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Adenosine and Hypoxia-Inducible Factor Signaling in Intestinal Injury and Recovery

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Abstract

The gastrointestinal mucosa has proven to be an interesting tissue in which to investigate disease-related metabolism. In this review, we outline some of the evidence that implicates hypoxia-mediated adenosine signaling as an important signature within both healthy and diseased mucosa. Studies derived from cultured cell systems, animal models, and human patients have revealed that hypoxia is a significant component of the inflammatory microenvironment. These studies have revealed a prominent role for hypoxia-induced factor (HIF) and hypoxia signaling at several steps along the adenine nucleotide metabolism and adenosine receptor signaling pathways. Likewise, studies to date in animal models of intestinal inflammation have demonstrated an almost uniformly beneficial influence of HIF stabilization on disease outcomes. Ongoing studies to define potential similarities with and differences between innate and adaptive immune responses will continue to teach us important lessons about the complexity of the gastrointestinal tract. Such information has provided new insights into disease pathogenesis and, importantly, will provide insights into new therapeutic targets.

Keywords

metabolism; inflammation; nucleotide; nucleoside; nucleotidase; mucosa; colitis; ischemia; neutrophil; epithelium; endothelium; murine model

INTRODUCTION

The primary functions of the gastrointestinal tract are the processing of ingested nutrients, waste removal, fluid homeostasis, and the development of oral tolerance to nonpathogenic antigens. These dynamic processes occur in conjunction with the constant flux of new antigenic material and require that the mucosal immune system appropriately dampen inflammatory and immunological reactions to harmless ingested antigens.

The intestinal epithelium lies juxtaposed to the mucosal immune system and lines the entire gastrointestinal tract. Covering a surface area of approximately 300 m² in the adult human, the intestinal epithelium consists of a monolayer of cells with intercellular tight junctions, a complex three-dimensional structure, and a thick mucous gel layer and provides a dynamic

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and regulated barrier to the flux of the luminal contents to the lamina propria (1, 2). The gastrointestinal tract exists in a state of low-grade inflammation. Such a state results from the constant processing of luminal antigenic material during the development of oral tolerance and the priming of the mucosal immune system for rapid and effective responses to antigens or microbes that may penetrate the barrier. The anatomy and function of the intestine provide a fascinating oxygenation profile, whereby even under physiological conditions, the intestinal mucosa experiences profound fluctuations in blood flow, oxygenation, and metabolic shifts. For example, less than 5% of total blood volume resides in the intestine (small and large combined) during fasting. This proportion can increase to as much as 30% of the cardiac output following ingestion of a large meal. Such changes in blood flow result in marked shifts in local partial pressure of oxygen (pO_2), and it is perhaps not surprising that the epithelium has evolved a number of features to cope with such large metabolic fluxes. Studies comparing functional responses between epithelial cells from different tissues have revealed that intestinal epithelial cells (IECs) seem to be uniquely resistant to hypoxia and that low pO_2 within the normal intestinal epithelial barrier (so-called physiological hypoxia) may be a regulatory adaptation mechanism in response to the steep O_2 gradient (3). Thus, the availability of O_2 in both health and disease regulates both the absorptive and barrier properties of the intestinal epithelium (4). Here, we discuss the signaling pathways involved in adaptation to hypoxia, with a particular focus on adenine nucleotide metabolism and signaling.

HYPOXIA AND THE MUCOSAL IMMUNE RESPONSE

Sites of mucosal inflammation are characterized by profound changes in tissue metabolism, including local depletion of nutrients, imbalances in tissue O_2 supply and demand, and the generation of large amounts of adenine nucleotide metabolites (5, 6). As shown in Table 1, the local generation of these adenine nucleotide metabolites is driven largely by hypoxia through mechanisms involving the transcriptional regulator hypoxia-inducible factor (HIF) (see below).

These inflammation-associated changes in metabolism can be attributed, at least in part, to the initial recruitment of cells of the innate immune system, including myeloid cells such as neutrophils [polymorphonuclear leukocytes (PMNs)] and monocytes. PMNs are recruited by chemical signals generated at sites of active inflammation as part of the innate host immune response to microorganisms. In transit, these cells expend tremendous amounts of energy. Large amounts of ATP, for example, are needed for the high actin turnover required for cell migration (7). Once PMNs reach inflammation sites, the nutrient, energy, and O_2 demands of the PMNs increase to accomplish the processes of phagocytosis and microbial killing. PMNs are primarily glycolytic cells, with few mitochondria and little energy produced from respiration (8). A predominantly glycolytic metabolism ensures that PMNs can function at the low O_2 concentrations (even anoxia) associated with inflammatory lesions.

Once at the inflammatory site, PMNs recognize and engulf pathogens and activate the release of antibacterial peptides, proteases, and reactive oxygen species (ROS) (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous acid) into the vacuole, which together kill the invading microbes (9). ROS are produced by phagocytes in a powerful oxidative burst that is driven by a rapid increase in O_2 uptake and glucose consumption, which in turn triggers further generation of ROS. When activated, PMNs can consume up to 10 times more O_2 than can any other cell in the body. Notably, the PMN oxidative burst is not hindered by even relatively low O_2 (as low as 4.5% O_2) (10), which is important in that ROS can be generated in the relatively low O_2 environments of inflamed intestinal mucosa (4).

In contrast to cells of the myeloid lineage, T and B cells utilize glucose, amino acids, and lipids as energy sources during oxidative phosphorylation. Mitogenic stimulation of thymocytes and naive T cells is a highly energy-demanding process. As lymphocytes proliferate, they become more and more dependent on glucose uptake. Stimulated proliferation of thymocytes can result in nearly 20-fold increases in glucose uptake, which is accomplished by high expression of glucose transporter-1 (11) and is tightly controlled by HIF (see below). For example, IL-7- and IL-4-dependent pathways instruct nutrient uptake in naive T cells (12). During periods of high proliferation, even in the presence of adequate O₂ concentration, lymphocytes shift toward aerobic glycolysis for ATP synthesis, and lactate production from glycolysis can increase by as much as 40-fold in mitogen-stimulated T cells (6). When glucose becomes limiting, as it often does at sites of high immune activity, T cells can utilize alternative energy sources, such as glutamine, within the TCA cycle (13). In the past 10 years we have begun to understand the nature of interactions between microenvironmental metabolic changes and the generation of recruitment signals and molecular mechanisms of leukocyte migration into these areas. The metabolic changes that occur as a result of the recruitment and activation of leukocytes during inflammation provide information about the potential sources of hypoxia at the intestinal epithelial barrier.

