Placenta growth factor expression is regulated by hydrogen peroxide in vascular smooth muscle cells

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Shaw JH, Xiang L, Shah A, Yin W, Lloyd PG. Placenta growth factor expression is regulated by hydrogen peroxide in vascular smooth muscle cells. Am J Physiol Cell Physiol 300: C349–C355, 2011. First published December 1, 2010; doi:10.1152/ajpcell.00374.2010.—When supply arteries become occluded, blood is diverted through preexisting collateral vessels. Shear stress arising from this increase in blood flow provides the initial physiological stimulus for expansion of the collateral circulation, a process termed arteriogenesis. Endothelial cells (EC) respond to increased shear stress by releasing a variety of mediators that can act on underlying smooth muscle cells (SMC). Placenta growth factor (PLGF) is known to mediate certain aspects of arteriogenesis, such as recruitment of monocytes to the vessel wall. Therefore, we tested whether SMC PLGF expression is influenced by mediators released by EC. We used A10 SMC cultured with medium that had been conditioned by EOMA EC for 4 days as a model. We found that EC-conditioned medium is able to upregulate PLGF gene expression in A10 SMC. Further experiments identified hydrogen peroxide (H2O2) as a key mediator of this response. We confirmed the physiological relevance of this mechanism in primary human coronary artery SMCs by demonstrating that exogenous H2O2 specifically upregulates PLGF gene and protein expression. We also demonstrated that the physiological stimulus of shear stress raises endogenous H2O2 levels in media into the range found to increase PLGF expression. In this study, we demonstrate that EC-released H2O2 acts as a positive regulator of PLGF gene and protein expression in vascular SMC. To our knowledge, this is the first study to describe H2O2 as a regulator of PLGF expression and therefore an upstream mediator of PLGF-driven arteriogenesis.

reactive oxygen species; arteriogenesis; angiogenesis; vascular endothelial growth factor

ARTERIOGENESIS, the process of expansion of collateral vessels in response to an arterial occlusion, improves tissue survival by restoring blood flow to the affected area (11). Placenta growth factor (PLGF) is a vascular endothelial growth factor (VEGF)-related protein. PLGF and VEGF-A are structurally similar; however, they have different receptor binding specificities and thus have marked functional differences. In particular, PLGF has been shown to cause arteriogenesis (11, 14), prostacyclin (PGI2) (19), and hydrogen peroxide (H2O2) (8, 10). Although PLGF was first identified in placental tissue (16), we and others have shown that it is also expressed in adult vasculature (27). Within the vasculature, PLGF is expressed by both endothelial cells (EC) and smooth muscle cells (SMC) (28). As discussed above, the ability of PLGF to induce arteriogenesis is dependent on the recruitment of monocytes to the vascular wall. Since the vascular wall is largely composed of SMC, we hypothesized that SMC PLGF expression may have an important role in arteriogenesis.

As a vessel becomes occluded, blood is diverted through preexisting collaterals. Shear stress arising from this increase in blood flow is the initial physiological stimulus for arteriogenesis. EC respond to increased shear stress by releasing a variety of soluble mediators that could potentially transduce the shear signal to the underlying smooth muscle. Thus we further hypothesized that SMC PLGF expression is influenced by mediators released by EC. This hypothesis is supported by the observation that ligation of the femoral artery (which produces an increase in shear stress through downstream collaterals) can elevate PLGF expression in collateral arteries in the absence of any additional arteriogenic stimulus, such as exercise training (27). The putative shear-dependent regulatory mechanism influencing PLGF in this setting remains undefined. However, mediators known to be produced by EC in response to shear stress include nitric oxide (NO) [which relaxes smooth muscle (20) and functions as a prerequisite for arteriogenesis (11, 14)], prostacyclin (PGI2) (19), and hydrogen peroxide (H2O2) (8, 10).

