Glucose Pathways Adaptation Supports Acquisition of Activated Microglia Phenotype

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With its capacity to survey the environment and phagocyte debris, microglia assume a diversity of phenotypes to respond specifically through neurotrophic and toxic effects. Although these roles are well accepted, the underlying energetic mechanisms associated with microglial activation remain largely unclear. This study investigates microglia metabolic adaptation to ATP, NADPH, H⁺, and reactive oxygen species production. To this end, in vitro studies were performed with BV-2 cells before and after activation with lipopolysaccharide + interferon-γ. Nitric oxide (NO) was measured as a marker of cell activation. Our results show that microglial activation triggers a metabolic reprogramming based on an increased glucose uptake and a strengthening of anaerobic glycolysis, as well as of the pentose pathway oxidative branch, while retaining the mitochondrial activity. Based on this energy commitment, microglial defense capacity increases rapidly as well as ribose-5-phosphate and nucleic acid formation for gene transcription, essential to ensure the newly acquired functions demanded by central nervous system signaling. We also review the role of NO in this microglial energy commitment that positions cytotoxic microglia within the energetics of the astrocyte–neuron lactate shuttle. © 2014 Wiley Periodicals, Inc.

Key words: glutamate–glutamine cycle; glycolysis; lactate; nitric oxide; pentose; phosphate pathway

In the central nervous system (CNS), quiescent microglia constitute the first line of defense (Falsig et al., 2008), reacting to acute damage or infectious agents. In a dynamic equilibrium between lesion progression and the environment, microglia adopt a diversity of phenotypes ranging from the proinflammatory M1 to the neurotrophic M2 (Luo and Chen, 2012). In response to a diversity of signals, the microglia transition to an activated state and migrate to the lesioned area, inducing phagocytosis, massive changes in gene expression, and reorganization of the cell phenotype to modify neuronal survival directly. Thus, in the same environment, activated microglia modify the responses of supporting cells through release of a diversity of factors (Kettenmann et al., 2011).

Microglia express a broad set of genes encoding proteins that include but are not limited to cytokines, chemokines, neurotrophins, neurotoxic factors, and proteases. Depending on the intensity of damage and the time postinjury, a cross-talk with neurons and astrocytes induces adaptation of the microglial phenotype to favor debris clearance, necrosis, and tissue repair or regeneration (De Yebra et al., 2006). For example, with proper timing and mode of activation, microglia work as efficient antigen-presenting cells that stimulate T cells and affect the balance between neurotrophism and cytotoxicity.

Reactive microglia mediate this diversity of processes in coordination with reactive astrocytes, and both cell types depend on glucose metabolism for feeding their activities. After glucose uptake, hexokinase (HK; E.C. 2.7.1.1) mediates glucose phosphorylation, yielding glucose-6-phosphate (G6P), a common precursor of glycolysis for energy and lactate production and of the pentose–phosphate pathway (PPP). In microglia, the PPP not only renders ribose-5-phosphate for nucleic acid synthesis but also NADPH + H⁺ redox equivalents through its oxidative branch, to be transformed at the external cytoplasm membrane by NADPH oxidase (E.C. 1.6.3.1) in superoxide ions for defense and oxidative stress (Wu et al., 2006; Yang et al., 2007). The rate-limiting step in glycolysis is catalyzed by 6-phosphofructokinase (PFK1; E.C. 2.7.1.1) and in PPP activity by glucose-6-phosphate dehydrogenase (6GPD; E.C. 1.1.1.49). The interconnected reactions between these two pathways facilitate a direct and rapid metabolic modulation that covers the cell demand in these diverse end products.

At present the adaptation of glucose pathways to feed microglia diversity of phenotypes remains unclear, and we

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hypothesized that it might in part mimic the adaptation described for astrocytes. In astrocytes, nitric oxide (NO) formation triggers a very rapid PFK1 activation that poten-
tiates anaerobic glycolysis vs. oxidative phosphorylation (OXPHOS). This metabolic transition protects astrocytes from ATP depletion and maintains their mitochondrial membrane potential (Almeida et al., 2004).

