Vascular proinflammatory responses by aldosterone are mediated via c-Src trafficking to cholesterol-rich microdomains: role of PDGFR

Glaucia E. Callera1*, Alvaro Yogi1, Ana M. Briones1, Augusto C.I. Montezano1, Ying He1, Rita C.A. Tostes2, Ernesto L. Schiffrin3, and Rhian M. Touyz1

1Ottawa Hospital Research Institute, University of Ottawa, Ottawa, ON, Canada; 2School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil; and 3Lady Davis Institute, SMBD-Jewish General Hospital, McGill University, Montreal, QC, Canada

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Aims
We demonstrated c-Src activation as a novel non-genomic signalling pathway for aldosterone in vascular smooth muscle cells (VSMCs). Here, we investigated molecular mechanisms and biological responses of this phenomenon, focusing on the role of lipid rafts/caveolae and platelet-derived growth factor receptor (PDGFR) in c-Src-regulated proinflammatory responses by aldosterone.

Methods and results
Studies were performed in cultured VSMCs from Wistar-Kyoto (WKY) rats and caveolin-1 knockout (Cav 12/2) and wild-type mice. Aldosterone stimulation increased c-Src phosphorylation and trafficking to lipid rafts/caveolae. Cholesterol depletion with methyl-β-cyclodextrin abrogated aldosterone-induced phosphorylation of c-Src and its target, Pyk2. Aldosterone effects were recovered by cholesterol reload. Aldosterone-induced c-Src and cortactin phosphorylation was reduced in caveolin-1-silenced and Cav 12/2 VSMCs. PDGFR is phosphorylated by aldosterone within cholesterol-rich fractions of VSMCs. AG1296, a PDGFR inhibitor, prevented c-Src phosphorylation and translocation to cholesterol-rich fractions. Aldosterone induced an increase in adhesion molecule protein content and promoted monocyte adhesion to VSMCs, responses that were inhibited an by cholesterol depletion, caveolin-1 deficiency, AG1296 and PP2, a c-Src inhibitor. Mineralocorticoid receptor (MR) content in flotillin-2-rich fractions and co-immunoprecipitation with c-Src and PDGFR increased upon aldosterone stimulation, indicating MR-lipid raft/signalling association.

Conclusion
We demonstrate that aldosterone-mediated c-Src trafficking/activation and proinflammatory signalling involve lipid rafts/caveolae via PDGFR.

Keywords
Aldosterone • Lipid rafts/caveolae • PDGFR • c-Src • Vascular smooth muscle cells

1. Introduction
Aldosterone, a mineralocorticoid hormone that is critically involved in electrolyte balance and blood pressure regulation, mediates its effects through mineralocorticoid receptors (MRs) via well-characterized genomic mechanisms. In addition to these long-term transcriptional mechanisms, aldosterone has important acute effects on vascular reactivity, endothelial function, inflammation, and oxidative stress. Many of these aldosterone actions extend beyond the classical genomic actions of MRs and involve non-genomic mechanisms through rapid activation of many signalling molecules and ion channels. Aldosterone modulates Na+/H+ exchanger activity and intracellular levels of calcium and cAMP independently of transcription and de novo protein synthesis. Aldosterone induces activation of various kinases, including protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-K), tyrosine kinases, epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), and mitogen-activated protein kinases (MAPKs). These pathways are critically involved in pathophysiological processes associated with vascular inflammation and injury.
We recently identified a novel non-genomic signalling pathway for aldosterone involving c-Src-regulated activation of p38MAPK and NAD(P)H oxidase in vascular smooth muscle cells (VSMCs).\(^{16}\) Src kinases are a family of non-receptor tyrosine kinases of which the prototype, c-Src, is the major isoform in the vasculature.\(^{17,18}\) As an important signalling molecule in VSMCs, c-Src signals to various downstream effectors, including Ras–MAPK, PI3–K–Akt, Pyk2, cortactin, integrins, and focal adhesion proteins, critically involved in cell growth, migration, inflammation, and survival.\(^{19–21}\) Src family kinases and their downstream effectors localize to membrane sphingolipids/cholesterol-rich domains termed ‘lipid rafts’, which are compartments that act as transient platforms for docking of interacting signalling molecules.\(^{22–24}\) Caveolae constitute a distinct subset of lipid rafts with morphologically defined cell surface invaginations that contain caveolin as a major structural protein.\(^{25}\) Cell stimulation by humoral or mechanical factors that modulate signalling events is associated with recruitment of receptors or molecules into membrane rafts.\(^{26}\) Of the many receptors localized within lipid rafts/caveolae, are receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR), which stimulates recruitment of multiple signalling molecules to these membrane microdomains.\(^{27,28}\) This phenomenon facilitates molecular proximity necessary for rapid, efficient, and specific regulation of downstream signalling events,\(^{29,30}\) including the stepwise activation of c-Src by PDGFR.\(^{31–33}\)

