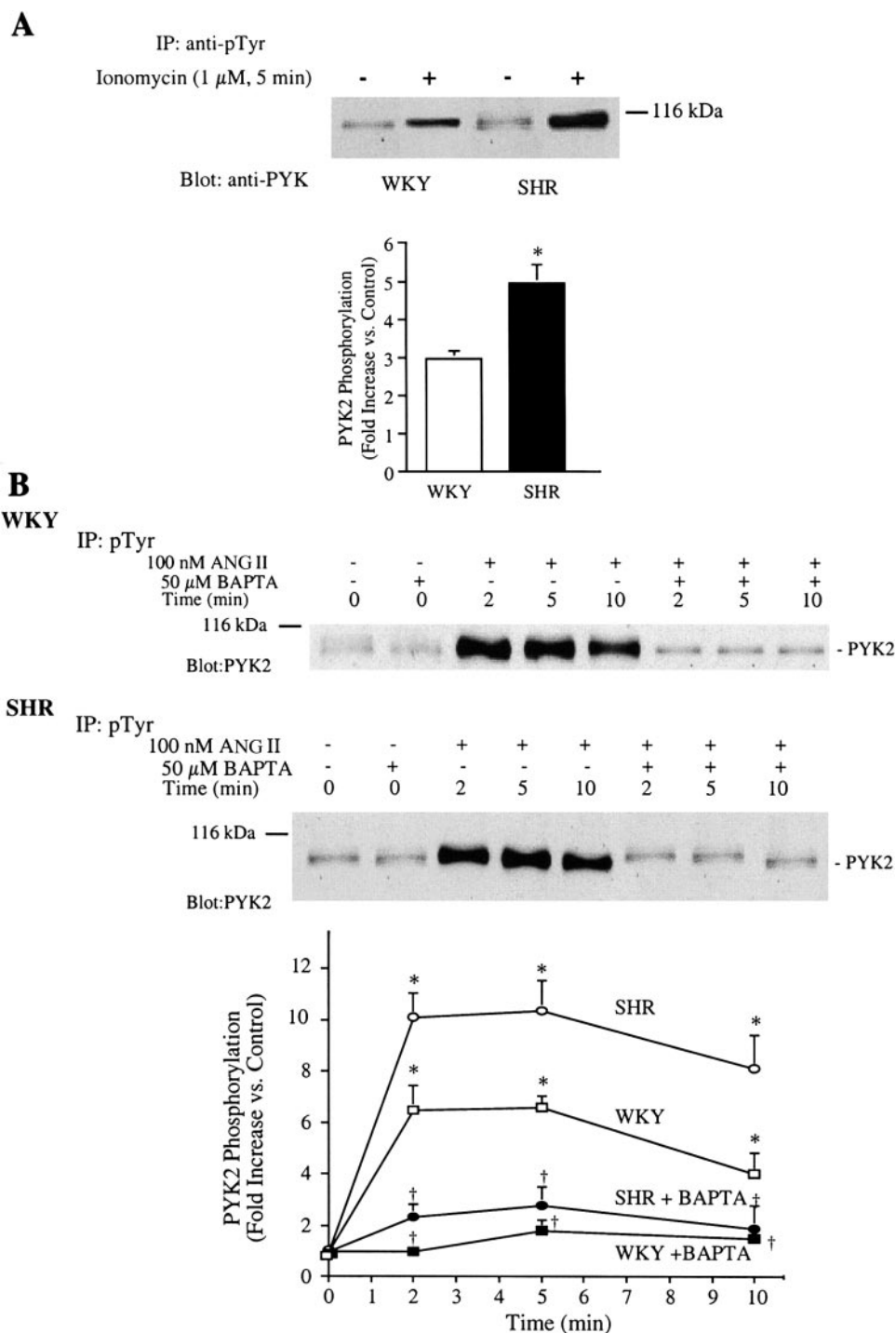


Fig. 3. PYK2 phosphorylation in SHR and WKY VSMC requires an increase in intracellular Ca^{2+} . *A*: VSMC were treated with 1 μ M ionomycin for 5 min. Cell lysates were immunoprecipitated using anti-pTyr antibodies. Immunoblot analysis was performed using anti-PYK2 antibodies. *Top*: representative immunoblot. *Bottom*: cumulative data from $n = 4$ experiments. * $P < 0.05$ vs. WKY VSMC. *B*: growth-arrested VSMC were pretreated with 50 μ M 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA) for 30 min before treatment with 100 nmol/l ANG II. Cell lysates were immunoprecipitated using anti-pTyr antibodies. Immunoblot analysis was performed using anti-PYK2 antibodies. *Top*: representative immunoblot. *Bottom*: cumulative data from of $n = 4$ experiments. * $P < 0.01$ vs. control; † $P < 0.01$ BAPTA treated vs. ANG II.

