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Review

Nitric oxide delivery to cancer: Why and how? ☆

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ABSTRACT

Hypoxia and blood flow heterogeneities are characteristics of solid tumours and are major obstacles for therapy. Exploiting the biology of nitric oxide (NO), a small radical with multiple functions, is particularly attractive to circumvent these sources of resistance and to sensitise tumour to cytotoxic treatments such as radiotherapy and chemotherapy. Indeed, while NO mediates angiogenic effects, NO may also promote tumour perfusion, drug delivery and oxygenation. Different strategies to deliver NO to tumours and pertaining to the FECS-EJC award laureate's work are reviewed, with a focus on their therapeutic potential. The development of techniques to monitor how and to which extent NO delivery influences the phenotype of a given tumour in a given patient is also discussed.

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1. Introduction

Hypoxia is a hallmark of solid tumours and, through both direct and indirect effects, a fundamental problem for therapy.[1] Two main paradigms pertaining to cancer treatment and related to pO_2 instability in tumours can be singled out: the 'oxygen effect' and heterogeneities in blood flow.

The 'oxygen effect' refers to a reaction in which dioxygen directly reacts with a radical DNA (formed in response to ionising radiations or some chemotherapeutic drugs) to yield stable adducts.[2] This reaction is of critical importance for the stabilisation of DNA lesions that would otherwise be fully reversible. The pO_2 limit below which tissues are considered as radioresistant (radiotherapeutic hypoxia) is around 10 mm Hg,[3] a threshold very often passed in tumours.[4] Hypoxic niches escaping therapeutic sterilisation constitute nodes for tumour recurrence after treatment completion.

A more indirect form of hypoxia-driven resistance results from the inefficient tumour vasculature. Indeed, the status of the tumour vasculature, in both its structural and dynamic dimensions, profoundly influences the biodistribution of drugs delivered via systemic routes, including conventional chemotherapeutic drugs, as well as all forms of treatments (including small molecules, DNAs, proteins, antibodies or cells) that require optimal tissue penetration to display full therapeutic benefits. Angiogenesis, the extension of a

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pre-existing vascular network, is the most famous and probably the best characterised process to be controlled by oxygen.[5] In cells becoming hypoxic, evolutionary conserved oxygen sensors initiate distress pathways (for example, activation of hypoxia-inducible transcription factors such as HIF-1) leading to the production and secretion of pro-angiogenic agents (such as vascular endothelial growth factor [VEGF]).[6] Angiogenic vascular activation and extracellular matrix remodelling ensue, leading to the progressive colonisation of hypoxic areas by neovessels. The persistent nature of angiogenesis is a hallmark of tumours arising from and maintained by the perpetual imbalance between the oxygen supply by local vessels and oxygen consumption by actively proliferating tumour cells (TCs).[1] A pernicious consequence is that a significant subset of neovessels fails to properly mature. Multiple vascular abnormalities (including abundant fenestrations, shunts, dead-ends and lack of contractile coating) cause suboptimal perfusion and restrict oxygen, nutrient and drug delivery to the tumour.[7] At the therapeutic level, persistent angiogenesis thus participates in radio- and chemoresistance. This influence is best evidenced by the observation that increased tumour pO_2, radio- and chemosensitisation are early responses to some anti-angiogenic agents that initially smooth the vascular irregularities in tumours.[8–10] This paradigm has been termed 'vascular normalisation'.[11]

Alteration in the capacity of tumour blood vessels to autoregulate flow is another insidious consequence of hypoxia. Physiologically, fine-tuning of local perfusion is under the control of the vasodynamic balance in which vasodilators and vasoconstrictors accommodate changes in tissue needs. In tumours, however, this balance is altered. Seminal papers by Algire et al.[12] and by Gullino and Grantham[13] reported that various types of carcinomas and sarcomas favour an increase in the diameter of peripheral host arteries. The claim that tumour vessels exist in a basal state of maximal dilation which cannot be enhanced any further derives from this finding as well as other observations such as the minimal response of the tumour vasculature to some vasodilators[14–17] and structural abnormalities at the post-capillary level leading to giant capillaries.[18] However, the development of technologies aiming to evaluate tumour haemodynamics (see below) together with a largely underestimated literature addressing heat- or drug-induced modulation of blood flow should nuance this view.[19–21] There are actually many examples of tumour vascular beds being subject to spontaneous vasomotion and/or dynamic changes in vascular tone (see Ref. in 21).

Mature tumour vessels may arise from the progressive incorporation of intact arterial vessels from host tissues (referred to as tumour-feeding arterioles) or from the maturation of post-angiogenic capillaries, especially in slow-growing tumours.[22] These mature tumour vessels may exist in a state of basal constriction caused by the production of vasoconstrictors that outcompete vasodilators.[23,24] Endothelin-1 (ET-1) is a good example of a vasoconstrictor chronically produced in tumours in response to hypoxia.[25,26] ET-1 mediates basal constriction of mature tumour blood vessels,[24] and precludes optimal delivery of chemotherapeutic drugs to tumours.[27] *A contrario*, the production of nitric oxide (NO), the main progenitor of local vasodilation, is defective in vascular endothelial cells (ECs) residing in the tumour microenvironment.[15,23,28] Resistance to flow in tumours is yet aggravated by an abnormal blood rheology that finds its origin in the metabolic activity of TCs yielding microenvironmental acidification. Low pH limits the deformability of red blood cells (RBCs), causing uneven partitioning at vascular branches and, sometimes, vascular occlusion.[29–31] The propensity of RBCs to stack is further increased in areas of poor lymphatic drainage where cell metabolites and cell debris of tumour origin, and materials that extravasate from leaky neovessels, drain water off vessels through osmotic effects. Fluid accumulation also results in vascular compression in the tumour oedema.[32]

Several strategies have been tested to increase tumour blood flow and oxygenation in combination with chemo- and/or radiotherapy. They include the delivery of hyperoxic gases,[33,34] modifiers of blood viscosity,[34] anti-angiogenic agents in their 'vascular normalisation' mode[35] and a variety of vasoactive drugs and treatments.[21,36] Although some of these approaches are indisputably endowed with a high clinical potential, this review, in the context of the 2007 FECS-EJC award to Dr. Pierre Sonveaux, will focus on the use of NO as a potential sensitiser of tumours to chemo- and radiotherapy. NO is a radical gas with multifaceted biological actions, some of which are potentially exploitable to mount new anticancer strategies. General concepts will be exposed and illustrated by selected examples from our own work. The key questions addressed here are why and how one should deliver NO to cancer.

2. Nitric oxide and nitric oxide synthases

NO is one of the smallest endogenous molecules involved in biological functions. It is a free radical gas which diffuses in water and through cell membranes. Its high reactivity, however, limits its half-life in biological fluids. NO acts directly or indirectly as a precursor of more stable reactive nitrogen species (RNS). In particular, peroxinitrites arise from the reaction of NO with superoxide in response to oxidative stress. Localisation, concentration and duration govern the biological activities of NO (Fig. 1).

NO is endogenously produced by a family of homodimeric enzymes termed NO-synthases (NOSs) that catalyse the O_2– and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidation of L-arginine to L-citrulline and NO in the presence of cofactors (tetrahydrobiopterin, flavin adenine dinucleotide [FAD] and flavin mononucleotide [FMN]). Besides the three canonical NOS isoforms (neuronal NOS [nNOS/NOS1], inducible NOS [iNOS/NOS2] and endothelial NOS [eNOS/NOS3]), a fourth enzyme, mitochondrial NOS (mtNOS), was recently identified as the α isoform of nNOS.[37] Subcellular NOS distribution is dynamically regulated by acetylations and/or protein interactions. Major locales for NOS are for instance the caveolar signal transduction platform for eNOS[38] and the inner mitochondrial membrane for mtNOS.[37] These specific assignments bring the localisation of NO production in line with its biological activities: vasodilation and angiogenesis (eNOS at the endothelial/smooth muscle cell interface), inhibition of mitochondrial respiration (mtNOS in the close vicinity of cytochrome c oxidase), neurotransmission (nNOS located in the post-synaptic terminal of neurons and

