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Antifibrotic Activity of an Inhibitor of Group IIA Secretory Phospholipase A\(_2\) in Young Spontaneously Hypertensive Rats

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The development of fibrosis in the chronically hypertensive heart is associated with infiltration of inflammatory cells and cardiac hypertrophy. In this study, an inhibitor of the proinflammatory enzyme, group IIA human secretory phospholipase A\(_2\) (sPLA\(_2\)-IIA), has been found to prevent collagen deposition as an important component of cardiovascular remodeling in a rat model of developing chronic hypertension. Daily treatment of young male spontaneously hypertensive rats (SHR) with an sPLA\(_2\)-IIA inhibitor (KH064, 5-(4-benzoyloxyphenyl)-4S-(phenyl-heptanoylamino)-pentanoic acid, 5 mg/kg/day p.o.) prevented increases in the content of perivascular (SHR 20.6 ± 0.9%, 5 = 5; SHR + KH064 14.0 ± 1.2%, 5 = 5) and interstitial (SHR 7.9 ± 0.3%, 5 = 6; SHR + KH064 5.4 ± 0.7%, 5 = 6) collagen in the left ventricle of rat hearts, but did not affect numbers of infiltrating monocytes/macrophages, left ventricular hypertrophy (SHR 2.88 ± 0.08, 5 = 12; SHR + KH064 3.09 ± 0.08 mg/g body weight, 5 = 9), increased systolic blood pressure, or thoracic aortic responses. This selective antifibrotic activity suggests that sPLA\(_2\)-IIA may have an important but specific role in cardiac fibrosis, and that its inhibitors could be useful in dissecting molecular pathways leading to fibrotic conditions. The Journal of Immunology, 2006, 176: 7000–7007.

Chronic hypertension often leads to excessive collagen deposition (fibrosis) as part of the process of cardiovascular remodeling that includes ventricular hypertrophy and endothelial dysfunction. This remodeling leads ultimately to a more rigid myocardium (1). Studies using different models of hypertension, such as spontaneously hypertensive rats (SHRs) (2), renal hypertensive rats (3), NO synthase inhibition by \(\text{l-NAME}\) (4), aldosterone-salt (5–7), deoxy-corticosterone acetate-salt (8), and aortic constriction (9), strongly suggest that the development of cardiac fibrosis is associated with inflammatory processes arising through recruitment of inflammatory cells such as macrophages into the left ventricle (8, 9).

Phospholipases A\(_2\) (PLA\(_2\)) are proinflammatory enzymes that could conceivably be associated with cardiac remodeling, but there are few reports to date linking these enzymes to fibrosis and none to cardiac fibrosis. One role of PLA\(_2\) enzymes in inflammatory diseases is to initiate inflammatory cascades by liberating arachidonic acid from the cell membrane through hydrolysis of the phospholipid sn-2 ester bond. Arachidonic acid is a substrate for cytochrome P450 monoxygenase (10, 11), and a precursor to many inflammatory eicosanoids, including PG and leukotrienes. Enzymes that use arachidonic acid as a substrate as well as arachidonic acid products such as 12-lipoxygenase, 5-lipoxygenase, cysteinyl leukotrienes, and thromboxane A\(_2\) (TXA\(_2\)) have well-defined profibrotic properties (12, 13). The precise isoform(s) responsible for arachidonate production in vivo in inflammatory conditions is still controversial, with evidence that cytosolic (c)PLA\(_2\) and secretory (s)PLA\(_2\) type V are more potent than sPLA\(_2\)-IIA in phospholipid degradation (14, 15). Nevertheless, the calcium-dependent human sPLA\(_2\)-IIA enzyme is implicated in a wide range of inflammatory diseases, including rheumatoid arthritis (16, 17), pancreatitis (18), and sepsis (19). Inhibitors of this enzyme have also shown therapeutic effects in many animal models of inflammatory diseases (15), providing compelling evidence for a causative role of sPLA\(_2\)-IIA.