We then examined the involvement of PYK2 in the regulation of the ERK1/2 MAP kinase signaling pathway. It has been shown that PYK2 and Src activation link G protein-coupled receptor activation to ERK1/2 MAP kinases (4). Furthermore, regulation of the MAP kinase pathway is Ca^{2+} dependent (14, 16) and PYK2 activation leads to the recruitment of Src (24, 26). Data from our laboratory (24) showed complex formation between PYK2, Src, Grb2, and Shc in VSMC.

Given the important role of $[Ca^{2+}]_i$ in the pathogenesis of hypertension, we then compared the relation-

ship between $[Ca^{2+}]_i$ and PYK2 activation between SHR and WKY VSMC. In theory, both changes in $[Ca^{2+}]_i$ mobilization or in the expression and/or the sensitivity of cellular signaling molecules to $[Ca^{2+}]_i$ could explain alterations in Ca^{2+} -dependent phenotypic modulation of hypertensive VSMC. There are conflicting reports about increased basal Ca^{2+} or agonist-induced Ca^{2+} transients in hypertensive vascular smooth muscle. Toyuz et al. (28) reported an increase in both basal Ca^{2+} as well as enhanced ANG II-dependent increases in Ca^{2+} in mesenteric artery VSMC.

A

WKY IP: pTyr

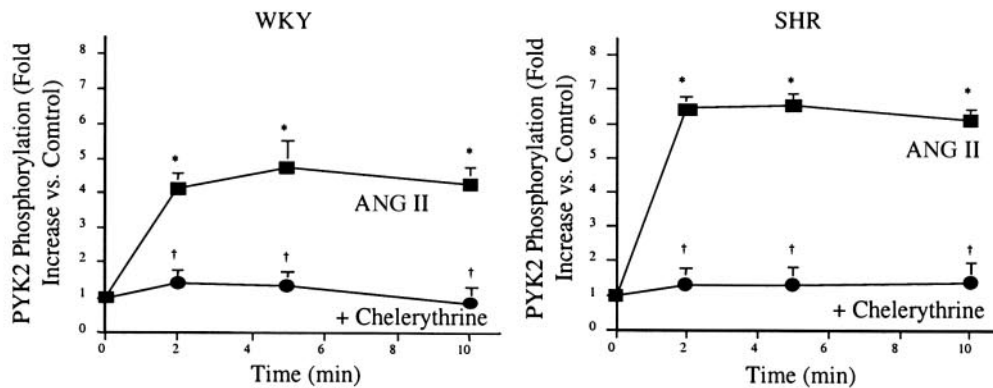
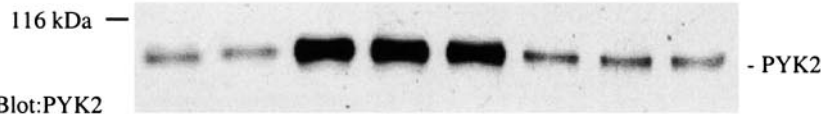
100 nM ANG II	-	-	+	+	+	+	+	+
10 μM Chelerythrine	-	+	-	-	+	+	+	+
Time (min)	0	0	2	5	10	2	5	10



SHR

Blot:PYK2

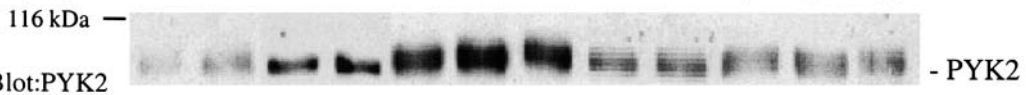
100 nM ANG II	-	-	+	+	+	+	+	+
10 μM Chelerythrine	-	+	-	-	-	+	+	+
Time (min)	0	0	2	5	10	2	5	10



