Lactate Engages Receptor Tyrosine Kinases Axl, Tie2, and Vascular Endothelial Growth Factor Receptor 2 to Activate Phosphoinositide 3-Kinase/Akt and Promote Angiogenesis*

Received for publication, April 3, 2013, and in revised form, May 27, 2013. Published, JBC Papers in Press, June 10, 2013, DOI 10.1074/jbc.M113.474619

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Background: The signaling events by which lactate instructs endothelial cells to undergo angiogenesis are incompletely resolved.

Results: Lactate activated Akt in endothelial cells by promoting ligand-dependent activation of multiple RTKs.

Conclusion: Metabolic products such as lactate engage established regulators of angiogenesis.

Significance: Our results advance our appreciation of the interface between metabolism and angiogenesis.

Although a high level of lactate is quintessential to both tumors and wound healing, the manner by which lactate impacts endothelial cells to promote angiogenesis and thereby create or restore vascular perfusion to growing tissues has not been fully elucidated. Here we report that lactate activated the PI3K/Akt pathway in primary human endothelial cells. Furthermore, activating this signaling pathway was required for lactate-stimulated organization of endothelial cells into tubes and for sprouting of vessels from mouse aortic explants. Lactate engaged the PI3K/Akt pathway via ligand-mediated activation of the three receptor tyrosine kinases Axl, Tie2, and VEGF receptor 2. Neutralizing the ligands for these receptor tyrosine kinases, pharmacologically inhibiting their kinase activity or suppressing their expression largely eliminated the ability of cells and explants to respond to lactate. Elucidating the mechanism by which lactate communicates with endothelial cells presents a previously unappreciated opportunity to improve our understanding of the angiogenic program and to govern it.

Angiogenesis, the development of blood vessels from the existing vasculature, is a fundamental process to both malignant tumors and physiological wound healing because it creates or restores vascular perfusion to growing tissues (1). Hypoxic tumor cells primarily use glucose for glycolytic energy production and release lactate (2). Oxygenated tumor cells also tend to convert glucose into lactate, which is known as aerobic glycolysis or the Warburg effect (3). Lactate facilitates tumor angiogenesis and helps establish a new vascular supply to support the fast growth of tumors. For instance, lactate mimics hypoxia by stabilizing hypoxia-inducible factor 1α (HIF1α)² and, thus, up-regulates expression of many proangiogenic genes such as VEGF (4–6). In wound tissues, reduced vascular perfusion generates tissue ischemia and hypoxia, and cells adapt by switching to anaerobic glycolysis, resulting in increased lactate production (7), which promotes angiogenesis to restore vascular perfusion to damaged tissues (1). In normal physiological conditions, the lactate level is ~1.8 mM (8), whereas in tumors and wound tissues, it may reach 10–15 mM (9, 10). A high level of lactate in tumors has been proposed to predict tumor metastases and reduced patient survival (11, 12).

Although the role of hypoxia in angiogenesis has been investigated extensively, the mechanism by which lactate promotes angiogenesis is incompletely understood. Previous studies indicate that lactate increases the level of VEGF in macrophages (13) and endothelial cells (14, 15), suggesting that VEGF is involved in lactate-mediated proangiogenic activity. The observation that lactate prevents prolyl-hydroxylase domain-mediated HIF1α degradation and, thereby, stabilizes HIF1α is a likely explanation for how lactate increases VEGF (4, 6, 16). In addition to elevating VEGF, lactate acts to promote angiogenesis by increasing the level of reactive oxygen species in endothelial cells and, thereby, stimulating NF-κB activity and interleukin 8 production (17). Reactive oxygen species also play an important role in VEGF/VEGF receptor 2 (VEGFR-2) signaling and angiogenic responses in endothelial cells (18, 19).

Receptor tyrosine kinases (RTKs) are cell surface receptors for extracellular agents such as growth factors, hormones, and cytokines. RTKs govern a wide variety of cellular processes, including proliferation, differentiation, survival, migration, metabolism, and cell cycle control (20). There are 58 RTKs in humans, which can be organized into 20 subfamilies (21). A small subset of RTKs contribute to angiogenesis, and these include VEGFR-2, Tie2, and FGF receptor 1 (FGFR1) (22). These angiogenic RTKs are activated by their ligands: VEGF, angiopoietin 1 (Ang1), and FGFRs, respectively, that trigger

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*This work was supported, in whole or in part, by National Institutes of Health Grant EY018344 (to A.K.). This work was also supported by Juvenile Diabetes Research Foundation Advanced Postdoctoral Fellowship 102011367 (to G.X.R.).

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2 The abbreviations used are: HIF1α, hypoxia-inducible factor 1α; VEGF, VEGF receptor 2; RTK, receptor tyrosine kinase; SFK, Src family kinase; HUVEC, human umbilical vein endothelial cell; DMSO, dimethyl sulfoxide.
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autophosphorylation at specific intracellular tyrosine residues and, thereby, engage Src homology 2 (SH2) domain-containing or phosphotyrosine binding domain-containing signaling proteins that initiate subsequent signaling cascades that direct appropriate cellular responses (23).

