Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A_2 in endothelial cells via p42/p44 mitogen-activated protein kinase

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Received 4 November 1997

Abstract Vascular endothelial growth factor (VEGF) stimulated a time- and concentration-dependent increase in PGI_2 synthesis in human umbilical vein endothelial cells with a mean maximum increase of 2-fold above basal levels at 25 ng/ml after 60 min. VEGF also rapidly stimulated the release of arachidonic acid and phosphorylation and activation of cytosolic phospholipase A_2 ($cPLA_2$). The VEGF-related factor, placenta growth factor (PlGF), had little effect on PGI_2 synthesis, arachidonic acid release or $cPLA_2$ activation. PD98059, a selective inhibitor of MAP kinase kinase, caused complete inhibition of VEGF-stimulated MAP kinase activity, PGI_2 synthesis and $cPLA_2$ gel retardation, but had no effect on VEGF-induced vWF secretion. These findings provide the first evidence that VEGF can stimulate PGI_2 synthesis via $cPLA_2$-mediated arachidonic acid release and indicate that VEGF stimulation of this biosynthetic pathway may occur, at least in part, via activation of p42/p44 MAP kinases.

Key words: Thrombin; Signal transduction; Endothelium; Arachidonic acid; Von Willebrand factor

1. Introduction

The endothelium of the arterial wall releases a variety of factors which play essential roles in the control of vascular tone and reactivity [1,2]. One of these factors, the prostanoid PGI_2, is an important regulator of vascular functions including vasodilatation, platelet aggregation, vascular permeability and vascular smooth muscle cell (VSMC) proliferation [3,4]. PGI_2 production is stimulated in cultured endothelial cells by a variety of agonists acting through receptors with seven transmembrane domains coupled to G-proteins, including thrombin, histamine, ATP and bradykinin [5–7]. In contrast to the well-established effects of agonists for G-protein coupled receptors, there have been few reports of endothelial PGI_2 production in response either to cytokines or polypeptide growth factors for receptor protein tyrosine kinases.

A major pathway leading to PGI_2 synthesis results from agonist-induced activation of the cytosolic form of PLA_2 ($cPLA_2$), the release of arachidonic acid from membrane phospholipids and its conversion into PGI_2 via cyclooxygenase and PGI synthase [8–10]. Recent evidence shows that thrombin-stimulated activation of $cPLA_2$ and PGI_2 synthesis involves phosphorylation by members of the mitogen-activated protein (MAP) kinase family, including p42/p44 Erks [11–13] and p38 MAP kinase [14–16].

The 46 kDa secreted polypeptide factor, vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis in vivo [17]. VEGF expression is upregulated by hypoxia and by cytokines and growth factors in arterial vascular smooth muscle cells (VSMC) [18,19] and recent findings suggest that VEGF may have a role in maintaining normal vascular function and inhibiting VSMC proliferation in vivo [20–25]. In particular, VEGF gene transfer inhibits intimal VSMC proliferation in models of arterial neo-intima formation [22–25] but the mechanism(s) involved in this effect are not clearly understood. The biological effects of VEGF are mediated by two protein tyrosine kinase receptors, the *fms*-like tyrosine kinase, Flt-1, and KDR/Flk-1 [26,27]. VEGF exhibits high-affinity binding to both receptors, and the closely related factor, placental growth factor (PlGF), is a specific ligand for Flt-1 and does not bind to KDR/Flk-1 [28].

In the present paper we show that VEGF induces PGI_2 generation via activation of $cPLA_2$. Further, we present evidence that PGI_2 production induced by VEGF is mediated by the MAP kinase pathway. These findings suggest one important mechanism by which vascular protective actions of this factor may be mediated.

2. Materials and methods

2.1. Cell culture

HUVECs were obtained from fresh umbilical cords by collagenase digestion and cultured on 1% gelatin-coated plates in medium 199 (M199) supplemented with 20% fetal calf serum and endothelial cell growth supplement [29]. Cells were used after 6–8 days or when the cells had formed a confluent monolayer.

2.2. PGI_2 assay

HUVECs were washed twice in serum-free M199 (pH 7.4) and after treatments the PGI_2 content of cell supernatants was quantified by radioimmunoassay of 6-keto $PGF_{1\alpha}$, the stable breakdown product of PGI_2 as described [30].

