Myoglobin in Brown Adipose Tissue: Novel Thermogenic Implications

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Brown adipose tissue (BAT) plays an important role in energy homeostasis by generating heat from chemical energy via uncoupled oxidative phosphorylation. Besides its high mitochondrial content and its exclusive expression of the uncoupling protein 1, another key feature of BAT is the high expression of myoglobin (MB), a heme-containing protein that typically binds oxygen, thereby facilitating the diffusion of the gas from cell membranes to mitochondria of muscle cells. In addition, MB also modulates nitric oxide (NO•) pools and can bind C16 and C18 fatty acids, which indicates a role in lipid metabolism. Studies in humans and mice implicated MB present in BAT in the regulation of lipid droplet morphology and fatty acid shuttling and composition, as well as mitochondrial oxidative metabolism.

brown adipose tissue | myoglobin | mitochondrial oxidative metabolism | energy metabolism

1. Introduction

Myoglobin (MB) was first discovered in 1872 by Ray Lankester as “intracellular hemo-globin” in the mammalian striated muscle [1], only to be later renamed monochrome by Hans Günther in 1921 [2]. In 1958, John C. Kendrew resolved the structure of MB via X-ray crystallography [3][4]. This later breakthrough provided the foundation for elucidating the mechanism of oxygen binding and release along with the role of structural changes within the protein as well as of the specific amino acids involved in ligand binding. For this pivotal work, Kendrew was awarded the Nobel Prize in Chemistry in 1962, shared with his colleague Max F. Perutz for the unraveling of the hemoglobin crystal structure. Today, MB is known to play a crucial role in oxygen transport and storage in muscle cells [5][6][7]. However, the complete story of MB has yet to be told, as much remains to be learned about this long-known protein and its multiple functional aspects.

Recent studies have proposed new functions for MB in brown adipose tissue (BAT) metabolism and thermogenesis. BAT is a specialized type of adipose tissue primarily involved in thermogenesis and energy expenditure. Unlike white adipose tissue (WAT), which stores energy in triglycerides (TGs), BAT contains numerous mitochondria and is enriched with small lipid droplets whose held stores can be rapidly mobilized for energy production. The fatty acid (FA) and lipid metabolism within BAT is tightly regulated and plays a critical role in determining its overall activity [8][9]. Brown adipocytes exhibit a myogenic transcriptional and mitochondrial signature [10][11], thus highlighting the functional proximity between BAT and skeletal muscle. In addition to its classical role of oxygen binding, MB has emerged as an essential regulator of lipid metabolism in different tissues, including BAT [12][13][14][15][16][17]. Recent research has furthermore implicated MB to play a crucial role in controlling

thermogenic activity in BAT [13][18][19]. Specifically, oxygenated MB (oxy-MB or MBO_2) has been reported to bind some FAs with physiological binding constants in vitro [14][20][21] and facilitate their transport and oxidation in the cell, processes necessary for heat generation in BAT. Other recent studies demonstrated MB to be involved in regulating the expression of genes that are critical for BAT thermogenesis [12][18][19], including genes involved in lipid metabolism, mitochondrial function, and energy expenditure. Finally, MB has also been shown to play a role in the sexual dimorphism observed in BAT activity, with females exhibiting higher thermogenic capacity than males [18].

2. Structure, Location, and Classical Functions of MB

2.1. Structure

The monomeric MB protein in of mice and men, in its mature state (after the removal of the initial methionine), is comprised of a single polypeptide chain of 153 amino acids and has a size of 17 kDa [4]. The globin superfamily, including hemoglobin, myoglobin, cytoglobin, neuroglobin, and globin X, carries out a variety of functions related to the ability of their prosthetic heme group to bind diatomic gaseous ligands [22]. Typical of members of this superfamily, the MB fold consists of a series of eight alpha helices that are tightly wrapped around the heme group. The central iron ion of the heme prosthetic group has six coordination sites. Four sites are bound to nitrogen atoms of the porphyrin ring, and the fifth is bound to the proximal histidine residue (His 93) of the globin protein [23]. Gaseous ligands such as oxygen (O_2), nitric oxide (NO•), and carbon monoxide (CO) reversibly bind at the sixth coordinate site of the ferrous heme iron (Fe^{2+}), where the distal histidine (His 64) of the globin facilitates the gas binding through hydrogen bonding [24].

2.2. Location

MB is typically known as the heme-binding globin in the cytoplasm of cardiac and skeletal myocytes. However, MB expression is not exclusive to myocytic cells, as it was later reported to also occur as a protein in the liver, brain, and gills of hypoxia-tolerant common carp [25]. Moreover, a distinct MB transcript has been detected in human and murine brain tissues, which differs from the previously seen neuroglobin, a member of the hemoprotein superfamily expressed in neural tissues [25][26]. In common carp, Fraser et al. showed comparable MB levels in the liver and muscle tissues [25], while Cossins et al. reported a discrepant MB expression pattern in different tissues of common carp and zebrafish, such as the liver, brain, kidney, gill, intestine, and eye [27]. With the strongest expression in the heart, the MB expression pattern in humans seems different relative to carp [27][28]. Human MB RNA levels were 333 times and 25 times higher in cardiac muscle than in healthy colon and breast tissues, respectively [28]. In carp and zebrafish, MB protein levels in the liver, gill, and brain comprised less than 1% of the heart levels [27]. Hence, MB is expressed in different lineages, but how the protein's function might depend on its abundance level and/or expression site is of fundamental relevance for textbooks to come. Much evidence has been accumulated for the muscle-independent expression of MB in cancerous tissues, suggesting a functional role of MB in malignant tissues [29][30][31][32][33][34][35][36][37][38][39][40][41]. Depending on the cancer context, MB can have a positive (tumor-suppressing) [29][30][37][39][40][42][43] or negative (tumor-promoting) [31][41] impact on patients' survival. Spanning from

alleged interplay with the tumor suppressor p53 to impacting mitochondrial respiration, crosstalk with hormonal receptors, and cell survival and/or death implications, MB might interfere with tumor metabolism. Thorough characterization is warranted to decipher the molecular function(s) of the endogenously over-expressed MB by addressing its exact bio-molecular mechanism(s) and the protein‘s interactions with other cancer hallmarks in tumor cells. These analyses should help to determine whether MB itself will turn out to be one new independent cancer hallmark one day.

