GPCR agonist-induced transactivation of the EGFR upregulates MLC II expression and promotes hypertension in insulin-resistant rats

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Aims
The presence of metabolic abnormalities such as insulin resistance and elevated levels of various vasoconstrictor G-protein-coupled receptor (GPCR) agonists contributes to the development of hypertension. Recent studies have suggested a link between disease progression and activation of growth factor receptor signalling pathways such as the epidermal growth factor receptor (EGFR) by matrix metalloproteinases (MMPs). We hypothesized that excessive stimulation of GPCRs such as α1-adrenergic receptors activates MMP-dependent EGFR transactivation and contributes to the development of hypertension by promoting increased synthesis of contractile proteins in vascular smooth muscle (VSM).

Methods and results
We tested this concept in experiments using insulin-resistant VSM cells (VSMCs) and fructose hypertensive rats (FHRs), a model of acquired systolic hypertension and insulin resistance. We found that insulin resistance and agonist stimulation increased the expression and activity of MMPs (MMP-2 and MMP-7), the EGFR, contractile proteins such as myosin light chain kinase and MLC II, and their transcriptional activators including P90 ribosomal kinase (P90RSK) and serum response factor, possibly via the activation of extracellular signal-regulated kinase (ERK1/2) in VSMCs. Further, in insulin-resistant VSMCs and arteries from FHRs, disruption of MMP-EGFR signalling either by a pharmacological or small interfering RNA approach normalized the increased expression and activity of contractile proteins and their transcriptional activators and prevented the development of hypertension in FHRs.

Conclusion
Our data suggest that the MMP-EGFR pathway could be a potential target in the treatment of hypertension in insulin resistance and/or hyperglycaemic conditions such as type 2 diabetes.

Keywords
Insulin resistance • Matrix metalloproteinase • Epidermal growth factor receptor • Hypertension • Vascular smooth muscle

1. Introduction
The presence of metabolic and haemodynamic abnormalities activates growth-promoting pathways and contributes to the development of hypertension both in insulin-resistant and non-insulin-resistant states.1 A growing body of evidence suggests that the characteristically elevated levels of G-protein-coupled receptor (GPCR) agonists such as catecholamines, endothelin-1 (ET-1), and angiotensin II (Ang II) promote hypertrophic growth as well as contribute to enhanced vascular tone in hypertension.2 Some of the molecular mechanisms that are associated with these changes in hypertension include upregulation of growth-promoting pathways and increased generation of reactive oxygen species.2,3 Until recently, the signalling events initiated by GPCR agonists were considered to be rapid, short lived, and divided into two separate linear but distinct pathways, the vasoconstrictory pathway involving the phospholipase C–diacylglycerol–Ca2+ axis and the growth-promoting pathway involving receptor tyrosine and mitogen-activated protein kinases (MAPKs). However, recent studies have suggested that these signalling pathways do actively engage in crosstalk.4 The resulting combinatorial signalling events allow GPCRs to take advantage of pathways downstream of growth factor receptors to influence cell function under varying physiological situations.5 One example of such crosstalk connecting GPCR activation with the MAPK signalling pathway is matrix
metalloproteinase (MMP)-dependent transactivation of the epidermal growth factor receptor (EGFR) in vascular smooth muscle cells (VSMCs). It has been proposed that MMPs transactivate the EGFR in VSMCs in response to stimulation by GPCR agonists such as catecholamines, Ang II, and ET-1. Further, overstimulation of the MMP-EGFR pathway leads to enhanced vascular tone, increased oxidative stress, and hypertrophic growth in hypertension. The EGFR could function as a convergent point for both mitogenic and non-mitogenic signals arising from various stimuli in VSMCs. Studies have demonstrated that inhibition of the activities of MMP or the EGFR promotes vasodilatation, reduces oxidative stress, improves insulin resistance, and reduces elevated blood pressure and cardiac hypertrophy. Although it is clear that EGFR activation triggers a series of complex but well-characterized signalling events, how these signalling events are integrated to regulate the expression of contractile proteins is poorly understood. Further, it is not clear whether MMP-dependent EGFR signalling is altered in insulin resistance and its role in regulation of contractile protein expression.

