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Nitric Oxide and Blood–Brain Barrier Integrity

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ABSTRACT

The blood–brain barrier (BBB) is comprised of the endothelial cells that line the capillaries of the brain. The unique characteristics of this barrier include tight intercellular junctions, a complex glycocalyx, a paucity of pinocytic vesicles, and an absence of fenestra. These properties allow for the selective exchange of substances between the systemic circulation and the extracellular fluid compartment of the brain. It is well established that there are many conditions, including those mediated by nitric oxide (NO), that can lead to an opening of the BBB, eventually leading to vasogenic edema and secondary brain damage. The precise molecular mechanisms mediating NO-induced tissue injury and the breakdown of the BBB are complex and not completely understood. NO is a soluble, easily diffusible gas that is generated by NO synthase. Two of the isoforms of NO synthase are constitutive, calcium-dependent enzymes that modulate many physiological functions, including the regulation of smooth muscle contraction and blood flow. The third isoform is calcium-independent and inducible and can be stimulated by stress, inflammation, and infection. Under these conditions, NO can be generated in large quantities and has detrimental effects on the CNS. NO has been shown to increase permeability of the BBB, allowing substances to enter into the brain passively. This review considers the role of NO and BBB integrity. Antioxid. Redox Signal. 3, 273–278.

INTRODUCTION

THE SO-CALLED BLOOD–BRAIN BARRIER (BBB) is localized at the single, continuous layer of endothelial cells that line the capillaries of the brain (9, 31). The unique properties of this barrier relative to the typical peripheral endothelium confer highly restricted exchange of blood-borne molecules between the systemic circulation and the extracellular fluid compartment. The distinguishing features of brain microvessel or capillary endothelium are illustrated in Fig. 1 and include the presence of tight intercellular junctions, a paucity of pinocytic vesicles, the absence of fenestra, and a complex glycocalyx (2). In addition, there are an abundance of mitochondria and numerous enzymes indicating substantial metabolic capacity. The expression of the multidrug resistant gene product 1 (MDR1) or P-glycoprotein, and the multidrug resistant associated protein (MRP) efflux systems contribute additional barrier mechanisms that restrict exchange of solutes between the blood and the central nervous system (CNS) (28, 32). Passive diffusion across the BBB is limited to rather lipophilic molecules. Water-soluble molecules such as amino acids, nucleosides, and hexoses utilize specific transporters that are present in the capillary endothelium (2). Most of the transporters present in the BBB are specific, and there are few transporters available for macromolecules (4).

It is well established that there are many pathophysiological conditions that disrupt the

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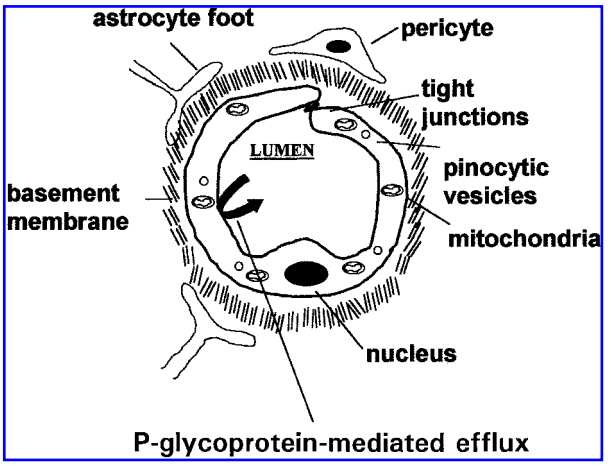

FIG. 1. Distinguishing features of the brain microvessel endothelial cell that forms the BBB.

integrity of the BBB and can lead to increased permeation of substances across the BBB (3). For instance, increased vesicular trafficking or an opening of the tight intercellular junctions present in the normal aging brain or pathological conditions such as stroke, ischemia, or Alzheimer's disease will compromise the BBB (26). Under these conditions, the CNS is highly vulnerable to free radical attack because of the high blood flow rate and the presence of easily oxidizable substances (11). Consequently, opening of the BBB can lead to vasogenic brain edema and subsequent secondary brain damage (36).

Stress can also cause an opening of the BBB and a subsequent change in permeability. For instance, soldiers who served in the Gulf War were treated with the acetylcholinesterase inhibitor, pyridostigmine, which is not expected to cross the BBB due to its quaternary ammonium structure. However, there was a reported threefold increase in CNS symptoms such as headaches, insomnia, and drowsiness in soldiers treated with the drug prior to departure for the battlefield (19). Friedman *et al.* (15) analyzed the effects of pyridostigmine on "normal" patients during peacetime. These subjects reported symptoms confined to the peripheral nervous system such as diarrhea and sweating. This suggests that the emotional effects and stress of the battlefield may have caused the BBB to become more permeable than normal (19).