2.3. Classic Gas-Binding Functions

In terrestrial mammals, MB occurs in high concentrations of ~350–700 µM, while in breath-holding deep-diving specialists (whales and large seals), its intracellular abundance can climb even to low millimolar concentrations. These high concentrations are required to sustain muscle contraction via aerobic metabolism. At millimolar levels, MB can temporarily store O_2 during the submergence of mammalian apnea divers. MB might also buffer short phases of exercise-induced increases in O_2 flux by supplying the gas to the respiring mitochondria of myocytes of terrestrial mammals [5][6][7]. As MB's O_2 affinity exceeds that of hemoglobin, the myocytic globin can acquire O_2 from hemoglobin and transport it from the cell membrane into mitochondria for energy production. While progressive MB desaturation is observed during hypoxia or exercise, the physiological significance of MB-derived O_2 in supporting mitochondrial oxidative metabolism remains uncertain. MB can only bind a single O_2 molecule in contrast to hemoglobin. Whether MB-derived O_2 can significantly aid in the amplified oxidative combustion of fuels in working muscles has been questioned from a stoichiometric perspective. However, the protein's high expression level in myocytes might put such skepticism to rest. While the loss of systemic MB expression (MB-knockout or MBko) did not alter the exercise capacities of mice [44], the resulting cardiac and vascular compensations supported the globin's O_2 supply role in vivo [6]. However, other studies showed these MBko mice to encounter faster fatigue and run shorter distances on a treadmill [45] or revealed no genotypic differences in a wheel-running paradigm [21].

Beyond O_2 binding, MB has also been reported to scavenge/detoxify reactive oxygen species (ROS) [46] as well as to maintain NO• homeostasis in cardiomyocytes by either scavenging (during normoxia, MB as MBO_2) or producing it (during low O_2 environments (hypoxia), MB as deoxy-MB) [47][48]. Similar to deoxygenated hemoglobin (deoxyhemoglobin) [49][50], which turns blood-borne nitrite into NO• to facilitate vasodilation, deoxy-MB exhibits nitrite reductase activity by converting ferrous (Fe^{2+}) myoglobin into metmyoglobin (Fe^{3+}) while NO• is generated (Formula (1)). Notably, nitrite reduction by deoxy MB occurs approximately 36 times faster than that by deoxyhemoglobin due to its low heme redox potential [51].

$$deoxyMb(Fe^{2+}) + NO_2^- + H^+ \rightarrow metMb(Fe^{3+}) + NO\cdot \quad (1)$$

In turn, NO• also reacts with MBO_2 under normal oxygen tensions. The ability of NO• to react with heme centers of MB (and other globins) is now recognized as one of its most important characteristics. When NO• reacts with MBO_2, the ferrous (Fe^{2+}) MB is converting into metmyoglobin (Fe^{3+}) and nitrate is generated in the process (Formula (2)) [52][53].

$$Mb(Fe^{2+})O_2 + NO\cdot \rightarrow metMb(Fe^{3+}) + NO_3^- \quad (2)$$

By fine-tuning intracellular NO• concentrations, MB might help regulate a broad spectrum of physiological processes, including the activity of mitochondria. NO• generated from nitrite by deoxy-MB inhibits cytochrome c oxidase (complex IV) and mitochondrial respiration at low O_2 conditions [52][54]. Because NO• competes with O_2 for binding to cytochrome c oxidase, this inhibition is especially pronounced in hypoxia [54]. In 2001, Brunori et al. proposed that the reaction of MBO_2 and NO• in normoxia (i.e., Formula (2)) is important to avoid inhibition of cytochrome c oxidase by NO• [52]. This MB-based protective effect turned out to be extremely vital to continuous energy demand due to ongoing contractive work in cardiac muscle. Therefore, mitochondria generate the necessary amount of adenosine triphosphate (ATP) to sustain such contractions. By regulating ROS and NO•, MB might play a crucial role across a wide range of physiological processes in mammalian cells and tissues (**Figure 1**).

