Abstract

Low tissue oxygen levels, known as hypoxia, characterize many solid cancers. Both clinical and experimental studies have demonstrated that intratumoral hypoxia is associated with malignant progression defined by increased tumour growth, metastasis and resistance to therapy. A major mechanism mediating malignant adaptive responses to hypoxia involves the activity of hypoxia-inducible factor 1 (HIF-1), a transcription factor composed of HIF-1α and HIF-1β subunits; HIF-1α is highly regulated by oxygen, such that its protein levels determine HIF-1 transcriptional activity. Accumulating evidence indicates that hypoxia-induced acquisition of malignant phenotypes is, in part, due to impaired nitric oxide (NO)-mediated activation of cyclic guanosine monophosphate (cGMP) signalling and that restoration of cGMP signalling prevents such hypoxic responses. The present study aimed to determine the downstream mechanism by which the NO/cGMP signalling regulates hypoxic responses. Using DU145 prostate cancer cells, studies were conducted to assess the effect of the NO mimic glyceryl trinitrate (GTN) and the cGMP analogue 8-Bromo-cGMP on hypoxic accumulation of HIF-1α. Results revealed that GTN, at a concentration known to primarily activate the NO/cGMP pathway, inhibited hypoxia-induced HIF-1α protein accumulation in a time-dependent manner; 8-Bromo-cGMP mimicked the effect of GTN on HIF-1α protein while levels of HIF-1α mRNA remained unaltered by exposure to either GTN or 8-Bromo-cGMP. Furthermore, treatment of cells with the calpain (Ca²⁺-activated proteinase) inhibitor calpastatin attenuated the effects of GTN and 8-Bromo-cGMP on HIF-1α protein accumulation. Collectively, these findings indicate a role for NO/cGMP signalling in the regulation of HIF-1α, and hence HIF-1-mediated hypoxic responses, via a mechanism that is likely dependent on calpain activity.
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List of Abbreviations

2-OG (2-oxoglutarate)
ADAM10 (A disintegrin and metalloproteinase domain-containing protein 10)
aHIF (antisense HIF)
AMF (Autocrine motility factor)
ANGPT2 (Angiopoietin 2)
ANGPTL4 (Angiopoietin-like 4)
ARNT (Aryl hydrocarbon receptor nuclear translocator)
BAK (BCL-2 antagonist/killer)
bHLH-PAS (Basic helix-loop-helix-per-arnt-sim)
BID (BH3 interacting domain death agonist)
CBP (CREB-binding protein)
cGMP (cyclic guanosine monophosphate)
CNG (Cyclic nucleotide-gated channel)
CTAD (C-terminal transactivation domain)
DETA/NO (Diethylenetriamine NO)
DFO (Desferrioxamine)
EC (Endothelial cell)
ECM (Extracellular matrix)
Egr1 (Early growth factor 1)
eNOS (endothelial NOS)
FIH-1 (Factor-inhibiting HIF-1)
GTN (Glyceryl trinitrate)
GTP (Guanosine triphosphate)
HIF-1 (Hypoxia-inducible factor 1)
HRE (Hypoxia response element)
HSP90 (Heat shock protein 90)
HuR (Human antigen R)
iNOS (inducible NOS)
L1CAM (L1 cell adhesion molecule)
LOX (Lysyl oxidase)
LRP (Lung resistance protein)
MAPK (Mitogen activated protein kinase)
MCL-1 (Myeloid cell leukemia 1)
MICA (MHC class I chain-related molecule A)
miRNA (micro RNA)
MMP (Matrix metalloproteinase)
MRP1 (Multidrug-resistance protein 1)
mTOR (mammalian target of rapamycin)
NF-κB (Nuclear factor-κB)
nNOS (neuronal NOS)
NO (Nitric oxide)
NOS (Nitric oxide synthase)
ODD (Oxygen-dependent degradation domain)
PDE (Phosphodiesterase)
PD-L1 (Programmed cell death ligand 1)
P-gp (Permeability glycoprotein)
PHD (Prolyl-hydroxylase domain)
PKG (cGMP-dependent protein kinase)
PSA (Prostate specific antigen)
PTB (Polypyrimidine tract-binding protein)
pVHL (von Hippel-Lindau tumour suppressor protein)
qRT-PCR (quantitative reverse transcription-polymerase chain reaction)
RACK1 (Receptor for activated C-kinase 1)
RNS (Reactive nitrogen species)
ROS (Reactive oxygen species)
sGC (soluble guanylyl cyclase)
SIAH (Seven in absentia homolog)
TCA (Tricarboxylic acid)
Topo IIα (Topoisomerase IIα)
uPAR (urokinase plasminogen activator receptor)
VDU2 (pVHL-interacting de-ubiquitylating enzyme 2)
VEGF (Vascular endothelial growth factor)
ZEB (Zinc finger E-box binding homeobox)
Chapter 1

Introduction

1.1 Cancer biology

Cancer is a major public health problem in Canada and worldwide. Nearly half of all Canadians will develop cancer, the most common of which is prostate cancer in men and breast cancer in women (excluding non-melanoma skin cancer), and one in four of all Canadians is expected to die from cancer in their lifetime [1]. Worldwide, an estimated 14.1 million cancer cases and 8.2 million cancer deaths occurred in 2012, and annual cancer cases are expected to rise to 22 million within the next two decades [2,3]. These surveillance data highlight the critical need for investigating anti-cancer mechanisms from which novel, effective cancer therapies may be developed.

There are more than 100 distinct types of cancer originating from most of the cell types and organs of the body. The complexity of these diverse neoplastic diseases, which are characterized by unrestrained proliferation of cells, may be better understood by recognizing a set of acquired capabilities integral to most forms of cancer known as the “hallmarks of cancer”; they include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis in addition to emerging hallmarks of reprogramming energy metabolism and evading immune destruction, all of which are enabled in large part by a succession of genomic alterations and resulting genetic instability in tumour cells [4,5]. Indeed, cancer development and progression proceeds via a process analogous to Darwinian evolution in which continuous acquisition of heritable genetic and epigenetic variations, each conferring a selective growth advantage, leads to progressive transformation of normal human cells to rapidly proliferating tumour cells that can
invade into local surrounding tissues and metastasize to distant organs; this evolutionary process is driven by selective pressures from the microenvironment [6,7].

There is an increased appreciation of the critical role of the tumour microenvironment in tumourigenesis, revealing that the biology of tumours is not simply a function of cell-autonomous properties of tumour cells. Of particular importance is the role of hypoxic microenvironment in promoting malignant progression and resistance to therapy, which remain a significant challenge in cancer treatment.

1.2 Tumour hypoxia

Hypoxia is commonly defined as decreased oxygen partial pressure that restricts or abolishes normal biological functions of organs, tissues or cells; however, such physiology-based definitions may not apply to tumour hypoxia because in contrast to normal tissues, neoplastic tissues already exhibit altered physiological functions [8]. Extreme inter- and intra-tumoral heterogeneity in oxygenation further adds to the complexity of tumour hypoxia and thus there does not appear to be a single, generally applicable hypoxic threshold [8]. Hypoxia in human tumours was first proposed more than 50 years ago [9] and direct evidence was shown using the Eppendorf probe, a polarographic electrode which makes accurate measurements of microregional tissue \( O_2 \) pressures [10]. It is now well known that regions with very low concentrations of \( O_2 \) (less than 5 mmHg \( pO_2 \) which corresponds to approximately 0.7% \( O_2 \) in the gas phase), often with much lower median \( pO_2 \) than their tissue of origin, characterize many solid cancers [11]. The development of tumour hypoxia is primarily due to structurally and functionally abnormal microcirculation that is unable to meet the oxygen demands of rapidly proliferating cancer cells [8]. Blood vessels in tumours are immature and often dilated, tortuous and hyperpermeable with excessive branching, blind ends and vascular shunts [12]. As a result of the
disorganized vascular network, tumour cells are typically located farther away from blood vessels than cells in normal tissues, often beyond the oxygen diffusion range which is up to 200 µM; this leads to diffusion-limited hypoxia, also known as chronic hypoxia. In addition, temporary obstruction or variant blood flow in these aberrant vessels may lead to transient episodes of severe hypoxia referred to as perfusion-limited or acute hypoxia [11]. Heterogeneous regions of both acute and chronic hypoxia likely contribute to the overall level of hypoxia in different tumours [12]. While prolonged exposure to complete deprivation of oxygen (anoxia) can ultimately lead to necrosis, viable hypoxic cancer cells are often adjacent to necrotic regions and acquire malignant phenotypes [13].

1.2.1 Hypoxia and malignant phenotypes

Multiple clinical studies have demonstrated that low tumour oxygenation (pO$_2$ < 10 mmHg) is an independent marker of poor patient prognosis for various types of cancers including carcinomas of the cervix, prostate and the head and neck, melanoma and soft tissue sarcomas [14-18]. Clinical evidence also indicates that tumour hypoxia is associated with increased tumour growth and metastasis as well as resistance to therapy [14,17-23], which are strongly supported by experimental evidence.