B

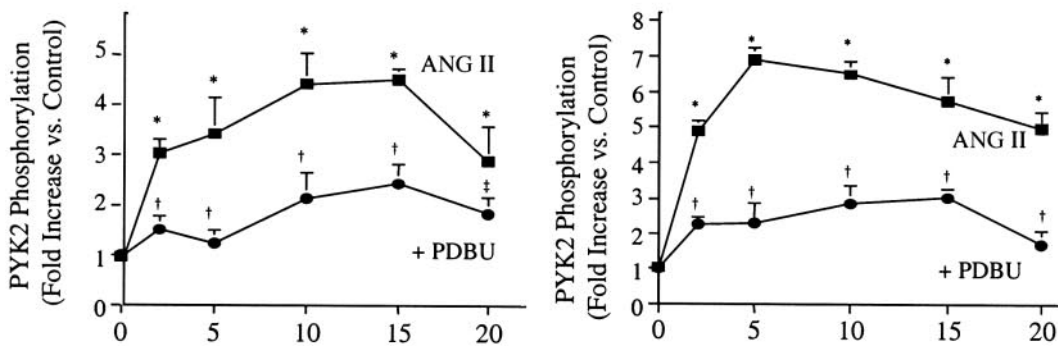
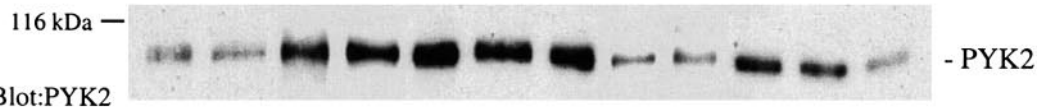
WKY IP:pTyr

100 nM ANG II	-	+	+	+	+	+	+	+	+	+	+	
100 nM PDBU	-	+	-	-	-	-	+	+	+	+	+	
Time (min)	0	0	2	5	10	15	20	2	5	10	15	20



SHR

100 nM ANG II	-	+	+	+	+	+	+	+	+	+	+	
100 nM PDBU	-	+	-	-	-	-	-	+	+	+	+	
Time (min)	0	0	2	5	10	15	20	2	5	10	15	20



