



Callera, G. E., Antunes, T. T., He, Y., Montezano, A. C., Yogi, A., Savoia, C., and Touyz, R. M. (2016) c-Src inhibition improves cardiovascular function but not remodeling or fibrosis in Ang II-induced hypertension. *Hypertension*, 68(5), pp. 1179-1190.
(doi:[10.1161/HYPERTENSIONAHA.116.07699](https://doi.org/10.1161/HYPERTENSIONAHA.116.07699))

This is the author's final accepted version.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

<http://eprints.gla.ac.uk/122659/>

Deposited on: 14 September 2016

Enlighten – Research publications by members of the University of Glasgow
<http://eprints.gla.ac.uk>

**c-Src inhibition improves cardiovascular function but not remodeling or fibrosis
in Ang II-induced hypertension**

Glauca E Callera PhD¹, Tayze T Antunes PhD¹, Ying He MSc¹, Augusto C Montezano PhD^{1,2},
Alvaro Yogi PhD¹, Carmine Savoia MD³, Rhian M Touyz MD, PhD^{1,2}.

¹Kidney Research Centre, Ottawa Hospital Research Institute, University of
Ottawa, Ontario, Canada; ²Institute of Cardiovascular and Medical Sciences, University of
Glasgow, UK; ³Clinical and Molecular Medicine Department, Cardiology Unit, Sapienza
University of Rome, Rome, Italy.

Running title: Role of c-Src in hypertension

Word count: Abstract:250; Text:5996

To whom correspondence should be addressed:

Rhian M Touyz MD, PhD

Institute of Cardiovascular and Medical Sciences,

BHF Glasgow Cardiovascular Research Centre,

University of Glasgow,

126 University Place, Glasgow, G12 8TA,

Phone: + 44 (0)141 330 7775/7774; Fax: + 44 (0)141 330-3360,

Email: rhian.touyz@glasgow.ac.uk.

Abstract

c-Src plays an important role in Ang II signaling. Whether this member of the Src family kinases is involved in the development of Ang II-induced hypertension and associated cardiovascular damage *in vivo* remains unknown. Here we studied Ang II-infused (400ng/Kg/min) mice in which c-Src was partially deleted (c-Src^{+/-}) and in wild-type (WT, c-Src^{+/+}) mice treated with a c-Src inhibitor (CGP077675(25mg/Kg/day)). Ang II increased blood pressure and induced endothelial dysfunction in WT mice, responses that were ameliorated in c-Src^{+/-} and CGP077675-treated mice. Vascular wall thickness and cross-sectional area (CSA) were similarly increased by Ang II in WT and c-Src^{+/-} mice. CGP077675 further increased CSA in hypertensive mice. Cardiac dysfunction (ejection fraction, fractional shortening) in Ang II-infused WT mice was normalized in c-Src^{+/-} mice. Increased oxidative stress (plasma TBARS, H₂O₂ and vascular superoxide generation) in Ang II-infused WT mice, was attenuated in c-Src-deficient and CGP077675-treated mice. Hyperactivation of vascular c-Src, ERK1/2 and JNK in hypertensive mice was normalized in CGP077675-treated and c-Src^{+/-} mice. Vascular fibronectin was increased by Ang II in all groups and further augmented by CGP077675. Cardiac fibrosis and inflammation induced by Ang II were amplified in c-Src^{+/-} and CGP-treated mice. Our data indicate that while c-Src downregulation attenuates development of hypertension, improves endothelial and cardiac function, reduces oxidative stress and normalizes vascular signaling, it has little beneficial effect on fibrosis. These findings suggest a divergent role for c-Src in Ang II-dependent hypertension, where c-Src may be more important in regulating redox-sensitive cardiac and vascular function than fibrosis and remodeling.

Key words: c-Src, angiotensin II, oxidative stress, hypertension, CGP077675, endothelial dysfunction.

Introduction

Hypertension is associated with vascular damage characterized by impaired endothelial function, and arterial remodeling (1,2). Many factors underlie these processes, including angiotensin II (Ang II), a particularly important vasoactive peptide in human and experimental hypertension. Ang II regulates vascular function and structure by activating complex signaling pathways inducing contraction, cell growth, inflammation and fibrosis (3,4). Common to these processes is generation of reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide (H_2O_2), and activation of redox-sensitive kinases (5,6). ROS function as intracellular second messengers to stimulate mitogen-activated protein kinases (MAPKs), tyrosine kinases and transcription factors, and inactivate protein tyrosine phosphatases (7,8). ROS also increase intracellular calcium and activate pro-inflammatory transcription factors (9,10). NADPH oxidases (Nox), the major source of ROS in the vasculature, are tightly regulated by Ang II in vitro and in vivo (11-13). Mechanisms linking Ang II to Nox and upstream signaling kinases in vascular cells have received considerable attention (14,15). Of particular importance in the vasculature is c-Src, a member of the Src kinase family of non-receptor tyrosine kinases, key proximal regulators of Nox-driven superoxide anion generation and MAPK activation (16-18).