Although our understanding of arteriogenesis and PLGF has increased over recent years, very little is known about the regulation of PLGF expression, particularly in vascular smooth muscle. To test our hypothesis that EC release mediators that influence SMC PLGF expression, we used A10 SMC cultured with medium that had been conditioned by EOMA EC for 4 days as a model. We found that EC-conditioned medium is able to upregulate PLGF gene expression in A10 SMC. Further experiments identified H2O2 as a key mediator of this response. We confirmed the physiological relevance of this mechanism in primary human coronary artery smooth muscle cells (CASM), in which both PLGF mRNA and protein were
specifically increased by H2O2 treatment. Finally, we showed that the physiological stimulus of shear stress elevates H2O2 in media to levels that can effectively induce PLGF expression.

In this study, we demonstrate that EC-released H2O2 acts as a positive regulator of PLGF gene and protein expression in vascular SMC. To our knowledge, this is the first study to describe H2O2 as a regulator of PLGF expression and therefore an upstream mediator of PLGF-driven arteriogenesis.

**MATERIALS AND METHODS**

**Cell lines.** Vascular SMC (A10) and EC (EOMA) were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in a humidified incubator (5% CO2) in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) containing 5–10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) until confluent. Confluence was defined as 85% coverage. A10 cells were serum starved (1% FBS, DMEM) for 48 h before treatments.

**Primary cells.** Human CASMC were purchased from Lonza (Walkersville, MD). Cells were grown in a humidified incubator containing 5% CO2 in Smooth Muscle Cell Basal Medium (SmBM, Lonza) plus Smooth Muscle Growth Medium (SmGM-2 SingleQuots, Lonza) until confluent. Culture medium was changed every other day. Cells were used for experiments between passages 6 and 13. Confluent A10 cells were serum starved (1% FBS, DMEM) for 48 h before treatments.

**Conditioned medium.** Culture medium was conditioned by allowing standard DMEM culture medium to remain in flasks containing confluent EOMA endothelial cells for 4 days.

**Gene expression.** At the conclusion of experimental treatment, culture medium was aspirated and cells were rinsed briefly in Dulbecco’s phosphate-buffered saline (D-PBS, Invitrogen). Total RNA was then extracted using TRIzol reagent (Invitrogen). RNA was assayed for quality and quantity in a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), treated to remove genomic DNA (Turbo DNAFree, Ambion, Austin, TX), and converted to cDNA (qScript cDNA SuperMix, Quanta BioSciences, Gaithersburg, MD). Real-time PCR was performed on an ABI 7500 Fast instrument for quantification of gene expression. Primers for rat and human PLGF and β-actin were designed using Primer Express and custom synthesized (Invitrogen). Primer sequences: rat PLGF forward 5'-GTGT-3' and reverse 3'-GGAGCTGACGTTCT-5'; human PLGF forward 5'-CTCTCTCCCGCTGTTT-3' and reverse 5'-GATCCGCATAATCTGCATGGT-3'; human VEGF-A forward 5'-ACGGGCTGGAGTTGT-3' and reverse 5'-GATCCGCATAATCTGCATGGT-3'. PCR product formation was detected using SYBR Green chemistry (PerfeCTa SYBR Green FastMix, Low ROX) (Quanta BioSciences). PLGF and VEGF gene expression was normalized to β-actin.

**Glucose assay.** Glucose concentration in culture medium was assayed enzymatically in a spectrophotometer. Ten microliters of culture medium were added to cuvettes containing 610 µl of glucose assay buffer (1 mM MgCl2, 25 mM Tris base, 0.5 mM dithiothreitol, 1.5 mM ATP, 0.3 U/ml glucose 6-phosphate dehydrogenase, 0.25 mM NADP, and 1 U/ml hexokinase; pH 8.1) (all reagents from Sigma, St. Louis, MO). Cuvettes were then incubated at 37°C for 1 h. Absorbance was measured at 340 nm and compared with a standard curve for calculation of glucose concentration.

**Lactate assay.** Media lactate concentration was assayed enzymatically. Twenty microliters of culture media were added to cuvettes containing 980 µl of lactate assay buffer (160 mM hydrazine, 400 mM glycine, 0.625 mM NAD, and 20 U/ml lactate dehydrogenase; pH 9.0) (all reagents from Sigma). Cuvettes were then incubated at 37°C for 1 h. Absorbance was measured at 340 nm and compared with a standard curve for calculation of lactate concentration.

