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Inflammatory Neurodegeneration and Mechanisms of Microglial Killing of Neurons

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Abstract Inflammatory neurodegeneration contributes to a wide variety of brain pathologies. A number of mechanisms by which inflammatory-activated microglia and astrocytes kill neurons have been identified in culture. These include: (1) acute activation of the phagocyte NADPH oxidase (PHOX) found in microglia, (2) expression of the inducible nitric oxide synthase (iNOS) in glia, and (3) microglial phagocytosis of neurons. Activation of PHOX (by cytokines, β-amyloid, prion protein, lipopolysaccharide, ATP, or arachidonate) causes microglial proliferation and inflammatory activation; thus, PHOX is a key regulator of inflammation. However, activation of PHOX alone causes little or no death, but when combined with iNOS expression results in apparent apoptosis via peroxynitrite production. Nitric oxide (NO) from iNOS expression also strongly synergizes with hypoxia to induce neuronal death because NO inhibits cytochrome oxidase in competition with oxygen, resulting in glutamate release and excitotoxicity. Finally, microglial phagocytosis of these stressed neurons may contribute to their loss.

Keywords Inflammation · Cell death · Nitric oxide · Peroxynitrite · Phagocytosis · iNOS · NADPH oxidase

Abbreviations

COX-2	Cyclooxygenase-2
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
iNOS	Inducible NOS
PHOX	Phagocytic NADPH oxidase
PS	Phosphatidyserine
RONS	Reactive oxygen and nitrogen species

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Introduction

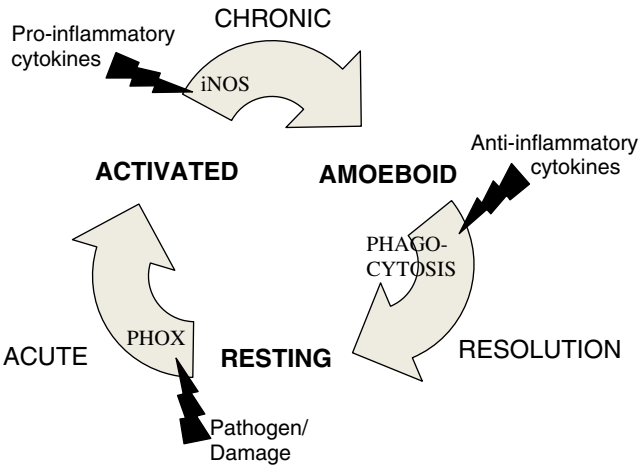
Inflammatory neurodegeneration is neuronal death/loss caused by inflammation and is a process (like energy depletion, protein aggregation, and excitotoxicity) contributing to neuronal loss or dysfunction in many different neurological diseases. In general, inflammation may have beneficial and/or detrimental effects in any particular disease and in any particular phase of a disease. The beneficial effects are mainly due to elimination of pathogens, clearing debris, and aiding repair, and the detrimental effects are probably unintended side effects of the beneficial processes. There is evidence that brain inflammation may contribute to the pathology of both chronic neurodegenerative diseases (Alzheimer's, Parkinson's, multiple sclerosis, and AIDS dementia) as well as acute pathologies such as stroke, brain trauma, and meningitis [1-6]. These pathologies have different causes and consequences, but they all involve brain inflammation, and there is evidence that blocking inflammation can either delay onset or reduce symptoms [1-6]. However, there are different types or modes of inflammation, and it is important to understand why inflammation is sometimes damaging and at other times protective so that interventions can be designed to prevent one but not the other.

Inflammation can damage the brain in a variety of ways, including: (1) inflammation in the vascular wall may drive

atherosclerosis, leading to stroke and vascular dementia; (2) inflammation in the blood–brain barrier may compromise barrier function and allow leukocytes and antibodies into the brain; (3) antibodies generated against brain antigens may induce immune attack as occurs in multiple sclerosis; (4) inflammation may induce brain edema (swelling); (5) some types of inflammation may suppress neurogenesis; and (6) pathogens, protein aggregates, or damaged neurons may inflammatory activate glia, which may then kill neurons. It is this last type of damage, common to many brain pathologies, that we shall be concerned with here.

Microglia, the brain's resident macrophages, are the predominant immune cells in the healthy brain. This is due to the brain being shielded by the blood–brain barrier (BBB) which limits the entry of peripheral cells and substances. Brain inflammation, which occurs behind the BBB, therefore differs from inflammation in the periphery by the relative absence of leukocytes (including neutrophils, monocytes, B cells, and T cells) and antibodies. However, it is now recognized that there is a limited traffic of these across the barrier, and this traffic can be increased by inflammation which can recruit leukocytes into the brain [7]. Nevertheless, microglia are key cells in brain inflammation and inflammatory neurodegeneration [5, 8].

