Published in final edited form as:

Free Radic Biol Med. 2014 November ; 0: 34–46. doi:10.1016/j.freeradbiomed.2014.07.033.

NADPH Oxidase- and Mitochondria-derived Reactive Oxygen Species in Proinflammatory Microglial Activation: A Bipartisan Affair?

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Abstract

Microglia are the resident immune cells of the brain and play major roles in central nervous system development, maintenance, and disease. Brain insults cause microglia to proliferate, migrate, and transform into one or more activated states. Classical M1 activation triggers the production of proinflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), nitric oxide (NO), and reactive oxygen species which, in excess, can exacerbate brain injury. The mechanisms underlying microglial activation are not fully understood, yet reactive oxygen species are increasingly implicated as mediators of microglial activation. In this review, we highlight studies linking reactive oxygen species, in particular hydrogen peroxide derived from NADPH oxidase-generated superoxide, to the classical activation of microglia. In addition, we critically evaluate controversial evidence suggesting a specific role for mitochondrial reactive oxygen species in the activation of the NLRP3 inflammasome, a multiprotein complex that mediates the production of IL-1 β and IL-18. Finally, the limitations of common techniques used to implicate mitochondrial ROS in microglial and inflammasome activation, such as the use of the mitochondrially-targeted ROS indicator MitoSOX and the mitochondrially-targeted antioxidant MitoTEMPO, are also discussed.

Keywords

Reactive oxygen species; NADPH oxidase; mitochondria; MitoSOX; MitoTEMPO; inflammation; NLRP3; microglia; macrophage; MAPK; NF-kappaB; TNF-alpha; IL-1beta; nitric oxide; permeability transition pore

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Introduction

Microglia are macrophage-like immune cells of the central nervous system which play major roles in both health and disease [1, 2]. In health, microglia are highly motile cells that survey their surrounding environment in order to maintain homeostasis [3], promote synaptogenesis, and regulate the integrity of the blood brain barrier [4, 5]. However, microglia become activated and proliferate following acute brain injury or in chronic neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [1, 2]. Activated microglia undergo drastic morphological changes, transforming from a ramified to an amoeboid morphology [1, 2]. This change in morphology is thought to favor mobility and increase phagocytic activity [6]. Microglia cordon off damaged neurons, and assist in the repair and regrowth of compromised cells by releasing various metabolites, growth factors, and cytokines. However, prolonged or excessive microglial activation becomes maladaptive [7]. This is partly because activation of microglia takes multiple shapes. Classical M1 microglial activation results in the proinflammatory state implicated in neurotoxicity, for instance, the degeneration of dopaminergic neurons in Parkinson's disease [3, 8-11] or of motor neurons in amyotrophic lateral sclerosis [12, 13]. In addition to the creation of a toxic proinflammatory milieu, activated microglia may also contribute to cell death following brain injuries via phagocytosis of live neurons [14-16]. In contrast to M1 activation, alternative microglial activation states, generally classified as M2 with further subclasses, counteract proinflammatory mediators and promote repair processes such as remodeling of the extracellular matrix and angiogenesis [17]. Impairing excessive M1 microglial activation and/or promoting a transition toward alternative M2 activation are promising neuroprotective treatment strategies receiving considerable attention [18, 19]. It is important to understand the M1 activation process in greater detail in order to successfully achieve this goal.

To this end, extensive progress has been made in elucidating the initial molecular signaling pathways underlying microglial activation, especially through activation of toll-like and cytokine receptors (see [9] and [20] for detailed reviews). Treatment with the bacterial endotoxin lipopolysaccharide (LPS) alone or in combination with the proinflammatory cytokine interferon- γ (IFN- γ) is frequently used to induce and study M1 microglial activation *in vitro*. These factors activate toll-like receptor 4 (TLR4, also called CD14) and IFN- γ receptor, respectively. LPS also induces TLR4-independent signaling by binding phagocytic scavenger receptors [21] and by binding macrophage antigen complex I (MAC1, also called CD11b/CD18), a pattern recognition receptor linked to the superoxide-generating enzyme NADPH oxidase (alternatively called Nox or Phox for phagocytic oxidase) [22]. Reactive oxygen species (ROS) production, activation of kinase cascades, and changes in gene transcription occur subsequent to receptor ligation. ROS such as hydrogen peroxide (H₂O₂) are suggested to act as second messengers in cytokine responses [23, 24]. H₂O₂ activates mitogen-activated protein kinase (MAPK) cascades, partly through oxidation of catalytic cysteines on MAPK-inactivating phosphatases [25-27], and induces nuclear factor kappa-B (NF- κ B) translocation from the cytosol to the nucleus [28, 29]. Initiation of NF- κ B-dependent gene transcription is a key step in the production of proinflammatory mediators [30, 31].

Here, we review evidence linking ROS to the proinflammatory M1 microglial activation state and the NLRP3 inflammasome complex, a mediator of caspase-1-dependent interleukin-1 β (IL-1 β) secretion. We also discuss the extent to which mitochondrial dysfunction can be incriminated. Importantly, although much can be extrapolated from macrophages and other immune cells that share many features of microglial activation, precise roles for ROS and the NLRP3 inflammasome in the activation of primary microglia require further elucidation.

A role for NADPH oxidase in the proinflammatory state

Early work linking ROS to the M1 microglial activation state used a pharmacological approach (Fig. 1, i). Kang et al. demonstrated that the superoxide dismutase (SOD)/catalase mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) or the NADPH oxidase/flavoprotein inhibitor diphenylene iodonium (DPI) reduced NF- κ B activation and IL-1 β transcription in the mouse microglial cell line BV2 in response to treatment with an Alzheimer's disease-relevant amyloid-beta (A β) peptide [32]. Additional experiments using the small molecule inhibitor DPI in primary rodent microglia implicated superoxide and downstream species originating from NADPH oxidase in the LPS-mediated induction of proinflammatory cytokines [33, 34]. However DPI is not a specific inhibitor of NADPH oxidase and can influence mitochondrial superoxide production, among additional targets [35-38]. Studies using microglial cultures from phagocytic oxidase (Phox) $\pm$ mice lacking the gp91 catalytic subunit of NADPH oxidase conclusively established NADPH oxidase as the source of LPS-stimulated extracellular superoxide production [8]. Much but not all of the intracellular ROS elevation in response to LPS was also eliminated by gp91^{phox} knockout [8]. Interestingly, while TNF- α secretion was diminished by gp91^{phox} deficiency, the production of NO was unaltered. This finding contrasts with a dominant negative approach to NADPH oxidase inhibition. Stable mutant p47^{phox} subunit overexpression to prevent functional NADPH oxidase assembly impaired both inducible nitric oxide synthesis (iNOS) induction and production of NO in rat HAPI microglial cells treated with LPS [34]. The role of the p47^{phox} and gp91^{phox} subunits of NADPH oxidase in microglial activation was further investigated by Choi et al. *in vivo* following intracerebroventricular injection of LPS or A β ₁₋₄₂ peptide [39]. Inhibition of NADPH oxidase by genetic knockout of p47^{phox} or gp91^{phox} or by the drug apocynin promoted microglial polarization toward an M2 anti-inflammatory phenotype while reducing release of pro-inflammatory mediators [39]. Knockout of p47^{phox} decreased TNF- α but not IL-1 β measured after intracerebroventricular injection of LPS. This finding is in agreement with the *in vitro* studies of Qin et al. [8] and Pawate et al. [34] showing that TNF- α secretion is dependent on NADPH oxidase activity but in contrast to the results of Kang et al. showing that A β ₂₅₋₃₅-induced IL-1 β is also impaired when blocking NADPH oxidase using DPI [32]. Finally, Loane et al. demonstrated both *in vitro* and *in vivo* that metabotropic glutamate receptor 5 agonists attenuate microglial NO and TNF- α production by inhibiting the expression and activity of NADPH oxidase [40, 41]. Collectively, these studies implicate microglial NADPH oxidase activity in the transition to a proinflammatory phenotype. However, these studies also suggest that NO, TNF- α , and IL-1 β may not always be regulated in tandem. Consequently, it is important to

