Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis

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ABSTRACT.

Lactate generated from pyruvate fuels production of intracellular NAD$^+$ as an end result of the glycolytic process in tumors. Elevated lactate concentration represents a good indicator of the metabolic adaptation of tumors and is actually correlated to clinical outcome in a variety of human cancers. In this study, we investigated whether lactate could directly modulate the endothelial phenotype and thereby tumor vascular morphogenesis and perfusion. We found that lactate could enter endothelial cells through the monocarboxylate transporter MCT-1, trigger the phosphorylation/degradation of IκBα and then stimulate an autocrine NFκB/IL-8 (CXCL8) pathway driving cell migration and tube formation. These effects were prevented by 2-oxoglutarate and reactive oxygen species (ROS) inhibitors, pointing to a role for prolyl-hydroxylase and ROS in the integration of lactate signaling in endothelial cells. PHD2 silencing in endothelial cells recapitulated the pro-angiogenic effects of lactate, whereas a blocking IL-8 antibody or IL-8-targeting siRNA prevented them. Finally, we documented in mouse xenograft models of human colorectal and breast cancer that lactate release from tumor cells through the MCT4 (and not MCT1) transporter is sufficient to stimulate IL-8-dependent angiogenesis and tumor growth. In conclusion, our findings establish a signaling role for lactate in endothelial cells and they identify the lactate/NF-kappaB/IL-8 pathway as an important link between tumor metabolism and angiogenesis.

PRECIS.

This study identifies an unsuspected lactate signaling pathway in endothelial cells within the tumor microenvironment that bridge cancer cell metabolism and angiogenesis.
INTRODUCTION.

Tumor cells fuel their metabolism with a variety of nutrients including glucose, glutamine and lactate to meet the bioenergetic and biosynthetic demands of proliferation (1, 2). Glycolysis coupling to the tricarboxylic acid (TCA) cycle and oxidative phosphorylations, is actually far to be the unique source of energy. Hypoxia and oncogenic mutations drive glucose to pyruvate transformation and then pyruvate to lactate conversion ensuring the regeneration of the NAD$^+$ pool necessary to support a high glycolytic flux to fulfil the demand for ATP and precursors (a phenomenon called the Warburg effect when occurring in the presence of sufficient oxygen). Also independently of a coupling to the oxidative phosphorylations, the TCA cycle may act as a biosynthetic hub fed by 2-oxoglutarate arising from glutaminolysis (3). Moreover, glycolysis and glutaminolysis share the capacity to generate NADPH, from the pentose phosphate pathway and indirectly through the malate conversion into pyruvate, respectively. The latter pathway may also contribute to an additional source of NAD$^+$ through the conversion of pyruvate into lactate (1). In tumors, both glycolysis and glutaminolysis therefore lead to the production of lactate. Interestingly, as previously documented in muscle fibres and brain (4, 5), lactate may in turn be taken up by oxygenated (tumor) cells to feed oxidative metabolism (1). We recently documented that this can contribute to reduce consumption of glucose (and probably glutamine) by tumor cells at the vicinity of blood vessels and thereby to increase the availability of these substrates for hypoxic tumor cells (6). One may thus propose that tumor lactate levels and fluxes reflect the extent of the adaptation of tumor cells to survive and proliferate.

A role of lactate in malignancy is supported by studies documenting a correlation between tumor lactate levels and clinical outcome in a variety of human cancers (7-13). In particular, high lactate levels were found to be associated with a high incidence of distant metastases already in
early stages of the disease and inversely, longer survival was associated with low lactate concentrations in tumors. Among the possible mechanisms supporting these clinical data, lactate is proposed to stimulate angiogenesis (14-17) through an activation of the VEGF/VEGFR2 signaling pathway (18-20); a parallel lactate-induced TGFβ-1-mediated pathway was also proposed to prevent maturation of neo-formed tubes (21). Because of the known HIF-mediated regulation of VEGF (22) and the capacity of glycolytic carboxylate intermediates including pyruvate and lactate to stimulate hypoxia-inducible factor (HIF) expression (23, 24), the effects of lactate on VEGF (and angiogenesis in general) are thought to be HIF-1α-dependent (16).