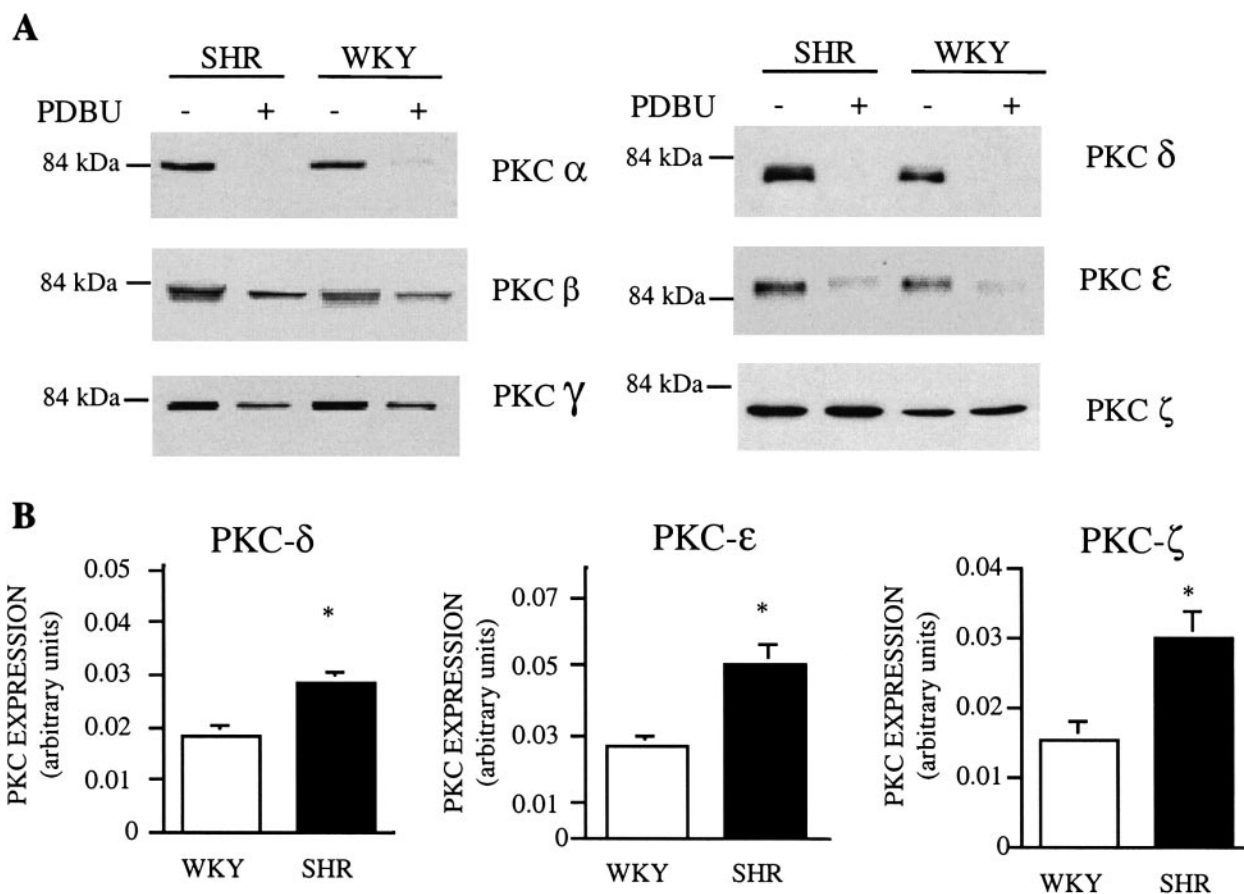


Fig. 5. Protein kinase C (PKC) expression in SHR and WKY VSMC. VSMC were treated with 1 μ M PDBU for 24 h. A: Western blot analysis using isoform-specific anti-PKC antibodies. B: cumulative data of $n = 3$ experiments. * $P < 0.05$ vs. WKY VSMC.

Bendhack et al. (1) demonstrated that basal and ANG II-stimulated increases in Ca^{2+} in cultured aortic VSMC from SHR were greater compared with WKY VSMC. On the other hand, we and others have shown that ANG II-induced Ca^{2+} transients were not different between SHR and WKY VSMC (16, 28). Reasons for these discrepancies are unclear but may be due to differences in the vessels used for VSMC isolation or in the passage or confluency of cultured VSMC.

There have been several reports of increased expression or sensitivity of Ca^{2+} -dependent signaling molecules in different models of hypertension. For example, Kato et al. (11) demonstrated enhanced Ca^{2+} sensitivity of the phospholipase C δ 1-isoform in the aorta of SHR compared with WKY. Several groups have reported increased expression and activation of Ca^{2+} -dependent PKC isoforms in SHR cardiac tissue and blood vessels (10).

These findings led us to determine whether Ca^{2+} -dependent activation of PYK2 was greater in SHR VSMC versus WKY VSMC. We and others (3, 24) have shown that PYK2 activation in VSMC is Ca^{2+} dependent. In the present study, we demonstrate that the Ca^{2+} ionophore ionomycin caused a significantly greater PYK2 phosphorylation in SHR VSMC compared with WKY VSMC (Fig. 3A). Moreover, chelation of intracellular Ca^{2+} with BAPTA also resulted in a greater degree of inhibition of ANG II-induced PYK2 activation in SHR than in WKY VSMC (Fig. 3B). In fact, the BAPTA pretreatment abrogated the differences in ANG II-induced PYK2 phosphorylation between SHR and WKY VSMC. These results are in close agreement with our previous study showing a greater Ca^{2+} dependency for ANG II-induced ERK1/2 MAP kinase activation in SHR versus WKY VSMC (16). Thus it is possible that differential PYK2 activation in

Fig. 4. A: effects of chelerythrine on PYK2 activation in response to ANG II. VSMC were pretreated with 5 μ M chelerythrine for 45 min before treatment with 100 nM ANG II. Cell lysates were immunoprecipitated using anti-pTyr antibodies. Immunoblot analysis was performed using anti-PYK2 antibodies. Top: representative immunoblot. Bottom: cumulative data from $n = 4$ experiments. * $P < 0.01$ vs. control; † $P < 0.01$ chelerythrine vs. ANG II. B: effects of phorbol dibutyrate (PDBU) on PYK2 activation in response to ANG II. VSMC were pretreated with 1 μ M PDBU for 24 h before treatment with 100 nM ANG II. Cell lysates were immunoprecipitated using anti-pTyr antibodies. Immunoblot analysis was performed using anti-PYK2 antibodies. Top: representative immunoblot. Bottom: cumulative data from $n = 5$ experiments. * $P < 0.01$ vs. control; † $P < 0.01$ PDBU vs. ANG II.