The role of PDGFR in vascular c-Src signalling by aldosterone is unclear. Moreover, the functional significance of lipid rafts/caveolae as a potential platform for PDGFR-c-Src interaction for non-genomic aldosterone signalling is unknown. Here, we investigate molecular mechanisms and biological responses of this phenomenon, focusing on the role of lipid rafts/caveolae and PDGFR in c-Src-regulated proinflammatory responses by aldosterone.

2. Methods

Please see Supplementary material online for detailed Methods section.

2.1 Cell culture

The study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. VSMCs from adult male Wistar-Kyoto rats (WKY), caveolin-1 knockout (Cav 1\(^{-/-}\)) and wild-type (Cav 1\(^{+/-}\)) mice were studied. Rats (16 weeks old) and mice (8–10 weeks old) were euthanized on the role of lipid rafts/caveolae and PDGFR in c-Src-regulated non-genomic signalling by aldosterone.

2.2 Electron microscopy

The presence of caveolae in cultured VSMCs was evaluated by electron microscopy. VSMCs were fixed in 2.5% glutaraldehyde, post-fixed in 2% OsO\(_4\), dehydrated in graded ethanol, and embedded in Spurr’s low viscosity epoxy resin. Ultrathin sections were cut and mounted on copper mesh grids. Sections were examined under transmission electron microscopy.

2.3 Protocols for cell stimulation

Growth-arrested VSMCs were stimulated with 10\(^{-7}\), 10\(^{-8}\), and 10\(^{-7}\) mol/L aldosterone for 30 min or 24 h. Aldosterone 10\(^{-7}\) mol/L was used for short (1–60 min) or long-term stimulations (2–24 h). In some experiments, cells were pre-exposed for 30 min to: PP2 (c-Src inhibitor, 10\(^{-5}\) mol/L), eplerenone (MR antagonist, 10\(^{-5}\) mol/L), or AG1296 (PDGFR inhibitor, 10\(^{-6}\) mol/L).

2.4 Cholesterol depletion/sequestration and cholesterol reloading

Depletion or sequestration of free cholesterol from the plasma membrane was carried out using two different agents, methyl-β-cyclodextrin (MCD) or nystatin, respectively. To deplete cholesterol, VSMCs were treated with 10\(^{-2}\) mol/L MCD. Cholesterol was reloaded by incubating cholesterol-depleted cells with 10\(^{-2}\) mol/L cholesterol:10\(^{-2}\) mol/L MCD complex. In some experiments, VSMCs were treated with cholesterol:MCD complex without previous cholesterol depletion. VSMCs were washed to remove the MCD and unincorporated cholesterol, and immediately used in experiments. For cholesterol sequestration, cells were incubated for 30 min with 50 μg/mL nystatin prior to aldosterone stimulation.

2.5 Filipin staining

To evaluate the membrane cholesterol manipulation, VSMCs were stained with filipin. Cells cultured on glass coverslips were subjected to the cholesterol-depletion/reload protocol, fixed in 1% glutaraldehyde and treated with 100 μg/mL filipin. Cells were mounted in anti-fade and immediately examined using a UV filter.

2.6 Protein fractionation by sucrose gradient centrifugation

A detergent-free method to purify rafts was used. Aldosterone-stimulated VSMCs were lysed in 500 mmol/L Na\(_2\)CO\(_3\) (pH 11). The homogenate containing 2 g of protein was adjusted to 45% sucrose in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered saline (MBS) (25 mmol/L MES and 150 mmol/L NaCl, pH 6.5) and overlayed with 6 mL of 35% sucrose and 2 mL of 5% sucrose. After centrifugation at 150 000 g for 18 h (SW 41, Beckman Instruments), twelve 1-mL fractions were collected. Proteins were precipitated with 10% trichloroacetic acid, and re-suspended in 0.1 mol/L NaOH and Laemmli buffer. Flotillin-2 was used as a marker of lipid rafts.