Since major metabolic pathways in tumors end up with lactate secretion and since this may occur under both hypoxic and non-hypoxic conditions (Warburg effect), we reasoned that lactate could act as a signaling molecule in endothelial cells through HIF-dependent but also -independent pathways. Furthermore, although hypoxia-independent expression of HIF-1 is possible in tumor cells in response to oncogenic or loss-of-function mutations pathways (22, 25), this is not occurring in genetically stable endothelial cells. Also, the paradigmatic HIF1-dependent angiogenic growth factor VEGF is produced to much larger extent by tumor cells or tumor-associated macrophages than by endothelial cells (10-50-fold more at the protein level, unpublished data). Thus, to examine a possible autocrine pathway in endothelial cells triggered by lactate arising from tumor cells, we first used a low density array strategy to identify possible actors. This initial exploration led us to document that lactate could promote a PHD2- and ROS-dependent NFκB activation in endothelial cells. We further identified the monocarboxylate transporter MCT1 as the main gate for lactate entry in endothelial cells and the consecutive production of IL-8/CXCL8 as an autocrine signal mediating the effects of lactate on angiogenesis and tumor perfusion.
MATERIALS AND METHODS.

Cell culture and treatments.

Human colorectal adenocarcinoma WiDr cells and MDA-MB231 breast cancer cells were obtained from the American Type Culture Collection Cell lines where they are regularly authenticated based on viability, recovery, growth, morphology, and isoenzymology. Cells were acquired in 2009 and 2010, respectively, stored according to the supplier's instructions and used within 6 months after resuscitation of frozen aliquots. Human umbilical vein endothelial cells (HUVEC) were used within 6 months of purchase from Sigma (Bornem, Belgium), acting as a distributor for ECACC (European Collection for Cell Cultures). Human umbilical vein endothelial cells (HUVEC) were routinely cultured in Endothelial Cell Basal Medium (Sigma) and used between passages 2 and 5. In some experiments, cells were treated with an anti-IL-8 neutralizing antibody (R&D, Minneapolis, MD, USA), evodiamine (Calbiochem, LaJolla, CA, USA) or N-acetyl-L-cysteine (Sigma) in glucose- or lactate-containing medium.

Mouse models.

For in vivo experiments, NMRI nude mice (Elevage Janvier, LeGenest-St-Isle, France) were injected subcutaneously with 400 µl Matrigel containing one of the following combinations: (i) \(5 \times 10^5\) tumor cells (WiDr or MDA-MB231) mixed with \(5 \times 10^4\) HUVEC cells, with or without IL-8 blocking antibody; (ii) \(5 \times 10^5\) WiDr shMCT1 or \(5 \times 10^5\) WiDr shMCT4 cells mixed with \(5 \times 10^4\) HUVEC cells; (ii) \(5 \times 10^5\) MDA-MB231 or \(5 \times 10^5\) MDA-MB231 shMCT4 cells mixed with \(5 \times 10^4\) HUVEC cells. Tumour sizes were tracked with an electronic calliper and tumors were collected after the animal sacrifice 14 days after injection. In some experiments, local tumor blood flow was measured with a Laser Doppler imager (Moor Instruments) on anesthetized mice as previously described (26).
**Real-time PCR and silencing experiments.**

PCR amplification was performed in an IQ5 Thermocycler (Bio-Rad); primers sequences are described in Supplementary data. For gene expression profiling, Taqman Angiogenesis Gene signature Arrays (Applied Biosystems Inc, Foster City, CA, USA) were used according to the manufacturer’s instructions. For siRNA silencing experiments, HUVEC cells were transfected (LipofectAMINE 2000, Invitrogen, Merelbeke, Belgium) with specific siRNA (Qiagen) while for shRNA silencing experiments, BLOCK-iT H1 RNAi Entry Vector Kit (Invitrogen) was used. Sequences are described in Supplementary data.

**IL-8 ELISA, NFκB luciferase reporter and DNA binding assays.**

The extent of IL-8 production by HUVEC and tumor xenografts was determined in an ELISA assay (QuantiGlo Chemiluminescent Immunoassay, R&D systems, Abingdon, UK). The luciferase reporter pNFκB-Luc plasmid (Stratagene, La Jolla, CA) was transfected 24 hours before cell treatments; a GFP-expressing vector was co-transfected to adjust for variations in transfection efficiencies. DNA binding of NFκB subunit p65 was determined using a TransAM ELISA kit (Active Motif, Carlsbad, CA, USA).

**Reactive oxygen species (ROS) measurements and lactate measurements.**

Intracellular ROS were assessed using oxidation-sensitive probe 2’,7’-dichlorofluorescein diacetate (DCFH-DA) (Sigma). Lactate concentration was determined via the lactate dehydrogenase (LDH)-catalysed oxidation of lactate into pyruvate in the presence of NAD⁺ (6).

**Endothelial cell migration and tube formation assays.**

HUVEC cell mobility and migration were evaluated in a modified Boyden chamber assay using transwell filters inserts with 8 µm pore size (Greiner Bio-one, Wemmel, Belgium). The formation
of capillary-like endothelial tubes was determined in an *in vitro* assay by plating endothelial cells on growth factor-reduced Matrigel (BD PharMingen), as previously reported (27, 28).