The PI3K/Akt pathway is activated by many proangiogenic growth factors in endothelial cells, and it regulates critical steps in angiogenesis, including survival, migration, and formation of capillary-like structures often called tubes (24, 25). Enhancing this pathway (by overexpression of PI3K or Akt) promotes angiogenesis, whereas angiogenic responses are suppressed when this pathway is antagonized (e.g. by overexpression of phosphatase and tensin homolog or by expressing dominant negative PI3K mutants) (26). Furthermore, PI3K/Akt is chronically activated in tumor blood vessels, and forced expression of a constitutively active mutant of Akt in endothelial cells leads to formation of hyperpermeable blood vessels in multiple non-tumor tissues that display the structural and functional abnormalities of tumor blood vessels (27). Thus, work from multiple laboratories using a vast array of experimental approaches has led to the prevailing appreciation that the PI3K/Akt pathway is central to angiogenesis. However, activation of PI3K/Akt is not sufficient for angiogenesis (28). Other signaling systems are known to make essential contributions to governing the angiogenic program (29).

The importance of the PI3K/Akt pathway in angiogenesis has motivated efforts to elucidate the mechanism by which angiogenic RTKs engage this pathway. In contrast to the relatively simple sequence of events by which the platelet-derived growth factor receptor activates PI3K/Akt, the emerging picture for angiogenic RTKs is considerably more complex. For instance, instead of directly recruiting PI3K to itself or to a tyrosine-phosphorylated adaptor protein, VEGFR-2 acts via Src family kinases (SFKs) to induce ligand-independent activation of Axl (a ubiquitously expressed RTK that was not widely studied in angiogenesis) (28). Other signaling systems are known to make essential contributions to governing the angiogenic program (29).

EXPERIMENTAL PROCEDURES

Reagents—The Axl inhibitor BGB324/R428 (R428) was kindly provided by Dr. Sacha J. Holland at Rigel Pharmaceuticals, Inc. (South San Francisco, CA). Gas6, Ang1, AxlFc, Tie2Fc, and the human phospho-RTK array kit were purchased from R&D Systems (Minneapolis, MN). Lucentis (Remibizumab) was obtained from Genentech (San Francisco, CA). LY294002 and antibodies against phospho-Akt (Ser-473), Akt, phospho-Erk, Erk, Axl, and Tie2 were purchased from Cell Signaling Technology (Beverly, MA). The Tie2 kinase inhibitor and mouse monoclonal antibody 4G10 were purchased from Millipore (Temecula, CA). The other anti-phosphotyrosine antibody PY20 and antibody against HIF1α were purchased from BD Biosciences. Rabbit polyclonal anti-RasGAP and anti-VEGFR-2 antibodies were produced in the Kazlauskas laboratory as described previously (30, 31). ZM 323881 hydrochloride; antibodies against Gas6 and Ang1; as well as HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent substrate for detection of HRP was purchased from Pierce Protein Research Products (Rockford, IL). All other chemicals and reagents, including lactate, were obtained from Sigma unless indicated otherwise.

Cell Culture—Human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD) were plated on tissue culture dishes that had been precoated with 0.2% gelatin and cultured in medium 199 (Sigma) supplemented with 20% bovine calf serum (HyClone, Logan, UT), 100 μg/ml heparin, 12 μg/ml bovine brain extract (Hammond Cell Tech, Windsor, CA), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Gemini BioProducts, West Sacramento, CA). 293T cells were cultured in high-glucose DMEM (Invitrogen) supplemented with 10% FBS (Lonza), 10 units/ml penicillin G, and 10 μg/ml streptomycin. The medium used to harvest lentiviral supernatant from 293T cells was high-glucose DMEM supplemented with 30% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified 5% CO2 atmosphere.

Lentiviral shRNA Knockdown of Axl, Tie2, and VEGFR-2 in HUVECs—The hairpin-pLKO.1 retroviral vector containing shRNA for Axl, Tie2, VEGFR-2, or GFP; the packaging plasmid (pCMV-ΔR8.91); the envelope plasmid (VSV-G/pMD2.G); and 293T packaging cells were obtained from iLab Solutions (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). The following target sequences were used: Axl, CTTTAGGT-TCTTTGCTGCAATT; Tie2, GCTCTTATACAAACCGGT-TAA; VEGFR-2, CGCTGACATGTACGCTTATG; and GFP, ACAACAGCCACAACGTCTATA. shRNA lentivirus production and infection were performed as described previously (28).

Western Blot Analysis—HUVECs plated in 24-well plates were grown to 80% confluence, serum-starved overnight, and then treated with reagents specified in each figure. The cells were washed twice with ice-cold PBS, and then lysed in sample buffer. The total cell lysates were subjected to SDS-PAGE and then treated with or without lactate (10 mM) for 2 h. For immunoblotting with desired antibodies. The resultant data were quantified densitometrically with Quantity One software (Bio-Rad). To reprobe a blot, the membrane was first stripped by incubating for 30 min at 60 °C in stripping buffer and then reprobed with the desired antibodies. The blotting data shown in this work are representative of at least three independent experiments.