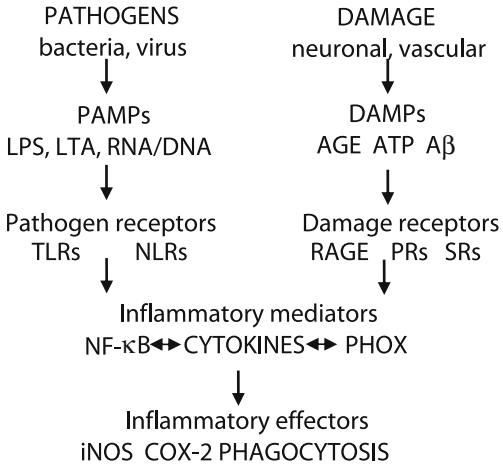
Brain inflammation may be divided into three phases (acute, chronic, and resolution) in which the microglia are largely in three different morphologies (resting, activated, and amoeboid/phagocytic, see Scheme 1). The healthy, non-inflamed brain contains almost entirely "resting" microglia which are highly ramified, with a small, static cell body, but with dynamic and branched processes actively seeking out (1) pathogens and (2) damage in the brain [9]. Pathogen receptors (pattern recognition receptors) include the Toll-like receptors (TLRs) and the NOD-like receptors (NLRs), and generally recognize cell wall components or RNA/DNA of pathogens (Scheme 2). Damage (or "danger") receptors include scavenger receptors, purinergic receptors, receptor for advanced glycation endproducts (RAGE), and TLRs, and these recognize components released from stressed or damaged host cells, including ATP, aggregated β-amyloid, and heat shock proteins [5]. Engagement of these receptors induces signal cascades that will (after several hours) produce the "chronically activated" state of microglia due to expression of new proteins, including iNOS, COX-II, and MHC-II, the latter enabling microglia to present antigens to T cells. Activation is accompanied by partial rounding up and mobility of the cells, proliferation, and the expression and release of pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6. These cytokines activate other microglia and astrocytes. Most of the expression changes are a result of activation of the transcription factor NF-κB via phosphorylation-induced activation of IκB kinase.



Scheme 1 Hypothetical relation of the three phases of inflammation to the three states of microglial activation, driven by three types of signal, acting through three effectors. Microglial PHOX activation by pathogens or damage drives acute activation, which results in pro-inflammatory cytokine production and iNOS expression. Chronic activation may result in amoeboid, highly phagocytic microglia and a switch to anti-inflammatory cytokines driving the resolution phase. Different phases may coexist in the brain, and PHOX activation may occur in the chronic phase, but (in the presence of iNOS) will then result in peroxynitrite production (rather than superoxide and hydrogen peroxide)

Prior to the induction of gene expression, the microglia are in the "acute" phase of activation triggered by receptor ligation. Receptor stimulation induces PKC activity, causing rapid activation of the phagocyte NADPH oxidase (PHOX) which in turn contributes to NF-κB activation. After induced gene expression, the microglia are in a chronic state of activation, which may be maintained by the pro-inflammatory cytokine release and the continued presence of pathogen/damage. However, the chronic state of activation may progress to a "resolution phase" where microglia are amoeboid, highly phagocytic, and produce anti-inflammatory cytokines (including IL-10 and TGFβ) in order to resolve the inflammation and clear up the mess. This three-phase division (acute, chronic, and resolution) of microglial inflammation is simplistic as there are alternative modes of activation and alternative ways of classifying microglial activation [8]. However, the progression through these three inflammatory states may be a mechanism common to microglial activation.

Activated microglia can kill and/or remove pathogens, but they may also kill neurons. The mechanisms by which they kill neurons are complex and not fully understood [5]. We will restrict ourselves to reviewing the roles of three different processes (NADPH oxidase activation, iNOS expression, and phagocytosis) corresponding to the three phases of inflammation (acute, chronic, and resolution phases), with particular emphasis on our own research in this area.