**Immunostaining and immunoblotting.**

Tumours were cryosliced and sections were probed with a rat monoclonal antibody against CD31 (BD PharMingen, Lexington, KY, USA) or rabbit polyclonal antibodies against MCT1 (Millipore) and MCT4 (Polypeptide) followed by a secondary antibody coupled to an Alexa Fluor 568 as previously described (6, 29). For immunoblotting, cells extracts were separated on SDS-PAGE and transferred onto PVDF membranes before incubation with the MCT1 (Millipore), IκBα (Millipore) and phospho-serine 32 and36 IκBα (Cell Signaling) antibodies; gel loading was normalized with a beta-actin antibody (Sigma).

**Statistics.**

Results are expressed as mean ± SEM. Student’s *t* test, 1-way ANOVA (Tukey’s post-hoc test), were used where indicated. *P*<0.05 or **P**<0.01 was considered statistically significant in the different experiments.
RESULTS.

**MCT1-driven lactate uptake in endothelial cells promotes IL-8 expression.**

We first determined the capacity of lactate to be taken up by endothelial cells and to influence cell proliferation. We found that independently of the presence or the absence of glucose, the addition of 10 mM lactate in the culture medium led to a net increase in intracellular lactate concentration reaching 6-fold the level of lactate observed in the presence of glucose-containing (lactate-free) medium (Figure 1A). This elevated level of cytosolic lactate was reached after one hour of exposure to extracellular lactate and then maintained itself around the newly set-up basal value, independently of the lactate concentration in the medium as long as comprised between 1 and 25 mM (not shown). Although no sign of cell death (ie, cell detachment) could be observed, the presence of lactate in the medium (instead of glucose) reduced endothelial cell proliferation rate by half when compared with glucose-containing medium (Figure 1B). The presence of lactate together with glucose in the culture medium however did not alter endothelial cell proliferation (Figure 1B). We then examined the influence of lactate on the endothelial expression profile of angiogenesis-related genes using dedicated TaqMan arrays on 96-well microfluidic cards. HUVEC were exposed to 10 mM lactate for 6 hours in order to detect early changes in gene expression. We found that 7 transcripts were upregulated more than two-fold in response to lactate and among them, IL-8/CXCL8 mRNA expression was the most responsive with a ~8-fold increase (vs control HUVEC maintained in glucose-containing medium) (Table 1). The increase in IL-8 gene expression was confirmed by qPCR analysis using (other) specific primers (Figure 1C) and further validated at the protein level in a dedicated ELISA (Figure 1D). To document a direct relationship between lactate influx and IL-8, we used a dedicated siRNA to silence the expression of the major monocarboxylate transporter MCT1 in HUVEC (unpublished...
A downregulation of more than 90% of MCT-1 expression (vs control siRNA conditions) was confirmed at the mRNA (Figure 1E) and protein (Figure 1F) levels. MCT1 silencing prevented lactate entry into endothelial cells (Figure 1A) as well as lactate-induced IL-8 mRNA and protein expressions (Figures 1C and 1D); MCT1 silencing reduced endothelial cell proliferation to a larger extent than lactate conditions (Figure 1B). Of note, the ELISA test also revealed that hypoxia (1% O2) was not able to induce IL-8 in our experimental set-up (Figure 1D).

**IL-8 induction by lactate depends on the NFκB pathway.**

The known regulation of IL-8 expression by NFκB led us to examine whether this transcription factor could mediate the lactate effects. As a first insight on the influence of lactate on NFκB activity, we performed a TransAM assay. This experiment confirmed a significant increase in DNA binding of the p65 subunit of the NFκB transcription complex in lactate-exposed endothelial cells (Figure 2A). We then used a NFκB reporter assay by transfecting HUVEC with a NFκB-responsive luciferase plasmid (together with a GFP-encoding plasmid for normalization). We found that lactate dramatically stimulated luciferase activity (17-fold increase vs control glucose medium, P<0.01) (Figure 2B). Also, as observed for the effects of lactate on IL-8 expression (Figures 1C and 1D), MCT-1 silencing blunted the increase in NFκB reporter activity (Figure 2B).

The recent demonstration of an autocrine IL-8-induced activation of NFκB in endothelial cells (30) led us to verify that in our model, IL-8 was acting downstream of the lactate-induced NFκB activity and not the converse. We therefore repeated the above experiments in the presence of an anti-IL-8 blocking antibody (10 µg/mL) (Figure 2C) or using endothelial cells wherein IL-8
expression was silenced using dedicated siRNA (Figure 2D); a downregulation of more than 90% (p<0.01, n=3) of IL-8 expression (vs control siRNA conditions) was confirmed by qPCR. We found that independently of the mode of IL-8 inhibition, lactate strongly stimulated NFκB activity in the luciferase reporter assays (Figures 2C and 2D). The use of evodiamine (500 nM) as a pharmacological inhibitor of NFκB (acting by blocking IκBα kinase activation (31)) further confirmed the stimulatory effect of lactate on this transcription factor in our experimental model (Figure 2C).