2.7 RNA interference and cell transfection

VSMCs were subjected to caveolin-1 gene knockdown with small interfering RNA (siRNA) prior to aldosterone stimulation. High-performance purity grade (>90% pure) siRNA was generated against mouse caveolin-1. Mouse VSMCs were exposed to transfection reagent (TR) alone or transfected with siRNA. A non-silencing siRNA oligonucleotide sequence that does not recognize any known homology to mammalian genes was also generated as a negative control (NS). Cells were seeded at a density of 2.5 × 10\(^{4}\) cells with DMEM containing 10% FBS and transfected with 10\(^{-8}\) mol/L siRNA using HiPerFect Transfection Reagent. Optimum gene silencing was observed at 72 h after transfection.

2.8 Western blotting

Total or fractionated proteins from VSMCs were separated by electrophoresis on a polyacrylamide gel, and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked with 5% skim milk. Membranes were then incubated with specific antibodies overnight at 4°C. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography and quantified densitometrically. Antibodies to non-phosphoproteins, β-actin, or GAPDH were used as internal controls.
2.9 Immunofluorescence microscopy

Membrane lipid rafts were localized with cholera toxin B-fluorescein isothiocyanate (CTB-FITC), and the antibodies anti-flotillin-2 and anti-caveolin-1. Living VSMCs in suspension were incubated with CTB-FITC 2 μg/mL for 15 min at 4°C, attached to poly-L-lysine-coated coverslips and fixed with 4% paraformaldehyde. Cells were then blocked with 2% BSA and incubated with the primary anti-MR antibody followed by incubation with Alexa Fluor secondary antibody. For further immunofluorescence staining with anti-MR anti-caveolin-1 or anti-flotillin-2, VSMCs were cultured on glass coverslips, fixed, blocked, and incubated with primary antibodies. Proteins were detected with Alexa Fluor secondary antibodies.

2.10 Immunoprecipitation analysis of MR and caveolin-1

Homogenates from aldosterone-stimulated VSMCs were adjusted to equal protein concentration (1 μg/μL), pre-cleared and then incubated with 2 μg/mL of anti-MR or anti-caveolin-1 antibodies overnight at 4°C. Caveolin-1 and MR immune complexes were immunoprecipitated with protein agarose beads for two additional hours. Immunoprecipitates

Figure 1 Methyl-β-cyclodextrin (MCD) and nystatin (NY) inhibit aldosterone-induced c-Src phosphorylation in WKY VSMCs. (A) Aldosterone effect (10^{-9} – 10^{-7} mol/L, 30 min) on c-Src phosphorylation. (B) Time-course effect of 10^{-7} mol/L aldosterone on c-Src phosphorylation in the absence and presence of 10^{-2} mol/L MCD or 50 μg/mL nystatin. (C) Aldosterone effects on c-Src phosphorylation after the following treatments: cholesterol depletion with 10^{-2} mol/L MCD and subsequent reloading with 10^{-3} mol/L cholesterol: 10^{-2} mol/L MCD complex; cholesterol:MCD complex without prior cholesterol depletion; MCD without subsequent cholesterol reloading. Representative immunoblots: c-Src [Tyr418], c-Src. Results are mean ± SEM of 4–8 experiments. *P < 0.05, vs. vehicle; (D) Side panels, representative images of VSMCs with filipin-stained free cholesterol (blue) in the following conditions: basal; cholesterol depleted with MCD; cholesterol depleted with MCD and reloaded with cholesterol:MCD complex.
were resolved in SDS–polyacrylamide gels. Immunoblottings of MR immunoprecipitates were undertaken with anti-caveolin-1, anti-c-Src, and anti-PDGFR antibodies. For caveolin-1 immunoprecipitates, the immunoblottings were performed with anti-MR. ExactaCruz system (Santa Cruz Biotechnology) was used to prevent interference of IgG chains in the immunoblot assays. Isotype-matched IgG control immunoprecipitations lacking primary antibody were performed on lysates to indicate background binding.