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cells; IgG, immunoglobulin G; MAP kinase, mitogen-activated protein kinase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PGI_2, prostacyclin; $cPLA_2$, cytosolic phospholipase A_2; PlGF, placenta growth factor; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; VSMC, vascular smooth muscle cells

PII S0014-5793(97)01481-6

2.3. Arachidonic acid release

Arachidonic acid release was determined essentially as described [31]. Confluent HUVEC cultures were incubated for 24 h with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (1 mCi/ml, 211 Ci/mmol). The cells were then washed twice with M199 and incubated in 1 ml of this medium supplemented with 0.3% BSA (essential fatty acid free) and other additions as indicated. The medium was then removed, centrifuged at 16000×*g* for 5 min, and the radioactivity in the supernatant was determined by counting in a scintillation counter.

2.4. Western blotting procedures

Assays of MAP kinase activity and $cPLA_2$ activation were performed by Western blotting of whole cell extracts using, respectively, an antibody which specifically recognizes p42 and p44 MAP kinases (Erk-1 and Erk-2) activated by phosphorylation at Tyr204 [32] and a polyclonal antiserum to $cPLA_2$ [15,16]. Immunoreactive bands were visualized by chemiluminescence using HRP-conjugated anti-mouse or anti-rabbit IgG and ECL reagent.

2.5. Assay of vWF secretion

vWF secretion was measured by ELISA as described [30] in samples of medium obtained from confluent cultures of HUVECs which had been treated with factors, as indicated. Plates were coated with the anti-vWF mAb CLBRAg35, and the lower detection limit of the assay was approximately 1.0 mU/ml.

2.6. Materials

Recombinant VEGF was obtained from R&D Systems. Recombinant PIGF was the gift of Professor Werner Risau and was also obtained from R&D Systems. Polyclonal $cPLA_2$ antiserum was the gift of Dr. Ruth Kramer (Eli Lilly, Indianapolis, IN). The anti-vWF mAb CLBRAg35 was the gift of Dr. J.A. Van Mourik (Central Blood Laboratory, Amsterdam, The Netherlands). Antibody to the activated phosphorylated form of p42/p44 MAP kinase was purchased from New England Biolabs Inc. [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, ECL reagents and HRP-conjugated anti-mouse IgG were from Amersham, UK. Goat anti-rabbit HRP-conjugated IgG was from Pierce Inc. Other reagents used were of the purest grade available.

3. Results

Confluent cultures of HUVEC were treated for various times up to 2 h with VEGF and the medium was removed and assayed for 6-keto $PGF_{1\alpha}$, a stable metabolic breakdown product of PGI_2. As shown in Fig. 1A VEGF caused a time-dependent increase in the production of PGI_2 which was detectable as early as 15 min after addition of the factor, continued to increase for up to 60 min, and was sustained for up to 2 h (Fig. 1A). VEGF stimulation of PGI_2 production was also concentration-dependent. After a 60 min incubation, an increase in PGI_2 synthesis was detectable at 5 ng/ml, was half-maximal at 10 ng/ml and reached a maximum at 15 ng/ml. It was consistently noted that VEGF-induced PGI_2 synthesis underwent a small decline at 25 ng/ml (Fig. 1B).

The effect of VEGF was weaker than that of thrombin, a potent inducer of PGI_2 synthesis and PIGF, a specific ligand for the Flt-1 receptor [28], induced a much weaker stimulation of PGI_2 synthesis compared to VEGF. The mean fold increases in 6-keto $PGF_{1\alpha}$ produced by 60 min incubations with 25 ng/ml VEGF, 60 ng/ml PlGF and 1 U/ml thrombin were, respectively, 2.0-, 1.3- and 4.4-fold above the mean control unstimulated level.