**Lactate stimulates the NFκB/IL-8 pathway in a ROS- and IκBα-dependent manner.**

To further dissect the pathway linking lactate stimulation and NFκB/IL-8 induction, we examined the role of reactive oxygen species (ROS) usually described as key triggers of NFκB activity. We first treated endothelial cells with the ROS scavenger N-acetylcysteine (NAC) and found that in these conditions, both lactate-induced NFκB activity (Figure 3A) and IL-8 expression (Figure 3B) were completely abrogated, as determined using the luciferase reporter assay and qPCR, respectively. The use of the ROS fluorescent dye 2’-7’-dichlorofluorescin diacetate (DCFDA) confirmed that the exposure of endothelial cells to 10 mM lactate led to a rapid increase in ROS production (vs control glucose conditions) (Figure 3C). Importantly, a similar increase in ROS production was observed when cells were exposed to both glucose and lactate (Figure 3C). Since ROS-mediated activation of NFκB is classically proposed to be dependent on IκBα phosphorylation/degradation (which favors the release of active NFκB subunits) (32), we then examined the effect of lactate on IκBα serines 32 and 36 phosphorylations. We found that lactate supplementation (alone or together with glucose) led to a net increase in IκBα phosphorylation, a
process that we also found to be associated with IκBα degradation, as documented by immunoblotting (Figure 3D).

2-oxoglutarate inhibits while PHD2 silencing mimics the effects of lactate on the NFκB/IL-8 signaling pathway.

A prolyl hydroxylase (PHD)-dependent activation of NFκB activity was previously reported to occur through the IκBα pathway (33-35). Considering that PHD activity requires 2-oxoglutarate as co-substrate and may thereby be influenced by other carboxylate metabolic intermediates (see Introduction), we examined whether in endothelial cells, lactate competition with 2-oxoglutarate could account for the observed increase in NFκB/IL-8 expression. We found that 2-oxoglutarate dose-dependently prevented lactate-induced NFκB activity (Figure 4A), as determined in a luciferase reporter assay. We also found that PHD2 silencing in endothelial cells led to a net increase in both NFκB activity (Figure 4B) and IL-8 expression (Figure 4C) under basal conditions (ie, in lactate-free glucose-containing medium) and failed to further stimulate the NFκB/IL-8 expression pathway in response to lactate (Figures 4B and 4C). Downregulation of more than 90% of PHD-2 expression (vs control siRNA conditions) was confirmed by qPCR (Figure 4D). We also documented that lactate-induced IL-8 expression was strongly inhibited by 2-oxoglutarate and diphenylene iodonium chloride (DPI), a potent inhibitor of NADPH oxidase (a major enzymatic source of ROS in endothelial cells) (Figure 4C). Interestingly, PHD2 silencing failed to stimulate IL-8 expression in glucose-containing medium when DPI was present (Figure 4C), suggesting that both PHD2 inhibition and ROS production are required to promote the NFκB pathway in endothelial cells.
MCT4-driven release of lactate from cancer cells stimulates IL-8 production together with tumor angiogenesis and perfusion.

To examine the NFκB/IL-8-driven effects of lactate in vivo, we used tumor cells genetically modified for their capacity to handle lactate. Accordingly, we established clones from the WiDr human colorectal cancer cell line selected for stable expression of shRNA directed against MCT1 or MCT4. We found that WiDr cells express both types of MCT transporters and that the use of dedicated shRNA led to the silencing of MCT1 expression (91.2± 6% down-regulation) (Figure 5A) and MCT4 (96.5± 9% downregulation) (Figure 5B), respectively. We found that while MCT1 silencing did not prevent the efflux of lactate induced under hypoxia (Figure 5A, lower panel), MCT4 silencing had a dramatic effect on the capacity of WiDr cells to release lactate in the same hypoxic conditions (Figure 5B, lower panel). Measurements of cell proliferation rate in vitro however did not reveal any difference between shMCT1 and shMCT4 WiDr cell clones (Figure 5C). Of note, the extents of lactate release in shMCT1 WiDr cells and sham-transfected WiDr were not significantly different, in agreement with a major role of this transporter in the influx –and not the efflux- of lactate in this cell type (6).

