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Regulation of vascular endothelial growth factor by metabolic context of the cell

V. B. Sameer Kumar · S. Binu · S. J. Soumya · Haritha K. · P. R. Sudhakaran

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Abstract Expression of vascular endothelial growth factor, major endothelial specific glycoprotein growth factor that promotes angiogenesis is regulated at transcriptional, post transcriptional and posttranslational levels. One of the key posttranslational modifications involved in regulating the angiogenic potential of VEGF is covalent modification involving polyADP ribosylation. Major factors contributing to the regulation of VEGF include factors relating to hypoxia, growth factors and cytokines and hormones. Apart from these, the metabolite status of the cell as sensed by various metabolite regulators can influence the angiogenic potential. Changes in the metabolite status of the cell occur during different conditions associated with excessive or insufficient angiogenesis contributing to pathology. Effect of metabolites, as exemplified by certain metabolites such as lactate, citrate, sarcosine, metabolites of arachidonic acid on angiogenesis through the regulation of expression of VEGF as well as its angiogenic potential through polyADP ribosylation is discussed.

Keywords VEGF · PolyADP ribosylation · Metabolites · Angiogenesis

V. B. S. Kumar · H. K.
Department of Biochemistry & Molecular Biology, Central University of Kerala, Kasaragod, Kerala, India

S. Binu · S. J. Soumya · P. R. Sudhakaran
Department of Biochemistry, University of Kerala, Karyavattom, Trivandrum, Kerala, India

S. J. Soumya · P. R. Sudhakaran
Inter-University Centre for Genomics and Gene technology, University of Kerala, Karyavattom, Trivandrum, Kerala, India

P. R. Sudhakaran (✉)
Department of Computational Biology and Bioinformatics, University of Kerala, Karyavattom, Trivandrum, Kerala 695 581, India
e-mail: prslab@gmail.com

Introduction

Angiogenesis is the process of formation of new blood vessels from the preexisting vasculature and is important in both physiological and pathological conditions. During angiogenesis quiescent endothelial cells (ECs) undergo a complex sequence of events that includes the secretion of metalloproteases and other proteases that degrade the extracellular matrix, cell migration into the newly created space, proliferation, and neo vessel formation [1]. There are a number of factors that promote or inhibit angiogenesis and a balance between both positive and negative regulators controls angiogenesis [2]. In angiogenesis the co-ordination between multiple participating cells occurs through the action of secreted factors such as growth factors, cytokines, chemokines, guidance molecules and their cognate receptors of which, one of the most studied glycoprotein is vascular endothelial growth factor (VEGF) and its receptor, VEGFR. Oxygen tension and metabolite status within the cell can be linked with the angiogenic state of the cell. When oxygen availability to the cell is limited, there occurs alteration in the oxidative process within the cell resulting in the accumulation of certain metabolites such as lactate. Apart from this there can be accumulation of metabolites due to their altered metabolism associated with certain pathological conditions. When the oxygen status is less or when the metabolite status is altered, cells sense the situation through sensing mechanisms such as hypoxia inducible factors (HIF) and metabolic regulators. This causes an increase in the expression of angiogenic factors particularly VEGF thereby inducing an angiogenic response. The role of VEGF in linking the metabolic microenvironment of the cell with its angiogenic potency is discussed below.