Src family protein kinases are proto-oncogenes involved in cell morphology, motility, proliferation and survival (19,20). There are 11 members of the Src-family in humans, of which c-Src, Fyn, and Yes are ubiquitously expressed. The 60-kDa c-Src, the prototype, is highly expressed in the vasculature, involved in redox signaling associated with vascular function, and rapidly activated by vasoactive agents, including Ang II (21,22). We previously reported that c-Src plays a major role in Ang II signaling (21-23). In vascular smooth muscle cells (VSMCs) from human resistance arteries, Ang II induces c-Src phosphorylation to produce superoxide

anion via Nox activation (24-26). Of clinical relevance, our studies demonstrated the contribution of c-Src in molecular and cellular processes underlying Ang II-mediated vascular changes that occur in human hypertension (24-27). In further support of a role for Src kinases in Ang II-dependent hypertension, Bo Qin and Zhou (28) demonstrated that pharmacological inhibition of Src family kinases prevented development of hypertension and reduced vascular smooth muscle myosin light chain phosphorylation in Ang II-infused mice. However, to our knowledge, it is still unclear whether c-Src is specifically implicated. Moreover, mechanisms whereby c-Src influences cardiovascular function *in vivo* remain to be elucidated.

In the present *in vivo* study, we focused on c-Src as a major player in vascular injury and target organ damage in Ang II-induced hypertension. We also explored putative molecular mechanisms, specifically ROS, pro-inflammatory and pro-fibrotic signaling pathways, whereby c-Src regulates cardiovascular function in hypertension.

Methods

See supplemental text

Animals

Genetic and pharmacological strategies were used to downregulate c-Src in mice. Firstly, we studied wild-type (WT) (c-Src^{+/+}) and heterozygous (c-Src^{+/-}) male mice (29) infused with Ang II (400 ng/kg/min, 10-12 days). c-Src^{-/-} mice have osteopetrosis with poor survival and hence were not studied. In some experiments we infused norepinephrine (NE; 3.8 µg/Kg/min) to assess whether c-Src effects are Ang II-specific or generalized. Secondly, we used a pharmacological approach to inhibit c-Src. Ang II-infused WT mice received daily injections of a c-Src inhibitor, CGP077675 (25 mg/Kg/day, 7 days) (30), started 5 days after initiation of Ang II infusion. At

study-end, plasma, aorta, mesenteric arteries and heart were collected. Systolic BP (SBP) was assessed by tail-cuff plethysmography and telemetry (11,31,).

Plasma Biochemistry

Plasma glucose, triglycerides, and cholesterol were determined by automated analyzer

Echocardiography

Cardiac geometry and function were evaluated by echocardiography (32).

Assessment of vascular function, structure and mechanics

Second order branches of the superior mesenteric artery were mounted in a pressure myograph and function, structure and mechanics studied (33).

ROS assessment

Vascular ROS generation was measured by lucigenin chemiluminescence and dihydroethidium fluorescence. Systemic oxidative stress was determined by quantifying plasma thiobarbituric acid-reactive substances (TBARS) levels and by amplex red (7,11).

Western blotting

Vascular protein was extracted and immunoblotted as described (7).

Histological and immunohistochemical analyses

Heart and aortic tissues were stained with Masson's trichrome or Sirius Red, and macrophages, labelled with CD68-antibody.

Statistics

Data are presented as means±SEM. Groups were compared using one-way ANOVA, two-way ANOVA or t-test as appropriate. P<0.05 was significant.

Results

Mouse characteristics

Table S1 demonstrates morphological and metabolic characteristics. Ang II increased heart mass in WT but not in c-Src^{+/-} mice. Metabolic parameters were similar between groups (Table S2).

Ang II-induced cardiac dysfunction is ameliorated in c-Src^{+/-} mice

Ang II-infused WT mice exhibited reduced fractional shortening and increased IVSd and LVPWd (Table S3).

Ang II-induced hypertension is blunted in c-Src-deficient mice

Ang II increased SBP in WT mice (Fig.1A). In c-Src^{+/-} mice, Ang II-induced BP increase was attenuated (Fig.1A). To evaluate whether c-Src effects are specific for Ang II, we also examined NE-induced pressor effects in c-Src-deficient mice. NE increased in SBP in both c-Src^{+/+} and c-Src^{+/-} mice, with similar maximal responses between groups (Fig.1B).

CGP077675 normalises blood pressure in Ang II-infused mice

In another series of experiments, c-Src^{+/+} mice were infused with Ang II in the absence and presence of CGP077675. Ang II-induced increase in SBP was reduced by CGP077675 (Fig.1C). Within 5 days of CGP077675, SBP was normalised to levels in vehicle-treated mice. BP responses observed by tail-cuff measurement were similar to those measured by telemetry (Fig.S1)

Endothelial dysfunction is ameliorated by c-Src downregulation in Ang II-induced hypertension

ACh-induced vasodilation was blunted in Ang II-infused c-Src^{+/+} mice, with maximal vasodilatory responses of 30% versus 70% in vehicle-infused counterparts. In c-Src^{+/-} mice, Ang II-induced maximal vasodilation was improved to 49.9% versus Ang II-infused controls (Fig.1D, Table S3). Ang II did not influence ACh-induced relaxation in c-Src^{+/-} mice versus vehicle-infused c-Src^{+/-} mice. In CGP077675-treated mice, ACh-mediated vasodilation was improved versus untreated mice (Fig.1D, Table S4).

SNP-induced endothelium-independent relaxation in Ang II-infused mice was unaltered (Fig.S2A) and responses to SNP were unaffected by CGP077675 (Fig.S2B).