consider the markers used to define activation as well as the activating stimulus when evaluating microglial polarization.

Superoxide produced by NADPH oxidase is predominantly extracellular (Fig. 1, ii). *In vivo*, extracellular superoxide dismutase 3 (SOD3) dismutates superoxide to form membrane permeable H_2O_2 [42]. *In vitro*, superoxide conversion to H_2O_2 can be enzymatically accelerated by addition of extracellular superoxide dismutase (SOD) while extracellular H_2O_2 derived from superoxide can be experimentally eliminated by catalase addition. The enzyme catalase degrades H_2O_2 to oxygen and water [43]. Interestingly, exogenous catalase, when added in combination with LPS/IFN- γ , impaired microglial release of proinflammatory mediators while SOD did not [34]. Similarly, catalase but not SOD prevented stimulation of microglial proliferation in response to IL-1 β or TNF- α [44]. These results suggest that H_2O_2 , not superoxide, is the primary ROS responsible for mediating microglial activation and proliferation in response to proinflammatory stimuli. It is important to note that arginase has been identified as a contaminant in some commercial catalase preparations [45]. As arginase degrades L-arginine, a precursor for NO synthesis, catalase purity is an important variable to consider, particularly when using NO as a marker for activation.

In addition to H_2O_2 removal, the effect of H_2O_2 addition on microglial phenotypes has been tested. Eguchi et al. demonstrated that H_2O_2 increased the LPS-induced expression of iNOS, responsible for production of NO, yet found that H_2O_2 alone was insufficient to upregulate iNOS [46]. Another recent study demonstrated that addition of H_2O_2 to LPS-treated microglial cells enhanced NO production [47]. Continuous H_2O_2 generation by two different ROS generating systems was able to stimulate microglial proliferation [44]. These studies, and observations that NADPH oxidase inhibition impairs but does not entirely block the release of proinflammatory mediators such as TNF- α , suggest that while H_2O_2 is involved in neuroinflammation, it may act more so to intensify inflammatory processes, rather than as a direct inducer of such processes. In addition, it is possible that ROS downstream of H_2O_2 , e.g. the reactive hydroxyl radical formed from H_2O_2 in the presence of iron [48], also contribute to the proinflammatory phenotype.

Importantly, H_2O_2 derived from NADPH oxidase-generated superoxide does not only regulate microglial activation in a cell autonomous manner. Zhang et al. described upregulation of neuronal NADPH oxidase following traumatic brain injury [49]. Inhibition of NADPH oxidase using apocynin or a gp91ds-tat peptide that competitively inhibits the p47^{phox} binding site on gp91^{phox} was neuroprotective and attenuated microglial activation following traumatic injury to the brain [49].

Reactive oxygen species, nitric oxide, and reactive microgliosis

Microglia *in vitro* continue to produce proinflammatory factors following removal of activating agent [47] and microglia *in vivo* frequently remain activated over long time periods following injury or onset of disease [50, 51]. This continued activation is likely due to a phenomenon termed reactive microgliosis, a self-propelling cycle mediated by intracellular ROS and NO that serves to maintain the M1 phenotype [47]. Hence, ROS play

a vital role in both the initial activation of microglia and in their continued activation through reactive microgliosis. CD11b, the MAC1 receptor which binds LPS [22], A β peptide [52, 53], α -synuclein [54], and high mobility group box 1 (HMGB1) [55] among several ligands, is a beta-integrin microglial cell surface molecule which demonstrates increased expression during activation. LPS and immunogenic stimuli associated with neurodegenerative disorders upregulate microglial CD11b expression [56] and increased CD11b expression corresponds to the severity of microglial activation in a variety of neuroinflammatory diseases [57]. Like the ability of antioxidants to decrease production of proinflammatory mediators, Roy et al. found that the antioxidants N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) prevented upregulation of CD11b *in vitro* and *in vivo* [56]. H₂O₂ alone, or a superoxide generating system consisting of hypoxanthine and xanthine oxidase, was sufficient to elevate CD11b expression in a catalase-sensitive fashion, implicating H₂O₂ in the CD11b increase [56]. Intriguingly, scavenging NO with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was able to prevent CD11b upregulation, suggesting that H₂O₂ upregulates CD11b indirectly through NO [56, 58]. As CD11b/MAC1 was required for NADPH oxidase activation in response to LPS [22], α -synuclein [54], or HMGB1 released by inflamed microglia and/or degenerating neurons [55], CD11b upregulation by an H₂O₂ and NO-mediated pathway is likely a major contributor to reactive microgliosis, as seen in a small animal model of Parkinson's disease [59]. Importantly, although compelling data suggest that ROS and NO participate in a self-amplifying loop during microglial activation, not all studies support the involvement of NO in proinflammatory cytokine production. In contrast to a role for NO in CD11b induction, Yoshino et al. observed that TNF- α produced by LPS-stimulated microglia was suppressed by the antioxidant NAC, but not by scavenging NO with cPTIO [60]. In addition, 3-(4-morpholinyl)sydnominine (SIN-1), a donor of superoxide and NO [61], induced microglial TNF- α production in an SOD-sensitive fashion, indicating that NO was incapable of stimulating TNF- α secretion in the absence of superoxide.

Although it is often convenient to think about microglial polarization in terms of linear pathways, microglial activation does not consist of a singular cell signaling cascade, and instead comprises a multitude of pathways that converge in the phenotype seen during excessive microglial activation. The activation phenotype undoubtedly depends on the proinflammatory trigger as well as the extracellular environment. As an interesting example, a transcriptional analysis recently found that microglia isolated from an ALS mouse model differed from LPS-activated microglia as well as from M1- or M2-polarized macrophages, including in the level of proinflammatory TNF- α and IL-1 β expression [62]. When investigating the role of ROS, NO, and other factors in microglial activation, it is also important to consider the experimental conditions used to measure activation. Serum promotes binding of LPS to TLR4 due to the presence of lipopolysaccharide binding protein (LBP) [63, 64]. *In vitro* studies variably use serum-containing or serum-free conditions to examine LPS-induced activation of microglia, potentially altering the ratio of TLR4-dependent to TLR4-independent signaling mechanisms and the activation phenotype.