INFLAMMATORY BOWEL DISEASE AS A MODEL DISEASE FOR INFLAMMATORY HYPOXIA AND NUCLEOTIDE METABOLISM

Inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis, is an interesting disease in which to study the metabolic events and metabolism of adenine nucleotides associated with inflammation, particularly the development of severe hypoxia within inflammatory lesions (4). Microvascular abnormalities that describe IBD patients have been associated with abnormal blood flow to the intestine, including increased production of tissue vasoconstrictor molecules and the reduced generation of nitric oxide by endothelial cells (14), as well as vascular endothelial growth factor-dependent pathological angiogenesis (15). In addition, studies of active inflammation in mouse models of IBD have shown the intestinal epithelial cell to be a primary target of hypoxia (16).

Prominent epithelial hypoxia in murine models of IBD was revealed using 2-nitroimidazole dyes, a class of compounds that undergo intracellular metabolism, depending on tissue oxygenation (Figure 1) (17). Tissue staining with these nitroimidazole dyes revealed two profound observations. First, in the healthy colon, physiological hypoxia predominates (Figure 1). These dyes adduct with proteins, peptides, and amino acids at a pO₂ of less than 10 mm Hg, and therefore such low O₂ levels may regulate basal gene expression in otherwise healthy IECs (5). Second, the inflammatory lesions seen in these mouse models are profoundly hypoxic or even anoxic, with levels of oxygen similar to those seen in some large tumors, and penetrate deep into the mucosal tissue. Multiple contributing factors, such as vasculitis, vasoconstriction, edema, and increased O₂ consumption, likely predispose the inflamed intestinal epithelia to decreased O₂ delivery and hypoxia (16). Although these 2-nitroimidazole compounds have not been used clinically to image inflammatory lesions, they have shown significant clinical utility in tumor imaging and in the identification of stroke regions within the brains of patients (18). As opposed to other imaging techniques, these compounds have the advantages that they image only viable tissue and are not active within necrotic or apoptotic regions of the tissue (19). Likewise, studies are under way to use these compounds as adjunct radiosensitizers for enhancing chemotherapy targeting (20).

HYPOXIA-INDUCIBLE FACTOR

The studies that identified inflammation-related hypoxia also revealed stabilization of HIF-1 α within the inflammatory lesions (16). Many cell types, including IECs (21), express

both HIF-1 α and HIF-2 α , and murine genetic studies suggest that these proteins have nonredundant roles (22). Some studies suggest that distinct transcriptional responses mediated by HIF-1 α and HIF-2 α may be integrated in ways that support particular adaptations to hypoxia. For example, the transcriptional responses that coordinate the glycolytic pathways include more than 11 target genes and seem to be more selective for the HIF-1 α isoform than for the HIF-2 α isoform (Figure 1) (22). Likewise, studies addressing the selectivity of the two isoforms for erythropoietin induction suggest a more prominent role for HIF-2 α (22). Currently, this specificity is not well understood. Some studies indicate that binding of HIF-1 α or HIF-2 α to other transcription factors at the site of DNA binding may determine such specificity (22), but findings are not conclusive.

Several studies demonstrate that HIF triggers the transcription of a number of genes that enable IECs to act as an effective barrier. Originally guided by microarray analysis of hypoxic IECs (23), these studies were validated in animal models of intestinal inflammation (16, 24–28) and in human intestinal inflammation tissues (29–31). Interestingly, the functional proteins encoded by a number of uniquely hypoxia-inducible genes in intestinal epithelia localize primarily to the most luminal aspect of polarized epithelia, providing significant support for the hypothesis that hypoxia supports a barrier-protective phenotype. Molecular studies of this hypoxia-elicited pathway(s) show a dependency on HIF-mediated transcriptional responses. Notably, epithelial barrier-protective pathways driven by HIF tend not to be the classical regulators of barrier function, such as the tight junction proteins occludin or claudins. Rather, the HIF-regulated molecules include molecules that support overall tissue integrity and promote increased mucin production (32), that modify mucin (e.g., intestinal trefoil factor) (3), that promote xenobiotic clearance via P-glycoprotein (33), that enhance nucleotide metabolism [by ecto-5'-nucleotidase (CD73)] (23, 34), and that drive nucleotide signaling [e.g., adenosine A_{2B} receptor (A_{2B}AR)] (34).

As an extension of the original studies identifying HIF stabilization within the intestinal mucosa, Karhausen et al. (16) generated transgenic mice expressing either mutant Hif1- α (causing constitutive repression of *Hif1- α*) or mutant von Hippel–Lindau (causing constitutive overexpression of HIF) targeted to IECs. Loss of epithelial HIF-1 α resulted in a more severe colitic phenotype than in wild-type animals, including increased epithelial permeability, enhanced loss of body weight, and decreased colon length. Constitutively active intestinal epithelial HIF (mutant von Hippel–Lindau) was protective for each of these individual parameters. These findings may be somewhat model dependent because another study found that epithelial HIF-based signaling also promotes inflammation (28). Nonetheless, the findings of Karhausen et al. (16) confirmed that IECs can adapt to hypoxia and that HIF may contribute to this adaptation.

Nonepithelial cell types within the gastrointestinal mucosa have also been studied for HIF expression and response to hypoxia. Activated T cells show increased expression of HIF-1 α , which prevents them from undergoing activation-induced cell death in hypoxic settings. T cell survival in hypoxia is, at least in part, mediated by the vasoactive peptide adrenomedullin (35). Other studies using chimeric mice bearing HIF-1 α -deficient T and B cells have revealed lineage-specific defects that result in increased autoimmunity, including autoantibodies, increased rheumatoid factor, and kidney damage (36). HIF function has also been studied in some detail in myeloid cells. Cre-*LoxP*-based elimination of HIF-1 α in cells of the myeloid lineage (lysozyme M promoter) has revealed multiple features that importantly implicate metabolic control of myeloid function (37). In particular, these studies show that PMN and macrophage bacterial killing capacities are severely limited in the absence of HIF-1 α , as HIF-1 α is central to the production of antimicrobial peptides and granule proteases. These findings are explained, at least in part, by the inability of myeloid cells to mount appropriate metabolic responses to diminished O₂ characteristic of infectious

sites (37). Finally, compelling evidence has revealed that HIF-1 α transcriptionally controls the critical integrin important in all myeloid cell adhesion and transmigration, namely, the β 2 integrin (CD18) (38). Such findings are important for our current understanding of the role of functional PMNs in IBD. A recent study, for example, used PMN depletion techniques to document a central role for PMNs in the resolution of inflammation in several murine IBD models (39).