Contractile responses in c-Src deficient mice

Contractile responses were similar in vehicle- and Ang II-infused mice (WT and c-Src^{+/-}) (Figs.S3A, Fig S3B). CGP077675 did not influence contractile responses (Fig S3A).

c-Src and small vessel structure

Morphologic parameters in small mesenteric arteries were evaluated at progressive increases in intraluminal pressure (Fig.S4). Mesenteric arteries from vehicle-infused c-Src^{+/+} and c-Src^{+/-} mice exhibited similar media thickness, lumen diameter, media:lumen ratio and cross sectional area. Ang II-infused c-Src^{+/+} mice displayed increased media thickness (Fig.2A) without changes in lumen diameter (Fig.2B), resulting in a higher media:lumen ratio (Fig.2C) versus c-Src^{+/+} vehicle-infused mice. Ang II did not alter the cross sectional area (Fig.2D), although a tendency towards an increase was observed. In CGP077675-treated Ang II-infused mice, media thickness, media:lumen ratio and cross-sectional area were significantly greater than that in vehicle-treated

control mice (Figs.2B-D). Arteries from Ang II-infused c-Src^{+/-} mice exhibited increased media thickness and media:lumen ratio (Figs.2E,2G), with no changes in lumen diameter or cross sectional area (Figs.2F,2H).

c-Src and vascular mechanics

Mechanical properties were evaluated at progressive increases in intraluminal pressure (Fig. S5). Mesenteric arteries from Ang II-infused c-Src^{+/+} and c-Src^{+/-} mice exhibited reduced mechanical stress versus vehicle-infused counterparts in response to stepwise increments of intraluminal pressure. No additional shift was observed in the mechanical stress curves from the CGP077675-treated Ang II-infused mice.

c-Src and aortic structure

Photomicrographs of Masson's trichrome-stained cross-sections of thoracic aorta showed a significant increase in the media thickness with Ang II infusion in c-Src^{+/+} and c-Src^{+/-} mice (Fig.3). Aortic thickening is evidenced by irregularities in the pattern and the distances between each concentric elastic lamina intermingled with smooth muscle cell layers (Fig.3A). Morphometric quantitative analysis showed that Ang II effects were attenuated by CGP077675 but not by c-Src deficiency (Fig.3B). Slight thickening of the adventitial layer with perivascular fibrosis surrounding the vessel wall was also observed in some Ang II-infused mice.

c-Src inhibition attenuates vascular and systemic oxidative stress in Ang II-induced hypertension

DHE fluorescence images show increased ROS generation in aortas from Ang II-infused mice, an effect attenuated by CGP077675 and c-Src deficiency (Fig.4A). Lucigenin-derived luminescence was increased in both aorta (Fig.4B) and mesenteric arteries (Fig.4C) from Ang II-

infused c-Src^{+/+} mice. CGP077675 reduced the increase of superoxide generation. Ang II infusion did not induce superoxide production vessels from c-Src^{+/-} mice. NE did not induce activation of vascular NADPH oxidase (Fig.4B). Plasma TBARS levels (products of lipid peroxidation), measured as an index of systemic oxidative stress, were higher in Ang II-infused c-Src^{+/+} mice versus vehicle-treated counterparts (Fig.4D). This increase was reduced by CGP077675. Ang II did not increase plasma TBARS levels in c-Src^{+/-} mice. Ang II-infused c-Src^{+/+} mice displayed increased plasma levels of H₂O₂ versus vehicle-infused Src^{+/+} mice (Fig.4E). CGP077675 reduced plasma H₂O₂ levels in Ang II-infused mice. Plasma H₂O₂ levels were similar in Src^{+/-} mice infused with either vehicle or Ang II.

c-Src, eNOS, eNOS [Ser¹¹⁷⁸] phosphorylation and caveolin-1 in Ang II-infused mice

Underlying molecular mechanisms whereby c-Src influences endothelium-dependent vasodilation were investigated by probing eNOS expression and phosphorylation. No differences were observed in eNOS protein expression (Fig.S6A) and phosphorylation at Ser¹¹⁷⁸ (activation residue) (Fig.S6B) in arteries from c-Src^{+/+} and c-Src^{+/-} mice treated with vehicle or Ang II. CGP077675 did not influence eNOS. Expression of caveolin-1, a negative regulator of eNOS, was increased in Ang II-infused c-Src^{+/+} mice, effects that were inhibited by CGP077675 (Fig.S6C). Caveolin-1 was unaffected by Ang II in c-Src^{+/-} mice versus vehicle-infused counterparts. Levels of urinary nitrite/nitrate (marker of systemic NO production), was not different between groups (Fig.S6D).

c-Src expression and phosphorylation

Total c-Src protein content was reduced in tissues from c-Src^{+/-} mice (Fig.S7A) and was not influenced by CGP077675 (Figs.S7B,S7C). Ang II-induced vascular c-Src phosphorylation

(Figs.5A,S8A) in WT mice was abrogated by CGP077675. Ang II failed to induce c-Src phosphorylation in vessels from c-Src^{+/-} mice.

c-Src differentially regulates phosphorylation of vascular MAPKs in Ang II-induced hypertension

ERK1/2 phosphorylation was increased in mesenteric arteries of Ang II-infused c-Src^{+/+} mice (Fig.5B). CGP077675 reduced ERK1/2 phosphorylation below levels observed in vehicle-infused c-Src^{+/+} mice. ERK1/2 phosphorylation was lower in Ang II-infused c-Src^{+/-} mice compared with vehicle-infused counterparts. In aorta Ang II-induced ERK1/2 phosphorylation was blunted by c-Src inhibition (Fig.S8B). In mesenteric arteries from c-Src^{+/+} and c-Src^{+/-} mice, p38MAPK phosphorylation was unaffected by Ang II. CGP077675 did not influence p38MAPK in Ang II-infused mice (Fig.5C). Ang II increased vascular JNK phosphorylation, an effect that was inhibited by CGP077675. Ang II failed to increase JNK phosphorylation in c-Src^{+/-} mice (Fig.5D).