**Exogenous glucose and lactate.** For studies on the effects of exogenous glucose and lactate, glucose-free DMEM (Sigma) was prepared and sterile-filtered. Stock solutions of glucose and lactate in H2O were prepared, syringe filtered, and added to DMEM to give final concentrations of glucose ranging from 0 to 20 mM and final concentrations of lactate ranging from 0 to 10 mM. A10 SMC were then exposed to the prepared media for 4 h, followed by RNA extraction and real-time PCR as described above.

**NO donor treatment.** The NO donor sodium nitroprusside (SNP) (Sigma) was used to assess the effect of NO on PLGF and VEGF-A expression in A10 SMC. A stock solution of SNP (100 mM) was made in PBS and then added to A10 culture vessels containing fresh culture medium to reach the final desired concentration. PBS alone was used as a vehicle control. Cells were incubated with vehicle or SNP for 4 h before total RNA was harvested for real-time PCR as described above.

**Peroxide assay.** Peroxide in culture medium was assayed spectrophotometrically using the Peroxoxquant kit (Pierce, Rockford, IL) according to manufacturer’s instructions.

**Peroxide treatment.** A10 SMC were serum starved for 48 h and then treated with varying doses of H2O2 (1–100 µM) for 4 h. Doses of >100 µM H2O2 were found to be cytotoxic (data not shown). RNA was harvested for real-time PCR as described above to determine whether upregulation of PLGF by H2O2 was dose dependent. A time course was performed on A10 SMC using 50 µM H2O2 (4 and 8 h data presented here). For treatment of primary cells, human CASMC were serum starved for 48 h followed by the addition of 50 µM H2O2 for 4–8 h.

**ELISA.** Medium was collected from culture vessels containing human CASMC following treatment with 50 µM H2O2 for 8 h. To protect the secreted PLGF or VEGF-A protein from degradation during sample processing, protease inhibitor cocktail (Sigma) was added (1:250) at the end of the 8-h H2O2 treatment. Media was then concentrated using molecular weight cutoff filters (Icon, 7 ml/9 K, Pierce). Total protein was quantified using the BCA Protein Assay (Pierce). PLGF and VEGF-A ELISAs was performed on the concentrated media using the Quantikine Human PLGF and VEGF-A ELISA kits (R&D Systems, Minneapolis, MN) to measure levels of the secreted proteins.

**Shear stress.** EOMA cells were grown until confluence in standard six-well culture plates, serum-starved for 48 h, and then exposed to shear stress in a custom-built dynamic cone and plate shearing device (37). The apparatus was designed to shear cells grown in a six-well culture plate. It consists of four high-density polyethylene cones attached to stepper motors. The motors were controlled by an Allegre two-axis programmable stepper motor control system (Optimal Engineering Systems, Los Angeles, CA). Shear stress applied to the cells was controlled by adjusting the speed of the cones. The center wells of the six-well plate were not fitted with cones and served as paired static control samples. Cells were exposed to either constant shear of 5 dyn/cm² or oscillatory shear of ±5 dyn/cm² for 2 h, after which media was collected for analysis of H2O2 levels as described above.

**Statistical analyses.** All data are expressed as means ± SE. A Mann-Whitney rank sum test was used to identify significant differences in gene expression and lactate concentration between control and conditioned-media treated cells and to identify significant effects of beraprost treatment. Differences in glucose concentration between control and conditioned media were analyzed by t-test. The effects of glucose and lactate on PLGF were analyzed by one-way ANOVA. The effect of SNP on PLGF and the effect of shear stress on peroxide levels in EOMA cell media were analyzed by one-way ANOVA on ranks.
Differences in peroxide levels between control and conditioned media were analyzed by t-test as well as differences between A10 and CASMC PLGF and VEGF-A gene expression and CASMC PLGF and VEGF-A protein expression following peroxide treatment. SigmaStat 3.5 (Systat) was used for all statistical analyses. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

Treatment of A10 SMC for 4 h with medium preconditioned for 4 days by EOMA EC strongly upregulated PLGF gene expression to 4.56 \( \pm 0.42 \)-fold of the level in untreated cells (Fig. 1; \( P < 0.001 \)). The increase in PLGF expression was transient and returned to baseline by 24 h (data not shown).