OXYGEN-SENSING PROLYL HYDROXYLASES AND HYPOXIA-INDUCIBLE FACTOR EXPRESSION

The molecular mechanisms of HIF stabilization have been clarified over the past decade. Three prolyl hydroxylases (PHDs) termed PHD1, PHD2, and PHD3 as well as factor-inhibiting HIF (FIH) contribute to HIF pathway regulation (40). As depicted in Figure 1, these hydroxylases are encoded by different genes, and their gene product enzymes demonstrate tissue-specific expression patterns (40). All three PHDs and FIH are found in the intestinal epithelium (24, 27, 41). Genetic studies have revealed significantly different phenotypes in mice lacking the individual isoforms of the PHDs. For example, studies in *Phd1*^{-/-} mice have revealed differences in basal metabolic profiles and decreased exercise performance (42). Disease models have demonstrated that these animals are protected against acute liver ischemia, muscle ischemia, and dextran sodium sulfate (DSS)-induced colitis (42-44). Homozygous PHD2 deletion is embryonic lethal due to developmental angiogenesis defects (45, 46). PHD2 heterozygous knockout animals show enhanced tumor angiogenesis but decreased metastasis (45). *Phd3*^{-/-} mice manifest reduced neuronal apoptosis, abnormal sympathoadrenal development, and reduced blood pressure (47). These diverse phenotypes strongly suggest distinct isoform-specific functions in vivo.

In the presence of 2-oxoglutarate, Fe²⁺, and molecular O₂, PHDs hydroxylate the α subunit of HIF and lead to subsequent ubiquitination and degradation via the proteasome. Hypoxia or pharmacological agents (such as DMOG) inhibit HIF hydroxylases that lead to HIF stabilization. The impact of HIF hydroxylase inhibitors on epithelial cell gene expression is not restricted to regulation via HIF. For example, HIF hydroxylases can regulate nuclear factor- κ B (NF- κ B) (40, 48). The transcriptional targets of HIF hydroxylases can impact epithelial barrier function in a number of ways. For example, HIF regulates the expression of a family of barrier-protective factors, including intestinal trefoil factor (3), the mucins (32), and actin cytoskeletal cross-linkers (49). Likewise, NF- κ B is thought to be largely protective in the intestinal epithelium via the inhibition of enterocyte apoptosis (24).

OXYGEN-SENSING PROLYL HYDROXYLASES IN THE CONVERGENCE OF HYPOXIA AND INFLAMMATION

The O₂-dependent regulatory role of PHDs is not restricted to HIF stabilization. For example, NF- κ B is activated during inflammation and may interact in fundamental ways with the HIF pathway. NF- κ B consists of either homodimers or heterodimers that, on activation, translocate to the nucleus and bind with the transcriptional coactivator CBP/p300 to begin transcription or repression of various genes. The inhibitory I κ B proteins regulate NF- κ B activity (50). The best-studied complex is I κ B α bound to the NF- κ B p50-p65 dimer (50). This interaction with I κ B α inhibits NF- κ B from binding to DNA and maintains the complex in the cytoplasm. On activation by various extracellular signals, I κ B kinase (IKK) is activated, resulting in phosphorylation (51) and polyubiquitylation of I κ B α (52). The S26 proteasome then selectively degrades polyubiquitinated I κ B α . Once dissociated from I κ B α , NF- κ B rapidly enters the nucleus and activates gene expression.

Recent studies have indicated that PHDs also regulate NF- κ B-dependent pathways and that PHD inhibitors for murine colitis also target the NF- κ B pathway. Indeed, hypoxia activates NF- κ B, and such activation is, at least in part, dependent on PHD-mediated hydroxylation of IKK β (41, 53). In normoxia, IKK β activity is held in check through LXXLAP-dependent hydroxylation by PHD1 and PHD2 (41). Conditional deletion of the NF- κ B pathway in IECs in mice leads to an increased susceptibility to colitis (54), a phenotype similar to that of the mice expressing homozygous mutant HIF-1 α (16). This implicates epithelial NF- κ B in a prominently protective role in colitis, probably through the expression of antiapoptotic genes in IECs and through enhanced epithelial barrier function. Some studies suggest that both the HIF and NF- κ B pathways may also be influenced by mediators found within inflammatory sites, including microbial products, cytokines, and even intact bacteria (37). NF- κ B is a classic transcriptional regulator activated by a spectrum of agonists, the activation of which drives a complex series of receptor-mediated signaling pathways. Recent studies indicate that NF- κ B-mediated signaling activates HIF-1 α transcription (55). Inflammation-associated upregulation of HIF-1 α mRNA occurs in an NF- κ B-dependent manner (55). HIF-1 may also promote increased NF- κ B activity in hypoxia (48). Thus, a cross-regulatory loop may exist between the HIF and NF- κ B pathways and may involve other transcriptional regulators that bear nonredundant PHD sensitivity, including activating transcription factor-4 and Notch (56, 57), both critical regulators of cell fate. Given that IECs are in an environment with constant exposure to potentially inflammatory stimuli, the cross-regulation of HIF and NF- κ B may have profound implications for intestinal epithelial cell function and survival under both homeostatic and disease conditions.

The identification of HIF-selective PHDs has provided unique opportunities for the development of PHD-based therapies (58, 59). Although there is wide interest in developing HIF-1 inhibitors as potential cancer therapies, opportunities also exist to selectively stabilize HIF in an attempt to promote inflammatory resolution in IBD. For example, 2-OG analogs effectively stabilize HIF- α (58). Although this approach is not selective for particular PHD isoforms, some in vitro studies suggest that marked differences in substrate specificity may exist and could be harnessed for selectivity. For example, all PHDs hydroxylate the C-ODD domain more efficiently than the N-ODD domain, and PHD2 hydroxylates the N-ODD domain less efficiently on HIF-2 α than on HIF-1 α . In addition, PHD3 does not hydroxylate the N-ODD domain of HIF-1 α (60, 61). Additionally, the protection afforded by PHD inhibitors (e.g., decreased tissue inflammatory cytokines, increased barrier function, decreased epithelial apoptosis) may involve both HIF and NF- κ B activity.

