Glucose metabolism in multiple sclerosis lesions: new insights in mitochondrial dysfunction and bioenergetic coupling

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CHAPTER 5

SUMMARY

Demyelinated axons in multiple sclerosis (MS) have an increased energy demand in order to maintain proper conduction. However, mitochondrial dysfunction and associated oxidative stress limit efficient glucose metabolism; a process that may play an important role in driving neurodegeneration in MS. Imaging and pathological studies indicate that glucose metabolism is altered in MS, although the underlying mechanisms remain elusive. In order to gain more insight into glucose metabolism in MS and to identify underlying pathways we investigated the expression of key enzymes involved in glycolysis, tricarboxylic acid (TCA) cycle and lactate metabolism in well characterized MS tissue. Expression levels of glycolytic enzymes were found to be highly increased in active and inactive MS lesions, whereas enzymes involved in the TCA cycle were differentially expressed. Importantly, we observed reduced expression levels of mitochondrial alpha-ketoglutarate dehydrogenase (αKGDH) in demyelinated axons. In inactive lesions, there is increased expression of lactate-producing enzymes in astrocytes, whereas axons mainly upregulate expression of lactate-catabolising enzymes. Our results implicate that glucose metabolism is highly increased in active MS lesions in both astrocytes and axons. In inactive MS lesions, we provide evidence for enhanced astrocyte-axon lactate shuttling. Bioenergetic coupling between astrocyte and axons may be pivotal for the survival of demyelinated axons. However, reduced axonal αKGDH expression may hamper adequate metabolism leading to mitochondrial dysfunction and associated axonal degeneration.
INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system where macrophages and T-cells infiltrate the brain and induce widespread demyelination. Over time, the number of newly formed lesions decreases and disease progression seems to be mainly driven by axonal and neuronal degeneration. Nowadays, evidence is emerging that mitochondrial dysfunction and associated oxidative stress are thought to play an important role in driving neurodegeneration. Demyelinated axons have enhanced energy demand in order to maintain proper conduction. As a result, axons in MS lesions have increased mitochondrial content, but, strikingly, decreased oxidative phosphorylation (OxPhos) activity and an increased number of mtDNA deletions, indicating that mitochondria are damaged and dysfunctional. To date, most studies have focussed primarily on OxPhos function to determine changes in energy metabolism. The OxPhos chain is the end stage of glucose metabolism, but is dependent on glycolysis and the tricarboxylic acid (TCA) cycle to provide electrons in order to maintain a proton gradient and produce ATP. Interestingly, essential glucose metabolites are increased in the cerebrospinal fluid (CSF), serum and the brain of MS patients compared to non-neurological controls. Moreover, glucose and lactate levels are increased in MS lesions, as determined with positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). Taken together, there is ample evidence that glucose metabolism is altered in MS brain tissue, however little is known about glycolysis and TCA cycle function in MS.

Glycolysis is the metabolic pathway in which glucose is metabolised into pyruvate by various enzymes, including the rate-limiting enzymes hexokinase (HK) and pyruvate kinase (PK). There are three different isoforms of HK of which hexokinase 2 (HK2) was shown to be the principal regulated isoform. HK rapidly phosphorylates glucose into glucose-6-phosphate, which is the initial step of the glycolysis, while the final step of the glycolysis is catalysed by PK. Pyruvate produced during glycolysis can be transported into mitochondria where it is converted into acetyl-CoA by pyruvate dehydrogenase (PDH) to fuel the TCA cycle. Two other rate limiting TCA cycle enzymes include, alpha-ketoglutarate dehydrogenase (αKGDH) and malate dehydrogenase (MDH), of which both enzymatic reactions result in the production of NADH.

Besides glucose, lactate can function as an important energy source in the brain. Lactate can be produced from pyruvate by lactate dehydrogenase (LDH), which forms a multiprotein complex consisting of the products of two different genes; LDHA and LDHB. LDHA converts pyruvate into lactate, whereas LDHB is involved in the generation of pyruvate. It has been proposed that lactate secreted by astrocytes via specific monocarboxylate transporters (MCT) can be taken up and oxidized into pyruvate by neighbouring cells. This metabolic coupling is also known as the astrocyte-neuron lactate shuttle (ANLS), and seems to be of particular importance in the grey matter. In the white matter, not astrocytes, but oligodendrocytes supply axons with lactate via MCT1 which is essential for proper axonal functioning.