In conditions of insulin resistance, many insulin target tissues, including VSMCs, display a significant defect of phosphatidylinositol 3-kinase (PI3K) signalling, but retain normal sensitivity to insulin via the MAPK signalling pathway. Since insulin resistance is also associated with hyperinsulinaemia and elevated levels of various GPCR agonists, we hypothesize that this results in activation of MAPK pathways, promoting increased synthesis of contractile proteins in insulin-resistant hypertension via the MMP-dependent transactivation of the EGFR. In the present study, we examined the role of MMP-EGFR pathway in agonist-induced expression of contractile proteins in insulin-resistant VSMCs in vitro and in vivo.

2. Methods

2.1 Cell culture

Rat aortic VSMCs were grown in complete Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum and 100 U/mL penicillin–streptomycin at 37°C and 95% O2/5% CO2. When cells were 80–90% confluent, they were made quiescent by incubation in DMEM containing normal glucose (NG, 5.5 mM), high glucose (HG, 25 mM) or manitol (19.5 mM mannitol), and 0.1% calf serum for 72 h. On the day of experiments, quiescent VSMCs were treated with inhibitors/agonists for the indicated times (see Section 3). The media and cells were collected for zymography and western blotting, respectively. See Supplementary material online for details.

2.1.1 Small interfering RNA experiments in VSMCs

Small interfering RNAs (siRNAs) specific to rat EGFR, MMP-2, and MMP-7 were transfected to VSMCs as described previously. See Supplementary material online for details.

2.1.2 Measurement of MMP activity by substrate zymography

MMP-2, MMP-9, and MMP-7 activities in cell culture releasates were measured using gelatin (for MMP-2 and MMP-9)– or carboxymethyl transferrin (CMT)-based substrate (for MMP-7) zymography as described previously. See Supplementary material online for details.

2.2 Western blotting

See Supplementary material online for details.

2.3 Animal studies

This study conforms with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the University of British Columbia Animal Care committee. Forty-eight male Wistar rats were randomly divided into six equal groups: control (C), control treated with doxycycline, a broad-spectrum MMP inhibitor (CD), or AG1478, the EGFR tyrosine kinase inhibitor (CA), fructose (60%)–, and fructose-fed and treated with doxycycline (FD, 20 mg/kg/day) or AG1478 (FA, 5 mg/kg/day). See Supplementary material online for details.

2.3.1 Oral glucose tolerance test and insulin sensitivity index

Oral glucose tolerance test (OGTT) in rats was performed as described previously. See Supplementary material online for details.

2.4 Statistical analysis

All values are expressed as mean ± SEM. ‘n’ denotes the sample size in each group. Statistical analysis was performed using either Student’s t-test, one- or two-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons. GraphPad Prism (GraphPad Software, CA, USA) software program was used for statistical analysis. For all results, the level of significance was set at P < 0.05.

3. Results

3.1 Modelling insulin resistance in VSMCs

As a first step to establish cell culture conditions that mimic insulin resistance, we adapted a previously described procedure by growing VSMCs in NG (5 mmol/L) or HG (25 mmol/L) for 72 h. Under these conditions, insulin-dependent phosphorylation of insulin receptor substrate (IRS-1) and Akt, the key markers of insulin resistance, was altered in VSMCs cultured in HG (Supplementary material online, Figure S1). Specifically, we detected increased IRS-1 and decreased Akt phosphorylation in response to insulin in VSMCs incubated in HG conditions. The phosphorylation of Ser-307, which results in insulin resistance, was markedly increased in VSMCs cultured in HG at 30 min after stimulation with insulin.