Given the central role of HIF-mediated signaling in erythropoietin production, PHD inhibitors have been developed and are in clinical trials for the treatment of anemia (62). Investigators have developed several PHD inhibitors, including direct inhibitors, analogs of naturally occurring cyclic hydroxamates, and antagonists of α -ketoglutarate (5). Each of these molecules serves as a competitive PHD inhibitor through the substitution for α -ketoglutarate in the hydroxylation reaction shown in Figure 1. Within the gastrointestinal tract, the PHD inhibitors DMOG and FG-4497 have been used effectively to reduce symptoms in at least two mouse models of colitis (24, 27). Indeed, these studies show that both DMOG and FG-4497 have an overall beneficial influence on multiple parameters studied in chemically induced trinitrobenzene sulfonic acid (TNBS) or DSS mouse models of colitis. In these mouse models, the drugs were well tolerated, with no significant adverse side effects. In our experience, both FG-4497 and DMOG can be delivered by multiple routes of administration (intraperitoneal, oral, and intravenous), and both FG-4497 and DMOG are absorbed orally, with only a slight loss of efficacy compared with intraperitoneal administration. To date, although no human trials have been initiated for the treatment of IBD, a recently published proof-of-principle study in end-stage renal disease demonstrates the efficacy of PHD inhibitors in elevating the HIF target erythropoietin (63).

ADENOSINE: A BIOMEDICAL DISCOVERY STORY DATING BACK TO THE 1920s

Although this review focuses on the contribution of hypoxia and adenine nucleotide metabolism to intestinal disease, the original discovery of adenosine as a signaling molecule stems from a completely different mindset. In fact, the first description of adenosine signaling dates back almost 90 years. In 1927, two scientists from the University of Cambridge, United Kingdom, were the first to observe specific signaling by adenosine. In their studies, Drury & Szent-Gyorgyi (64) performed intravascular injections of an extract derived from cardiac tissues in an intact animal. They were somewhat surprised to observe a robust and transient slowing of the heart rate upon intravascular injection of this tissue extract (64). Utilizing what were then the state-of-the-art chemical purification methods of the early twentieth century, the authors identified the biological activity within the cardiac tissue extract as an “adenine compound” (64). Following this ground-breaking discovery, almost 50 years passed until the heart rate–slowing influences of extracellular adenosine were translated from bench to bedside. In the 1980s, the heart rate–slowing influence of adenosine was considered for the treatment of patients with supraventricular tachycardia (a disturbance of cardiac rhythm) (65-68). Intravenous treatment with a bolus of adenosine results in a transient complete heart block, leading to a complete standstill of the heart for typically 5 to 10 s. When adenosine is cleared, the heart rate recovers, and if treatment is successful, a normal sinus rhythm prevails (66). Today, studies in gene-targeted mice for individual adenosine receptors (ARs) provide convincing evidence that the transient slowing of the heart rate is due to the activation of adenosine A₁ receptors (A₁ARs) expressed on cardiac tissues (69-71). Other clinical applications of the direct or indirect influences of adenosine include its role as an arterial vasodilator during pharmacologically induced stress echocardiography (72) or as an inhibitor of platelet aggregation (73, 74). Moreover, the nonspecific AR antagonist caffeine has been used to treat headaches, whereas the AR antagonist theophylline has been used to treat obstructive airway disease (75). Thus, adenosine-based medical therapy plays an important role in the treatment of medical or surgical patients. The more recent discoveries of adenosine generation and signaling as potential therapeutic targets for the treatment of inflammatory diseases are less well developed as therapies for human diseases.

STRUCTURAL AND BIOLOGICAL RELATIONSHIP BETWEEN EXTRACELLULAR NUCLEOTIDES AND ADENOSINE

Adenosine belongs to the chemical group of nucleosides and is structurally composed of the purine-based nucleobase adenine bound to a ribose sugar moiety via a β -N₉-glycosidic bond. In the extracellular compartment, adenosine is generated predominantly through the phosphohydrolysis of extracellular nucleotides, particularly ATP or ADP. Enzymatic conversion of ATP or ADP by the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) 1 (CD39) to AMP and subsequent conversion of AMP to adenosine by CD73 represent the major pathway for extracellular adenosine generation (76-78). Extracellular adenosine can activate any of four distinct ARs (A₁AR, A_{2A}AR, A_{2B}AR, or A₃AR), and signaling is terminated by the relatively short half-life of extracellular adenosine. Passive transport from the extracellular compartment into the intracellular space through adenosine transporters is responsible for the short half-life of adenosine in circulation (68, 79-81). Within the cytosol, adenosine is deaminated to inosine by adenosine deaminase or is rephosphorylated to AMP by adenosine kinase (82, 83). As alluded to above and as depicted in Table 1, hypoxia directly influences many aspects of this pathway.

SOURCES OF NUCLEOTIDES DURING INJURY

During inflammation or hypoxia, a number of cell types actively release adenine nucleotides, particularly in the form of ATP or ADP (68, 80, 84). Likewise, as the intracellular concentrations of ATP are high (approximately 5–7 mM), cellular necrosis, lysis, or programmed cell death (apoptosis) is associated with the liberation of large amounts of ATP. For example, recent studies investigated the role of ATP released by apoptotic cells as a so-called find-me signal for promoting phagocytic clearance (85, 86). In this context, a very elegant study provided several lines of evidence for extracellular nucleotides as a critical apoptotic cell find-me signal. Enzymatic removal of ATP (by apyrase or the expression of ectopic CD39) abrogated the ability of apoptotic cell supernatants to recruit monocytes *in vitro* and *in vivo*. Subsequent studies identified the ATP receptor P2Y₂ as a critical sensor of nucleotides released by apoptotic cells. The results from this study pinpointed nucleotides as a critical find-me cue released by apoptotic cells to promote P2Y₂-dependent recruitment of phagocytes (86). Interestingly, an additional study identified (a) a specific mechanism of nucleotide release from apoptotic cells, (b) the plasmamembrane channel pannexin 1 (PANX1) as a mediator of find-me signal and nucleotide release from apoptotic cells and selective plasma membrane permeability during apoptosis, and (c) a new mechanism of PANX1 activation by caspases (85).