Brain glucose metabolism is subjected to changes during aging and an altered glucose metabolism has been suggested to contribute to different neurodegenerative disorders including Alzheimer’s disease (AD) and Huntington disease (HD). It was shown that the activity and expression of different metabolic enzymes was altered in the brain of AD patients. Experimental studies demonstrated that increased glycolytic activity induces a pro-inflammatory phenotype.
in astrocytes and can induce cell death in neuronal cells. Moreover, dysfunction of specific TCA cycle enzymes such as, PDH and aKGDH, have also been associated to neurodegeneration. These studies illustrate the impact of altered glycolysis and TCA cycle function on brain functionality.

In order to gain more insight into the pathways involved in glucose metabolism in MS brain tissue we investigated the expression of key enzymes involved in glycolysis, TCA cycle and lactate metabolism in well characterized MS tissue. We show that the expression of glycolytic and TCA cycle enzymes is highly increased in active MS lesions. In inactive MS lesions, astrocytes evidently increase the expression of key glycolytic and lactate-producing enzymes, whereas axons mainly upregulate lactate-catabolising enzymes. Finally, we observed reduced expression levels of mitochondrial aKGDH in demyelinated axons. Taken together, we provide evidence for increased glycolysis, astrocyte-axon lactate coupling and axonal mitochondrial dysfunction in MS, which may contribute to the ongoing axonal degeneration.

**MATERIALS AND METHODS**

**Brain tissue**

Brain samples were obtained from 13 MS patients and 4 non-neurological controls, in collaboration with the Netherlands Brain Bank, Amsterdam, The Netherlands (coordinator Dr. Huitinga). Detailed clinical data are summarized in table 1. The study was approved by the institutional ethics review board (VU University Medical Center, Amsterdam, The Netherlands) and all donors or their next of kin provided written informed consent for brain autopsy, use of material and clinical information for research purposes.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously. In short, paraffin sections were deparaffinised in a series of xylene and ethanol. Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.3% H₂O₂ and the antigens were retrieved in citrate buffer (pH=6). Primary antibodies (see supplementary table 1) were diluted in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Roche diagnostics GmbH, Mannheim, Germany) and 0.05% Tween-20 (SigmaAldrich, St. Louis, MO). The sections were subsequently stained with EnVision horseradish peroxidase (DAKO, Glostrup, Denmark) followed by 3,3’ diaminobenzidine-tetrahydrochloridedihydrate (DAB; DAKO, Glostrup, Denmark). Sections were counterstained with haematoxylin for 1 min, washed with tap water and dehydrated in a series of ethanol and xylene baths and mounted with Entellan (Merck, Darmstadt, Germany). Negative controls were essentially blank.

To reveal the cellular localization, deparaffinised sections were incubated for 20 minutes in PBS containing 1% BSA, 0.05% Tween-20 and 10% Normal Goat Serum (NGS) followed by incubations with the primary antibody. Alexa Fluor® (Life technologies, Vienna, Austria) labelled secondary antibodies were used for fluorescent labelling. To reduce autofluorescence, sections were counterstained with Sudan Black (0.3% in ethanol 70%) (Sigma Life Science). Finally, sections were stained with Hoechst (diluted 1:1.000) (Molecular Probes, Invitrogen) to visualize cellular
nuclei and mounted with mounting medium (DAKO, Glostrup, Denmark). Images were taken with confocal microscope (TCS SP2, Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) equipped with an Ar/Kr laser (488 nm) and three HeNe lasers (543, 594 and 633 nm). In order to quantify the amount of axonal colocalization at least 9 images from active lesions and 9 from inactive lesions obtained from 3 different MS patients were used. All images were taken with 40x objectives except the images of αKGDH expression in axonal mitochondria which were taken with 63x objectives. The images were analyzed with ImageJ software, using the intensity correlation plug-in.

To visualize astrocyte-axon interactions, 8μm thick paraffin sections were used and treated as described above. Z-stacks were made with the confocal microscope and used for 3D reconstructions with IMARIS® software (Bitplane, Zurich, Switzerland).

**Statistical analysis**

1-way ANOVA with Bonferroni post-hoc test was used to assess difference in colocalization between the NAWM, active rim and inactive lesion center.