The sample buffer contained 100 mM Tris-HCl (pH 6.8), 5 mM EDTA, 100 mM DTT, 10% glycerol, 2% SDS, 0.5% bromophenol blue, 100 mM 2-mercaptoethanol, 2 mM sodium orthovanadate, and 1 mM PMSF. The stripping buffer contained 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol.

Immunoprecipitation—HUVECs plated on 10-cm culture dishes were grown to 80% confluence, serum-starved for 3 h, pretreated with 100 μM sodium orthovanadate for 30 min, and then treated with or without lactate (10 mM) for 2 h. For immu-
noprecipitation with the anti-Axl or anti-VEGFR-2 antibody, HUVECs were lysed in extraction buffer. The precipitating antibody (anti-Axl or anti-VEGFR-2) was added to the pre-cleared cell lysates, and the mixture was incubated overnight at 4 °C on a rocking platform. Protein A/G PLUS-agarose (Santa Cruz Biotechnology) was then added and incubated at 4 °C for another 80 min. Beads were then sedimented and washed three times with extraction buffer. For immunoprecipitation with the anti-Tie2 antibody, HUVECs were lysed in radioimmune precipitation assay buffer instead of extraction buffer to eliminate non-specific binding proteins. Anti-Tie2 antibody was added to the pre-cleared cell lysates, and the mixture was incubated overnight at 4 °C on a rocking platform. Protein A/G PLUS-agarose (Santa Cruz Biotechnology) was then added and incubated at 4 °C for another 80 min. Beads were then sedimented, washed twice with radioimmune precipitation assay buffer, and once with extraction buffer. Bound proteins were eluted by boiling in sample buffer, separated by SDS-PAGE, and immunoblotted with the indicated antibodies.

The extraction buffer contained 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/ml aprotinin, 2 mM sodium orthovanadate, and 1 mM PMSF. The radioimmune precipitation assay buffer contained 150 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 20 μg/ml aprotinin, 50 mM NaF, 14 mM 2-mercaptoethanol, 2 mM sodium orthovanadate, and 1 mM PMSF.

**Tube Formation/Collagen Gel Sandwich Assay**—Tube formation was monitored using our collagen gel sandwich assay described previously (32) with some modifications. Briefly, a collagen gel mixture consisting of 80% (by volume) bovine collagen pureCol (Advanced Biomatrix, San Diego, CA), 1.6 mg/ml NaOH, 2 mg/ml NaHCO₃, 20 mM HEPES (Lonza), 0.5 μg/ml fibronectin, 0.5 μg/ml laminin, and 10.4 mg/ml RPMI powder (Invitrogen) was prepared. For the lower gel layer, 200 μl of the gel mixture was added to each well of a 48-well plate and incubated at 37 °C for 1 h. After polymerization, 10⁶ HUVECs were seeded in each well and incubated with EB medium (Lonza) containing 2% horse serum (Sigma) and 12 μg/ml bovine brain extract for 24 h at 37 °C. The medium was then removed, and 80 μl of the gel mixture was added to each well. The plate was incubated at 37 °C for 1 h to polymerize the upper gel layer. 500 μl of EB medium containing 12 μg/ml bovine brain extract and the reagents specified in each figure was then added to each well. Tubes were photographed 17 h after treatment with a SPOT camera attached to a Nikon Eclipse TE2000-S inverted microscope. After calibration with a stage micrometer, the total tube length within a field was quantified using ImageJ software (National Institutes of Health).

**Aortic Ring Assay**—An aortic ring assay was performed as described previously (33) with some modifications. In brief, aortas were isolated from 8- to 12-week-old male C57BL/6 mice. All animal use was approved by the Schepens Animal Care and Use Committee and conducted in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals. Aortas were transferred into 5 ml of Opti-MEM (Invitrogen). The protective fatty layer and all accessory vessels were removed carefully.

Blood was flushed out from the lumen with a 30-gauge needle fixed to a 1-ml syringe filled with Opti-MEM, and the aortas were cross-sectioned into ~0.5-mm-long rings. After overnight serum starvation in Opti-MEM, the rings were embedded into a collagen gel mixture containing 1 volume of 10× minimum Eagle’s medium (Invitrogen), 1.5 volumes of NaHCO₃ (15.6 mg/ml), 3.75 volumes of H₂O, 3.75 volumes of type I collagen (BD Biosciences), and a few drops of 1 N NaOH to turn the mixture pink (pH 7.5). The rings were treated with drugs in the presence or absence of 10 mM lactate in Opti-MEM supplemented with 2.5% FBS, 50 units/ml penicillin G, and 50 μg/ml streptomycin. The medium was subsequently changed every other day. Aortic rings were photographed at day 6 post-treatment with a SPOT camera attached to a Nikon Eclipse TE2000-S inverted microscope using a ×4 objective lens. Quantification was performed by live phase-contrast microscopy with a ×10 objective lens and by counting the number of sprouts originating from the aortic rings.