Analysis of the EOMA-conditioned medium showed that glucose was depleted from a starting concentration of 4.61 \( \pm 0.14 \) to 0.79 \( \pm 0.07 \) mM during the conditioning phase. There was a concomitant increase in lactate from undetectable levels to 8.17 \( \pm 0.29 \) mM (Fig. 2A; \( P < 0.01 \) for both). To determine whether glucose depletion contributed to increased PLGF expression following conditioned medium treatment, cultured A10 SMC were exposed to medium containing 0–20 mM glucose for 4 h. Glucose concentration had no significant effect on PLGF mRNA levels over this range (Fig. 2B). Likewise, treatment of A10 SMC with exogenous lactate (0–10 mM) also had no significant effect on PLGF gene expression (Fig. 2C).

Since the effect of EOMA-conditioned medium on PLGF expression in A10 cells suggested that soluble EC-derived mediators could regulate PLGF, we next tested the effect of the endothelial-derived mediators NO and prostaglandin I\(_2\) (PGI\(_2\); prostacyclin) on PLGF expression. A10 SMC were treated with the NO donor sodium nitroprusside (SNP; 100 and 250 \( \mu \)M). SNP treatment did not significantly affect PLGF gene expression in A10 SMC at either concentration tested (Fig. 3A). Interestingly, the prostacyclin analog beraprost (100 nM) significantly inhibited PLGF gene expression (Fig. 3B; \( P < 0.05 \)). Thus neither NO nor prostacyclin appears to mediate the increased PLGF expression seen following treatment of A10 SMC with EOMA-conditioned medium.

Further analysis of EOMA-conditioned medium revealed that \( \text{H}_2\text{O}_2 \) levels were significantly increased following the conditioning phase, relative to fresh medium (Fig. 4, \( P < 0.001 \)). To assess whether \( \text{H}_2\text{O}_2 \) regulates PLGF gene expression, exogenous \( \text{H}_2\text{O}_2 \) (50 \( \mu \)M) was added to culture vessels containing A10 cells. RNA was isolated following 4 and 8 h \( \text{H}_2\text{O}_2 \) treatment for real-time PCR. Peroxide treatment increased PLGF gene expression to 1.53 \( \pm 0.18 \)-fold of control at 4 h and 3.08 \( \pm 0.90 \)-fold of control at 8 h (Fig. 5, \( P < 0.05 \)). The upregulation of PLGF was transient and returned to baseline by 24 h (data not shown).
To determine whether the response of SMC to H$_2$O$_2$ was dose dependent, SMC were treated with exogenous H$_2$O$_2$ at concentrations ranging from 1 to 100 µM. PLGF gene expression was dose dependently upregulated by H$_2$O$_2$ treatment ($R^2=0.78$, $P < 0.01$). Doses of >100 µM H$_2$O$_2$ were found to be cytotoxic (data not shown), consistent with other studies (9).