Norepinephrine (NE) does not induce c-Src or ERK1/2 phosphorylation

The effect of NE infusion on c-Src and ERK1/2 phosphorylation was evaluated in aortas from c-Src^{+/+} and c-Src^{+/-} mice (Fig.S9). NE had no effect on vascular c-Src or ERK1/2 phosphorylation.

Molecular markers of inflammation and fibrosis in Ang II-induced hypertension.

Pro-inflammatory pathways were investigated by probing for COX2, VCAM-1 and PAI-1. As shown in Figure 6A, COX2 was increased by Ang II, effects that were inhibited by CGP077675. COX2 expression was similar in vehicle- and Ang II-treated c-Src^{+/-} mice. Ang II failed to increase expression of pro-inflammatory molecules in c-Src^{+/-} mice. Expression of VCAM-1 (Fig.6B) was not altered by Ang II, although CGP077675 reduced basal expression of the

adhesion molecule. There were no differences in VCAM-1 expression in c-Src-deficient mice infused with vehicle or Ang II. PAI-1 expression was not influenced by either CGP077675 or c-Src deficiency (Fig.6C). Figure 6D shows that vascular fibronectin expression is increased by Ang II in Src^{+/+} and c-Src^{+/-} mice, effects that were augmented by CGP077675 in c-Src^{+/+} mice. Increased fibronectin expression was also evident in Ang II-infused c-Src^{+/-} mice versus control counterparts

Molecular markers of growth and contraction are unaltered by c-Src inhibition or deficiency.

In mesenteric arteries from c-Src^{+/+} mice, PCNA expression, a molecular marker of cell growth, was unaffected by Ang II infusion alone or in association with CGP077675 treatment (Fig.S10A). PCNA expression was similar in c-Src^{+/-} mice infused with vehicle or Ang II. In mesenteric arteries from c-Src^{+/+} and c-Src^{+/-} mice, phosphorylation of MLC, an index of vascular contraction, was unaffected by Ang II infusion (Fig.S10B). CGP077675 or c-Src deficiency did not influence phosphorylation of MLC in Ang II-infused mice.

Histopathology of the heart

Hypertension-associated cardiac damage was also assessed by evaluating cardiac collagen accumulation and interstitial fibrosis by Sirius Red and Masson's trichrome staining respectively. Representative heart sections are shown in Figure S11. Hearts from Ang II-infused mice displayed excessive deposition of collagen with fibrotic areas and positive staining for CD68, identifying the presence of mononuclear inflammatory cell infiltrate. Pharmacological inhibition of c-Src exacerbated the fibrotic and inflammatory processes induced by Ang II, since hearts from mice treated with CGP077675 stained highly positive for CD68 and presented more

extensive areas of collagen accumulation and fibrosis. Increased collagen deposition and fibrosis were also evident in Ang II-infused c-Src^{+/-} mice.

Discussion

Major finding from the present study demonstrate that pharmacological and genetic inactivation of c-Src prevents development of hypertension in Ang II-infused mice. Endothelial and cardiac dysfunction were ameliorated by c-Src inhibition, processes associated with reduced oxidative stress and decreased activation of ERK1/2, JNK and pro-inflammatory signaling pathways. Whereas vascular functional responses were ameliorated by c-Src downregulation, Ang II-induced cardiovascular remodeling and fibrosis were amplified by c-Src inactivation or deficiency, as evidenced by increased vascular media thickness, cross-sectional area and fibronectin expression in resistance and conduit arteries. Similar trends were evident in the hearts of Ang II-induced hypertensive mice where cardiac fibrosis was also exaggerated by CGP077675 or c-Src deficiency. These findings suggest that while c-Src inhibition prevents development of hypertension, improves endothelial and cardiac function, reduces oxidative stress and normalizes vascular signaling, it has little beneficial effect on cardiovascular remodeling or fibrosis. Our study suggests a divergent role for c-Src in Ang II-dependent hypertension, where c-Src may be more important in regulating redox-sensitive cardiac and vascular function rather than processes associated with cardiovascular fibrosis and structure. These novel insights underscore the complex role of c-Src in Ang II-induced hypertension and suggest that c-Src inhibition as a potential therapeutic modality should be considered with caution, because while it improves endothelial and cardiac function, it may aggravate cardiovascular fibrosis.

Ang II, an important vasoactive hormone, mediates effects through multiple complex signaling pathways that regulate vascular cell contraction/relaxation, growth, apoptosis, senescence,

fibrosis, and inflammation, processes involved in the regulation of vascular function and structure (8-11). Central to these events is c-Src and ROS, which are tightly interlinked (21-23). We previously demonstrated in human and rodent VSMCs that Ang II-induced increase in $[Ca^{2+}]_i$, phosphorylation of MAPKs, regulation of RhoA/Rho kinase and activation of Nox are c-Src-dependent (23-25). While extensive *in vitro* evidence indicates a critical role for c-Src in Ang II signaling in cardiovascular cells, the pathophysiological significance of c-Src in hypertension and associated cardiovascular injury, *in vivo*, requires further investigation.

Using pharmacological and genetic approaches to inhibit c-Src activation, we interrogated the role of this non-receptor tyrosine kinase in the development of hypertension. Ang II induced a significant increase in BP in c-Src^{+/+} mice, responses that were associated with increased activation of vascular c-Src. These effects were inhibited when c-Src activity was downregulated. To support these findings, CGP077675, a pharmacological c-Src inhibitor of the pyrrolopyrimidine class that acts on the autophosphorylation site of the tyrosine kinase suppressing its activity (30) exhibited anti-hypertensive effects in Ang II-infused mice. Similar results were reported for SU6656, a non-specific Src family kinase inhibitor, (28). c-Src-associated pressor actions seem to be highly regulated by specific vasoactive agents, since NE-induced hypertension was unaffected by c-Src-deficiency.

