Biochimica et Biophysica Acta 1852 (2015) 92–103

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

In vitro study of the direct effect of extracellular hemoglobin on myelin components

Vladimir V. Bamm, Danielle K. Lanthier, Erin L. Stephenson [1], Graham S.T. Smith, George Harauz *

Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

ARTICLE INFO

Article history:
Received 26 August 2014
Received in revised form 26 September 2014
Accepted 9 October 2014
Available online 18 October 2014

Keywords:
Multiple sclerosis
Myelin
Myelin basic protein
Oxidative stress
Hemoglobin
Globin radical

ABSTRACT

There is a relationship between cerebral vasculature and multiple sclerosis (MS) lesions: abnormal accumulations of iron have been found in the walls of dilated veins in MS plaques. The sources of this iron can be varied, but capillary and venous hemorrhages leading to blood extravasation have been recorded, and could result in the release of hemoglobin extracellularly. Extracellular hemoglobin oxidizes quickly and is known to become a reactive molecule that triggers low-density lipoprotein oxidation and plays a pivotal role in atherogenesis. In MS, it could lead to local oxidative stress, inflammation, and tissue damage. Here, we investigated whether extracellular hemoglobin and its breakdown products can cause direct oxidative damage to myelin components in a peroxidative environment such as occurs in inflamed tissue. Oxidation of lipids was assessed by the formation of fluorescent peroxidized lipid–protein covalent adducts, by the increase in conjugated diene and malondialdehyde. Oxidation of proteins was analyzed by the change in protein mass. The results suggest that the globin radical could be a trigger of myelin basic protein oxidative cross-linking, and that heme transferred to the lipids is involved in lipid peroxidation. This study provides new insight into the mechanism by which hemoglobin exerts its pathological oxidative activity towards myelin components. This work supports further research into the vascular pathology in MS, to gain insight into the origin and role of iron deposits in disease pathogenesis, or in stimulation of different comorbidities such as cardiovascular disease.

1. Introduction

Multiple sclerosis (MS) has been studied for more than 150 years, but this devastating disease remains frustratingly enigmatic and has been compared with the mythological Daedalus' labyrinth that has not yet been conquered [1]. The inflammatory face of MS, together with use of anti-inflammatory drugs to relieve the associated symptoms, strongly reflects the autoimmune nature of the disease. This hypothesis invokes the idea of an "outside-in" mechanism of disease, where autoantibodies directed to myelin may be responsible for demyelination and subsequent damage to neuropil [2]. However, other recent studies have provided histopathological evidence that MS may also be considered a degenerative disorder rather than solely an autoimmune disease, and the concept of an "inside-out" model of disease progression has arisen [3]. This model suggests that the autoimmune and/or inflammatory responses are the secondary reaction to the primary process that causes death of oligodendrocytes and demyelination of axons in the central nervous system (CNS) [4,5].

Since Charcot's first histological characterization of multiple sclerosis [6], a relationship has been observed between cerebral vasculature and MS lesions [7–9]. An abnormal iron accumulation has been shown histologically and biochemically in MS [7,8,10,11] and, particularly, has been found in the walls of the dilated veins in cerebral MS plaques [12]. Studies of MS patients show evidence of iron accumulation in grey matter structures such as the thalamus, globus pallidus, red nucleus, substantia nigra, putamen, caudate nucleus, and hippocampus, that go beyond any age-related effects [13–16]. The brains of patients with MS show iron staining in macrophages and microglia, around sites of inflammation, and near demyelinated plaques in gray and white matter [17]. In general, the extent of iron accumulation in gray matter structures and lesions has been shown to be a good predictor of disability progression in MS, as well as the extent of lesion accumulation and level of cell death [17–20]. Moreover, it has been observed that iron-deficient mice fail to develop EAE, demonstrating that iron deficiency