Scheme 2 How inflammation is induced by pathogens or damage. Pathogens are recognized by pathogen receptors, mainly Toll-like receptors (*TLRs*) or NOD-like receptors (*NLRs*), on host cells which recognize pathogen-associated molecular patterns (*PAMPs*) on the pathogen, such as bacterial cell wall components lipopolysaccharide (*LPS*) or lipoteichoic acid (*LTA*), or bacterial/viral RNA or DNA. Damage produces damage-associated molecular patterns (*DAMPs*) such as advanced glycation endproducts (*AGE*) that are recognized by the receptor for AGE (*RAGE*) or extracellular nucleotides such ATP that are recognized by certain purinergic receptors (*PRs*) or aggregated proteins such as beta-amyloid (*Aβ*) that are recognized by RAGE and scavenger receptors (*SRs*). Engagement of receptors results in the activation of inflammatory pathways such as the phagocyte NADPH oxidase (*PHOX*) and NF-κB, and causes cytokine and reactive oxygen production and release, which feeds back to cause further and spreading inflammation. This in turn causes expression of inflammatory effectors: inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), as well as phagocytosis

The Phagocyte NADPH Oxidase

Phagocytic cells such as neutrophils, macrophages, and microglia have a specific NOX (NADPH oxidase) known as PHOX (phagocytic oxidase), consisting of subunits gp91, p22, p47, p67, p40, and Rac. In the healthy, non-inflamed brain, PHOX is expressed at high levels in microglia (and possibly at low levels in astrocytes and neurons). However, PHOX is not active unless acutely stimulated by, for example, TNF-α, IL-1β, chemokines, arachidonate, β-amyloid, lipopolysaccharide (LPS), ATP, or phagocytosis. When activated, it rapidly produces high levels of superoxide extracellularly, which may either dismutate to hydrogen peroxide (catalyzed by extracellular superoxide dismutase) or react with NO to produce peroxynitrite [10]. These oxidants contribute to the killing of pathogens by phagocytes, but may also damage neurons.

Importantly, however, activation of PHOX alone causes no apparent neuronal death [11], but stimulates the microglia to proliferate [12], produce TNFα and IL-1β [13, 14], and express iNOS [14]. For example, we showed that fibrillar β-amyloid induces microglia to proliferate and produce cytokines via the activation of PHOX [13]. In general, H_2O_2 from PHOX leads to cell proliferation and activation by oxidation of the active site cysteine residue of protein tyrosine phosphatases, causing inhibition of these phosphatases and thus amplification of mitotic and pro-inflammatory tyrosine kinase cascades [15]. H_2O_2 may also activate matrix metalloproteinases which can amplify inflammation. PHOX has been shown by several labs (particularly Block et al. [5]) to be a key regulator of inflammatory activation of microglia. Correspondingly, inhibition of PHOX is sufficient to prevent activation of microglia and thus is a target for anti-inflammatory strategies.

Inducible NO Synthase

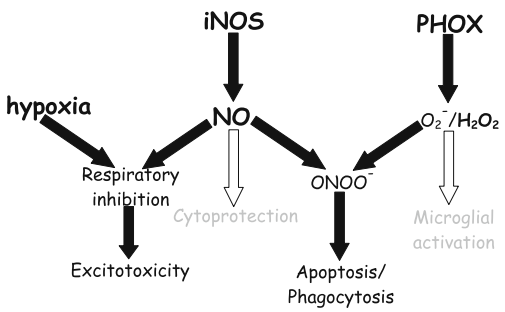
Inducible NO synthase (iNOS) is not normally expressed in the brain, but inflammatory mediators such as LPS and cytokines cause its expression in microglia and astrocytes [16] and possibly in neurons [17]. Once expressed, iNOS produces high levels of NO continuously (up to 1 μM NO from microglia or astrocytes [10, 18]). We showed that these high levels of NO induce neuronal death by causing inhibition of mitochondrial cytochrome oxidase in neurons [18, 19]. NO inhibition of neuronal respiration caused neuronal depolarization and glutamate release, followed by excitotoxicity via the NMDA receptor [18, 20-23]. This excitotoxicity may be potentiated by a second mechanism as NO from iNOS results in glutamate release from astrocytes via calcium release from intracellular stores stimulating exocytosis of vesicular glutamate [24]. Thus, inflammatory-activated astrocytes maintained a relatively high extracellular glutamate level [24]. This level of glutamate may be insufficient to induce excitotoxicity alone, but it has been shown that non-toxic levels of extracellular glutamate can become toxic to neurons if in addition the mitochondrial respiratory chain is inhibited, possibly because such inhibition may partially depolarize the neurons, activating the NMDA receptor if glutamate is present outside [25]. However, high glutamate may impose an increased metabolic demand on neurons so that if metabolism is inhibited, this may suffice to push the cell over some toxic threshold. Such mechanisms may contribute to how NO kills neurons.