2.11 Adhesion assay

Growth-arrested VSMCs from WKY or Cav$^{-/-}$ and Cav$^{+/-}$ mice were stimulated with aldosterone. For cell fluorescent labelling, rat-derived NR8383 and murine-derived J774A.1 macrophages (105 cells/mL) were stimulated with aldosterone. For cell fluorescent labelling, rat-derived completely abolish aldosterone-induced c-Src and cortactin phosphorylation in VSMCs without affecting c-Src and cortactin content in VSMCs. Inhibition of c-Src phosphorylation with PP2 (Figure 3C) prevented its recruitment to the low-density fractions, suggesting that c-Src is phosphorylated prior to its translocation to cholesterol microdomains.

2.12 Data analysis

Aldosterone-stimulated effects were determined as the per cent increase over control, with the control normalized to 100%. Results are presented as mean ± SEM and compared by ANOVA one way or by Student’s t-test when appropriate. Values of P < 0.05 were considered to be significant.

3. Results

3.1 Functionally intact cholesterol microdomains are essential for c-Src trafficking by aldosterone in VSMCs

To probe the role of cholesterol-rich domains in aldosterone non-genomic signalling, we studied cultured WKY VSMCs in which cholesterol content was manipulated pharmacologically. As shown in Figure 1A and B, aldosterone concentration- and time-dependently increased c-Src phosphorylation. This effect was inhibited by depletion or sequestration of free cholesterol from the plasma membrane with cholesterol-disruptors, MCD, and nystatin. To strengthen the above observation, additional experiments were performed to deplete and then reload cholesterol to VSMCs. As observed in Figure 1C, c-Src phosphorylation induced by aldosterone was recovered by cholesterol reloading after its depletion with MCD. Treatment with cholesterol/MCD complex without previous cholesterol depletion did not affect aldosterone actions. Effectiveness of the procedure for cholesterol depletion and reload was confirmed by filipin staining (Figure 1D). The presence of caveola in VSMCs was confirmed by electron microscopy (Figure 2A). To evaluate the role of caveola in the phosphorylation of c-Src and its downstream effector, cortactin, VSMCs, in which caveolin-1 was down-regulated by siRNA, were stimulated with aldosterone. As shown in Figure 2B and C, caveolin-1 siRNA, but not scrambled siRNA, reduced caveolin-1 protein content in VSMCs without affecting c-Src and cortactin protein levels. Caveolin-1 siRNA significantly inhibited, but did not completely abolish aldosterone-induced c-Src and cortactin phosphorylation. Supporting caveolin-1 siRNA gene knockdown data, VSMCs from Cav 1$^{-/-}$ mice displayed decreased c-Src (Figure 2D) and cortactin (Figure 2E) phosphorylation in response to aldosterone stimulation.

3.2 Aldosterone promotes c-Src trafficking into cholesterol-rich domains

We next examined whether aldosterone, in addition to stimulating c-Src phosphorylation, induces trafficking of this non-receptor tyrosine kinase into cholesterol-rich domains. VSMCs fractionation by sucrose gradient centrifugation to isolate cholesterol-rich membranes showed presence of c-Src and flotillin-2 in the low-density fractions. Aldosterone stimulation increased c-Src phosphorylation (Figure 3A) and protein content (Figure 3B) in flotillin-2-rich fractions indicating activation and movement of the kinase to these membrane microdomains of VSMCs. Inhibition of c-Src phosphorylation with PP2 (Figure 3C) prevented its recruitment to the low-density fractions, suggesting that c-Src is phosphorylated prior to its translocation to cholesterol microdomains.

3.3 Aldosterone-induced PDGFR phosphorylation mediates c-Src activation

Caveolae have been identified as enriched sites of PDGFR, which stimulates the recruitment of multiple signalling molecules to lipid rafts. PDGFR inhibition with AG1296 prevented aldosterone-induced c-Src translocation to cholesterol-rich domains (Figure 3D). Aldosterone-induced PDGFR phosphorylation in a concentration- and time-dependent manner; an effect inhibited by MR antagonism (Figure 4A and B). PDGFR phosphorylation by aldosterone was inhibited in MCD-treated cells and cholesterol reload reversed this effect (data not shown). AG1296 inhibited c-Src phosphorylation by aldosterone within 5–60 min (Figure 4C). Figure 4D and E demonstrates that aldosterone stimulation increased PDGFR phosphorylation levels in cholesterol-rich fractions without modifying the protein content of the tyrosine receptor. These findings indicate that PDGFR activation contributes to c-Src translocation to cholesterol-rich domains in its active form.