Tumour hypoxia can drive malignant progression in various ways involving changes in the proteome, genome and clonal selection of tumour cells. It has been shown that hypoxia promotes genomic instability (through point mutations, gene amplifications and chromosomal rearrangements), increasing the number of genetic variants [24-26]; concurrently, hypoxia exerts a strong selection pressure which leads to clonal selection and expansion of tumour cells with mutations that confer survival advantage under hypoxic conditions, resulting in malignant tumour phenotypes [8,20,24-26]. In addition to the genomic alterations, hypoxia can induce substantial changes in the proteome by modulating gene expression. In response to hypoxic conditions,
tumour cells undergo changes in gene expression and subsequent phenotypic changes necessary for their survival and/or escape from the hostile microenvironment [27]. A major mechanism mediating cellular adaptations to hypoxia involves the regulation of transcription by hypoxia-inducible factor 1 (HIF-1) [28]. Tumour cells, which harbour genetic mutations and epigenetic changes that alter normal feedback mechanisms, co-opt these physiological responses to hypoxia and consequently acquire aggressive phenotypes [29].

Indeed, it has been extensively shown that hypoxia increases invasive and metastatic capacities of various cancer cell types [30-37] and further studies revealed that these hypoxic responses require HIF-1 transcriptional activity [38,39]. In support of these findings, there is evidence that prometastatic consequences and hence failure of certain anti-angiogenic therapies designed to destroy tumour vasculature may be due to increased intratumoral hypoxia and HIF-1 activity [40,41]. Tumour hypoxia is also implicated in resistance to various forms of therapy including radiotherapy, chemotherapy and more recently immunotherapy. It has been known for many years that hypoxia in solid tumours reduces the efficacy of radiation therapy [42], as this treatment modality relies on oxygen for the formation of free radicals that cause DNA damage and hence tumour cell death [11]. Similarly, some chemotherapeutic agents, such as bleomycin, require oxygen to achieve maximal cytotoxicity [43]. Apart from the oxygen-dependent drug activity, hypoxia may lead to increased resistance to a range of chemotherapeutics by altering the phenotype of tumour cells through multiple mechanisms [44-49]. In particular, HIF-1 activity is required for many of the mechanisms of hypoxia-induced chemoresistance in various cancer cell types [48-55]. Accumulating evidence also indicates that tumour hypoxia may promote resistance to immunotherapy by facilitating tumour cell-mediated immune evasion strategies, such as avoiding immune detection [56,57] and inactivating immune effector cells [58-60]. Studies have also shown a direct effect of hypoxia on the development and function of immune effector and immunosuppressive cells, which may further promote tumour immune evasion [61-64]; in fact, it
has been proposed that physiological mechanisms that prevent excessive collateral tissue damage during an immune response, which often occur under hypoxic conditions, may be involved in protecting the hypoxic cancerous tissue from immune destruction [65]. As observed in other hypoxia-induced malignant phenotypes, HIF-1 accounts for many of the hypoxia-dependent effects on tumor immune evasion that have been described to date [57,59,60,64,66]. Therefore, hypoxia has a key negative role in tumor prognosis as it promotes the acquisition of invasive and metastatic properties along with resistance to therapy, all of which can be mediated by HIF-1. Thus, it is imperative to further understand the precise role of HIF-1 in hypoxia-induced tumor malignancy and to explore the mechanisms by which HIF-1 is regulated.

1.3 Hypoxia-Inducible Factor 1 (HIF-1)

HIF-1, also known as the master regulator of oxygen homeostasis, is a heterodimeric transcription factor composed of HIF-1α (120 kDa; chromosome 14) and HIF-1β (91-94 kDa; chromosome 1) subunits, both of which contain basic helix-loop-helix (bHLH)-per-arnt-sim (PAS) domains that mediate heterodimerization and DNA binding [67,68]. HIF-1β, originally identified as the aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed and is present in excess while HIF-1α is highly regulated such that its protein levels determine HIF-1 transcriptional activity [67]. Together, the HIF-1 complex binds to hypoxia response elements (HREs) and activates the transcription of multiple genes that facilitate adaptation and survival of cells under hypoxic conditions [28]. HIF-2α and HIF-3α isoforms which can dimerize with HIF-1β to form HIF-2 and HIF-3 complex, respectively, have also been described. HIF-2α shares a number of structural and biochemical similarities with HIF-1α, including 48% amino acid identity; however, in contrast to the ubiquitously expressed HIF-1α, HIF-2α shows a more restricted expression pattern. Furthermore, HIF-1α and HIF-2α transactivate overlapping but distinct target
genes in a cell type-dependent manner [28,69]. Less is known about the more distantly related HIF-3α, which is expressed as multiple splice variants; evidence indicates that it may function primarily as a negative regulator of HIF-1 activity in a feedback control system [70,71]. Thus, HIF-1 is the most prominent and well characterized of the three and it is a key mediator of cellular adaptations to hypoxia not only during development and normal physiological functions but also during pathological processes such as cancer [12,28].

1.3.1 HIF-1 and malignant adaptations to hypoxia

HIF-1α, often used as an endogenous marker of hypoxia, is overexpressed in the majority of common human cancers and their metastases [72]. The critical role of HIF-1 in cancer progression and malignancy is reflected by a large body of clinical data that correlates increased HIF-1α expression with poor clinical outcomes in a wide range of human cancers including breast, cervical, colorectal, head and neck, prostate, pancreatic and non-small-cell lung cancers [73-79].

HIF-1 exerts its effects on tumour progression by transactivating genes encoding proteins that play key roles in many critical aspects of cancer biology including invasion and metastasis, resistance to therapy and immune evasion (Table 1). Metastasis is a complex, multistep process involving a succession of changes in tumour cell-cell and cell-extracellular matrix (ECM) interactions. The initial step requires disruption of adhesive interactions with surrounding cells, degradation of ECM and increase in motility which enable migration through the stroma and invasion of adjacent tissues; this is followed by entry into blood and lymphatic vasculature (intravasation), survival in the circulation, movement out of the circulation (extravasation) and colonization of distant tissues [80]. The expression of molecules that are required at various steps of the metastatic process is regulated by HIF-1 both directly and indirectly. The latter is demonstrated by the finding that HIF-1 activates the transcription of genes encoding repressors (ZEB1 and ZEB2) that block the expression of E-cadherin cell-cell adhesion molecule, the loss of
which is a key step in the metastatic cascade [81]. HIF-1 also transactivates genes encoding matrix metalloproteinases (MMPs), urokinase plasminogen activator receptor (uPAR) and lysyl oxidase (LOX) which are components of proteolytic enzyme systems that degrade or remodel the ECM and thus facilitate tumour growth and invasion at primary and metastatic sites [67,82,83]. Furthermore, HIF-1 up-regulates the expression of molecules that enhance tumour cell motility such as autocrine motility factor (AMF) and c-MET, and factors that increase microvascular permeability and thereby promote tumour cell intravasation such as vascular endothelial growth factor (VEGF) and angiopoietin 2 (ANGPT2); in addition, HIF-1-dependent expression of L1 cell adhesion molecule (L1CAM) and angiopoietin-like 4 (ANGPTL4) facilitates tumour cell extravasation by increasing tumour cell adhesion to endothelial cells (ECs) and inhibiting EC-EC interactions, respectively [84].

Likewise, HIF-1 contributes to hypoxia-induced chemoresistance by mediating various aspects of drug resistance mechanisms that include drug efflux, alterations in cell survival and proliferation, and protection against DNA damage. HIF-1 activates the expression of genes encoding drug transporters such as P-glycoprotein (P-gp), which is an ATP-dependent multidrug efflux pump that can decrease intracellular concentrations of a range of chemotherapeutics such as anthracyclines and taxanes [85]. Along with P-gp, HIF-1-mediated expression of other drug transporters such as multidrug-resistance protein 1 (MRP1) and lung resistance protein (LRP) has been implicated in hypoxia-induced chemoresistance in various tumour cell types including hepatocarcinoma and gastric cancer cells [53,55]. It has also been shown that HIF-1 functions as a suppressor of apoptosis in various tumour cell types by activating and inhibiting the expression of anti-apoptotic factors (e.g. MCL-1) and pro-apoptotic factors (e.g. BAK and BID), respectively, thereby mediating protective effects of hypoxia on chemotherapy-induced apoptosis [51,86,87]. Furthermore, HIF-1 is an important mediator of hypoxia-induced autophagy, which is a self-degradative process that functions primarily as a survival mechanism under cellular stress.
including that caused by anti-cancer agents [88,89]. In addition to apoptosis and autophagy, cellular senescence is another key player in the regulation of cell survival and proliferation; it is characterized by an irreversible cell cycle arrest that can be induced by various forms of stress including DNA damage caused by anti-cancer activities of some chemotherapeutic agents [85]. Studies in our laboratory have demonstrated that HIF-1-dependent protective effect of hypoxia on drug-induced senescence contributes to the development of hypoxia-mediated drug resistance, highlighting yet another mechanism by which hypoxia via HIF-1 activity confers drug resistance phenotype in tumour cells [48]. Further studies in our lab revealed that the hypoxia-induced increase in tumour cell survival may be due to the prevention of drug-induced DNA damage, partly via HIF-1-dependent down-regulation of topoisomerase IIα (topo IIα) expression, an enzyme that mediates DNA damage, specifically DNA strand breaks, induced by topo II-targeting anti-cancer agents such as etoposide [49].

