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Review

Therapeutic Targets for Hypoxia-Elicited Pathways

Cormac T. Taylor[1] and Sean P. Colgan[1,2]

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Diminished oxygen supply to tissues (hypoxia) can stem from many sources, and is a contributing factor to diverse disease processes. Cell and tissue responses to hypoxia are diverse and include dramatic changes in metabolic demand, regulation of cellular gene products, and release of lipid and protein mediators. Surprisingly little attention has been paid to targeted development of therapeutics for hypoxia-related disease processes. This review will focus on recent advances in cellular and molecular biology pertaining to the hypoxia response, and will discuss paradigms used to study hypoxia and the potential targets for therapeutic intervention.

KEY WORDS: ischemia; therapeutics; cyclic nucleotide; proteasome; angiogenesis; lipid mediator.

INTRODUCTION

The chemical reduction of oxygen is the primary source of metabolic energy for all eukaryotic cells (1). Diminished tissue oxygen supply (hypoxia) is a common physiologic and pathophysiologic occurrence in nature, and for this reason, mammalian cells have evolved elaborate compensatory mechanisms for adapting to hypoxia. At the tissue level, hypoxia can emanate from a number of sources. Such events include frank vascular occlusion such as occurs with stroke. Tissue fibrosis and microvascular breakdown associated with chronic inflammation also result in localized tissue hypoxia/ ischemia. Alternatively, diminished oxygen delivery to tissues may occur in shock, hypotension or in cases where the oxygen carrying capacity of blood is compromised (e.g., carbon monoxide poisoning). Finally, solid tumors have been demonstrated to form hypoxic cores, and respond accordingly by induction of hypoxia-responsive gene products. The basic mechanism(s) by which cells "sense" oxygen are not well understood at the molecular level. While significant insight has been gained by the discovery of specific oxygen regulated gene products (1–4), the fundamental principles are not well delineated. Importantly, many parallels exist between hypoxia-adaptive responses and the acute inflammatory response. In this brief review, we will address recent developments in molecular targets related to cell and tissue hypoxia, particularly as they contribute to inflammation.

An aspect which will not be addressed in this review is the activation of signaling pathways which result in acute cell death during hypoxia/ischemia. In short, these responses are mediated primarily through acute energy depletion and severe mitochondrial dysfunction. These aspects of hypoxia differ sufficiently in scope and substance from the transcriptional response described here to warrant separate emphasis, and have been expertly reviewed elsewhere (5).

MOLECULAR SIGNALING BY HYPOXIA

Hypoxia Inducible Factor-1 (HIF-1)

At the tissue and cellular level, an array of genes pivotal to survival in low oxygen states are activated (1–4). A number of elegant studies, exemplified by those defining induction of the erythropoietin (EPO) gene (6,7), have utilized multidisciplinary approaches to elucidate basic hypoxia-adaptive responses. Studies of hypoxia-induced EPO production concentrated on the regulation of gene transcription. Sequences from the EPO promoter were identified and assessed for oxygen-sensitivity in transformed cell lines. Semenza and Wang (8) identified a potent regulatory sequence in the EPO enhancer which bound a transcription factor, the abundance of which was elevated in response to hypoxia through suppression of proteolytic breakdown (9). The factor was identified as a heterodimer with independently regulated subunits termed hypoxia inducible factor-1 (HIF-1), a member of the rapidly growing Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors (10,11). HIF-1 exists as an $\alpha\beta$ heterodimer, the activation of which is dependent upon stabilization of an O_2-dependent degradation domain of the α subunit by the ubiquitin-proteasome pathway (9). While not clear, HIF-1 appears to reside in the cytoplasm of normoxic cells, and like a number of other transcription factors (e.g., NF-κB, see later), HIF-1

[1] Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115.