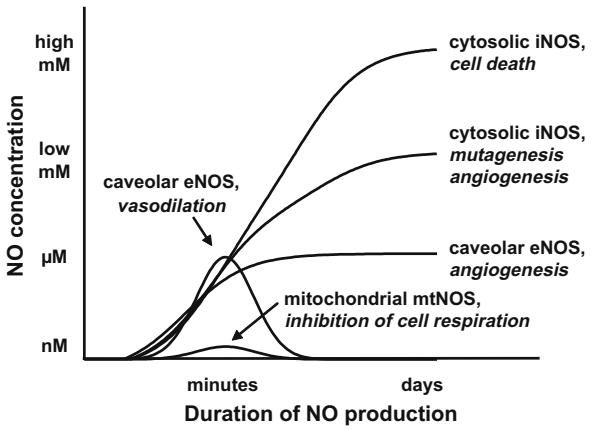

Fig. 1 – Concentration, duration and location govern the biological effects of nitric oxide (NO). NO is endogenously produced by nNOS, eNOS and iNOS. In mitochondria, the α chain of nNOS, mtNOS, is activated in an ATP-sensitive manner to produce nanomolar levels of NO. Through competition with oxygen for cytochrome c oxidase, mitochondrial NO acts as a reversible inhibitor of mitochondrial respiration and as a guardian of cellular energy homeostasis. Located in endothelial cell (EC) caveolae, eNOS produces micromolar concentrations of NO following calcium–calmodulin activation. Transient NO production accounts for vasodilation. A further phase of activation involving Akt1 recruitment and eNOS phosphorylations allows sustained NO production and the associated pro-angiogenic effects, notably in response to vascular endothelial growth factor (VEGF) stimulation. In macrophages and inflammed cells, iNOS constitutively releases millimolar amounts of NO. Persistently high NO promotes cytotoxic damage and cell death, whereas the low millimolar levels of NO produced during chronic inflammation rather promote mutagenesis and angiogenesis.

at neuromuscular junctions) and inflammation (iNOS in the cortical area of immune cells).[39,40] The regulation of these functions, of high relevance for cancer progression and treatment, is discussed below.

NOS activation requires the binding of calcium–calmodulin (CaM) to a recognition motif that accelerates the electron flux from a reductase to an oxygenase domain across the two protein monomers.[41] Structural differences account for a differential affinity of NOS isoforms for CaM: whereas iNOS tightly binds CaM at ambient calcium levels and is thereby constitutively active,[42] the activation of nNOS and eNOS requires an elevation in intracellular calcium owing to a lower affinity for CaM.[43–45] iNOS distinguishes from other NOS by its prolonged production of millimolar amount of NO.[42] It is also proposed to be part of a protein aggregate in which superoxide production by NADPH oxidase and NO production by iNOS readily yield peroxinitrites that, rather than NO, would be the main product of the iNOS reactome.[46] When produced at high millimolar levels, peroxinitrites and other RNS react with proteins and DNA to cause cell death (Fig. 1).[47] However, when oxygen and cofactors become limiting as it is the case during chronic inflammation, lower levels of NO and RNS are formed and promote mutagenesis and angiogenesis,[48] thereby bridging chronic inflammation and cancer initiation at the molecular level. iNOS-deficient mice show some degree of cancer protection (see Ref. in 49). The termination of iNOS activity normally proceeds through enzyme degradation by the calpain and ubiquitin-proteasome pathways.[50,51]

In contrast to the constitutively active iNOS whose activity is regulated at the transcriptional level, the activities of the constitutively expressed NOSs are finely regulated post-translationally. Upon activation, eNOS and nNOS produce micromolar and mtNOS nanomolar levels of NO (Fig. 1).

In ECs under basal conditions, eNOS is kept inactive because of a physical interaction with caveolin-1 (cav-1), the structural protein of caveolae[52] and a basal phosphorylation of a threonine residue (Thr495 in human eNOS sequence).[53,54] Acute eNOS activation is typically caused by agents that induce the release of calcium from intracellular stores.[55] Calcium activates the protein phosphatase 1 (PP1) to cause Thr495 dephosphorylation, thereby unravelling a docking site for CaM.[53,54] CaM recruitment displaces eNOS from its inhibitory interaction with cav-1, thus allowing short-term enzyme activation.[56] A burst of NO is produced, which, upon diffusion, stimulates the soluble guanylate cyclase (sGC) in the neighbouring smooth muscle vascular cells, causing cGMP-mediated relaxation.[57] NO-induced vasodilation is transient, mostly because the turnover rate of cGMP is high. Interestingly, however, the binding of CaM to eNOS also recruits the chaperon protein hsp90 to the complex, thereby providing a docking platform for the kinase Akt1 (also known as PKB).[58] Sustained eNOS activation proceeds through phosphorylation of a serine residue (Ser1177 in human eNOS sequence) by Akt1.[58–60] Other kinases including adenosine monophosphate protein kinase (AMPK) were also reported to catalyse Ser1177 phosphorylation.[61] Similar to iNOS, eNOS can also catalyse the formation of superoxide and RNS under circumstances of oxygen and/or cofactor depletion (uncoupled eNOS).[62] However, in contrast to iNOS, these species at low micromolar levels could convey physiological activities of eNOS (including sGC activation) through protein S-nitrosylation rather than cell death or mutagenesis.[63,64]

The regulation of nNOS activity shares some similarities with that of eNOS. In skeletal muscle cells, for example, CaM binding to nNOS displaces a basal inhibitory interaction with caveolin-3,[65] which subsequently allows the recruitment of hsp90 to the heterocomplex and enhances NO formation.[66] In the rat synaptosome, phosphorylations of nNOS on Ser1412 by Akt2 and on Ser847 by CaM-dependent kinase II (CaMKII) have been recently identified to switch the activity of the enzyme on and off, respectively.[67] mtNOS was also described as a client protein of Akt2,[68] which supports a physiological coupling between ATP levels and mtNOS-dependent inhibition of cell respiration to fine-tune energy production.[69]

The exquisite regulation of NOS isoforms and their distribution in different cell subpopulations in tumours offer opportunities to selectively modulate NO production to treat cancer. For example, acute eNOS activation (leading to transient vasodilation) and mtNOS inhibition (restricting mitochondrial oxygen consumption) may radiosensitise

tumours. Conversely, sustained iNOS activity could be of bad influence as it may promote mutagenesis and angiogenesis (Fig. 1).

3. Why should we deliver NO to tumours?

3.1. NO as a vasodilator

As stated before, tumour heterogeneities extend to the tumour vasculature that comprises leaky angiogenic vessels, immature neovessels that lack a coat of contractile cells, and vessels mature enough to autoregulate blood flow. The distribution of vessels between these subpopulations is quite variable. In human tumours, for example, histological analyses have revealed that the ratio of mature (i.e. pericytes-covered) *versus* immature vessels ranged from ~10% in glioblastomas and renal cell carcinomas to ~70% in mammary and colon carcinomas.[70] In a given tumour, angiogenesis is generally restricted to areas of active growth, whereas maturation is possible in resting areas where endogenous anti-angiogenic factors behave as physiological terminators of angiogenesis.[71] In fact, most experimental and human tumours contain a significant population of mature blood vessels with contractile cells and neural junctions that are able to regulate blood flow.[72–76] Their existence is of therapeutic interest because exploitation of the vascular reactivity offers a way to modulate blood flow together with vascular permeability, oxygenation and drug delivery.[77] The strategy consisting in exploiting the reactivity of mature tumour vessels to improve the efficacy of conventional therapy has been termed 'provascular' to contrast with anti-angiogenic and antivascular approaches that are destructive by nature.[21]

Evaluations of a broad range of vasoactive substances (reviewed in Ref. 78) have led to identify the ideal provascular treatment. First, this treatment should induce a vasodilation restricted to tumour blood vessels. Vasodilators lacking tumour specificity have indeed unpredictable effects on tumour perfusion and oxygenation inherent to (1) the lower density of functional vessels in tumours *versus* healthy surrounding tissues and (2) the spatial arrangement between host and tumour vessels (Fig. 2).[79] Identification of a differential reactivity between mature tumour and host vessels that could be targeted therapeutically is thus a necessary step to overcome the steal effect. Second, the ideal provascular treatment should have transient effects to limit in time the gain in oxygen and nutrients supplied to the tumour. Finally, to be clinically exploitable, the treatment should be applicable to a wide range of tumours at different stages of development.

3.2. NO as an inhibitor of cell respiration

Through different mechanisms, eNOS and mtNOS collaborate to modulate the oxygen input for optimal cell energy production: while eNOS-derived NO promotes oxygen delivery from dilated vessels, NO generated in mitochondria by mtNOS acts as an inhibitor of cell respiration (Fig. 1).