MAPK and NF- κ B as hydrogen peroxide-sensitive regulators of microglial activation

The precise mechanisms by which ROS, and H₂O₂ in particular, promote microglial proliferation and the M1 transition remain incompletely characterized. Much of the work on the proinflammatory role of ROS, both in non-microglial and microglial cell types, has focused on the MAPK signaling pathway and the downstream transcription factor NF- κ B (Fig. 1, iii). Although most reports do not examine exactly how ROS increase MAPK phosphorylation during the activation process, oxidation of catalytic cysteines on MAPK-inactivating phosphatases may be involved [25-27]. A study in rat epithelial cells demonstrated that LPS treatment induces phosphorylation of the MAPKp38 subunit which was required for LPS-stimulated TNF- α production [29]. The antioxidant NAC was able to ablate p38 phosphorylation and TNF- α release while exposure to a superoxide-generating system or H₂O₂ was able to induce MAPK p38 phosphorylation [29]. In peripheral blood mononuclear cells, inhibition of superoxide production with DPI impaired translocation of the p50 subunit of NF- κ B from the cytosol to the nucleus and subsequent production of cytokines IL-6 and IL-8 [65].

The importance of multiple MAPKs and NF- κ B to the activation process specifically in microglial cells, and their regulation by ROS, has also been demonstrated through multiple approaches. Inhibition of p38, extracellular signal-related kinase (ERK)1/2, or c-Jun N-terminal kinase (JNK) in primary rat microglia impaired LPS- or A β ₁₋₄₂ stimulated production of IL-1 β [66]. Similarly, treatment with the flavonoid antioxidant isoorientin or with NAC decreased LPS-induced MAPK activation, nuclear NF- κ B translocation, and the production of IL-1 β , TNF- α , and NO in BV2 microglial cells [67]. Multiple groups used the p47^{phox} dominant negative approach discussed earlier to demonstrate a specific requirement for NADPH oxidase activity in p38 and ERK phosphorylation during proinflammatory microglial activation [34, 68]. Another study with BV2 cells demonstrated that fructose-1,6-bisphosphate, a glycolytic intermediate that preserves cellular antioxidant capacity through stimulation of the pentose phosphate pathway [69], decreased LPS-induced iNOS expression to a similar extent as NAC by inhibiting JNK and p38 MAPK phosphorylation [70]. The antioxidant protein peroxiredoxin I was recently identified as an endogenous negative regulator of NF- κ B-mediated microglial activation [71], providing further support for the redox-sensitivity of NF- κ B. Strikingly, constitutive activation of NF- κ B in microglia *in vivo* achieved by genetic means was sufficient to induce microgliosis and neuronal death [13], confirming the central role of this transcription factor in classical M1 microglial activation.

Although most of the attention has focused on NF- κ B as a redox-sensitive transcriptional regulator of microglial activation, other transcription factors are undoubtedly involved. The transcription factor p53 is a master regulator of cell-cycle control and apoptosis [72]. It responds to a variety of stressors, including oxidative damage. Inhibiting p53 in microglia using the small molecule pifithrin- α decreased LPS- or A β ₂₅₋₃₅-induced iNOS expression and ameliorated microglia-mediated neurotoxicity [73]. The involvement of p53 in microglial activation was also recently confirmed by genetic knockout. p53^{-/-} microglia showed a decrease in production of proinflammatory mediators in response to IFN- γ , and

displayed increased expression of genes associated with alternative activation [74]. Although it was hypothesized that p53 activation during microglial activation is induced by ROS [74], the necessity and/or sufficiency of ROS in p53 activation leading to microglial activation has yet to be established. Studies testing the effects of anti- and pro-oxidants on p53 in microglia with and without activating stimuli should prove enlightening. Finally, evidence suggests that Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional activator of genes regulated by antioxidant response elements (ARE), is a negative regulator of microglial activation in response to LPS [75] or the Parkinson's disease-inducing toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [76]. Drugs which activate the Nrf2 pathway such as sulforaphane [77] may mediate neuroprotection in part by preventing excessive M1 microglial polarization [75, 76, 78]. Figure 2 illustrates some of the main connections between H₂O₂ and proinflammatory microglial activation.

Rotenone and microglial activation – is mitochondrial superoxide involved?

To this point we have focused on studies pointing to a role for NADPH oxidase in microglial activation. However, mitochondria are a possible source of the component of LPS-induced intracellular ROS that was not eliminated by gp91^{phox} knockout [8]. Superoxide anion is the primary ROS produced by mitochondria, mostly in the matrix, but it does not readily cross biological membranes [79]. MnSOD (SOD2) efficiently converts mitochondrial matrix superoxide into H₂O₂ which diffuses into the cytoplasm. Although the preponderance of evidence supports the involvement of NADPH oxidase-derived H₂O₂ in microglial activation, the possibility that mitochondria-derived H₂O₂ contributes to the M1 transition under some scenarios has also been considered. For example, the direct microglial activation by the mitochondrial toxin rotenone observed by some was attributed to mitochondria-derived ROS [80, 81].

Rotenone inhibits complex I of the electron transport chain and stimulates mitochondrial superoxide production during the oxidation of complex I substrates [79]. Systemic rotenone administration recapitulates features of Parkinson's disease in rats, including highly selective nigrostriatal dopaminergic degeneration [82]. Early studies by Gao et al. using primary brain cell cultures suggested that microglia may contribute to rotenone-induced dopaminergic neurodegeneration [83, 84]. Low dose rotenone treatment (0.5-20 nM) did not cause significant neurotoxicity in neuron-enriched cultures, yet induced robust neurotoxicity in neuron/glia mixed cultures [83]. Although mitochondrial superoxide production is frequently cited as the link between rotenone and neurotoxicity [85, 86], inhibition of rotenone neurotoxicity by apocynin in mixed cultures suggested a surprising involvement of microglial NADPH oxidase [83, 84]. Low dose rotenone treatment (5-10 nM) elicited an increase in superoxide production by wild type microglia, but not by cells derived from gp91^{phox}-deficient mice [84]. Furthermore, apocynin had no effect on superoxide levels or low dose rotenone neurotoxicity in Phox^{-/-} mixed cultures, confirming the participation of microglial NADPH oxidase in rotenone neurotoxicity [84]. Even more surprisingly, radiolabeled ligand binding assay revealed an affinity of rotenone for the catalytic gp91^{phox} subunit of NADPH oxidase. Rotenone-gp91^{phox} binding, which was inhibitable by DPI,

induced membrane translocation of the p67^{phox} subunit and led to NADPH oxidase-induced superoxide production [87].