More recently, findings from our lab have demonstrated that HIF-1 is also involved in mechanisms of hypoxia-mediated tumour cell escape from both innate and adaptive immunity. Results demonstrated that exposure of DU145 prostate and MDA-MB-231 breast cancer cells to hypoxia enhances their resistance to lysis mediated by innate immune effectors via HIF-1-dependent up-regulation of ADAM10 proteolytic enzyme (a disintegrin and metalloproteinase domain-containing protein 10) and consequent cleavage and shedding of immune recognition molecule MICA (MHC class I chain-related molecule A) from tumour cell surface [57]. Further work revealed that HIF-1-dependent increase in tumour cell expression of B7-H1 immune inhibitory ligand contributes to hypoxia-induced resistance to adaptive immunity (i.e. cytotoxic T lymphocyte-mediated lysis) [60]. In addition to these malignant adaptations, HIF-1 plays key roles in mediating many other hypoxic responses necessary for tumour cell survival and proliferation including angiogenesis, autocrine growth factor signalling and metabolic reprogramming [67]. Given the broad and crucial role of HIF-1 in cancer biology and its value as
a therapeutic target, it is of compelling importance to understand the molecular mechanisms underlying HIF-1 regulation.

### Table 1. HIF-1-mediated regulation of key molecules involved in cancer progression

<table>
<thead>
<tr>
<th>Role in malignant progression</th>
<th>Target gene products</th>
</tr>
</thead>
</table>
| Invasion<sup>1</sup>           | Autocrine motility factor  
c-MET  
Matrix metalloproteinase 2, 9 and 14  
Urokinase plasminogen activator receptor |
| Metastasis<sup>1</sup>         | Angiopoietin 2  
Angiopoietin-like 4  
L1 cell adhesion molecule  
Lysyl oxidase  
Vascular endothelial growth factor  
ZEB1, ZEB2 |
| Drug resistance<sup>2</sup>    | BCL-2 antagonist/killer  
BH3 interacting domain death agonist  
Lung resistance protein 1  
Multidrug resistance protein 1  
Myeloid cell leukemia 1  
P-glycoprotein  
Topoisomerase IIα |
| Immune evasion<sup>3</sup>     | ADAM10  
B7-H1 (PD-L1) |

<sup>1</sup>Reviewed in [67] and [80]  
<sup>2</sup>Reviewed in [85]  
<sup>3</sup>[57] and [60]
1.3.2 HIF-1 regulation

The principal mechanism by which O$_2$ regulates HIF-1$\alpha$, and hence HIF-1 activity, occurs at the level of protein stability [90]. Under well-oxygenated conditions, HIF-1$\alpha$ protein is highly unstable with a half-life of less than 5 min; this rapid degradation is mediated via hydroxylation of two conserved proline residues (Pro402 and Pro564 in human HIF-1$\alpha$) in the oxygen-dependent degradation domain (ODD) of HIF-1$\alpha$ by the prolyl-hydroxylase domain (PHD)-containing enzymes (PHD1, PHD2 and PHD3) [91]. The hydroxylation of HIF-1$\alpha$ is required for the binding of the von Hippel-Lindau tumour suppressor protein (pVHL), the substrate recognition component of an E3 ubiquitin ligase complex. This interaction leads to HIF-1$\alpha$ ubiquitylation and consequent degradation by the 26S proteasome [90]. The PHDs have an absolute requirement for O$_2$ as a co-substrate; thus, prolyl hydroxylation of HIF-1$\alpha$ is inhibited under hypoxic conditions, allowing HIF-1$\alpha$ to escape recognition by the pVHL ubiquitin ligase complex and to accumulate in the cell. Stabilized HIF-1$\alpha$ is able to translocate to the nucleus, dimerize with HIF-1$\beta$, bind HRE sequences in target gene promoters and induce gene expression (Figure 1). In addition to protein stability, O$_2$-dependent hydroxylation events also regulate the transactivation function of HIF-1$\alpha$; hydroxylation of an asparagine residue (Asn803 in human HIF-1$\alpha$) in the C-terminal transactivation domain (CTAD) of HIF-1$\alpha$ by factor-inhibiting HIF-1 (FIH-1) prevents interactions between HIF-1$\alpha$ and transcriptional co-activators CREB-binding protein (CBP)/p300, impairing HIF-1 transcriptional activity [90]. FIH-1, like the PHDs, requires O$_2$ for enzymatic activity and thus HIF-1 transactivation is increased under hypoxic conditions (Figure 1). These well-described mechanisms are a part of a complex, integrative network of multiple signalling pathways and players governing HIF-1$\alpha$ regulation directly and indirectly at different levels, including transcription, mRNA stability, translation and post-translational modification, in an O$_2$-dependent as well as O$_2$-independent manner.
Figure 1. A major mechanism by which O$_2$ regulates HIF-1α stability and transactivation function.

Under well-oxygenated conditions, prolyl-hydroxylase (PHD) enzymes hydroxylate two specific proline residues (P$_{402}^{\alpha}$ and P$_{564}^{\alpha}$) in the oxygen-dependent degradation domain (ODD) of hypoxia-inducible factor 1α (HIF-1α). These hydroxylation events are required for the binding of the von Hippel-Lindau tumour suppressor protein (pVHL), the substrate recognition component of an E3 ubiquitin ligase complex, and subsequent ubiquitylation and proteasomal degradation of HIF-1α. PHD enzymes have an absolute requirement for O$_2$ as a co-substrate; thus, prolyl hydroxylation of HIF-1α is inhibited under hypoxic conditions, allowing HIF-1α to accumulate, translocate to the nucleus, dimerize with HIF-1β, bind hypoxia response elements (HREs) and induce expression of target genes. Hydroxylation of an asparagine residue (N$_{803}^{\alpha}$) in the C-terminal transactivation domain (CTAD) of HIF-1α by factor-inhibiting HIF-1 (FIH-1) blocks interactions between HIF-1α and transcriptional co-activators CREB-binding protein (CBP)/p300, impairing HIF-1 transcriptional activity. FIH-1 also requires O$_2$ for enzymatic activity and hence HIF-1 transactivation is increased under hypoxic conditions. bHLH, basic helix-loop-helix; PAS, per-arnrt-sim.
In addition to PHDs and FIH-1, mitochondria have been reported as ‘O$_2$ sensors’ transducing decreased O$_2$ concentration into increased HIF-1 activity [92]. This has been attributed to hypoxia-mediated mitochondrial production of reactive oxygen species (ROS), which may directly or indirectly inhibit PHD activity and promote HIF-1$\alpha$ stabilization; however, precise mechanisms of ROS generation and its role in O$_2$ sensing and HIF-1$\alpha$ regulation have been controversial [92]. With its requirement of the tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (2-OG) as a co-substrate, PHD activity is also intricately linked to metabolic pathways and can be modulated by changes in the relative levels of metabolic intermediates which in turn influence HIF-1$\alpha$ stabilization [93]. In addition, availability of the PHD co-factor Fe$^{2+}$ can affect HIF-1$\alpha$ levels by regulating PHD activity; in fact, an iron chelator often used as a hypoxia mimetic, desferrioxamine (DFO), can inhibit PHD activity and increase HIF-1$\alpha$ accumulation. The HIF-1$\alpha$-stabilizing effect of transition metal ions, such as Co$^{2+}$, may also be explained by their ability to substitute the PHD active-site Fe$^{2+}$ and consequent inhibition of PHD activity [94]. Furthermore, PHDs and consequently HIF-1$\alpha$ protein levels are subject to regulation by other factors including intracellular Ca$^{2+}$ concentrations [95] and ubiquitin ligases of the seven in absentia homolog (SIAH) family [96].

Likewise, pVHL is subject to regulation by several mechanisms including those that modulate its stability by targeting it for ubiquitin-mediated degradation [97] and those that stabilize its interactions with HIF-1$\alpha$ [98], thereby hindering and promoting HIF-1$\alpha$ ubiquitylation, respectively. Interestingly, while the prolyl hydroxylase reactions are irreversible, pVHL-interacting de-ubiquitylating enzyme 2 (VDU2) can reverse the ubiquitylation of HIF-1$\alpha$ and salvage it from proteasomal degradation; VDU2, in turn, is subject to pVHL-mediated degradation and thus, the interplay between pVHL and VDU2 may contribute to HIF-1$\alpha$ regulation [99]. HIF-1$\alpha$ protein stability is also modulated by an O$_2$/PHD/pVHL-independent, proteasome-dependent pathway involving the competitive binding of HIF-1$\alpha$ by two oppositely
acting proteins, heat shock protein 90 (HSP90) and receptor for activated C-kinase 1(RACK1) which promote HIF-1α stabilization and degradation, respectively [100]. In addition to hydroxylation and ubiquitylation, other post-translational modifications of HIF-1α have been reported such as phosphorylation, which exerts influences on HIF-1α protein stability as well as nuclear localization and transcriptional activity, and plays an important role in mediating HIF-1α-inducing effects of growth factor signalling pathways that act independently of or at times in cooperation with low O_2 tension [101-105].