The BP lowering effect of CGP077675 was rapid, occurring within 2 days after treatment, indicating that c-Src inhibitory effects likely occur through increased endothelium-dependent vasodilation, typically associated with acute BP changes. In support of this the reduced Ach-induced vasorelaxation in c-Src^{+/+} mice was improved in CGP077675-treated mice, indicating vasoprotection by c-Src inhibition. Molecular processes underlying these actions may relate to oxidative inactivation of NO as previously reported (34), and is supported by our findings of no

change in eNOS phosphorylation with concomitant increase in ROS production. It may also be possible that c-Src influences eNOS indirectly by upregulating caveolin-1, a negative modulator of eNOS (35). This may be especially important in the endothelium, which is rich in caveolin-1 and eNOS (36). CGP077675 normalized caveolin-1 expression in Ang II-infused mice with associated improvement in endothelial function and blood pressure reduction. Our findings differ to those previously reported where c-Src was found to induce activation of eNOS through PI3-kinase/Akt. However, those studies were performed in cultured endothelial cells (37).

Ang II-induced hypertension was associated with increased systemic and vascular ROS generation as evidenced by increased levels of plasma TBARS and H₂O₂ and enhanced vascular NADPHoxidase-derived superoxide production. These findings confirm previous studies demonstrating an important pathophysiological role for oxidative stress in the development of hypertension and associated vascular dysfunction. CGP077675 normalized redox status in Ang II-infused mice, indicating a role for c-Src in oxidative stress in hypertension. Since endothelial function is regulated not only by NO-dependent mechanisms but also by redox-sensitive pathways, the improved endothelial function in CGP077675-treated mice may relate, at least in part, to reduced oxidative stress (34). We previously demonstrated a close interaction between Nox, ROS and c-Src in human and rat VSMCs and found that c-Src was both upstream and downstream of Nox (16,21-23). We showed that this occurs through c-Src-induced phosphorylation of p47phox and increased abundance of NADPHoxidase subunits (16). The c-Src-ROS link in VSMCs has recently been highlighted (38) and is increasingly being recognized as a feedforward loop whereby oxidative stress is amplified when c-Src is activated. Interrupting this loop by inhibiting c-Src dampens activation of downstream redox signaling including MAPK and pro-inflammatory transcription factors. This is evidenced here in our *in vivo* study

where increased ROS generation, MAPK phosphorylation and enhanced COX2 expression in hypertensive mice, were normalized or attenuated by c-Src downregulation. c-Src seems to be especially important in Ang II-induced ERK1/2 activation, because c-Src inhibition reduced phosphorylation of ERK1/2 below basal levels. The critical role of c-Src in these events is underscored by the fact that even partial reduction of c-Src (in heterozygous mice) is enough to normalize signaling in Ang II-infused mice.

c-Src regulatory effects of Ang II are not generalized phenomena, because phosphorylation of p38MAPK was not altered by c-Src deficiency. These findings are in contrast to our earlier studies in aldosterone-treated vascular smooth muscle cells, where we found that p38MAPK activation is c-Src-sensitive (39). Hence, c-Src actions are agonist/receptor-sensitive, highly regulated and target protein-specific.

At the vascular level, hypertension is characterized not only by endothelial dysfunction, but also by vascular structural (remodeling) and mechanical changes (40,41). In particular small arteries in hypertension undergo inward eutrophic remodeling and/or hypertrophic remodeling with associated impaired mechanical properties (2,40). Increased collagen deposition, fracturing of elastin, calcium deposition, proliferation, rearrangement and hypertrophy of VSMCs and inflammation contribute to these processes (40-42). Mechanically, vessels become stiffer and less distensible. In hypertension vascular remodeling has an important Ang II-dependent component as evidenced by clinical investigations demonstrating that ACE inhibition or AT₁ receptor blockers prevented or reversed structural and mechanical changes (42-44). In our study, significant vascular alterations were observed in both resistance and conduit arteries. Small mesenteric arteries of Ang II-infused c-Src^{+/+} mice exhibited increased media thickness, cross-sectional area and vascular stiffness, responses that also present in c-Src^{+/-} mice. These findings

suggest that Ang II-induced remodeling may be independent of c-Src activation. In aorta, media thickness was also increased by Ang II in both c-Src^{+/+} and c-Src^{+/-} mice. CGP077675 did not improve structural and mechanical changes and in fact hypertrophy was exaggerated in small arteries. However, differential effects were observed in aorta, where CGP077675 prevented the increase in media thickness. At the molecular level, vascular fibronectin expression was similarly increased by Ang II in c-Src^{+/+} and c-Src-deficient mice. Moreover CGP077675 exacerbated fibronectin expression in Ang II-infused mice. To further support our findings that Ang II-induced remodeling is likely c-Src independent, we observed that collagen deposition, fibrosis and inflammation were variably increased in hearts from c-Src-deficient mice and in mice treated with CGP077675. These findings suggest that c-Src may not play a major role in cardiovascular growth or fibrosis and may not contribute significantly to structural changes elicited by Ang II, at least in the model studied here.