Recent studies using BV2 mouse microglial cells found that rotenone treatment alone was sufficient to induce a proinflammatory activation phenotype as assessed by induction of iNOS, TNF- α , and IL-1 β [80, 81]. Activation was mediated by ROS, p38 MAPK, and the NF- κ B pathway [81]. Nevertheless, activation of microglia by rotenone has not always been recapitulated in primary microglial cultures ([83, 88, 89], but see [90] and [91]). The concentrations of rotenone employed in these studies varied over a 10,000-fold range (0.1 nM - 1 μ M). Studies implicating rotenone in cytokine production by macrophages have ranged even higher (e.g. 5-40 μ M [92, 93]). Toxicity due to rotenone has sometimes but not always been measured. This is important, as compromised cells release damage-associated molecular patterns which can promote microglial activation. As noted earlier, HMGB1 is one factor that can be released from damaged cells which propagates an activation phenotype [55]. Since ROS generally appear to augment rather than initiate activation, full microglial proinflammatory cytokine production in response to rotenone may require NADPH oxidase- and/or mitochondria-derived ROS plus a second “hit” that is cell culture-condition or model-dependent. A rigorous establishment of dose-response relationships among rotenone-induced inhibition of mitochondrial oxygen consumption, superoxide production, toxicity, and activation markers is required and should help explain discrepant results. Nevertheless, reliance on mitochondrial inhibitors is an inherently flawed approach for determining the role of mitochondrial superoxide in microglial activation as it will likely prove impossible to completely separate out effects on mitochondrial superoxide generation from other effects on mitochondrial function. It is also generally not appreciated that rotenone, in contrast to the complex I inhibitor piericidin A [94], impairs microtubule assembly in the low nanomolar range [94-97]. A comparison of rotenone, piericidin A, and other, structurally distinct, complex I inhibitors should help untangle true contributions of mitochondrial superoxide/respiratory inhibition from effects on NADPH oxidase, microtubule polymerization, or other targets.

Importantly, in addition to ROS, altered mitochondrial and glycolytic energy metabolism were linked to BV2 microglial activation [98-100]. BV2 microglial cells significantly decreased mitochondrial oxygen consumption [98, 100] and increased glycolysis [98] upon stimulation with LPS or LPS plus IFN- γ . Consistent with other studies linking ROS to proinflammatory cytokine production, a combination of the antioxidants Trolox and edaravone or overexpression of the ROS-lowering protein Grp75/mtHsp70/mortalin was able to suppress NF- κ B activation and release of TNF- α and IL-6 [98]. Inhibition of the mitochondrial ATP synthase by the drug oligomycin overcame the ability of antioxidants to attenuate secretion of these proinflammatory cytokines. Elevated glycolytic lactate production in the presence of oligomycin was suggested to be the crucial factor supplanting the effect of antioxidants on activation; sodium lactate (5 mM) mimicked the effect of oligomycin [98]. This study underscores the importance of considering effects on metabolism in addition to ROS when using mitochondrial inhibitors to modulate the activation phenotype.

A proinflammatory amplification loop initiated by mitochondrial superoxide? Lessons from macrophages

Not long ago, Kasahara et al. took an interesting alternative approach to address whether there is a mitochondrial contribution to the activation phenotype in macrophages, the peripheral “cousins” of microglia. Rho zero (ρ^0) cells lacking mitochondria DNA were created from the RAW 264.7 macrophage cell line. The cells exhibited defective oxygen consumption, diminished ROS production in response to LPS, and attenuated TNF- α and IL-6 secretion [101]. An H_2O_2 -generating system consisting of glucose plus glucose oxidase partially rescued LPS-stimulated TNF- α secretion in ρ^0 cells while having no effect on their mitochondria-competent counterparts. In addition, NAC but not apocynin suppressed mitochondrial ROS levels and TNF- α secretion in LPS-treated RAW 264.7 cells, suggesting that at least in this particular cell line under the tested experimental conditions, mitochondria-derived superoxide—instead of or in addition to NADPH oxidase-derived superoxide—was required for proinflammatory cytokine production. While taking a similar approach, Nakahira et al. found that a different ρ^0 macrophage cell line, J774A.1, exhibited normal TNF- α secretion in response to LPS plus ATP (rather than LPS alone), but impaired IL-1 β release [93], discussed further below). Although compelling evidence as outlined above implicates ROS originating from NADPH oxidase in microglial activation, it would be interesting to test whether mitochondria-defective ρ^0 microglia exhibit impaired activation, suggesting a possible secondary contribution of mitochondria to the activation process.

Studies in macrophages have also indirectly implicated mitochondrial ROS in proinflammatory activation via knockout of the putative uncoupling protein UCP2. Data from multiple groups suggested that UCP2 $-/-$ macrophages produce more mitochondrial ROS compared to wild type [102-105]. UCP2 $-/-$ macrophages were more sensitive to induction of proinflammatory factors, with evidence for ROS-mediated protein tyrosine phosphatase inhibition [105], MAPK phosphorylation [104, 105], iNOS induction [103], and conflicting evidence on NF- κ B involvement [103, 104]. In contrast to UCP2 knockout, UCP2 overexpression lowered intracellular ROS and attenuated induction of iNOS in response to LPS [106]. Rapid downregulation of UCP2 was observed in wild type macrophages stimulated with LPS; it was suggested that this decrease was necessary for a proinflammatory amplification loop mediated by mitochondrial ROS and resulting in accelerated p38 and ERK activation [104]. Consistent with this possibility, mitochondrial superoxide, as assessed by decreased activity of the superoxide-sensitive matrix enzyme aconitase, was basally elevated in the absence of UCP2, and UCP2 $-/-$ macrophages failed to respond to LPS with a further increase in mitochondrial ROS [104].

There is limited work on the involvement of UCP2 in microglial activation (as opposed to macrophage activation), however one study found an increase rather than a decrease in UCP2 mRNA in microglia following mouse ischemic brain injury [107]. Evidence from UCP2 knockout mice suggested that UCP2 may regulate mitochondrial uptake of the antioxidant glutathione rather than superoxide production directly [107]. While the role of UCP2 in mitochondrial ROS production is still debated, increasing evidence suggests that

UCP2 does not even function as a proton transporter, instead likely acting as a C4 metabolite transporter [108-110]. Of note, de Bilbao et al. observed a steady-state upregulation of MnSOD in UCP2 mouse brain [107], indicating that secondary, potentially complicating changes to the proteome should be considered when using these constitutive knockout mice to assign protein function.

Mitochondrial superoxide and the NLRP3 inflammasome – a murky, yet intriguing story

NADPH oxidase-independent reactive oxygen species and NLRP3

Our discussion to this point has centered mostly on TNF- α , however IL-1 β is also one of the major proinflammatory cytokines in the brain. Release of IL-1 β , in contrast to TNF- α , is regulated enzymatically through caspase-1 activity. The NOD-like receptor family, pyrin domain-containing 3 (NLRP3, also called NALP3) inflammasome is a protein scaffolding complex that induces the secretion of the cytokines IL-1 β and IL-18 from their pro-forms by activating caspase-1 (Fig. 1, iv) [111]. Present in most immune cells including microglia, the NLRP3 inflammasome is stimulated by diverse damage-associated molecular patterns and pathogen-associated molecular patterns, such as extracellular ATP, bacterial toxins, and particulate matter [111, 112]. In microglia, fibrillar or oligomeric A β peptide [113, 114], fibrillar α -synuclein [115], and a neurotoxic prion peptide [116] were all shown to induce NLRP3 inflammasome-dependent IL-1 β secretion. Although regulation of IL-1 β release by the inflammasome appears to be similar in macrophages and microglia, microglia, in contrast to macrophages, may regulate IL-18 release at least partly by an NLRP3-independent pathway [112].