Growth factor signalling, mainly through mammalian target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) pathways, can also stimulate the synthesis of HIF-1α protein under normoxic conditions by modulating translational initiation processes [106]. Under hypoxic conditions, general protein translation is decreased in an effort to reduce energy consumption while certain proteins essential for survival, including HIF-1α, continue to be translated; this preferential HIF-1α translation may be mediated by RNA-binding proteins such as human antigen R (HuR) and polypyrimidine tract-binding protein (PTB) [106-108]. Negative regulators of HIF-1α mRNA stability have also been identified including antisense HIF (aHIF) [109], micro RNAs (miRNAs) such as miR-17-92, miR-155 and miR-20b [110-112], and mRNA-destabilizing protein tristetraprolin [113], some of which appear to participate in negative feedback control of HIF-1α. Although HIF-1α mRNA is constitutively expressed regardless of O_2 levels in many cell types, hypoxia-induced transcriptional up-regulation of HIF-1α was described and further shown to be mediated by hypoxia-activated transcription factors, nuclear factor-κB (NF-κB) and early growth factor 1 (Egr1) [114,115]. Furthermore, HIF1A gene promoter contains several putative HREs, suggesting a positive auto-regulatory loop whereby HIF-1α induces its own expression [106]; indeed, this has been reported to occur in colon cancer cells in which aberrant demethylation of the HRE enables HIF-1α binding to its own promoter and facilitates auto-transactivation [116].
Thus regulation of HIF-1α levels, and hence HIF-1 activity, involves interactions between multiple, interconnected pathways and factors, reflecting the complex nature of molecular responses to hypoxia. A significant addition to this regulatory network is the crosstalk with nitric oxide (NO), a molecule with considerable similarities to O₂, which in part enables it to interfere with O₂ sensing mechanisms and HIF-1 activity [117]. Findings from our lab have demonstrated a role for NO signalling in the regulation of tumour cell adaptive responses to hypoxia, further implicating an intricate link between NO and hypoxia. Better understanding of the mechanism and function of NO signalling in the modulation of tumour hypoxic responses is needed to fully unravel its therapeutic potential.

1.4 NO signalling

Nitric oxide is a simple diatomic, free radical gas that plays a complex role in cellular signal transduction. It is produced endogenously by the nitric oxide synthase (NOS) enzyme family, which consists of three isoforms: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). These isoforms can be classified based on their regulation of expression (constitutive versus inducible), Ca²⁺ dependency and subcellular localization [118]. Although NOS I, NOS II and NOS III were originally identified in brain, macrophages and endothelium, respectively, they have been found in various other tissues and cells including tumour cells [119-121]. All three NOS enzymes catalyze the formation of NO and L-citrulline from L-arginine using O₂ and NADPH as co-substrates and multiple co-factors including FAD, tetrahydrobiopterin (BH₄) and heme [122]. Once NO is produced, it can participate in the regulation of a vast array of biological functions including vasodilation, platelet adhesion and neurotransmission. With the well-characterized role of NO in the cardiovascular system, NO donors are in clinical use as nitrovasodilators, one of the oldest and most commonly
employed of which is glyceryl trinitrate (GTN; nitroglycerin). GTN has been used for over a century in the treatment of angina pectoris and congestive heart failure. It is the prototype of the organic nitrate class of NO donors which require bioactivation to release NO although the precise mechanism is not fully understood; both non-enzymatic and enzymatic bioactivation pathways have been proposed including reactions with sulfhydryl groups, and catalysis by different enzymes such as glutathione S-transferase, cytochrome P450, xanthine oxidase and mitochondrial aldehyde dehydrogenase, respectively [123]. Its current use and safety for clinical application make GTN an attractive NO donor to explore as a therapeutic agent for many more pathological states in which NO plays an important role, such as cancer.

The involvement of NO in cancer biology was observed as early as 1988 when NO was identified as a molecular effector of macrophage cytotoxicity against tumour cells [124,125]. Beginning with these initial findings, NO has been shown to affect various aspects of cancer biology including cellular proliferation, apoptosis, metastasis, angiogenesis and resistance to therapy; however, the precise role of NO in tumour progression has been controversial with studies suggesting either tumour-promoting or tumour-suppressing effects [27,126,127]. The apparent dichotomy of NO-mediated effects may be explained by the presence of multiple pathways through which NO can regulate cellular processes depending on the local concentration of NO and the molecular environment (e.g. redox status) [122]. At high concentrations (>1 µM), NO can undergo reactions with oxygen or superoxide radicals, resulting in the formation of reactive nitrogen species (RNS) such as dinitrogen trioxide and peroxynitrite, which in turn mediate nitrosylation and nitration, respectively, among many other reactions [122,128]. Nitrosylation of thiols (S-nitrosylation), such as those found in cysteine residues, and nitration of tyrosine can alter the function of various proteins including signalling molecules and transcription factors [122]. When concentrations of NO are relatively low (<1 µM) interactions with transitions metals, such as iron within heme-containing enzymes, can occur [122,128]. The heme-protein
soluble guanylyl cyclase (sGC) is the main target of NO that mediates most of its downstream effects [122]. While the membrane-bound forms of guanylyl cyclase (particulate guanylyl cyclases) are not generally activated by NO, sGC is activated upon NO binding and catalyzes the conversion of guanosine 5'-triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP). The second messenger cGMP, in turn, activates downstream effectors including cGMP-dependent protein kinase (PKG), cyclic nucleotide-gated channels (CNG) and phosphodiesterases (PDEs); of these, PKG is thought to be responsible for the majority of cellular effects of cGMP via phosphorylation of various target molecules involved in regulation of gene expression and cell function [27,122]. Studies in our laboratory have demonstrated that this low concentration cGMP-dependent NO signalling pathway (i.e. classical NO signalling) plays an important role in the regulation of hypoxia-induced tumour malignancy [35,36,47,56,57,60,120,121] and warrant further exploration.

1.4.1 NO signalling and malignant adaptations to hypoxia

A growing body of evidence indicates that decreased endogenous NO/cGMP signalling is a critical aspect of the mechanism underlying hypoxic induction of adaptive responses in tumour cells. Studies conducted in our laboratory demonstrated that hypoxia-induced acquisition of malignant properties in tumour cells is attenuated by activation of the NO/cGMP signalling pathway whereas inhibition of the pathway in well-oxygenated cells results in phenotypes similar to those induced by hypoxia [27,35,36,47,56,57,60,120,121]. Specifically, it was shown that low concentrations (≤ 1 µM) of NO donors, such as GTN, could abrogate hypoxic up-regulation of uPAR expression and in vitro invasiveness of human MDA-MB-231 breast carcinoma cells; in a manner similar to the NO-donor treatment, a non-hydrolyzable cGMP analogue (8-Br-cGMP) was also able to inhibit the hypoxia-induced uPAR expression and invasiveness [35]. Furthermore, pharmacological inhibition of endogenous NO production (i.e. inhibition of NOS)
in well-oxygenated cells resulted in an increase in uPAR expression that could be attenuated by treatment with GTN [35]. Pharmacological inhibition of cGMP signalling pathway constituents, such as sGC and PKG, similarly increased uPAR expression in well-oxygenated conditions; interestingly, GTN was unable to inhibit uPAR expression when sGC activity was blocked, further demonstrating the involvement of cGMP signalling in mediating the effects of NO on hypoxia-induced invasiveness [35]. Enhanced tumour cell invasion is a well-known prerequisite for metastasis. Indeed, a subsequent study revealed that administration of low concentrations of NO donors, including GTN and diethylenetriamine NO adduct (DETA/NO), prevented hypoxic up-regulation of B16F10 murine melanoma metastasis; 8-Br-cGMP was also able to abrogate the hypoxia-induced metastatic potential and NOS inhibition elicited a similar response (i.e. increased metastasis) to that of hypoxia, which could be attenuated by NO donors [36]. Further studies demonstrated that GTN or DETA/NO at low concentrations could also attenuate hypoxia-mediated acquisition of chemoresistance via the cGMP-dependent pathway in various cell lines including MDA-MB-231, B16F10 and human and mouse prostatic cancer cells (DU145, PC-3 and TRAMP-C2); direct activation of cGMP signalling with 8-Br-cGMP abrogated the hypoxia-induced chemoresistance while pharmacological inhibition of NO production or of sGC or PKG activity enhanced tumour cell resistance in well-oxygenated conditions [47,120,121]. Recent work revealed a similar mechanism of action of NO in the regulation of hypoxia-mediated tumour cell immune escape involving ADAM10-dependent shedding of MICA immune recognition molecule [56,57].

Collectively, these findings suggest that hypoxia-induced phenotypes in tumour cells is in part due to impaired NO/cGMP signalling and that re-establishment of this signalling inhibits such effects of hypoxia as enhanced invasiveness, metastatic potential, drug resistance and immune escape. This concept is further supported by the fact that endogenous NO production requires O2 [122] and that exposure of cells to low oxygen conditions limits NO synthesis by up
to 90% [129,130]. The reduced NO levels associated with hypoxia as well as pharmacological inhibition of NOS may lead to decreased sGC activity and a consequential reduction in cGMP production. Indeed, studies have found that both hypoxia and NOS inhibition can result in significant decreases in cellular cGMP levels [131,132]; our previous study similarly showed a marked reduction in cGMP accumulation in tumour cells exposed to hypoxic conditions which could be restored by treatment with GTN [35]. Thus there appears to be an intricate cross-regulatory link between NO/cGMP and O\textsubscript{2} signalling pathways that plays an important role in the regulation of tumour cell adaptations to hypoxia. Given the central role of HIF-1 in mediating these hypoxic responses, HIF-1\textalpha, and hence HIF-1 activity, may be a potential downstream target of the NO/cGMP signalling pathway and an important component of the mechanism by which NO modulates the hypoxia-induced phenotypes.