PCNA, a marker of cell proliferation was not significantly altered in c-Src^{+/-} or CGP077675-treated mice. Reasons for the additional trophic and pro-fibrotic effects of CGP077675 are unclear, but may relate to effects on cell cycle regulators other than PCNA, since expression and activity of the cyclin-dependent kinase (CDK)-inhibitor p21WAF1/CIP1 are reduced by the c-Src inhibitor resulting in cell cycle progression (45). Moreover we can not exclude the possibility that CGP077675 may have some non-specific effects, since it can also inhibit other Src isoforms (46).

Perspectives

Our results provide *in vivo* evidence to show that c-Src plays an important role in the pathophysiology of Ang II-dependent hypertension through processes that involve ROS and MAPK. While vascular and cardiac functional alterations and redox-dependent processes are c-

Src-dependent, arterial remodeling and cardiac fibrosis are c-Src-independent. Although c-Src may be an interesting potential therapeutic target to reduce blood pressure and improve cardiovascular function, caution is warranted regarding the possible pro-fibrotic cardiovascular effects of c-Src inhibition.

Acknowledgements

This study was funded by a grant from the Canadian Institute of Health Research (CIHR, 44018). RMT was supported through a Canada Research Chair/Canadian Foundation for Innovation award and a British Heart Foundation Chair (CH/12/4/29762).

Disclosure: None

References

1. Montezano AC, Nguyen Dinh Cat A, Rios FJ, Touyz RM. Angiotensin II and Vascular Injury *Curr Hypertens Rep* 2014;16: 431-436.
2. Savoia C, Burger D, Nishigaki N, Montezano A, Touyz RM. Angiotensin II and the vascular phenotype in hypertension. *Expert Rev Mol Med*. 2011;13:e11.
3. Kimura K, Eguchi S. Angiotensin II type-1 receptor regulates RhoA and Rho-kinase/ROCK activation via multiple mechanisms. Focus on "Angiotensin II induces RhoA activation through SHP2-dependent dephosphorylation of the RhoGAP p190A in vascular smooth muscle cells". *Am J Physiol Cell Physiol*. 2009;297:C1059-1061.
4. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82-97.
5. Montezano AC, Tsiropoulou S, Dulak-Lis M, Harvey A, Camargo Lde L, Touyz RM. Redox signaling, Nox5 and vascular remodeling in hypertension. *Curr Opin Nephrol Hypertens*. 2015;24:425-433.
6. Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal*. 2013, 19:1110-11120.
7. Tabet F, Schiffrin EL, Callera GE, He Y, Yao G, Ostman A, Touyz RM. Redox-sensitive signaling by angiotensin II involves oxidative inactivation and blunted phosphorylation of protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells from SHR. *Circ Res*. 2008;103:149–158.

8. Touyz RM, Cruzado M, Tabet F, Yao G, Salomon S, Schiffrin EL. Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells –role of receptor tyrosine kinase transactivation. *Can J Physiol Pharmacol.* 2003;81:159–167.
9. Tabet F, Savoia C, Schiffrin EL, and Touyz RM. Differential calcium regulation by hydrogen peroxide and superoxide in vascular smooth muscle cells from spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 2004;44: 200–208.
10. Wenzel S, Taimor G, Piper HM, Schluter KD. Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF-beta expression in adult ventricular cardiomyocytes. *FASEB J.* 2001;15: 2291–2293.
11. Viridis A, Neves MF, Amiri F, Touyz RM, Schiffrin EL. Role of NAD(P)H oxidase on vascular alterations in angiotensin II-infused mice. *Journal of Hypertension* 2004;22:535–542.
12. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griending KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone. *J Clin Invest.* 1996;97:1916–1923.
13. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol.* 2003;285:R277–R297.
14. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griending KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res.* 2002;91:406–413.
15. Briones AM, Tabet F, Callera GE, Montezano AC, Yogi A, He Y, Quinn MT, Salaices M, and Touyz RM. Differential regulation of Nox1, Nox2 and Nox4 in vascular smooth muscle cells from WKY and SHR. *J Am Soc Hypertens.* 2011;5:137–153.

16. García-Redondo AB, Briones AM, Martínez-Revelles S, Palao T, Vila L, Alonso MJ, Salices M. c-Src, ERK1/2 and Rho kinase mediate hydrogen peroxide-induced vascular contraction in hypertension: role of TXA₂, NAD(P)H oxidase and mitochondria. *J Hypertens*. 2015;33:77-87.
17. Ishida M, Ishida T, Thomas SM, Berk BC. Activation of extracellular signal regulated kinases (ERK1/2) by angiotensin II is dependent on c-Src in vascular smooth muscle cells. *Circ Res* 1998;82:7–12.
18. MacKay CE, Knock GA. Control of vascular smooth muscle function by Src-family kinases and reactive oxygen species in health and disease. *J Physiol*. 2015;593:3815-3828.
19. Yeatman TJ. A renaissance for SRC. *Nature Reviews Cancer* 2004, 4:470-480.
20. Martin GS. The hunting of the Src. *Nat Rev Mol Cell Biol*. 2001;2:467–475.
21. Oda Y, Renaux B, Bjorge J, Saifeddine M, Fujita DJ, Hollenberg MD. c-Src is a major cytosolic tyrosine kinase in vascular tissue. *Can J Physiol Pharmacol*. 1999;77:606–617.
22. Godeny MD, Sayeski PP. Ang II-induced cell proliferation is dually mediated by c-Src/Yes/Fyn regulated ERK1/2 activation in the cytoplasm and PKC-controlled ERK1/2 activity within the nucleus. *Am J Physiol Cell Physiol*. 2006;291:C1297–C1307.
23. Touyz RM, Wu XH, He G, Salomon S, Schiffrin EL. Increased angiotensin II-mediated Src signaling via epidermal growth factor receptor transactivation is associated with decreased C-terminal Src kinase activity in vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension*. 2002;39:479–485.
24. Touyz RM, He G, Wu XH, Park JB, Mabrouk ME, Schiffrin EL. Src is an important mediator of extracellular signal-regulated kinase 1/2–dependent growth signaling by

- angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients. *Hypertension*. 2001;38:56–64.
25. Touyz RM, Wu XH, He G, Park JB, Chen X, Vacher J, Rajapurohitam V, Schiffrin EL. Role of c-Src in the regulation of vascular contraction and Ca²⁺ signaling by angiotensin II in human vascular smooth muscle cells. *J Hypertens*. 2001;19:441-449.
 26. Touyz RM, Yao G, Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23:981-987.
 27. Montezano AC, Dulak-Lis M, Tsiropoulou S, Harvey A, Briones AM, Touyz RM. Oxidative stress and human hypertension: vascular mechanisms, biomarkers, and novel therapies. *Can J Cardiol*. 2015;31:631-641.
 28. Qin B, Zhou J. Src Family Kinases (SFK) Mediate Angiotensin II-Induced Myosin Light Chain Phosphorylation and Hypertension. *PLoS One*. 2015;10:e0127891.
 29. Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-Src proto-oncogene leads to osteoporosis in mice. *Cell*. 1991;64:693–702.
 30. Missbach, M. Jeschke, J. Feyen, K. Müller, M. Glatt, J. Green, M. Susa. A Novel Inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption in vitro and in rodent models in vivo. *Bone* 1999;24:437–449.
 31. Carlson SH, Wyss JM. Long-term telemetric recording of arterial pressure and heart rate in mice fed basal and high NaCl diets. *Hypertension* 2000;35:E1-E5.

32. Mercure C, Yogi A, Callera GE, Aranha AB, Bader M, Ferreira AJ, Santos RA, Walther T, Touyz RM, Reudelhuber TL. Angiotensin (1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. *Circ Res.* 2008;103:1319-1326.
33. Schiffrin EL, Park JB, Intengan HD, Touyz RM. Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation* 2000;101:1653–1659.
34. Cai H, Davis ME, Drummond GR, Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. *Arterioscler Thromb Vasc Biol.* 2001;21:1571-6.
35. Mineo C, Shaul PW. Circulating cardiovascular disease risk factors and signaling in endothelial cell caveolae. *Cardiovascular Research.* 2006;70:31-41.
36. Rath G, Dessy C, Feron O. Caveolae, caveolin and control of vascular tone: nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF) regulation. *J Physiol Pharmacol.* 2009;60:105-109.
37. Liu S, Premont RT, Rockey DC. Endothelial nitric-oxide synthase (eNOS) is activated through G-protein-coupled receptor kinase-interacting protein 1 (GIT1) tyrosine phosphorylation and Src protein. *J Biol Chem.* 2014;289(26):18163-74.
38. Knock GA, Ward JP. Redox regulation of protein kinases as a modulator of vascular function. *Antioxid Redox Signal.* 2011;15:1531-1547.
39. Callera GE, Touyz RM, Tostes RC, Yogi A, Ying He, Sam Malkinson, Ernesto L. Schiffrin. Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src. *Hypertension.* 2005;45:773-779.

40. Intengan HD, Schiffrin EL. Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. *Hypertension*. 2001;38:581-587.
41. Briet M, Boutouyrie P, Laurent S, London GM. Arterial stiffness and pulse pressure in CKD and ESRD. *Kidney International*. 2012;82:388–400.
42. Touyz RM, Deng LY, He G, Wu XH, Schiffrin EL. Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases. *J Hypertens* 1999; 17:907–916.
43. Touyz RM, Savoia C, He Y, Endemann D, Pu Q, Ko EA, DeCicceis C, Montezano A, Schiffrin EL. Increased inflammatory biomarkers in hypertensive type 2 diabetic patients: improvement after angiotensin II type 1 receptor blockade. *J Am Soc Hypertens*. 2007;1:189-199.
44. Savoia C, Touyz RM, Endemann DH, Pu Q, Ko EA, De Cicceis C, Schiffrin EL. Angiotensin receptor blocker added to previous antihypertensive agents on arteries of diabetic hypertensive patients. *Hypertension*. 2006;48:271-277.
45. Recchia I, Rucci N, Funari A, Migliaccio S, Taranta A, Longo M, Kneissel M, Susa M, Fabbro D, Teti A. Reduction of c-Src activity by substituted 5,7-diphenyl-pyrrolo[2,3-d]-pyrimidines induces osteoclast apoptosis in vivo and in vitro. Involvement of ERK1/2 pathway. *Bone*.2004;34:65-79.
46. Dincer S, Cetin KT, Onay-Besikci A, Ölgün S. Synthesis, biological evaluation and docking studies of new pyrrolo[2,3-d] pyrimidine derivatives as Src family-selective tyrosine kinase inhibitors. *J Enzyme Inhib Med Chem*.2013;28:1080-1087.

Novelty and significance

What is new?

- Genetic and pharmacological inhibition of c-Src reduces blood pressure and ameliorates endothelial and cardiac dysfunction in Ang II-induced hypertension.
- c-Src effects may be Ang II-specific, because NE-induced hypertension was unaffected by c-Src inhibition.
- Ang II-induced cardiovascular fibrosis and remodelling are not protected by c-Src inhibition.

What is relevant?

- In Ang II-induced hypertension c-Src plays a role in functional but not structural changes in the cardiovascular system.
- c-Src may have differential cardiovascular effects, both protective and injurious, in Ang II-induced hypertension.