ROS have been heavily implicated as an upstream event in the activation of the NLRP3 inflammasome [117]. For example, reduction of IL-1 β production was observed following treatment with antioxidants such as NAC or DPI, while in some cases H₂O₂ alone was sufficient to induce NLRP3 activation [118-120]. Although NADPH oxidase-derived ROS were initially suggested to be important for NLRP3 inflammasome activation [121], mouse macrophages deficient in NADPH oxidase subunits gp91^{phox} or gp22^{phox} remained activation-competent [122, 123]. In addition, gp91^{phox}-defective, gp47^{phox}-defective, and gp22^{phox}-defective macrophages from human patients with chronic granulomatous disease all exhibited active inflammasomes in response to multiple triggers [124].

In 2011, a series of three reports implicated ROS specifically derived from mitochondria in the activation of the NLRP3 inflammasome [92, 93, 123]. Additional reports, e.g. [111, 125-127], have surfaced since. Other reports have failed to replicate the necessity of ROS in NLRP3 inflammasome activation [128] or suggested that ROS are necessary only for an LPS “priming” step involving induction of NLRP3 and pro-IL-1 β protein expression, but not for actual NLRP3-dependent caspase-1 activation [129]. In contrast, a rare study performed in microglial cells rather than peripheral immune cells found that the antioxidant NAC had no effect on LPS priming while inhibiting caspase-1 dependent IL-1 β secretion in response to A β ₁₋₄₂ peptide oligomers [114].

MitoSOX and MitoTEMPO—not always mitochondria-specific

Three techniques have been used often but not exclusively to implicate ROS derived from mitochondria in NLRP3 inflammasome activation: a temporal correlation between activation and increased mitochondrial ROS measured using the mitochondrially-targeted fluorescent indicator MitoSOX, inhibition of NLRP3-dependent IL-1 β secretion by a mitochondrially-targeted antioxidant, e.g. MitoTEMPO or MitoQ, and inflammasome activation or amplification by a superoxide-producing mitochondrial toxin such as rotenone (Fig. 1, v). While there is clearly an increase in intracellular ROS associated with NLRP3 activation, there are several complicating factors in the implementation of these techniques that for the most part have not been appreciated. In primary macrophages or macrophage cell lines, rotenone was reported to stimulate IL-1 β release [92], augment LPS plus ATP-induced IL-1 β release [93], or inhibit LPS plus ATP-induced IL-1 β release [130]. Difficulties associated with using mitochondrial toxins such as rotenone were discussed above and will not be elaborated further here other than to note that the metabolic consequences of respiratory inhibition in addition to increased superoxide generation merit careful consideration. Regarding MitoSOX and mitochondrially-targeted antioxidants such as MitoTEMPO, there are serious concerns that limit their utility for assigning a mitochondrial origin to ROS during the activation process.

Conjugation to the lipophilic triphenylphosphonium cation (TPP⁺) is a strategy for targeting drugs and fluorescent probes to the negatively charged matrix of polarized mitochondria [131-133]. Unfortunately, once reagents are labeled as “mitochondria-specific,” there is a tendency to overlook the fact that the degree of mitochondrial targeting depends on the potential across the mitochondrial inner membrane (Fig. 3). MitoSOX (also called “mito-dihydroethidium” or “mito-hydroethidine”) accumulates in the mitochondrial matrix due to a TPP⁺ moiety conjugated to ROS-sensitive dihydroethidium [132]. When oxidized by superoxide, MitoSOX forms mito-2-hydroxyethidium that fluoresces red upon interaction with nucleic acids—predominantly DNA in the mitochondria matrix [132, 134-136]. MitoSOX oxidation by ROS other than superoxide yields additional oxidation products that also contribute to the red fluorescence detected in cells [137].

Mitochondrial electron transport chain inhibitors such as rotenone and antimycin A depolarize mitochondria and cause MitoSOX redistribution to the cytoplasm and nucleus [136]. ATP synthase reversal maintains a partial negative membrane potential by pumping protons out of the matrix at the expense of ATP—for as long as ATP levels remain sufficient—however a correction for MitoSOX redistribution in response to electron transport chain inhibition is still required [136]. Mitochondrial respiratory inhibition occurs early in the NLRP3 inflammasome activation process in response to multiple triggers, inevitably causing mitochondrial depolarization [93, 111, 128, 138]. This depolarization will lead to partial to near complete redistribution of MitoSOX out of the matrix depending on the extent to which the negative potential is lost; thus ROS detected by total cellular MitoSOX fluorescence do not necessarily originate in mitochondria. Indeed, Voloboueva and colleagues noted that MitoSOX imaging needed to be performed within a short period of time in activated BV2 microglial cells due to a time-dependent migration of the fluorescent signal to the nucleus [98]. This caveat is of particular concern because MitoSOX

fluorescence in immune cells is frequently quantified by flow cytometry [134], a method that does not allow for assessment of intracellular dye distribution. We suggest that fluorescent imaging for dye distribution, positive and negative controls, and confirmation of effects using secondary methods be implemented in future studies using this problematic dye.

Like MitoSOX, MitoTEMPO depends on a TPP⁺ conjugate to deliver the antioxidant to the matrix of polarized mitochondria [139]. During the initial development and characterization of MitoTEMPO as a mitochondria-specific ROS scavenger, addition of only 25 nM MitoTEMPO increased mitochondrial matrix superoxide dismutation three-fold without impacting the cytoplasmic dismutation of superoxide [140]. Although early studies employing MitoTEMPO used this scavenger in the 1 nM to 5 μ M range, concentrations as high as 500 μ M have been employed in immune cells, usually with limited dose-response analysis, no controls employing untargeted TEMPO or TPP⁺ alone, and no assessment of the impact of MitoTEMPO on nonmitochondrial sources of ROS. For example, two of the main studies implicating mitochondrial ROS in NLRP3 inflammasome activation found that 500 μ M MitoTEMPO impaired NLRP3 inflammasome-dependent IL-1 β secretion while 100 μ M MitoTEMPO was relatively ineffective [93, 111]. Even provided mitochondria are still polarized during the activation process and initially accumulate mitochondrially-targeted antioxidant, these high concentrations of MitoTEMPO will undoubtedly disrupt mitochondrial function since as little as 1 μ M of the closely related antioxidants Mito-TEMPOL or MitoQ caused respiratory inhibition in cells [140]. Consequently, the ability of 500 μ M MitoTEMPO to inhibit IL-1 β secretion likely has nothing to do with modulation of mitochondria-derived ROS. Additional studies on how MitoTEMPO influences specific reactive oxygen species within cells (e.g. superoxide, H₂O₂, etc.) and characterization of non-ROS targets are clearly needed. Unfortunately, non-pharmacological approaches, such as the overexpression of SOD or catalase enzymes targeted to various subcellular compartments, have been under-utilized when assessing the contribution of mitochondria-derived superoxide and H₂O₂ to inflammasome activation. However, the commercially available MCAT mouse (Strain name: B6.Cg-Tg(CAG-OTC/CAT)4033Prab/J), which transgenically overexpresses catalase in the mitochondrial matrix, was recently used to show that elevating mitochondrial H₂O₂ degrading activity did not in fact attenuate NLRP3 inflammasome activation in bone marrow-derived macrophages in response to the most commonly used activating trigger, LPS plus extracellular ATP, although it did in response to *Salmonella* mutants [141]. Therefore, it appears that mitochondria-derived H₂O₂ downstream of matrix superoxide is not a general requirement for NLRP3 inflammasome activation, but may contribute to activation in specific contexts.