To this end, in vitro metabolic studies were performed in BV-2 microglia, a suitable alternative model of primary culture (Henn et al., 2009), before and after activa-
tion with lipopolysaccharide (LPS) + interferon-γ (IFNγ), and NO production measured under each condition (Blasi et al., 1990). Here we provide evidence that, in association with NO formation, activation of microglia triggers a meta-
abolic reprogramming based on an increased glucose uptake and a potentiation of both the anaerobic glycolysis and the oxidative branch of PPP, while retaining a mito-
ochondrial activity. Essential to ensuring the new functions of activated microglia, ribose-5-phosphate availability for nucleic acid synthesis and gene transcription increases rap-
idly, as do ATP, lactate, and NADPH + H+. In the Dis-
cussion, the role of microglia lactate formation and glutamate uptake is considered within the neuroenergetics of the astrocyte–neuron lactate shuttle (ANLS; Bouzier-
Sore et al., 2003; Magistretti and Chatton, 2005; Magis-
tretti, 2006; Pellerin and Magistretti, 2012) of a four-part synapse, in which lactate is shuttling to neurons not only from astrocytes but also from microglia (Rodriguez, 2013).

MATERIALS AND METHODS

Materials

Murine BV-2 microglial cell line was purchased from Cell Bank (Interlab Cell Line Collection [ICLC], Geneva, Italy). RPMI medium supplemented with L-glutamine was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was from VWR Scientific (San Francisco, CA). Culture plates and flasks were purchased from Nunc (Roskilde, Denmark). LPS from Escherichia coli 0111:B4, IFNγ, and all metabolites and enzyme reagents were purchased from Sigma (St. Louis, MO).

BV-2 Cell Culture

BV-2 cells were cultured in RPMI 1640 medium with L-glutamine and supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidifier cell incubator containing 5% CO2 at 37°C. Before activation, cells were cultured at a density of 5 × 104 cells/ml for 24 hr and flasks were divided into three groups: 1) one group with no further manipulation, called control; 2) the LPS + IFNγ group, in which BV-2 cells were activated with LPS + IFNγ (0.1 μg/ml and 0.5 ng/ml, respectively) for 24 hr; and 3) the IL-4 group, in which BV-2 cells were stimulated with IL-4 (0.5 μg/ml) for 24 hr.

Inhibition of NO Synthesis in LPS- + IFNγ-Stimulated BV-2 Cells

To inhibit NO formation, BV-2 cells activated with LPS + IFNγ were incubated with 2.5 mM Nω-nitro-l-arginine (NLA), a potent inhibitor of nitric oxide synthase (NOS; Molnár and Hertelendy, 1992).

Quantification of NO and Tumor Necrosis Factor-α Production by BV-2 Cells

NO production was assessed in culture supernatants by the Griess reaction, a colorimetric assay that detects nitrite (NO2−) as a stable reaction product of NO with molecular oxygen (Green et al., 1982). Briefly, 50 μl of each sample was incubated with 25 μl Griess reagent A (1% sulfanilylamide, 5% phosphoric acid) and 25 μl of Griess reagent B (0.1% N-1-naphthylenediamine) for 5 min. Sample optical density was measured at 540 nm with a microplate reader (Sunrise-Basic Reader; Tecan). The nitrite concentration was determined from a sodium nitrite standard curve. Tumor necrosis factor-α (TNFα) released in the cell culture supernatant was determined by an ELISA murine TNFα kit (PeproTech, London, United Kingdom), following the manufacturer’s guidelines.

Real-Time RT-PCR

Total RNA from microglial cells was isolated using the NucleoSpin RNA/Protein kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Then, 2 μg first-
strand cDNA was synthesized with random primers using the First Strand cDNA Synthesis kit. The RT reaction was performed at 42°C for 60 min and then at 70°C for 5 min. Real-
time PCR was conducted using SensiFAST SYBR No-ROX One-Step mix and Applied Biosystems (Foster City, CA) Stem One Plus Real Time PCR Systems according to the manufac-
turer’s instructions. The PCR program was 2 min at 95°C for denaturation, subsequently 45 cycles of 15 sec at 95°C for amplification, and 1 min at 60°C for final extension. The level of mRNA expression was determined with a standard curve and normalized to the mRNA level of B2m. The PCR primers of B2m endogenous control; glucose transporters (GLUT) −1, −2, −3, −4, and −5; and glucokinase (GK) target genes were purchased from RealTimePrimers (Elkins Park, PA). The ΔΔCt method was used to analyze the data as described by Bookout et al. (2006). Primer sequences for target gene and endogenous controls are presented in Table I.