1.4.2 NO signalling and HIF-1\textalpha

Studies have reported regulatory effects of NO on HIF-1\textalpha accumulation and HIF-1 activity, many of which have been attributed to cGMP-independent mechanisms. Based on these studies, it is also evident that the regulatory capacities of NO are highly complex and dependent on various factors including O\textsubscript{2} availability and NO concentration [117]. It has been found that NO, derived from either endogenous or exogenous sources, induces HIF-1\textalpha stabilization and HIF-1 activity under well-oxygenated conditions; these effects on HIF-1 were found to take place via high concentrations of NO involving S-nitrosylation of HIF-1\textalpha or pVHL [133-138]. Under hypoxic conditions, NO appears to have an opposite effect, attenuating HIF-1\textalpha accumulation and HIF-1 transactivation [139-146]. Mechanisms that have been proposed to explain inhibitory effects of NO on hypoxic accumulation of HIF-1\textalpha have primarily centred on the regulation of PHD activity. It has been suggested that under hypoxic conditions, NO inhibits mitochondrial respiration at cytochrome c oxidase (complex IV of the respiratory chain) resulting in decreased
O₂ consumption and hence redistribution of O₂ such that more O₂ becomes available for re-
activation of PHD enzymes and consequent degradation of HIF-1α protein [140,147]. In addition
to altering O₂ availability, NO may also increase the availability of PHD cofactor iron and
activate PHDs in hypoxia via mechanisms involving reactive nitrogen species [144,145]. Direct
activation of PHDs [148] and induction of PHD expression [142] by NO have also been proposed
to attenuate HIF-1α accumulation in hypoxia. Nevertheless, modulation of PHD activity may not
fully explain the inhibitory effects of NO on hypoxic accumulation of HIF-1α. Zhou et al. [146]
revealed that an alternative, PHD/pVHL/proteasome-independent mechanism involving the
calpain (Ca²⁺-activated protease) system mediates NO-induced HIF-1α degradation [146]. Using
pVHL-deficient renal cell carcinoma (RCC4) cells, they found that exposure to hypoxia with the
addition of the NO donor DETA/NO could inhibit HIF-1α accumulation and that calpain
inhibitors could reverse this effect; calpain was further shown to bind HIF-1α suggesting that
calpain may directly induce HIF-1α degradation. Moreover, modulation of intracellular Ca²⁺
using Ca²⁺ chelators and mobilizers demonstrated that the calpain-mediated degradation of HIF-
1α is Ca²⁺-dependent [146]. Interestingly, cGMP signalling has been associated with increases in
intracellular Ca²⁺ and/or calpain activity [149-152] although its role in the regulation of calpain-
mediated HIF-1α degradation has yet to be determined.

1.5 Calpain

Calpains constitute a family of intracellular cysteine proteinases that generally require
Ca²⁺ for enzymatic activity. There are at least 14 human calpain isoforms identified to date, of
which μ-calpain (calpain I) and m-calpain (calpain II) are the most extensively studied,
ubiquitously expressed archetypical members of the calpain family [153,154]. The third well-
characterized player of the calpain system is calpastatin, an endogenous inhibitor of μ- and m-
calpain. Although the physiological function of calpains remains to be fully elucidated owing in part to their broad substrate specificity, studies have demonstrated important roles for calpains in multiple cellular processes including cell motility, apoptosis, cell cycle progression, signal transduction and long-term potentiation [153,154]. Deregulated calpain expression and activity have also been implicated in various pathological conditions including cancer, type 2 diabetes, multiple sclerosis and myocardial infarction [153,155]. Thus, calpains have an extensive influence on cellular processes via controlled proteolysis of many specific substrates including HIF-1α.

1.6 Hypothesis

NO signalling via cGMP generation attenuates hypoxia-induced HIF-1α accumulation in human DU145 prostate tumour cells in a calpain-dependent manner (Figure 2).
Figure 2. Proposed model for NO/cGMP signalling pathway leading to inhibition of HIF-1α accumulation and malignant adaptations to hypoxia.

Based on previous findings indicating that impaired endogenous nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signalling is a key aspect of the mechanism underlying hypoxia-induced phenotypes and that re-establishment of the signalling attenuates such hypoxic responses, the present thesis aims to determine the downstream mechanism by which the cGMP-dependent pathway inhibits malignant adaptations to hypoxia. As a central mediator of these hypoxic responses, hypoxia-inducible factor 1 (HIF-1) is likely targeted by the NO/cGMP signalling, which may be an important part of the mechanism by which NO regulates hypoxic responses. Regulation of HIF-1 occurs primarily at the level of the HIF-1α subunit protein stability; a well-established mechanism by which oxygen regulates HIF-1α levels and hence HIF-1 activity involves the canonical prolyl hydroxylase (PHD)-von Hippel-Lindau protein (pVHL) axis. We propose an alternative mechanism of HIF-1α regulation by NO/cGMP signalling involving calpain-mediated degradation of HIF-1α. A mechanistic link between calpain and HIF-1α modulation has been shown previously. In addition, cGMP signalling has been associated with increases in calpain activity and in intracellular calcium levels, the primary activator of calpain; however, the role of cGMP signalling in the regulation of calpain-mediated HIF-1α degradation has not been addressed. Solid lines represent established pathways whereas dotted lines indicate specific areas to be investigated.
1.7 Objectives

1. To characterize the effect of NO on hypoxic accumulation of HIF-1α by assessing concentration-dependent and temporal relationships between NO exposure, through administration of the NO donor GTN, and HIF-1α expression in hypoxic DU145 tumour cells.

2. To determine the role of cGMP-dependent signalling in the regulation of hypoxia-induced HIF-1α accumulation by examining the effect of pharmacological activation of the pathway, using cGMP analogue 8-Br-cGMP, on HIF-1α expression in hypoxic DU145 tumour cells.

3. To determine whether calpain is involved in NO/cGMP-mediated regulation of hypoxia-induced HIF-1α accumulation by blocking calpain activity using specific calpain inhibitor calpastatin peptide.
Chapter 2

Materials and Methods

2.1 Cells

Human prostate carcinoma cell line DU145 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in monolayer culture in RPMI 1640 medium (Life Technologies Invitrogen Corporation, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). DU145 was the first prostate cancer cell line to be established in tissue culture and one of the three most widely used prostate cancer cells along with LNCaP and PC-3 cells [156]. This cell line was derived from a brain metastasis of prostatic cancer in a 69-year-old patient [157]. DU145 is characterized as androgen receptor-negative and prostate specific antigen (PSA)-negative cell line with a doubling time of approximately 34 hours in tissue culture [156]. Previous studies have provided evidence, using DU145 cells, that restoration of NO/cGMP signalling prevents hypoxic responses (i.e. hypoxia-induced chemoresistance and immune evasion) [47,56,57]. Also, DU145 cells express components of the calpain system, including m-calpain and calpastatin [158]. Furthermore, DU145 cells can form tumours in vivo, facilitating the transition from in vitro to in vivo studies.

2.2 Culture conditions

Cells were cultured in six-well plates at 60-70% confluence (to avoid pericellular hypoxia resulting from high-density cultures [50]) at the start of all experiments. Following a 24-h incubation in standard culture conditions, the culture medium was changed and the cultures were placed in standard (20% O₂) or hypoxic (0.2% O₂) conditions. For incubations in standard
conditions, cells were placed in a Thermo Forma CO\textsubscript{2} incubator (5\% CO\textsubscript{2} in air at 37\°C) whereas for incubations in hypoxia, cells were placed in airtight chambers that were flushed with a gas mixture of 5\% CO\textsubscript{2}/95\% N\textsubscript{2} (BOC, Kingston, ON, Canada) and incubated at 37\°C in a standard incubator. Oxygen concentrations within these chambers were maintained at 0.2\% O\textsubscript{2} using Pro-Ox model 110 O\textsubscript{2} regulators (Biospherix, Redfield, NY, USA).

To assess dose-dependent effects of NO on hypoxia-induced HIF-1\textalpha accumulation, randomly selected culture plates were incubated with various concentrations (10 nM-1 mM) of the NO mimetic agent GTN (Omega Laboratories Ltd., Montreal, QC, Canada) administered at the beginning of the 24-h exposure to standard or hypoxic conditions. Subsequently, temporal effects of NO were determined by exposing cells to 1 \mu M GTN for 4, 8, 16 and 24 h in 20\% or 0.2\% O\textsubscript{2}.

To further examine the role of NO/cGMP signalling pathway in the regulation of HIF-1\textalpha accumulation, cells were incubated with the non-hydrolysable analogue of cGMP, 8-Br-cGMP (1 \mu M; Sigma-Aldrich Canada), for 4, 8, 16 and 24 h in 20\% or 0.2\% O\textsubscript{2}. The involvement of calpain in the NO/cGMP-mediated regulation of HIF-1\textalpha was analyzed by incubating cells with GTN (1 \mu M; 4 h) or 8-Br-cGMP (1 \mu M; 4 h), or in combination with the selective calpain inhibitor calpastatin peptide (2 \mu M for 4 h; Calbiochem/EMD Biosciences, San Diego, CA, USA) in 20\% or 0.2\% O\textsubscript{2}. The concentration of calpastatin used in this study was previously shown to effectively inhibit calpain-mediated degradation of HIF-1\textalpha [146].

2.3 Western blot analysis

Following incubation under various conditions, cells were frozen immediately by rapidly discarding the medium, rinsing in PBS and placing the culture plates in liquid nitrogen. Cells were lysed with a buffer containing 2\% SDS, 10 mM Tris, 0.15 M NaCl (pH 7.6) and Complete
Protease Inhibitor Cocktail (Roche Diagnostics Canada, Laval, QC, Canada). Lysates were subjected to sonication at 40 Hz for 12 s followed by centrifugation for 15 min at 10,000 × g. The supernatant was collected and protein content was determined using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Protein samples of 3-7 µg were resolved on 7.5% SDS-polyacrylamide Next Gels (Amresco/Cedarlane Laboratories, Burlington, ON, Canada) and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) via a wet transfer system (Bio-Rad Laboratories).