Summary

- c-Src may be more important in regulating redox-sensitive cardiovascular function than fibrosis and remodeling.
- While inhibition of c-Src may be an attractive strategy to reduce blood pressure, improve endothelial function and prevent target organ damage, caution is warranted because of the potential pro-fibrotic consequences of c-Src inhibition.

Figure Legends

Figure 1. c-Src plays a role in Ang II-induced hypertension and vascular dysfunction. Time course of SBP in: (A) c-Src^{+/+} and c-Src^{+/-} mice infused with vehicle or Ang II; (B) c-Src^{+/+} and c-Src^{+/-} mice infused with vehicle or NE; (C) c-Src^{+/+} mice infused with vehicle or Ang II, with the arrow indicating the time of CGP077675 treatment. (D) c-Src inhibition improves endothelium-dependent vasodilation in Ang II-infused mice. Concentration-response curves to ACh (1nmol/L-100 μ mol/L) were performed in endothelium-intact arteries. Results are mean \pm SEM of 5-15/group. **P* < 0.05 vs. vehicle-infused counterparts, [†]*P* < 0.05 CGP077675-treated Ang II-infused c-Src^{+/+} mice vs. Ang II-infused c-Src^{+/+} mice, ***P* < 0.05 Ang II-infused c-Src^{+/-} mice, +++*P* < 0.05 vs. Ang II-infused c-Src^{+/+} mice.

Figure 2. c-Src inhibition influences structural properties of mesenteric resistance arteries in Ang II-infused mice. Small mesenteric arteries were mounted in a pressure myograph. Measurements of media and lumen were taken at stepwise increments of luminal pressure (3-140 mmHg) in zero Ca²⁺ Krebs solution containing 10 mM EGTA. Line graphs: media, lumen, media:lumen ratio, and cross-sectional area (CSA) at increasing luminal pressure in arteries from c-Src^{+/+} (A, B, C, D) and c-Src^{+/-} (E, F, G, H) mice. Results are mean \pm SEM of 5-11 mice/group. **P* < 0.05 vs. vehicle-infused mice, ***P* < 0.05 vs. Ang II-infused c-Src^{+/+} mice.

Figure 3. Effects of CGP077675 and c-Src-deficiency on aortic hypertrophy in Ang II-induced hypertension. Paraffin-embedded thoracic aorta sections were processed for Masson's trichrome staining. For morphometric analysis in cross-sections of aorta, the wall thickness was measured in four regions and values averaged. Upper panels, representative photomicrographs of Masson's trichrome-stained thoracic aorta sections, original magnifications x400. Bar graph,

wall thickness of aortic segments. Results are presented as mean±SEM of 5 mice/group **P* <0.05 vs. vehicle-infused c-Src^{+/+} mice, †*P* <0.05 vs. vehicle-infused c-Src^{+/-} mice.

Figure 4. c-Src plays a role in Ang II-induced increase in vascular reactive oxygen species (ROS) generation. (A) Upper panels, representative images of ROS levels assessed by dihydroethidium (DHE) fluorescence in aortic rings. Original magnification, x320. Images are representative of five independent experiments. Bar graphs, NADPH-dependent lucigenin-derived luminescence in homogenates of (B) aorta and (C) mesenteric arteries; (D) plasma TBARS levels; (E) plasma H₂O₂ levels. Values are mean±SEM of 4-12 mice/group. **P* <0.05 vs. vehicle-infused c-Src^{+/+} mice, ***P* <0.05 vs. Ang II-infused c-Src^{+/+} mice.

Figure 5. c-Src inhibition influences vascular signaling in Ang II-infused mice. Phosphorylation levels of c-Src (A), ERK1/2 (B), p38MAPK (C), and JNK (D) were analyzed in mesenteric arteries from vehicle or Ang II-infused c-Src^{+/+} and c-Src^{+/-} mice, and CGP077675-treated Ang II-infused c-Src^{+/+} mice and presented as the a ratio to β-actin. Upper panels, representative immunoblots of c-Src [Tyr⁴¹⁸], ERK1/2 [Thr²⁰²/Tyr²⁰⁴], p38MAPK [Thr¹⁸⁰/Tyr¹⁸²], JNK [Thr¹⁸³/Tyr¹⁸⁵], and β-actin. Bar graphs represent mean±SEM of 4-6 mice/group. **P* <0.05 vs. vehicle-infused c-Src^{+/+} mice, ** *P* <0.05 vs. Ang II-infused c-Src^{+/+} mice, †*P* <0.05 vs. vehicle-infused c-Src^{+/-} mice.

Figure 6. c-Src inhibition influences vascular expression of pro-inflammatory and pro-fibrotic markers. Protein expression levels of COX-2 (A), VCAM-1 (B), PAI (C), and (D) fibronectin were analyzed in mesenteric arteries from vehicle or Ang II-infused c-Src^{+/+} mice, and CGP077675-treated Ang II-infused c-Src^{+/+} mice and presented relative to β-actin or GAPDH. Upper panels, representative immunoblots of COX-2, VCAM-1, PAI-1, fibronectin, β-actin and GAPDH. Bar graphs represent mean±SEM of 4-9 mice/group. **P* <0.05 vs. vehicle-

infused c- $\text{Src}^{+/+}$ mice, ** $P < 0.05$ vs. Ang II-infused c- $\text{Src}^{+/+}$ mice, $^{\dagger}P < 0.05$ vs. vehicle-infused c- $\text{Src}^{+/-}$ mice.