3.4 Aldosterone-induced c-Src signalling is mediated via lipid rafts/caveolae and PDGFR

We also evaluated the contribution of cholesterol-rich domains and PDGFR in aldosterone-induced c-Src-mediated signalling. The non-receptor tyrosine kinase proline-rich tyrosine kinase 2 (Pyk2), a member of focal adhesion kinase family, is activated by c-Src. Aldosterone time-dependently increased Pyk2 phosphorylation (Supplementary material online, Figure SIA). This effect was inhibited by MCD and recovered by cholesterol reload (Supplementary material online, Figure SIB). This effect was also blunted by PP2 and AG1296 while eplerenone partly inhibited aldosterone-induced Pyk2 phosphorylation (Supplementary material online, Figure SIC).

3.5 Proinflammatory responses by aldosterone involve c-Src PDGFR and cholesterol-rich domains

To investigate the functional significance of cholesterol-rich domains in aldosterone signalling, we evaluated the expression of proinflammatory markers in VSMCs. Aldosterone long-term stimulation increased the protein content of ICAM-1 (Figure 5A, Supplementary material
Figure 2 Caveolin-1 siRNA and caveolin-1 deficiency inhibit aldosterone-induced c-Src and cortactin phosphorylation. (A) Presence of caveolae in cultured VSMCs evaluated by electron microscopy. Caveolae and caveolar remnants are marked by arrowheads. Effect of 10^{-7} mol/L aldosterone on c-Src and cortactin phosphorylation in mouse VSMCs transfected and non-transfected with caveolin-1 siRNA (B and C) or in VSMCs from Cav 1^{+/+} and Cav 1^{-/-} mice (D and E). Representative immunoblots: caveolin-1, c-Src[Tyr^{418}], c-Src, cortactin[Tyr^{421}] and cortactin. Results are mean ± SEM of 5–9 experiments. *P < 0.05, vs. vehicle; †P < 0.05, vs. corresponding aldosterone stimulation in non-transfected cells; vs. corresponding aldosterone stimulation in Cav 1^{+/+} cells. (TR), VSMCs exposed to transfection reagent alone; (NS), VSMCs exposed to non-silencing siRNA.
online, Figure SIIA) and VCAM-1 (Figure SIIIB). Figure 5 shows that inhibition of c-Src and PDGFR with PP2 and AG1296, respectively, prevented these aldosterone actions. Proinflammatory actions of aldosterone were blunted in MCD-treated cells and cholesterol reload reversed these effects (Figure 5, Supplementary material online, Figure SIIC and SIIID). To further evaluate the contribution of caveolae to aldosterone-induced ICAM-1 and VCAM-1 protein content increase, VSMCs from Cav 1<sup>+/+</sup> and Cav 1<sup>−/−</sup> mice were studied. Caveolin-1 deficiency significantly reduced the effects of aldosterone on ICAM-1 (Supplementary material online, Figure SIIIA) and VCAM-1 (Supplementary material online, Figure SIIIB) protein levels. In order to confirm the functional significance of cholesterol-rich domains with respect to aldosterone-mediated proinflammatory responses, monocyte adhesion to VSMCs was assessed by fluorimetry (Figure 5C) and imaging (Figure 5D and E). Aldosterone-induced cell adhesion to WKY VSMCs was blocked by PP2 and AG1296. MCD decreased monocyte adhesion to VSMCs and cholesterol reload recovered the aldosterone effect. The contribution of cholesterol-rich domains to aldosterone-induced cell adhesion was confirmed by using caveolin-1 deficient cells (Supplementary material online, Figure SIIIC and D). Aldosterone induced monocyte adhesion to Cav 1<sup>+/+</sup>...
Figure 4 PDGF mediates c-Src phosphorylation by aldosterone in WKY VSMCs. (A) Aldosterone effect (10^{-9}–10^{-7} mol/L, 30 min) on PDGFR phosphorylation. Time-course effect of 10^{-7} mol/L aldosterone on: (B) PDGFR phosphorylation in absence and presence of 10^{-5} mol/L eplerenone, MR antagonist; (C) c-Src phosphorylation in absence and presence of 10^{-6} mol/L AG1296, PDGFR inhibitor. WKY VSMCs were subjected to discontinuous sucrose density gradient after stimulation with 10^{-7} mol/L aldosterone (5 and 30 min). (D) Aldosterone-induced PDGFR phosphorylation in flotillin-2-enriched membrane fractions 2 and 3, represented as ratio of flotillin-2 expression. (E) Distribution of PDGFR protein content across the sucrose gradient in aldosterone-stimulated VSMCs. PDGFR protein content in each individual fraction is expressed relative to the sum of the intensity of the total fractions taken as 100%. Representative immunoblots of PDGFR, PDGFR [Tyr^{849/857}], c-Src[Tyr^{418}], c-Src, and flotillin-2. Results are mean ± SEM of 6–8 experiments. *P < 0.05 vs. vehicle; †P < 0.05, vs. corresponding aldosterone stimulation times in the absence of inhibitors.
VSMCs, whereas no effect was observed in Cav $1^{-/}$ cells. Aldosterone increased PAI-1 protein content (Supplementary material online, Figure SI). PP2 and MCD did not influence aldosterone-induced increase of PAI-1, at least within 8 h of stimulation.