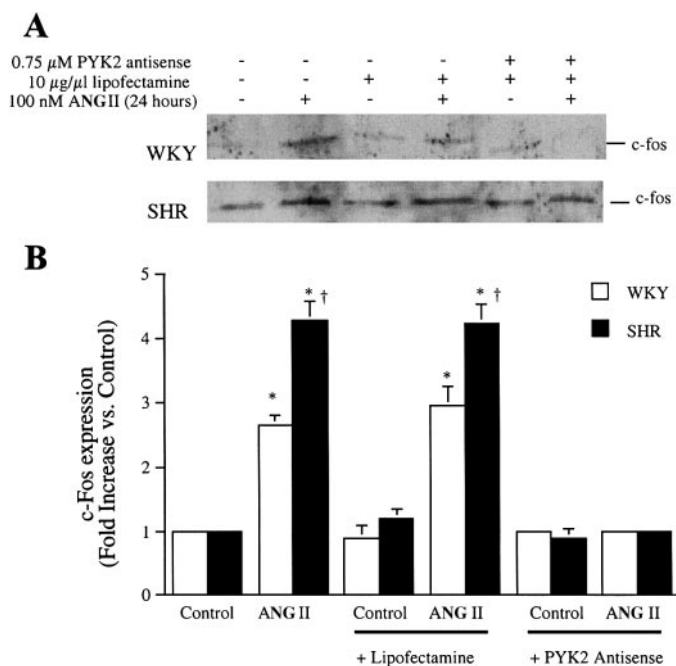


Fig. 6. Increased c-Fos expression in SHR VSMC is blocked by PYK2 antisense oligodeonucleotides (AS-ODNs). SHR and WKY VSMC were treated with ANG II for 24 h and with PYK2 AS-ODNs or lipofectamine alone. *A*: representative Western blots using anti-c-Fos antibodies. *B*: cumulative data from $n = 3$ experiments. * $P < 0.05$ vs. control; † $P < 0.05$ vs. WKY VSMC.

SHR VSMC may represent one Ca^{2+} -dependent upstream regulator of ERK1/2 MAP kinases.

There is mounting evidence that alterations in PKC isoform expression or activation are involved in the progression of hypertension. Both ERK1/2 activation as well as PYK2 activation in VSMC has been shown to be PKC dependent (15, 24). We now provide evidence that PKC inhibition by chelerythrine completely blocks PYK2 phosphorylation in both SHR and WKY VSMC (Fig. 4A). These results are in close agreement with our previous studies (24) in VSMC derived from the Sprague-Dawley rat aorta and suggest that one or more PKC isoforms are upstream of PYK2.

PKC isoforms have been classified into three groups; the conventional Ca^{2+} -dependent phorbol ester-sensitive isoforms (PKC- α , - β , and - γ), the novel Ca^{2+} -independent phorbol ester-sensitive isoforms (nPKC- δ , - ϵ , - η , and - θ) and the atypical Ca^{2+} -independent phorbol ester-insensitive isoforms (PKC- λ , - ι , - μ , and - ζ). We pretreated VSMC for 24 h with PDBU to downregulate both classic and novel PKC isoforms. PDBU led to a significant but incomplete inhibition of ANG II-induced PYK2 phosphorylation in SHR and WKY VSMC (Fig. 4B), which is in agreement with our previous findings for ERK1/2 MAP kinases (16).

Given this incomplete inhibition by prolonged PDBU treatment, we can conclude that both phorbol ester-sensitive and -insensitive PKC isoforms are involved in mediating ANG II-induced PYK2 phosphorylation. Interestingly, we noticed an increase in the expression of the novel Ca^{2+} -insensitive phorbol ester-sensitive PKC

isoforms δ and ϵ and the atypical PKC isoform PKC- ζ (Fig. 5). Therefore, it is tempting to speculate that both novel and atypical PKC isoforms are upstream regulators of PYK2 in SHR VSMC. These data are in agreement with previous findings indicating increased activation of PKC- δ and - ϵ SHR VSMC and with increased activity and expression of PKC- α mRNA in the SHR aorta (9, 10) and PKC- ϵ in cardiac myocytes from deoxycorticosterone acetate-salt-sensitive hypertensive rats (6). Moreover, at least one of those PKC isoforms (PKC- ζ) is involved in ANG II-stimulated ERK1/2 activation in VSMC (15).