Disease conditions such as ischemia, stroke, multiple sclerosis, or Alzheimer's disease evoke an inflammatory response in the CNS. This response can contribute to neurological damage due to the release of cytokines, such as tumor necrosis factor or interleukin-1β (IL-1β) (35). These types of mediators can be released from astrocytes and neurons, as well as blood-derived cells, and therefore can initiate immune signaling from both apical and basolateral sides of the capillaries (18). In addition, the release of these same mediators may also exhibit some neuroprotective effects (35). Nitric oxide (NO) is a transient product of the inflammatory processes, generated from L-arginine utilizing the enzyme NO synthase (NOS). Indeed, NO has been implicated in both the damage (10) and protection (21) of the brain, similar to that seen with inflammatory modulators. NO appears to be involved in numerous vital cellular functions including neurotransmission, blood-pressure control, and the regulation of vascular tone. Our purpose herein is to present an overview of the role of NO functions and mechanisms specific to modulation of the permeability properties of the BBB, the gateway to the CNS.

NO AT THE BBB

Over the past 20 years, NO and its signaling properties have been extensively investigated and are one of the most rapidly growing areas of biology (29). NO serves a multitude of physiological purposes and reacts with a diverse number of cellular targets. The basal production of NO appears to be required for biological regulation, and yet an excess of this same molecule can be cytotoxic to the organism.

NO is synthesized by the conversion of L-arginine to L-citrulline through the actions of NOS. There are three distinct known isoforms of NOS, and they are classified according to their order of isolation and characterization (27). These isoforms share an overall amino acid sequence homology of ~55%, and there is strong sequence conservation in the regions of the proteins that are important in catalysis (27). The NOS isoforms carry out a five-electron re-

duction process utilizing oxygen, and cofactors such as reduced NADPH, tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide participate in the formation of NO.

Endothelial NOS (eNOS)

Endothelial cells can produce NO in small amounts that are released to control the local blood flow. This NO is synthesized by a calcium/calmodulin-dependent isoform of NOS termed NOSIII or eNOS (30). Due to the dependence on calcium/calmodulin binding, eNOS is very sensitive to changes in calcium levels in the cell. The release of NO from endothelial cells results in the activation of the guanylate cyclase signaling cascade (20). Once activated, the cytosolic, soluble form of guanylate cyclase can then convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which is a second messenger molecule. In vascular smooth muscle cells, cGMP activates a Ser/Thr protein kinase that dephosphorylates myosin light chains, inhibiting contraction that results in vasodilation. This aids in blood flow during conditions such as stroke. Therefore, it is thought that eNOS has a protective effect due to these vasodilation properties of the endothelial cells (20).

A link between the cGMP signaling pathway and the release of NO has been shown using primary cultures of brain microvessel endothelial cells (BMECs). The cells were treated with the NO donor sodium nitroprusside in the presence or absence of the nonhydrolyzable GTP analogue 5′-*O*-3′-thiotriphosphate (24). This analogue prevents the formation of cGMP, and hence the commencement of the second messenger signaling pathway. Detectable levels of NO were measurable in the cells treated solely with sodium nitroprusside. However, the BMECs treated with both sodium nitroprusside and 5′-*O*-3′-thiotriphosphate did not produce detectable levels of NO, indicating a link between the cGMP signaling pathway and NO production (24). There are also cases of NO effects that are independent of the cGMP cascade as well (29). For instance, NO activates cyclooxygenase and lipoxygenase, which leads to the production of prostaglandin E_2.

Other evidence also suggests NO is involved in BBB permeability and its regulation (34). NOS has been shown to be enriched in caveolae of cultured cells by Garcia-Cardena *et al*. (16). The caveolae are thought to be the initial structures that bud off the membrane during receptor-mediated endocytosis and transcytosis through the BBB and are regulated pathways molecules can utilize to enter into the brain. These investigators also showed that the palmitoylation of the enzyme is required for its targeting to the caveolae structure (16). Schnitzer *et al*. (33) demonstrated that the hydrolysis of GTP is involved in the fission of the caveolae structures away from the plasma membrane. Thus, it appears that NO and the activation of cGMP via GTP appear to be important in the regulation of transendothelial permeability (14).

Recent evidence from *in situ* rat perfusion studies indicates that certain forms of NO will mediate the opening of the BBB to different extents, depending on the NO donor used for treatment (7). NO itself caused a moderate disruption of BBB permeability, but the greatest disruption was caused when the rats were treated with an NO donor that produced multiple redox species of NO (7).