Vascular endothelial growth factor

VEGF is an endothelial cell –specific mitogen that stimulates proliferation,migration and tube formation of endothelial cells and functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels [3, 4]. Several types of cells particularly, cancer cells, endothelial cells and vascular smooth muscle cells produce VEGF and modulate the endothelial function through autocrine and paracrine pathways [5, 6]. VEGF A is produced in multiple forms of which the common form is VEGF165 which is a heparin binding glycoprotein with a single glycosylation site and secreted as a homodimer of approximately 45 kDa size, stabilized by intra and interchain disulphide bonds [7]. Activity of VEGF is regulated transcriptionally, translationally and post translationally. Glycosylation and poly ADP ribosylation are the two major post translational modifications which affect the biological activity of VEGF as an angiogenic factor [8]. The biologically active form of VEGF165 is glycosylated at Asn 74 and appears as homodimers of 23 kDa monomeric units [9]. Experiments using inhibitors of glycosylation and mutation of the N-glycosylation site suggest that glycosylation of VEGF is critical for its secretion [10]; but it does not appear to be critical for its binding to the VEGFR2 receptor. Though the glycosylation status of VEGF does not appear to be important for receptor engagement or biological function, a recent report on VEGF- independent tumor angiogenesis suggest that glycosylation of VEGFR2 is important in galectin 1 dependent cell surface retention of the receptor and its activation [11]. The binding of VEGF165 to endothelial cells is strengthened by glycosaminoglycan (GAGs) and the cell surface heparan sulphate is a coreceptor for VEGF. Glycosylated VEGF165 binds to heparin with higher affinity than non-glycosylated one [12].

The most important post-translational modification of VEGF, which also affects its angiogenic potency, is poly-ADP ribosylation (PAR) [8, 13, 14]. PolyADP ribose (PAR) is a polymer of ADP ribose units linked by unique 1, 2 ribose phosphate–phosphate linkages and display several branching points [14]. PAR shows structural heterogeneity with respect to the length and the extent of branching and may consist of upto 200 ADP ribose units with approximately one branch per 20–50 monomeric units. Polymerisation of ADP ribose moiety of NAD is catalysed by poly (ADP-ribose) polymerase. It catalyses the transfer of ADP ribose moieties from NAD to acceptor protein producing PAR linked proteins. PAR modification of VEGF produced by endothelial cells and macrophages has been observed. The extent of PAR modification of VEGF produced by endothelial cells was dependent on the microenvironment of the cells as evidenced by a reduction in PAR modification in cells maintained in culture on fibronectin or laminin [15, 16]. The extent of PAR modification of VEGF is important for its biological activity; a reciprocal relation between the extent of PAR modification and angiogenic potential has been observed. Decrease in PAR modification of VEGF caused an increase in its angiogenic potential. In murine macrophages VEGF produced by the constitutive pathway is normally ADP ribosylated nonangiogenic form whereas VEGF produced by LPS activated cells was less ribosylated with greater angiogenic potential [8]. It has been reported that PAR modification of VEGF in macrophages is regulated by nitric oxide and also that nitric oxide activates cytosolic ADP ribosyl transferase [8, 17]. But VEGF being a secretory protein processed through the endoplasmic reticulum and golgi, the subcellular site of ADP ribosylation of VEGF is not clear. Though ER pool of NAD has been reported, no PARP isoform has been reported yet to ADP ribosylate proteins in the microsomal fraction. NAD being the substrate, changes in the energy homeostasis and oxidation reduction status can affect the level of PAR modification of VEGF in an NAD dependent manner . Oxygen tension and the level of oxidised substrates can affect the ratio of NAD/NADH. Higher the relative level of NAD more amount of the PAR modified form of VEGF which is less angiogenic will be formed; relatively lower levels of NAD produce less PAR modification of VEGF, increasing its angiogenic potential . Thus NAD dependent PAR modification of VEGF can play a critical role in linking metabolite status and oxygen tension with the angiogenic potential of a cell.

The expression as well as the post translational modification of VEGF can be modulated by the metabolic microenvironment of the cells. Hypoxia, growth factors such as EGF, PDGF, TGF β, and TNF α, hormones such as estrogens and progestins have been reported to regulate the expression of VEGF gene. The regulation of the expression and the levels of VEGF and its biological activity by various metabolites is discussed in the following sections.