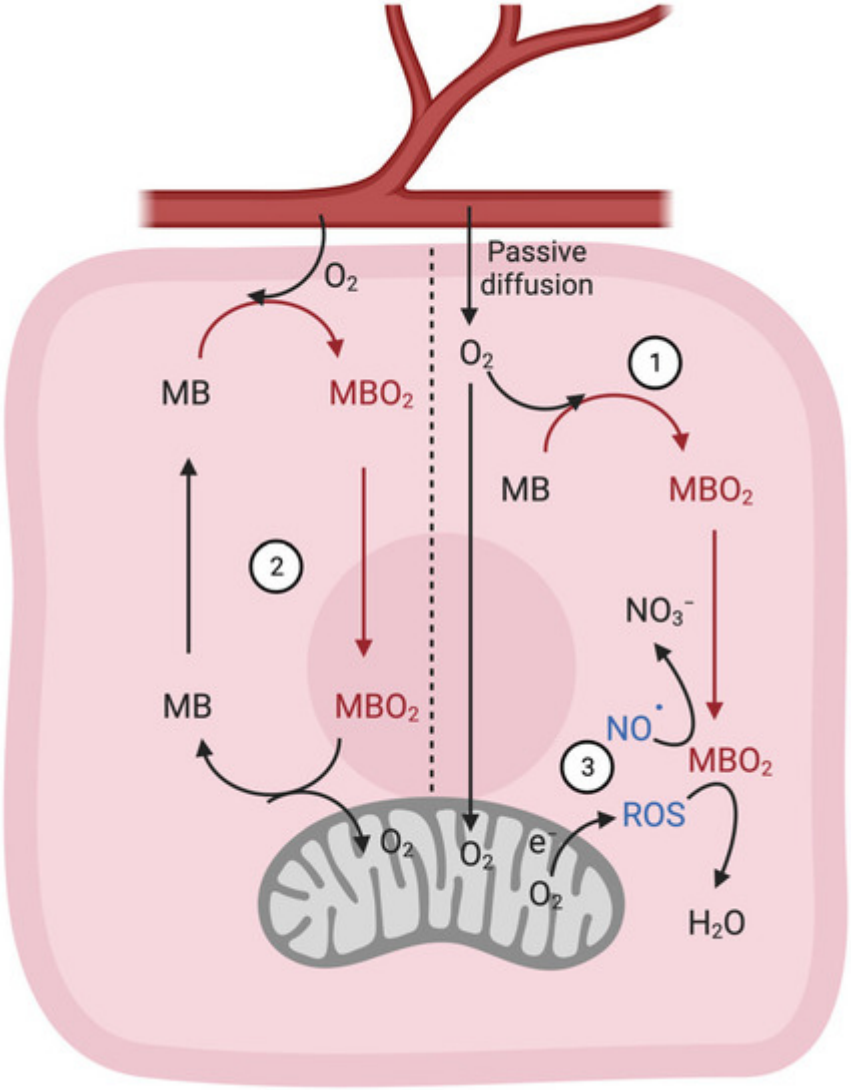

Figure 1. Typical gas binding-related functions of MB in muscle cells. (1) MB acts as a temporary store or reservoir for O_2 storage, especially in breath-holding diving mammals. (2) MB buffers short phases of exercise-induced increases in O_2 flux by supplying it to the mitochondria of myocytes via facilitated diffusion [5][6][7]. (3) MB impacts the homeostasis of important mediators of cell signaling. Reactive oxygen species (ROS) generated from mitochondria are rapidly detoxified/scavenged by MB. Under normoxic conditions, oxygenated MB (MBO_2) scavenges nitric oxide (NO•), thus preventing its inhibitory effect on cytochrome c oxidase and allowing for

sustained mitochondrial respiration. Under hypoxic conditions, deoxy MB (MB) produces (NO•) that works dually to stimulate vasodilation (i.e., bring more blood and O_2) as well as to inhibit cytochrome c oxidase and thus mitochondrial respiration to spare the limited O_2 in the cell for other metabolic processes [47][48]. The figure was created with Biorender.com.

3. Fatty Acid Homeostasis in BAT Thermogenesis and Novel Roles of MB in Lipid Metabolism

By maintaining body temperature, BAT has been reported to contribute considerably to whole-body energy expenditure (EE) in small mammals. BAT thermogenic capacity is canonically cold- and/or diet-induced by ligand-dependent activation of β-adrenergic G protein-coupled receptors (GPCRs) [55][56][57][58], which signal via increased cyclic AMP (cAMP) and ultimately improve metabolic homeostasis to generate heat [59]. During this so-called non-shivering thermogenesis of BAT metabolic substrates, mainly lipids, but also glucose, and to a lesser extent, branched-chain amino acids and Krebs cycle metabolites, are consumed [60][61][62] to fuel uncoupling protein 1 (UCP1)-dependent respiration [63] to ultimately convert chemical energy to heat. Hence, the two main energy-expending determinants of brown adipocytes are mitochondrial density and multiple lipid droplets per cell as large-surface energy depots accounting for the plurilocular phenotype of this cell type (**Figure 2**A). These characteristics underlie the high metabolic rate required for heat production with FA substrates [64]. FA metabolism and homeostasis are fundamental during BAT thermogenesis, since FAs are required to activate UCP1 proton transport activity [65][66]. Moreover, elevated levels of free saturated fatty acids (SFAs) likely increase UCP1 expression, thus inducing and fueling the uncoupling of oxidative phosphorylation [66][67]. **Figure 2**B illustrates the processes of FA metabolism in BAT, which is essential for the tissue's thermogenic activity. Besides high demands of lipids and glucose flux to the mitochondria, thermogenesis requires a continuous flux of O_2. Naturally, the O_2 expenditure required to dissipate heat in small-bodied mammals (e.g., mice, rats, and human infants) is enormous, as witnessed by the 2- to 4-fold increase in the O_2 consumption rate of rodents challenged by both acute and chronic cold exposure (4 °C) [68][69].