Inducible NOS (iNOS)

Unlike eNOS, the inducible isoform of NOS called NOSII or iNOS is not normally expressed in healthy cells. iNOS is stimulated and produced via cytokines or inflammatory responses. There is a lag time of ~8 h between the induction of the iNOS enzyme and subsequent production of NO (13). It is calcium-independent and is therefore not affected by levels of calcium in the cell like its constitutive isoform counterparts. Cytokines such as IL-1β, interleukin-6, interferon-γ, and tumor necrosis factor have been shown to modulate BBB integrity, and are inducers of iNOS (18). Once initiated, the production of NO will continue until the stimulatory signal inducing the enzyme is diminished.

Interestingly, although not detectable in normal healthy brain, iNOS can be detected in aged conditions (26). Astrocytes in the aging brain have high levels of IL-1β. IL-1β induces iNOS, which in turn produces a large quantity

of NO. Similarly, in AIDS lymphocytes, IL-1β is released, which can influence the BBB. Indeed, there is evidence that the AIDS brain appears to look very similar to an aged brain (26).

It is not clear as to whether these observations are solely due to NO, or to the action of NO with other molecules or free radicals. Reactive oxygen species are generated by many different sources such as the environment (ultraviolet rays) or simply the normal functioning of the cell (mitochondrial metabolism). Due to the high volume of blood flow and exchange, it is common for blood vessels to generate free radical species including NO, as well as superoxide anion ($O_2^{\bullet -}$) (8). Under many conditions such as reoxygenation after a stroke or injury, NO and $O_2^{\bullet -}$ can react to form peroxynitrite. The rate-limiting step of the peroxynitrite formation is dependent on the molecule's diffusion rate. Peroxynitrite can isomerize to form nitrate, which has minimal biological activity and may act as a mechanism to inactivate the free radicals NO and $O_2^{\bullet -}$ (1). However, peroxynitrite is also a powerful oxidant that can cause extensive cellular damage by oxidizing proteins, lipids, and DNA (6). Peroxynitrite can induce toxicity through nitrosylation of tyrosine residues on proteins, thereby critically inactivating them (20). NO and peroxynitrite have been implicated in the pathogenesis of multiple sclerosis where NO was found to be cytoxic to oligodendrocytes and neurons, and inhibited the mitochondrial respiratory chain (17). Elevated levels of nitrate and nitrite are found in the cerebrospinal fluid of patients with multiple sclerosis, and the cerebrospinal fluid levels of nitrite and nitrate correlate with the breakdown of the BBB (17). It is also possible for NO itself to nitrosylate thiol groups in proteins as well.

When produced in excessive amounts due to stimuli such as infection, NO production may no longer be beneficial to the cell. Instead, the large quantities of NO become neurotoxic. The mitochondrial electron transport chain has been shown to be impaired by excessive NO, and therefore diminishes the cellular energy production levels of adenosine triphosphate (ATP). This ATP depletion is a hallmark of neuronal cell death (12). Direct evidence that NO and $O_2^{\bullet -}$ appear to be involved in cellular toxicity involves studies of transgenic animals. Cu/Zn superoxide dis-mutase is a cytosolic scavenger enzyme that removes reactive $O_2^{\bullet -}$, thus preventing the formation of peroxynitrite (25). The overexpression of Cu/Zn superoxide dismutase in transgenic mice reduces the infarct volume in the middle cerebral artery occlusion model of focal ischemia compared with that in wild-type mice (25). These findings are strengthened by outcomes in other studies utilizing neuronal NOS or iNOS knock-out mice where there was reduced damage to focal ischemia compared with controls (22, 23).

SUMMARY

The BBB tightly regulates the passage of molecules between the systemic circulation and the brain interstitial fluid. However, there are many conditions that can alter the permeability characteristics of the BBB.

The soluble gas NO is crucial for a variety of normal physiological functions concerned with maintenance of the CNS. NO has dual duties in the CNS, acting as both a neuroprotectant against viruses and a cytotoxic molecule, aiding in the mediation of tissue damage. The inappropriate release of this mediator has been linked to a variety of pathologies and subsequent opening of the BBB (20). What is apparent from this discussion is that NO generated through the constitutive eNOS is generally protective. By contrast, the dramatic elevation of NO generated through induction of iNOS is usually part of a biochemical cascade stimulated by injury or inflammatory conditions. Therefore, agents that modulate the activity of NO may be of considerable interest and therapeutic value (20). Attempts have been made in the pharmaceutical industry to capitalize on NO and NOS mechanisms to enhance drug delivery across the BBB (5). We anticipate that the basic characterization of biochemical processes involved in opening the BBB could also be applied in reducing permeability as either a therapeutic treatment or potentially prophylactic action for cerebrovascular dysfunctions. The current availability of more selective inhibitors

of each of the NOS isoforms and availability of transgenic animals should aid in this type of research.

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ABBREVIATIONS

ATP, adenosine triphosphate; BBB, blood–brain barrier; BMECs, brain microvessel endothelial cells; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; eNOS, endothelial nitric oxide synthase; GTP, guanosine triphosphate; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\bullet -}$, superoxide anion.

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