### 3.6 Mineralocorticoid receptor is a raft-associated protein

Immunofluorescence image analysis demonstrated that in the basal state, MR is detected in the cytoplasmic and membrane regions in VSMCs (Supplementary material online, Figure S5). GM1 gangloside, a marker for lipid rafts and caveolae, was detected with cholera toxin subunit B labelled with FITC and colocalized with MR immunostaining (Supplementary material online, Figure SVA). Co-staining of flotillin-2 (Supplementary material online, Figure SVB) or caveolin-1 (Supplementary material online, Figure SVC) and MR antibodies demonstrated co-localization of these proteins in the cell periphery. We further evaluated whether aldosterone induces increase in MR content in cholesterol-rich domains. Supplementary material online, Figure SVD, demonstrates the presence of MR in flotillin-2-rich cell fractions under basal conditions. MR expression was not detected in the presence of blocking peptide. Aldosterone stimulation increased MR protein content in the cholesterol-rich fractions within 5 min (Figure 6A). Immunoprecipitation was performed to evaluate the interaction between MR and raft-associated proteins. MR immunoprecipitates were analysed for the presence of caveolin-1, c-Src, and PDGFR in aldosterone stimulated VSMCs. Caveolin-1 immunoprecipitates were also evaluated for the presence of MR. As shown in Figure 6B and C, MR co-immunoprecipitated with caveolin-1 and aldosterone stimulation had no effect on this interaction. MR co-immunoprecipitated with c-Src (Figure 6D) and PDGFR (Figure 6E). Aldosterone increased the interaction of MR with the signalling proteins within 5 min.

### 4. Discussion

Lipid rafts are key players in the integration of cellular responses. Here, we show that (i) aldosterone is reliant on cholesterol-rich microdomains to mediate activation of c-Src downstream signalling, (ii) aldosterone-mediated c-Src trafficking/activation and proinflammatory signalling involve lipid rafts/caveolae and PDGFR, (iii) MR localizes in flotillin-2-rich domains in the plasma membrane and interacts with signalling molecules. Lipid rafts can include or exclude proteins creating a restricted and integrative microenvironment by promoting reactions between raft partitioning proteins. The mechanisms whereby lipid raft association occurs are variable. Lipid modifications of weak raft affinity proteins would determine their trafficking into lipid rafts/caveolae and/or increase residency time in this microenvironment. Extracellular stimuli drive the partitioning dynamics of the signalling proteins towards lipid rafts to organize complexes of receptors, protein kinases, adapters and G proteins, triggering signal transduction from the cell surface. $^{34}$ The role of cholesterol-rich domains in G protein-coupled receptor and tyrosine kinase receptor signalling has recently been highlighted in VSMCs. Studies demonstrate receptor trafficking into lipid rafts/caveolae and interaction with caveolin-1 as well as with respective caveolae-associated signalling components in a specific subcellular environment. $^{35–38}$ However, little is known about lipid raft/caveolae association with intracellular receptors, such as MR. We previously demonstrated c-Src activation as novel non-genomic signalling pathway for aldosterone in VSMCs from mice and rats. $^{16}$ Of importance, Src family kinases are abundant in lipid rafts/caveolae through their lipid modifications and through direct binding to caveolin. $^{1,2,23}$ The attribution of cellular functions to lipid raft/caveolae remains a challenge. Here, we show, by modifying the cholesterol content of cell membrane, that lipid rafts/caveolae play a role in aldosterone-induced c-Src signalling. Our findings are supported by the fact that membrane cholesterol disruption also inhibits aldosterone-induce phosphorylation of Pyk2, downstream target of c-Src. We also employed two strategies to examine cells in which functionally active caveolae are absent. First, we used siRNA to knockdown caveolin-1 in VSMCs and second, we studied VSMCs isolated from caveolin-1-deficient mice. In response to aldosterone, the phosphorylation of c-Src and its downstream target contactin was reduced, but not abolished in both caveolin-1 silenced and caveolin-1 deficient VSMCs. These observations suggested that both caveolar and non-caveolar processes are involved in aldosterone-mediated c-Src signalling.