Although historically the mitochondrion has been singled out to be the primary target of the cytotoxic effects of NO, several lines of evidences concur to support that endogenous NO is also an important *physiological* regulator of cell respiration.[69] Mitochondrial NO is produced by mtNOS, strategically located at the inner mitochondrial membrane in the close vicinity of cytochrome *c* oxidase/complex IV, the terminal enzyme of the mitochondrial electron transfer chain that dictates the rate of oxidative energy production. Upon mtNOS activation by physiological stimuli (such as insulin),[68] mitochondrial NO inhibits cytochrome *c* oxidase at subunit II through a high-affinity, nanomolar competition for oxygen occupancy.[80,81] The local NO/O_2 ratio thereby provides a sensing mechanism that regulates oxygen consumption at the locale where it is used, and serves to accommodate cell energy production to energetic needs.[82] Of particular relevance for this mode of regulation is the absolute requirement of ATP for the phosphorylative activation of mtNOS by Akt2: Akt2 could serve as an ATP sensor to inhibit respiration when ATP is produced in excess.[69] This negative feedback, analogous to the 'Pasteur effect', could also help to average oxygen utilisation between cells at different distances from blood vessels, thereby allowing optimal oxygen penetration into tissues.[69]

Delivering physiological concentrations of NO to mitochondria is an attractive option for cancer treatment because it could redirect oxygen from a respiratory fate to the fixation of DNA damages occurring in response to radiotherapy. It is likely that NO coming from other sources than mtNOS could also act as an inhibitor of cytochrome c oxidase, as documented for the NO donor isosorbide dinitrate[83] or for eNOS activators such as insulin.[84]

3.3. NO as a direct radiosensitiser

Perhaps one of the most striking properties of NO is that it can radiosensitise hypoxic cells as efficiently as oxygen at concentrations that do not modify aerobic radiosensitivity.[85] For example, hypoxic mammary carcinoma cells exposed to the NO donors 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) or spermine (SPER)/NO before irradiation were reported to be almost equally as radiosensitive as aerobic cells.[86] Because it has a similar potential to oxygen in its ability to bind to other free radicals, NO was suggested to behave as an intrinsic radiosensitiser that could mimic the 'oxygen effect' in a hypoxic environment.[87] Using different NO-generating treatments, we recently provided indirect evidence that NO may have radiosensitising properties, based on a mismatch between the amplitude of the tumour reoxygenation effectively achieved and the radiotherapeutic response observed *in vivo*.[88] *In vitro* and *in vivo*, however, the lack of correlation between the measured levels of free NO and the radiosensitising activity may reflect a contribution of intracellular NO adducts and/or the additive nature of the effects of NO and oxygen on tumour radiosensitivity.[88,89] An approach to exploit the NO effect while avoiding hypotension would be to generate relatively high levels of NO selectively in tumours. This may be achieved, at least *in vitro*, by priming iNOS in hypoxic macrophages with pro-inflammatory cytokines.[90] High levels of NO can also be achieved through iNOS gene transfer after intratumoural injection.[91]

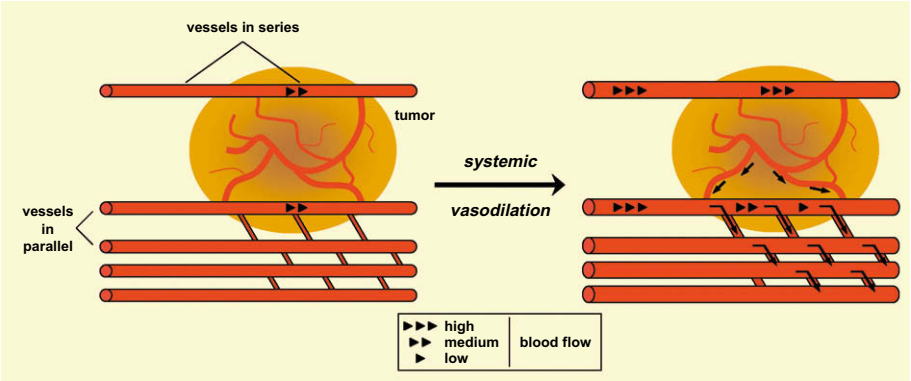

Fig. 2 – Vascular networking influences the tumour response to vasodilators. Changes in tumour blood flow caused by systemic vasodilators are fundamentally influenced by the structural relationship between the tumour vascular bed and the vascular bed in surrounding normal tissues. According to this model, an increase in the blood flow in normal tissues yields increased tumour perfusion if vascular beds are in series. However, if vascular beds are in parallel, an increase in the blood flow in normal tissues reduces tumour perfusion (vascular steal). In most tumours, beds in parallel and in series are interlaced, rendering unpredictable the overall effects of systemic vasoactive drugs on tumour perfusion and oxygenation.

3.4. *NO as a modulator of tumour immunity*

Tumour-associated macrophages (TAMs) use arginine to synthesise NO through iNOS or to produce ornithine through arginase activity.[92] Contrasting effects may arise from the balance between either products of the L-arginine metabolism: NO is primarily cytotoxic whereas polyamines derived from ornithine promote TC proliferation. Classically, NO-producing macrophages, whose prototypical activating stimuli are interferon gamma (IFNγ) and lipopolysaccharide (LPS), are termed M1. The tumouricidal activity of M1 is, however, dampened by the alternative activation regulated by Th2 cytokines, such as interleukin (IL)-4, -10 and -13.[92] These anti-inflammatory signals favour the arginase activity and the associated immunosuppression. TAMs are therefore usually described as M2-type macrophages. TAMs were also reported to promote TC invasion *via* secretion of proteases and promotion of angiogenesis, both processes further facilitating metastasis dissemination.[93]

Favouring the conversion of M2 macrophages into NO-producing M1 macrophages would represent an elegant modality aiming to exploit host cells present in the tumour microenvironment to exert cytotoxic effects on TCs and abrogate angiogenesis. The principle of this approach was recently validated with a combination of CpG oligodeoxynucleotides and IL-10 receptor antibody which triggered an innate response.[94] Further understanding of the macrophage balance hypothesis is, however, needed to transfer this strategy in humans. The M2 subtype of TAM is indeed not a strict paradigm: iNOS expression was identified in several tumours together with arginase. Interestingly, blocking the peroxinitrite production resulting from the co-expression of these two enzymatic entities can also be exploited therapeutically. Accordingly, coupling a NO-releasing moiety to aspirin was recently shown to provide feedback inhibition of both iNOS activity and associated peroxinitrite generation, leading to a correction of immunosuppression in tumours.[95]

It should be mentioned, however, that simultaneous activation of iNOS and arginase in tumours is associated with increased protein tyrosine nitration, which accounts for the unresponsive status of tumour-infiltrating lymphocytes.[96] Inhibition of iNOS together with arginase activity was therefore logically documented to restore lymphocyte responsiveness.[97] The adhesion of lymphocytes to tumour blood vessels is also directly influenced by NOS activity in angiogenic ECs. We have documented that VEGF is associated with a defect in the clustering of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) at the EC surface in a NO-dependent manner.[98] Interestingly, overexpression of cav-1 could overcome VEGF-mediated anergy by preventing eNOS activation. Similar observation was reported using the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) and led us to identify EC cytoskeleton rearrangement as the target of locally produced NO in angiogenic ECs.

3.5. *NO as a modulator of angiogenesis*

Angiogenesis is an adaptative response to hypoxia under the master command of HIF-1, a heterodimeric transcription factor composed of an α and a β subunit. HIF-1 controls the expression of a variety of genes encoding proteins that mediate cell survival and/or contribute to restore tissue oxygenation. Among these proteins, the production and release of VEGF and basic fibroblast growth factor (bFGF/FGF2), the main progenitors of angiogenesis, are directly and indirectly promoted by HIF-1, respectively.[99,100] NO acts both as a regulator of HIF-1 activation and as a downstream effector of VEGF, which places the small radical at a central position in the angiogenic process (Fig. 3).

Although HIF-1α and HIF-1β are constitutively transcribed, the overall activity of HIF-1 is determined by the stability of its HIF-1α subunit which is continuously degraded through the von Hippel-Lindau (pVHL)-proteasome pathway in aerobic cells but escapes destruction in hypoxic cells.[101] The oxygen sensor HIF-1 prolyl hydroxylase-2 (PHD2), a Fe^{2+}- and 2-oxoglutarate-dependent dioxygenase, has been identified as the molecular switch for HIF-1 activation.[102] This enzyme indeed