However, the above mechanism requires relatively high levels of NO or iNOS expression, as occurs in the presence of IFNγ which dramatically potentiates iNOS expression [26] and changes microglial phenotype in multiple ways. In contrast, iNOS can be expressed at low levels in vitro [11] or in vivo [27] apparently with little or no neuronal death. Indeed, NO from iNOS may be protective by blocking

brain cell death [28, 29]. On the other hand, low levels of iNOS expression may synergise with other conditions to induce cell death [30]. For example, hypoxia strongly synergises with NO or iNOS expression to induce neuronal death via respiratory inhibition [31]. This is because NO is a competitive inhibitor of cytochrome oxidase, the NO competing with oxygen for binding to cytochrome oxidase [19, 31] so that NO greatly increases the apparent K_M of neuronal respiration for oxygen. This sensitisation to hypoxia is potentially important in stroke, trauma, vascular dementia, Alzheimer's, and brain aging where both inflammation and hypoxia may coexist (Scheme 3). Note, however, that O_2 is a substrate for all NO synthases with apparent K_M of 4, 130, and 350 μM O_2 for eNOS, iNOS, and nNOS, respectively, [32] so that nNOS and iNOS may be substrate-limited by oxygen. On the other hand, O_2 also promotes NO consumption by NO reaction with oxygen, superoxide, oxyhemoglobin, or oxymyoglobin [33] so that the dependence of NO level on O_2 is complex and poorly understood [34].

iNOS and PHOX

As described above, neither activation of NADPH oxidase (NOX/PHOX) nor production of (moderate levels of) nitric oxide through iNOS expression is sufficient to induce neurotoxicity. However, we found that when both were activated together, this resulted in apparent apoptosis of most neurons mediated by peroxynitrite (Scheme 3). We showed that inflammatory neurodegeneration induced by TNFα, IL-1β, prion peptide, LPS, IFNγ, arachidonate, ATP, and/or PMA was mediated by this dual-key (iNOS and PHOX) mechanism of inflammatory neurodegeneration in particular conditions [11]. Simultaneous activation of PHOX and iNOS in microglia resulted in the disappearance of NO [10], appearance of peroxynitrite [10, 11], and apoptosis of co-cultured neurons that was prevented by inhibitors of iNOS or PHOX or by scavengers of superoxide or peroxynitrite [11].



Scheme 3 Mechanisms of inflammatory neurodegeneration. Hypoxia, iNOS expression, or PHOX activation may be relatively benign, or even cytoprotective, when present alone. However, hypoxia combined with NO results in neuronal death via respiratory inhibition, and PHOX activation combined with iNOS expression results in peroxynitrite production, inducing neuronal apoptosis at high levels, but loss of neurons by microglial phagocytosis at lower levels

Phagocytosis

Lipoteichoic acid (LTA) is a cell wall component of Gram-positive bacteria which may trigger inflammatory neurodegeneration in bacterial meningitis. We showed that LTA induces iNOS in glia via Toll-like receptor 2 [26] and results in loss of neurons in glial–neuronal co-cultures. This neuronal loss is prevented by scavengers of superoxide or peroxynitrite [35]. However, intriguingly, there is no measurable neuronal death in these cultures, only neuronal loss presumably due to phagocytosis [35].

How apoptosis causes cells to die is, surprisingly, not always clear, but in vivo, the main means is by triggering exposure of "eat me" signals (such as phosphatidylserine) on the cell surface which induce phagocytes to eat the cell. Reactive oxygen and nitrogen species (RONS) can induce PS exposure directly via activation of the phospholipid scramblase and inactivation of amino phospholipid translocase (flippase) [36] or indirectly via induction of apoptosis [37, 38]. However, it is important to realize that phosphatidylserine exposure can be reversible [39] including NO-induced phosphatidylserine exposure of neurons [40]. Furthermore, RONS-induced PS exposure by neurons may induce activation of microglia [37, 38], which may then phagocytose the neurons. Thus, it is possible that microglial phagocytosis may actively contribute to neuronal death.

Conclusions

We have discussed the nature of inflammatory neurodegeneration and outlined the contributions of PHOX, iNOS, and phagocytosis to microglial killing of neurons during inflammation. A variety of other neurotoxic factors released from activated microglia have been identified, including glutamate, TNFα, Fas ligand, cathepsin B, and other proteases. Much future work is required to elucidate the contribution of all these processes to particular brain diseases. This would be greatly aided by drugs that specifically inhibit these targets within the brain without toxicity. We also need to know the relative contributions of inflammation, energy depletion, protein aggregation, and excitotoxicity to the pathology of different brain diseases and to assess the extent to which blocking these processes individually or in combination blocks pathology.

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