The importance of sPLA\(_2\)-IIA in the pathology of the cardiovascular system has been highlighted by the description of this particular enzyme as an independent risk factor in cardiovascular disease (20). PLA\(_2\) levels are elevated following myocardial infarction in humans (21–23), with sPLA\(_2\) localized to the infarct area (22) and involved in progressing nonlethally affected ischemic cardiac myocytes to late stage apoptotic or necrotic cardiac myocytes (24, 25). Furthermore, mice transfected with the human sPLA\(_2\) gene have increased collagen in atherosclerotic lesions (26). Together, these studies suggest the possibility that inhibitors of sPLA\(_2\)-IIA may have therapeutic value in preventing cardiac fibrosis in the hypertensive heart.

To date, there are no reports of the effects of inhibitors of sPLA\(_2\)-IIA on cardiovascular remodeling. The present study has investigated whether the sPLA\(_2\)-IIA inhibitor 5-(4-benzoyloxyphenyl)-4S-(phenyl-heptanoylamino)-pentanoic acid (designated here as KH064 but referred to as compound “2b” in Ref. 27) prevents cardiovascular remodeling in young male SHR during the development of hypertension. KH064 is an orally active, competitive and reversible inhibitor with high potency (IC\(_{50}\) 29 nM) and selectivity (sPLA\(_2\)-V: IC\(_{50}\) > 5 \(\mu\)M) for the human sPLA\(_2\)-IIA enzyme (27, 28). This inhibitor was effective at 5 mg/kg/day p.o. as an anti-inflammatory drug in rat models of adjuvant-induced arthritis (27), intestinal ischemia-reperfusion injury (28), and inflammatory bowel disease (29). KH064 also reduces NOX production from the myocardiun, myocardial damage, and cardiomyocyte

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¶Abbreviations used in this paper: SHR, spontaneously hypertensive rat; l-NAME, \(\text{l-nitroarginine methyl ester}\); cPLA\(_2\), cytosolic PLA\(_2\); sPLA\(_2\), secretory PLA\(_2\); \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin; MMP, matrix metalloproteinase; WKY, Wistar-Kyoto.

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death during cold ischemia associated with donor heart preservation, and sPLA₂ activation has been implicated in up-regulation of NO synthase-2 during cardiac ischemia (25).

The results of the present study demonstrate that KH064 treatment prevents collagen deposition in the left ventricle of the hypertensive rat heart, without affecting left ventricular hypertrophy, vascular reactivity, or hypertension. This result suggests that inhibition of sPLA₂-IIA may represent a novel approach to preventing the development of cardiac fibrosis.

Materials and Methods

**SHR and Wistar-Kyoto (WKY) rats**

Male SHR and WKY rats were bred in the University of Queensland animal housing facility from breeders purchased from the Animal Research Centre (Perth, Australia). All experimental protocols were approved by the Animal Experimentation Ethics Committee of the University of Queensland, under the guidelines of the National Health and Medical Research Council of Australia. Rats were fed pelleted rat chow and were housed in 12-h light/dark conditions. Body weight was measured daily.

SHR and WKY rats were treated with KH064 (5 mg/kg/day by oral gavage, suspended in olive oil) beginning at 4 wk of age before the onset of hypertension, and continued through to 12 wk of age when systolic blood pressure begins to plateau in our strain of SHR (L. Brown, unpublished data). This dose was chosen because it was effective in the rat models described above (27–29). Olive oil-treated SHR and WKY rats served as vehicle controls. Systolic blood pressure was measured in selected rats lightly sedated via i.p. injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments).

**Echocardiographic studies**

Echocardiography was performed at the Prince Charles Hospital small animal theater by trained sonographers. Rats were anesthetized via i.p. injection with Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg) and Ilum Xylazil (xylazine 10 mg/kg). A Hewlett-Packard Sonos 5500 echocardiography machine using a 12-MHz neonatal transducer with an image depth of 3 cm and 2 focal zones was used. Measurements of left ventricular posterior wall thickness and internal diameter were made using two-dimensional M-Mode taken at midpapillary level (30).