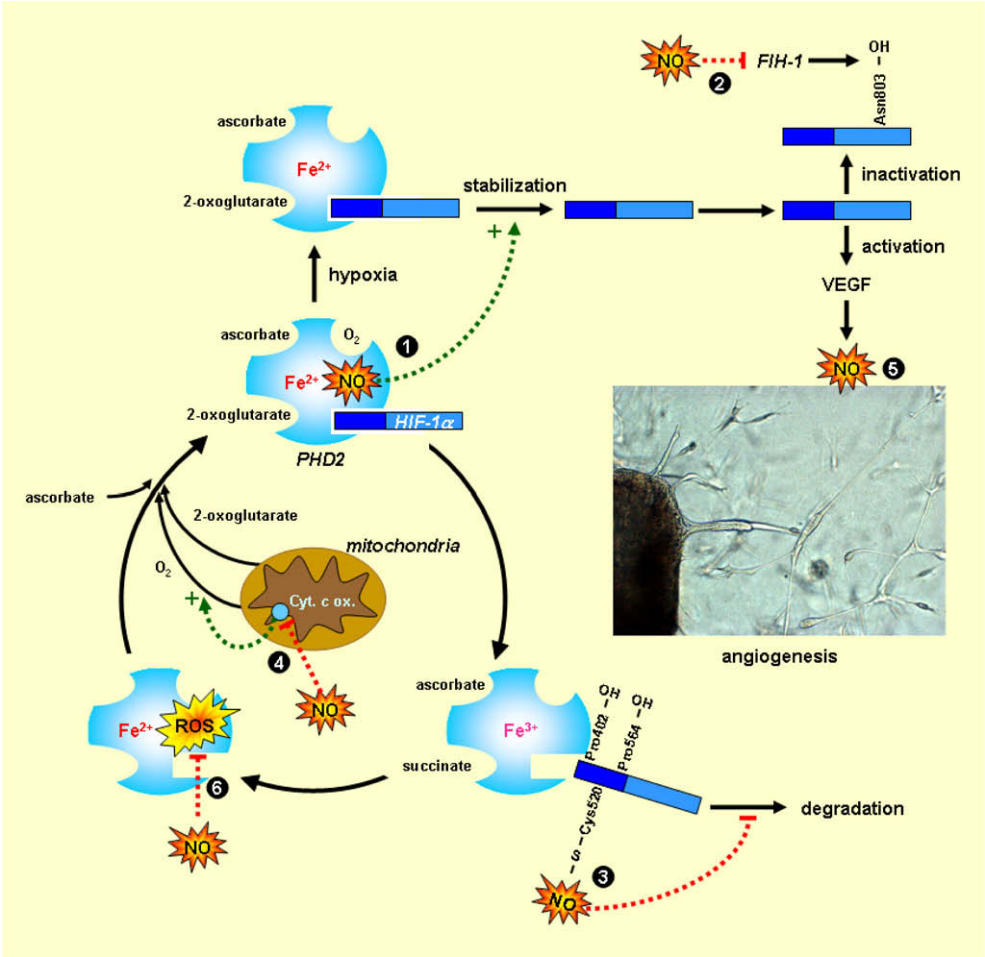

Fig. 3 – NO regulates HIF-1 activation and angiogenesis. HIF-1-mediated angiogenesis is controlled by PHD2, an oxygen-sensor prolyl hydroxylase that normally addresses HIF-1α for proteasomal degradation in normoxic cells. Redox transfer of two hydroxyl residues on the oxygen-dependent domain of HIF-1α (*dashed/deep blue*) requires O_2 and 2-oxoglutarate as substrates and Fe^{2+} at the catalytic site of the enzyme. Hydroxylated HIF-1α (on Pro402 and Pro564), succinate and CO_2 are products of the reaction, whereas the oxidised iron is further reduced by ascorbate for enzyme recycling. The low affinity of PHD2 for oxygen couples local pO_2 to HIF-1α stabilisation and HIF-1 activity: at high pO_2, hydroxylated HIF-1α is addressed for degradation through the von Hippel-Lindau (pVHL)-proteasome pathway; at low pO_2, however, HIF-1 is stabilised and transcriptionally activated to promote VEGF expression and angiogenesis (the inserted microphotograph shows endothelial cells networking from an explanted tumour microvessel cultured on a Matrigel matrix). Coupling pO_2 and angiogenesis is further regulated by factor inhibiting HIF-1 (FIH-1), an oxygen- and 2-oxoglutarate-dependent asparagine hydroxylase that inactivates HIF-1α under normoxia. NO influences this pathway at several levels. Under normoxia, NO may act as an hypoxia-mimetic through inactivating the catalytic Fe^{2+} site of PHD2 (1), inactivating FIH-1 probably via S-nitrosylation (2), directly S-nitrosylating HIF-1α on Cys520, which prevents ubiquitylation (3), and inhibiting mitochondrial respiration at the cytochrome c oxidase (Cyt. c ox.) to elevate free O_2 (4). It also acts as a downstream pro-angiogenic effector of VEGF in ECs (5). Conversely, NO may repress angiogenesis under conditions of oxidative stress, most probably because the high reactivity of NO for ROS neutralises the PHD2-inactivating activity of both species (6). It is currently not known whether NO could affect the mitochondrial production of 2-oxoglutarate.

catalyses the oxygen-dependent hydroxylation of HIF-1α at two proline residues (Pro402 and Pro564, human sequence), thereby tagging HIF-1α for degradation, a reaction becoming inoperative at low pO_2 merely because of the low affinity of PHD2 for its O_2 substrate.[103] Stabilised HIF-1α migrates to the cell nucleus where it binds HIF-1β, cofactors and the hypoxia-responsive elements of genes targeted for overexpression. In addition to regulating the stability of HIF-1α, oxygen affects the transcriptional activation of HIF-1 by promoting the hydroxylation of a critical asparagine residue of HIF-1α (Asn803, human sequence), which renders it unable to bind to the transcription co-activator p300.[104] This reaction is catalysed by the dioxygenase FIH-1 (factor inhibiting HIF-1).[105]

The contribution of NO to the complex regulation of HIF-1 is Janus-faced since NO is reported to stabilise HIF-1α in normoxic cells whereas it promotes HIF-1α degradation under hypoxia.[106] These antagonistic behaviours are inherent to the impact of the cell redox status on intrinsic NO reactivity. NO

has a particularly high reactivity for iron within iron–sulphur centres and haemoproteins, especially when a free ligand position is available as it is the case in sGC, cytochrome *c* oxidase, haemoglobin (Hb), but also PHD. It competes with oxygen for the catalytic iron centre of PHD2, thereby reducing HIF-1α ubiquitylation and degradation to mimic hypoxia under aerobic conditions.[107] NO also readily reacts with superoxide to produce RNS, which consequently reduces the bioavailability of both NO and superoxide. Superoxide is a byproduct of the mitochondrial activity when electrons escape from the respiratory chain (as it is the case during cell hypoxia) and a known inhibitor of PHDs. The cogeneration and inter-reactivity of both species thus compromises HIF-1 activation by either one. Accordingly, (1) NO-mediated activation of HIF-1 is attenuated by superoxide donors but restored in the presence of superoxide dismutase,[108] and (2) a progressive increase in superoxide generation in cells exposed to steady-state NO first attenuates and then promotes HIF-1α expression.[109] An additional layer of complexity comes from the modulatory effect of NO on mitochondrial respiration, reported to influence oxygen availability for PHD2 and FIH-1 at intermediate cell oxygenation.[110]

The biological significance of endogenously produced NO in activating HIF-1 has been particularly well documented in cells overexpressing iNOS and in transwell coculture experiments pointing to a paracrine action of NO on the stabilisation of HIF-1α.[111] We have recently validated this paradigm *in vivo* by showing that the recruitment of iNOS-expressing M1 macrophages in irradiated tumours accounts for increased expression of a HIF-1α reporter transgene and HIF-1 activation in normoxic tumour areas.[112] This model also allowed us to identify a new mechanism by which iNOS-derived NO reacts with a single cysteine residue of HIF-1α (Cys520, human sequence) to form an *S*-nitrosothiol that prevents pVHL binding and the normoxic degradation of HIF-1α. A similar reaction was reported to inactivate FIH-1[113] through a new PHD2-independent pathway. By either direct *S*-nitrosylation of HIF-1α or inhibition of dioxygenase-mediated hydroxylation(s), NO generated by iNOS in M1 macrophages promotes angiogenesis, wound repair and the growth of solid tumours, making iNOS inhibitors good candidates for anti-angiogenic interventions.[112,113] This strategy would be particularly well suited to oppose radiation-induced angiogenesis.

VEGF is a HIF-1-target gene and the main transducer of the pro-angiogenic signal to ECs. Angiogenesis is indeed initiated through binding of VEGF to the tyrosine-kinase receptor VEGFR2 (also known as KDR in human and flk-1 in mouse), which promotes vascular permeability, vasodilation, EC survival, proliferation, migration and angiogenic sprouting.[114] Interestingly, these phenotypic changes have been reported to be significantly impaired in *eNOS*$^{-/-}$ *versus* wild-type mice, although similar levels of VEGF were detected in the ischemic tissues of both strains.[115] Deletion of the *iNOS* gene had not such an effect.[116] VEGF-dependent tumour angiogenesis and tumour growth were found to be inhibited by the pharmacological blockade of eNOS or its genetic deletion.[117,118] The inability of recombinant VEGF to restore angiogenesis in *eNOS*$^{-/-}$ mice further validated eNOS as a key transducer of the pro-angiogenic signal of VEGF.[115] We have recently characterised the underlying pathway and found that VEGF binding to VEGFR2 leads to the phosphorylative activation of eNOS by Akt1 (itself stimulated by PI3K activation) and sustained production of micromolar levels of NO in ECs.[58] This cascade of reactions also mediates the pro-angiogenic effects of statins[59] and angiopoietin-1.[119] Because PI3K and hsp90 are necessary for long-term eNOS activation, their inhibition offers potential therapeutic options to block eNOS-mediated angiogenesis while preserving systemic vascular homeostasis.