Since the A10 SMC line is well-established in culture and has a less-differentiated SMC phenotype than primary SMC, we next treated primary CASMC with H$_2$O$_2$ to determine whether this regulatory mechanism is operative in differentiated SMC. Human CASMC were treated with 50 µM exogenous H$_2$O$_2$ for either 4 or 8 h. PLGF gene expression increased to 3.4 ± 0.7-fold of control (at 4 h) and 5.1 ± 0.1-fold of control (at 8 h) in H$_2$O$_2$-treated CASMC, compared with untreated CASMC (Fig. 7A, $P < 0.05$ for both time points). As observed in A10 SMC, PLGF upregulation was transient and returned to baseline by 24 h (data not shown). To determine whether the effect of H$_2$O$_2$ on PLGF gene expression was specific, we also measured VEGF-A mRNA levels. Peroxide increased VEGF-A gene expression to 4.5 ± 1.1-fold of control at 4 h; however, VEGF-A expression had returned to control by 8 h of treatment (1.0 ± 0.4-fold of control), whereas PLGF expression remained elevated at the 8-h time point (Fig. 8A). To determine whether the upregulation of PLGF gene transcription produces a corresponding increase in PLGF protein, we measured PLGF protein levels in culture medium from flasks containing human CASMC that had been treated with 50 µM exogenous H$_2$O$_2$ for 8 h. PLGF protein expression was slightly, but significantly increased by H$_2$O$_2$ treatment, from 91 to 131 pg/ml (Fig. 7B, $P < 0.05$). ELISA confirmed that VEGF-A protein was not significantly increased 8 h post-H$_2$O$_2$ (Fig. 8B).

Finally, to determine whether a physiological stimulus (shear stress) could potentially activate this signaling mechanism, we exposed EOMA endothelial cells to two different shear stress patterns using a cone and plate shearing device.
After 2 h of exposure, both constant shear of 5 dyn/cm² and oscillatory shear of ± 5 dyn/cm² significantly increased peroxide levels in EOMA cell media (static control, 47.9 ± 0.6 μM; constant shear, 65.6 ± 5.6 μM; oscillatory shear, 105.0 ± 16.5 μM, n = 8/group) (Fig. 9, *P < 0.05 vs. static control).

Thus brief exposures to low levels of shear stress increased H₂O₂ production by endothelial cells to levels that our data show are sufficient to elevate PLGF expression in vascular smooth muscle cells.

DISCUSSION

In this report we present the first evidence identifying H₂O₂ as a novel EC-released, positive regulator of PLGF expression in vascular SMCs. Physiological concentrations of H₂O₂ in the vasculature in vivo are estimated to range between 0.1 and 60 μM (13, 26, 31). Our data suggest that H₂O₂, at a level that reflects mild oxidative stress, functions as a physiological signaling molecule that can transduce a shear signal from the endothelium to the underlying smooth muscle to promote arteriogenesis via upregulation of PLGF. Our data show an acute, early response of PLGF expression to H₂O₂. The response was specific, as shown by continued upregulation of PLGF, but not the related protein VEGF-A, at 8 h post-H₂O₂ treatment. PLGF levels were no longer elevated 24 h post-H₂O₂ (data not shown). There are two potential explanations for this lack of sustained response. First, it is possible that H₂O₂ has a transient effect on PLGF levels and that this mechanism contributes to acute regulation of PLGF expression. Transient upregulation of PLGF could set the arteriogenic sequence of events in motion by favoring recruitment of monocytes to the vessel wall, where they can release additional...
arteriogenic mediators. As-yet-unknown negative feedback mechanisms may then act to return PLGF to normal levels in the absence of additional stimulatory inputs. If upregulation of PLGF by H$_2$O$_2$ is transient, sustained reactive oxygen species generation may not enhance PLGF expression.

A second, alternative explanation is that the H$_2$O$_2$ signal may have been lost at time points >8 h. Indeed, analysis of culture media 8 h post-H$_2$O$_2$ treatment showed that H$_2$O$_2$ levels in media had returned to control (data not shown). It is clear that an endothelium-dependent mechanism for sustained upregulation of PLGF in remodeling collateral arteries must exist, since PLGF gene expression remains elevated in collateral arteries downstream of an occlusion for up to 24 days in rats with hindlimb ischemia (27). In this model, an early spike in PLGF was followed by a lower, but nevertheless sustained, increase. Additional experiments with repeated H$_2$O$_2$ administration or H$_2$O$_2$-generating systems are needed to determine whether the upregulation of PLGF in vascular SMC by H$_2$O$_2$ is transient, or whether it can be sustained in the presence of continued low levels of reactive oxygen species.