[2] To whom correspondence should be addressed. (e-mail: colgan@zeus.bwh.harvard.edu)

ABBREVIATIONS: cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; CRE, cAMP response element; CREB, CRE-binding protein; EPO, erythropoietin; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia responsive element/enhancer; IL, interleukin; MHC, major histocompatibility complex; NF-κB, nuclear factor kappa-B; NOS, nitric oxide synthase; NSAID, non-steroidal anti-inflammatory; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

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translocates to the nucleus to form a functional complex (12). Binding of HIF-1 to consensus domains in the EPO enhancer results in the transcriptional induction of HIF-1-bearing gene promoters (4). A series of experiments by Wang and Semenza (13) and Maxwell et. al. (14) demonstrated that reporter genes containing the EPO enhancer were induced by hypoxia in a variety of cell types that did not normally produce EPO. Subsequently it was determined that HIF-1 is widely expressed and that consensus HIF-1 binding sequences exist in a number of genes other than that of EPO, and are termed hypoxia responsive enhancers (HRE) (4). Interestingly, HIF-1 mediated transcription is inhibited by nitric oxide activity and the tumor supressor, p53 (15–17). Thus, the discovery of HIF-1 represented a major advance in the understanding of erythropoietin regulation as well as a general cellular response to hypoxia.

Genes induced by HIF-1 include those necessary for cellular, whole tissue and whole animal adaptive responses to hypoxia (see Fig. 1) (18,19). These proteins include a large number of enzymes involved in anaerobic metabolism (1–4). Vascular endothelial growth factor (VEGF), an angiogenic cytokine, is HIF-1-responsive and may serve to increase blood supply to hypoxic tissues through neo-vascularization (20,21). Vasodilation is increased in response to hypoxia through increased expression of inducible nitric oxide synthase, and recent evidence has also linked HIF-1 to the control of cellular apoptosis (22). Taken together, induction of HIF-1 responsive genes drives altered cellular metabolism, increased vascular mass and diameter and increased oxygen carrying capacity of the blood, all events which are conducive to an adaptive response to diminished oxygen supply (18,19,23,24). Consequently, HIF-1 represents a potential therapeutic target in the treatment of a number of important diseases related to hypoxia.

Nuclear Factor-kappa B in Hypoxia

Because hypoxia and inflammation often occur coincidentally, it is imperative to understand how induction pathways of inflammation and hypoxia overlap. Nuclear factor-kappa B (NF-κB) is an extensively studied transcription factor with demonstrated importance in stress and inflammatory responses (25). NF-κB exists as a cytoplasmic protein associated with an inhibitory sub-unit IκB. Activation of NF-κB through degradation of the IκB subunit (and subsequent translocation to the nucleus where transcription is initiated) occurs in response to a broad array of stimuli including, but not limited to, cytokines, oxidants, viral and bacterial infection and ultraviolet light (26). A number genes bear NF-κB binding motifs, including proinflammatory cytokines (e.g., TNFα), chemokines (e.g., IL-8), inflammatory enzymes (e.g., inducible nitric oxide synthase) and leukocyte adhesion molecules (e.g., intercellular adhesion molecule-1) (25). Thus, NF-κB is a potentially pivotal transcription factor in mediating the pathogenesis of acute and chronic inflammation.

A number of studies have demonstrated that hypoxia activates NF-κB *in vitro* and *in vivo* (25). Such an event may be pivotal in determining cellular expression of pro-inflammatory signals and consequently, represents a potential site for therapeutic intervention in hypoxia associated inflammation (see Fig. 1). As an aside, rather than serving as a stand alone activator, hypoxia may promote stimulus-dependent induction of NF-κB. For instance, a number of studies have determined that hypoxia fails to induce endothelial-leukocyte adhesion molecules, such as ICAM-1 and E-selectin (27–29). However, the combination of hypoxia and other soluble mediators (e.g., LPS, cytokines, etc.) potentiates induction by inflammatory mediators. It was subsequently determined that a contributing factor to the hypoxia component may be metabolic acidosis, conditions which promote proteasome activation and nuclear localization of NF-κB (30,31). Importantly, stimuli such as bacterial endotoxin, can significantly influence metabolic processes and promote acidosis, thus hypoxia may simply potentiate an established pathway (32,33). Consequently, we found that dampening intracellular acidosis during hypoxia effectively dampened this relative contribution (30), unveiling a potential therapeutic strategy (see below).