4. How should we deliver NO to tumours?

4.1. *NO donors*

In the tumour context, NO donors may be categorised as 'classical' NO donors (that donate NO systemically) and 'selective' NO donors (that release NO preferentially in tumours). Tumour-restricted NO delivery offers the theoretical advantage of leaving systemic vasodynamics unaltered, thereby preventing hypotension and limiting the influence of the vascular steal phenomenon (see above).

NO was initially investigated for its vasodilatory activity and NO-donors were anticipated to improve the therapeutic efficacy of chemo- and radiotherapy upon combinational delivery (provascular strategy, Table 1).[21,120] In a study evaluating the effects of 34 vasodilators, we identified four NO donors (isosorbide dinitrate, nitroglycerin, sodium nitroprusside, and molsidomine) as particularly efficient to increase tumour oxygenation.[36] However, the response of the tumour vasculature was dependent on the chemical mechanism of NO generation, the administered dose as well as the nature and size of the treated tumour.[121] These limitations are well exemplified by the preclinical evaluation of two drugs, DEA/NO and 3-morpholinosydnonimine hydrochloride (SIN-1). *In vivo*, DEA/NO spontaneously and continuously releases NO with a half-life of about 2 min.[122] In a landmark study, Shan et al.[123] monitored the systemic and local effects of increasing doses of DEA/NO delivered intravenously to rats bearing a mammary R3230Ac adenocarcinoma. At low dose (100 nmol/kg), DEA/NO reduced the mean arterial blood pressure and constricted tumour arterioles by a yet unidentified mechanism. At higher doses (500 and 1000 nmol/kg), it caused systemic hypotension and a transient dilation of healthy blood vessels at the tumour periphery, whereas the reaction of tumour blood vessels ranged from no change to vasomotion (i.e. rhythmic diameter changes) in about one half of the animals. Thus, by different mechanisms, namely a relative increase in tumour resistance to flow at low dose and vascular steal at high doses, DEA/NO invariably reduced tumour perfusion and pO_2. In another study, Wood et al.[124] tested SIN-1, an oxygen-activated NO donor, as a potential radiosensitiser. They reported that low dose (2 mg/kg) SIN-1 given immediately before irradiation increased TC killing by two- to fourfold over radiotherapy alone in SCCVII/Ha squamous cell carcinoma-bearing mice. A decrease in ATP turnover was shown to mirror increased tumour perfusion and pO_2. However, these changes were not observed when the same treatment was delivered to RIF-1 sarcoma-bearing mice. Moreover, the effects of SIN-1 on TC metabolism turned out to be highly variable in function of time and dose: with the administration of 2 mg/kg SIN-1, energy levels peaked after 5 min and returned

Table 1 – Principal characteristics of NO-donors and NO-synthase agonists in tumours.

	Mode of administration	Mechanism of NO production	Tumour-selective NO production	Tumour-selective vasodilation	Inhibition of mitochondrial respiration	Tumour reoxygenation	Stabilisation of DNA damage by NO	Chemosensitisation	Radiosensitisation	Stimulation of angiogenesis
NO donors	i.v.	Chemical release	–	–	+/–	+/–	ND	+/–	+/–	ND
SNO-Hb	i.v. + O_2 or i.a.	SNO release under hypoxia	+/–	+	ND	ND	ND	ND	ND	ND
Nitrites	i.v.	Disproportionation at low pH	+	–	+	+	ND	ND	+	ND
Ionising radiations	Local	eNOS activation In ECs	+	+	+	+	ND	+	+	+
Insulin	i.v. + glucose	eNOS activation	+	–	+	+	+	ND	+	ND
Electrical nerve stimulation	Local	eNOS activation	+	+	+	+	+	ND	+	ND

Abbreviations: ECs, endothelial cells; eNOS, endothelial NO synthase; i.a., intra-arterially; i.v., intravenously; ND, not determined; NO, nitric oxide; SNO-Hb, Cysβ93-S-nitroso-haemoglobin; TCs, tumour cells.

to basal levels 20 min later, whereas the drug at 10 mg/kg induced no early effect but rather increased ATP levels 1 h after injection. Thus, using classical NO donors in provascular applications appears perilous. In some cases, however, radiotherapy may benefit from additional effects of NO. For example, we recently evidenced in two experimental models that the NO donor isosorbide dinitrate improved tumour pO_2 in part through a NO-mediated decrease in TC oxygen consumption.[83]

Although topical delivery of classical NO donors or local iNOS gene transfer (reviewed in Ref. 91) may be provascular options in some cases, the development of tumour-selective drugs could broaden the range of application. We have been involved in the evaluation of S-nitrosohaemoglobin (SNO-Hb) and nitrites as potential tumour radiosensitisers (Table 1).

Hb is a protein composed of four iron haeme-containing monomers. Its physiological role depends on its ability to selectively bind oxygen at its iron haemes. Oxygen binding is governed by a cycle of allosteric transitions in which Hb assumes the R (relaxed) conformation to bind oxygen with high-affinity in the lungs, and the T (tense, low oxygen affinity) conformation to selectively release oxygen in less oxygenated peripheral tissues.[64] The conformational change is regulated by the level of haeme oxygenation, making Hb an oxygen sensor. However, because oxygen binding is cooperative, all-or-nothing responses are usually described with Hb. The same allosteric transitions govern NO binding to Hb (preferentially in the lungs) and the release of NO equivalents from Hb (preferentially in less oxygenated peripheral tissues). We recently proposed that this unique property of Hb could be used to selectively deliver NO to tumours, the release of oxygen and NO equivalents being facilitated under low oxygen or hypoxia (T state Hb).[125]

SNO-Hb is the product of the endogenous reaction of NO (and NO-related species) with oxy-Hb in RBCs. In oxy-Hb, Cysβ93 has indeed a high reactivity towards NO, allowing its capture in the form of an S-nitrosothiol through complex reactions.[64] Then, in peripheral tissues, de-oxygenation induces the R to T conformational change rendering SNO-Cysβ93 available to release NO equivalents, which reach the vasculature *via* intermediate SNO carriers. Unlike Hb historically known as a NO scavenger,[126,127] SNO-Hb is thus naturally poised to selectively deliver NO to hypoxic tissues, and could therefore be used as a selective tumour vasodilator. We tested this possibility in a prospective study in which we administered a low dose of cell-free human SNO-Hb (~6.5 μmol/L, to match the Hb concentration in the plasma of patients with haemolytic diseases) to tumour-bearing rats.[125] By contrast to Hb that decreased tumour perfusion, we found that SNO-Hb stabilised tumour blood flow through an exquisitely oxygen-dependent mechanism. Intravenous SNO-Hb infusion with concomitant pure O_2 delivery, or alternatively intra-arterial infusion, indeed both circumvented the vasoconstricting action of Hb. Blood hyperoxygenation prolonged by approximately two-fold the half-life of the SNO moiety on Hb and concomitantly increased tumour perfusion, confirming that SNO-Hb bears the intrinsic potential to radio- and chemosensitise tumours. Clinical exploitation of this modality could benefit from the development of

drugs (such as ethyl nitrite gas) that directly nitrosylate Hb in RBCs, thereby preserving the optimal coupling between Hb de-oxygenation and NO release in hypoxic tissues.[128–130]

Nitrites may also serve as tumour radiosensitisers. They are not only natural catabolites of NO,[131] but amazingly also NO precursors when mild tissue acidification favours disproportionation.[132] The conversion of nitrites back to NO is promoted by the catalytic activity of Hb, xanthine oxidase and eNOS, which all behave as nitrite reductases under hypoxia.[133–135] This pathway is likely to account for the so-called hypoxic vasodilation.[136] Because acidic pH and hypoxia are hallmarks of many tumours, we recently tested nitrites as potential radiosensitisers.[137] We found that a bolus nitrite administration (100 μmol/L) to tumour-bearing mice induced a transient (30 min window) and selective increase in tumour pO_2 that was exploited to improve the response to radiotherapy. *In vitro*, we further identified that nitrite disproportionation at low pH (pH 6.8) could lead to both NO-mediated vasodilation and inhibition of TC respiration. The nanomolar concentrations of NO produced under our experimental conditions may account for specific effects on cell respiration but not on haemodynamics *in vivo*. Although mechanistic tenets still remain to be characterised, the low cost and safety profile of nitrites[138] support their use as selective tumour radiosensitisers.

