Free Radical Biology and Medicine 112 (2017) 494–503

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Potential role of ferric hemoglobin in MS pathogenesis: Effects of oxidative stress and extracellular methemoglobin or its degradation products on myelin components

Vladimir V. Bamm[a], Mary E.L. Henein[a,b], Shannon L.J. Sproul[a,c], Danielle K. Lanthier[a], George Harauz[a,*]

[a] Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1
[b] Department of Human Genetics, McGill University, Montréal, Québec, Canada
[c] Department of Cell and Developmental Biology, University of British Columbia, Vancouver, BC, Canada

ARTICLE INFO

Keywords:
Multiple sclerosis
Myelin
Myelin basic protein
Oxidative stress
Hemoglobin
Lipid oxidation
Protein oxidation
Hemin
Globin radical

ABSTRACT

There is a well-documented relationship between cerebral vasculature and multiple sclerosis (MS) lesions: abnormal accumulations of iron have been found in the walls of the dilated veins in cerebral MS plaques. The source of this iron is unknown, but could be related to the recognized phenomenon of capillary and venous hemorrhages leading to blood extravasation. In turn, hemorrhaging leading to hemolysis results in extracellular release of hemoglobin, a reactive molecule that could induce local oxidative stress, inflammation, and tissue damage. Our previous studies with a reduced form of hemoglobin (oxyHb) have demonstrated its ability to cause extensive lipid and protein oxidation *in vitro*, which would result in membrane destabilization. Here, we investigated in further detail the mechanism by which the more abundant oxidized form of extracellular hemoglobin (metHb), and dissociated hemin, cause direct oxidative damage to myelin components, specifically membrane-mimetic lipid vesicles and myelin basic protein (MBP), a highly-abundant protein in the CNS. Oxidation of lipids was assessed by the formation of conjugated diene/triene and malondialdehyde, and oxidation of MBP was demonstrated by the bityrosine formation and by the change in protein mass. Our results show that metHb causes oxidative damage to MBP and myelin lipids, partly by transferring its hemin moiety to protein and lipid, but mostly as an intact protein possibly *via* formation of a ferryl radical. These results elucidating the mechanism of extracellular hemoglobin-induced oxidative damage to myelin components support the need for further research into vascular pathology in MS pathogenesis, to gain insight into the role of iron deposits and/or in stimulation of different comorbidities associated with the disease.

1. Introduction

One of the major determinants of health in all aerobes is the delicate balance between production of reactive species (RS) and antioxidant defense systems. However, when this balance is not maintained properly, RS production overwhelms the antioxidant capacity and contributes to oxidative damage. Indeed, the biomedical literature is full of claims that these RS are involved in many, if not all human diseases, but the real question in this case is the causality. In other words, some diseases are caused by oxidative stress, but in most disorders, RS and oxidative stress are a consequence of the disease. If so, what is their role? Do they contribute to pathology or, in contrast, could they be beneficial in triggering a rapid adaptive response?

Multiple sclerosis (MS), an inflammatory demyelinating disorder [1], is an example of the above paradigm. The disease has been extensively studied for over 150 years, since it was first recognized and

Abbreviations: Amp, ampicillin; a.u., arbitrary units; BBB, blood-brain barrier; Cam, chloramphenicol; CNS, central nervous system; cytLUV, cytoplasmic large unilamellar lipid vesicle; DMSO, dimethylsulphoxide; EDTA, ethylenediaminetetraacetic acid; H_2O_2, hydrogen peroxide; Hb, hemoglobin; HO, heme-oxygenase; Hpt, haptoglobin; Hpx, hemopexin; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside;; EAE, experimental autoimmune/allergic encephalomyelitis; ferrylHb, ferryl-hemoglobin ($Hb(Fe^{4+})$); FPLC, fast protein liquid chromatography; heme, ferrous (Fe^{2+}) protoporphyrin IX; hemin, ferric (Fe^{3+}) protoporphyrin IX; LB, lysogeny broth; MBP, unmodified 18.5-kDa recombinant murine myelin basic protein isoform; metHb, met-hemoglobin (ferric $Hb(Fe^{3+})$); MDA, malondialdehyde; MOPS, 3-[*N*-morpholino]propanesulphonic acid; MS, multiple sclerosis; oxyHb, oxyhemoglobin (ferrous $Hb(Fe^{2+})O_2$); PBS, phosphate-buffered saline; ROS, reactive oxygen species; RS, reactive species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; TBST, Tris-buffered saline + Tween-20