To examine the effect of VEGF on the mobilization of arachidonic acid, HUVECs were preincubated with radiolabelled arachidonic acid for 24 h and challenged with VEGF for different times. Arachidonic acid release experiments were

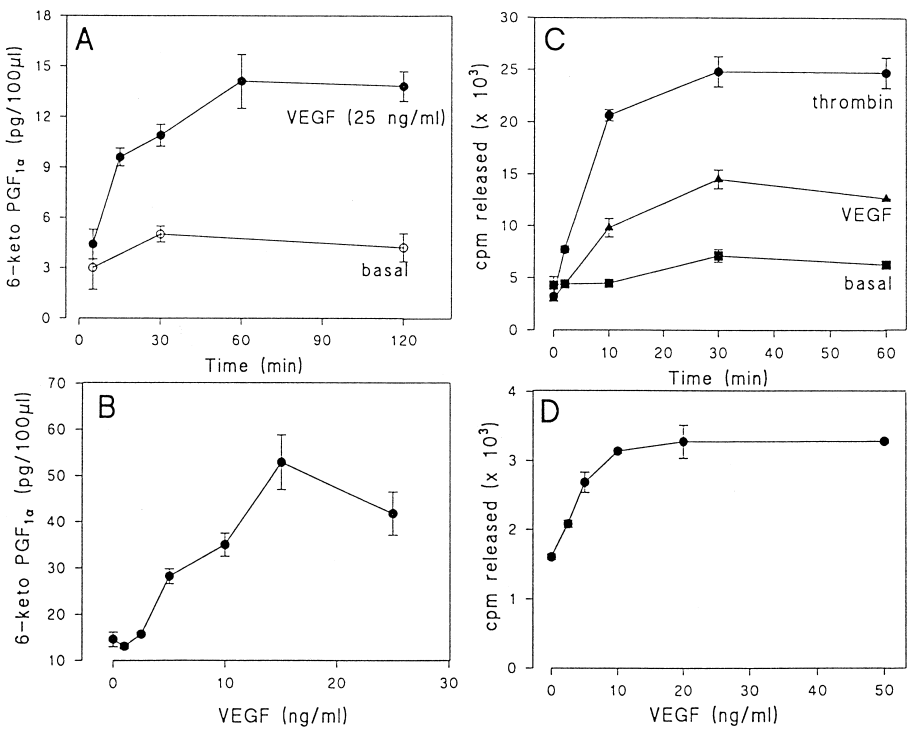

Fig. 1. VEGF-induced PGI_2 synthesis and arachidonic acid release in HUVEC. A and B: HUVECs were incubated in the absence (open circles) or presence (closed circles) of 25 ng/ml VEGF for the times indicated (A) or in the presence of different concentrations of VEGF for 60 min (B). 6-keto $PGF_{1\alpha}$ was assayed as described. The results shown are representative of three experiments. Values are presented as the mean ± S.E.M. of 3–4 determinations. C and D: Cells prelabelled with [^{3}H]arachidonic acid were incubated for different times (C) with 1.0 U/ml thrombin (closed circles) or 25 ng/ml VEGF (closed triangles) or without addition. The concentration-dependence for VEGF (60 min incubation) is shown in D. The results shown are representative of three experiments and values are presented as the mean ± S.E.M. of triplicate determinations.

conducted in the presence of 0.3% BSA which binds avidly to the free fatty acid and acts as a extracellular 'trap' for arachidonic acid. As shown in Fig. 1C, VEGF caused an increase in label released into the medium of prelabelled cells which was evident at 10 min and reached a maximum at 30 min, after addition of the factor. As measured in parallel cultures, the time-course for VEGF-stimulated arachidonic acid release was very similar to that for thrombin (Fig. 1C). The concentration-dependence for VEGF-stimulated arachidonic acid release was very similar to that obtained for PGI_2 production, with a half-maximal effect at 2.5–5 ng/ml and a maximum at 10–20 ng/ml (Fig. 1D). Similar to the relative abilities of thrombin and VEGF to stimulate PGI_2 production, maximum VEGF-induced arachidonic acid release was consistently lower than that obtained for thrombin. In four experiments, VEGF and thrombin caused mean increases in the release of labelled arachidonic acid of 1.6- and 3.4-fold above the basal unstimulated level, respectively. PlGF caused no detectable increase in arachidonic acid release.