**Real-time RT-PCR**—Total RNA was extracted from vehicle- or lactate-treated HUVECs using the RNeasy Mini extraction kit (Qiagen, Valencia, CA). Reverse transcription of 300 ng of RNA was carried out with the iScript cDNA synthesis kit (Bio-Rad) following the protocols of the manufacturer.
The relative expression levels of Gas6, Ang1, and VEGF were studied by real-time PCR. PCR was performed with the Eppendorf Mastercycler ep realplex 2 (Eppendorf, Hauppauge, NY) using the FastStart Universal SYBR Green Master (ROX) kit (Roche Applied Science). The following primers were used in real-time PCR: Gas6, CGACCCCGAGACGGATTATTT (forward) and GCTTGGGTCCCCTTCCTATC (reverse); Ang1, AGCGCCGAAGTCCAGAAAAC (forward) and TACTCTACGACAGTTGCCAT (reverse); VEGF, CTACCTCCACACTGCAATT (forward) and TGCCAAGT (reverse); and β-actin, CTTCTACATGAGCTGCTGTC (forward) and GTCTCAACATGATGCTGTC (reverse). Real-time PCR was performed with the following cycle profile: 10 min of preincubation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. At the end of each reaction, a melting-curve analysis was performed to confirm the absence of primer dimmers. Gas6, Ang1, VEGF, and β-actin cDNAs were amplified in duplicate for every sample, and two independent experiments were performed. mRNA levels of target genes in each sample were calculated by the standard curve method, and relative expression levels of Gas6, Ang1, and VEGF were normalized to that of β-actin.

**Statistical Analysis**—Data are presented as the mean ± S.E. Comparisons between two groups were performed using Student’s t test. Comparisons between multiple groups were performed using one-way analysis of variance followed by Tukey’s honestly significant difference (HSD) post-hoc test.

**RESULTS**

**Lactate Activates Akt in Primary Endothelial Cells**—Because engaging the PI3K/Akt pathway is essential for angiogenesis (26, 27), we considered whether lactate activated PI3K/Akt in primary HUVECs. In normal physiological conditions, the lactate level is ~1.8 mM (8), whereas in angiogenic settings (tumors and wound tissues), the concentration of lactate can reach 10–15 mM (9, 10). Consequently, we tested whether 10 mM lactate (lactic acid) increased phosphorylation of Akt. It did, and the maximal response was observed consistently by 2 h (Fig. 1A). Consequently, this time point was used in all subsequent biochemical studies.

**FIGURE 2. Lactate activates the receptor tyrosine kinases Axl, Tie2, and VEGFR-2.** A, a human phospho-RTK array was used to examine lactate-induced RTK phosphorylation levels in HUVEC lysates. In this array, antibodies against 49 RTKs were spotted in duplicate on nitrocellulose membranes. The RTK array in the top panel was incubated with lysate from vehicle-treated HUVECs, whereas the array in the bottom panel was incubated with lysate from lactate-treated HUVECs (10 mM for 2 h). Both arrays were immunoblotted with anti-phospho-tyrosine HRP detection antibody and exposed to x-ray film for the same length of time. The RTKs whose tyrosine phosphorylation levels were most increased by lactate are as follows: Axl (B21 and B22, 1.9-fold), Tie2 (D1 and D2, 1.6-fold), and VEGFR-2 (D21 and D22, 1.4-fold). In addition, lactate slightly increased tyrosine phosphorylation of VEGFR-1 (D9 and D10) and EphA2 (D19 and D20). B, immunoprecipitation (IP) experiments confirm that lactate promotes tyrosine phosphorylation of Axl, Tie2, and VEGFR-2. Serum-starved HUVECs were pretreated with 100 μM sodium orthovanadate (an inhibitor of protein tyrosine phosphatases) for 30 min and then either left resting or stimulated with lactate (10 mM) for 2 h. The clarified lysates were immunoprecipitated with the antibodies directed against Axl, Tie2, or VEGFR-2. The resulting samples were subjected to Western blot analysis using the indicated antibodies. pY, 1:1 mixture of anti-phosphotyrosine antibodies PY20 and 4G10.

The relative expression levels of Gas6, Ang1, and VEGF were studied by real-time PCR. PCR was performed with the Eppendorf Mastercycler ep realplex 2 (Eppendorf, Hauppauge, NY) using the FastStart Universal SYBR Green Master (ROX) kit (Roche Applied Science). The following primers were used in real-time PCR: Gas6, CGACCCCGAGACGGATTATTT (forward) and GCTTGGGTCCCCTTCCTATC (reverse); Ang1, AGCGCCGAAGTCCAGAAAAC (forward) and TACTCTACGACAGTTGCCAT (reverse); VEGF, CTACCTCCACACTGCAATT (forward) and TGCCAAGT (reverse); and β-actin, CTTCTACATGAGCTGCTGTC (forward) and GTCTCAACATGATGCTGTC (reverse). Real-time PCR was performed with the following cycle profile: 10 min of preincubation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. At the end of each reaction, a melting-curve analysis was performed to confirm the absence of primer dimmers. Gas6, Ang1, VEGF, and β-actin cDNAs were amplified in duplicate for every sample, and two independent experiments were performed. mRNA levels of target genes in each sample were calculated by the standard curve method, and relative expression levels of Gas6, Ang1, and VEGF were normalized to that of β-actin.