3.2 Agonist stimulation increases MMP and EGFR activities in insulin-resistant VSMCs

Activation of GPCRs such as α1-adrenergic receptor (AR) by their putative agonists has been linked to MMP-dependent transactivation of the EGFR in VSMCs. To understand whether the activity or expression of vascular MMPs such as MMP-2, MMP-9, and MMP-7 are altered in response to HG and agonist stimulation, we treated VSMCs cultured in NG and HG conditions with phenylephrine (PE) for 60 min. We chose the 60 min time point to mimic a condition of sustained stimulation of α1-AR to study changes in protein synthesis or expression. We found a significantly increased expression of all major vascular MMPs including both pro- and active forms of MMP-2, MMP-9, and MMP-7 in VSMCs grown in HG conditions and in response to PE stimulation (Supplementary material online, Figure S2). Further, as shown in the zymograms (Figure 1A), MMP-2 activity was also significantly elevated in VSMCs cultured in HG compared with NG and stimulation with PE increased MMP activity further in cells cultured in HG conditions. However, we did not detect MMP-7 and MMP-9 activities in our experimental settings. Treatment of VSMCs to HG as well as stimulation with PE significantly increased the levels of phospho-EGFR (Figure 1B). Tyr-1173, a major site for
autophosphorylation of the EGFR is crucial for the initiation of downstream signalling and is often used as an index of EGFR activation.\(^6\)

### 3.3 Blockade of the MMP-EGFR pathway reduces EGFR activity in insulin-resistant VSMCs

To determine whether inhibition of MMP activity or suppression of its expression reduces the EGFR phosphorylation induced by HG and PE stimulation, we conducted experiments using both pharmacological inhibition and siRNA approaches. Pharmacological inhibition of MMPs by GM6001, a broad-spectrum MMP inhibitor, reduced MMP-2 activity both in HG-treated and PE-stimulated VSMCs (Figure 1A). Similarly, treatment of cells with GM6001 decreased the activation of the EGFR induced by both HG and PE stimulation (Figure 1B). As expected, GM6001 did not affect the expression of MMP-2, MMP-9, or MMP-7 (data not shown). Knockdown of MMP-2 and MMP-7, which we have previously shown to decrease the expression of MMP-2 and MMP-7 by 60 and 50%, respectively,\(^1^8\) reduced the phosphorylation of the EGFR induced both by HG and PE stimulation (Figure 1C and D). The observation that knockdown of either MMP-2 or MMP-7 nearly completely blocked EGFR activity suggests that these two MMPs form a sequential rather than a parallel axis in the transactivation process. It has been reported that MMP-7 activates the proform of MMP-2,\(^2^3,2^4\) and therefore, it is possible that knockdown of MMP-2 or its upstream activator MMP-7 reduced EGFR activity. Likewise, suppression of the EGFR expression by its siRNA also decreased both HG- and PE-induced increase in EGFR activity in insulin-resistant VSMCs (Supplementary material online, Figure S3).
3.4 Agonist stimulation enhances MAPK activity in insulin-resistant VSMCs

Activation of MAPK signalling pathways is linked to mitogenic effects including the synthesis of contractile proteins downstream of growth factor receptors such as insulin and the EGFR.\(^{20,25}\) We tested the effect of HG and PE stimulation on the classical MAPKs such as the ERK, c-Jun-N-terminal kinase (JNK), and the p38 MAPK. Incubation of VSMCs in HG alone activated ERK (Figure 2) but not other MAPKs (Supplementary material online, Figure S4). Further, stimulation with PE increased ERK activity as determined by increased phosphorylation of Thr-202 and Tyr-204 in VSMCs cultured in HG compared with NG conditions (Figure 2). PE stimulation of VSMCs also increased the phosphorylation of p38 MAPK but not JNK-1 in HG conditions (Supplementary material online, Figure S4). These data suggest that activation of ERK may be the most important signalling event in response to HG as well as agonist stimulation in insulin-resistant VSMCs.

3.5 Inhibition of MMP-EGFR pathway reduces PE-induced ERK activation in insulin-resistant VSMCs

Because ERK has been previously implicated in the synthesis of proteins involved in contraction in response to vasoactive agents,\(^{26}\) we next wanted to investigate whether MMP and EGFR inhibitors can modulate ERK activity. Treatment of cells with either GM6001 or AG1478 significantly reduced the activation of ERK produced by both HG and agonist stimulation (Figure 2A). To confirm the involvement of specific MMPs and the EGFR in mediating PE-induced increase in ERK activity, we transfected VSMCs with MMP-2, MMP-7, and EGFR siRNA and studied the effect of HG and PE stimulation. Knockdown of the EGFR by siRNA not only reduced the expression of the EGFR and its activity but also the ERK activation induced by both HG and PE stimulation (Figure 2B). Similarly, knockdown of MMP-2 and MMP-7 by their corresponding siRNA significantly decreased ERK activation (Figure 3).