**Left ventricular wet weight and hydroxyproline assay**

Following excision of the heart, the atria and right ventricle were dissected away from the septum and left ventricle. Left ventricular and septum were then blotted and weighed. Furthermore, left ventricular tissue samples were snap frozen in liquid nitrogen and stored at −80°C. Hydroxyproline assessment was conducted using modified methods of Stegemann and Stalder (31). Absorbance was read at 550 nm, and hydroxyproline content was calculated from a hydroxyproline standard curve.

**Picrosirius red-staining**

Left ventricular perivascular and interstitial collagen content was determined by analysis of picrosirius red-stained sections. Left ventricles were cut transversely at midpapillary level and fixed in 100 ml Telly's fixative (100 ml 70% ethanol until blocked.}

For immunostaining, 5-μm-thick paraffin-embedded sections of left ventricle were dewaxed, then Ag retrieval was conducted by microwave tissue sections in 0.01 M citrate buffer for 15 min followed by pepsin digestion (0.05 M) for a further 15 min at 37°C. Samples were then incubated for 20 min at room temperature in blocking solution (PBS containing 5% sheep serum and 1% BSA) before application of primary Abs for rat macrophages (ED1; Serotec mouse anti-rat ED1 diluted 1/15) or α-smooth muscle actin (α-SMA) (1A4; Sigma-Aldrich; diluted 1/400) and incubation overnight at 4°C. Omission of primary Abs, and staining with an irrelevant mouse Ig of the same isotype, served as negative controls. Following PBS washes, samples were incubated with IgG-fluorescein-conjugated secondary Ab (Chemicon; dilution of 1/200). Sections were mounted with gelvat (polyvinyl alcohol/glycerol) mounting medium containing n-propyl gallate (5 mg/ml) as an antifade agent and visualized with a Bio-Rad MRC-1024 confocal laser-scanning microscope using an objective lens of ×40 magnification. A 0–4 grading scale was used to classify the extent of ED1-positive monocyte/macrophage infiltration in the left ventricle: 0, no monocytes/macrophages present; 1, low levels of singular monocyte/macrophages located throughout the left ventricle; 2, moderate numbers of monocytes/macrophages located throughout the left ventricle; 3, moderate numbers of monocytes/macrophages located in groups throughout the left ventricle and/or sites of infarct; 4, large numbers of monocytes/macrophages located in groups throughout the left ventricle and scar sites, and associated directly with areas of fibrosis. α-SMA was qualitatively assessed for spatial location in the left ventricle.

**Organ bath studies**

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath chamber with a resting tension of 10 mN (32). Cumulative concentration-response curves were performed for noradrenaline and either acetylcholine or sodium nitroprusside in the presence of a submaximal (~70%) contraction to noradrenaline.

**Statistical analysis**

All values are presented as mean ± SEM. The negative log EC₅₀ of the change in force of contraction in mN was determined from the concentration giving half-maximal responses in individual concentration-response curves. Statistical comparisons between groups were made using a one-way ANOVA with a Bonferroni post test. A p value of <0.05 was considered statistically significant.

**Results**

**Biometrics**

Systolic blood pressure was significantly increased in vehicle-treated SHR when compared with WKY rats from 8 wk of age onwards and was unchanged by KH064 treatment (Fig. 1). SHR

![FIGURE 1. Tail-cuff measurement of systolic blood pressure recorded at 4, 6, 8, 10, and 12 wk of age for WKY with vehicle, WKY treated with KH064, SHR with vehicle, and SHR treated with KH064 (∗, p < 0.05 vs WKY+vehicle).](https://www.jimmunol.org/doi/abs/10.4049/jimmunol.0900130)

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also showed significant left ventricular hypertrophy at 12 wk of age, compared with WKY rats (Table I), and KH064 treatment had no effect in either rat strain. There were no significant differences in body weights between SHR and WKY rats at 12 wk of age (Table I), and this difference was also unaltered by KH064 treatment.