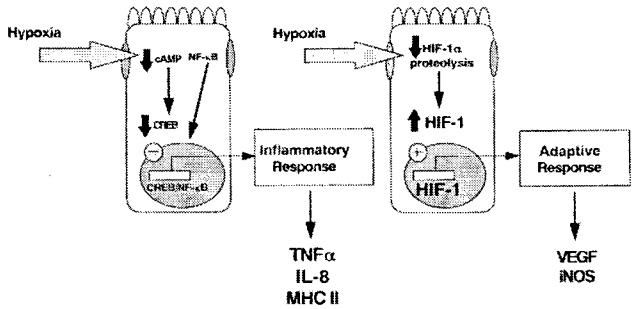

Fig. 1. Molecular signaling by hypoxia. Cellular responses to hypoxia involve the activation or supression of a number of transcription factors. Activation of NF-κB and decreased expression of CREB results in the induction of transcription of a number of inflammatory signals. Alternatively, hypoxia-elicited activation of HIF-1 results in transcriptional induction of a number of genes which mediate a hypoxia-adaptive response.

Cyclic AMP Response Element Binding Protein (CREB) and Hypoxia

A number of genes which contain NF-κB regulatory elements are not activated by hypoxia. Furthermore, transcription of a number of genes which contain neither NF-κB- nor HIF-1-binding sites can be activated by hypoxia, suggesting an additional level of regulation. Recent studies have defined critical transcriptional signaling pathways regulated by changes in intracellular concentrations of cyclic nucleotides, such as cAMP (34). Sensitivity of these genes to cyclic nucleotide regulation depends on the existence of an eight base pair consensus sequence known as the cyclic AMP response element (CRE). To date, more than 15 distinct sequences function as CREs (34). Phosphorylation of the nuclear transcriptional regulator CREB by PKA or PKC induces a binding affinity for CRE sequences. Once bound to CRE, phospho-CREB complexes with transcriptional co-activators such as CBP (CREB binding protein), ATF-2 (activating transcription factor 2) or CREM (cyclic AMP responsive element modulator protein) forming a functional complex capable of regulating transcription. Depending on the gene, such regulation can activate or repress transcription (34).

The relationship between hypoxia and cAMP have been extensively studied (31,35–42). As a general observation, cellular hypoxia diminishes both basal and stimulated cAMP and results in attenuated PKA activity. Given the importance of PKA in regulating CRE-responsive gene products, we have

studied the impact of hypoxia on CREB signaling in endothelia and epithelia. Initial studies revealed a distinct parallel between hypoxia inducibility and the presence of one or more CRE at or near the gene promoter (e.g., TNFα, IL-8, E-selectin, COX-2, see Fig. 1). Direct evidence for PKA in this response were provided by the findings that: 1) levels of intracellular cAMP directly correlate with the hypoxia response; 2) elevation of intracellular cAMP during hypoxia dampens such responses; and 3) specific PKA inhibitors recapitulate the hypoxia response (42). Subsequent studies have addressed the role of CREB in this response. Gel shift and western blot analysis revealed that hypoxia elicits a profound loss of CREB protein during hypoxia (42). This diminution in CREB is, at least in part, blocked by maintenance of intracellular cAMP, and retroviral mediated overexpression of CREB serves to rescue the hypoxia phenotype (42). These studies define a novel, CREB-mediated pathway for induction of proinflammatory gene products by hypoxia, and importantly, reveal CREB as a potential target for therapeutic intervention (see later).