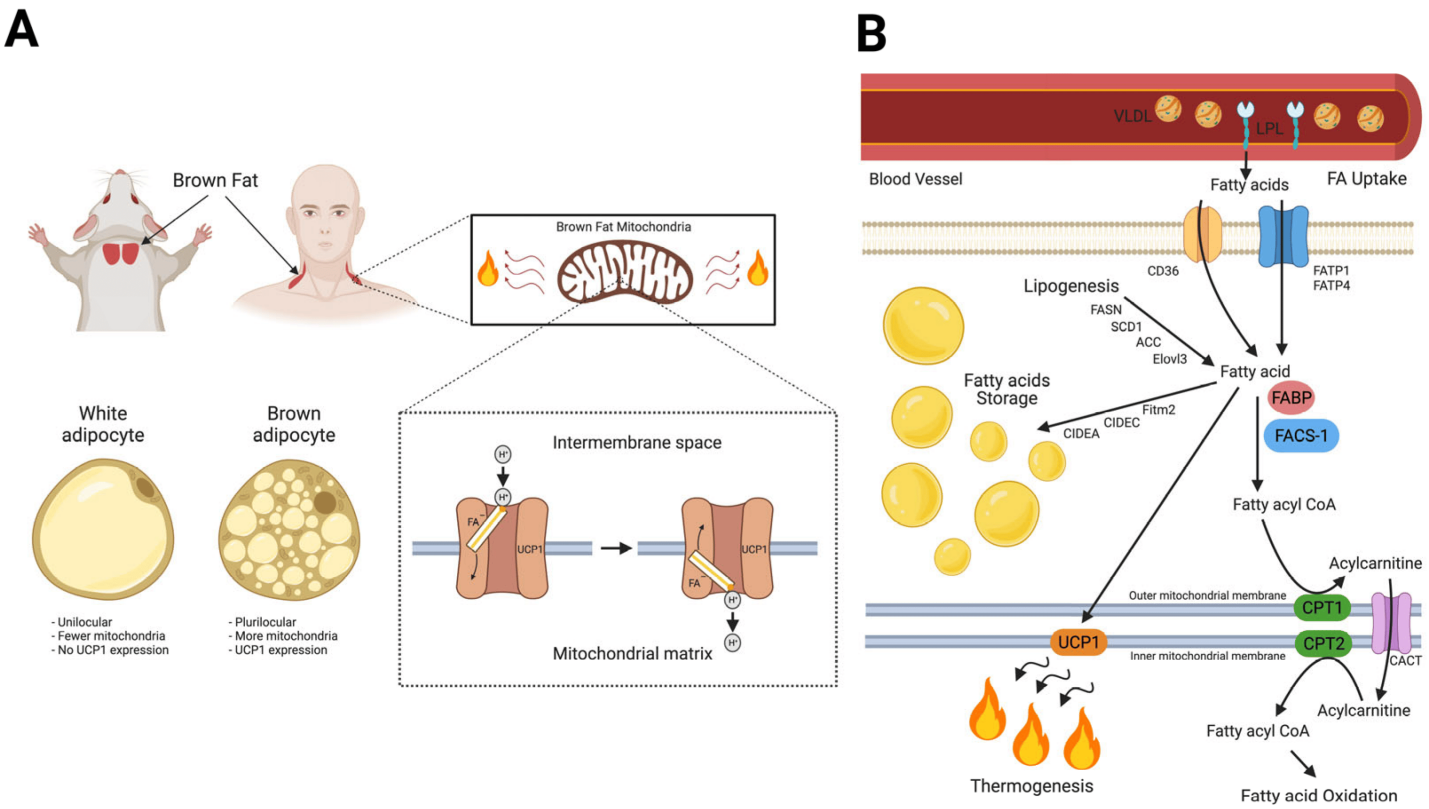

Figure 2. Fatty acid metabolism in BAT. (**A**) Active BAT is located in interscapular and cervical–supraclavicular sites in mice [70] and humans [71][72][73], respectively. Brown adipocytes are characterized by the presence of multiple small lipid droplets (plurilocular phenotype) and a high content of mitochondria, unlike white adipocytes which have one large lipid droplet (unilocular appearance) and far fewer mitochondria [74]. Moreover, only the mitochondria of brown adipocytes express the uncoupling protein 1 (UCP1). UCP1 is located at the inner mitochondrial membrane and facilitates the runback of protons along their gradients without the formation of ATP but with the dissipation of heat instead [75][76]. Free fatty acids (FAs), bound to UCP1, are essential for this uncoupling activity [65][66]. (**B**) Fatty acids (Fas) are released from very low-density lipoproteins (VLDLs) carried in the bloodstream by the action of the endothelial lipoprotein lipase (LPL) on the triglycerides contained within VLDLs. Fatty acid transporter proteins 1 and 4 (FATP1 and 4) as well as cluster of differentiation 36 (CD36) take up Fas into brown adipocytes. Proper fat storage into smaller and numerous lipid droplets is achieved via the activity of the following lipid droplet membrane proteins genes: the lipolytic regulator cell death-inducing DNA fragmentation factor- a-like effector A (CIDEA) and the fat-specific protein 27 (FSP27 or CIDEC), which ultimately increase the surface area of lipid droplets by storing FAs within numerous small lipid droplets (i.e., increasing surface area of energy expenditure by providing lipids more efficiently to mitochondria). The levels of the saturated free FAs (SFAs) determine UCP1 expression and thermogenic activity. The second mechanism determining FA abundance in brown adipocytes is de novo synthesis (lipogenesis) via the following enzymes: fatty acid synthase (FASN), elongation of very long chain fatty acid 3 (ELOVL3), and stearoyl-CoA desaturase1 (SCD1). FA-binding proteins (FABP) aid in binding FAs and hence prevent lipotoxicity. Regarding FA oxidation: FAs are converted into fatty acyl-CoA via the action of fatty acyl-CoA synthetase-1 (FACS1), then converted to acylcarnitine via the action of carnitine palmitoyltransferase-1 (CPT1). Acylcarnitine is transported to the mitochondrial matrix by carnitine/acylcarnitine translocase (CACT), followed by reconversion to fatty acyl CoA via the action of carnitine

palmitoyltransferase-2 (CPT2) before being oxidized in presence of O_2 [77]. The figure was created with BioRender.com.