Abbreviations: Amp, ampicillin; a.u., absorbance units; BBB, brain–blood barrier; Cam, chloramphenicol; CNS, central nervous system; cytLUV, cytoplasmic large unilamellar lipid vesicle; 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 allergic encephalomyelitis; ferrylHb, ferryl-hemoglobin; FPLC, fast protein liquid chromatography; LB, Luria broth; MBP, unmodified 18.5-kDa recombinant murine myelin basic protein isoform; metHb, met-hemoglobin; MDA, malondialdehyde; MS, multiple sclerosis; oxyHb, oxy-hemoglobin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; TBST, Tris-buffered saline + Tween-20

* Corresponding author. Tel.: +1 519 824 4120x52535; fax: +1 519 837 1802.
E-mail address: gharauz@uoguelph.ca (G. Harauz).
[1] Present address: Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada.

http://dx.doi.org/10.1016/j.bbadis.2014.10.009
0925-4439/

is more effective than any currently known pharmaceutical drug in preventing EAE [21]. The deleterious effects of abnormal iron accumulations on MS metabolism have been recently reviewed [15,20,22].

The origin of the elevated iron deposits in MS is unknown, but they are not necessarily simply a consequence of release from glial intracellular ferritin stores [23] or inflammation [14,15]. In general, the most abundant source of iron in the human body is heme, and iron released from it plays a central role in multiple pathologies, such as reperfusion after ischemia, trauma, and hemolysis. The major source of heme is hemoglobin, the major protein in erythrocytes. Thus, any type of cerebrospinal blood vessel abnormality [24] or abnormally permeable blood–brain barrier [25,26] can potentially lead to intravascular hemolysis, or to extravasation of erythrocytes and extravascular hemolysis [27,28]. This situation resembles several pathologies that are completely different from MS: atherosclerotic lesion formation (intravascular hemolysis), chronic venous disorders, and chronic hemorrhagic micro-stroke (intra- or extravascular hemolysis), all resulting in the release of hemoglobin extracellularly [29]. Under these conditions, as a result of hemolysis, hemoglobin will appear in its ferrous (Fe^{2+}) form that can readily be oxidized to the ferric (Fe^{3+}) or ferryl (Fe^{4+}) forms [30–32]. Due to its nature, oxidized extracellular hemoglobin is a highly-reactive molecule. This reactivity is operative in numerous pathologies, and could be derived from three major properties: the oxidative activity of intact hemoglobin (and/or its chains); the oxidative activity of heme released from hemoglobin (and/or its chains); and the oxidative reactivity of free iron released from heme.

Each or all three of these pathways can be involved also in MS pathogenesis. For instance, intact hemoglobin can act as a peroxidase under oxidative stress or inflammatory conditions [33]. Also, heme can be released from oxidized hemoglobin and, being a hydrophobic molecule, can readily enter the hydrophobic domains of biological membranes [34]. Thus, free heme can enter the myelin sheath and cause oxidative damage directly to the constituent lipids and proteins [35]. Those oxidatively-damaged proteins could initiate a secondary autoimmune response. Lastly, heme is not a stable molecule, and deteriorates or can be enzymatically oxidized to produce biliverdin, ferrous iron (Fe^{2+}), and carbon monoxide (CO). Under this last scenario, free iron can give rise to oxidative stress and generation of free radicals [36]. Although mechanistically these three ways of hemoglobin-induced damage are different, the outcome could be the same, namely local oxidative stress, inflammation, and tissue damage. Thus, considering the fact that the hemoglobin extravasation is a minor but chronic process, over a certain period of time the disease can progress to its clinical stage when severe demyelination and inflammation become noticeable.

Myelin, with its high proportion of polyunsaturated lipids, is an especially susceptible target for hemoglobin-induced oxidative attack [37]. Disruption of lipid homeostasis can affect the integrity of myelin, and promote neurodegeneration [38–40]. Oxidized lipoproteins can be a source of pro-inflammatory and pro-atherogenic effects, induce oxidative stress, and cause neuronal degeneration [41]. Lipid peroxidation products can also lead to protein modification [18]. Myelin basic protein (MBP), a highly positively-charged (pI > 10.5) peripheral protein of the myelin sheath, has a crucial role in the formation and maintenance of the myelin sheath [42]. Oxidized cholesterol has been shown to increase the exposure of MBP to the cytoplasm, enhancing its susceptibility to proteolytic cleavage [43]. The MBP is also a major candidate auto-antigen in MS, and the increased exposure of immunodominant epitopes of MBP may allow recognition by the immune system [43–46].