**Echocardiography**

Echocardiography was used to provide in vivo measurements of the dimensions of the left ventricle, allowing a more detailed characterization of the left ventricle. Echocardiographic analysis of vehicle-treated SHR at 12 wk of age showed increased thickening of the left ventricular posterior wall without a change in chamber diameter (Table I). Treatment with KH064 did not prevent the left ventricular wall thickening or alter the internal diameter in the SHR. KH064 did not change cardiac dimensions in WKY rats.

**Left ventricular collagen**

Assessment of left ventricular hydroxyproline content was performed to evaluate total collagen content within the left ventricle. Left ventricular hydroxyproline content was significantly increased in vehicle-treated SHR compared with WKY rats. Treatment with KH064 prevented this increase in left ventricular hydroxyproline content in the SHR, but did not alter hydroxyproline content in WKY rats (Table I). To further evaluate the spatial distribution of collagen within the left ventricle, picrosirius redstaining was used. Both perivascular (20.6 ± 0.9%, n = 5; Fig. 2) and interstitial (7.9 ± 0.3%, n = 6; Fig. 3) collagen content were significantly increased in the left ventricles of vehicle-treated SHR compared with WKY rats (perivascular, 14.8 ± 0.7%, n = 4; interstitial, 4.6 ± 0.2%, n = 5) at 12 wk of age. These increases were prevented by treatment with KH064 in the hypertensive rats (SHR+KH064 14.0 ± 1.2%, n = 5; 5.4 ± 0.7%, n = 6). Neither component was altered by KH064 in the nonhypertensive WKY left ventricle (WKY+KH064 14.3 ± 1.8%, n = 5; 4.7 ± 0.2%, n = 5).

**Left ventricular monocyte/macrophage infiltration grading**

Spatial location of ED1-positive monocyte/macrophages was determined by immunofluorescence. This was done to confirm previous findings in various rat models of hypertension, and to evaluate whether sPLA₂-IIA inhibition influences ED1-positive monocyte/macrophage recruitment into the left ventricle. Results for left ventricular ED1-positive monocyte/macrophage infiltration are displayed in Fig. 4. ED1-positive monocyte/macrophages were found in the left ventricle of WKY rats in very low numbers and always as single cells. The density of ED1-positive monocyte/macrophages found in the left ventricle of the SHR was significantly greater than in the WKY, and these cells were usually found as clusters of cells located at scar sites and throughout the interstitium; they were often, but not always, localized in areas of fibrosis. Perivascular areas in the SHR left ventricle did not always contain ED1-positive monocyte/macrophages. Treatment with KH064 did not alter macrophage infiltration within the left ventricle of either SHR or WKY rats.

**FIGURE 2.** Representative images of picrosirius red-stained left ventricular perivascular collagen (magnification, ×40): A, WKY + vehicle; B, SHR + vehicle; C, WKY + KH064; D, SHR + KH064. E, Graphical representation of left ventricular perivascular collagen (*, p < 0.05 vs WKY + vehicle; #, p < 0.05 vs SHR + vehicle).
Left ventricular α-SMA

α-SMA in the left ventricle was qualitatively assessed as a marker of fibroblast differentiation into contractile myofibroblasts. α-SMA was found to surround coronary vessels of the left ventricle in all groups and was detected in small quantities in interstitial areas of the left ventricle of SHR. α-SMA distribution was unchanged by KH064 treatment.

Organ bath studies

Vascular reactivity may be altered in hypertension. Organ bath studies using isolated thoracic aortic rings can provide insight to the contractile and relaxant properties of blood vessels. Thoracic aortic rings showed a decreased contractile response to noradrenaline in vehicle-treated SHR compared with WKY rats, with no change in potency (Fig. 5A). KH064 treatment did not alter noradrenaline responses in the SHR or WKY. Relaxant responses to the endothelium-dependent vasodilator, acetylcholine, and the endothelium-independent vasodilator, sodium nitroprusside, were similar in vehicle-treated SHR and WKY (Fig. 5, B and C). KH064 treatment did not alter these relaxant responses.