Molecular Crosstalk in Hypoxia

Although each of the pathways described above may individually be important in altering cellular physiology and/or phenotype, crosstalk does exist at the level of transcriptional activation. For example, CREB and associated proteins have been demonstrated to interact at the level of DNA binding with HIF-1 (43). Similarly, cyclic AMP-dependent transcriptional events may be intimately associated with NF-κB signaling under some conditions (see Fig. 1). The cyclooxygenase-2 (COX-2) gene promoter, for instance, bears binding sites for both NF-κB and CREB (44), and as a result, is inducible by both hypoxia and inflammatory stimuli (45,46). NF-κB is specifically integrated by the nuclear coactivator CREB binding protein (47), providing yet another level of potential site of crossover for these pathways. Additionally, it is important to note that these crosstalk pathways may be temporal events. Studies with intestinal epithelia have revealed, for example, that hypoxia induces the production and release of soluble TNFα, which in turn activates surface TNFα receptors. This observation provides a temporal autocrine signaling loop with potential for therapeutic intervention (see Fig. 2) (48).

THERAPEUTIC INTERVENTION

Potential Sites for Drug Development

Through a variety means, hypoxia contributes to many disease processes. In the classic example of solid tumors, induction of hypoxic genes due to undervascularization of core tumor cells may be of great importance in promoting neovascularization and subsequent tumor development (24). Alternatively, in conditions of chronic inflammation such as Crohn's disease or rheumatoid arthritis, hypoxia may potentiate ongoing tissue damage through a number of well defined pathways (see above). Therefore, the use of existing drugs and the development of novel therapies hold promise in effectively treating of a plethora of disorders. Discussed below are potential therapeutic targets for hypoxia-related disease processes (see Table I).

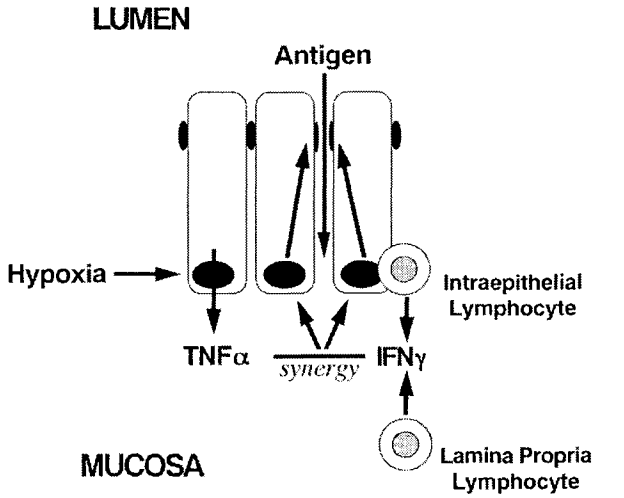

Fig. 2. Hypoxia-elicited TNF-α synergizes with IFN-γ in an autocrine manner. Under conditions of chronic inflammation, hypoxia occurs coincidentally with inflammatory events. In an intestinal epithelial cell-culture model, hypoxia-elicits the polarized release of TNF-α which synergizes with immune-derived cytokines such as interferon-γ resulting in decreases in epithelial barrier function.

Proteasome Inhibitors

As summarized above, proteasomes are central to the activation of both NF-κB and HIF-1. Proteasome inhibitors represent a class of drugs with significant potential. A primary site at which HIF-1 signaling may be altered is the inhibition of proteasome activation (which leads to the accumulation of HIF-1α subunit (9)). Aspirin and aspirin-like drugs (e.g., sulfasalazine) have been shown to inhibit proteasome activation and resultant NF-κB activation, and may in part explain the anti-inflammatory actions of aspirin (49,50). Recently, a number of low molecular weight, cell permeant proteasome inhibitors have been identified (51–53). The most widely used are the peptide aldehydes, such as CBz-leu-leu-leucinal (MG132), CBz-leu-leu-norvalinal (MG115) and acetyl-leu-leu-norleucinal