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Because lactate enters endothelial cells through the monocarboxylate transporter MCT1 (17), we expected that the MCT1 inhibitor α-cyano-4-hydroxycinnamate would antagonize lactate-dependent activation of Akt. This is indeed what
we observed (Fig. 1B). Thus lactate activated Akt in primary human endothelial cells, and entry into cells appeared to be a prerequisite for this response. In contrast to lactate, glucose, pyruvate, or the metabolic product alanine was unable to activate Akt in HUVECs (Fig. 1C). Similarly, reducing the pH of the medium (which occurs following addition of lactate to a final concentration of 10 mM) did not consistently activate Akt (data not shown).

**Lactate Activates Multiple RTKs That Are Capable of Engaging PI3K/Akt**—Because RTKs induce the activation of the PI3K/Akt pathway, we considered whether the lactate-mediated mechanism to engage the PI3K/Akt pathway involved activation, i.e. phosphorylation of RTKs. To this end, we subjected control and lactate-stimulated lysates of HUVECs to phospho-RTK array analysis. As shown in Fig. 2A, there was a readily detectable increase in phosphorylation of three of the 49 RTKs that were analyzed: Axl (1.9-fold), Tie2 (1.6-fold), and VEGFR-2 (1.4-fold). Lactate also induced a modest and reproducible increase in tyrosine phosphorylation of VEGFR-1 and ephrin type A receptor 2 (EphA2). In light of the previous report that lactate increased production of basic FGF (bFGF) in HUVECs (4), we were surprised that lactate did not detectably increase the phosphorylation of any of the FGF receptors (FGFR1–4) (Fig. 2A). We decided to focus on Axl, Tie2, and VEGFR-2 because of the magnitude of the lactate-induced response and for their documented ability to activate the PI3K/Akt pathway (34–36). Phosphotyrosine Western blot analysis of Axl, Tie2, and VEGFR-2 immunoprecipitates prepared from control and lactate-stimulated HUVECs confirmed that lactate stimulates tyrosine phosphorylation of these three RTKs (Fig. 2B).

**Lactate-mediated Activation of RTKs Is Required for Stimulating Akt and Promoting Tube Formation**—We deployed a combination of pharmacological and molecular approaches to determine whether stimulation of Axl, Tie2, and VEGFR-2 was required for lactate-dependent activation of PI3K/Akt. This response was greatly attenuated by either the Axl kinase inhibitor BGB324/R428 (R428) (37) or the Tie2 kinase inhibitor and slightly reduced by the VEGFR-2 kinase inhibitor ZM 323881 (Fig. 3A). The combination of all three inhibitors nearly abolished lactate-induced activation of Akt, indicating that Axl, Tie2, and VEGFR-2 were the major mediators of PI3K/Akt acti-
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It is noteworthy that the dose of R428 used in these studies (100 nM) is likely to at least partially reduce Tie2 activity (IC\textsubscript{50} = 14 nM for Axl and 42 nM for Tie2, IC\textsubscript{50} > 100 nM for other RTKs that were tested) (37).

To complement these pharmacological studies, we individually silenced expression of Axl, Tie2, or VEGFR-2 using shRNA (Fig. 3B) and again found that lactate-dependent activation of Akt was attenuated greatly in Axl or Tie2 knockdown HUVECs and reduced slightly in VEGFR-2 knockdown HUVECs (Fig. 3C). Taken together, these data indicate that Axl, Tie2, and VEGFR-2 are intrinsic to the mechanism by which lactate activated PI3K/Akt and that Axl and Tie2 play a more prominent role than VEGFR-2.

To test the importance of the three RTKs for lactate-mediated tube formation, we used our previously characterized collagen gel sandwich assay in which primary endothelial cells are plated within a collagen gel sandwich and organized into lumen-containing chords, i.e. tubes in response to VEGF (32). Overlaying the collagen gel sandwich with lactate-containing medium promoted tube formation (Fig. 4A). Our biochemical data predict that lactate-dependent tube formation will be compromised when Axl, Tie2, and/or VEGFR-2 are inhibited. Indeed, the Axl inhibitor R428 and the Tie2 kinase inhibitor significantly reduced lactate-stimulated tube formation, whereas only a trend toward inhibition was observed in the presence of the VEGFR-2 inhibitor ZM 323881 in this pure endothelial cell system (Fig. 4B). The combination of all three inhibitors showed a similar potency in inhibiting lactate-induced tube formation as the PI3K inhibitor LY294002 (Fig. 4B).