4. Myoglobin in BAT; State-of-the-Art Research

4.1. Expression

In murine BAT, the basal expression levels of MB transcripts ranked fourth among tissues with the most abundant MB transcript levels, only preceded by the heart, skeletal muscle, and non-lactating mammary glands [78]. As one essential first indication that MB contributes to regulating thermogenesis in BAT, the expression of MB during brown adipogenesis and cold exposure in mice (in vivo: male C57BL/6N mice [79] and ex vivo: female NMRI mice [12]) and rats [80], as well as in immortalized brown adipocytes (imBA), originally described in [81]), was strongly upregulated. Browning of human and murine white adipocyte cell lines (i.e., induction of differentiation) induced MB expression but by one order of magnitude less [19]. In line, male and female C57BL/6N mice exhibited a significant temperature-dependent increase in MB mRNA and protein expression in BAT in cold-exposed animals compared to littermates housed at thermoneutrality. In addition, MB protein ELISA measurements from BAT lysates of NMRI mice housed at 30 °C and 8 °C showed a 3-fold induction of MB protein content in cold-exposed animals [19]. In contrast, β-adrenergic stimulation did not increase MB expression [19]. Hence, MB expression in BAT seems to be independent of canonical adrenergic stimulation by β3 adrenergic agonists such as CL 316,243 (CL) and TRPM8-activating menthol, as well as the peroxisome proliferator-activated receptor-gamma (PPARγ)-activating rosiglitazone [19]. However, extracellular purine signaling, and intracellular lipolysis, provide signals that are associated with cold-induced or endocrine activation of the tissue [82][83] and, possibly, might also drive MB expression. On a side note, myostatin, via its action on activin receptor ActRIIB, not only negatively regulates muscle growth but also inhibits brown adipocyte differentiation and brown adipogenesis as well as BAT growth [79]. The pharmacological inhibition of ActRIIB resulted in enhanced non-shivering thermogenesis, which was accompanied by an upregulation of both MB and the PPARγ coactivator (PGC1-α) in brown adipocytes [79]. As PGC1-α was shown to robustly and directly stimulate MB expression in C2C12 myoblasts as well as in type I muscle fibers [84], a positive feed-forward loop might exist between MB and PGC1-α to ultimately drive mitochondrial biogenesis and respiration, yet this remains to be furthermore demonstrated (Research gap#3). Interestingly, MB expression in mouse BAT was under transcriptional control of the PGC1-α-regulated nuclear respiratory factor-1 (Nrf1) [19], which, in thermogenic fat cells, is considered a metabolic guardian, preventing tissue stress and inflammation and mediating the proteasomal homeostatic activity that is required for thermogenic adaptation [85]. Proteasomal inhibitors for cancer treatment would worsen the condition affecting HB blood level, which merits further study (Research gap#4). Unlike in muscle [86] or cancer cells [37][39][42], hypoxia did not upregulate MB expression in primary brown adipocytes [12]. Therefore, future studies are needed to characterize the cis- and trans-elements that confer cold-induced MB transcriptional regulation. Potential epigenetic changes to the hypoxia response elements (HREs) that were characterized in breast cancer cells [28] should also be examined in brown adipocytes (Research gap#5).

4.2. Regulation of Mitochondrial Metabolism and UCP1 Expression

MB's role in BAT has been hypothesized to effectively supply O_2 to BAT mitochondria for the oxidation of respiratory substrates (FAs and glucose) to generate heat via the activity of UCP1. Previous studies have demonstrated mitochondrial localization of MB in the skeletal muscle [87][88] and partially in BAT as well [19]. In female MBko mice fed a standard chow diet, interscapular BAT (iBAT) explants exhibited a significant reduction in mitochondrial respiration rates, particularly the maximal and Complex I-/Complex II-mediated oxidative respiration [12]. This observation was associated with fewer and/or smaller mitochondria, as evidenced by fewer mitochondrial to nuclear DNA copy numbers and reduced abundance of mitochondrial loading marker VDAC1 as well as OXPHOS proteins [12]. Interestingly, the lack of MB correlated with the reduced expression of PPARα and PGC-1α, the latter being a key marker of mitochondrial biogenesis [89] and cold-induced thermogenesis [90]. Hence, the respiration differences between MB-proficient and -deficient brown adipocytes were mainly explained by the mitochondrial mass rather than activity per mitochondrion [12]. The cytochrome c oxidase subunit 4 (COX4) protein expression was reduced in MBko BAT of females and to a lesser extent in male mice fed with a high-fat diet [18]. The knockdown of MB in immortalized brown adipocytes (imBA) revealed lower maximal respiration in the presence or absence of the adrenergic agent forskolin as compared to control cells [19]. Similar results were obtained using differentiated primary brown adipocytes from female MBko and control NMRI mice [19]. Moreover, overexpressing MB levels in the thermogenic adipocytes increased mitochondrial respiration after treatment with forskolin [19]. Furthermore, MB seems to interact with UCP1, the key protein responsible for uncoupling oxidative phosphorylation, in BAT. Loss of MB function in mouse models was associated with a reduction in both basal [12] and cold-induced expression levels of UCP1 [19]. Moreover, MB and UCP1 mRNA expression levels correlated positively in female C57BL/6N mice housed at 8 °C for one week [19]. However, mice fed a high-fat diet showed no correlation between MB and UCP1 expression levels in brown adipocytes [18]. Nevertheless, the overexpression of MB in differentiated imBA cells induced UCP1 and CD36 thermogenic transcript expression levels in response to β3 adrenergic agonist [19]. Human transcriptome data indicate that the MB expression in WAT is regulated differently in obesity and correlates with UCP1 and other markers of adipose tissue browning, suggesting the functional significance of MB expression in human adipose tissue [19]. Thus, MB-mediated regulation of UCP1 activity may be an important determinant of overall energy expenditure and metabolic health.