Glucose Consumption and Lactate Production

Culture medium was collected from control and LPS-
+ IFNγ-activated BV-2 cells at 0 and 24 hr. Then, glucose and lactate levels were determined with an ADVIA 2400 Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarry-
town, NY) that uses the glucose oxidase method described by Barham and Trinder (1972) and the method of Shimnojo et al. (1989) for lactate measurement. For statistical analysis of lactate release, a normal distribution of data was assumed (Voisin et al., 2010).

Lysate Preparation and Enzyme Activity Determination

Cells were grown to confluence, transferred to a 15-ml Falcom tube (BD Biosciences, San Jose, CA), and centrifuged at 1,000g at RT for 10 min. Pelleted cells were washed in 10 ml PBS 10 mM, pH 7.4; counted in a Neubauer counting
chamber; and centrifuged again. The pellet was suspended in enough ice-cold lysis buffer (50 mM Tris-HCl, 4 mM EDTA, 50 mM FK, 0.1% [v/v] Triton X-100, pH 7) to have 10^6 cells/ml, transferred to a 1.5-ml centrifuge tube, and homogenized (Potter’s homogenizer) at 11,000 min^-1. Then, the cell lysate was centrifuged at 14,000 g for 15 min at 4°C. The supernatant fraction was transferred to a centrifuge tube and stored at 4°C until the enzymatic assay was performed.

The enzyme activities of HK, PFK1, glucose 6-phosphate dehydrogenase (G6PDH), and lactate dehydrogenase (LDH; E.C. 1.1.1.27) were estimated spectrophotometrically in BV-2 lysate supernatants. The assays were performed at 30°C in 1 ml final volume of incubation buffer added to 100 μl cell supernatant in a Beckman Coulter (Brea) recording spectrophotometer. Then, the concentration of NADH or NADPH was monitored by measuring absorbance at 340 nm. One unit of enzyme activity (U) is defined as μmol/min, and specific activity is expressed as U/No. cells.

HK activity was assayed by measuring the rate of reduction of NADP^+ by G6PDH (Bergmeyer, 1983). The assay medium contained Tris-HCl 50 mM, MgSO4 80 mM, EDTA 20 mM, KCl 1.5 mM, mercaptoethanol 2 mM, NADP^+ 3 mM, ATP 2.5 mM, triethanolamine 200 mM, glucose 1 mM. PFK1 activity was measured by evaluating changes in the absorbance of NADH in a coupled enzyme assay (Beutler, 1975). The assay buffer consisted of Tris-HCl 50 mM, pH 7.4; MgCl2 100 mM; EDTA 5 mM; aldolase (mouse muscle, 0.04 U/ml); triose phosphate isomerase (mouse muscle, 2.4 U/ml); GAPDH (mouse muscle, 0.32 U/ml); and 1 mM fructose-6-phosphate and ATP.

LDH activity was measured by using the method previously described by Hakala et al. (1956). The assay buffer consisted of Tris-HCl 50 mM, pH 7.5; NADH 2 mM; and pyruvate 20 mM. G6PDH activity was assayed as described by Bishop (1966). The assay medium contained 380 mM glycerine, 304 mM hydrazine, 83 mM lactate, and 2.6 mM NAD disodium salt.