Discussion

This study has provided compelling evidence that the orally active sPLA$_2$-IIA inhibitor known as KH064 (27–29) significantly prevented cardiac fibrosis during the development of hypertension in the young male SHR. This antifibrotic property was manifested in substantially reduced levels of perivascular and interstitial collagen deposition in the left ventricle of rat hearts following daily oral treatment with KH064. Interestingly, this occurred without altering ED1-positive monocyte/macrophage infiltration, hypertrophy of the left ventricle, or vascular reactivity, and was independent of systolic blood pressure. No previous studies have reported antifibrotic activity for sPLA$_2$ inhibitors in cardiac remodeling in the hypertensive heart, nor investigated whether sPLA$_2$-IIA is required for the development of cardiac fibrosis.
FIGURE 5. Concentration-response curves to noradrenaline (A) for WKY with vehicle (log EC\textsubscript{50} = 7.8 ± 0.1, n = 13), WKY treated with KH064 (log EC\textsubscript{50} = 7.8 ± 0.1, n = 10), SHR with vehicle (log EC\textsubscript{50} = 7.8 ± 0.2, n = 6), SHR with KH064 (log EC\textsubscript{50} = 7.8 ± 0.1, n = 9), and SHR treated with KH064 (log EC\textsubscript{50} = 7.8 ± 0.1, n = 9). Concentration-response curves to acetylcholine (B) for WKY with vehicle (log EC\textsubscript{50} = 7.2 ± 0.1, n = 10), WKY treated with KH064 (log EC\textsubscript{50} = 6.9 ± 0.2, n = 5), SHR with vehicle (log EC\textsubscript{50} = 7.3 ± 0.1, n = 9), and SHR treated with KH064 (log EC\textsubscript{50} = 7.5 ± 0.1, n = 8). Concentration-response curves to sodium nitroprusside (C) for WKY with vehicle (log EC\textsubscript{50} = 8.3 ± 0.2, n = 8), WKY treated with KH064 (log EC\textsubscript{50} = 7.9 ± 0.2; n = 5), SHR with vehicle (log EC\textsubscript{50} = 8.0 ± 0.1, n = 8), and SHR treated with KH064 (log EC\textsubscript{50} = 8.1 ± 0.1, n = 7). (*, p < 0.05 vs WKY+vehicle).

By preventing perivascular and interstitial fibrosis, the sPLA\textsubscript{2}-IIA inhibitor provides evidence that sPLA\textsubscript{2}-IIA may be an important enzyme in the signaling pathway that leads to cardiac fibrosis. The target specificity of any drug in vivo can never be completely certain, but there is considerable evidence that KH064 is a highly target-selective drug. We have reported its crystal structure (pdb code: 1kq) in complex with sPLA\textsubscript{2}-IIA (27), and identified its specific interactions within the unusual active site of that enzyme. In vitro radioligand binding and fluorometric/calorimetric functional assays (D.P. Fairlie, unpublished results) also did not reveal any appreciable binding to, or inhibition of (IC\textsubscript{50} > 5 \mu M), a wide range of receptors (adenosine, adrenergic, angiotensin, Bradykinin, calcium or potassium channels, dopamine, estrogen, \gamma-aminobutyric acid, glucocorticoid, glutamate, glycine, histamine, insulin, muscarinic, neuroepetide Y, nicotinic, opiate, phorbol ester, protegerone, serotonin, tachykinin) including two other human PLA\textsubscript{2} isofoms, cPLA\textsubscript{2} (IC\textsubscript{50} > 100 \mu M) and sPLA\textsubscript{2-V} (IC\textsubscript{50} > 5 \mu M). Furthermore, the sPLA\textsubscript{2}-IIA inhibitor KH064 inhibited IL-1\beta or TNF-\alpha induced release of PGE\textsubscript{2} in vitro from WI38 human lung fibroblasts or human HeLa S3 cells, respectively (IC\textsubscript{50} < 1 \mu M; D.P. Fairlie, unpublished data). This in vitro information, together with the in vivo anti-inflammatory activity of KH064 in rat models of diverse sPLA\textsubscript{2}-IIA mediated inflammatory conditions such as adjuvant arthritis (27), intestinal ischemia/reperfusion injury (28), myocardial ischemia (25), inflammatory bowel disease (29), oxytocin-induced rat uterine contractions (33), and preservation of bone structure following ovariectomy (34), strongly suggests that sPLA\textsubscript{2}-IIA is the likely target in vivo for this drug.