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Table 1. Clinical data of MS patients and non-neurological controls. SP = secondary progressive MS; RR = relapse remitting MS; PP = primary progressive MS; ND = not determined; NA = non applicable; M = male; F = female; A = active lesions; CA = chronic active lesions; CIA = chronic inactive lesions
RESULTS

**Increased expression of key glycolytic enzymes in active and inactive MS lesions**
Active MS lesions are characterized by the presence of densely packed macrophages throughout the lesion area, whereas in inactive MS lesions inflammation has abated. The expression of HK2 and PK was strikingly upregulated in both active and inactive MS lesions compared to the surrounding normal appearing white matter (NAWM), and predominantly localized to reactive astrocytes (Figure 1A-F). There were no marked differences observed in HK2 and PK immunoreactivity between control white matter and the NAWM (data not shown). Chronic active lesions are characterized by a rim of activated microglia and macrophages and a center devoid of inflammatory cells. The expression pattern of HK2 and PK in the active rim of chronic active lesions was comparable to that observed in active lesions. HK2 and PK expression levels in the inactive lesion center of chronic active lesions were similar as seen in inactive lesions (data not shown). To quantify the cellular colocalization of the different metabolic enzymes, we used chronic active sections, which allows comparison of active and inactive lesion areas within the same section. Quantification of HK2 and PK immunofluorescent double labelling with a pan-neurofilament marker (SMI312) illustrated that the expression of these key glycolytic enzymes was evidently increased in demyelinated axons, most notably in the inactive center of CA MS lesions (Figure 1G-N).

**Increased expression of key TCA cycle enzymes in active MS lesions**
PDH, αKGDH and MDH expression was distinctly increased in active MS lesions compared to the surrounding NAWM and predominantly localized to astrocytes (Figure 2 A-B, D-E, G-H). No marked differences were observed in PDH, αKGDH and MDH immunoreactivity between control white matter and the NAWM (data not shown). In inactive MS lesions, expression levels of PDH and αKGDH were similar as observed in the NAWM (Figure 2C,F). In contrast, MDH immunostaining was increased in inactive lesions compared to NAWM, albeit less pronounced compared to active lesions (Figure 2I). Axonal colocalization of PDH was marginally increased in active lesions and inactive lesions compared to NAWM. In contrast, axonal MDH immunoreactivity was significantly higher in inactive lesions than in the surrounding NAWM (Figure 3A-L). αKGDH expression in axons was unchanged in both active and inactive lesions compared to NAWM (Figure 3E-H). In line with previous studies, we observed increased expression of the mitochondrial marker porin in astrocytes and axons in both active and inactive lesions (data not shown)\(^6,34\). Despite an increased number of mitochondria, αKGDH expression remained unaltered, suggesting that αKGDH levels are reduced in axonal mitochondria. Triple immunofluorescent staining of αKGDH with porin and SMI312 demonstrated that αKGDH expression was significantly reduced in mitochondria in demyelinated axons compared to mitochondria in myelinated axons in the NAWM (Figure 3M-P).
Taken together, these results indicate that the expression of key glycolytic and TCA cycle enzymes is highly increased in active MS lesions. In inactive MS lesions, expression of glycolytic enzymes is increased in both astrocytes and axons, whereas the TCA cycle enzymes investigated show a differential expression pattern.
Increased astrocyte-axon lactate coupling in inactive MS lesions

Increased expression of HK2 and PK in inactive MS lesion might indicate enhanced glycolytic rate(s), which is generally associated with increased production and secretion of lactate. To determine whether lactate is rather metabolized or catabolized in MS lesions we analyzed the expression levels of LDHA and LDHB. Both LDHA and LDHB immunoreactivity were evidently increased in active MS lesions compared to the surrounding NAWM and predominantly observed
in astrocytes (Figure 5A-B,D-E). In inactive lesions, LDHA and LDHB expression levels were comparable to the NAWM (Figure 5C,F). Since LDH forms a multiprotein complex consisting of LDHA and LDHB we determined the ratio of LDHA/LDHb expression in astrocytes and axons in MS lesions in order to learn whether lactate is rather produced or catabolized. Astrocytic LDHA/LDHb ratio was increased in inactive lesions compared to the NAWM (Figure 5G). In contrast, the ratio of LDHA/LDHb immunoreactivity was decreased in axons in inactive MS lesions compared to the NAWM (Figure 5H). Thus, in inactive MS lesions, astrocytes express more lactate-generating enzymes, whereas axons contain enhanced levels of lactate-utilizing enzymes.