Tumour-specific NO production from SNO-Hb and nitrites exploits tumour hypoxia and acidity, which are well-recognised cancer hallmarks.[1] However, tumours are not uniformly hypoxic and acidic, both parameters being interconnected and highly variable in space and time. Chronic hypoxia originates from the high oxygen consumption rate of TCs, creating a radial gradient of oxygen deprivation from blood vessels towards the tumour mass and a steep longitudinal gradient of blood deoxygenation from the tumour periphery to the tumour core.[139] In addition, as stated before, intermittent hypoxia arises from the uneven partitioning of RBCs at vascular branches and other perfusion instabilities,[30,31] contributing to tumour resistance to radiotherapy.[140] TC metabolism, the major source of tumour acidity through CO_2 (from respiration) and lactic acid (from glycolysis) release, is largely coupled to local pO_2.[141] The balance between glycolysis and respiration is actually finely-tuned by the negative feedback of energy metabolites on glycolysis (the Pasteur effect), which favours respiration when oxygen is available and glycolysis under hypoxia. This coordinated balance may cause tumour acidity to fluctuate in parallel with tumour oxygenation, whereas other cells function using glycolysis irrespectively of local pO_2 (Warburg phenotype)[142] constitute continuous sources of protons from lactic acid. Oxygen and acid heterogeneities should thus be taken into account for the design of SNO-Hb and nitrite-based therapies. As for fractionated radiotherapy, regimens of repeated treatment could, to some extent, overcome the fluctuant sources of resistance.

4.2. *Ionising radiations*

Conventional radiotherapy is usually delivered per daily dose fractions. The time interval between doses was determined empirically. Interestingly, however, accelerated radiotherapy and dose hyperfractionation provide an additional clinical benefit in some circumstances.[143] In this context, we have shown that radiotherapy itself acts as a radiosensitiser and highlighted mechanisms to rationalise dose fraction intervals for a maximal tumour response.[23,144] We found that tumour reoxygenation after irradiation is biphasic, with essentially NO-independent early effects (first hours)[144] relayed by later NO-mediated vasodilation and reoxygenation (24–48 h, Fig. 4).[23] We also documented that the latter effect is exploitable to improve the biodistribution of circulating antitumour agents in tumours.[28] Finally, we observed that radiotherapy promotes tumour angiogenesis on the long-term (several days),[145] a side-effect that can be opposed with anti-angiogenic drugs (Fig. 4).

Pioneering studies have revealed that tumour reoxygenation is an early response to ionising radiations characterised by a significant decline in the hypoxic fraction as soon as 1 h after a single dose irradiation.[146,147] It was interpreted to coincide with the *de novo* acquisition of sensitivity by surviving cells that were initially confined in hypoxic niches. Although the post-radiation reoxygenation paradigm was consecutively substantiated,[148,149] little was known about the supporting mechanisms. We addressed part of these concerns by monitoring tumour pO_2 during and after *in vivo* tumour irradiation at a clinically relevant dose (2 Gy).[144] In two models, early reoxygenation was observed to peak at ~4 h, concomitantly with an increase in tumour blood flow, a decrease in the rate of oxygen consumption by TCs and a decrease in interstitial fluid pressure (IFP). We found that inflammation was the main mediator of changes in blood flow and oxygen consumption, whereas decompression following TC death is likely to account for decreased IFP.[150] Early tumour reoxygenation could be antagonised by diclofenac but not by a NOS inhibitor, ruling out a major contribution of endogenous NO to this process.[144]

In the same models, early tumour reoxygenation was followed by a later phase of increased pO_2 peaking between 24 and 48 h (i.e. a time corresponding to the interval of clinical fractionation). We have identified mature tumour vessels as the key contributors for this late effect.[23] At the molecular level, irradiation upregulates the expression of eNOS and decreases that of cav-1 in ECs, thereby promoting the release of the caveolin inhibitory clamp. We showed that a consecutive 2.5-fold increase in NO production supported the vasorelaxation of mature tumour arterioles otherwise totally insensitive to eNOS agonists. As shown in Fig. 5, the increase in eNOS expression in response to ionising radiations is triggered by ROS. In experimental mouse models, we have evidenced that late tumour reoxygenation arises from active vasodilation of mature tumour vessels following eNOS activation by X-rays in ECs.[23] Such effect is of critical importance for therapy (Table 1). It first offers a rationale for clinical dose fractionation, as evidenced by our observation that eNOS inhibition between two irradiation doses results in a total loss of therapeutic efficacy of the second dose. Increased perfusion may also be exploited to selectively increase drug accessibility to tumours. We have documented that low-dose irradiation constitutes a pretreatment promoting cationic lipid-driven delivery of cDNA to tumours *in vivo*.[28] Interestingly, ECs are the primary

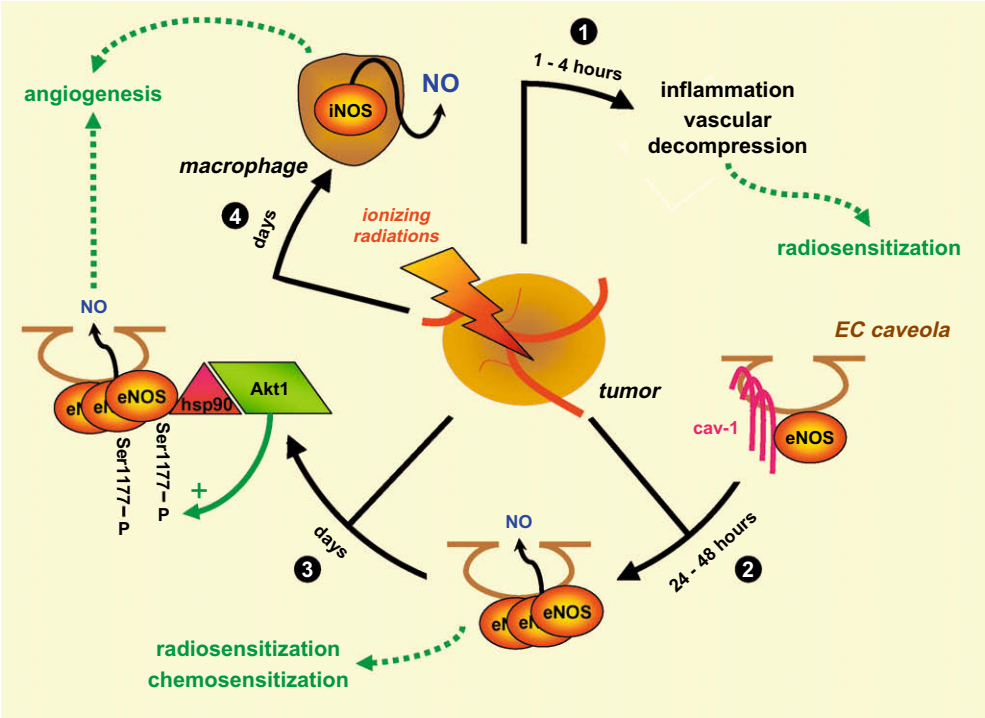

Fig. 4 – Biological agenda of NO in irradiated tumours. After irradiation, a tumour undergoes a series of modifications impacting growth control. In the early hours, reoxygenation occurs through vascular decompression (following tumour cell death) and an inflammatory response downregulating tumour cell respiration. These responses are essentially NO independent. Peaking at ~4 h, early reoxygenation might be exploited in protocols of hyperfractionated radiotherapy (1).[144] It is relayed by a second phase of reoxygenation originating from irradiated ECs (2).[23] There, upregulation of eNOS expression and decreased cav-1 expression create a molecular imbalance that releases eNOS from its inhibitory interaction with cav-1, promotes NO synthesis and causes vasodilation. Improvements in tumour blood flow and pO_2 favour drug delivery and account at least in part for the efficacy of fractionated radiotherapy.[23,28] Radiotherapy may, however, also chronically secure eNOS activation through Ser1177 phosphorylation (3),[145] and promote the recruitment of M1 macrophages expressing iNOS (4).[112] Sustained NO production from either source stimulates angiogenesis, tumour growth, and might assist tumours to resist radiotherapy and regrow. Hsp90, PI3K, Akt1 and iNOS inhibitors could be used therapeutically to antagonise late angiogenesis while preserving the beneficial effects of NO in irradiated tumours.

target for *in vivo* transfection of irradiated tumours, offering a way to selectively antagonise survival pathways at the tumour vasculature. Because one vessel supports the survival of hundreds of TCs, this strategy has a high therapeutic potential.

The reverse side of the coin is that radiotherapy promotes angiogenesis on the long-term. It acts both upstream and downstream of HIF-1α. We first detected a progressive increase in HIF-1α expression in 4T1 mammary tumours starting 3 days post-irradiation (6 Gy), peaking after 6–7 days, and resuming around Day 10.[112] It coincided with the recruitment of iNOS-expressing M1 macrophages at the irradiated tumour site. Consecutive elevated NO production promoted HIF-1α *S*-nitrosylation in bystander TCs, thereby supporting HIF-1 activation independently of changes in PHD2 activity. Macrophage depletion in mice, the delivery of the selective iNOS inhibitor *N*-3-aminomethyl-benzyl-acetamidine (1400 W), or the use of *iNOS*$^{-/-}$ mice prevented the irradiation-induced activation of HIF-1.[112] A delayed tumour regrowth was observed in this model after the delivery of a single 6 Gy dose to mice treated with a NOS inhibitor.