The expression of various sPLA\textsubscript{2} isofoms (IIA, IID, IIE, IIF, V, and X) in human hearts has been recently reported (35, 36). In normal hearts, sPLA\textsubscript{2}-IIA was detected by immunohistochemistry in coronary vascular smooth muscle cells and sPLA\textsubscript{2-V} in cardiomyocytes and ischemic myocytes beneath the endocardium. Both enzymes were markedly increased in infarcted hearts and damaged cardiomyocytes. sPLA\textsubscript{2-IIA} mRNA has been detected in the normal rat heart (37) and, more specifically, in cardiac myocytes and fibroblasts (38). Although Nyman et al. (37) found no evidence of the sPLA\textsubscript{2} enzyme itself in the rat heart, McHowat et al. (39) detected sPLA\textsubscript{2} in the cytosolic fraction of the rat heart.

Ghesquiere et al. (26) have shown that transfection of the human sPLA\textsubscript{2} gene in mice resulted in increased collagen in atherosclerotic lesions. Furthermore, Nijmeijer et al. (23) have speculated that sPLA\textsubscript{2}-IIA may be involved in scarring in myocardial infarction because sPLA\textsubscript{2-IIA} was observed to form a border zone around the infarcted area. They subsequently found that sPLA\textsubscript{2-IIA} was involved in progressing nonlethally affected ischemic cardiac myocytes to late stage apoptotic or necrotic cardiac myocytes (24). We had earlier reported histopathological evidence that treatment of hearts harvested from Lewis rats, subjected to 10 h of cold ischemia, with the sPLA\textsubscript{2-IIA} inhibitor KH064 (3 mg/kg) significantly reduced production of NOx (nitrite/nitrate) from the myocardium, cardiomyocyte damage, and apoptotic cardiomyocyte death in cardiac allografts (25). Activation of sPLA\textsubscript{2} and the arachidonate cascade causes induction of NO synthase, which, in turn, is known to be up-regulated during cardiac ischemia and to cause myocyte death by apoptosis, decreased myocyte function, and cardiac damage that characterize ischemia/reperfusion injury during heart transplantation.
Following the release of arachidonic acid by PLA2 enzymes, the efficiency of cyclooxygenase-2-PGE2 synthase coupling is increased, increasing PGE2 release (40). PG (especially PGE3) induces expression of matrix metalloproteinases (MMPs), especially MMP-2 (41, 42). It is therefore conceivable that KH064 exerts its antifibrotic effects through regulation of PGE2 release, which in turn affects MMP that directly regulate collagen deposition. The reduction of fibrosis by cyclooxygenase inhibitors such as aspirin (43) lends support to this idea. However, substantial further detailed studies are required to determine any links between individual sPLA2 enzymes, release of specific arachidonate metabolites, activation of specific MMPs, collagen deposition, and cardiac fibrosis.

A sPLA2-dependent mechanism for the antifibrotic properties of KH064 is supported by other studies relating to the actions of arachidonic acid metabolites. TXA2 (44) and 12-lipoxygenase (45) are elevated in young SHR. Increased TXA2 is associated with pulmonary fibrosis (12), whereas experiments using fibroblasts overexpressing 12-lipoxygenase have shown that these cells synthesize greater amounts of collagen I (13), the predominant type of collagen found in the heart and primarily responsible for cardiac fibrosis and increased myocardial stiffness (1). Furthermore, antagonism of LTD2/E4 receptors during myocardial reperfusion reduced the size of the necrotic site and preserved cardiac function (46).