Other studies have investigated the contributions of inflammatory cells to extracellular nucleotide release. Given the association of neutrophils (PMNs) with adenine nucleotide/nucleoside signaling in the inflammatory milieu, we hypothesized that PMNs may represent an important source of extracellular ATP (87, 88). Initial studies using high-performance liquid chromatography (HPLC) and luminometric ATP detection assays revealed that PMNs release ATP through activation-dependent pathways. After excluding lytic ATP release, pharmacological strategies revealed a mechanism involved in PMN ATP release via connexin 43 (Cx43) hemichannels. Cx43 molecules assemble as hexadimers (so-called connexons) that form junctional complexes between different cell types. More recently, and in addition to their role as gap-junctional proteins, studies implicate Cx43 connexons as intercellular signaling channels via ATP release (89, 90). In the above studies defining ATP release from human PMNs (87, 88), the authors confirmed their findings in PMNs derived from induced Cx43^{-/-} mice, whereby activated PMNs released less than 15% of ATP relative to littermate controls and Cx43 heterozygote PMNs were intermediate in their capacity for ATP release. This study implicated Cx43 in activated PMN ATP release, therein contributing to the innate metabolic control of the inflammatory milieu (87). Subsequent studies by others revealed that human neutrophils release ATP predominantly from the leading edge of their cell surface as a mechanism to amplify chemotactic signals and direct cell orientation by feedback through P2Y₂ nucleotide receptors (91, 92).

An additional source of extracellular ATP release comes from platelets, which release nucleotides at high concentrations upon activation by ADP or collagen via dense granule release (93). In this context, a recent study highlighted the interaction between PMNs and platelets in regulating intestinal inflammation and fluid transport via nucleotide release (94). Mucosal diseases are often characterized by a mixed inflammatory infiltrate that includes PMNs and platelets. These studies showed that platelets migrate across intestinal epithelia in a PMN-dependent manner. Furthermore, platelet-PMN comigration occurred in intestinal tissue derived from human patients with IBD. The translocated platelets release large quantities of ATP, which is metabolized to adenosine via a two-step enzymatic reaction involving CD73 and CD39-like molecules expressed on IECs. Subsequent studies revealed a mechanism involving adenosine-mediated activation of electrogenic chloride secretion, with concomitant water movement into the intestinal lumen, originally described by Madara et al. (95). Together, these studies demonstrated that E-NTPDases are expressed on IECs and

interact with platelet-derived nucleotides through a mechanism involving platelets that piggy back across mucosal barriers while attached to the surface of PMNs (Figure 2) (94).

HYPOXIA PROMOTES THE PHOSPHOHYDROLYSIS OF EXTRACELLULAR ATP/ADP TO AMP

As discussed above, there is strong evidence that mucosal inflammation is characterized by the release of extracellular nucleotides from multiple cell types. Extracellular nucleotides either activate extracellular ATP receptors or are rapidly converted to adenosine in a two-step enzymatic process, including phosphohydrolysis to AMP and the conversion of AMP to adenosine. This metabolic pathway can be readily detected through the use of non-native etheno-nucleotide derivatives using HPLC (Figure 3). AMP generation from ATP/ADP is achieved mainly enzymatically by the E-NTPDases, a recently described family of ubiquitously expressed membrane-bound enzymes (76, 96). The catalytic sites of plasma membrane-expressed E-NTPDases 1–3 and 8 are exposed to the extracellular milieu, whereas the other E-NTPDases are intracellular (76). The presumptive biological role of plasma membrane-bound E-NTPDases is to fine-tune extracellular nucleotide levels. For example, E-NTPDase 1 (CD39) plays an important role in vascular endothelial function by blocking platelet aggregation via the phosphohydrolysis of ATP and ADP from the blood to maintain vascular integrity (97–99). At the same time, E-NTPDase 1 is also important in the maintenance of platelet functionality by preventing platelet P2Y₁ receptor desensitization. As such, mice gene targeted for E-NTPDase 1 (*Cd39*^{−/−} mice) show prolonged bleeding time with minimally perturbed coagulation parameters (100) and increased vascular permeability as measured by Evan's blue dye extravasation (Figure 4) (3, 23, 33, 34).

Several studies have provided evidence that the CD39 transcript, protein, and function are under the direct control of hypoxia-dependent signaling pathways. The first evidence comes from two studies that subjected vascular endothelia or intestinal epithelia to hypoxia and observed robust increases in the expression and function of CD39 (and CD39-like molecules) (78, 88). To define these molecular principles, a recent study determined whether the human CD39 (hCD39) promoter was hypoxia responsive (101). In view of the likelihood of transcription-mediated induction of CD39 during hypoxia, attention was directed to the 5'-UTR for potential hypoxia-regulated transcription factor sequences. 5'-RACE (5'-rapid amplification of cDNA ends) results confirmed the findings of Maliszewski et al. (102), who identified the transcription start site at 81 bp upstream of the start codon. Sequence comparison of this region in the hCD39 and mouse CD39 promoter regions revealed only a single-base-pair difference and an identical expression profile for both specificity protein-1 (Sp1) and GATA3 binding sites, suggesting that this region is highly conserved. Subsequent studies with site-directed mutagenesis of the central transcription factor binding site in Sp1 or GATA-3 in the hCD39 promoter and analysis of hypoxia inducibility revealed that only Sp1 contributes to this response (101).

As CD39 hydrolyzes nucleotides to generate adenosine, another pathway examined the role of hypoxia-induced CD39 in inflammatory bowel disease. Here, Friedman et al. (103) hypothesized that CD39 might protect against IBD. They studied these possibilities in a mouse model of colitis using mice with global CD39 deletion and tested whether human genetic polymorphisms in the CD39 gene might influence susceptibility to Crohn's disease. *Cd39*^{−/−} mice were highly susceptible to chemically induced colitis; heterozygote mice showed an intermediate phenotype. Moreover, these researchers identified a common single-nucleotide polymorphism (SNP) that tags CD39 mRNA expression levels in humans. The SNP tagging low levels of CD39 expression was associated with increased susceptibility to Crohn's disease in a case-control cohort composed of 1,748 Crohn's patients and 2,936 controls. These data indicate that CD39 deficiency exacerbates murine

colitis and suggest that CD39 polymorphisms are associated with IBD in humans (103). Other studies identified CD39 as a specific marker for regulatory T cells and implicate CD39-dependent ATP/ADP breakdown in autocrine enhancement of the anti-inflammatory functions of this group of T cells (104).