Table I. Potential Therapeutic Sites in Hypoxia-Elicited Signaling

Therapeutic target	Candidate drugs	Outcome
Proteasomes	Peptide aldehydes, β-lactins	Altered NF-kB/HIF-1 response
Intracellular nucleotides	Phosphodiesterase Inhibitors	Decreased TNFα release
Angiogenesis	VEGF, Thalidomide/analogs	Increased Blood/O_2 supply
COX II inhibition	NSAIDs	Anti-inflammatory
Intracellular pH	Proton pump inhibitors, Na/H exchange inhibitors	Cytoprotection
Adenosine	A_2 receptor agonists	Ischemic preconditioning
TNFα	TNFα mAb, Thalidomide/analogs	Anti-inflammatory

(ALLN), which act as substrate analogues and transition-state inhibitors of the chymotrypsin activity of the proteasome (51). These peptide aldehyde inhibitors are highly potent (e.g. MG132 K_i <10nM for chymotryptic activity) but lack specificity for proteasomes and exhibit some inhibition of calpains and lysosomal enzymes (51). The β-lactone inhibitors, including lactocystin, are naturally occurring proteasome pseudosubstrates which are generally more specific for the proteasome than peptide aldehydes and have the advantage of increased water solubility (53). More recently, vinyl sulfone- and boronate-derived peptides have been tested and shown to exhibit a potent and high degree of specificity compared to peptide aldehydes, and in some cases may be reversible (e.g., borate derivatives) (51). Thus, development of potent and specific proteasome inhibitors hold promise in effective treatment of a number of disorders, including those pathways induced by hypoxia.

Regulation of Angiogenesis

As alluded to above, VEGF expression is tightly linked to oxygen supply. Hypoxia-dependent angiogenesis has recently been hailed as a potentially powerful anti-tumor therapeutic approach. The development of solid tumors involves the formation of a cell mass, the core of which becomes hypoxic due to the lack of microvasculature (24). Hypoxia-elicited activation of "adaptive genes" such as VEGF, iNOS and enzymes allowing anaerobic metabolism, are conducive to the survival and growth of the tumor. For this reason interruption in hypoxia-elicited HIF-1-dependent signaling represents a potential tumor repressive therapeutic target (4,24). O'Reilly et al. have demonstrated complete tumor remission in mice treated with the endogenous anti-angiogenic factors angiostatin and endostatin in a manner which is not sensitive to drug resistance (54,55). Moreover, recent studies indicate the tumor suppressor protein VHL (mutated in von Hippel-Lindau disease) mediates the breakdown of HIF-1α. In the absence of functional VHL, accumulated HIF-1α results in increased angiogenesis (presumably via VEGF) and increased tumor formation (56). Alternatively, inhibitors of HIF-1-dependent gene products may be employed to inhibit adaptive responses to hypoxia. Gene products which may act as potential targets include vascular endothelial growth factor (57–59), the inhibition of which represents a potential anti-angiogenic approach (60). Similarily, selective inhibition of inducible nitric oxide synthase by specific pharmacological inhibitors (61,62) should reduce hypoxia-dependent vasodilation and further inhibit the supply of blood to tumors (63). Finally, the drug thalidomide has demonstrated qualities as an angiogenesis inhibitor (64). The mechanism(s) of thalidomide action on angiogenesis and tumor development remain to be clarified, but this drug, and it's analogs, are potent inhibitors of TNF-α (65). Moreover, hypoxia-elicited induction of TNF-α is specifically inhibited by the native thalidomide compound (48). Thus, inhibition of the "adaptive response" to hypoxia has tremendous potential as a therapeutic intervention in tumor progression.

Alternatively, pro-angiogenic therapies may prove beneficial in hypoxia-related disease processes. For instance, in patients with narrowed coronary blood vessels or myocardial infarction, induction of new blood vessel formation clearly may enhance cardiac perfusion. Gene transfer angiogenesis therapy may be a particularly viable option (66,67). Viral and non-viral vectors exist to specifically introduce foreign DNA or RNA into cells and such strategies can effectively induce or inhibit gene and protein expression (66,67). Vector improvement, efficacy standards and safety are among the present challenges for these therapies.