The release of sPLA2-IIA from cardiac myocytes and fibroblasts may be stimulated by macrophages recruited into the left ventricle following release of cytokines such as TNF-α and IL-1β. Type II sPLA2 was up-regulated in the presence of TNF-α in cultured neonatal cardiac myocytes (39), whereas stimulation of cardiac myocytes with IL-1β caused an increase in sPLA2-IIA mRNA (47). As indicated above, the sPLA2-IIA inhibitor KH064 inhibited TNF-α and IL-1β-mediated release of PGE2. Macrophages are also known to release sPLA2-IIA (48, 49), and Ghersiencier et al. (26) demonstrated that mice overexpressing the human sPLA2 gene in macrophages have increased collagen in atherosclerotic lesions.

sPLA2-IIA can also mediate TNF-induced up-regulation of ICAM-1 (50). This may indicate a potential role for sPLA2-IIA in the infiltration of inflammatory cells into the left ventricle by influencing cell adhesion. However, in the current study, the sPLA2-IIA inhibitor KH064 failed to reduce the infiltration of ED1-positive macrocyte/macrophages into the left ventricle of young SHR, suggesting that sPLA2-IIA may not play a significant role in this process in the hypertensive heart. ED1-positive macrocyte/macrophages were localized in areas of interstitial fibrosis, as well as scar sites. However, ED1-positive monocyte/macrophages were also found throughout the left ventricle, which presumably were cells that had infiltrated the interstitium before initiating fibrosis. Conversely, areas of perivascular fibrosis did not always include ED1-positive monocyte/macrophages. By 12 wk of age, macrophage-induced fibrosis may have already occurred in the perivascular areas, and other signals may continue the progression.

α-SMA within nonvascular regions of the left ventricle can be used as a marker of fibroblast differentiation into contractile myofibroblasts (51). Macrophages are localized with myofibroblasts in the L-NAME model of fibrosis (52) and isoproterenol-induced model of myocardial injury (53). In addition, macrophage infiltration has been shown to precede the presence of myofibroblasts, and clusters of macrophages are located in close proximity to myofibroblasts (54). Myofibroblasts have been identified in myocardial scars as the predominant source of collagen (55). The finding of the current study indicates that in the early stages of cardiac fibrosis in the young SHR, myofibroblasts were detectable by immunofluorescence staining. Presumably, normal cardiac fibroblasts also contribute to fibrosis, particularly because macrophages are also associated with increased numbers of fibroblasts (9). KH064 did not alter myofibroblast detection within the left ventricle of young SHR, suggesting that although involved in fibrosis, sPLA2-IIA may not be involved in fibroblast differentiation to myofibroblast in the left ventricle of the young SHR.

The current study also shows that the increase in systolic blood pressure in the young SHR is unaltered by KH064 treatment, in agreement with previous studies showing that inhibition of inflammation of the left ventricle does not prevent increases in blood pressure (9, 52). In isolated thoracic aortic rings, endothelial function and direct NO-induced vasodilation were not different between SHR and WKY in the current study, as illustrated in a previous study (57). However, arachidonic acid metabolites are clearly involved in the regulation of blood pressure. 12-lipoxygenase (45), cysteinyl leukotrienes (58, 59), and 20-hydroxyeicosatetraenoic acid (60) all influence blood pressure in the SHR, whereas TXA2, a potent vasoconstrictor (61), is elevated in young SHR (44). Arachidonic acid released as the precursor to these products is therefore likely to be liberated by another PLA2 enzyme, probably cPLA2-IV either from endothelial or vascular smooth muscle cells.

In summary, our results indicate that sPLA2-IIA inhibitors may be particularly valuable molecular probes for dissecting the signaling pathway leading to cardiac fibrosis. They suggest an important and previously unrecognized role for sPLA2-IIA in cardiac fibrosis during development of hypertension in the rat heart. This may point to sPLA2-IIA as a potential new therapeutic target, with the possibility that sPLA2-IIA inhibitors may selectively prevent cardiac fibrosis in a clinical setting. However, sPLA2-IIA inhibitors seem unlikely to be useful in curbing excessive infiltration of macrophages, development of ventricular hypertrophy, chronic hypertension, or altered vascular contractile responses.

Disclosures
The authors have no financial conflict of interest.

References
sPLA2 INHIBITION AND CARDIAC FIBROSIS