We conclude that Axl, Tie2, and VEGFR-2 are required for lactate to promote tube formation and appear to account for the PI3K/Akt-dependent portion of this response. Furthermore, because neither the combination of the three RTK inhibitors nor LY294002 completely abolished lactate-dependent tube formation, there appear to be PI3K/Akt-independent pathways (e.g. those driven by interleukin 8 (17) and bFGF (4)) that also contribute to lactate-driven tube formation.

Lactate Increases the Level of RTK Ligands, and This Event Is Necessary for Activation of Akt and Sufficient for Lactate-mediated Tube Formation—RTKs can be either activated directly when ligands engage their extracellular domain or indirectly by other stimuli involving an intracellular mechanism (21, 28). To determine whether direct (ligand-mediated) activation of the three RTKs was required for lactate-dependent activation of PI3K/Akt, we tested the effect of agents that neutralized their ligands: AxlFc (a trap for the Axl ligand Gas6), Tie2Fc (a trap for the Tie2 ligand angiopoietin), and/or ranibizumab/Lucentis (a neutralizing anti-VEGF Fab used clinically). AxlFc and Tie2Fc greatly attenuated lactate-induced activation of Akt, whereas Lucentis slightly reduced this response (Fig. 5A). The combina-
tion of all three neutralizing reagents nearly abolished lactate-induced Akt activation, suggesting that ligand-mediated activation is a predominant mechanism for engaging the three RTKs in response to lactate.

To complement these neutralization studies, we investigated whether lactate increased the amount of Gas6 and Ang1, the angiopoietin trap Tie2Fc (4 μg/ml), the VEGF-neutralizing antibody fragment Lucentis (10 μg/ml), or a combination of the three reagents (Combo) for 30 min and then either left resting or treated with lactate (10 mM) for 2 h. Cell lysates were subjected to Western blot analysis using the indicated antibodies. Neutralizing the ligands for Axl, Tie2, and VEGFR-2 reduced lactate-induced Akt activation. B, lactate induces the production of Gas6 and Ang1. Serum-starved HUVECs were left resting or treated with lactate (10 μM) for 2 h. Ang1 was detected in the medium, whereas Gas6 was detected in cell lysates. Purified human recombinant Gas6 (1 ng) and Ang1 (1 ng) served as positive controls.

C, quantitation of Gas6 and Ang1 protein levels. The levels of Gas6 and Ang1 were normalized by RasGAP and expressed as an arbitrary unit in which the control group value equaled 1.0. The data shown are mean ± S.E. of five independent experiments. *, p < 0.05; **, p < 0.01 (paired Student’s t test).

D, relative Gas6, Ang1, and VEGF mRNA expression in vehicle- or lactate-treated HUVECs. Serum-starved HUVECs were either left resting or treated with lactate (10 mM) for 2 h. mRNA expression levels were determined by quantitative real-time RT-PCR. The relative levels of Gas6, Ang1, and VEGF mRNA were normalized by β-actin and expressed as an arbitrary unit in which the control group value equaled 1.0. The data shown are mean ± S.E. of three different samples. NS, no significant difference; *, p < 0.05. Lactate significantly changed the relative mRNA expression of VEGF but not Gas6 or Ang1.

E, lactate increases the level of HIF1α. Serum-starved HUVECs were either left resting or treated with lactate (10 mM) for 2 h. Cell lysates were subjected to Western blot analysis using the indicated antibodies.

The observation that Gas6 and Ang1 were required for lactate-stimulated tube formation begged the question of whether the combination of Gas6 and Ang1 would be sufficient for this response. Indeed, although either Gas6 or Ang1 alone did not promote tube formation, the combination mimicked the response induced by lactate (Fig. 6). Importantly, neither the VEGF-neutralizing antibody fragment Lucentis nor the VEGFR-2 activator HIF1α and the mRNA level of VEGF but not the level of VEGF protein in HUVECs. In accordance to these findings of others, we observed that lactate increased the mRNA level of VEGF and the protein level of HIF1α in HUVECs (Fig. 5, D and E). These results indicate that the amount of endogenously produced VEGF is low and further support the idea that VEGF is not making a major contribution to lactate-driven PI3K/Akt activation and tube formation when endothelial cells are the only cell type present. Taken together, these studies indicate that lactate increases the amount of Gas6 and Ang1 and, thereby, activates Axl and Tie2 to engage the PI3K/Akt pathway. In contrast, the impact of lactate on the level of VEGF and subsequent contribution to activating VEGFR-2 were less pronounced in endothelial cells.
inhibitor ZM 323881 significantly changed Gas6- and Ang1-induced tube formation (Fig. 6B), suggesting that Gas6 and Ang1 are able to drive angiogenesis in a VEGF-independent manner.