To validate the functional consequences of a deficit in lactate release, we first used the conditioned medium (CM) collected from either shMCT1 or shMCT4 WiDr cells to treat endothelial cells. We found that IL-8 expression (as determined by qPCR) was stimulated by CM collected from hypoxic shMCT1 cells but not altered by the exposure to CM from hypoxic shMCT4 cells (Figure 5D). In another set of experiments, we injected s.c. plugs of Matrigel containing 5x10^4 HUVEC together with either 5x10^5 WiDr shMCT1 cells or 5x10^5 WiDr shMCT4 cells. We found that tumor growth from the plug containing shMCT4 WiDr cells was reduced (vs shMCT1 cells-containing plug) (Figure 5E). We also verified that tumor lactate
(Figure 5F) and IL-8 (Figure 5G) contents were reduced in shMCT4-expressing tumors (vs shMCT1 tumors). Importantly, this was associated with a dramatic inhibition of angiogenesis as determined by CD31 immunostaining of vascular structures, together with a reduction in tumor blood flow as measured by laser Doppler imaging (Figure 5H, see lower panels for quantification). We repeated these experiments using a MDA-MB231 breast cancer cell clone wherein MCT4 was also silenced and found a similar phenotype (ie, reduced CD31-labelled microvascular density and tumor perfusion; see Figure 5I). For both tumors, the use of a CD31 antibody selective for mouse tissues (36) did however not lead to any specific staining indicating that the vascular signal was of human origin and thus corresponded to HUVEC.

**Lactate-dependent induction of IL-8 expression supports angiogenesis and tumor growth.**

To dissect the NFκB/IL-8-driven pro-angiogenic effects of lactate, we first measured the migration of HUVEC in a modified Boyden chamber. Migration was determined after 24 hours by counting DAPI-stained cells on the bottom face of the filter. To first validate the IL-8 capacity to drive cell migration in this assay, recombinant IL-8 protein was added to glucose-containing medium in the upper part of the well. A 10-fold increase in the number of migrated endothelial cells was observed (vs untreated conditions) (Figure 6A). Replacement of glucose by lactate (10 mM) in the medium of the upper chamber led to a similar stimulation of endothelial cell migration. This pro-migratory effect of lactate was IL-8-dependent since migration was completely blocked by either a dedicated IL-8 siRNA cell pretreatment or the presence of a blocking anti-IL-8 antibody in the upper well compartment (Figure 6A). The use of NAC and evodiamine similarly reduced the capacity of lactate to promote the motility of endothelial cells (Figure 6A)
We next examined how interfering with lactate cellular uptake and consecutive increase in IL-8 expression could influence the capacity of endothelial cells to form tubes when plated on Matrigel. We first showed that lactate exposure stimulates the formation of an endothelial network in these experimental conditions (Figure 6B). We then documented that siRNA directed against either MCT-1 or IL-8 prevented lactate-driven formation of tubes, respectively (Figures 6B and 6C for quantification). Importantly, the addition of recombinant IL-8 to IL-8-siRNA-treated endothelial cells rescued tubulogenesis (Figures 6B and 6C). As observed in Figure 6A, the use of NAC and evodiamine confirmed a role for ROS/NFκB in the lactate-dependent IL-8-driven formation of endothelial tubes (Figure 6C). Also, in adequation with results of Figures 4A and 4B, 2-oxoglutarate blunted the tubulogenic response to lactate and PHD2 silencing in glucose-containing medium recapitulated the effects of lactate with a strong stimulation of tube formation (Figure 6C).

Finally, to further validate the in vivo relevance of the lactate/IL-8 pro-angiogenic pathway, we injected s.c. two plugs of Matrigel containing $5 \times 10^5$ wild-type WiDr cells and $5 \times 10^4$ HUVEC supplemented with of without $100 \mu\text{g/mL}$ IL-8-blocking antibody. In this tumor model of lactate-driven angiogenesis, we found that the presence of anti-IL-8 blocking antibodies within the Matrigel plug delayed tumor growth (Figure 6D) and considerably reduced the development of the tumor vasculature, as revealed by anti-CD31 immunostaining (Figure 6E, upper panel) (-75±13 % vs control, P<0.01, n=5). Finally, to prove the role of MCT1 in vivo, we similarly co-injected WiDr cells and HUVEC pre-treated with MCT1 siRNA. We found that tumor growth and development of the microvascular network (-66 ± 21 % vs control, P<0.05, n=5) were both inhibited in response to MCT1 silencing in endothelial cells (Figures 6D and 6E, lower panels).
DISCUSSION

Major findings of this study are the identification of a lactate/NFκB signaling pathway in endothelial cells, the existence of a lactate-driven feed-forward IL-8 autocrine loop driving angiogenesis in tumors and the key roles of monocarboxylate transporters MCT1 and MCT4 in this lactate-based dialog between cancer cells and endothelial cells. Our data indeed support a model according to which lactate released from tumor cells through MCT4 may be taken up by endothelial cells via the MCT1 transporter and consecutively stimulate angiogenesis through NFκB/IL-8 signaling (Supplementary Figure 1); all these lactate-driven signaling events may importantly occur in the presence of glucose.