The increased expression of these Ca^{2+} -independent PKC isoforms cannot account for the greater Ca^{2+} dependence of PYK2 activation in SHR VSMC. There is a slight trend for increase expression of the Ca^{2+} -sensitive PKC- β_2 in SHR VSMC, but the relatively weak sensitivity of the anti-PKC- β_2 antibodies available cautions the interpretation of these data. It is also possible that the Ca^{2+} -sensitive step lies between PKC and PYK2 activation because several laboratories have reported that differences in Ca^{2+} (17) sensitivity between SHR and WKY VSMC occur downstream of PKC activation (18). Future studies are required to characterize the Ca^{2+} -dependent signaling molecules that regulate ANG II-induced PYK2 activation in hypertensive VSMC.

To determine the functional consequences of increased PYK2 activation in SHR VSMC, we examined the effect of PYK2 downregulation by PYK2 antisense oligodeonucleotides on ANG II-induced c-Fos expression. We have previously shown that PYK2 antisense oligonucleotides reduce PYK2 expression by >80% and completely inhibited ANG II-induced protein synthesis in an ERK1/2- and PI3-kinase-dependent manner (22). Our results demonstrate that treatment with PYK2 antisense oligonucleotides completely inhibits ANG II-induced c-Fos expression in both SHR and WKY VSMC (Fig. 6). Schiffrin's laboratory (29) recently demonstrated that pharmacological inhibitors of Src blocked c-Fos mRNA expression in SHR and WKY VSMC. Sabri et al. (24) demonstrated an ANG II-dependent complex formation between PYK2 and Src in VSMC. Thus these data are in agreement with our hypothesis that the functional consequences of enhanced PYK2 activation in SHR VSMC include enhanced ERK1/2 activation leading to increased expression of key transcription factors, including c-Fos.

In summary, our data suggest that PYK2 lies upstream of one or more signaling pathways that have been implicated in the phenotypic modulation of hypertensive VSMC. The precise role this kinase plays in mediating these processes remains to be elucidated. Future studies with PYK2 antisense oligonucleotides will determine the precise role of this kinase in altered VSMC function during the development and progression of hypertension.