The conversion of $cPLA_2$ to its activated form can be monitored by a shift in its mobility in SDS-PAGE gels from a fast- to a slow-migrating form [15,16]. In Western blots of extracts of control HUVECs, antibody to $cPLA_2$ recognized two distinct bands of approximately equal intensity and migrating with approximate M_r 97 000 (Fig. 3). Though $cPLA_2$ has a predicted molecular weight of 85 kDa, this protein has previously been reported to migrate in SDS-PAGE as a 97 kDa band [15,16]. VEGF at 25 ng/ml caused a marked increase in the immunoreactivity of the slow-migrating form of $cPLA_2$ and a concomitant relative decrease in the faster-migrating form, which was detectable after 2 min, reached a maximum after 15 min, and was sustained for up to 60 min after VEGF addition (Fig. 2, upper panel). In five independent experiments, VEGF consistently caused a marked increase in the immunoreactivity of the slower-migrating form of $cPLA_2$. The VEGF-induced decrease in the electrophoretic mobility of $cPLA_2$ was also concentration-dependent with a noticeable increase in the slow-migrating form at 2.5 ng/ml and a maximum increase at 5–10 ng/ml, the highest concentration tested (Fig. 2, middle panel). Thrombin also caused a striking shift in the mobility of $cPLA_2$ from a faster- to a slower-migrating form and, similar to the results obtained for PGI_2 synthesis and arachidonic acid release, the effect of thrombin was greater than that of VEGF with virtually complete disappearance of the faster-migrating form of the enzyme (Fig. 2, middle panel). PlGF caused no detectable shift in the mobility of immunoreactive $cPLA_2$ from faster to slower-migrating forms (results not shown).

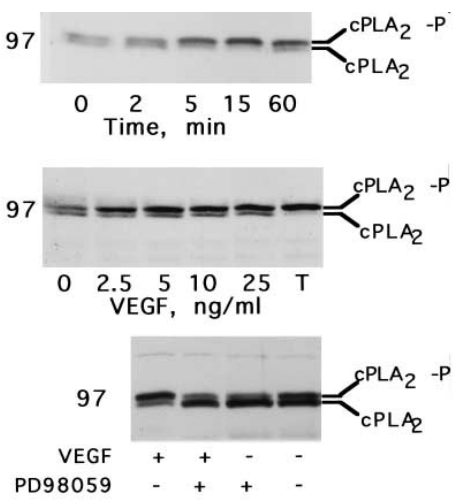

Fig. 2. Effect of VEGF treatment on mobility of $cPLA_2$ in SDS-PAGE. HUVECs were incubated in the presence of 25 ng/ml VEGF for the times indicated (upper) or in the presence of the indicated concentrations of VEGF for 60 min, or with 1.0 U/ml thrombin (T) for 20 min (centre). In other experiments (bottom) cells were pretreated as indicated for 30 min with 25 μM PD98059 and then incubated with 25 ng/ml VEGF for 15 min as indicated. Whole-cell lysates were Western blotted using an anti-$cPLA_2$ antiserum. The positions of molecular weight markers ($M_r \times 10^{-3}$) and of phosphorylated ($cPLA_2$-P) and faster-migrating ($cPLA_2$) forms of $cPLA_2$ are indicated. The results shown are representative of 2–3 experiments.