4.3. Regulation of Lipid Metabolism

Histologically, iBAT in two different MBko mouse models (female NMRI [12] and both genders of C57BL/6N [18]) displayed larger but fewer lipid droplets than control mice, representing less of the plurilocular phenotype of active BAT with substrates less readily available for oxidation from the relatively smaller surface area energy depot. In addition, loss of MB was associated with reduced expression of PPAR-α and other genes involved in lipid storage [12]. However, another study reported no obvious differences in BAT histology between MBko and NMRI control mice held at thermoneutrality and after cold exposure (8 °C, 23 °C, and 30 °C). In contrast, white adipocytes from inguinal and epididymal fat stores of MBko male mice were significantly larger at all temperatures [19]. The overexpression of MB in brown and white adipocytes resulted in smaller but more numerous lipid droplets along with the increased responsiveness to adrenergic activation and lipolysis [19]. Moreover, it also induced phosphorylation of protein kinase A (PKA) that regulates adipose depot and energy expenditure [91]. In addition, Christen et al. confirmed that fatty acids bind to oxy-MB and demonstrated that overexpression of a non-lipid

binding mutant of MB could not increase respiration in cultured thermogenic adipocytes [19]. This suggests oxyMB to act as a lipid chaperone and shuttle, similar to fatty acid binding proteins, and to deliver fatty acids to mitochondria for UCP1 activation and β-oxidation. Regarding a possible impact of lipid composition by MB, researchers reported that the lipid content of all major lipid subgroups (triglycerides, diglycerides, total cholesterol, phospholipids, and total lipids) in iBAT was not affected by MB deficiency. However, iBAT in MBko animals encompassed more palmitate incorporated into diglycerides and less as a free FA, possibly underlying the diminished UCP1 expression. This downregulated UCP1 expression was associated with increased transcript expression levels of genes involved in FAs synthesis, elongation, and desaturation, all promoting lipogenesis [12]. Taken together, MB contributes to regulating lipid synthesis and storage as well as FA metabolism in BAT. **Figure 3** illustrates the different roles of MB in regulating lipid and mitochondrial metabolism in brown adipocytes.

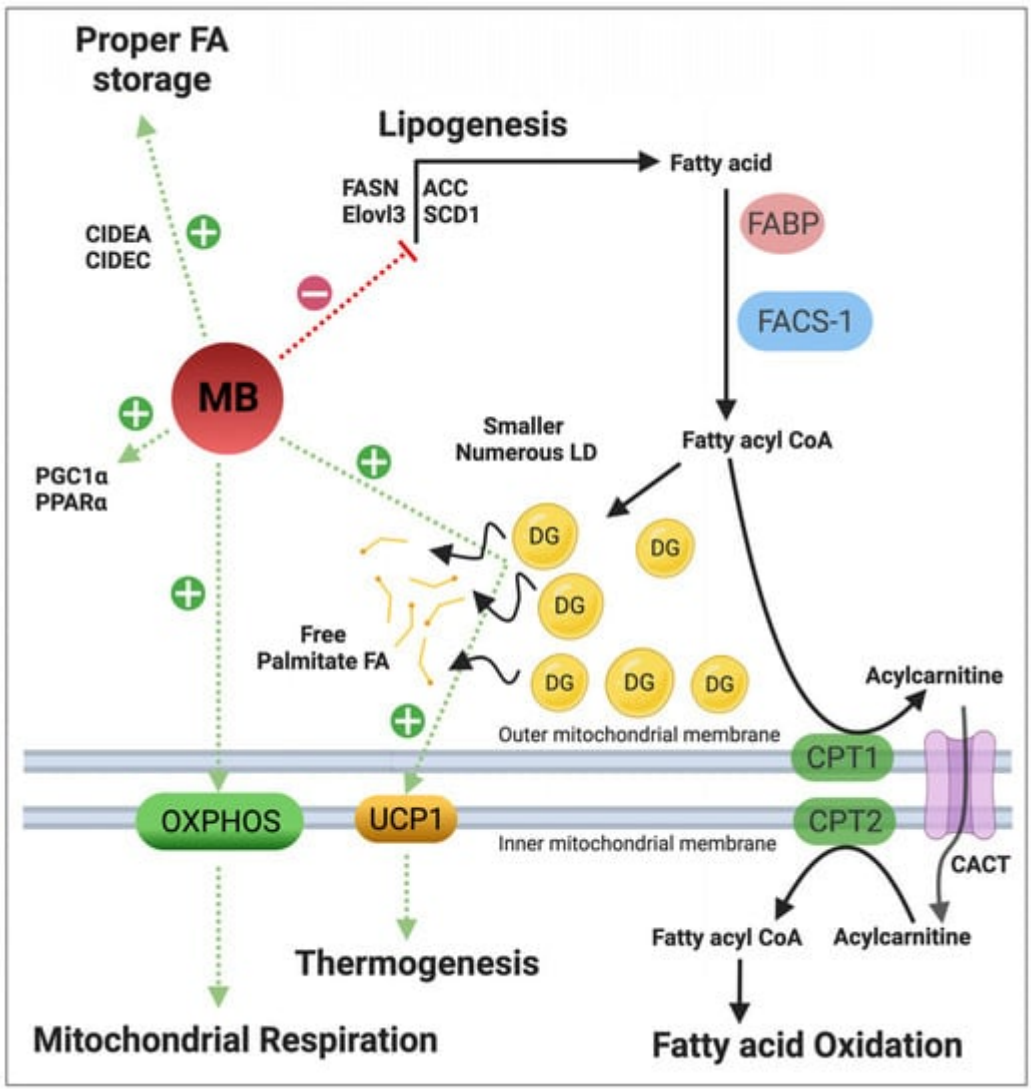

Figure 3. Illustrative scheme of proposed roles of MB in regulating lipid and mitochondrial metabolism in BAT. MB enhances mitochondrial respiration, positively stimulates expression of oxidative phosphorylation proteins (OXPHOS) and upregulates abundance of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and peroxisome proliferator activated receptor α (PPARα). MB expression was also associated with enhanced expression of the thermogenic marker uncoupling protein 1 (UCP1). MB aids in proper fat storage into smaller and