**Lactate-mediated Neovessel Formation Is Dependent on Axl, Tie2, and VEGFR-2**—To consider the relevance of our tube assay results in a more physiologically relevant context, we tested the ability of lactate to promote vessel sprouting from mouse aortic rings and whether Axl, Tie2 and VEGFR-2 were involved. As shown in Fig. 7, lactate triggered vessel sprouting from mouse aortic ring explants. This response was attenuated by each of the three RTK inhibitors, indicating that every one of the RTKs was making an essential contribution. The increased (relative to the tube assay) importance of VEGFR-2 may reflect the presence of cell types that produce VEGF. The combination of the three inhibitors blocked lactate-induced sprouting completely (Fig. 7), which suggests that these RTKs are major contributors to the response. The importance of PI3K for lactate-dependent neovessel sprouting was apparent from the minimal response in the presence of the PI3K inhibitor LY294002 (Fig. 7). The observation that blocking Axl (with R428) and Tie2 (with the Tie2 inhibitor) was as effective as the combination of all three inhibitors is consistent with the fact that VEGF/VEGFR-2 engages PI3K via Axl (28). This concept was reinforced by the observation that blocking both Axl and VEGFR-2 (R428 + ZM 323881) was no more effective than inhibiting Axl alone (R428) (Fig. 7). We conclude that like the tube assay, lactate-driven neovessel formation from aortic ring explants depends on multiple RTKs. Furthermore, the presence of cell types capable of producing VEGF (e.g. perivascular cells and fibroblasts) is a likely reason for the enhanced importance of VEGFR-2 in this more complex experimental system.

**DISCUSSION**

Although lactate-mediated angiogenesis plays an important role in tumors and wound healing and lactate has been shown recently to directly communicate with endothelial cells, the signaling pathways that lactate engages in endothelial cells to enable angiogenesis are not fully resolved. We discovered that lactate stimulated the PI3K/Akt pathway and, thereby, promoted organization of endothelial cells into tubes as well as sprouting of neovessels from aortic rings. The underlying mechanism involved augmenting the level of ligands for a specific complement of RTKs that are associated with angiogenesis (Fig. 8).

This study expands our appreciation of the relationship between endothelial cells and hypoxic tissue. In the case of tumors, tumor cells produce both proangiogenic growth factors and metabolic products. The growth factors instruct endothelial cells to engage various facets of the angiogenic program. Our results indicate that metabolic products stimulate endothelial cells to further increase the pool of proangiogenic growth factors.

Because the amount of angiomodulators that endothelial cells produce is likely to be far less than the quantities generated by other cell types (such as tumor or infiltrating immune cells), the endothelial contribution may not be relevant. However, there are reports indicating that locally produced agents are

**FIGURE 6.** The combination of Gas6 and Ang1 promotes tube formation independently of VEGF/VEGFR-2. A, HUVECs were embedded within a collagen gel sandwich, and then culture medium containing vehicle, Gas6 (100 ng/ml), Ang1 (25 ng/ml), Gas6 + Ang1, Lucentis (10 μg/ml), Gas6 + Ang1 + Lucentis, ZM 323881 (100 μM), Gas6 + Ang1 + ZM 323881, or lactate (10 mM) was added. 17 h later, the tubes were photographed with an inverted microscope. Scale bar = 500 μm. B, quantification of tube formation. Although either Gas6 alone or Ang1 alone was unable to induce tube formation, the combination of the two promoted tube formation comparable with lactate, which was not inhibited either by the VEGF-neutralizing antibody fragment Lucentis or by the VEGFR-2 inhibitor ZM 323881. Neither Lucentis nor ZM 323881 significantly changed basal tube formation. The data shown are mean ± S.E. (n = 5). ***, p < 0.001.
for indispensable. For instance, although the amount of VEGF produced by endothelial cells does not substantially contribute to the circulating level of VEGF (and is therefore considered to be small), it is nonetheless essential (38). Similarly, endogenously produced lysophosphatidic acid makes a vital contribution to instructing regression of endothelial cell tubes (39). These findings raise the possibility that angiomodulators that are produced by endothelial cells in response to lactate may make an important contribution to the angiogenic response despite the relative small quantity.

One surprising observation of this study is that although lactate increased HIF1α/H9251, the protein level of VEGF was unchanged in endothelial cells. A trivial explanation is that our approaches to detect VEGF were insufficiently sensitive. Alternatively, this observation suggests that signaling events, in addition to elevating HIF1α/H9262, are necessary to increase the amount of VEGF. Even without understanding why VEGF was so low in our experimental system, its paucity is a likely explanation for why VEGFR-2 made only a supporting (instead of a major) contribution to lactate-driven Akt activation and tube formation. It seems likely that in tumors and healing wounds, wherein the tumor cells and infiltrating macrophages can produce relatively high levels of VEGF, the VEGF/VEGFR-2 contribution will be more prominent than what we observed using pure HUVECs. Consistent with this idea is our observation that the VEGFR2 kinase inhibitor, ZM323881, significantly attenuated lactate-
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mediated sprouting of neovessels from aortic rings. The presence of non-endothelial, VEGF-producing cells within the aortic rings is likely to increases the amount of VEGF present and hence the relative contribution of VEGF to neovessel formation. The strength of VEGF-deficient systems is their opportunity to identify the VEGF-independent mediators of angiogenesis.