Regulation of Cyclic Nucleotide Levels

As described above, hypoxia may induce genes containing NF-κB response elements, provided the gene also contains a CRE sequence. Consequently, interference with hypoxia-elicited alterations in the cAMP signaling pathway is a potential site of therapeutic intervention. Since cellular hypoxia decreases intracellular cAMP (40,48), pharmacological elevation of intracellular cAMP (using cAMP analogs or specific phosphodiesterase inhibitors) in hypoxic tissues may be of benefit. Some work has been done in this regard. *In vitro* studies indicate that maintenance or elevation of cAMP during exposure to hypoxia reverses TNFα release (48), increased vascular permeability (36), increased leukocyte-mediated epithelial permeability (41), and increased agonist-induced E-selectin expression (31). *In vivo*, Stern and colleagues have demonstrated a beneficial influence of elevated cAMP/cGMP on pulmonary function and cardiac function in an orthotopic transplant models (68,69). Thus, drugs which alter cyclic nucleotide levels may provide a reasonable approach for hypoxia-related tissue damage.

Cyclooxygenase Inhibitors

Since hypoxia elicits the expression of a number of NF-κB and CREB-dependent signals which may be important in inflammation, pharmacological inhibition of these signals may be another therapeutic target. COX-2 facilitates the metabolic genesis of a number of bioactive lipid molecules. Since the expression of COX-2 is induced by hypoxia, inhibitors may be useful at several different levels. First, aspirin and related salicylates have demonstrated qualities of inhibiting NF-κB activation (50), the multiple benefits of which are discussed above. Second, specific inhibition of cyclooxygenase-derived prostacyclin (PGI_2) may prove beneficial in treating hypoxia-related disorders. As an example, it was recently demonstrated that intestinal epithelia bear receptors for the stable prostacyclin hydrolysis product 6-keto-$PGF_{1\alpha}$ (see Fig. 3) (46). Ligation of epithelial surface receptors for 6-keto-$PGF_{1\alpha}$ activates electrogenic chloride secretion, the transport event responsible for mucosal hydration, and in excess, massive fluid loss symptomatic of diseases with a distinct hypoxia component (e.g., septic shock, adult respiratory distress syndrome, ischemia-reperfusion injury). It is possible, therefore, that diminished prostacyclin liberation through COX inhibition could inhibit excessive fluid loss in these disorders. Third, the beneficial actions of cyclooxygenase inhibitors may, at least in part, be related to the liberation of by products termed lipoxygenase-derived interactive products (lipoxins) (70). Lipoxins are tetraene eicosanoids derived from membrane arachidonic acid by the combined action of 5-lipoxygenase (LO) and 12-LO or 15-LO (71). In particular, one lipoxin termed 15-epi-LXA4, is generated *in vivo* in the presence of aspirin (72), and may contribute in part to the anti-inflammatory actions of aspirin. 15-epi-LXA4 and it's synthetic analog 15 (R/S)-methyl-LXA_4 (73), are potent