3.6 Agonist stimulation promotes increased expression of contractile proteins in insulin-resistant VSMCs

We next examined the effects of HG and agonist stimulation on signalling pathways that are involved in VSM contraction and regulatory proteins that influence contraction. The most important proteins involved in smooth muscle contraction are myosin light chain kinase (MLCK), the 20 kDa regulatory light chain of myosin-II (MLC20), myosin phosphatase, the enzyme involved in dephosphorylation of MLC20, and Rho kinase, an enzyme involved in inactivating myosin phosphatase, all of which have been implicated in some way in the development of hypertension.\(^{26–28}\) Incubation of VSMCs in HG significantly increased the expression of MLC II, MLCK, RhoA, ROCK1, and ROCK2. Further, stimulation with PE increased the expression levels of MLC II and MLCK and other regulatory proteins in insulin-resistant VSMCs (Supplementary material online, Figure S5). Pharmacological inhibition of MMPs or the EGFR did not change the expression of MLC II or MLCK (Figure 4A) or other regulatory proteins induced by HG or PE in VSMCs (data not shown). However,
knockdown of MMP-2 (Figure 4B), MMP-7 (data not shown), or the EGFR (Figure 4C) by siRNA significantly reduced both phospho- and total MLC II expression in HG- and PE-treated cells. The decrease in phospho-MLC II levels in these cells is most likely due to the reduced synthesis of MLC II. The expression of MLCK or other regulatory proteins was not affected by knockdown of MMP-2 or the EGFR (data not shown).

3.7 Inhibition of MMP-EGFR pathway reduces serum response factor activation induced by insulin resistance and PE stimulation in VSMCs

Activation of ERK promotes serum response factor (SRF)-dependent expression of early response genes in VSMCs. SRF is known to bind to 2 CC (A/T-rich) GG elements, also referred to as CArG boxes, a DNA sequence within the regulatory regions of smooth muscle genes. Further, it has been reported that nearly all smooth muscle genes contain one or more CArG boxes, underscoring the importance of SRF in regulating gene expression in VSMCs. Increased expression and activation of such a gene product could have downstream effects that may result in hypertension. Exposure of VSMCs to HG alone markedly increased activation of SRF as determined by the increased phosphorylation of Ser-103 (Figure 5). The ribosomal S6 kinase P90RSK, a growth factor-inducible kinase, is known to phosphorylate SRF at Ser-103, resulting in the enhancement of the affinity of SRF to its binding site, the serum response element (SRE), within the c-fos promoter. In parallel with increased phosphorylation of SRF, incubation of VSMCs in HG increased the activation of P90RSK as determined by increased levels of Ser-380 on P90RSK. Further, stimulation with PE increased the levels of p-P90RSK further in VSMCs cultured in HG compared with NG conditions (Figure 5A).

Inhibition of MMPs by GM6001 and the EGFR tyrosine kinase activity by AG1478 reduced the phosphorylation of SRF induced by both HG and PE stimulation. Similarly, MMP and EGFR inhibitors also inhibited the phosphorylation of P90RSK in VSMCs cultured in HG (Figure 5A). Suppression of MMP-2 (Figure 5B) or MMP-7 (data not shown) or the EGFR (Figure 5C) expression by their corresponding siRNAs also significantly reduced the activation of SRF induced by HG and PE stimulation in VSMCs. Further, in agreement with previous studies, we also found that inhibition of ERK1/2 (by U0126, an MEK inhibitor) decreased the phosphorylation of SRF in response to HG or agonist stimulation (data not shown).