We identified key players acting upstream and downstream of this pathway in endothelial cells. We found that ROS were produced in endothelial cells in response to lactate and that ROS inhibitors prevented lactate-induced activation of NFκB and IL-8 expression. Also, we showed that the PHD co-substrate 2-oxoglutarate could prevent the effects of lactate on NFκB activation and that conversely, PHD2 silencing recapitulated them in normal glucose conditions. We further documented the stimulatory effects of lactate on IκBα phosphorylation and consecutive degradation in endothelial cells, a process known to promote translocation of freed NFκB activation into the nucleus for transcriptional regulation (32). Using siRNA directed against IL-8 (rescued or not with recombinant IL-8), we showed that this autocrine pathway accounts for most of the pro-angiogenic effects of lactate in endothelial cells. Importantly, we validated in vivo that the release of lactate by cancer cells can effectively contribute to the development of the tumor vasculature by stimulating the IL-8 pathway. A blocking antibody directed against IL-8 could indeed prevent the stimulation of angiogenesis (Figure 6E) and reduce tumor growth (Figure 6D).
in a model where lactate efflux through the MCT4 transporter is a key trigger of the development of tumor vasculature (Figures 5H and 5I).

Lactate was previously documented to stimulate angiogenesis (14-16) through activation of the VEGF/VEGFR2 signaling pathway (16-20). While it may be difficult to discriminate between the role of hypoxia and lactate in triggering VEGF signaling in vivo, our finding of NFκB acting as a lactate-responsive transcription factor unravels a pathway which may link tumor cell metabolism and angiogenesis independently of the O2 conditions. An interesting parallel may be drawn with previous studies by Verma and colleagues who reported that pyruvate, lactate and several TCA cycle intermediates can stabilize HIF-1 through inhibition of PHD enzymes (23, 24). These data are proposed to support the Warburg effect, i.e. the capacity of non-hypoxic tumor cells to exploit glycolysis without a need for coupling to oxidative phosphorylations to support the high biosynthetic and energy demands of proliferating cells. Our study allows to extend this paradigm to tumor angiogenesis and supports a central role of lactate bridging avid glucose metabolism and key aspects of malignancy, namely tumor cell proliferation and angiogenesis (Supplementary Figure 1).

Interestingly, reduction in PHD activity appears to be the common denominator in the stabilization of HIF and NFκB observed in response to lactate. In particular, our data are in line with the work of Cummins et al. (34) who previously showed that hypoxia may activate NFκB through a decreased PHD1-dependent hydroxylation of IKKβ and consecutive phosphorylation-dependent degradation of IκBα. In a more recent study, Chan and colleagues, starting from the observation of a frequent loss of PHD2 expression in cancers, documented that the tumor suppressor gene potential of this hydroxylase was associated with an increased NFκB activation in tumor cells (33). To reproduce the loss of PHD2 expression, these authors used PHD2-silenced
cancer cells and documented that mouse injection with these modified tumor cells led to the development of more vascularized tumors. The effects of lactate observed in our study therefore mimic the effects of a downmodulation of PHD2, leading in fine to the activation of NFκB-driven angiogenic cascades. Interestingly, we found that lactate-driven angiogenesis leads in vivo to a net increase in tumor perfusion indicating that the stimulated NFκB/IL-8 pathway also contributes to the maturation of the tumor neovasculature. These data may be related to a recent study by Mazzone et al. who documented that heterozygous deficiency of PHD2 normalized the tumor endothelial lining, resulting in improved tumor perfusion (37). The same authors further documented that haplodeficiency of PHD2 restores tumor oxygenation and thus possibly induces a metabolic shift to a more oxidative, less malignant phenotype. Similarly, our data support the hypothesis that in response to lactate exposure, endothelial cells may exhibit alterations in their metabolic phenotype leading to increased cell survival in glucose-free conditions (Figure 1B). The ROS production observed in endothelial cells in response to lactate could actually arise from the activation of the NAD(P)H oxidase by NADH accumulation resulting from the conversion of lactate into pyruvate, as we previously reported in tumor cells (6).

In conclusion, our study provides a new rationale for associating elevated lactate concentrations in tumors and negative outcomes for patients. These results further support the current enthusiasm for new cancer treatments targeting metabolic pathways (38). In particular, inhibitors of MCT1 and/or MCT4 bear the promise of interfering with the subtle exchanges of lactate between tumor cells and endothelial cells, and to indirectly attenuate the IL-8 effect on tumor vascular development and associated tumor burden.
REFERENCES.