HYPOXIA-INDUCIBLE FACTOR CONTROLS ADENOSINE GENERATION BY REGULATING ECTO-5'-NUCLEOTIDASE (CD73)

In the extracellular compartment, AMP phosphohydrolysis to adenosine is achieved primarily by the ecto-enzyme CD73, which is the pacemaker enzyme for extracellular adenosine production (77). CD73 is bound to the extracellular compartment of the membrane via a glycosylphosphatidylinositol anchor (77). This anatomic localization within the extracellular membrane and orientation toward the extracellular compartment would allow CD73 to be released from the cell membrane during injurious conditions. However, the function of circulating CD73 and its potential as a biomarker of human disease have yet to be established. CD73 is expressed ubiquitously, with the highest expression levels in the intestine (105). Consistent with this notion, several studies have implicated CD73 in dampening hypoxia-elicited inflammation of the intestine. For instance, pharmacological inhibition or gene-targeted deletion of CD73 is associated with intestinal or vascular permeability dysfunction upon exposure of mice to ambient hypoxia (8% over 4 h) (78, 105). During experimental colitis induced by the hapten TNBS, *Cd73*^{-/-} mice developed a more severe phenotype (106). Cytokine profiling revealed similar increases in both interferon (IFN)- γ and tumor necrosis factor- α mRNA in colitic animals, independent of genotype. However, IL-10 mRNA increased in wild-type mice on day 3 after TNBS administration, whereas *Cd73*^{-/-} mice mounted no IL-10 response. This IL-10 response was restored in the *Cd73*^{-/-} mice by exogenous IFN- α A. Further cytokine profiling revealed that a transient IFN- α A induction precedes IL-10 induction. Together, these studies indicate a critical regulatory role for CD73-modulated IFN- α A in the acute inflammatory phase of TNBS colitis, thereby implicating IFN- α A as a protective element of adenosine signaling during mucosal inflammation (106). Other studies have revealed a protective role for CD73-dependent adenosine generation during intestinal ischemia-reperfusion injury (107) and in hypoxic preconditioning. These studies highlight an important role of CD73 in dampening inflammatory responses in the context of tissue hypoxia.

To dissect the direct consequences of hypoxia on CD73 expression and function, a study exposed IECs to hypoxia and observed robust induction of CD73 transcript, protein, and enzymatic activity (78). Examination of the CD73 gene promoter identified at least one binding site for HIF-1, and the inhibition of HIF-1 α expression by antisense oligonucleotides resulted in significant inhibition of hypoxia-inducible CD73 expression. Studies using luciferase reporter constructs revealed a significant increase in activity in cells subjected to hypoxia; no such increase was seen in truncated and mutated constructs lacking a functional HIF binding site. In vivo studies in a murine hypoxia model revealed that hypoxia-induced CD73 may protect the epithelial barrier because the CD73 inhibitor $\alpha\beta$ -methylene ADP promoted increased intestinal permeability. These results identify a HIF-1-dependent regulatory pathway for CD73 and indicate that CD39 and CD73 protect epithelial barrier function during hypoxia. Studies of intestinal ischemia-reperfusion injury demonstrate that gene-targeted mice for HIF-1 α suffer from a more severe phenotype that is associated with attenuated CD73 levels (108). Conversely, treatment with the pharmacological HIF activator DMOG provides potent protection from intestinal ischemia-reperfusion injury in wild-type mice but is ineffective in *Cd73*^{-/-} mice (108).

A recent study identified mutations in the gene encoding CD73 (*5NTE*) that cause human disease (109). This study revealed a severe vascular phenotype wherein mutations of CD73

result in arterial calcification. This study identified nine persons with calcifications of the lower-extremity arteries and hand and foot joint capsules: all five siblings in one family, three siblings in another family, and one patient in a third family. All mutations resulted in nonfunctional CD73. Genetic rescue experiments normalized the CD73 and alkaline phosphatase activity in patients' cells, and adenosine treatment reduced the levels of alkaline phosphatase and calcification. The authors conclude that mutations in the *NT5E* gene are associated with symptomatic arterial and joint calcifications, supporting a role for this metabolic pathway in inhibiting ectopic tissue calcification. In this study (109), the authors developed a complex model proposing that vascular cells produce adenosine via the conversion of ATP to AMP and pyrophosphate by ecto-nucleotide pyrophosphatase phosphodiesterase 1 (ENPP1), with subsequent hydrolysis of AMP to adenosine by CD73. Decreased levels of adenosine secondary to lower CD73 activity boost alkaline phosphatase activity, which clears pyrophosphate. Although deletion of *Enpp1* in mice recapitulates disease in infancy with arterial calcifications and ectopic osteochondral differentiation (110), there is no evidence that CD73 deletion in mice is associated with arterial calcifications (77, 105). This work raises many interesting issues and provides an important basis for further study (111).

ADENOSINE RECEPTORS AND HYPOXIA

Extracellular adenosine exerts its biological signaling actions through the activation of any of four ARs. Whereas activation of the A₁AR or the A₃AR leads to attenuation of intracellular cAMP levels, activation of the high-affinity A_{2A}AR or the low-affinity A_{2B}AR is associated with elevation of cAMP levels (68). The crystal structure of agonist- and antagonist-bound A_{2A}AR was recently solved (112).

As we discuss in the paragraph above, hypoxia shifts the balance to nucleotide signaling by enhancing the phosphohydrolysis of adenosine precursor nucleotides. This shift from ATP toward adenosine signaling involves Sp1-dependent induction of CD39 and HIF-dependent induction of CD73 during conditions of hypoxia. In addition, hypoxia directly influences adenosine signaling events. In fact, investigators have described two hypoxia-elicited, transcriptionally regulated pathways for ARs, including HIF-1 α -dependent induction of A_{2B}AR (113) and HIF-2 α -dependent induction of A_{2A}AR (114). A_{2B}AR has the lowest affinity of the ARs. However, extracellular adenosine levels that are sufficient to activate A_{2B}AR can be achieved, particularly during conditions of hypoxia or ischemia (84, 115).