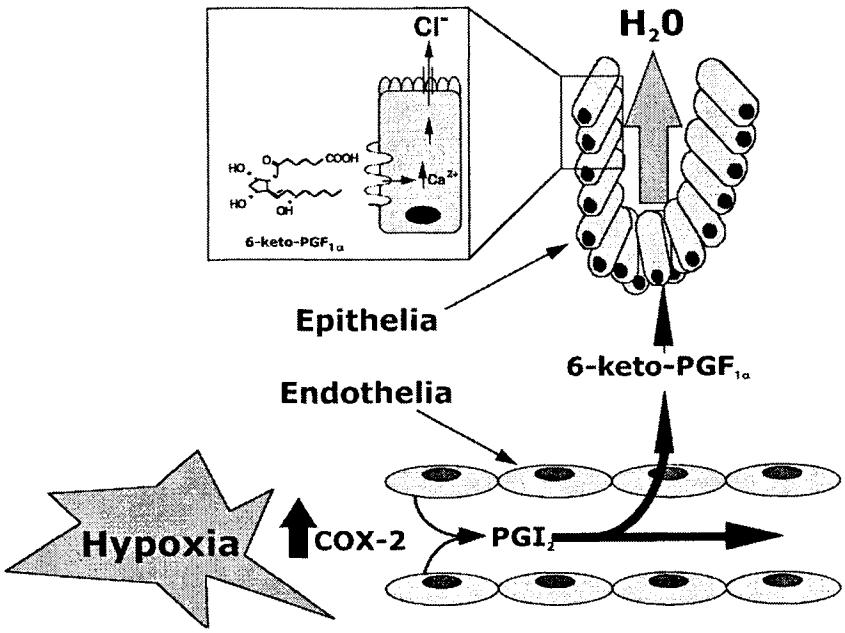

Fig. 3. Model of hypoxia-induced activation of mucosal epithelial Cl^- secretion and water transport. Hypoxia activates COX-2 expression in vascular endothelial cells. Induced COX-2 drives endothelial production of prostacyclin (PGI_2), which hydrolyses to 6-keto-$PGF_{1\alpha}$. Epithelia express a functional, calcium-coupled 6-keto-$PGF_{1\alpha}$ receptor on the basolateral surface which, when activated by 6-keto-$PGF_{1\alpha}$, activates electrogenic Cl^- secretion (inset), the ion transport event responsible for mucosal hydration. See text for more details.

anti-inflammatory agents and effectively inhibit cell proliferation (74). Thus, several lines of evidence indicate beneficial influences of cyclooxygenase inhibition in hypoxia-related disorders.

Other Anti-inflammatory Agents

Tissue injury resulting from ischemia-reperfusion has been shown to be mediated, at least in part by recruitment of leukocytes (75,76). Leukocyte recruitment across the intact endothelium or epithelium occurs through a concerted series of adhesion and de-adhesion events involving a number of cell surface adhesion proteins (77,78). The hypoxic epithelium and endothelium appear to direct leukocyte trafficking through a mechanism involving the liberation of soluble chemokines (79,80). Moreover, leukocyte-epithelial interactive pathways are significantly influenced by locally generated lipid mediators. Of particular interest are lipoxins (70). A number of recent *in vitro* and *in vivo* studies have revealed that lipoxins, and specifically lipoxin A_4 (LXA_4), function as an innate "stop signals", acting to control local inflammatory processes (73,74,81,82). At nanomolar concentrations, LXA_4 has been demonstrated to inhibit PMN transmigration across confluent epithelia and endothelia (73,81). It is likely that the action(s) of lipoxins are on leukocytes and involve activation of protein kinase C, since original studies revealed that inhibition required PMN preexposure to LXA_4 and that such activity was sensitive to staurosporine (81). For these reasons, therapeutic agents directed against leukocyte accumulation may prove effective in the treatment of hypoxia-related disease processes. Lipoxins are rapidly (minutes) converted to inactive compounds by myeloid cells, and for this reason, stable lipoxin analogs have be synthesized and biochemically and functionally studied in detail (73). Strategies to alter the native LXA_4 molecule have primarily utilized bulk (methoxy, cyclohexyl, or phenoxy groups) at the carbon 15 and/or carbon 20 positions. As a general finding, the synthetic lipoxin analogs exhibit greater potency for these counter-regulatory actions than the native compound(s), likely due to decreased metabolism to inactive compounds (73,83). In particular, the synthetic LXA_4 analog 15 (R/S)-methyl-LXA_4, the structure of which resembles that of 15-epi-LXA_4, a native lipoxin generated *in vivo* in the presence of aspirin (74), may contribute in part to the anti-inflammatory actions of aspirin. 15 (R/S)-methyl-LXA_4 is a potent inhibitor of PMN transmigration across epithelia and effectively blocks PMN adhesion to vascular endothelia (73). Other analogs which have proven effective include 16-phenoxy-LXA_4 and 15-cyclohexyl-LXA_4 (73). *In vivo*, both the native compound and analogs to LXA_4 has been demonstrated to block PMN diapedesis within the microcirculation of the hamster cheek pouch (84), depress contraction of the guinea pig ileum (85) and inhibit increases in vascular permeability (86).