Under normal conditions oligodendrocytes supply axons with lactate. Here, we hypothesized, that in the absence of oligodendrocytes, astrocytes might provide demyelinated axons with lactate. Double-labeling of astrocytes and axons illustrated a tight interaction between astrocyte processes and demyelinated axons within inactive MS lesions. This cellular interaction was less prominent in the surrounding NAWM (Figure 5I-J). 3D reconstruction revealed that astrocyte processes can ensheath demyelinated axons almost completely (Figure 5K-L). We previously described that astrocytes in inactive MS lesions express increased levels of
GLUCOSE METABOLISM IN MS LESIONS

Figure 3. Axonal PDH, αKGDH and MDH expression in MS lesions. PDH (A-C), αKGDH (E-G) and MDH (I-K) expression is indicated in green and SM1312 positive axonal structures in red. The axonal colocalization of PDH (D), αKGDH (H) and MDH (L) was quantified. High magnification immunofluorescent staining of αKGDH (red), porin (green) and SM1312 (magenta) (M-O) and related quantification (P). *P<0.05, **P<0.01, as determined by two-way ANOVA with post-hoc Bonferroni correction.

monocarboxylate transporter (MCT) 1, which is involved in the secretion of lactate. Remarkably, axonal MCT2 expression, which is essential for lactate uptake, is reduced in inactive MS lesions. To determine whether demyelinated axons are able to take up sufficient lactate we investigated the axonal MCT2 expression in more detail. Although overall expression levels of axonal MCT2 are reduced in MS lesions, MCT2 remains expressed along the length of the demyelinated axons, including axonal structures in close contact with astrocytes (Figure 5M-P). In sum, our data shows increased expression of lactate producing enzymes in astrocytes in inactive MS lesions.
Axons have increased expression of lactate consuming enzymes and are more closely associated with astrocytes in inactive lesions.

**DISCUSSION**

In this study we provide a comprehensive overview of the expression of key glycolytic, TCA cycle and lactate-metabolising enzymes in MS lesions. We showed that the expression levels of these metabolic enzymes were consistently upregulated in astrocytes and to a lesser extent in axons in active MS lesions. Astrocytes and axons in inactive MS lesions displayed enhanced expression of glycolytic enzymes compared to NAWM. In inactive lesions, astrocytes express increased levels of lactate-producing enzymes, whereas the level of lactate-consuming enzymes was predominantly upregulated in axons. Finally, we observed that the expression of mitochondrial αKGDH was significantly decreased in demyelinated axons.

Our immunohistochemical analyses revealed increased expression of key glucose metabolising enzymes (GMEs) in active MS lesions, suggesting that the activity of the glycolytic and TCA cycle pathway is increased. However, the activity of GMEs are predominantly regulated at the post-translational level allowing cells to quickly adapt their glucose metabolism to changes in the environment. Hence, future experiments should aim to determine the activity of GMEs. On the other hand, previous studies have indicated that changes in the activity of TCA cycle and glycolytic enzymes are generally much higher compared to changes in expression levels. As such, altered expression of GME may reflect more apparent changes in activity, but further research is needed to verify that claim. Interestingly, it has been shown that increased expression of HK2 alone is sufficient to enhance the glycolytic flux. Increased expression levels of GME corroborates previous studies, which observed increased mitochondrial mass and OxPhos activity in astrocytes and axons in active MS lesions. Our results are also in line with imaging studies which revealed increased glucose and lactate metabolism in active MS lesions. Thus, although we only investigated GME protein level, substantial evidence suggests that the observed metabolic alterations are reflected by changes in the glycolytic and TCA cycle flux.

The expression of GME was most evidently increased in astrocytes, which is in line with our previous study where we demonstrated increased expression of GLUTs and MCTs in reactive astrocytes in active MS lesions. Active MS lesions are characterized by abundant inflammation, which can lead to enhanced glucose metabolism, since an intricate relationship between cellular metabolism and inflammation exists. In vitro experiments demonstrated that exposure of cultured astrocytes to pro-inflammatory cytokines increases glucose uptake and TCA cycle flux to much greater levels compared to cultured neurons. Moreover, since neuronal cell bodies reside at great distance from the axon, axonal structures can not increase protein expression as quickly as astrocytes, making them more dependent on the regulation of activity rather than transcription of GME. This may explain why the most prominent changes in GME expression levels in active MS lesions were observed in astrocytes.