The primary objective of this study was to investigate whether hemoglobin can be considered as a player in the ensemble of different factors that together damage myelin protein and lipids, and lead to the progression of MS. An intact molecule of oxyHb was incubated with lipid and protein components of myelin, and its direct oxidative potential was assessed. Also, hydrogen peroxide was used in the low micromolar range (10 μM) to mimic oxidative stress as occurs under inflammatory conditions [47–49]. Our results clearly indicate that Hb causes oxidative stress/damage to cultured oligodendrocytes, and to MBP and myelin lipids *in vitro*, and that the mechanism of this oxidative damage could involve the formation of a globin radical and heme transfer. Therefore, we conclude that extracellular hemoglobin can play an important role in the pathogenesis of MS.

2. Materials and methods

2.1. Purification of unmodified 18.5-kDa recombinant murine myelin basic protein isoform (MBP) and oxyhemoglobin (oxyHb)

An unmodified 18.5-kDa recombinant murine MBP isoform (MBP, 168 residues), without any tags or other recombinant additions, was expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RP cells (Stratagene, La Jolla, CA) and purified by ion exchange chromatography as previously described [50]. Pure fractions were collected and their purity was confirmed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), then extensively dialyzed (6000–8000 Da dialysis tubing) against 2 × 2 L of Buffer A (50 mM Tris-base, 250 mM NaCl, pH 7.4), 2 × 2 L of Buffer B (100 mM NaCl), and 4 × 2 L MilliQ water. Following dialysis, the purified samples were lyophilized until dry and then stored at −20 °C. Before use, the frozen MBP stock was resuspended in MilliQ water. Protein concentration was determined by measuring the absorbance at 280 nm, using the extinction coefficient $\varepsilon = 0.705\ L\ g^{-1}\ cm^{-1}$ (as calculated by SwissProt for protein in 6.0 M guanidine hydrochloride, 0.02 M phosphate buffer, pH 6.5).

Hemoglobin was purchased from Sigma-Aldrich (Oakville, Canada). Contaminants (mainly catalase) were removed by CM-52 ion exchange chromatography as previously described [51], and spectrophotometrically verified to be a mixture of oxyHb and metHb. MetHb was produced by the addition of 10% molar excess of potassium ferricyanide followed by extensive dialysis against water [52], aliquoted, and frozen at −80 °C. OxyHb was prepared from the metHb stock by gel filtration on a G25 column (25 × 1.5 cm) and fast protein liquid chromatography (FPLC) at 4 °C [53]. Briefly, 0.2 mL of 0.1 g/mL of sodium dithionite was added to the column at 1 mL/min, followed by 1 mL of ~18 mM metHb when all of the sodium dithionite had completely entered the gel bed. The product, deoxy-ferrous hemoglobin, was visible as a purple band at the top of the column, and changed its color to red (oxyHb) while moving through the column. The concentration of oxyHb was determined using the absorbance at wavelengths 541, 577, and 415 nm with extinction coefficients of 13.8, 14.6, and 125 $cm^{-1}\ mM^{-1}$, respectively [52]. Concentrations of Hb are expressed in heme equivalents throughout this study. OxyHb was analyzed for potential contamination with endotoxin using an Endosafe®-PTS Reader equipped with the Endosafe®-PTS cartridge-0.05 EU/mL sensitivity (Charles River Laboratories, Sherbrooke, QC). Using this method, we determined that the concentration of endotoxin under experimental conditions was ≤3.47 ng/mL.

2.2. Formation 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 44:27:13:11:3:2 mole ratio, which is characteristic of the cytoplasmic leaflet of myelin [54]. Lipids (in chloroform) were combined 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 double-distilled water to yield an ~20 mM concentration, and layered under argon gas. Vesicles were subjected to three freeze (−20 °C) and thaw (1 h at 45–50 °C) cycles with occasional