**TABLE 1. Influence of lactate (10 mM) on the mRNA expression profile of endothelial cells using TaqMan Gene Signature Array.** Data (mean ± s.e.m; n=3) are presented as fold-change vs control condition (glucose-containing medium); n.d. = not detect

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FIGURE LEGENDS.

Figure 1. MCT1-dependent lactate uptake in endothelial cells stimulates IL-8 expression. Endothelial cells were exposed to medium containing glucose, lactate (10 mM), glucose + lactate or none of the above; in some experiments, cells were first transfected with a MCT1-targeting siRNA (or a control siRNA) or incubated under 1% O₂ for the same period of time. Graphs represent for these different conditions, (A) the intracellular lactate concentrations determined after 6 and 24 hours of treatment, (B) the extent of cell proliferation after 24, 48 and 72 hours, (C) IL-8 mRNA levels as determined by qPCR and (D) IL-8 protein expression as determined by ELISA. **P<0.01, n=3-4. MCT1 silencing with dedicated siRNA (vs sham conditions and control siRNA) was confirmed (72 hours post-transfection) by qPCR (E) and by immunoblotting as shown in a representative Western blot (F); β-actin signal is used as a gel loading control. **P<0.01, n=3-4.

Figure 2. Lactate stimulates NFκB activity in endothelial cells in a MCT1-dependent manner. A. Bar graph represents the extent of p65 subunit detected in a NFκB TransAM assay from nuclear extracts of endothelial cells exposed for 6 hours to glucose- or lactate (10 mM)-containing medium (P<0.01, n = 3). Other bar graphs represent the NFκB activity (normalized per cell numbers) determined using a dedicated luciferase reporter assay (see Methods) from endothelial cells cultured for 6 hours in glucose- (open bars) or lactate- (black bars) containing medium, (B) after transfection with a MCT1-targeting siRNA (vs sham conditions and control siRNA), (C) after treatment with 10 µg/mL anti-IL-8 blocking antibody or 500 nM Evodiamine,
(D) after transfection with a IL-8-targeting siRNA (vs control siRNA); each bar represents the mean ± sem of three independent experiments (**P<0.01).

Figure 3. Lactate induces the NFκB/IL-8 pathway in endothelial cells in a ROS- and IKK-dependent manner. Bar graphs represent (A) NFκB activity determined using a dedicated luciferase reporter assay and (B) IL-8 mRNA expression determined by qPCR from endothelial cells cultured in the presence (or not) of 10 mM N-acetylcysteine (NAC) for 6 hours in glucose- (open bars) or lactate- (black bars) containing medium. Each bar represents the mean ± sem of three to four independent experiments (**P<0.01). (C) Graph represents the production of ROS (arbitrary units, a.u.), as determined using the cell permeant fluorescent indicator DCF-DA (25 μM), from endothelial cells exposed for the indicated periods of time to fresh medium containing glucose, lactate or both; n = 3, s.e.m are smaller than symbols. (D) Representative phospho-IκBα (serine 32 and 36) and total IκBα immunoblotting from endothelial cells exposed for 15 min to glucose, lactate or both; mean changes in signal intensity resulting from three independent experiments are indicated.

Figure 4. Lactate induces the NFκB/IL-8 pathway in endothelial cells in a 2-oxoglutarate/PHD2-dependent manner. Bar graphs represents NFκB activity determined using a dedicated luciferase reporter assay in the presence of (A) increasing concentrations of 2-oxoglutarate (2-OG) and (B) after PHD2-targeting siRNA transfection in glucose- (open bars) or lactate- (black bars) (**P<0.01 vs corresponding glucose condition, §§P<0.01 vs corresponding sham glucose condition, n =3). (C) Bar graph represents IL-8 mRNA expression determined by qPCR from endothelial cells treated as above (PHD2 siRNA or 1 mM 2-OG), in the presence (or
not) of flavoenzyme inhibitor diphenyleneiodonium chloride (DPI) (10 µM) for 6 hours in glucose- (open bars) or lactate- (black bars) containing medium (**P<0.01, §§P<0.01 as above, n =3). (D) PHD2 silencing with dedicated siRNA (vs sham conditions and control siRNA) was confirmed (24 hours post-transfection) by qPCR (p<0.01, n=3).