NLRP3 and brain injury

Caveats aside, the possibility that mitochondria contribute to NLRP3 activation in response to at least some triggers remains intriguing and the NLRP3 inflammasome is emerging as a new target for neuroprotection. Intracerebral hemorrhage or injection of rotenone into the mouse brain basal ganglia induced NLRP3 expression which was mitigated by MitoTEMPO injection *in vivo* [142]. This finding is consistent with but does not prove the proposed involvement of ROS in NLRP3 inflammasome priming [129]. NLRP3 knockdown by

siRNA reduced brain edema and improved neurological functions at 24-72 hours after intracerebral hemorrhage [142], suggesting a deleterious role for the NLRP3 inflammasome following acute brain injury. Interestingly, glibenclamide (also known as glyburide), a diabetes drug proposed as a novel treatment for acute brain injury due to its ability to inhibit the Sur1-Trpm4 channel involved in brain edema [143], was also shown to inhibit the NLRP3 inflammasome [144]. Similar to NLRP3 knockdown, glibenclamide reduced neuroinflammation and cognitive impairment following subarachnoid brain hemorrhage [145].

In addition to acute brain injury, the NLRP3 inflammasome is implicated in the pathogenesis of Alzheimer's disease [146]. An increase in the cleaved form of caspase-1 was detected in post-mortem hippocampal and frontal cortex brain samples of patients with Alzheimer's disease relative to controls [147], implying increased inflammasome activity. Strikingly, knockout of either NLRP3 or caspase-1 ameliorated memory loss and β -amyloid plaque burden in an Alzheimer's disease transgenic mouse model [147]. Microglia in these mice were skewed toward an M2-like phenotype, displaying increased M2 markers such as arginase-1 and IL-4 concomitant to a reduction in the M1 marker iNOS. Surprisingly, production of the proinflammatory mediators TNF- α and NO in response to fibrillar A β peptide in microglia or macrophages depended on NLRP3 inflammasome-dependent IL-1 β production, as shown using knockout cells for NLRP3, caspase-1, or the IL-1 receptor [113]. This TNF- α /NO dependence on the NLRP3 inflammasome in response to fibrillar A β is in contrast to studies performed in macrophages which indicate that the NLRP3 inflammasome is not required for TNF- α /NO secretion in response to most activators, e.g. a high concentration of LPS [113] or LPS plus ATP [93]. Data showing normal TNF- α but blunted IL-1 β response following LPS plus ATP treatment of ρ^0 macrophage cells demonstrated that IL-1 β but not TNF- α was influenced by the presence or absence of respiration-competent mitochondria [93]. Pathways controlling NLRP3 inflammasome activation in response to various triggers, including the necessity of mitochondrial superoxide generation and interaction with other proinflammatory changes such as NF- κ B-dependent transcription, plainly require further elucidation. Parajuli et al. recently found that even fibrillar and oligomeric A β peptides activated the NLRP3 inflammasome through different pathways, additionally showing a small but significant contribution of NADPH oxidase activity to oligomeric A β -induced IL-1 β secretion by demonstrating suppression with a cell permeable gp91ds-tat peptide inhibitor of NADPH oxidase [114].

NLRP3 activation—is a mitochondrial signal required?

Even if one assumes that mitochondria-derived ROS participate in NLRP3 inflammasome activation, how also remains a contentious question. In macrophages, NLRP3 expression during the priming step depended on NF- κ B [148]. Thus one possibility is that mitochondria-derived H₂O₂ participates in NLRP3 priming by amplifying MAPK signaling as discussed above (Fig. 1, iii). However, many studies suggest that ROS are necessary not only for inflammasome priming but also for activation. An early proposal was that ROS induce thioredoxin-interacting protein (TXNIP) dissociation from thioredoxin, allowing it to bind and activate NLRP3 [119]. However, a subsequent study failed to reproduce this finding [149]. A series of more recent studies raised the possibility that release of a mitochondrial

signal is required to trigger activation of pro-caspase-1 by the NLRP3 inflammasome [93, 130, 138, 150], similar to apoptosome-mediated pro-caspase-9 activation that is triggered by the release of mitochondrial cytochrome *c* during apoptosis [151].

Nakahira et al. provided initial evidence that the release of mitochondrial DNA from the matrix to the cytoplasm subsequent to mitochondrial compromise is the NLRP3 trigger [93]. They and others showed that cyclosporin A, inhibitor of a large conductance mitochondrial inner membrane channel called the permeability transition pore (PTP), suppressed the release of IL-1 β by activated macrophages [93, 130, 138]. The PTP is a ROS-sensitive channel that opens in response to the oxidation of thiols on pore components [152], including cysteine 203 of the cyclosporin A target cyclophilin D [153]. Therefore, in this model ROS would be acting in the mitochondrial matrix rather than the cytoplasm to initiate PTP-dependent release of mitochondrial DNA as an activating ligand of NLRP3. However, a just published study strongly challenged this model by demonstrating that knockout of cyclophilin D had no effect upon NLRP3 inflammasome activity while cyclosporin A impaired inflammasome activation equally well in wild type and cyclophilin D knockout macrophages [154]. Although it remains possible that mitochondrial DNA is released by mitochondrial damage independent of the classical cyclophilin D-dependent permeability transition pore, Allam et al. also found normal NLRP3 inflammasome activation in cells deficient in pro-apoptotic Bax and Bak or in cells overexpressing Bcl-2. These findings indicate that such mitochondrial membrane disruption would have to also occur independently of central mechanisms regulating mitochondrial permeability during apoptosis. Nevertheless, in an interesting twist, opening of the mitochondrial permeability transition pore was impaired in NLRP3 knockout macrophages [93], suggesting an upstream role for NLRP3 in mitochondrial damage, perhaps independent of the inflammasome or as part of a feedback loop. In a variation of the mitochondrial DNA efflux model, Shimada suggested that rather than simply cytoplasmic mitochondrial DNA, oxidized mitochondrial DNA was the specific trigger of inflammasome activation [138]. Thus, ROS were required both for the oxidation of mitochondrial DNA and for triggering a mitochondrial pore-dependent efflux to the cytoplasm. Subsequently, Iyer and colleagues discovered an NLRP3 inflammasome trigger, linezolid, that caused the release of IL-1 β from LPS-primed macrophages in the absence of a detectable increase in mitochondrial ROS [130]. Cyclosporin A but not MitoTEMPO impaired IL-1 β release. The mitochondria-specific inner membrane phospholipid cardiolipin was identified as a direct NLRP3 activating ligand, apparently without a requirement for mitochondrial ROS or mitochondrial DNA release [130]. Cardiolipin, along with microtubules, appeared to also be acquired for the recruitment of NLRP3 to mitochondria, where NLRP3 bound the adaptor protein Apoptosis-associated speck-like protein containing CARD (ASC) to form functional inflammasome complexes [130, 150].