3.8 Treatment of insulin-resistant rats with AG1478 decreases the activation of the EGFR, ERK, SRF, and MLC II and prevents the development of hypertension

Feeding rats with a high fructose diet induced insulin resistance as determined by lower insulin sensitivity indices in an OGTT and rendered them hypertensive (Table 1). Fructose feeding did not affect the body weights or plasma glucose in these rats. However, fasted plasma insulin, triglyceride, and cholesterol levels were significantly higher in fructose-fed rats compared with control rats. Treatment with doxycycline or AG1478 did not affect any of these parameters in either control or fructose-fed rats (Table 1). Insulin resistance in these rats was associated with increased levels of phospho-EGFR, phospho-ERK, phospho-P90RSK, phospho-SRF, and phospho- and total MLC II in their superior mesenteric arteries (SMAs) (Figure 6). In addition, both MMP-2 and MMP-7 activities were significantly...
elevated in the SMA of fructose hypertensive rats (FHRs) and were inhibited by doxycycline treatment (data not shown). Treatment of fructose-fed rats with AG1478 normalized the activities of EGFR (Figure 6A), ERK1/2 (Figure 6B), P90RSK (Figure 6C), SRF (Figure 6D), and MLC II (Figure 6E) and prevented the development of hypertension without any improvement in insulin resistance (Table 1).

4. Discussion

We previously have shown that the maintenance of adrenergic vascular tone by the MMP-EGFR pathway requires PI3K activation and mitochondrial ATP synthesis in normal physiological conditions. Because in insulin resistance, VSMCs retain the normal MAPK response to insulin despite defects in PI3K signalling, we reasoned that in such conditions, elevated levels of various vasoactive molecules could promote transactivation of the EGFR via MMPs and contribute to increased expression of contractile proteins and perhaps high blood pressure. In support of this idea, we found increased expression and activity of contractile proteins and their transcriptional activators in insulin-resistant VSMCs that were normalized by blockade of the MMP-EGFR pathway. Moreover, inhibiting EGFR activity prevented the development of hypertension as well as normalized activities of ERK, P90RSK, SRF, and MLC II in SMA in insulin-resistant rats.

Induction of insulin resistance by HG significantly increased the expression of all major MMPs (MMP-2, MMP-9, and MMP-7) in VSMCs. Our data are consistent with previous studies that have reported increased expression and activity of MMPs in conditions of insulin resistance, diabetes, and hypertension. The exact nature of signals that contribute to altered activity and expression of MMPs is unclear but may include growth factors, cytokines, and neurohormones. MMP activity is regulated at both transcriptional and post-transcriptional levels by mechanisms such as cleavage of pro-peptide and regulation by tissue inhibitors of MMPs. Hyperglycaemia per se can increase the expression and activity of MMPs by mechanisms such as oxidative stress and advanced glycation end products formation. Further, MMP promoters are known to host response elements to transcriptional activators such as activator protein-1 and NF-κB that are upregulated by hyperglycaemia. Regardless of the mechanisms involved in MMP activation, HG-exposed VSMCs were also insulin resistant as determined by increased and decreased phosphorylation of Ser-307 and Tyr-989, respectively, in response to insulin. Since hyperglycaemia is closely associated with insulin,

Figure 4 Effect of MMP and EGFR inhibition on PE-induced expression of MLCK and MLC II in VSMCs. (A) Representative western blots showing the expression of MLC II and MLCK in VSMCs. Representative western blots and quantitative analysis of MLC II expression in VSMCs transfected with (B) MMP-2 siRNA or (C) the EGFR siRNA and cultured in NG (open bars) or HG (closed bars). $P < 0.05$ vs. all groups, *$P < 0.05$ vs. their respective control groups and all MMP or EGFR siRNA treated groups, and #*$P < 0.05$ vs. all MOCK siRNA-treated groups. $n = 4$ independent experiments.
resistance, our cell culture system may mimic the conditions of diabetes wherein insulin resistance and hyperglycaemia co-exist and provides a model to examine the role of MMP-EGFR pathway in the aetiology of hypertension via increased synthesis or activity of contractile proteins.

VSMCs cultured in HG showed increased expression of various proteins involved in vascular contraction such as the smooth muscle-specific MLCK, MLC II, Rho A, ROCK1, and ROCK2. PE stimulation further increased the expression of these proteins in HG conditions. Our data are consistent with previous studies that have reported increased expression of MLCK in VSMCs isolated from the arteries of spontaneously hypertensive rats (SHRs) or normal Wistar–Kyoto rats stimulated with Ang II. Inhibition of MEK, the upstream activator of ERK1/2 by U0126, has been shown to reduce ERK1/2 activation and normalize elevated blood pressure in SHRs. In the present study, we observed a significant increase in ERK activity in response to both HG and agonist stimulation. Pharmacological or genetic inhibition of the MMPs or the EGFR prevented agonist-induced ERK activity, suggesting a role for MMP-EGFR pathway in the regulation of ERK activity in insulin-resistant VSMCs. We next investigated the mechanisms by which ERK, in response to MMP-EGFR activation, contributes to the synthesis of contractile proteins in VSMCs.