It also remains to be seen whether pathologically high levels of reactive oxygen species (oxidative stress) stimulate or inhibit PLGF expression. Rocic et al. (29) demonstrated that coronary collateral growth is dependent on optimal levels of reactive oxygen species, with either abnormally low or abnormally high levels causing inhibition of arteriogenesis. We speculate that PLGF expression in vascular SMC existing in an environment of high oxidative stress (e.g., diabetes or hyperlipidemia) may be refractory to further activation of this novel H$_2$O$_2$ signaling pathway, consistent with several studies in animal models linking elevated oxidative stress to impaired arteriogenesis (3, 29). Furthermore, PLGF expression has been shown to be reduced in wounds of streptozotocin-treated diabetic mice (6) and in the placenta of streptozotocin-treated rats (12). In contrast, mounting evidence shows that lower, physiological concentrations of reactive oxygen species contribute to pro-arteriogenic signaling (1, 29, 32, 33).

In these studies we also examined other potential factors that could influence PLGF expression in our culture system and/or in vivo. There was a sharp decrease in glucose and a concomitant increase in lactate in EC-conditioned media. Thus we tested the hypothesis that glucose deprivation or an increase in lactate could mimic the onset of ischemia and induce PLGF expression. However, we found that neither glucose nor lactate (across a wide range of concentrations consistent with those found in conditioned media) had any effect on PLGF expression. Thus we conclude that changes in glucose and lactate levels are not involved in the specific regulatory pathway operating in our model system.

Likewise, and contrary to our initial expectations, neither NO nor prostacyclin appear to be involved in EC-dependent upregulation of PLGF expression in SMC in our experimental model. NO and prostacyclin are key mediators released from the endothelium in response to shear stress that work via the cGMP or cAMP pathways, respectively, to drive relaxation of vascular smooth muscle. NO and prostacyclin also have pro-arteriogenic effects (14, 17). The apparent lack of involvement of NO or prostacyclin in upregulating PLGF supports the specific nature of the signaling pathway activated by H$_2$O$_2$. However, it is important to note that the studies described here were done in cultured vascular cells grown under static conditions. It is possible that NO and prostacyclin may contribute to PLGF regulation in a more physiological setting, such as an intact vessel experiencing flow. Indeed, Mohammed et al. (17) found that cyclic stretch upregulated PLGF expression in bronchial airway epithelial cells via a NO-dependent mechanism. The possibility that other EC-derived mediators such as NO and prostacyclin contribute to the putative PLGF regulatory cascade induced by shear stress is an important area for future research.

Although H$_2$O$_2$ strongly and reproducibly upregulated PLGF gene expression in SMC, PLGF protein expression was only slightly elevated by H$_2$O$_2$ treatment. One possible explanation for this observation is that PLGF protein expression is only partially regulated at the transcriptional level. Posttranscriptional mechanisms such as micro-RNA regulation of PLGF mRNA may also be involved. Thus H$_2$O$_2$ may be necessary, but not entirely sufficient, to fully upregulate PLGF. Further studies are underway to investigate the possibility that PLGF protein expression is subject to posttranscriptional control.

We are only aware of one other study to date that identifies an EC-derived molecule that acts on vascular SMC to increase PLGF expression. Recent work by Pan et al. (21) showed an increase in PLGF expression in vascular SMC following treatment with exogenous angiotensin II via an ERK (1/2) and phosphoinositol-3-kinase-dependent pathway.

Finally, we assessed H$_2$O$_2$ production by EOMA cells exposed to constant and oscillatory shear stress to determine whether this physiological stimulus for arteriogenesis can elevate H$_2$O$_2$ concentrations to the range shown to be effective at upregulating PLGF expression. These experiments confirmed that the concentrations of H$_2$O$_2$ tested in this study are physiological.

Continued unraveling of the details of the molecular mechanism by which H$_2$O$_2$ elicits upregulation of PLGF expression will provide important new insights into the understanding of PLGF-driven arteriogenesis. Such insights may suggest ways in which expression of this important arteriogenic signaling protein can be stimulated or inhibited in patients who could benefit from pro-arteriogenic (ischemic cardiovascular disease) or anti-angiogenic (cancer) therapies.

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