**Imaging of Mitochondrial Membrane Potential**

Mitochondrial membrane potential (Δψm) was measured by confocal imaging of BV-2 cells loaded with the fluorescent probe tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR), as previously described (Baczkó et al., 2004). In brief, cells were first incubated in six-well plates for 30 min in medium containing TMRE (50 nM) and then washed with fresh medium. Afterward, cells were incubated in the presence of 50 nM TMRE during the imaging process. Cell fluorescence was acquired by sequential scanning at the 510-nm emission wavelength. Cell average pixel intensity was obtained as an average of measurements in four fields of each of three culture wells. Images were obtained with a Zeiss Observer.Z1 microscope coupled to a Retiga EXi Fast 1394 camera, objective LD ×20/0.4 DICII (resolution 0.83 μm).

**Software and Data analysis**

Statistical studies were performed and graphics were generated using Graphpad Prism (GraphPad Software, La Jolla, CA). For each parameter, kurtosis and skewness moments were calculated to test the normal distribution of data. One-way ANOVA was used to analyze differences between the groups. In all cases P < 0.05 was considered statistically significant. Image acquisition was performed in Fluov4 software (Exploranova, Bordeaux, France). Figure 5 was produced in Servier Medical Art archive (Les Laboratoires Servier, Suresnes, France).

**RESULTS**

**BV-2 Cell Activation Increases Uptake and Phosphorylation of Glucose**

BV-2 cell activation was monitored by estimation of NO and TNFα release 24 hr after activation with LPS + IFNγ. At this time, LPS + IFNγ had increased NO production by sixfold (from 4.89 ± 0.85 to 29.62 ± 1.96, P < 0.0001) and TNFα by 3.5-fold (from 534.88 ± 113.94 to 1,948.58 ± 333.32, P < 0.0001) compared with controls.

BV-2 glucose consumption was compared between control and LPS- + INFγ-activated cells. In both situations, glucose was measured in the culture medium at times 0 and 24 hr, and results were referred to the BV-2 cell number. After activation, glucose consumption...
increased significantly with a fold change of 1.37 (Figs. 1A, 2D).

An increased energy demand requires greater microglial usage of glucose, and in other cells this is facilitated by the increased activity of HK to retain glucose within the cell and also by the increased expression of GLUT. To estimate the effect of cell activation on the glucose uptake and consumption by microglia, we determined the HK-specific activity and the expression of GK and the five main species of GLUT. Relative to control, LPS + IFNγ promoted a significant 1.9-fold increase of HK-specific activity in the soluble fraction of cell lysate (Figs. 1B, 2D). BV-2 glucokinase expression was analyzed by RT-qPCR. mRNA of this enzyme was amplified from total RNA isolated from control BV-2 cells, and no change was found after LPS + IFNγ activation (data not shown).

To identify which of the five members of the GLUT family are expressed in microglia, mRNA of GLUT-1–5 was analyzed in control cells by RT-qPCR. GLUT-1 expression was very predominant (91% of total), far from GLUT-4, second in abundance (8.2% of total). The very low expression of GLUT-2, GLUT-3, and GLUT-5 (<0.2% of total) was considered negligible. Twenty-four hours after LPS + IFNγ activation, both GLUT-1 and GLUT-4 expression had similarly increased, but this increase was significant for GLUT-4 ($P = 0.034$) and not for GLUT1 ($P = 0.057$). GLUT-2, GLUT-3, and GLUT-5 levels remained negligible.

**BV-2 Cell Activation Induces a Switch of Glucose Metabolism to Anaerobic Glycolysis and the Pentose Phosphate Pathway**

The increased glucose uptake and its phosphorylation measured in activated BV-2 cells may indicate an enhancement of anaerobic glycolysis, supported by the increased activity of PFK1, the key regulatory enzyme of the glycolytic rate. This enhancement is sometimes associated with an increased ROS production at the plasma membrane facilitated by the increased activity of G6PDH, the regulatory enzyme of the PPP.

Consistent with this possibility, G6PDH-specific activity was determined in the soluble fraction of cell lysate, together with PFK1- and LDH-specific activities and lactate extracellular concentration. Relative to control, LPS + IFNγ activation promoted at 24 hr a significant increase of G6PDH specific activity in a 1.95-fold change ($P = 0.004$; Fig. 2A,D). PFK1-specific activity was also significantly increased by 1.14-fold ($P = 0.0014$; Fig. 2B,D), and the same occurred with LDH activity, which increased by 1.4-fold ($P < 0.0001$; Fig. 2C,D).