Our findings demonstrate that aldosterone is an important promoter of c-Src trafficking and activation in VSMCs. The significance of c-Src compartmentalization may relate to aldosterone signalling efficiency. If the signalling event occurs within lipid rafts/caveolae, the protein phosphorylation state can be modified by local kinases and phosphatases, resulting in target-directed downstream signalling. $^{34,39}$ In addition to stimulating c-Src movement into lipid rafts/caveolae, aldosterone induced activation of c-Src in these domains as shown by increased phosphorylation of the protein tyrosine kinase in cholesterol-rich cell fractions. However, prior c-Src phosphorylation appears to be necessary for the kinase recruitment to lipid rafts/caveolae. The exact mechanisms underlying this process are not totally understood and transactivation through receptor tyrosine kinases may be important.

It has become clear that multiple levels of cross talk actually exist between MR and membrane receptor systems at early points in the signal transduction pathway. $^{11,12,40}$ This is underscored by our findings that aldosterone induces PDGFR phosphorylation in cholesterol-rich domains without interfering with the receptor tyrosine kinase trafficking. PDGFR plays a versatile role in c-Src autophosphorylation and trafficking in the cell membrane. PDGFR regulates the switch between the inactive and active conformations of c-Src. $^{41–43}$ Phosphorylation of c-Src in the SH2 domain by PDGFR determines displacement of intramolecular interactions that lock the enzyme in an inactive form, resulting in maximal autophosphorylation of Tyr 418 residue on the activation site. c-Src can also be directly recruited to the plasma membrane by PDGFR activation. $^{44–46}$ possibly through Rac and actin filaments. $^{37,46}$ Rapid transactivation of PDGFR by aldosterone/MR leading to c-Src activation may be the first step in the cascade of events for the kinase recruitment to lipid rafts/caveolae. Expression of adhesion molecules is a key step upon monocyte recruitment and inflammation. The ability of monocytes to adhere to vascular cells is critical for invasiveness during the inflammatory process. In order to confirm the functional importance of cholesterol-rich domains in aldosterone-mediated proinflammatory responses, we evaluated monocyte adhesion and protein levels of adhesion molecules as well. From molecular to functional viewpoints, we show that aldosterone increases ICAM-1 and VCAM-1 protein content.
Aldosterone induces monocyte adhesion and increases ICAM-1 and VCAM-1 protein content in WKY VSMCs. Long-term (4–24 h) effect of $10^{-7}$ mol/L aldosterone on ICAM-1 (A) and VCAM-1 (B) protein levels in absence and presence of $10^{-5}$ mol/L PP2, $10^{-2}$ mol/L MCD, and $10^{-6}$ mol/L AG1296. Representative immunoblots of ICAM-1, VCAM-1, and GAPDH. Monocyte adhesion was evaluated by fluorimetry (C) and fluorescence microscopy (D). Effect of $10^{-7}$ mol/L aldosterone (24 h) on adhesion of fluorescence labelled rat-derived NR8383 monocytes to VSMCs after the following treatments: cholesterol depletion with $10^{-2}$ mol/L MCD; cholesterol depletion with $10^{-3}$ mol/L MCD and subsequent reloading with $10^{-3}$ mol/L cholesterol; $10^{-2}$ mol/L MCD complex; $10^{-5}$ mol/L PP2; $10^{-6}$ mol/L AG1296. Representative images of adhered fluorescence labelled monocytes (E). Results are means ± SEM of 6–7 experiments. *P < 0.05, vs. vehicle.
Figure 6  MR protein content in raft fractions and MR interaction with signalling proteins in aldosterone-stimulated VSMCs. Distribution of MR protein content across the sucrose gradient in 10^{-7} mol/L aldosterone stimulated (5 and 30 min) VSMCs (A). MR protein content in each individual fraction is expressed relative to the sum of the intensity of the total fractions taken as 100%. Results are mean ± SEM of 12 experiments. Protein samples from 10^{-7} mol/L aldosterone-stimulated (5 and 30 min) VSMCs were subjected to immunoprecipitation (IP) and immunoblotting (IB) with the following antibodies: (B) IP with anti-caveolin-1 and IB with anti-MR; (C) IP with anti-MR and IB with anti-caveolin-1; (D) IP with anti-MR and IB with anti-c-Src; (E) IP with anti-MR and IB with anti-PDGFR; (F) Input: IP and IB with anti-MR or anti-caveolin-1. Results are mean ± SEM of 4–9 experiments. Representative immunoblots of MR, caveolin-1, c-Src, and PDGFR. *P < 0.05 vs. vehicle; †P < 0.05 vehicle vs. corresponding aldosterone counterpart fractions.
and stimulates monocyte adhesion to VSMCs, responses that are inhibited by cholesterol depletion or caveolin-1 deficiency. PP2 and AG1296 also inhibited these effects, indicating the importance of PDGFR/c-Src in aldosterone-induced cell adhesion. Not all proinflammatory mediators are influenced by c-Src and cholesterol-rich domains in aldosterone responses. Despite the fact that PAI-1 expression is activated by aldosterone, PP2, as well as manipulation of membrane cholesterol content, failed to inhibit this response. Together these data underlie a significant role of lipid rafts/caveolae in mediating PDGFR/c-Src-dependent proinflammatory responses by aldosterone.