Figure 5 Effect of MMP and the EGFR inhibition on PE-induced activation of P90RSK and SRF in VSMCs. (A) Representative western blots showing the expression of phospho-P90RSK and phospho-SRF in VSMCs cultured in NG (5.5 mmol/L) or HG (25 mmol/L). Representative western blots and quantitative analysis of SRF activity as measured by the ratio of phospho-SRF to total SRF expression in VSMCs transfected with (B) MMP-2 siRNA or (C) the EGFR siRNA and cultured in NG (open bars) or HG (closed bars). $P < 0.05$ vs. all groups, *$P < 0.05$ vs. their respective control groups and all MMP/EGFR siRNA-treated groups, **$P < 0.05$ vs. all MOCK siRNA-treated HG and HG + PE groups, and ***$P < 0.05$ vs. all MOCK siRNA-treated groups. $n = 4$ independent experiments.

sought to investigate the role of ERK pathway in the expression of contractile proteins in response to HG and agonist stimulation.

A number of studies have previously reported increased ERK activity in response to hyperglycaemia and agonist stimulation of ras and receptor tyrosine kinases. Inhibition of MEK, the upstream activator of ERK1/2 by U0126, has been shown to reduce ERK1/2 activation and normalize elevated blood pressure in SHRs. In the present study, we observed a significant increase in ERK activity in response to both HG and agonist stimulation. Pharmacological or genetic inhibition of the MMPs or the EGFR prevented agonist-induced ERK activity, suggesting a role for MMP-EGFR pathway in the regulation of ERK activity in insulin-resistant VSMCs. We next investigated the mechanisms by which ERK, in response to MMP-EGFR activation, contributes to the synthesis of contractile proteins in VSMCs.

Agonist stimulation of ERK is known to activate the expression of immediate early genes that are linked to cellular growth and muscle differentiation. Such genes are characterized by the presence of SREs in their promoters, which bind to SRF and recruit ternary
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All values are expressed as mean ± SEM. C, control; F, fructose-fed; CD and FD, control and fructose treated with doxycycline; CA and FA, control and fructose treated with AG1478.

*Different from C, CD, and CA groups.
#Different from all groups (P < 0.05).

Complex factors such as Elk-1, 48 Elk-1 is phosphorylated by ERK and recruited to the c-fos SRE. 49 However, before recruitment, these accessory factors require prior assembly with SRF to form a binary complex. SRF is a 508-amino-acid-long protein, consisting of a central core that contains the DNA-binding domain, a C-terminal transcriptional activation domain, and an N-terminal domain that can be phosphorylated by ribosomal S6 kinase. 29 The P90RSK, a growth factor-inducible kinase, phosphorylates SRF at Ser-103 and significantly enhances the affinity and rate with which SRF associates with its binding site, the SRE, within the c-fos promoter. 50 In the present study, we found that exposure of VSMCs to HG or agonist stimulation significantly increased the activity of SRF and P90RSK. Further, inhibition of MMP-EGFR activity decreased the phosphorylation of SRF and P90RSK, suggesting that MMPs may regulate SRF and P90RSK activities via the ERK downstream of the EGFR in insulin-resistant VSMCs.

Although HG and GPCR agonists increased the expression of various contractile and regulatory proteins, inhibition of the MMP-EGFR pathway only decreased the expression of MLC II. The preferential inhibition of MLC II by MMP-EGFR pathway suggests that this pathway is specific to the synthesis of MLC II induced by HG and agonist stimulation. However, further studies are required to investigate this observation. Taken together, all of these data suggest that the MMP-EGFR pathway mediates the synthesis of MLC II in response to HG and agonist stimulation by regulating ERK and downstream transcriptional regulators such as SRF and P90RSK.