* Corresponding author.
E-mail address: gharauz@uoguelph.ca (G. Harauz).

http://dx.doi.org/10.1016/j.freeradbiomed.2017.08.022
Received 5 July 2017; Received in revised form 22 August 2017; Accepted 28 August 2017
Available online 31 August 2017

histologically characterized [2]. Inflammation is one of the key factors in the progression of MS, and RS are considered to be involved in the observed tissue injury [3]. Oligodendrocytes are particularly susceptible to oxidative stress due to the high presence of iron and polyunsaturated fatty acids, and the low concentration of glutathione [4,5]. Additionally, oligodendrocytes have the highest metabolic rate in the central nervous system (CNS) [6], and produce high levels of H_2O_2 that is efficiently removed in healthy mature cells [7]. However, unregulated production of H_2O_2 can induce oxidative stress and cell death [8]. Although uncontrolled H_2O_2 production is damaging enough by itself, it can also be converted by the action of metal ions such as Fe^{2+} or Cu^{2+} to the even more active oxidant, HO^-. Therefore, it is not surprising that the magnitude of iron accumulation in MS grey or white matter, as detected by magnetic resonance imaging (MRI), correlates with disease severity, the extent of lesion load, and the level of cell death [9–12]. Clearly, abnormal iron accumulation may contribute to the MS pathogenesis; however, it remains unclear whether iron accumulation is a causative factor or a result of chronic inflammation, and the origin of this abnormal iron deposition is also unknown.

The relationship between cerebral vasculature and MS lesions is well-documented [13–15], and abnormal accumulations of iron have been found in the walls of the dilated veins proximal to demyelinated plaques in the white and grey matter [9,12,16–18]. There is definitely more than one scenario that can explain abnormal iron deposition in the MS brain, including but not limited to: *(i)* abnormalities in the function of blood-brain barrier [19,20]; *(ii)* inefficient iron clearance [11]; and *(iii)* chronic subclinical cerebral microbleeds [21,22]. In this theme, we have proposed that unprotected extracellular hemoglobin (Hb) might invade the parenchyma of the CNS as a result of intravascular hemolysis or extravasation of red blood cells, followed by their rupture [23–25]. Under these circumstances, the release of extracellular Hb (being a highly-reactive molecule) could lead to local oxidative stress, inflammation, and tissue damage. In support of this proposition, we have demonstrated experimentally that extracellular Hb causes direct oxidative damage to myelin protein and lipid components, in an *in vitro* myelin-mimetic system [24]. In that first study, we determined the ability of oxyHb (reduced oxygen-bound form, specifically ferrous $Hb(Fe^{2+})O_2$) to induce oxidative damage to lipids and proteins of the myelin sheath by using intact human Hb, large unilamellar lipid vesicles (LUVs) mimicking the cytoplasmic leaflets of the myelin membrane, and purified myelin basic protein (MBP, specifically the 18.5-kDa splice isoform that predominates in the adult human CNS). The addition of H_2O_2, to simulate the peroxidative environment (such as occurs in inflamed tissue [26,27]) increased the oxidation potential of Hb, possibly via a redox reaction of the heme prosthetic group. Our data thus showed conclusively that, indeed, free Hb causes oxidative damage to MBP and myelin lipids *in vitro*, and support our hypothesis that extracellular Hb can exacerbate the myelin damage and neurodegeneration observed in MS.

More recently, Lewin et al. [28] observed that free serum Hb is associated with brain atrophy in a cohort of patients with secondary progressive MS. Their results supported the scenario that we had proposed, in which chronic low-grade intravascular hemolysis causes release of Hb into the circulation, followed by its translocation through a leaky blood-brain barrier to the CNS. Therefore, extravasated Hb could explain, at least in part, the abnormal iron overload observed in MS brains, and even more so it can contribute to neurodegeneration and brain atrophy during the course of disease. Under this scenario, the extracellular Hb will readily oxidize into its ferric form (metHb, specifically ferric $Hb(Fe^{3+})$). In this current study, we focus on the mechanisms of metHb oxidative damage on myelin components using our previously-designed *in vitro* system.