Upregulated VEGF production by irradiated TCs was originally reported to account for post-irradiation angiogenesis.[151,152] We showed that irradiated ECs may, however, acquire an angiogenic phenotype in the absence of inflammatory cells or TCs.[145] *In vivo*, angiogenesis was detected in Matrigel plugs containing ECs 5 days after a 6-Gy irradiation. EC migration, tube formation and endothelial sprouting from tumour vessels were all observed in response to sustained eNOS activation in irradiated ECs. Hence, in addition to increasing eNOS and decreasing cav-1 abundances,[23] irradiation dose dependently activated the PI3K–Akt1 pathway, leading to eNOS phosphorylation on Ser1177, long-term NO production and angiogenesis.[145]

The above mechanistic dissection allows to propose new treatment modalities. To oppose angiogenesis, delivering anti-angiogenic drugs after the course of radiotherapy would be an attractive option. iNOS inhibitors (such as 1400 W) and inhibitors of eNOS phosphorylation (such as the PI3K inhibitor wortmannin or the hsp90 inhibitor geldanamycin) are of particular interest because they would repress angiogenesis while preserving irradiation-induced, NO-mediated vasodilation.

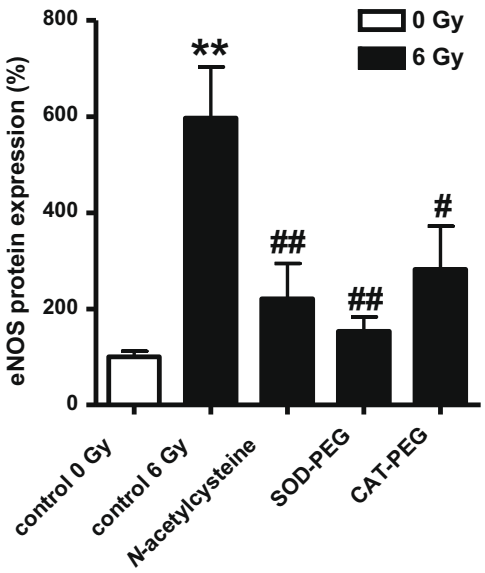

Fig. 5 – Reactive oxygen species mediate the irradiation-induced increase in eNOS protein expression. In this experiment, bovine aortic endothelial cells (BAECs, Clonetics) were used to exemplify that reactive oxygen species (ROS) mediate increased eNOS protein expression in response to X-ray irradiation. The graph shows eNOS protein expression detected by Western blot using a previously published protocol.[145] A significant increase in eNOS expression was observed 24-h after a single 6-Gy irradiation (RT-250 device, Philips). Increased expression was blocked when the general antioxidant N-acetylcysteine (5 μM), PEGylated superoxide dismutase (SOD–PEG 100 U/ml, Sigma–Aldrich) that catalyses the disproportionation of superoxide, or PEGylated catalase (CAT-PEG, 1000 U/ml, Sigma–Aldrich) that catalyses the decomposition of hydrogen peroxide, was added to the cell culture at the time of irradiation. Irradiation-induced ROS production thus promotes the expression of eNOS protein. (Results are expressed as % eNOS ±SEM. $^{**}P < 0.01$ *versus* Control 0 Gy; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* Control 6 Gy; $n = 3–4$ (one-way ANOVA.)

4.3. *Insulin*

NO was recently evidenced to play a critical role in insulin signalling in muscle cells.[68] Insulin indeed promotes Akt2 activation, phospho-Akt2 targeting to mitochondria, subsequent mtNOS activation and NO-mediated inhibition of cytochrome c oxidase. Reversible inhibition of mitochondrial respiration by insulin may therefore act as a switch between increased oxidative ATP production and glucose energy storage in the form of glycogen or fat.

Interestingly, insulin further couples metabolism to vascular homeostasis by a direct action on the endothelium. In ECs, binding of insulin to its cell-surface tyrosine kinase receptor activates the PI3K–Akt1 cascade.[153] A first consequence is downstream eNOS activation through phosphorylation on Ser1177, further favoured by associated Thr495 dephosphorylation and the formation of an eNOS–hsp90–Akt1 complex.[154–156] PI3K activation also acts as a triggering stimulus for eNOS transcription through yet unidentified mechanisms.[157] Insulin distinguishes from other vasodilators in that eNOS phosphorylation is mandatory for insulin-mediated vasodilation. Accordingly, NO production in response to insulin is abolished in ECs expressing a dominant-negative Akt1 or a mutant eNOS lacking the Akt1 phosphorylation site.[154,158]

That the effects of insulin converge to increase tissue pO_2 was confirmed by documenting that insulin (400 mU/kg) could radiosensitise tumours in three independent syngeneic mouse models.[84,88] Radiosensitisation was conveyed by NO in a combination of direct and indirect effects. Insulin first induced a net increase in tumour pO_2 that could be fully blocked either by a pharmacological NOS inhibitor or when the tumour was implanted in *eNOS*$^{-/-}$ mice.[88] Interestingly, despite a noticeable reduction in blood flow through vascular steal, the increase in tumour oxygenation was found to be the consequence of a decrease in TC respiration.[84] Mechanistically, insulin was documented to stimulate eNOS activation through Ser1177 phosphorylation in ECs, thereby contributing to a paracrine source of NO to compete with oxygen to control energy production.[153] The metabolic response to insulin in tumours, however, diverged from the muscle situation where NO, through the interplay with mitochondrial respiration, influences oxidative phosphorylations and favour glucose sparing and storing.[159] In tumours, insulin rather reoriented glucose to a glycolytic fate, clearly evidenced by rising ATP and lactate production.[84]

Surprisingly, the oxygen effect of insulin accounted only for part of the radiosensitisation observed. In FSAII tumours, insulin increased pO_2 from ~3 mm Hg to ~9 mm Hg and carbogen (95% O_2/5% CO_2) breathing up to ~21 mm Hg, but insulin was more efficient as a radiosensitiser than carbogen.[88] Carbogen having no influence on tumour NO content, the superior therapeutic output of insulin might reflect the intrinsic radiosensitising property of NO (Table 1).

Based on these observations, we evaluated the potential toxicity of insulin when combined with radiation therapy in preclinical settings.[160] At 400 mU/kg, insulin did not potentiate radiation toxicity in a mouse jejunum crypt survival assay. Similarly, late radiation damage in musculoconnective tissues was not affected by insulin up to 120 days after irradiation. Finally, addition of glucose to the insulin solution prevented hypoglycaemia while preserving tumour reoxygenation and radiosensitisation. Clearly, further preclinical evaluation of insulin in combination with fractionated radiotherapy is now warranted.

4.4. *Electric stimulation*

During physical exercise, muscle perfusion increases to adjust the nutrient supply to the metabolic demand and remove waste catabolites. The contribution of NO to muscle exercise is well documented and has been extensively reviewed elsewhere (see Ref. 161). Both eNOS in ECs and nNOS in skeletal muscle cells fulfil this function by promoting retrograde vasodilation.

To exploit the physiology of exercise for the purpose of tumour reoxygenation, we developed a model in which electrical stimulation (5 Hz, 0.2 ms pulses) of the sciatic nerve was tested for its ability to radiosensitise tumours implanted in

mouse hindlimb.[162] Tumour perfusion and pO_2 steeply increased as soon as stimulation was initiated. Interestingly, although perfusion immediately fell down to basal at the end of stimulation, increased pO_2 was maintained during an additional period of 30 min, coinciding with a persistent inhibition of TC respiration. Changes in pO_2 were totally abolished by the co-administration of a NOS inhibitor[162] or when tumours were grown in an *eNOS*$^{-/-}$ background,[88] emphasising the critical role played by NO in both perfusion and consumption processes.

Although we measured similar gains in tumour pO_2, a 15-min sciatic stimulation during irradiation efficiently radiosensitised tumours, whereas the same treatment completed 30 min before irradiation had no effect.[88] The therapeutic benefit actually mirrored changes in tumour NO levels (that were high during electrical stimulation but back to basal right after) indicating that the intrinsic property of NO to stabilise DNA damage, not changes in tumour pO_2, is the main progenitor of exercise-based radiosensitisation.