Compared with control, lactate production was doubled in the activated cells, as evidenced at 24 hr by its 2.02-fold increased concentration in the culture medium ($P = 0.019$). In addition to the increased glucose uptake of the activated cells, the lactate level represents an increased and massive transformation of G6P into lactate, supported by a glucose/lactate ratio from 1.266 in controls to 1.874 in activated cells. This massive lactate production could be attributed to reduced mitochondrial activity and a switch to anaerobic glycolysis of the activated cells. This was assessed through a time-lapse confocal imaging of the inner membrane potential ($\Delta \Phi_{m}$) at 24 hr in control and activated cells with the mitochondrial membrane potential-sensitive dye TMRE (Fig. 3A). In both situations, the frequency distribution of mitochondrial fluorescence followed a normal distribution and was similar in absolute value and relative frequency distribution (Fig. 3B). The lack
of $\Delta \Phi_m$ difference found here indicates that the activated cells maintain their electron transport chain usage besides their anaerobic glycolysis switch and their increased PPP.

**NO Formation Participates in the Activated BV-2 Cells Glycolitic Activation**

Involvement of NO in the metabolic reprogramming of LPS-+IFNγ-activated BV-2 cells was studied after 2–5 mM Nω-nitro-arginine inhibition of iNOS for 24 hr. Glucose and lactate were measured in the culture medium at times 0 and 24 hr, and results were referred to the BV-2 cell number. iNOS inhibition resulted in a decreased of both glucose consumption (fold change of 0.82, $P = 0.02$) and lactate release (fold change of 0.63, $P < 0.001$; Fig. 4A,B) back to control values. No significant change in G6PDH was found (Fig. 4C), indicating that its activation is independent of NO.

**BV-2 Cell Adoption of a Phagocytic Phenotype Activates a New Metabolic Reprogramming**

To determine whether our results also apply to the adoption of a phagocytic phenotype, BV-2 cells were stimulated with IL-4 (0.5 μg/ml) for 24 hr. Glucose and lactate were both measured in the culture medium at times 0 and 24 hr and G6PDH activity in the homogenate as usual. With regard to control, glucose consumption was 30% reduced ($P < 0.001$) and lactate production 43% reduced ($P < 0.006$), whereas G6PDH activity was unchanged. Under these conditions, the maintenance of the PPP ensures that NADPH+H⁺ redox equivalents will be transformed into superoxide ions for phagocytosis, because G6DPH strongly increases its activity with higher NADP⁺ level. The new biomolecules delivered by phagocytosis directly available to the BV-2 cells would reduce at least in part the need for anabolic reactions and thus the cell energy demand. This possibility requires further experiments for validation.
DISCUSSION

BV-2 cells were used to investigate the microglial adaptation to an excitotoxic phenotype. This cell line is considered a suitable alternative model of primary culture or of many animal studies (Henn et al., 2009; Stansley et al., 2012). For example, 90% of the genes induced in BV-2 by LPS activation are also found in primary microglia, including the iNOS gene. Twenty-four hours after incubation with LPS + IFNγ, BV-2 cells had increased NO and TNFα production that was related to an enhancement of cell inflammatory activity (Ortega et al., 2013). With these conditions, the main finding of this study is the coordinated response of the glucose pathways necessary to cover the cell demand and allow the microglial response to activation. This metabolic reprogramming is different with microglial adoption of a phagocytic phenotype, because the delivery of new biomolecules may reduce the cell energy demand. In astrocytes, LPS stimulation induced NO production, leading to an increased glucose consumption and lactate release (Bolaños et al., 1994) and an increased PPP activity to prevent glutathione depletion and increase astrocyte resistance to NO-mediated cellular damage (García-Nogales et al., 1999). After exposure to peroxinitrite, the maintenance of astrocyte mitochondrial activity and its reduction in neurons also demonstrate the astrocytic resistance capacity to NO damage (Bolaños et al., 1995). The direct NO participation in the microglial metabolic reprogramming shown by our data reinforces the similarity to the astrocyte metabolic adaptation to excitotoxicity. As such, the increased HK activity and increased GLUT1 and GLUT4 expression ensure enough glucose availability to the cell. GLUT1 expression in control microglia was much higher than that of GLUT4, with similar changes 24 hr after activation. Because of this, these two transporters can be considered constitutive of microglia.