Whether aldosterone acts via the classical MR to mediate the described actions or via a novel receptor remains controversial. In previous studies and in this report, we show that aldosterone-mediated c-Src signalling and PDGFR phosphorylation in VSMCs is inhibited by eplerenone, a MR antagonist, indicating the importance of the classical receptors in this responses. Here, we also extend our findings by demonstrating the presence of MR in the cell plasma membrane and cytosol. The membrane-associated MR co-localized with lipid raft markers, suggesting that in addition to its cytosolic location, MR are associated with cholesterol-rich domains. To further support these findings, we showed that MR co-immunoprecipitate with caveolin-1. The interaction between MR and caveolin-1 has been described in heart tissue supporting our findings here in VSMCs. Aldosterone induces the increase in MR content in cholesterol-rich domains. This similar spatial pattern of c-Src and MR translocation to cholesterol-rich domains may suggest that molecular interactions and biological functions are dependent on their subcellular localization. In fact, MR interacts with raft-associated signalling, since we observed co-immunoprecipitation also with c-Src and PDGFR in VSMCs. These interactions increased upon aldosterone stimulation. The site-directed activation of PDGF and c-Src by aldosterone may be facilitated by the MR translocation to cholesterol-rich domains.

In summary, using a multidisciplinary approach we demonstrate that proinflammatory effects of aldosterone in VSMCs involve c-Src activation and trafficking to cholesterol-rich domains via PDGFR. Localization of MR in these microdomains may facilitate rapid and efficient signalling. These novel findings identify lipid rafts/caveolae as dynamic scaffolding systems for aldosterone/MR in VSMCs and stimulation of a proinflammatory MR translocation to cholesterol-rich domains. This similar spatial pattern of c-Src and MR translocation may suggest that molecular interactions and functional properties are dependent on their subcellular localization. In fact, MR interacts with raft-associated signalling, since we observed co-immunoprecipitation also with c-Src and PDGFR in VSMCs. These interactions increased upon aldosterone stimulation. The site-directed activation of PDGF and c-Src by aldosterone may be facilitated by the MR translocation to cholesterol-rich domains.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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