Membranes were incubated for 1 h in 1% (w/v) non-fat milk (reconstituted from powder) in TBS (20 mM Tris, 140 mM NaCl, pH 7.6) to block non-specific binding sites. Subsequently, blots were incubated with primary antibodies in 1% milk/TBS for 1.5 h; antibodies used included mouse monoclonal anti-human HIF-1α antibody (1:250 dilution, BD Biosciences, Mississauga, ON, Canada) and mouse monoclonal anti-β-actin antibody (1:5000 dilution, Sigma-Aldrich Canada). Membranes were washed three times for 5 min each with TBST (TBS containing 0.1% Tween-20). Blots were then incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:5000 in 1% milk/TBS; Bio-Rad Laboratories) for 1 h and washed three times for 5 min each with TBST. HRP activity was detected by enhanced chemiluminescence (Perkin-Elmer Life Sciences Inc., Boston, MA, USA) and exposure to Kodak X-Omat film (Eastman Kodak, Rochester, NY).

2.4 Real-time reverse transcription-PCR (qRT-PCR)

Following incubation under various conditions, cells were frozen immediately as described in 3.3. Total RNA was isolated using Total RNA Mini Kit (Geneaid Biotech Ltd./Frogga Bio, Toronto, ON, Canada) according to the manufacturer’s protocol and quantified using a Nano-Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One
microgram of total RNA was reverse-transcribed using random hexamers (100 µM; Cortec DNA Service Laboratories Inc., Kingston, ON, Canada) and Omniscript RT Kit (Qiagen Inc., Toronto, ON, Canada) according to the manufacturer’s guidelines. The resultant cDNAs were used as templates for real-time PCR amplification using a LightCycler 480 (Roche) and the KAPA SYBR FAST Master Mix (Kapa Biosystems/D-Mark Biosciences, Toronto, ON, Canada).

Primer sets were designed from published NIH Genbank mRNA sequences (NIH Centre for Biotechnical Information, Bethesda, MD, USA) using Primer Design 2.01 software (Scientific & Educational Software, Cary, NC, USA). To prevent genomic DNA contamination, intron-spanning primers were constructed. Additional primer design criteria included similarity in melting temperature ($T_m$) levels (up to 1°C difference in $T_m$), optimal primer length (18-20 bp), $T_m$ (65-70°C) and product size (150-200 bp) as well as avoidance of primer secondary structures and mispriming (minimum 3’end matches and runs of bases). The primer sequences were as follows: 

$HIF$-$1a$, forward 5’-CGACACAGCCTGGATATGAA-3’ and reverse 5’-

TCCTGTGGTGACTTGTCCTT-3’ ($T_a= 63$ ºC, 200 nM; Eurofins mwg/operon, Huntsville, AL, USA); $\beta$-actin, forward 5’-CTGGACTTCGAGCAAGAGAT-3’ and reverse 5’-

GATGTCCACGTCACACTTCA-3’ ($T_a= 63$ ºC, 200 nM; Eurofins mwg/operon). Primer specificity and amplification cycling conditions were tested and optimized using melt curve analyses. PCR products from each primer set were subjected to agarose gel electrophoresis (1% agarose gel) to further confirm the production of a single amplicon of the correct size. The cycling conditions consisted of a denaturation step (95ºC for 5 min) followed by 40 cycles at 95ºC for 15 s, 63ºC for 20 s and 72ºC for 5 s. Levels of gene expression were calculated using the standard curve method for each gene. The primers had an efficiency range between 1.7 and 2.0.
2.5 Calculations and statistical analysis

To quantify the level of HIF-1α expression from Western blot experiments, x-ray films were scanned and densitometric analysis was performed using Image Processing and Analysis in Java (ImageJ, National Institute of Mental Health, Bethesda, Maryland, USA). The relative expression levels of HIF-1α protein and mRNA were measured by HIF-1α/β-actin ratio to account for sample loading differences. To pool results from independent experiments, data were further normalized against the normoxic or hypoxic control group (20% or 0.2% O₂) within each experiment.

Results are presented as means ± the standard error of the mean (SEM). All statistical analyses were performed using Prism 6.0 Software (GraphPad Software Inc., La Jolla, CA, USA). Based on the experimental design, statistical significance was determined using one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at P < 0.05.
Chapter 3

Results

3.1 Effect of GTN on HIF-1α expression

To assess the effect of NO on hypoxia-induced HIF-1α accumulation, DU145 prostate cancer cells were exposed to hypoxic (0.2% O₂) or control (20% O₂) conditions in the presence or absence of the NO donor GTN, followed by Western blot analysis of HIF-1α. Incubation of cells in 0.2% O₂ for 24 hours resulted in a significant increase (p < 0.0001) in HIF-1α protein levels compared with cells incubated in 20% O₂, while administration of GTN (10 nM-1 mM) at the onset of the 24-hour hypoxic exposure prevented the accumulation of HIF-1α in a concentration-dependent manner (Figure 3A); a significant inhibitory effect of GTN on HIF-1α accumulation was observed at concentrations of 1 µM (p=0.0061), 10 µM (p=0.0032) and 1 mM (p<0.0001). In contrast, GTN did not affect HIF-1α protein levels in cells incubated in 20% O₂ at all concentrations tested (Figure 3B).
Figure 3. Effect of various concentrations of GTN on HIF-1α protein levels in DU145 cells. Shown are the representative Western blots and densitometric analyses of HIF-1α protein levels in DU145 cells exposed to various concentrations of GTN (10 nM-1 mM) for 24 hours in 0.2% O₂ (A) or 20% O₂ (B). Bars represent mean ± SEM. **, P<0.01; ****, P<0.0001, one-way ANOVA followed by Bonferroni multiple comparison post hoc test. Results represent pooled data from three to six independent experiments. The separating vertical dotted lines indicate where the image was cut and reordered to facilitate description of the data.
To further characterize the effect of NO on hypoxic accumulation of HIF-1α, temporal relationship between NO exposure and HIF-1α protein was investigated. DU145 cells treated with GTN (1 µM) were exposed to 0.2% or 20% O₂ for various durations (4-24 hours), followed by Western blot analysis of HIF-1α. Results showed that the inhibitory effect of GTN on hypoxia-induced HIF-1α accumulation was most robust at the 4-hour time point (p=0.0102) (Figure 4, A and B). In cells incubated in 20% O₂, GTN did not significantly alter HIF-1α protein levels at all time points examined (Figure 4, C and D).

![Figure 4. Time course of effects of GTN on HIF-1α protein levels in DU145 cells.](image)

Shown are the representative Western blot (A) and densitometric analysis (B) of HIF-1α protein levels in DU145 cells exposed to various durations (4-24 hours) of hypoxia (0.2% O₂) in the presence or absence of GTN (1 µM) (n=4). Representative blot (C) and quantification (D) of HIF-1α protein in cells incubated in 20% O₂ for various times in the presence or absence of 1 µM GTN are also presented (n=3). Control (Con) cells were incubated for 24 hours in 20% O₂ (A and B) or 0.2% O₂ (C and D). Bars represent mean ± SEM. *, P<0.05, two-way repeated measures ANOVA followed by Bonferroni post hoc test.
To determine whether changes in HIF-1α mRNA levels account for the observed variations in HIF-1α protein levels following GTN treatment, HIF-1α mRNA was analyzed by qRT-PCR. In contrast to the hypoxic induction of HIF-1α protein accumulation, DU145 cells exposed to 0.2% O₂ for 24 hours resulted in a significant decrease in HIF-1α mRNA levels (p=0.0002) compared with cells incubated in 20% O₂ and administration of GTN did not affect HIF-1α mRNA expression at all concentrations (10 nM-10 µM) tested (Figure 5A). Similarly, time course analysis revealed that various durations of hypoxic exposure (4-24 hours) significantly decreased HIF-1α mRNA levels (p<0.0001 for 4, 8 and 16 hours and p=0.0001 for 24 hours) and addition of GTN (1 µM) did not alter HIF-1α mRNA expression in either 0.2% O₂ or 20% O₂ at all time periods examined (Figure 5B).

**Figure 5.** Effect of GTN on HIF-1α mRNA levels in DU145 cells.
A, qRT-PCR analysis of HIF-1α mRNA expression in DU145 cells exposed to 0.2% or 20% O₂ in the presence or absence of various concentrations (10 nM-1 mM) of GTN for 24 hours (n=3). B, qRT-PCR analysis of HIF-1α mRNA in cells incubated in 0.2% or 20% O₂ in the presence or absence of 1 µM GTN for various periods of time (4-24 hours) (n=3). Bars represent mean ± SEM. ***, P<0.001; ****, P<0.0001, one-way ANOVA (A) or two-way repeated measures ANOVA (B) followed by Bonferroni post hoc test.
3.2 Effect of 8-Br-cGMP on HIF-1α expression

To determine the role of cGMP-dependent signalling in the regulation of hypoxia-induced HIF-1α accumulation, DU145 cells were incubated in 0.2% or 20% O₂ in the presence or absence of the non-hydrolysable cGMP analogue 8-Br-cGMP. In a similar manner to that of the GTN time course experiment, a time course (4-24 hours) of the effect of 8-Br-cGMP (1 µM) on hypoxic accumulation of HIF-1α protein was analyzed (Figure 6, A and B). Interestingly, 8-Br-cGMP mimicked the effect of GTN and significantly reduced HIF-1α accumulation in cells exposed to hypoxia (0.2% O₂) for four hours (p=0.0222). HIF-1α protein levels in cells incubated in 20% O₂ were unaffected by exposure to 8-Br-cGMP at all time points examined (Figure 6, C and D).