To examine the expression of contractile proteins in vivo in insulin-resistant conditions, we used FHRs, a model of diet-induced systolic hypertension. Previous studies from our laboratory have reported elevated levels of various GPCR agonists in FHRs including Ang II, 50 ET-1, 51 and TXA 2, 52 all of which are known to transactivate the EGFR. 10, 20 In the present study, we found increased activities of SRF, P90RSK, and MLC II in addition to increased MMP, EGFR, and ERK activities in SMAs. Treatment of FHRs with AG1478 not only prevented the development of hypertension but also attenuated the elevated activities of the EGFR, ERK, SRF, and MLC II. Treatment of fructose-fed rats with AG1478 or doxycycline significantly prevented the development of hypertension but not insulin resistance.

Our in vivo data suggest that the MMP-EGFR pathway may not be involved in the development of insulin resistance. However, this is in contrast to a study by DeLano et al. 12 that showed improvement in insulin resistance in SHR treated with doxycycline. This discrepancy could be due to the differences in dosage of doxycycline used (5.4 vs. 20 mg/kg/day), the subtype of MMP enzyme involved (MMP-9 vs. MMP-2/7) and/or the animal model used (FHR vs. SHR). The inherent differences between these two models, particularly in the aetiology of insulin resistance, could explain the differential effects of MMP inhibition with doxycycline on insulin resistance. 53 Similarly, Arellano-Plancarte et al. 54 demonstrated that inhibition of EGFR tyrosine kinase by AG1478 improved insulin sensitivity in vitro experiments using hepatic cells. These authors demonstrated that Ang II specifically inhibited insulin-induced Akt phosphorylation (Thr-308) and this was associated with increased IRS-1 phosphorylation of Ser-636/Ser-639 that was blocked by AG1478. Although we did not study the effect of MMP-EGFR inhibitors on insulin resistance in vitro, it is likely that observed differences between these two studies are due to the differences in the cell type studied (hepatic cells vs. whole-body insulin resistance) and also the model system used, in vitro vs. in vivo.
The association between increased expression of contractile proteins, hypertrophy, and vascular remodelling and the development of hypertension is unclear. Treatment of SHRs with either an ERK inhibitor or a selective inhibitor of MLCK resulted in a decrease in blood pressure associated with regression of hypertrophy. On the other hand, despite the increased expression of MLC II, hypertensive FHRs do not appear to exhibit vascular remodelling. It is possible that increased expression and phosphorylation of MLC II detected in the present study contributes to the development of hypertension in FHRs by enhancing vasoconstriction. It is also likely that increased expression of MLC II precedes the development of hypertension and the presence of chronically elevated blood pressure may then predispose arteries to abnormal vascular remodelling. However, further studies involving long-term fructose feeding may be required to understand the sequence of events involved.

Our data suggest that vasoactive GPCR stimulation in the presence of insulin resistance or hyperglycaemia increases the expression of MLC II by mechanisms involving MMP-dependent transactivation of the EGFR. These results provide new insights into the mechanisms by which GPCR agonists regulate the expression of MLC II by involving MMP-EGFR pathways, and thereby influence the haemodynamic outcomes in insulin resistance and possibly in type 2 diabetes. Although it is unclear whether hypertension is the cause or consequence of increased MMP and EGFR activities, the presence of elevated levels of GPCR agonists in both clinical and animal models of hypertension activates this pathway to form a vicious cycle involving enhanced vascular tone and hypertrophic growth of cardiovascular tissues. Because it is virtually impossible to reduce the levels of GPCR agonists, an alternative way to break this cycle is to inhibit MMP-EGFR pathway, a potential target for the treatment of hypertension.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Figure 6 In vivo effects of AG1478 on the activities of EGFR, ERK, P90RSK, and SRF and MLC II expression in the SMAs from control (C), fructose (F), control treated (CA) and fructose treated (FA) with AG1478. Representative western blots and quantitative analysis of (A) phospho-EGFR, (B) phospho-ERK1, (C) phospho-P90RSK, (D) phospho-SRF, and (E) MLC II expression. *P < 0.05 vs. all groups. All values are expressed as mean ± SEM (n = 8).