2. Materials and methods

2.1. Preparation of proteins and hemin

An unmodified 18.5-kDa recombinant murine MBP isoform (MBP, 168 residues), without any extraneous tags or other modifications was subcloned into a pET22b vector, expressed in *E. coli* BL21-CodonPlus (DE3)-RP cells (Stratagene, La Jolla, CA) and purified by ion-exchange chromatography as previously described [29]. Pure fractions were confirmed by SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis), and then extensively dialyzed (6000–8000 Da dialysis tubing) against 2 × 2 L of 50 mM Tris-base, 250 mM NaCl, pH 7.4. Following dialysis, the protein was further purified using reversed-phase high-performance liquid chromatography (HPLC) as previously published for purification of MBP-derived peptides, with minor modifications [30]. Briefly, purification was done using a Waters (Mississauga, ON) apparatus with a Symmetry 300 C18, 5 μm, 4.6 × 250 mm column. Acetonitrile constituted the mobile phase, and 0.1% TFA (trifluoroacetic acid) was the ion-pairing agent. Detection was at 214 nm, the flow rate was 1 mL/min, and the column was maintained at 40 °C. The elution gradient was begun at 75% Solvent A (ddH_2O with 0.1% TFA) and 25% Solvent B (acetonitrile with 0.1% TFA), and run at a rate of 1% Solvent B/min for 15 min, followed by 15% Solvent B/min for 4 min. The pure protein was lyophilized, solubilized in water, and the concentration was determined at 276 nm using $\varepsilon = 0.469\ g^{-1}\ cm^{-1}\ L$ and a molecular mass of 18,356.4 $mol^{-1}\ g$ [31]. Protein was stored at − 20 °C until use.

Hemoglobin was isolated from red blood cell lysates by ion-exchange chromatography using CM-52 cellulose [32]. This Hb was verified spectrophotometrically as oxyHb, and metHb was prepared by treating it with 10% molar excess of $K_3Fe(CN)_6$ followed by extensive dialysis against water [33]. The concentration of metHb throughout this study was quantified and expressed in heme equivalents, using the extinction coefficient $\varepsilon_{405} = 179\ mM^{-1}\ cm^{-1}$ at pH 6.3 [33].

Hemin (EMD Chemicals Inc., San Diego, CA) was prepared freshly as a stock solution by dissolving in 5 mM NaOH or in 80% dimethylsulphoxide (DMSO). Undissolved remains were removed by centrifugation at 18,000 *g* for 10 min. The concentration of hemin in this stock solution was determined using the extinction coefficient $\varepsilon_{385} = 58.4\ mM^{-1}\ cm^{-1}$ in 5 mM NaOH [34]. Dilutions for experimental use were done in 50 mM 3-[*N*-morpholino]propanesulphonic acid (MOPS buffer, pH = 7.4) or in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

2.2. Preparation of cytoplasmic large unilamellar lipid vesicles (cytLUVs)

All lipids (cholesterol, phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin (SM)) were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at − 20 °C until use. Aliquots of chloroform solutions of cholesterol, PE, PS, PC, SM, and PI were combined in a 44:23:13:11:3:2 mol ratio, which is characteristic of the cytoplasmic leaflet of myelin [35], in a glass tube, and solvent was evaporated under a steady stream of nitrogen gas, followed by an over-night lyophilization. The lipid film was resuspended in 5 mL of MilliQ™ water to yield ~ 20 mM concentration, and layered with argon gas. Vesicles were subjected to three cycles of freeze (− 20 °C) and thaw (1 h at 45–50 °C), with occasional vortexing to break the multilamellar structures, and were then frozen into aliquots. To prepare unilamellar vesicles, frozen samples were thawed and extruded (at 45 °C) to ensure size homogeneity of vesicles with a 100-nm diameter. After extrusion, lipids were stored at 4 °C until use. The Modified Micro-Bartlett Phosphorus Assay was used to determine the molar concentration of the lipid vesicles [36].