**Figure 6. Time course of effects of 8-Br-cGMP on HIF-1α protein levels in DU145 cells.** Shown are the representative Western blot (A) and densitometric analysis (B) of HIF-1α protein levels in DU145 cells exposed to various durations (4-24 hours) of hypoxia (0.2% O₂) in the presence or absence of 8-Br-cGMP (1 µM) (n=3). Representative blot (C) and quantification (D) of HIF-1α protein in cells incubated in 20% O₂ for various periods of time in the presence or absence of 1 µM 8-Br-cGMP are also presented (n=3). Control (con) cells were incubated for 24 hours in 20% O₂ (A and B) or 0.2% O₂ (C and D). Bars represent mean ± SEM. *, P<0.05, two-way repeated measures ANOVA followed by Bonferroni post hoc test.
To assess whether the inhibitory effect of 8-Br-cGMP on hypoxic accumulation of HIF-1α occurs at the mRNA level, qRT-PCR analysis of HIF-1α mRNA was conducted. Exposure of DU145 cell to 8-Br-cGMP (1 µM) in 0.2% or 20% O₂ for various time periods (4-24 hours) did not result in a significant change in HIF-1α mRNA levels in either 0.2% or 20% O₂ compared with controls (cells incubated in 0.2% or 20% O₂ alone) at all time points tested (Figure 7).

![Figure 7. Effect of 8-Br-cGMP on HIF-1α mRNA levels in DU145 cells.](image)

Cells were incubated in 0.2% or 20% O₂ in the presence or absence of 1 µM 8-Br-cGMP for various periods of time (4-24 hours), followed by qRT-PCR analysis of HIF-1α mRNA levels (n=3). Bars represent mean ± SEM. ****, P<0.0001, two-way repeated measures ANOVA followed by Bonferroni post hoc test.

3.3 Effect of hypoxia on HIF-1α expression

Interestingly, analysis of HIF-1α protein and mRNA in DU145 cells revealed a distinct pattern of expression in response to various durations of hypoxic exposure (Figure 8). While HIF-1α protein was nearly undetectable in 20% O₂, exposure to hypoxia (0.2% O₂) for four hours
resulted in a significant increase in HIF-1α protein abundance (p=0.0005) (Figure 8, A and B). Prolonged (8-24 hours) hypoxia progressively decreased HIF-1α protein levels, which still remained higher than base-line levels although this difference did not gain statistical significance (Figure 8, A and B). In contrast to HIF-1α protein, four hours of hypoxic exposure significantly reduced HIF-1α mRNA levels (p=0.0011) and sustained hypoxia (8, 16 and 24 hours) progressively decreased HIF-1α mRNA (p=0.0002, p<0.0001 and p=0.0001 for each time point, respectively) (Figure 8C).

A.

<table>
<thead>
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<th>4</th>
<th>8</th>
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<td>β-Actin</td>
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B.

C.

Figure 8. Time course of effects of hypoxia on HIF-1α protein and mRNA levels in DU145 cells.

Shown are the representative Western blot (A) and quantification (B) of HIF-1α protein levels in DU145 cells exposed to various durations (4-24 hours) of hypoxia (0.2% O₂) (n=8). qRT-PCR analysis of HIF-1α mRNA in cells incubated in 0.2% O₂ for various periods of time was also conducted (n=3) (C). Bars represent mean ± SEM. **, P<0.01; ***, P<0.001; ****, P<0.0001, one-way ANOVA followed by Bonferroni post hoc test.
3.4 Calpain and NO/cGMP-mediated inhibition of HIF-1α protein accumulation

Subsequent to the earlier analyses suggesting that both GTN and 8-Br-cGMP inhibit hypoxic accumulation of HIF-1α protein at the translational or post-translational level, the involvement of calpain protease was investigated. To determine a direct role of calpain in the NO/cGMP-mediated attenuation of HIF-1α accumulation, DU145 cells were subjected to treatment with GTN (1 µM) or 8-Br-cGMP (1 µM), or co-treatment with the specific calpain inhibitor calpastatin (2 µM) at the onset of a 4-hour incubation in 0.2% or 20% O₂. While GTN alone significantly reduced HIF-1α protein accumulation in cells exposed to hypoxia (p=0.0019), co-incubation with calpastatin significantly abolished the inhibitory effect of GTN on HIF-1α accumulation (p=0.0489) (Figure 9). Similarly, 8-Br-cGMP significantly decreased HIF-1α protein levels in cells under hypoxic conditions (p=0.0081) while addition of calpastatin significantly blocked the effect of 8-Br-cGMP (p=0.0365) (Figure 10).
Figure 9. Effect of GTN/calpastatin on hypoxia-induced HIF-1α protein accumulation in DU145 cells.
Shown are the representative Western blot and densitometric analysis of HIF-1α protein levels in cells incubated for four hours in 0.2% or 20% O₂ in the presence or absence of GTN (1 µM) alone or in combination with the calpain inhibitor calpastatin peptide (2 µM) (n=4). The separating vertical dotted line indicates that irrelevant lanes on the original blot were cut out. Bars represent mean ± SEM. *, P<0.05; **, P<0.01, one-way ANOVA followed by Bonferroni post hoc test.
Figure 10. Effect of 8-Br-cGMP/calpastatin on hypoxia-induced HIF-1α protein accumulation in DU145 cells.
Shown are the representative Western blot and densitometric analysis of HIF-1α protein levels in cells incubated for four hours in 0.2% or 20% O\textsubscript{2} in the presence or absence of 8-Br-cGMP (1 µM) alone or in combination with the calpain inhibitor calpastatin peptide (2 µM) (n=4). The separating vertical dotted line indicates that irrelevant lanes on the original blot were cut out. Bars represent mean ± SEM. *, P<0.05; **, P<0.01, one-way ANOVA followed by Bonferroni post hoc test.
Chapter 4

Discussion

The role of NO in cancer biology is extensive and multifaceted owing in part to the complex chemistry of NO that determines its cellular effects depending on such factors as the local NO concentration and duration of exposure as well as the specific molecular environment (e.g. redox status) [27,126,127]. This is further complicated by the intricate cross-regulatory relationship between NO and O$_2$ signalling which is relevant in pathological states, particularly those associated with hypoxic microenvironment, such as cancer [27,138]. A growing body of evidence indicates that an important aspect of the mechanism underlying hypoxia-induced phenotypes in tumour cells is the impairment in cGMP-dependent NO signalling and that re-establishment of NO signalling attenuates such hypoxic responses as enhanced invasiveness, metastatic potential, drug resistance and immune escape [35,36,47,56,57,60,120,121]. The work of this thesis sought to characterize the downstream mechanism by which the NO/cGMP signalling regulates adaptive responses to hypoxia in tumour cells. Using human DU145 prostate cancer cells, for which there is evidence that restoration of NO/cGMP signalling prevents hypoxic responses such as hypoxia-induced immune evasion and chemoresistance [47,56,57], studies were conducted to assess the effect of the NO donor GTN and the cGMP analogue 8-Br-cGMP on hypoxic accumulation of HIF-1$\alpha$ and to determine the role of calpain in the NO/cGMP-mediated regulation of HIF-1$\alpha$. Results from these studies revealed that GTN at a relatively low concentration significantly attenuated hypoxia-induced HIF-1$\alpha$ protein accumulation in a time-dependent manner. Furthermore, 8-Br-cGMP mimicked the effect of GTN on HIF-1$\alpha$ protein while neither GTN nor 8-Br-cGMP significantly altered HIF-1$\alpha$ mRNA levels. Interestingly, the inhibitory effects of GTN and 8-Br-cGMP on HIF-1$\alpha$ protein accumulation were significantly
attenuated in the presence of the calpain inhibitor calpastatin peptide. These findings point to a role for NO/cGMP signalling in the regulation of HIF-1α via a calpain-dependent mechanism.

Both concentration and duration of exposure to NO are critical determinants of the quality and magnitude of the biological response to exogenously administered NO [159]. Analysis of the effect of GTN on HIF-1α accumulation at various concentrations and exposure times revealed that a relatively low concentration (1 µM) as well as high concentrations of GTN (10 µM, 1 mM) were able to significantly attenuate hypoxic accumulation of HIF-1α protein and that the inhibitory effect of the low concentration of GTN was rapid and possibly transient, peaking at 4 hours of treatment. Although NO production was not directly measured and hence caused difficulties in comparing with results of other studies using NO donors with variable kinetics of NO release, the effects observed with >1 µM of GTN are likely due to high concentration actions of NO. These may possibly involve cytostatic/cytotoxic events as observed by Adami et al. [160] in MCF-7 breast cancer and U251 glioblastoma cells treated with high concentrations of GTN; it was shown that 200-300 µM and 1 mM GTN caused half-maximal and maximal inhibition of thymidine incorporation (i.e. cytostasis), respectively, and 500 µM GTN exhibited progressive, time-dependent cytotoxicity [160].