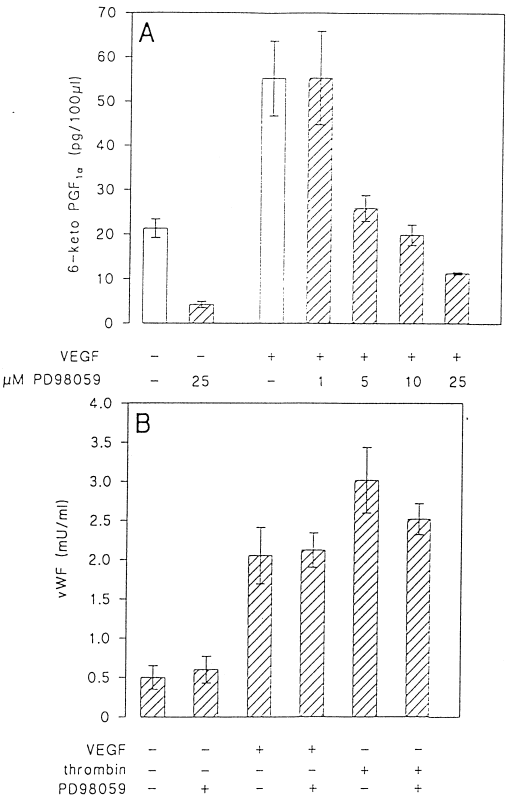

Fig. 3. Effects of PD98059 on PGI_2 synthesis and vWF secretion induced by VEGF and thrombin. HUVEC were pre-treated for 30 min with the indicated concentrations of PD98059 (A) or in the absence (−) or presence (+) of 25 μM PD98059 (B) and subsequently incubated as indicated with 25 ng/ml VEGF or 1.0 U/ml thrombin for 60 min. Assays for 6-keto $PGF_{1\alpha}$ content (A) or vWF content (B) were as described. The results shown are representative of three similar experiments. Data are given as mean ± S.E.M. of triplicate determinations.

It was next investigated whether VEGF-induced PGI_2 synthesis and $cPLA_2$ activation were mediated via activation of p42/p44 MAP kinases. We have previously reported that VEGF induces MAP kinase activation in HUVECs [29]. VEGF induced a detectable increase in activity of p42/p44 MAP kinases in confluent HUVEC at a concentration as low as 0.5 ng/ml, a half-maximal increase between 1 and 5 ng/ml, and a maximum effect at 10 ng/ml (results not shown). PlGF in the concentration range 1–60 ng/ml caused no significant increase in MAP kinase activity in HUVEC (results

not shown). In accord with our previous findings [29], VEGF stimulation of p42/p44 MAP kinases was completely inhibited by a 30 min pretreatment with 10 μM PD98059, a selective inhibitor of MAP kinase kinase [33], the dual-specificity threonine and tyrosine kinase which specifically phosphorylates and activates p42/p44 MAP kinases. As shown in Fig. 2, pretreatment with PD98059 at 25 μM completely blocked the VEGF-stimulated increase in the slow-migrating form of $cPLA_2$. It was noted that the inhibitor not only reversed the VEGF-dependent decrease in $cPLA_2$ gel mobility, but also decreased the immunoreactivity of the slower-migrating form below, and increased that of the faster-migrating form above control levels.

Pretreatment of HUVEC with 30 μM PD98059 for 30 min completely inhibited PGI_2 production induced by a subsequent 60 min incubation with either 25 ng/ml VEGF or 1 U/ml thrombin (Fig. 3A). The effect of PD98059 on VEGF-induced PGI_2 synthesis was concentration-dependent with a half-maximal effect at approximately 5 μM and a maximum inhibitory effect on stimulated PGI_2 production at 10 μM (Fig. 3A). This correlated closely with the concentration-dependence for the effect of the inhibitor on VEGF-stimulated MAP kinase activity (results not shown). It was also noted that PD98059 reduced PGI_2 production in VEGF-treated cells to below the values measured in control cells, though this effect was only apparent at concentrations of the inhibitor greater than 10 μM (Fig. 3A). VEGF induced a striking increase in vWF secretion (Fig. 3B) which was concentration- and time-dependent (results not shown). In contrast to its effect on $cPLA_2$ activation and PGI_2 synthesis, pretreatment of HUVECs with the inhibitor at 25 μM had no effect on vWF secretion caused by a subsequent addition of either VEGF or thrombin (Fig. 3B).

4. Discussion

The results presented here show that VEGF stimulates a striking time- and concentration-dependent increase in PGI_2 production in HUVEC. We also show for the first time that VEGF stimulates the rapid mobilization of arachidonic acid from human endothelial cells and, as judged by a gel retardation assay, the rapid activation of $cPLA_2$. The concentration-dependencies for VEGF-induced PGI_2 production, arachidonic acid release and $cPLA_2$ activation were similar to each other (all within the range 5–10 ng/ml) and are also similar to the concentration-dependence reported for the effects of VEGF on mitogenesis, chemotaxis and other early signalling events in HUVECs [29,34]. These results indicate that VEGF stimulation of arachidonic acid release and PGI_2 synthesis are mediated through high-affinity receptors for this factor in HUVECs. Since PlGF induced PGI_2 synthesis more weakly than VEGF and also failed to significantly increase either arachidonic acid release or $cPLA_2$ mobility shift, these results are most consistent with the conclusion that stimulation of PGI_2 production by VEGF in HUVECs is mediated primarily by KDR/Flk-1 receptors.