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REFERENCES

1. **Bendhack LM, Sharma RM, and Bhalla RC.** Altered signal transduction in vascular smooth muscle cells of spontaneously hypertensive rats. *Hypertens* 19, *Suppl*: II142–II148, 1992.
2. **Berk BC, Vallega G, Muslin AJ, Gordon HM, Canessa M, and Alexander RW.** Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na^+/H^+ exchange. *J Clin Invest* 83: 822–829, 1989.
3. **Brinson A, Harding T, Diliberto P, He Y, Li X, Hunter D, Herman B, Earp H, and Graves L.** Regulation of a calcium-dependent tyrosine kinase in vascular smooth muscle cells by angiotensin II and platelet-derived growth factor. *J Biol Chem* 273: 1711–1718, 1998.
4. **Dikic I, Tokiwa G, Lev S, Courtneidge SA, and Schlessinger J.** A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* 283: 547–550, 1996.
5. **Duff JL, Marrero MB, Paxton WG, Schieffer B, Bernstein KE, and Berk BC.** Angiotensin II signal transduction and the mitogen-activated protein kinase pathway. *Cardiovasc Res* 30: 511–517, 1995.
6. **Fareh J, Touyz R, Schiffrin E, and Thibault G.** Altered cardiac endothelin receptors and protein kinase C in deoxycorticosterone-salt hypertensive rats. *J Mol Cell Cardiol* 32: 665–676, 2000.
7. **Giasson E and Meloche S.** Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells. *J Biol Chem* 270: 5225–5231, 1995.
8. **Govindarajan G, Eble DM, Lucchesi PA, and Samarel AM.** Focal adhesion kinase is involved in angiotensin II-mediated protein synthesis in cultured vascular smooth muscle cells. *Circ Res* 87: 710–716, 2000.
9. **Kanashiro C, Alexander B, Granger J, and Khalil R.** Ca^{2+} -insensitive vascular protein kinase C during pregnancy and NOS inhibition. *Hypertension* 34: 924–930, 1999.
10. **Kanayama Y, Negoro N, Okamura M, Konoshi Y, Nishimura M, Umetani N, Inoue T, and Takeda T.** Modulation of protein kinase C in aorta from spontaneously hypertensive rats with enalapril treatment. *Osaka City Med J* 40: 83–97, 1994.
11. **Kato H, Fukami K, Shibasaki F, Homma Y, and Takenawa T.** Enhancement of phospholipase C delta 1 activity in the aortas of spontaneously hypertensive rats. *J Biol Chem* 267: 6483–6487, 1992.
12. **Kortenjann M, Thomae O, and Shaw PE.** Inhibition of v-ras-dependent c-Fos expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. *Mol Cell Biol* 14: 4815–4824, 1994.
13. **Kubo T, Ibusuki T, Saito E, Kambe T, and Hagiwara Y.** Vascular mitogen activated protein kinase activity is enhanced via angiotensin system in spontaneously hypertensive rats. *Eur J Pharmacol* 372: 279–285, 1999.
14. **Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio J, Plowman G, Rudy B, and Schlessinger J.** Protein tyrosine kinase PYK2 involved in Ca^{2+} -induced regulation of ion channel and MAP kinase function. *Nature* 376: 737–745, 1995.
15. **Liao DF, Monia B, Dean N, and Berk BC.** Protein kinase C mediates angiotensin 2 activation of ERK1/2 in vascular smooth muscle cells. *J Biol Chem* 272: 6146–6150, 1997.
16. **Lucchesi PA, Bell JM, Willis LS, Byron KL, Corson MA, and Berk BC.** Calcium-dependent MAP kinase activation in SHR defines a hypertensive signal transduction phenotype. *Circ Res* 78: 962–979, 1996.
17. **Marche P, Herembert T, and Zhu D.** Molecular mechanisms of vascular hypertrophy in the spontaneously hypertensive rat. *Clinic Experiment Pharm Physiol* 22, *Suppl*: S114–S116, 1995.
18. **Neusser M, Tepel M, and Zidek W.** Angiotensin II responses after protein kinase C activation in vascular smooth muscle cells from spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 21: 749–753, 1993.
19. **Owens GK.** Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 257: H1755–H1765, 1989.
20. **Rocic P, Govindarajan G, Sabri A, and Lucchesi PA.** A role for PYK2 in regulation of ERK1/2 MAP kinases and PI3-kinase by ANG II in vascular smooth muscle. *Am J Physiol Cell Physiol* 280: C90–C99, 2001.
21. **Rocic P, Griffin TM, McRae CN, and Lucchesi PA.** Increased expression and activation of PYK2 in hypertensive vascular smooth muscle (Abstract). *FASEB J* 13: A1008, 1999.
22. **Rocic P and Lucchesi PA.** Downregulation by antisense oligonucleotides establishes a role for the proline rich tyrosine kinase PYK2 in angiotensin II-induced signaling in vascular smooth muscle. *J Biol Chem* 276: 21902–21906, 2001.
23. **Rosendorff C.** Vascular hypertrophy in hypertension. *Mt. Sinai J Med* 65: 108–117, 1998.
24. **Sabri A, Govindarajan G, Griffin T, Samarel A, Byron K, and Lucchesi P.** Calcium and Protein kinase C dependent-activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. *Circ Res* 83: 841–851, 1998.
25. **Saward L and Zahradka P.** Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle cells. *Circ Res* 81: 249–257, 1997.
26. **Takenawa T, Miki H, and Matuoka K.** Signaling through Grb2/Ash-control of the Ras pathway and cytoskeleton. *Curr Top Microbiol Immunol* 228: 325–342, 1998.
27. **Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, and Nadal-Ginard B.** Angiotensin II induces c-Fos expression in aortic smooth muscle. *J Biol Chem* 264: 526–530, 1989.
28. **Touyz R, Tolloczko B, and Schiffrin E.** Mesenteric vascular smooth muscle cells from spontaneously hypertensive rats display increased calcium in response to angiotensin II but not to endothelin-1. *J Hypertens* 12: 663–673, 1994.
29. **Touyz RM, He G, El Mabrouk M, Diep Q, Mardigyan V, and Schiffrin EL.** Differential activation of extracellular signal-regulated protein kinase 1/2 and p38 mitogen activated-protein kinase by AT1 receptors in vascular smooth muscle cells from Wistar-Kyoto rats and spontaneously hypertensive rats. *J Hypertens* 19: 553–559, 2001.
30. **Wilkie N, Ng L, and Boarder M.** ANG II responses of vascular smooth muscle cells from hypertensive rats; enhancement at the level of p42 and p44 mitogen activated protein kinase. *Br J Pharmacol* 122: 209–216, 1997.