Oxygen sensors and regulation of VEGF expression

Under normal physiological conditions, ECs remain quiescent; however they exhibit a remarkable phenotypic dynamicity, in terms of their ability to divide, migrate, and form neo vessels during exposure to hypoxic or other pro-angiogenic conditions. The various aspects relating to oxygen sensing and relation of metabolism to the process of angiogenesis has been reviewed by Fraisi *et al.* [18]. The reduced oxygen tension is sensed by the ECs by a variety of mechanisms which include, oxygen sensitive NADPH oxidases, endothelial nitric oxide synthase (eNOS), and heme oxygenase. In addition to these oxygen sensing mechanisms, the most important sensory mechanism involves the family of hypoxia inducible factors (HIF), which is regulated by a family of O_2-dependent prolyl hydroxylase domain-containing enzymes (PHD 1–3) that act as an oxygen-sensing/transducing factor

[19, 20]. Hydroxylation of a specific proline in hypoxia-inducible factor-1α (HIF-1α) by the PHD enzyme directs its constitutive degradation [21, 22]. Under hypoxic condition, failure of the oxygen-dependent PHD to hydroxylate the proline interrupts the targeting of HIF for proteasomal degradation; further, failure to hydroxylate an asparagine residue in the transactivating domain of HIF results in an increased transactivating potential [23]. As the degradation of HIF- 1α is inhibited under hypoxic condition, it dimerizes with HIF-1β and causes binding to HIF responsive elements (HREs) within target genes. HIF directly activates the VEGF gene by binding to its HRE causing upregulation of the expression of VEGF [24]. Apart from responding to limited oxygen availability through aforementioned oxygen sensing mechanisms leading to production of VEGF by the cells, as discussed in detail in a following section, limited oxygen availability can affect oxidative metabolism leading to alteration in the levels of several metabolites that can affect angiogenesis.

Reactive oxygen species (ROS) are naturally produced by cells through aerobic metabolism, and high levels of ROS in the cells are associated with pathogenesis of several diseases. The major source of ROS in cells is NADPH oxidase which consists of Nox1, Nox2 (gp91phox), Nox4, p22phox, p47phox, p67phox and the small G protein Rac1 [25]. Xia *et al.* reported that ROS regulated hypoxia-inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) expression in ovarian cancer cells. Elevated levels of endogenous ROS were shown to be required for inducing angiogenesis and tumor growth. Further they showed that NOX4 knockdown in ovarian cancer cells could decrease the levels of VEGF and HIF-1α and tumor angiogenesis [26].

Metabolic sensors as key regulators of VEGF expression

There are several metabolic regulators that sense alteration in the status of different metabolites in the cell. Some of the key metabolic regulators that modulate angiogenesis include PGC-1α, PPAR γ, AMPK, FOXO *etc.* among which, PGC-1α is a potent modulator of oxidative phosphorylation, mitochondrial biogenesis and respiration [27, 28]. Arany *et al.* [29] reported that PGC-1 α stimulates angiogenesis by inducing VEGF expression through interacting with estrogen receptor related (ERR- α) pathway. It also regulates energy production in skeletal muscle and the heart by stimulating mitochondrial biogenesis and cellular respiration [30]. A recent study also showed increase in PGC-1 α on local overexpression of VEGF-A in brown adipose tissue indicating a probable metabolic regulatory function of VEGF [31]. AMP-dependent kinase (AMPK) is important in regulating the metabolism and angiogenesis [32] which may act through its downstream effector, mTOR. During conditions of energy starvation AMPK is activated leading to upregulation of VEGF expression. Chatterjee *et al.*, [33] has identified an autocrine feed-forward loop in tumor cells where, tumor-derived VEGF stimulates VEGF production *via* VEGFR2-dependent activation of mTOR, substantially amplifying the initial pro-angiogenic signal. Disruption of this feed-forward loop by chemical perturbation or knockdown of VEGFR2 in tumor cells dramatically inhibited production of VEGF *in vitro* and *in vivo*. It senses energy deprivation, and promotes utilization of the fuel and also induces angiogenesis through VEGF expression [34]. Peroxisome proliferator-activated receptors (PPARs) are transcription factors that regulate nutrient utilization and energy homeostasis by modulating glucose and lipid metabolism. While the PPARα and PPARγ inhibit angiogenesis, PPARβ activates angiogenesis. Panigrahy *et al.* [35] reported that PPARα has a role in inhibition of tumor cells, endothelial cell proliferation, angiogenesis and inflammation. PPARβ, a regulator of lipid oxidation, increased the expression of VEGF and thereby enhanced EC proliferation, microvessel sprouting, and tube formation [36, 37]. Forkhead transcription factors (Foxo), Foxo1 and foxo3 which are predominant transcription factors expressed in endothelial cells are involved in lipid metabolism [38]. FOXO family of transcription factors are regulated by NAD dependent deacetylase, SIRT-1. Balayia *et al.* reported that elevated SIRT1 levels resulting from hypoxic challenge activate HIF-2α and promote VEGF transcription. Increased VEGF levels after hypoxia, and the subsequent decrease after inhibiting SIRT1 activity as reported in this study, establish a causal relation between SIRT1 and HIF-2α [39]. It has thus become amply evident that changes in metabolite status can be sensed by different sensing mechanisms within the cell . This can modulate signaling pathways and transcription factors leading to modulation of VEGF expression in both endothelial and non endothelial cells which become source of stimuli for endothelial cell activation and induction of angiogenic phenotype. Alteration in the levels of certain metabolites causing changes in the levels and biological activity of VEGF is discussed in a following section.