Regulation of Adenosine Levels

A consistent finding from a number of studies indicate that hypoxic tissues release adenosine (87). Depending on the cell/tissue, adenosine influences may be protective or detrimental under these conditions, but for the most part are not well understood. The protective role of adenosine in cardiac ischemic preconditioning has been studied in detail (88–90). In general, adenosine, adenosine receptor agonists and nucleotide uptake inhibitors have proven beneficial in enhancing cardiac function following episodes of hypoxia/ischemia. Alternatively, drugs

which modulate activity of the surface enzyme 5′-ecto-nucleotidase (converts 5′-AMP to adenosine) may be effective therapies. First, a significant source of extracellular adenosine is 5′-AMP, and thus, regulation of 5′-ecto-nucleotidase serves as an effective means to control extracellular adenosine levels. Second, 5′-ecto-nucleotidase can exist in surface bound and soluble forms (91). The soluble form of the molecule has been proposed to be an effective "decoy" for 5′-AMP in a number of tissues. Third, 5′-ecto-nucleotidase activity correlates directly with the extent of ischemic preconditioning (92). Finally, 5′-ecto-nucleotidase activity contributes to vascular permeability during leukocyte-endothelial interactions. In this model, surface 5′-ecto-nucleotidase activity is the rate limiting step in conversion of leukocyte-derived 5′-AMP to adenosine (93). This action liberates adenosine, which in turn binds and activates endothelial adenosine A_{2b} receptors and promotes endothelial barrier function. Such findings provide the framework for targeted use of existing adenosine antagonists/agonists and the development of new strategies to utilize adenosine metabolism as a drug target.

Gene Therapy

A great deal of interest has developed in defining novel approaches for application of gene therapy. A recent strategy which has gained attention is the "two-step" approach, whereby it is possible to tissue-specifically deliver a putative therapeutic gene, in a dormant state, and then control gene activity by administration of a second treatment (e.g., orally delivered drug). This "second-step" organizes an active transcriptional complex capable of inducing expression of the dormant gene product (94,95). In theory, the applications for this gene delivery system are well designed for HRE-dependent gene transcription (i.e., physiologic hypoxia as the "second step"). Some work has been done in this regard. For example, Prentise et. al. selectively expressed a luciferase gene construct bearing tandem HRE in rabbit heart tissue and tested the hypothesis that cardiac ischemia *in vivo* might activate the transgene (96). These experiments revealed a rapid (minutes) and sustained (hours) gene induction specifically in tissues which were made ischemic, providing feasibility for such therapies in the future. Current limitations include tissue specificity for vector delivery, gene stability at tissue sites, and basal activity of target gene(s) at these tissue sites *in vivo*.

CONCLUSIONS AND FUTURE PERSPECTIVES

The complex cellular responses to hypoxia have, and will continue to teach important lessons regarding basic physiologic stress responses. The recent discovery of specific hypoxia-responsive pathways pave the way for better defining the present voids in our knowledge. In the drug discovery arena, a number of hurdles exist for effective development of hypoxia-related pharmaceuticals. The absence of an identified cell oxygen sensor(s) significantly blurs the targeted development of experimental therapeutics. Without the focus of a "lock and key" mechanism, we are placed in a position to better refine the existent pathways and continue to examine biochemical and cellular crosstalk during hypoxia. Moreover, the very nature of disorders related to hypoxia/ischemia (chronic inflammation, diminished blood flow, cardiac and pulmonary disease, tissue edema, etc.) establish particularly difficult barriers on the drug delivery front. Strategies to overcome these barriers will require forward thinking in drug delivery, state of the art drug discovery tools and multidisciplinary approaches to identification of potential targets.

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