Recent findings have implicated the p42/p44 MAP kinases in the phosphorylation and activation of $cPLA_2$. The finding that VEGF causes a rapid shift in the electrophoretic mobility of $cPLA_2$ is most consistent with increased phosphorylation, and hence activation, of the enzyme. This inference is supported by the finding that thrombin, which is known to stimulate $cPLA_2$ phosphorylation and activation in platelets [15,16], caused a similar shift in $cPLA_2$ mobility in HUVECs. The results presented here also show that VEGF activates p42/p44 MAP kinases and that inhibition of this pathway with a selective inhibitor of MAP kinase kinase, PD98059, blocks PGI_2 production and the increased gel retardation of $cPLA_2$ induced by VEGF. PD98059 was previously reported to block thrombin-induced PGI_2 production in HUVECs [13]. Though it is impossible to preclude non-specific effects of the inhibitor, the effect of PD98059 was at least partially selective since it had no effect on vWF secretion stimulated either by VEGF or by thrombin. Furthermore, the inhibitory effect of PD98059 on PGI_2 synthesis could be reversed by addition of exogenous arachidonic acid to the cells (results not shown).

Compared to the effect of the potent inducer of p38 kinase activator, anisomycin, VEGF did not significantly increase p38 kinase activity in HUVECs (results not shown) suggesting that p38 kinase is unlikely to play a major role in VEGF-induced $cPLA_2$ activation. It is plausible that other signalling mechanisms may contribute to VEGF stimulation of PGI_2 synthesis and arachidonic acid release, including elevation of intracellular Ca^{2+}. $cPLA_2$ is sensitive to physiological Ca^{2+} concentrations [8] and VEGF is able to induce the mobilization of Ca^{2+} from intracellular stores [26,27,35]. Since the Ca^{2+} ionophore ionomycin can stimulate PGI_2 synthesis in HUVECs without activating the MAP kinase pathway (C.J.W. Wheeler-Jones et al., unpublished results), any Ca^{2+}-dependent effect of VEGF is likely to occur independently of the MAP kinase-dependent pathway. The finding that a MAP kinase kinase inhibitor blocks VEGF-stimulated PGI_2 synthesis but does not affect vWF secretion suggests that these two biological responses to VEGF are triggered by distinct intracellular signalling pathways. The precise relationship between Ca^{2+} fluxes, MAP kinase activation and PGI_2 synthesis in the action of VEGF requires more detailed delineation.

The mechanisms involved in VEGF-dependent inhibition of intimal VSMC hyperplasia are unclear but have been proposed to be primarily due to the stimulation of endothelial re-growth. Given that prostanoid-stimulated elevation of intracellular cyclic AMP is a potent inhibitor of VSMC mitogenesis [2], it is attractive to hypothesize that VEGF stimulation of PGI_2 has other biological effects in the vascular system, including vasodilatation, increased vascular permeability and the inhibition of platelet aggregation [4–6], and it is entirely possible that VEGF-induced PGI_2 production may have implications for the regulation of other aspects of vascular reactivity. For example, the reported effects of VEGF on vasodilatation of blood vessels [21,22] may be mediated in part by PGI_2.

The finding that VEGF stimulates PGI_2 production, arachidonic acid mobilization and $cPLA_2$ activation probably through a MAP kinase-dependent pathway identifies both a novel function and a new mechanism of action for VEGF in endothelial cells. Activation of this pathway by VEGF is consistent with a role for this factor in the protection of the intact vessel wall. The ability of VEGF both to augment endothelial cell responses and to promote endothelial regeneration makes this factor ideally suited for a role in the maintenance and renewal of normal blood vessel functions.

Acknowledgements: This work was supported by the British Heart Foundation and by European Community Biomed II Grant

PL950329. C.W-J., R.C. and I.Z are supported by the British Heart Foundation. J.M. is BHF Professor of Cardiovascular Science.

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