Angiogenesis is a rate-limiting process in tumor growth (40). Thus, targeting tumor angiogenesis could be an effective strategy to treat cancer (41). VEGF is thought to be the most important proangiogenic growth factor because it promotes all steps of angiogenesis. In the past decade, VEGF has received great attention as a target for therapeutics. However, anti-VEGF therapy results in only a transient response in the tumor treatment (42), suggesting that VEGF-independent angiogenic pathways exist and are responsible for resistance to anti-VEGF therapy. In a rat insulin promoter-Simian Virus 40 T antigen (RIP-Tag) mouse model, FGF-1 and FGF-2 are up-regulated in tumors treated with anti-VEGFR antibodies (43), suggesting that they constitute one such VEGF-independent pathway. In this study, we found that Axl and Tie2 were the major contributors to lactate-dependent activation of PI3K/Akt and that they were required for lactate to stimulate tube formation and promote neovessel formation. In addition, Gas6 and Ang1 sufficed to stimulate tube formation, and the anti-VEGF antibody fragment Lucentis and the VEGFR-2 inhibitor ZM 323881 did not significantly attenuate this response. Thus, the combination of Gas6 and Ang1 may constitute a second VEGF-independent mode of driving angiogenesis.

Axl is a member of the TAM family (Tyro3, Axl and Mer) of RTKs. The ligand for all three TAM family members is Gas6. Axl controls diverse cellular responses essential for angiogenesis, including endothelial cell proliferation, migration, and tube formation (44). We discovered recently that VEGF/VEGFR-2 acts through SFKs to activate Axl and engage the PI3K/Akt pathway (28). Thus, Axl is an angiogenic RTK that can be engaged by multiple stimuli, including Gas6, VEGF, lactate, and probably other stimuli that activate SFKs. In addition, transcription of axl is under the control of HIF-1α, and the mRNA level of Axl in endothelial cells is increased by hypoxia (45). Therefore, Axl may serve as a central player in integrating multiple proangiogenic signals to activate the PI3K/Akt pathway and promote angiogenesis.

In addition to endothelial cells, Axl is also expressed in tumor cells and vascular smooth muscle cells. Overexpression of Axl is associated with increased invasiveness of human cancers and has been reported in a wide variety of solid human tumor types, whereas inhibition of Axl in tumor cells reduces the growth of mouse tumors, including breast and lung tumors as well as glioblastomas (44, 46). Thus, targeting Axl signaling may represent a promising therapy to treat tumors by simultaneously targeting endothelial cells, tumor cells, and vascular smooth muscle cells.

Tie2 is a RTK that plays a critical role in both vascular development and pathological angiogenesis (47). Ang1 is a Tie2 receptor agonist, whereas Ang2 normally functions as a Tie2 antagonist. Tie2 expression is up-regulated in the endothelial cells of the neovascularature of skin wounds (48) and tumors (49, 50). Similar to Axl, Tie2 expression in endothelial cells is elevated by hypoxia (51). This study revealed that lactate, another stress factor in tumors and healing wounds, is able to activate Tie2 and that Tie2 is required for lactate to activate PI3K/Akt and promote angiogenesis.

Comparing the effect of kinase inhibitors with agents that neutralized ligands indicates that the kinase inhibitors were more effective at blocking lactate-dependent activation of Akt (Fig. 3A versus Fig. 5A). This phenomenon suggests that ligand-independent pathways are also involved in lactate-induced activation of the three RTKs. We recently discovered that VEGF/VEGFR-2 acts through SFKs to activate Axl and engage the PI3K/Akt pathway (28). Thus, it is possible that lactate acts through SFKs to indirectly activate Axl and, thereby, activate PI3K/Akt. However, this mechanism is likely to make only a small contribution under these experimental conditions.

This study did not address which downstream effectors of the PI3K/Akt pathway are engaged by lactate to promote angiogenesis. It has been reported recently that lactate activates the NF-κB/interleukin 8 pathway, which is required for lactate-dependent endothelial cell migration and tube formation (17). Activated Akt can phosphorylate IκB, which, in turn, frees NF-κB and allows it to translocate to the nucleus where it binds and subsequently activates target genes. Thus, it is possible that NF-κB/interleukin 8 is the downstream effector of PI3K/Akt for promoting angiogenesis in response to lactate. Further clarification and understanding of the mechanism by which lactate promotes angiogenesis may provide new opportunities for treating pathological angiogenesis.

Acknowledgments—We thank members of the Kazlauska laboatory, Sarah Melissa P. Jacob, Steven Pennock, Ruta Motiejunaite, Hetian Lei, and Zhigang Cai for their constructive input. We also thank Dr. Sacha J. Holland at Rigel Pharmaceuticals, Inc. for providing R428.

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