numerous lipid droplets by upregulating expression of lipid droplet membrane proteins: the lipolytic regulator cell death-inducing DNA fragmentation factor- a-like effector A (CIDEA), fat-specific protein 27 (FSP27 or CIDEC), ultimately increasing surface area of energy expenditure. MB promotes shuttling of C16:0 palmitate fatty acid (FA) from diglycerides (DG) to the cytosolic pools (represented by free FAs). The increased levels of the saturated free FAs might be the reason behind stimulating UCP1 expression and uncoupling activity. Lack of MB was correlated with increased abundance of enzymes controlling de novo synthesis of FAs (lipogenesis): fatty acid synthase (FASN), elongation of very long chain fatty acid 3 (ELOVL3) and stearoyl-CoA desaturase1 (SCD1), probably as compensatory mechanism to increased long chain (limited) FA oxidation seen in absence of MB. Conversely, MB has no impact on expression of FA binding proteins (FABP) or on regulators of FA oxidation: acyl-CoA synthetase-1 (FACS-1/ACSL1), carnitine palmitoyltransferase-1 B isoform (CPT1B), carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyltransferase-2 (CPT2). Figure created by BioRender.com.

4.4. Regulation of NO• Metabolism

NO• and its downstream effector, cyclic guanosine monophosphate (cGMP), play a significant role in promoting the differentiation of brown adipocytes and their thermogenic gene signature via regulation of protein kinase G (PKG) [92][93][94][95]. Moreover, NO• plays a role in hypoxia-inducible factor-1 (HIF-1) stabilization and hence the expression of its target genes [96]. As detailed above, MB has NO• scavenging and producing activities at normoxic and hypoxic conditions, respectively, acting as an oxygen sensor [47]. At hypoxia, deoxy-MB-produced NO• inhibits cytochrome c oxidase and regulates mitochondrial biogenesis in different tissues [97][98][99]. By switching between oxy- and deoxy-form, MB might serve to regulate oxidative phosphorylation through NO• in muscle and heart. Since activated BAT has a high oxygen demand and NO• turnover, aspects of MB-NO• biology could be shared between muscle, heart, and BAT. Cold stimulation in rats leads to localized reductions in BAT oxygen partial pressure (pO_2) [100] and increased accumulation of the hypoxia marker pimonidazole [101]. Hence, a drop in pO_2 during BAT activation might stimulate MB expression, which, in turn, triggers NO• synthesis and accumulation through deoxy-MB to finally drive cGMP- and PKG-mediated thermogenesis [102][103][104][105]. Because MB expression levels did not affect gene expression of the NO• synthases (NOS1, NOS2, and NOS3) in BAT [12], an additional NO• source might also participate in regulating BAT metabolic phenotype (i.e., xanthine oxidoreductase [106]). On the other hand, NO• produced by deoxy-MB might act as a vasodilator and/or stimulate angiogenesis and, therefore, indirectly improve the supply of O_2 to the brown adipocytes [107], where it is required for FA biosynthesis. It would be interesting to examine whether NO• produced by deoxy-MB interferes with the molecular pathways regulating BAT phenotype as well as FA biosynthesis (Research gap#6). **Figure 4** summarizes the different functions carried out by MB as deoxy- and oxy-MB in brown adipocytes.