Unlike high NO concentrations, most of the low concentration effects of NO are attributable to the activation of the NO/cGMP signalling pathway in which NO binds sGC and subsequently induces cGMP production and activation of downstream effectors [122]. Thus, the observed inhibitory effect of GTN, at such low concentration as 1 µM, on hypoxia-induced HIF-1α accumulation suggests that this effect occurs via activation of the cGMP-dependent signalling pathway. This is further supported by results of previous studies showing that levels of NO in cells treated with ≤1 µM of NO donors, including GTN, were undetectable using standard assays.
that measure nitrate and nitrite formation as an index for NO production [47]; this indicated that NO levels were lower than those required to produce the reactive nitrogen species nitrate/nitrite and, as such, exerted their effects predominantly through the low-concentration NO/cGMP pathway [47]. Based on the observed effect of GTN on HIF-1α accumulation and given the central role of HIF-1α in mediating hypoxic responses, it is conceivable that inhibitory effects of low concentrations of GTN on hypoxia-induced phenotypes observed in previous studies [35,36,47,56,60,120,121] are, in part, a result of interfering with HIF-1α accumulation. Interestingly, while induction and attenuation of these previously reported phenotypes (i.e. hypoxia-induced increase in invasion and metastasis, drug resistance and immune escape) by hypoxia and by low concentrations of GTN, respectively, were evident following 24-hour exposures, a similar pattern of effect of hypoxia and GTN on HIF-1α protein accumulation was observed as early as and most prominently at four hours. This may perhaps reflect the time it takes to effect a change in transcription and protein expression to finally manifest phenotypic alterations, once HIF-1α accumulation and hence HIF-1 activity is modified.

In the course of the experiments, particularly those examining the effect of GTN on hypoxic accumulation of HIF-1α protein at 24 hours of treatment, some degree of inconsistency in the results was noted wherein GTN inhibited or did not alter HIF-1α accumulation. This may be explained, in part, by the complexities of the multiple mechanisms governing HIF-1α regulation and the multifaceted actions of NO. In addition to the canonical PHD-pVHL regulatory pathway, a number of other mechanisms appear to be involved in the modulation and fine-tuning of HIF-1α accumulation/activity including negative feedback control of HIF-1α. Indeed, time course analysis of hypoxic induction of HIF-1α protein revealed that a rapid increase in HIF-1α protein levels (i.e. after 4 hours of hypoxia) was followed by a progressive decrease in its levels during prolonged hypoxia (8-24 hours). These results are in line with previous studies showing
that hypoxia-induced HIF-1α protein accumulation progressively decreases with sustained hypoxia in various types of cells [109,111,113,161], and confirm those of recent time course studies conducted on DU145 cells by Ravenna et al [162]. Such phenomena have been attributed to several mechanisms of negative feedback regulation of HIF-1α mRNA whereby HIF-1α mRNA stability is reduced and consequently leads to decreases in HIF-1α protein levels during prolonged hypoxia; negative regulators of HIF-1α mRNA, such as natural antisense HIF (aHIF) [109], miRNA-155 [111] and tristetraprolin mRNA-destabilizing protein [113], have been implicated in these mechanisms. Consistent with these studies, results revealed that HIF-1α mRNA levels, along with its protein levels, progressively decreased during sustained hypoxia (8-24 hours); interestingly, the effect of acute hypoxia (four hours) on HIF-1α mRNA was opposite to that on HIF-1α protein levels, suggesting that acute hypoxic induction of HIF-1α protein involves translational or post-translational changes. Based on these findings, it is evident that HIF-1α regulation and response to hypoxia is highly dynamic and complex, which may partly explain the observed inconsistency in the results; the negative regulatory mechanism stimulated under prolonged hypoxia may have at times masked the inhibitory effect of NO signalling on HIF-1α accumulation. Given the differential effects of NO depending on its concentration [117,122], it is also important to consider the resulting NO concentration experienced by the cells, which is likely determined by the amount of NO produced and already present in the cells as well as the mechanism and kinetics of exogenous NO delivery; these factors may have contributed to the observed variability in the effects of GTN on HIF-1α accumulation and hence direct measurement of NO may be needed in future studies to ensure that NO concentrations are within the desired range.

In addition to the results showing that GTN, at a concentration known to primarily activate the cGMP-dependent pathway, inhibited hypoxic accumulation of HIF-1α, the
participation of the cGMP signalling pathway in HIF-1α regulation was further demonstrated by the finding that 8-Br-cGMP (i.e. cGMP analogue) similarly attenuated hypoxia-induced HIF-1α accumulation. These results are in agreement with previous studies [35,36,47] showing that NO via cGMP production prevents hypoxia-mediated acquisition of malignant phenotypes in tumour cells and suggest that modulation of HIF-1α may be an important aspect of the mechanism by which NO/cGMP signalling regulates hypoxic responses. Although many of the studies examining the regulatory effects of NO on HIF-1α accumulation and/or HIF-1 activity have proposed cGMP-independent mechanisms of HIF-1 regulation, Tsuruda et al. [163] have found in cultured cardiomyocytes that activation of sGC/cGMP signalling decreased hypoxia-induced HIF-1α protein accumulation; this further supports the notion that the cGMP-dependent signalling interferes with hypoxic induction of HIF-1α accumulation and suggests that such mechanism of HIF-1α modulation may apply to a wide variety of both normal and transformed cells.

Interestingly, in contrast to its effects on HIF-1α accumulation in hypoxia, neither GTN nor 8-Br-cGMP altered HIF-1α protein levels in DU145 cells under oxygenated conditions (20% O₂). This selective action of GTN and 8-Br-cGMP is in line with previous studies showing that such activation of NO/cGMP signalling inhibited malignant phenotypes of hypoxic tumour cells without affecting well-oxygenated cells (i.e. cells with normal NO production) [36,47,120,121], highlighting its potential to selectively target the more malignant hypoxic cells.

The present study revealed that GTN or 8-Br-cGMP did not alter the levels of HIF-1α mRNA in either hypoxic (0.2% O₂) or oxygenated (20% O₂) conditions, suggesting that the NO/cGMP-mediated attenuation of hypoxia-induced HIF-1α accumulation occurs via translational or post-translational mechanisms. It has been reported that NO decreases HIF-1α protein abundance and hence HIF-1 activity via a PHD/pVHL/proteasome-independent mechanism that involves calpain (Ca²⁺-activated protease)-mediated degradation of HIF-1α [146];
this report did not address the role of the cGMP-dependent NO signalling in the proposed mechanism of HIF-1α regulation. In accordance with and extending these findings, results indicated that the NO/cGMP-induced inhibition of HIF-1α accumulation requires calpain activity. It is possible that activation of the cGMP-dependent NO signalling pathway, in turn, activates calpains and hence HIF-1α degradation. Indeed, cGMP has been shown to increase intracellular levels of Ca²⁺ [149,150,152], the primary activator of calpain, and this has been linked to increases in calpain activity [151]. Furthermore, it has been found that the downstream effector of cGMP, PKG, is required for NO/cGMP-mediated generation of the Ca²⁺ signal and activation of μ-calpain [164]. Interestingly, PHDs are also subject to regulation by Ca²⁺ concentrations and it has been shown that chelation of intracellular Ca²⁺ induces HIF-1α accumulation and HIF-1 transactivation by inhibiting PHD activity under oxygenated conditions [95]. Based on the results presented in this thesis, the potential role of PHD/pVHL/proteasome pathway in the NO/cGMP-mediated attenuation of HIF-1α accumulation cannot be excluded and further studies may be needed to investigate the possibility that PHDs and calpains may synergistically mediate HIF-1α degradation. In addition to HIF-1α, calpain has been found to mediate the degradation of HIF-2α [165], suggesting that HIFs are among the many calpain substrates and that calpain plays an important role in the regulation of HIFs and hence hypoxic responses.

4.1 Concluding Remarks

The present results indicate that NO/cGMP signalling attenuates hypoxic accumulation of HIF-1α protein via a mechanism dependent on calpain activity. These results support the concept that tumour hypoxic responses involving HIF-1 activity may be prevented by activation of the cGMP-dependent NO signalling pathway.
Taken together, the findings presented in this thesis provide new mechanistic insights into the NO-mediated regulation of hypoxia-induced HIF-1α accumulation and a better understanding of the mechanism by which NO modulates cellular hypoxic responses. This mechanism may apply to various systems characterized by low oxygen levels during both normal biological processes such as placentation and under pathological conditions such as cancer and cardiovascular diseases. The proposed pathway of HIF-1α modulation may serve as a potential therapeutic target and offer an approach to selectively target hypoxic cells and perhaps cells that are inherently deficient in NO/cGMP signalling. In fact, Zhu et al. [166] found that glioma cells have impaired NO/sGC/cGMP signalling and that pharmacological or genetic restoration of this signalling inhibits glioma cell growth and malignant progression; this raises the possibility that HIF-1α, which is found at high levels in glioma cells under normoxic conditions [167], is subject to regulation by the cGMP-dependent pathway in these cells and that normalization of the signalling may interfere with HIF-1α accumulation and hence HIF-1-mediated malignant phenotypes. The present findings on the effects of NO signalling on HIF-1α, taken together with the wide applicability and clinical relevance [168] of NO-mediated regulation of hypoxic responses, should prompt further exploration into the mechanism of action and therapeutic potential of NO as a HIF-1 inhibitor.
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