The first evidence for hypoxia-dependent enhancement of A_{2B}AR signaling comes from a study that examined expressional and transcriptional responses of ARs during hypoxia (88). The authors profiled the relative expression of ARs in normoxic or hypoxic endothelial cells by microarray analysis. These experiments identified selective induction of A_{2B}AR. Several studies validated these microarray results, and subsequent analysis revealed a mechanism involving direct HIF-1 α -dependent regulation of the A_{2B}AR promoter (113). Induction of A_{2B}AR in hypoxia has translated to a strong anti-inflammatory phenotype that, at least in part, includes barrier protection in several different tissues. Consistent with these findings, other studies demonstrated induction of A_{2B}AR in intestinal ischemia-reperfusion injury (108, 116) and experimental colitis (117) in conjunction with attenuated inflammation (118) and improved organ function (68, 79, 84, 119).

A second transcriptional pathway that is under the control of hypoxia signaling involves A_{2A}AR. Genetic and pharmacological studies strongly implicated A_{2A}AR in attenuation of inflammatory responses in a wide range of models (36, 104, 120-122). Studies that examined the transcriptional control of A_{2A}AR during conditions of hypoxia focused on HIF-2 α (128). Unlike the case for HIF-1 α , which regulates a unique set of genes, most

genes regulated by HIF-2 α overlap with those induced by HIF-1 α . Thus, the unique contribution of HIF-2 α remains largely obscure. By using adenoviral mutant HIF-1 α or adenoviral mutant HIF-2 α constructs, in which the HIFs are transcriptionally active under normoxic conditions, a study from the White laboratory (114) demonstrated that HIF-2 α , but not HIF-1 α , regulates A_{2A}AR expression in primary cultures of human lung endothelial cells, suggesting nonredundant, tissue-specific roles for HIF-1 α and HIF-2 α in the regulation of ARs.

In addition to directly influencing AR expression, hypoxia was recently implicated in a pathway that examined mechanisms of indirect AR amplification during conditions of hypoxia. A previous study had demonstrated that the neuronal guidance molecule netrin-1 requires interactions with A_{2B}AR for axon outgrowth and cAMP production (123). On the basis of other studies showing that signaling events involving netrin-1 can attenuate acute inflammatory responses (124), a subsequent study investigated the contribution of netrin-1 signaling to hypoxia-induced inflammation (125). The authors detected HIF-1 α -dependent induction of expression of the gene encoding netrin-1 (*Ntn1*) in hypoxic epithelia. Neutrophil transepithelial migration studies showed that by engaging A_{2B}ARs on neutrophils, netrin-1 attenuated neutrophil transmigration. Moreover, exogenous netrin-1 suppressed hypoxia-elicited inflammation in wild-type but not in A_{2B}AR-deficient mice, and inflammatory hypoxia was enhanced in *Ntn1*^{+/-} mice relative to that in *Ntn1*^{+/+} mice. These studies demonstrate that HIF-dependent induction of netrin-1 attenuates hypoxia-elicited inflammation at mucosal surfaces by enhancing signaling events through A_{2B}AR (125).

OTHER INFLUENCES OF HYPOXIA ON ADENOSINE METABOLISM

As discussed above, hypoxia elicits a coordinated response that results in increased enzymatic production and signaling events of adenosine. Other studies have implicated hypoxia signaling in the regulation of the extracellular half-life of adenosine through transcriptional control of adenosine transporters (26, 81, 126). The short half-life of extracellular adenosine is attributable to its uptake via nucleoside transporters. Two groups of nucleoside transporters have been described: the equilibrative nucleoside transporters (ENTs), which allow for passive diffusion of adenosine following its gradient across the cell membrane, and the active concentrative nucleoside transporters, which transport adenosine in exchange for Na⁺ (81, 127-129). The ENTs have been implicated in the functional regulation of adenosine signaling during conditions of hypoxia (26, 81, 126, 130-134).

Hypoxia also strongly influences intracellular adenosine metabolism. Within the intracellular space, adenosine can undergo deamination by adenosine deaminase to inosine (82) or phosphorylation to AMP by adenosine kinase (83, 135). Despite its intracellular location, adenosine kinase regulates extracellular adenosine signaling, most likely through hypoxia-mediated inhibition of adenosine kinase (135-138); such inhibition involves HIF-1 α -regulated repression of adenosine kinase (83).

MECHANISMS OF ANTI-INFLAMMATION MEDIATED BY ADENOSINE

Surprisingly little is known about the actual mechanisms of adenosine-mediated anti-inflammation. Although signal transduction through the various ARs is well characterized, less is known about postreceptor events. One particularly intriguing mechanism suggests that adenosine inhibits NF- κ B through actions on proteasomal degradation of I κ B proteins (139). These findings were based on previous work suggesting that commensal bacteria inhibit NF- κ B through Cullin-1 (Cul-1) deneddylation (140). Studies addressing adenosine signaling mechanisms revealed that adenosine and adenosine analogs display a dose-

dependent deneddylation of Cul-1, with rank order of receptor potencies as follows: A_{2B}AR > A₁AR ≫ A_{2A}AR = A₃AR (139). Regulated protein degradation is an essential feature of cell signaling for many adaptive processes. The proteasomal degradation of IκB proteins that inhibit NF-κB is one such example of a rapid response by the cell to signal for cell growth, differentiation, apoptosis, or inflammation. The E3 SCF ubiquitin ligase, which is specific to IκB-family members and comprises SKP1, Cul-1, and the F-box domain of β-TrCP, is responsible for the polyubiquitination of IκB (141). E3 SCF requires the COP9 signalosome (CSN) to bind Nedd8 to Cul-1 in order to be active, and deneddylated Cul-1 is incapable of IκB ubiquitination and, hence, of the inactivation of NF-κB (142). Deneddylation reactions on Cullin targets via CSN-associated proteolysis are increasingly implicated as a central point for Cullin-mediated E3 ubiquitylation (143). Notably, other pathways for deneddylation have been reported. For example, the identification of the Nedd8-specific proteases NEDP1 and DEN1 has provided new insights into this emerging field. NEDP1/DEN1 have isopeptidase activity capable of directly deneddyating Cullin targets (144, 145). How adenosine influences NEDP1/DEN1 activity is not known.