**Figure 5. Lactate efflux through tumor cell MCT4 stimulates IL-8 expression in endothelial cells and promotes tumor vascular development in vivo.** WiDr colorectal cancer cells were sham or stably transfected with a MCT1- or MCT4-targeting shRNA. Representative pictures of (A) MCT1 and (B) MCT4 immunostaining. Graphs represent the time course of lactate release from shMCT1 WiDr cells (A, lower panel) and shMCT4 WiDr cells (B, lower panel) cultured under normoxic (open symbols) or 1% O2-hypoxia (black symbols) (*P<0.01, n=4). (C) Graph represents the in vitro proliferation rate of shMCT1- or shMCT4 WiDr cells (n=3). (D) Bar graph represents IL-8 mRNA expression determined by qPCR from endothelial cells cultured for 6 hours in the presence of conditioned medium (CM) of shMCT1- or shMCT4 WiDr cells maintained (for 72 hours) under normoxia (open bars) or hypoxia (black bars) (*P<0.01, n=3). (E) Representative pictures of tumors removed from mice injected (14 days before) with Matrigel plug containing 5x10^4 endothelial cells mixed either with 5x10^5 shMCT1 WiDr cells or 5x10^5 shMCT4 WiDr cells. Bar graph (E, lower panel) represents the mean tumor volume of corresponding tumors (n = 5; P<0.01). Bar graphs represent the intratumor lactate (F) and IL-8 (G) concentrations in the tumors described in E. Representative pictures of CD31 immunostaining, laser Doppler imaging (including reconstituted tumor photograph) of WiDr (H) and MDA-MB231 (I) tumors. Quantifications of corresponding microvascular density and perfusion are presented in bar graphs (H and I, bottom panels).
Figure 6. Lactate stimulates IL-8-dependent angiogenesis in vitro and in vivo in a MCT1-dependent manner. (A) Bar graph shows the extent of (24 h) migrated endothelial cells (per microscopic field) through Matrigel coated-filters in response to 5 ng/mL recombinant IL-8 (in glucose medium) or in response to lactate (added to the upper well compartment), including after cell transfection with a dedicated IL-8 targeting siRNA or concomitant incubation with a blocking anti-IL-8 antibody, 10 mM N-acetylcysteine (NAC) or 500 nM evodiamine (***P<0.01, n=4). (B) Representative pictures of (24 h) tube formation in glucose- or lactate-containing medium from Matrigel-plated endothelial cells after transfection with a control siRNA, a MCT1-targeting siRNA or an IL-8-targeting siRNA with or without addition of recombinant IL-8 (C) Bar graph represents the quantification of tubulogenesis experiments as described in Figure 6B as well as in similar assays performed in the presence of evodiamine, NAC or 2-oxoglutarate and after transfection with PHD2-targeting siRNA (***P<0.01 vs corresponding glucose conditions, §§P<0.01 vs sham condition, n=4). (D) Bar graph represents the mean volumes of tumors removed from mice injected (14 days before) with Matrigel plug containing 5x10^5 wild-type WiDr cells mixed with 5x10^4 endothelial cells in the presence (or the absence) of an anti-IL-8-blocking antibody or following pre-treatment of HUVEC with a MCT1-targeting siRNA (n = 5-6 mice per group; **P<0.01). (E) Representative pictures of CD31 immunostaining of corresponding tumor sections.
Figure 1

A

B

C

D

E

F

[Images of bar graphs and line graphs showing cellular lactate levels, cell density over time, IL-8 mRNA expression, and protein expression for different conditions.]
Figure 2

A

NFκB transAM
p65 binding (a.u.)

Glucose  Lactate

0.0  1.0  2.5

B

NFκB activity
(fold induction)

Sham  Ctrl siRNA  siMCT1

Glucose  Lactate

0  5  10  15  20

C

NFκB activity
(fold induction)

Control  anti-L3 Ab  adriamycin

Glucose  Lactate

0  5  10  15  20

D

NFκB activity
(fold induction)

Ctrl siRNA  MCT1 siRNA  IL-6 siRNA

Glucose  Lactate

0  5  10  15  20
Figure 3

A. NFκB activity (fold induction) over time with Glucose and Lactate.

B. IL8 mRNA expression (fold induction) with Glucose and Lactate.

C. ROS production (a.u.) with Glucose, Lactate, and Glucose + Lactate.

D. Western blot analysis showing P-IkBα and IkBα with Glucose and Lactate treatments.
Figure 4

A

NFKB activity (fold induction)

[oxoglutarate] (mM)

sham 0.03 0.1 0.3 1 3

Glucose Lactate

** **

B

NFKB activity (fold induction)

sham siPHD2

Glucose Lactate

**

C

IL-8 mRNA (fold induction)

sham siPHD2 DPI DPI + siPHD2 2-O-G

Glucose Lactate

** $\$\$

D

PHD2 mRNA expression (%)

sham ctrl siRNA siPHD2

**
Figure 6

A

B

C

D

E

ctl siRNA  siMCT1  siIL-8  siIL-8 + rec IL-8

glucose  lactate  glucose  lactate  glucose  lactate  glucose  lactate

Tube formation (intersections #)  Mean tumor volume (mm³)

control  anti-IL8 Ab  ctl siRNA-EC  siMCT1-EC
Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis

Frédérique Végran, Romain Boidot, Carine Michiels, et al.

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