Unfolded protein response associated changes in VEGF expression

Signaling networks controlling the metabolic turnover of nutrients, such as amino acids and glucose, are also integrated with ER function. The ER is particularly sensitive to glucose availability and energy fluctuations. Disruption of ER homeostasis causes accumulation of unfolded and misfolded proteins in the ER resulting in a condition referred to as ER stress. Cells cope up with ER stress by activating the Unfolded Protein Response (UPR) [40, 41]. The UPR is initiated by three ER transmembrane proteins: Inositol Requiring 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor 6

(ATF6). These three master regulators sense and interpret protein folding conditions in the ER and translate this information across the ER membrane to regulate downstream effectors. During the UPR the regulation of metabolic pathways in the cell is affected [42]. Glucose synthesis or breakdown pathways are transcriptionally regulated during the Unfolded Protein Response (UPR). For example, genes involved in glucose output and glycogen synthesis such as glycogen synthase 1 are targets of XBP1 [43]. Ghosh *et al.*, showed that IRE1α, PERK, and ATF6α regulate VEGFA mRNA expression under various stress conditions. They further proved that the induction of VEGFA by IRE1α, PERK and ATF6 involves activation of transcription factors, spliced-XBP-1, ATF4 and cleaved ATF6 respectively [44]. Although changes in the availability of precursors due to dysregulation of metabolic pathways can affect the glycosylation reaction, no direct link between changes in metabolite status associated with ER stress and alteration in VEGF production has been demonstrated. However studies using inhibitors of protein glycosylation have shown alteration in the production of VEGF during UPR.

Regulation of VEGF by metabolites

As indicated before, ECs are equipped with machinery to sense oxygen and metabolite status. They are also equipped with metabolic mechanisms to survive stress and hypoxic conditions while sprouting in avascular tissues and to generate sufficient energy for the biosynthesis of macromolecules required for rapid cell proliferation. Cells in avascular tissue experience oxygen and nutrient insufficiency and associated metabolic alteration and activate oxygen and metabolite sensing mechanisms. Apart from alteration in the levels of normal metabolites accumulation of unusual metabolites may occur in pathological conditions. The role of certain such metabolites in angiogenesis by modulating the expression of VEGF and its biological activity is discussed below (Fig. 1).