5. Clinical perspectives

5.1. *Defining treatment protocols*

Till recently, no clinical studies in the field of cancer had addressed how the modulation of NO production could impact tumour blood flow. In 2007, however, Ng et al.[163] reported the first phase I dose-escalation study where a single dose of the NOS inhibitor *N*-nitro-L-arginine was administered to cancer patients. A sustained reduction in tumour blood volume was observed in patients undergoing dynamic computed tomography (CT)-scan, and toxic effects were found to be self-limiting cardiovascular changes (hypertension, arrhythmia). Although the goal behind the above study was to identify a new antivascular strategy, these data identify eNOS as a modulatory target which could thus also be stimulated to favour local NO production and be exploited in combination with radio- and chemotherapy. This gives credential to strategies aiming to pharmacologically impact the local eNOS activity instead of unselectively increasing NO through the systemic administration of NO donors. One may indeed anticipate that bypassing NOS activation (with a NO donor) will consistently lead to major cardiovascular side-effects, including hypotension and shock.

Priming radiotherapy is likely to be the most easily applicable approach to locally increase NO production and favour either drug delivery or the therapeutic efficacy of irradiation through transient tumour reoxygenation. The impact of irradiation on tumour blood flow and oxygenation is likely to account for a part of the advantages of fractionated radiotherapy over single dose administration, as documented by our group in preclinical studies.[23,28] A second approach consists in exploiting the tumour microenvironment to confer selectivity to a systemically administered drug. Nitrite is a good example of a relatively safe approach which may lead to a selective reconversion into NO in the tumour vasculature under the low pO_2- and low pH-dependent conditions observed in many tumours.[137] Furthermore, nitrites are historically used as a treatment for cyanide poisoning[138] and were shown to prevent delayed cerebral vasospasm in a primate model of subarachnoid haemorrhage without clinical or pathological evidence of toxicity.[164] Finally, as detailed above, insulin in combination with glucose also presents a relatively safe and efficient profile to be exploited in combination with radiotherapy in humans.

5.2. *Imaging*

To establish a clear relationship between an increase in NO concentration in tumours and a possible therapeutic benefit for cancer patients, a major challenge will be to develop clinical tools able to determine the dynamic evolution of NO production and tumour pO_2. Imaging technologies and surrogate markers are warranted to identify those patients who could benefit from a NO-mediated treatment.

In preclinical settings, the direct measurement of NO in tissues can be carried out using several techniques. Invasive methods, such as chemiluminescence and the use of electrochemical sensing electrodes, differ in sensitivity and specificity. They do not allow to straightforwardly attribute measured NO to local production, calling therefore to question their clinical utility. To circumvent this limitation, non-invasive methods, such as *in vivo* electron paramagnetic resonance (EPR) spectroscopy and imaging, have been developed and validated in rodents for their ability to spatially map NO production in tumours and tissues. These techniques use a spin trapping method based on a reaction between NO and a trapping molecule yielding a radical adduct with a longer half-life than NO, essentially analogous to a NO fingerprint. Recent advances in *in vivo* EPR spectroscopy and imaging using diethyldithiocarbamate derivatives offer the best perspective to quantify and image local NO production.[165] Yet, the clinical implementation of these methods still faces important challenges as how to increase their fairly limited sensitivity[166] (most clinical studies were dealing with the detection of a large amount of NO in septic shock),[167,168] and which spin trap agent has the best profile compatible with rapid approval by regulation authorities.

If the detection of NO in human tissues is still far from routine clinical applications, monitoring oxygen has comparatively received much more attention in the cancer imaging community in the recent years. For clinical applications purposed to track dynamic changes of tumour oxygenation, it is evident that invasive methods such as *in situ* implantation of Eppendorf electrodes[169] or immunostaining (for example, detection of HIF-1α or carbonic anhydrase IX)[170] are inappropriate to perform repeated (acute or chronic) pO_2 measurements over the time of a treatment. The use of histological markers is further confronted to an appreciable mismatch between their expression and local pO_2.[171] Among non-invasive techniques, nuclear medicine, nuclear magnetic resonance (NMR) and EPR offer pragmatic alternatives to evaluate the dynamics of tumour oxygenation.

Nuclear medicine allows to assess tumour hypoxia non-invasively using radiolabeled nitroimidazoles that are selectively trapped in hypoxic cells through bioreductive reactions involving reductases, primarily the cytochrome P450 reductase.[172] Intracellular trapping thus increases as pO_2 decreases. Several radiolabelled nitroimidazoles have been developed: fluoromisonidazole (FMISO), 2-(2-nitro-1H-imida-

zol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) and copper(II) diacetyl-di(N^4-methylthiosemicarbazone) (Cu-ATSM).[173] It was found that the tumour retention of FMISO correlated with the radiobiological hypoxic fraction of the tumour,[174] and that accumulation of EF5 was higher in tumour areas with a pO_2 lower than 10 mm Hg.[175] Conversely, the validity of Cu-ATSM as an hypoxic marker is still under debate since oxygen-independent events could additionally modulate its accumulation.[176] In general, however, uneven repartition of nitroimidazole-activating reductases in tumours accounts, at least in part, for a high degree of variability in tracer trapping.

Oxygen-sensitive NMR techniques could also be useful in the context of NO modulation within tumours. As far as the mechanism of reoxygenation is related to changes in tumour perfusion, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a very valuable tool to assess changes in tumour haemodynamics.[177] It is, however, unhelpful if NO-dependent reoxygenation primarily depends on an effect on oxygen consumption. The same holds true for blood oxygen level-dependent (BOLD) MRI, which is sensitive to the balance between paramagnetic deoxy-Hb and diamagnetic oxy-Hb. On one hand, it was suggested that Hb oxygenation may serve as an endogenous marker of tumour oxygenation due to the effect on the transverse relaxation rates of blood and surrounding tissues.[178] On the other hand, however, this source of contrast is complex,[179] and we have documented that BOLD-MRI is insensitive to increases in tumour oxygenation due to changes in oxygen consumption, including the NO-mediated effect of insulin.[180] Conversely, a careful analysis of the source of contrast using T2*-weighted gradient-echo (T2*-w GRE) MRI allows to produce 3D parametric maps that are proportional to oxygen and blood flow fluctuations in tumours. This technique was used to non-invasively predict the vascular response to anti-angiogenic and antivascular agents.[181,182] Similarly, we used T2*-w GRE MRI to determine the ratio of mature arterioles and venules and the relative proportion of functional vessels (i.e. those carrying oxygenated blood) in experimental tumours.[183] The approach is based on the principle that vascular reactivity to hypercapnia (5% CO_2–air) occurs in mature vessels, whereas a carbogen (95% O_2, 5% CO_2) breathing challenge produces acute changes in blood oxygenation selectively in functional vessels (BOLD contrast effect). Our non-invasive estimation of vascular maturity and functionality correlated nicely with histological data, including α smooth muscle actin and Hoechst 33342 perfusion staining. T2*-w GRE MRI sequences are usable on clinical MR scanners and carbogen breathing is used in clinical studies of head and neck cancer therapies. Therefore, the assessment of the functional status of the tumour vasculature is directly translatable to the clinic.

Among MRI techniques, ^{19}F-relaxometry appears to be the most promising method to monitor changes in tumour pO_2. The ^{19}F NMR spin lattice relaxation rate is indeed highly sensitive to oxygen and, after intratumoural administration of hexafluorobenzene, fluorocarbon relaxometry can be used for dynamic oxygen mapping.[184] The clinical implementation of this strategy will likely require the development of related methods using approved biocompatible perfluorocarbons.[185]

The most robust technique so far to monitor dynamic changes in tumour pO_2 is *in vivo* EPR oximetry.[186,187] This method, based on the oxygen-dependence of the EPR signal of probes (such as charcoals or inks) implanted inside tissues, has been used in preclinical models to evaluate the influence of NO-mediated treatments on tumour oxygenation.[23,36,83,84,88,160,162] It has the unique capability of allowing repeated measurements of the pO_2 at the exact same site over time, which can be adjusted from seconds to months to years. Several studies were recently conducted to improve the biocompatibility of the oxygen sensors.[188–190] Moreover, instrumental developments have provided the first whole body EPR systems for clinical application.[191] Initial clinical trials conducted in volunteers and in cancer patients indicate the high value of the technique to provide highly sensitive measurements of pO_2.[192,193] Future developments may provide a powerful tool to monitor the effects of NO-mediated treatments in cancer patients.

6. Concluding remarks

Exploitation of the biology of NO offers the opportunity to improve the efficacy of conventional anticancer treatments such as chemo- and radiotherapy. Different NO donors and NOS agonists have now been identified and tested in preclinical settings, revealing a variety of mechanisms that contribute to transiently improve tumour blood flow and pO_2, thereby increasing drug delivery and the stabilisation of radiation-driven DNA damages (see Table 1). The development of techniques able to image the response of human tumours to these drugs will help to select patients and the best time frame for combinational treatments in the clinical situation.

Conflict of interest statement

None declared.

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