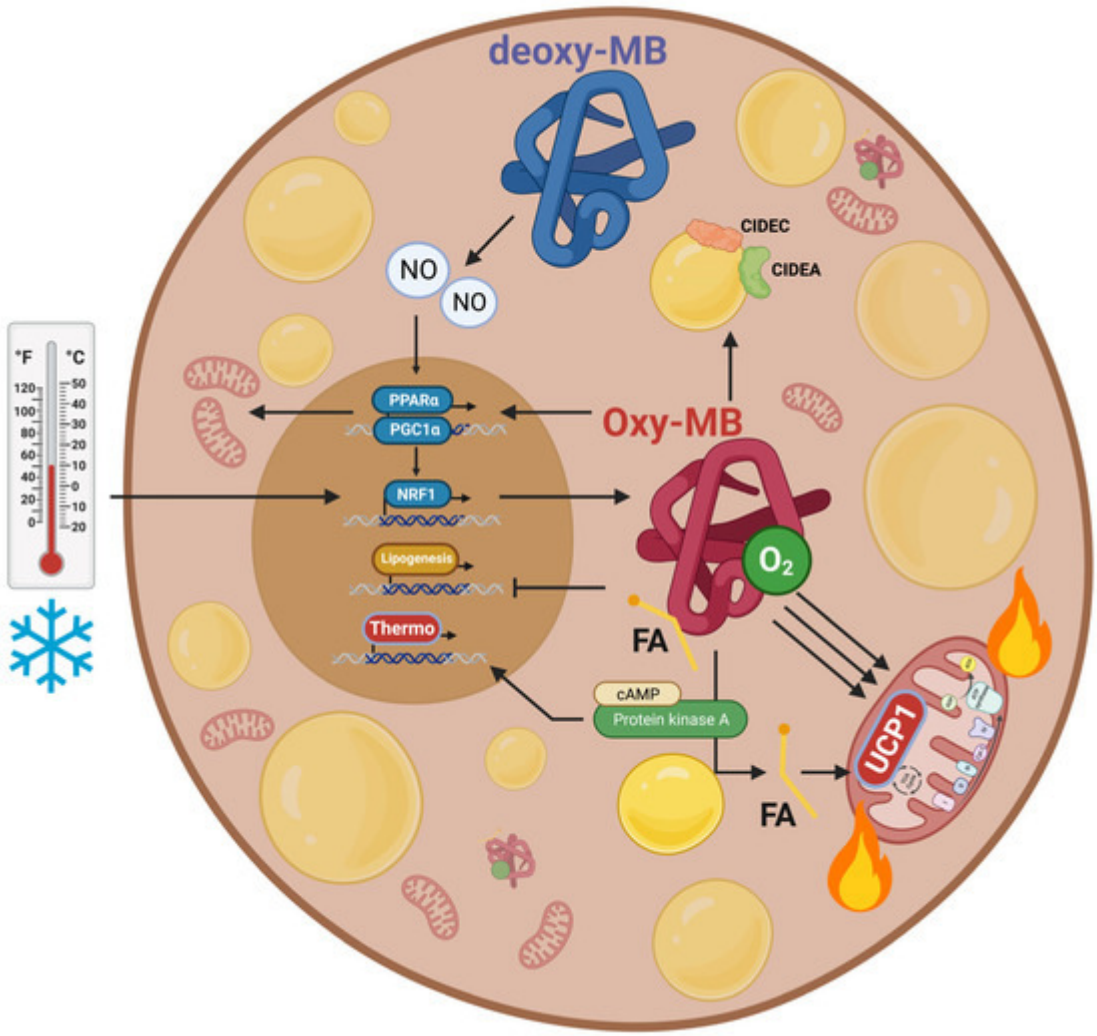

Figure 4. Oxy- vs. deoxy-MB roles in BAT metabolism. Oxygenated myoglobin (oxy-MB) can bind and shuttle fatty acids (FAs), enhance mitochondrial respiration, stimulate the expression of oxidative phosphorylation proteins (OXPHOS), and upregulate the abundance of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and peroxisome proliferator-activated receptor α (PPARα). MB expression was correlated with an elevated thermogenic marker uncoupling protein 1 (UCP1) expression. MB helps to properly store fat into smaller and numerous lipid droplets by upregulating the expression of lipid droplet membrane proteins: the lipolytic regulator cell death-inducing DNA fragmentation factor- a-like effector A (CIDEA), fat-specific protein 27 (FSP27 or CIDEC), ultimately increasing surface area of energy expenditure. MB promotes the shuttling of C16:0 palmitate FA from diglycerides to the cytosolic pools (represented by free FAs) by stimulating protein kinase A (PKA)-dependent lipolysis. The increased levels of the saturated free FA might be the reason behind the stimulated UCP1 expression and uncoupling activity. Lack of MB, in turn, was correlated with an increased abundance of enzymes controlling de novo synthesis of FA (lipogenesis). MB expression in brown adipocytes is under the transcriptional control of nuclear respiratory factor-1 (Nrf1) and is upregulated during cold. On the other hand, deoxygenated Mb can generate NO• from nitrite (NO_{2-}) when O_2 partial pressure drops. A sufficient degree of hypoxia is likely to occur under conditions of increased O_2 demand and O_2 flux, as seen in thermogenically activated BAT under conditions of β3-adrenergic stimulation or cold. NO• can induce mitochondrial biogenesis by impacting PGC1a and NRF1 transcription. NO• can also regulate hypoxia-inducible factor-1 (HIF-1) and increase cGMP levels, ultimately

regulating genes and pathways that support thermogenesis and brown adipocyte function. Hence, MB might impact brown adipocyte phenotype and activity through its O_2 and FA-sensing qualities. Figure was created with BioRender.com.

4.5. Regulation of Energy Expenditure and Clinical Implications

As discussed, MB seems to participate in controlling molecular and metabolic cellular mechanisms that govern BAT activity. Indeed, female NMRI mice with systemic MB expression deficiency increased body weight at 20 weeks of age, mainly due to increased WAT content [12]. Similarly, female C57BL/6N MBko mice gained more WAT mass than their wild-type counterparts despite equal energy intake and EE as estimated from indirect calorimetry [108]. Additionally, body and BAT temperatures tended to be lower in MBko than wild-type controls under thermoneutral conditions. These differences between control and ko mice reached significance at sub-thermoneutral temperatures (23 °C and 8 °C) [19]. Since NMRI MBko mice showed impaired adaption to cold, they exhibited a significant drop in body temperature and EE 6 h after transitioning from thermoneutrality to 8 °C with less locomotor activity and rearing [19]. These differences in the adaptability to cold were attributed to non-shivering thermogenesis because indirect calorimetry-measured oxygen consumption revealed MB-dependent differences in response to acute injection of the β3 adrenergic agonist CL [19]. However, another study investigated the effects of a high-fat diet and cold conditions on male and female mice with and without MB expression. This time, no differences in the respiratory exchange ratio, blood glucose levels, and energy expenditure estimates from indirect calorimetry were observed between the two groups [108]. As a consequence of this study, the presence of MB does not seem to significantly impact whole-body fuel choice and oxidation. However, despite similar energy intake and EE estimates, there was a significant increase in adiposity in female MBko mice compared to female control mice after 13 weeks of high-fat feeding. This was also true in another study when female mice were fed a standard chow diet [12]. Taken together, the cumulative evidence suggests that the absence of MB may have subtle metabolic effects that are difficult to measure in vivo, which could affect net energy balance and complicate interpretations based on the whole-body indirect calorimetry [108]. The loss of MB might induce metabolic alterations in BAT that impair BAT activation and thermoregulation in MBko mice housed at temperatures below thermoneutrality or after treatment with adrenergic agonists. However, the loss of MB did not translate into clear changes in whole-body energy expenditure.

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