Reactive oxygen species and NLRP3 activation—challenges and final thoughts

Overall, the evidence that mitochondria are involved in NLRP3 inflammasome activation in some way is intriguing. Nevertheless, a unifying model is needed to assimilate the interesting but not always consistent evidence implicating their participation. Challenging all of the work implicating mitochondria-derived ROS in inflammasome activation, Muñoz-

Planillo et al. found that pore-forming gramicidin, which collapses the plasma membrane sodium and potassium gradients, initiated NLRP3-dependent IL-1 β release from macrophages prior to any mitochondrial dysfunction and without ROS production [128]. They failed to reproduce NLRP3 inflammasome activation by H₂O₂, rotenone, or the mitochondrial complex III inhibitor antimycin A [128]. Furthermore, several antioxidants including NAC did not inhibit LPS priming or gramicidin-triggered NLRP3 activation in their hands. In addition, Jabaut et al. found a poor correlation between the effect of mitochondria-targeted treatments on NLRP3 inflammasome activation and their effect on ROS production [127].

All tested NLRP3 inflammasome activators cause cytoplasmic potassium efflux [128, 155]. Muñoz-Planillo et al. demonstrated that the removal of extracellular potassium alone was sufficient to cause activation of the NLRP3 inflammasome by causing a drop in cytoplasmic potassium below a critical threshold, in the apparent absence of mitochondrial ROS or a mitochondria-derived activating ligand [128].

In striking contrast to the stimulatory role proposed for mitochondrial matrix superoxide, evidence suggests that cytoplasmic superoxide is a negative regulator of NLRP3-dependent IL-1 β secretion [156]. Macrophages deficient in the cytoplasmic Cu/Zn SOD enzyme (SOD1) exhibited impaired caspase-1 activation mediated by reversible oxidation and glutathionylation of cysteine residues on caspase-1 [156]. Thus, the spatial and temporal distribution of ROS may profoundly affect how they influence priming and/or activation of the NLRP3 inflammasome. Ever improving techniques to measure site-specific ROS production within individual intracellular compartments should help to unravel the many complexities of how these species regulate NLRP3 inflammasome activation.

Microglia vs. Macrophages

The vast majority of studies on NLRP3 inflammasome activation were performed in macrophages or other peripheral immune cells, particularly with regard to mitochondrial involvement. It is accepted by most that microglia are simply the resident macrophages in the brain and indeed the two phagocytic cell types are quite similar. For example, antigens that are commonly used to identify microglia, such as CD11b or CD68, are also present in macrophages [157]. However, although both cell types are of myeloid origin, differences in resting environment (brain vs. periphery) have led to some genetic and phenotypic differences between microglia and macrophages. Utilizing direct RNA sequencing of microglia and macrophages isolated from the same mouse, Hickman et al. found substantial differences in the expression of transcripts between the two cells types, with microglia expressing higher levels of several genes associated with the sensing of endogenous activating ligands [158]. The interplay between NO and ROS was discussed above, and may account for important differences in the reaction of macrophages or microglia to proinflammatory stimuli. Intriguingly, a study by Toku et al. found that IFN- γ stimulated NO production in macrophages, but failed to elicit an NO response in microglia [159]. Chao et al. demonstrated that the flavonoid naringenin inhibited iNOS and cyclooxygenase-2 expression more effectively in macrophages than in microglia [160]. While macrophages share many characteristics with microglia, further study into the role of ROS, particularly

mitochondrial superoxide, in the activation of microglia and its NLRP3 inflammasome need to be completed.

M1 vs. M2 activation

The majority of this review has focused upon proinflammatory microglial activation. This mechanism of activation is referred to as a 'classical' phenotype, or M1 activation [161]. However, microglia can also exhibit an 'alternative' M2 activation [19], which serves to reduce production of proinflammatory mediators and promote brain repair. While M1 polarization may be beneficial immediately following brain injury by phagocytic clearing of debris, M2 polarization may help to resolve a neuroinflammatory response by "putting the breaks" on proinflammatory cytokine production before levels become harmful. IL-4 is often employed to initiate M2 polarization in cultured microglia and induces prototypic M2 markers such as arginase expression [89]. Although possible roles for mitochondrial dysfunction and ROS production in M2 activation have not yet been widely investigated, one study found that low, non-toxic doses of the mitochondrial electron transport chain inhibitors rotenone and 3-nitropropionic acid (3-NP) impaired IL-4-induced M2 microglial activation *in vitro*, as assessed by arginase activity. IL-4 treatment also decreased LPS-induced production of proinflammatory factors, consistent with a role for anti-inflammatory IL-4 in checking the M1 response. Interestingly, addition of rotenone or 3-NP, to inhibit complex I or II of the mitochondrial electron transport chain, respectively, prevented IL-4 from decreasing LPS-induced proinflammatory cytokines [89]. As inhibition of the electron transport chain is likely to produce superoxide, it is possible that superoxide or downstream ROS plays an inhibitory role in alternative (M2) microglial activation; i.e. ROS may in part promote M1 activation by downregulating the M2 "breaks" on the M1 response. Microglia within p47^{phox} deficient mice defective in NADPH-mediated superoxide production acquired an M2 rather than an M1 phenotype in response to LPS, consistent with an inhibitory role for ROS in M2 polarization [39]. Further studies on how mitochondrial dysfunction, ROS production, and NADPH oxidase interact following anti-inflammatory microglial stimulation are clearly needed before any conclusions on the precise role(s) of ROS production in M2 activation can be drawn.