We observed marked astrocytic expression of enzymes involved in glycolysis in chronic lesions suggesting that astrocytes may become more glycolytic in inactive MS lesions. This is supported by our observation of an increased LDHA/LDHB ratio in astrocytes in inactive MS lesions compared to surrounding NAWM, since high glycolytic rates coincide with enhanced production of lactate,
Figure 4. astrocyte-axon lactate coupling in MS lesions. LDHA (A,C) and LDHB (D,F) were moderately expressed throughout the NAWM and inactive MS lesions. In active MS lesions intense LDHA (B) and LDHB (E) immunoreactivity was observed. LDHA (Inset B) and LDHB (Inset E)(green) mainly localized to astrocytes as indicated by double labeling with GFAP (red). Quantification of immunofluorescent double labeling of LDHA and LDHB with GFAP (G) or SMI312 (H). Double-labeling of GFAP (green) and SMI312 (red) illustrates astrocytes-axon interaction in the NAWM (Inset I) and inactive lesions (J). 3D reconstruction of GFAP (green) and SMI312 (red) staining (K-L). Staining of MCT2 (M, magenta), SMI312 (N, green), GFAP (O, red) and corresponding overlay (P) in inactive MS lesions. *P<0.05 as determined by two-way ANOVA with post-hoc Bonferroni correction.
also known as the Warburg effect. Moreover, lactate levels are enhanced in inactive MS lesions and MCT1, which is involved in lactate secretion, is markedly increased in astrocytes. Taken together, our data suggest, that astrocytes in inactive MS lesions may become more glycolytic resulting in enhanced production of lactate. Lactate levels are also increased in the CSF and serum of MS patients and correlate to disease progression. Here, we identified astrocytes as the major source of lactate in MS lesions, hence, serum lactate levels may partially reflect astrocyte activity making it an interesting biomarker to monitor astrocyte function. In inactive MS lesions, axons upregulated the expression of HK2, PK, as well as PDH and MDH, which suggests that axonal glucose metabolism is increased. Axons do not only depend on glucose to fulfil their energy needs, but also lactate is essential for proper axonal function. Under normal conditions oligodendrocytes supply axons with lactate, however most oligodendrocytes are lost during the inflammatory attack in MS lesions. Axons in inactive MS lesions have a reduced LDHA/LDHB ratio compared to the NAWM, which suggests increased lactate utilization by demyelinated axons. Moreover, we showed that demyelinated axons express abundant MCT2, despite an overall decrease in protein expression. Since extracellular concentrations of lactate are increased in inactive MS lesions this may result in ample uptake and metabolism of lactate. Interestingly, we observed that astrocytes and axons are more closely associated in inactive MS lesions compared to the NAWM. This is in line with a previous study which showed tight interactions between astrocytes and axonal membranes in MS lesions using electron microscopy. It has to be noted that we only used GFAP as an astrocytic marker. Since GFAP expression is increased in reactive astrocytes, this may contribute to the increased astrocyte-axon interactions we observed. Therefore, different astrocyte markers should be included to verify our results. Taken together, our observations suggest, that in MS lesions, there may be increased astrocyte-axon lactate shuttling in the absence of oligodendrocytes. Demyelinated axons require increased amounts of energy to maintain proper conduction, which likely explains the upregulation of GME. Remarkably, we observed reduced αKGDH expression in axonal mitochondria in MS lesions. αKGDH is considered to be one of the rate-limiting enzymes of mitochondrial metabolism because it has the lowest activity and is the most regulated mitochondrial enzyme. Therefore, reduced expression of αKGDH may hamper sufficient mitochondrial energy production. Increased glucose metabolism may result in increased oxidative stress since ROS are formed during OxPhos. In fact, there is extensive oxidative damage in MS lesions, which could lead to reduced levels of αKGDH. αKGDH activity is blocked by oxidative stress, which serves as a mechanism to limit ROS production by reducing TCA cycle flux and OxPhos activity. Interestingly, αKGDH is also known to produce ROS and recent findings suggest it may even be the principal source of ROS in the mitochondria. Mahad et al. previously showed there is decreased complex IV activity in a subset of SMI32-positive axons despite an increased number of axonal mitochondria. It would be interesting to determine whether αKGDH levels are reduced in this subset of axons with decreased OxPhos activity. Thus, increased glucose metabolism in demyelinated axons results in oxidative stress, which may reduce αKGDH levels and OxPhos activity leading to axonal degeneration.

In conclusion, we show for the first time that enzymes involved in glycolysis, TCA cycle and lactate metabolism are differentially expressed in MS lesions. Future research is warranted to determine
the activity of the different metabolic pathways. Our results implicate that glucose metabolism is highly increased in active MS lesions in both astrocytes and axons. In inactive MS lesions, we provide evidence for increased astrocyte-axon lactate shuttling, however reduced axonal αKGDH expression may hamper adequate metabolism, leading to mitochondrial dysfunction and associated axonal degeneration.

REFERENCES


60. Ng, LF, Gruber, J, Cheah, IK, et al. The mitochondria-targeted antioxidant MitoQ extends lifespan and improves healthspan of a transgenic Caenorhabditis.


**SUPPLEMENTARY DATA**

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**Supplementary table 1. Antibody details.** \(^1\)Sources: Abcam, Cambridge, UK; Covance, Emeryville, CA; Dako, Glostrup, Denmark; Novus Biologicals, Littleton, CO; Sigma-Aldrich, St Louis, MO;