CONCLUSION

The interplay of metabolic pathways in health and disease defines an elegant lesson in biology. Studies in model disease systems and human patients have allowed for the identification of metabolic changes that have proven fundamental to our understanding of disease pathogenesis. The interdependence of HIF and adenosine shows how these biochemical and physiological pathways yield insights both to better understand tissue function and to define new targets as templates for the development of novel therapies that promote the resolution of inflammatory disease (Figure 5).

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Glossary

IECs	intestinal epithelial cells
HIF	hypoxia-inducible factor
PMNs	polymorphonuclear leukocytes
IBD	inflammatory bowel disease
CD73	ecto-5'-nucleotidase
A_{2B}AR	adenosine A _{2B} receptor
PHD	prolyl hydroxylase
A₁AR	adenosine A ₁ receptor
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
CD39	ecto-nucleoside triphosphate diphosphohydrolase 1
A_{2A}AR	adenosine A _{2A} receptor
A₃AR	adenosine A ₃ receptor
ENT	equilibrative nucleoside transporter

SUMMARY POINTS

1. The microenvironment of an inflammatory lesion is depleted of O₂ (e.g., is hypoxic).
2. Hypoxia-inducible factor (HIF) functions as one of the master regulators of O₂ homeostasis.
3. HIF prolyl hydroxylases are a primary sensor of O₂ in both health and disease.
4. Adenine nucleotides represent a dominant metabolic fingerprint of hypoxia.
5. HIF coordinates adenine nucleotide metabolism and regulates adenosine signaling.

Figure 1.

Localization of hypoxia and mechanism of hypoxia-inducible factor (HIF) stabilization. (a) Tissue sections from healthy control or trinitrobenzene sulfonic acid (TNBS) colitis were examined for localization of the 2-nitroimidazole compound 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,2,3,3-pentafluoropropyl) acetamide (EF5) (red). Nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI) is shown in blue. Hematoxylin and eosin staining of an adjacent section is shown for orientation. (b) General features of HIF. Overlapping functions of HIF-1α and HIF-2α include the regulation of tissue development, vascular angiogenesis, cell proliferation, and multiple inflammation targets and genes that promote or suppress tumorigenesis. Specific target genes for HIF-1α include those genes involved primarily in metabolism, including genes within the glycolytic pathways. HIF-2α-specific targets include erythropoietin and gene targets involved in duodenal iron transport. (c) The biochemical pathway of HIF hydroxylation by the combination of α-ketoglutarate (αKG), molecular oxygen (O₂), and the prolyl hydroxylase (PHD) enzymes in normoxia. When O₂ or prolyl hydroxylation becomes limiting (due to hypoxia or PHD inhibition), the HIF-1α subunit and/or HIF-2α subunit stabilize and bind to the HIF-1β subunit within the nucleus, where the dimer becomes transcriptionally active upon binding to the hypoxia-response element (HRE) DNA consensus sequence.

Figure 2.

Platelet-polymorphonuclear leukocyte (PMN) cotransmigration in crypt abscesses from human inflammatory bowel disease. (a) A merged fluorescence image localizing PMNs in green [anti-myeloperoxidase (MPO)], platelets in red (anti-CD41), and nuclei 4',6-diamidino-2-phenylindole (DAPI) stain in blue from a human ulcerative colitis crypt abscess. (b) A model of facilitated platelet translocation and activation of epithelial electrogenic Cl⁻ secretion during PMN transmigration. During active inflammation, platelets are caught in the flow of PMN transmigration. PMN- and platelet-derived ATP is selectively metabolized to adenosine by a two-step enzymatic reaction involving CD39 and CD73. Adenosine binding to apical adenosine A_{2B} receptors (A_{2B}AR) results in activation of electrogenic Cl⁻ secretion and the paracellular movement of water.

Figure 3.

Biochemical analysis of intestinal epithelial CD73 activity. (a) A representative high-performance liquid chromatography (HPLC) tracing demonstrating peak resolution between etheno-AMP (structure shown in *inset*) and etheno-adenosine. (b) T84 intestinal epithelial monolayers were exposed to normoxia (pO_2 147 torr) or hypoxia (pO_2 20 torr) for 36 h and washed. Surface CD73 activity was determined by HPLC analysis of etheno-AMP conversion to etheno-adenosine at the indicated sampling times.

Figure 4.

Analysis of colonic vascular leak in CD39-deficient (*Cd39*^{-/-}) mice. (a) Wild-type (*Cd39*^{+/+}) and *Cd39*^{-/-} mice were administered intravenous Evans blue (EB) solution (0.2 ml of 0.5% in phosphate-buffered solution) and were exposed to normobaric hypoxia (8% O₂, 92% N₂) or room air for 4 h. Animals were sacrificed, and their colons were harvested and imaged. (b) A plot of extracted EB, expressed as optical density (OD) at 610 nm mg⁻¹ tissue.

Figure 5.

Model of cooperation between nucleotide and nucleoside receptors in inflammation. In areas of ongoing inflammation, diminished O₂ supply (inflammatory hypoxia) coordinates the metabolism of nucleotides to adenosine and subsequent signaling via P1 adenosine receptors. Much of the inflammatory nucleotide signaling occurs via P2 receptors, whereas P1 receptors contribute significantly to the resolution of ongoing inflammation.

Table 1Hypoxia and HIF targets in adenine nucleotide metabolism and signaling^a

Gene product	Function	Regulation	Reference
CD39	ATP/ADP → AMP	Sp1	101
CD73	AMP → Ado	HIF-1	78
A ₁ AR	Signaling	Unknown	146
A _{2A} AR	Signaling	HIF-2	114
A _{2B} AR	Signaling	HIF-1	113
ENT-1	Ado transport	HIF-1	147
Ado kinase	Ado → AMP	HIF-1	83
Ado deaminase	Ado → inosine	Unknown	82

^a Abbreviations: Ado, adenosine; A₁AR, adenosine A₁ receptor; A_{2A}AR, adenosine A_{2A} receptor; A_{2B}AR, adenosine A_{2B} receptor; CD39, ecto-nucleoside triphosphate diphosphohydrolase 1; CD73, ecto-5'-nucleotidase; ENT, equilibrative nucleoside transporter; HIF, hypoxia-inducible factor.