Regulation by metabolites of carbohydrate metabolism The most important metabolic product of glycolysis regulating the process of angiogenesis is lactate. Under hypoxic conditions, (the condition existent at any of the angiogenic source site) cells switch over to anaerobic metabolic pathways, with concomitant

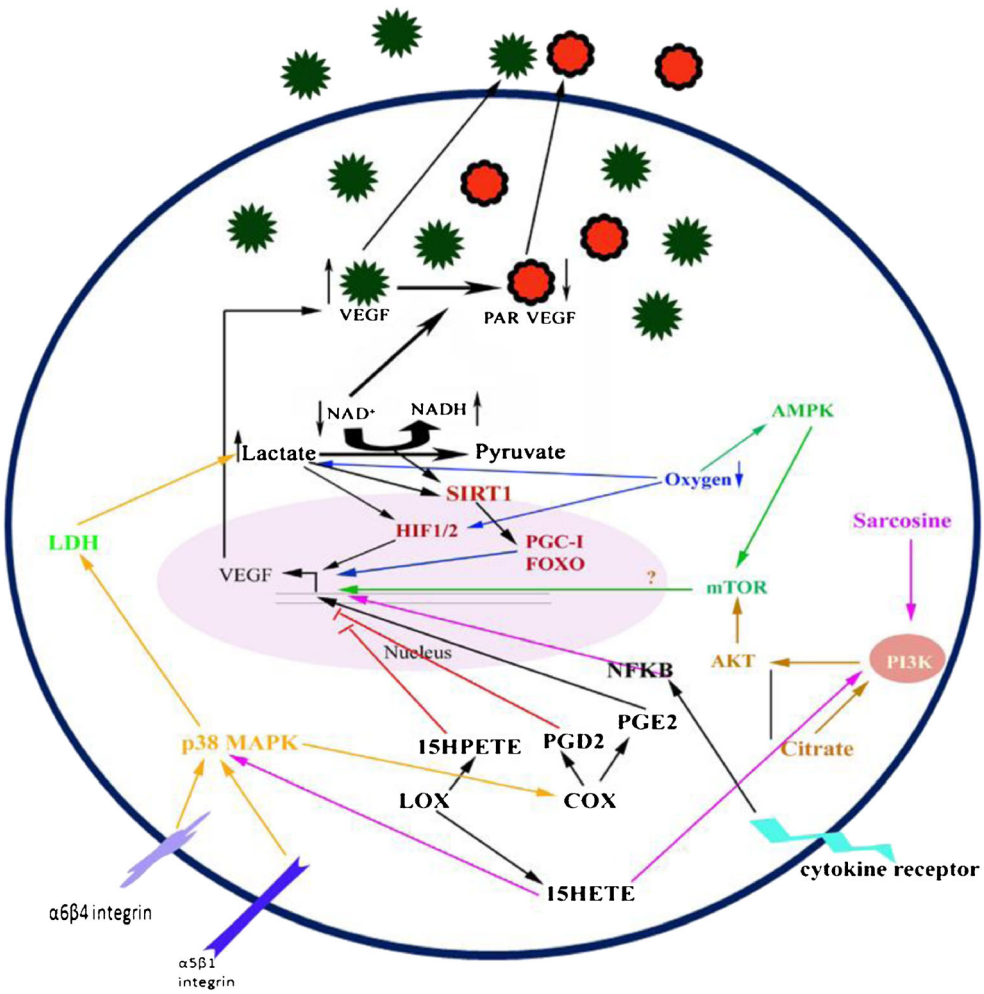

Fig. 1 Regulation of VEGF by various metabolites Cells sense the metabolite status within its microenvironment using several metabolic sensors and regulate angiogenic glycoprotein, VEGF. The secreted VEGF elicits an angiogenic response either in an autocrine or a paracrine manner. In autocrine signaling,secreted VEGF binds to the receptor VEGFR expressed on the surface of ECs