Concluding Remarks

Although it is generally accepted that reactive oxygen species, and in particular H₂O₂, play a role in M1 microglial activation, there is still disagreement as to the extent. A number of studies indicate that pro-oxidants cause microglial activation, while even more demonstrate that antioxidants impair activation. However, concerns exist with the methodology used to quantify and modulate ROS, particularly superoxide of mitochondrial origin. It is noteworthy that flawed approaches such as the use of non-specific inhibitors have been overwhelmingly used to implicate mitochondrial ROS in microglial or macrophage activation whereas the involvement of NADPH-derived superoxide and H₂O₂ is supported by *in vitro* and *in vivo* gene knockout and dominant negative experiments. The recent demonstration that inhibition of NLRP3 inflammasome activation by cyclosporin A is not due to inhibition of cyclophilin D-dependent mitochondrial permeability transition pore opening refutes a major line of evidence linking mitochondrial superoxide-induced pore

formation to IL-1 β production [154]. Nevertheless, as NADPH oxidase knockout cells still produce proinflammatory factors such as IL-1 β , it is important that additional studies are performed on the sources and species of ROS implicated in microglial activation, particularly with regard to the NLRP3 inflammasome. Modification of mitochondrial ROS in microglia by ectopic alteration of MnSOD and catalase expression should help in this regard.

Inflammasome-independent functions for NLRP3 is also an emerging area of investigation that may impact brain disorders such as Alzheimer's disease and intracerebral hemorrhage where aberrant NLRP3 expression is implicated. In addition to a possible upstream role for NLRP3 in mitochondrial permeability transition pore opening [93], new evidence suggests that NLRP3 can increase ROS production independent of inflammasome activation [162]. As the permeability transition pore is a ROS-sensitive channel [152], NLRP3 may indirectly activate pore opening by elevating mitochondrial matrix superoxide. Establishment of the subcellular localization of NLRP3 and its proximity to the electron transport chain will be crucial to understanding if and how it regulates mitochondrial and/or cellular ROS production. It is also essential that more microglia-specific studies are performed, particularly with regard to NLRP3 inflammasome activation, as the biochemical events associated with changes in ROS and M1 polarization may not always be identical in macrophages and microglial cells.

Finally, we would be remiss if we did not mention that NADPH oxidase enzymes and mitochondria are not the exclusive producers of ROS in activated microglia. Cyclooxygenase enzymes and nitric oxide synthase [163] are but two of several potential additional culprits.

Acknowledgments

The authors acknowledge funding from NIH R01 NS085165 to B.M.P. In addition, the authors thank Dr. David Loane for helpful feedback during the preparation of the review.

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Abbreviations

Aβ	amyloid-beta
ARE	antioxidant response element
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DPI	diphenylene iodonium

ERK	extracellular signal-related kinase
H₂O₂	hydrogen peroxide
HMGB1	high mobility group box 1
IFN-γ	interferon- γ
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAC1	macrophage antigen complex 1
MAPK	mitogen-activated protein kinase
$\delta\psi$	mitochondrial membrane potential
NAC	N-acetylcysteine
NF-κB	nuclear factor kappa-B
NLRP3	NOD-like receptor family pyrin domain-containing 3
NO	nitric oxide
Nox	NADPH oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
PDTC	pyrrolidine dithiocarbamate
Phox	phagocyte oxidase
PTP	permeability transition pore
ρ^0	rho zero
ROS	reactive oxygen species
SOD	superoxide dismutase
TLR	toll-like receptor
TNF-α	tumor necrosis factor- α
TPP⁺	triphenylphosphonium cation

Highlights

- Reactive oxygen species, particularly H_2O_2 , participate in microglial activation.
- This H_2O_2 is primarily of NADPH oxidase rather than of mitochondrial origin.
- Common techniques used to study mitochondrial superoxide are critically evaluated.
- Evidence linking mitochondria to NLRP3 inflammasome activation is not conclusive.

Fig. 1.

NADPH oxidase- and mitochondria-derived reactive oxygen species pathways in microglial activation. The antioxidants DPI, apocynin, NAC, MnTMPyP, gp91ds-tat peptide, and MitoTEMPO, decrease reactive oxygen species (i), leading to a decrease in release of proinflammatory factors. Exogenous catalase but not superoxide dismutase also blocks release, suggesting that superoxide ($O_2^{\cdot-}$) produced by NADPH oxidase requires dismutation to hydrogen peroxide (H_2O_2) before it can mediate intracellular signaling (ii). Cell permeable H_2O_2 activates the MAP kinase signaling pathway and induces NF- κ B translocation from the cytosol to the nucleus (iii), where it promotes synthesis of TNF- α and iNOS, leading to increased secretion of TNF- α and NO. Evidence also exists that reactive oxygen species upregulate and activate the NLRP3 inflammasome, initiating release of the proinflammatory cytokines IL-1 β and IL-18 from their pro-forms subsequent to cleavage by caspase-1 (iv). Induction of mitochondrial superoxide by rotenone, measurement of mitochondrial reactive oxygen species by MitoSOX, and scavenging of mitochondrial reactive oxygen species by MitoTEMPO are three techniques commonly used to implicate mitochondrial reactive oxygen species in NLRP3 inflammasome activation (v). DPI, diphenylene iodonium; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin; NAC, N-acetylcysteine; NO, nitric oxide; NLRP3, NOD-like receptor family pyrin domain-containing 3; SOD, superoxide dismutase; TNF- α , tumor necrosis factor- α .

Fig 2.

Impact of reactive oxygen species on inflammatory pathways. Following an inflammatory stimulus (i.e. LPS, A β peptide), activation of NADPH oxidase and/or impairment of mitochondrial respiration results in elevated superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These reactive oxygen species are implicated in $NF-\kappa B$ translocation to the nucleus, upregulation of CD11b/Mac1, p53 activation, and activation of the NLRP3 inflammasome. NO and TNF- α production occur subsequent to $NF-\kappa B$ activation. Cleavage of pro-IL-1 β to the active form occurs subsequent to NLRP3 inflammasome activation. Reactive oxygen species produced by NADPH oxidase or mitochondria may also constitute an inflammatory stimulus in itself or lead to proinflammatory amplification. A β , amyloid-beta; IL-1 β , interleukin-1 β ; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family pyrin domain-containing 3; NO, nitric oxide; TNF- α , tumor necrosis factor- α .

Fig. 3.

MitoSOX and MitoTEMPO are not always mitochondria-specific. A positively charged lipophilic triphenylphosphonium cation (TPP^+) conjugate within the reactive oxygen species-sensitive dye MitoSOX or the antioxidant MitoTEMPO mediates their accumulation in the matrix of functional mitochondria exhibiting negative membrane potential ($\Delta\Psi$). This charge-based accumulation results in several-fold higher concentrations of MitoSOX or MitoTEMPO in the mitochondrial matrix compared to the cytoplasm, with their relative concentrations indicated by font size within the figure (i). However, during the microglial/macrophage activation process, loss of $\Delta\Psi$ due to respiratory inhibition, mitochondrial uncoupling, or opening of the mitochondrial permeability transition pore (PTP) results in charge equilibration across the mitochondrial membrane, sometimes accompanied by mitochondrial swelling and loss of outer membrane integrity. Along with other unbound, charged molecules, MitoSOX and MitoTEMPO re-equilibrate across the mitochondrial membrane into the cytoplasm and their charge-based preferential sequestration in mitochondria is lost (ii). Thus, although they may still report or scavenge reactive oxygen species, respectively, their mitochondria-specific mechanism of action is no longer maintained.