increase in lactate. Wound healing and tumor development are two important conditions where angiogenesis plays an important role. In both the conditions, the level of lactate in tissue increases up to 10–15 mM as compared to 1.8–2 mM under normal physiological conditions [45–47]. Thus, lactate is an anaerobic metabolite in the presence of anoxia, a hypoxic metabolite in the presence of dyoxia, and an aerobic metabolite under conditions of adequate oxygen supply [48]. Studies reported from our laboratory and that from others showed lactate to exert proangiogenic effect in different angiogenic model systems [49, 50]. Our findings suggest that the pro-angiogenic effect of lactate is mediated by at least two distinct mechanisms *viz* upregulation of angiogenic factors, most importantly VEGF and by post translational modification of VEGF, ie the poly ADP ribosylation of VEGF. Lactate induces the expression of VEGF in both macrophages and endothelial cells [8, 13]. Lactate exerts its effect by inactivating PHDs [51] and thereby triggering HIF driven VEGF expression [52, 13]. We have also observed that treatment of HUVECs with lactate caused a reduction in the level of PAR modification of VEGF, thereby enhancing its biological activity [13]. The decrease in the level of PAR modification of VEGF is dependent on cellular NAD^+ levels which can be modulated by the presence of metabolites such as lactate. Further, under conditions where there was shift in the lactate dehydrogenase isoenzyme pattern in favour of utilisation of NAD, PAR modification of VEGF was reduced and its angiogenic potency was high [15, 53]. Though it is known that PAR modification can reduce the angiogenic potency of VEGF, the precise mechanism is not clear yet.

Citrate is a key metabolite that contributes to both mitochondrial energy metabolism and as a source of carbon for cytosolic synthesis of fatty acid and cholesterol critical for membrane biogenesis. Under normoxic conditions, citrate is synthesized from acetyl CoA formed from glucose, but in hypoxic conditions, the much needed citrate is formed by rerouting glutamine metabolism in mitochondria to citrate synthesis [54]. Citrate is reported to have altered metabolism in tumor cells particularly in prostate tumor cells. The prostate gland cells are characterized by high levels of citrate (40–150 μmol/g tissue) [55]. Recently we observed that increased concentration of citrate cause endothelial sprouting and induction of angiogenic phenotype in different angiogenic model systems. Citrate accumulation in ECs promotes activation of PI3K-Akt-mTOR pathway and increased VEGF production [56]. Citrate caused these effects only at high concentrations suggesting that the angiogenic effect may be produced only in conditions of excessive accumulation of citrate.

Regulation by metabolites of Lipid metabolism The major metabolites derived from lipids capable of influencing angiogenesis include lipid mediators of inflammation. Eicosanoids, the metabolites of cyclo-oxygenase (COX) and lipoxygenase (LOX) action on arachidonic acid, act as key modulators of angiogenesis by regulating the expression of pro- and/or anti-angiogenic factors [57, 58]. They include prostaglandins and thromboxanes generated through the COX pathway, and 15 (*S*)- hydroperoxyeicosatetraenoic acid [15 (*S*)-HPETE] and 15 (*S*)-hydroxyeicosatetraenoic acid [15 (*S*)-HETE] generated through the 15-LOX pathway [59]. Recent reports suggest that 15-LOX metabolites, 15 (*S*)- HPETE and 15 (*S*)-HETE, exhibit opposing angiogenic responses in different model systems. It appears that the relative rate of conversion of 15 (*S*)-HPETE to 15 (*S*)-HETE during inflammation can affect angiogenesis, in a manner dependent on the cellular level of peroxidases, which catalyse the reduction of 15 (*S*)-HPETE to 15 (*S*)-HETE [60]. 15 (*S*)-HETE promotes angiogenesis by inducing the expression of VEGF through the activation of PI3K-AkT-mTOR pathway while 15 (*S*)-HPETE inhibits angiogenesis probably by inducing apoptosis [60, 61]. Similar divergent effects of COX metabolites on angiogenesis have been reported. Prostaglandin E2 is proangiogenic and prostaglandin D2 is anti-angiogenic in nature [62]. PGE2 is reported to induce the expression of VEGF. The relative rate of conversion of prostaglandin H2 by PGE synthase and PGD synthase is known to contribute to the opposing effects [63].

Apart from this, steroids such as 17ß-estradiol (E2) has been reported to directly regulate VEGF gene transcription in endometrial cells and in Ishikawa adenocarcinoma cells. Estradiol-induced gene transcription is ER dependent and is activated through a variant ERE localized ’1.5 kb upstream from the VEGF transcription start site [64]. Further oxysterol, the ligand for LxR can induce the expression of VEGF through PPAR/LxR heterodimerisation and activation.

Regulation by metabolites of protein/aminoacid metabolism Although amino acid deprivation (AAD) - induced VEGF expression has been reported, the results are conflicting. Drogat *et al.* [65] reported that although glutamine deprivation potently induces VEGF mRNA expression, it leads to the decrease of VEGF expression at the protein level in A549/8 human carcinoma cells. In contrast, Marjon and colleagues found that glutamine deprivation caused a marked induction in both transcription and the secretion of VEGF in a human breast adenocarcinoma cell line (TSE cells) [66]. A recent study showed that the GCN2/ATF4 pathway is involved in amino acid deprivation-induced expression of proangiogenic mediators in tumors. Suppressing GCN2 inhibited tumor growth and blood vessel formation in xenograft tumors grown in SCID mice. It is suggested that AAD acts through a distinct kinase, GCN2 to phosphorylate eIF-2α, which in turn leads to reduced global translation with the exception of select genes such as *ATF4*. Activation of GCN2, PERK, and their downstream target ATF4 is central for tumors to adapt to nutrient deprivation in the TME (Tumor microenvironment), eliciting gene expression that enhances tumor angiogenesis, thereby promoting tumor survival and proliferation [67].

Sarcosine, an intermediate of glycine metabolism, is a normal physiological metabolite, the concentration of which in normal blood is reported to be 1.59±1.08 μM/L [68]. However, sarcosine level increases several fold in tissue and urine of prostate cancer patients [69, 70]. Recently we observed the angiogenic effect of sarcosine at concentrations from 0.1 mM and no major effect was observed at lower concentrations. Sarcosine produced upregulation of VEGF mediated through PI3K/Akt/mTOR pathway [71]. Further, there was decrease in PAR modification of VEGF. During ischemia, purine and pyrimidine nucleotides are degraded and their metabolites accumulate, of which adenosine and nicotinamide have been reported to induce vascular growth; but their effects, if any on PAR modification of VEGF have not been examined.

Conclusion

Metabolic status of the cell can alter angiogenic potential of the cell by activating signaling pathways relevant to angiogenesis or by modulating VEGF biology particularly its PAR modification or both. As indicated in the figure, the expression levels of VEGF as well as its biological activity are regulated by metabolites. Metabolic intermediates of glycolysis, amino acid metabolism, lipid metabolism as well as TCA cycle that may accumulate in certain tumors, wound healing process, ischemia and hypoxic states, are capable of modulating angiogenesis through VEGF. Moreover when metabolites such as lactate, citrate and sarcosine accumulate within ECs, the cells upregulate VEGF which elicits an autocrine effect through VEGFR expressed by ECs. ECs sense accumulation of citrate and sarcosine by the nutrient sensing kinase mTOR (mammalian target of rapamycin). These metabolites cause upregulation of VEGF expression and at posttranslational level decrease PAR modification of VEGF thereby increasing its angiogenic potency. Further, cytokine signalling as well as integrin signalling can also culminate in regulation of key metabolic pathways, which in turn regulate the expression or post transcriptional modification of VEGF.

Though glycosylation at the ER is critical for secretion of VEGF, it is not clear where the PAR modification occurs. NAD being the principal substrate required for PAR modification, changes in the metabolite and cellular redox status that can affect NAD levels, can affect PAR modification. Lactate is capable of regulating the cellular redox status by changing the levels of NAD^+ in the NAD/NADH pool. Although NAD pool is reported in ER [72], it is not clear how NAD status in ER is related to that in cytosol and mitochondria. Though an isoform of PARP is reported to be localised in ER membrane [73], the details of PARP involved in PAR modification of VEGF are not available.

A reciprocal relation between PAR modification and angiogenic activity of VEGF is observed. But it is not clear how PAR modification can regulate the angiogenic potential of VEGF. It is possible that introduction of a highly negatively charged PAR moiety may affect its binding to VEGF receptor on the cell surface and/or receptor activation and signaling .

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