GLUT1 is a high-affinity glucose transporter, whose expression in tumor cells is stimulated under hypoxic conditions. This adaptation also includes enhancement of the glycolysis rate to support the increased energy demand of the proliferative cells (Bashan et al., 1992; Haber et al., 1998). The HK and GLUT1 increases found here would then reflect the microglial adaptation to an enhanced glycolytic demand with a high lactate production and high metabolic rate. GLUT4 expression is modulated in brain and pancreas by ATP-dependent potassium (KATP) channels whose activity depends on glucokinase (Choeiri et al., 2006). In agreement with previous brain studies (Alvarez et al., 2002), the low glucokinase Km that renders the enzyme unable to detect a glucose concentration increase may explain the unchanged glucokinase expression in activated microglia. The increased KATP channel expression described for activated microglia (Ramonet et al., 2004b; Ortega et al., 2013) may then participate in the GLUT4 expression increase. If this is true, the similar changes of GLUT1 and GLUT4 shown by our results suggest a coordinated microglial control of their expression that may depend, at least in part, on KATP channel activity. As observed in taste cells (Yee et al., 2011), this would represent a fine coordination between activation of microglia and increased glucose availability.

Microglial metabolic reprogramming led to a potentiation of anaerobic glycolysis reflected by the increased PFK1 and LDH activities and a massive lactate formation. In astrocytes, increased NO stimulates PFK1 activity through allosteric activation with fructose-2,6-bisphosphate (Fru-2,6-P2) produced by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Moreover, NO inhibition of cytochrome c oxidase switches the glycolytic rate on, with pyruvate conversion into lactate recovering NAD+ in the cytosol for glycolysis feedback (Almeida et al., 2004). The supply of glucose-derived lactate by activated microglia would support active neurons, whose elevated energy demand relies mostly on mitochondrial ATP production. Thus, with the astrocytes and microglia adapted to a major glucose anaerobic oxidation, lactate represents the major neuronal energy source in damaged CNS (Fig. 5).

The NO-mediated downregulation of mitochondrial energy production observed in neurons and initially
in astrocytes (Bolaños et al., 1994), but afterward considered to be unaffected by the same authors, with similar activities of the enzymatic complexes of the Krebs cycle (Bolaños et al., 1995), was not truly observed in microglia. The similar 

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of control and activated BV-2 cells might reflect the ATP synthase reversed reaction fed by ATP from glycolysis and other factors (Almeida et al., 2001) or could indicate an active electron transport chain energy production. In microglia of damaged brain tissue, the higher numbers of mitochondria labeled with \( [\text{H}] \text{PK11195} \) (De Yebra et al., 2006; Bernal et al., 2009) and their higher \( K_{\text{ATP}} \) channel expression (Virgili et al., 2011; Rodríguez, 2013) argue in favor of a significant mitochondrial production of energy to help ensure sufficient bioenergetics to execute the new transcription programs derived from excitotoxic activation. If this is true, feeding of the Krebs cycle to maintain mitochondria activity cannot rely on pyruvate because of its transformation into lactate but relies on 2-oxoglutarate formation from glutamate and glutamine deamination that increases progressively from apoptotic to necrotic damage (Ramonet et al., 2004a).

Upon activation, microglia express the two glutamate transporters EAAT-1 and −2 for glutamate and glutamine synaptic extrusion as well as glutamine synthase (Gras et al., 2006). Thus, in excitotoxicity, the fast microglial uptake of glutamate and glutamine, coordinated with the astrocytic glutamate removal, promotes a quick deactivation of the increased synaptic glutamate signal. For astrocytes, the early work by Pellerin et al. (1994) describes the glutamate-induced glycolysis as facilitating lactate to neurons during physiological activation and hypothesized for the first time the ANLS. With excitotoxicity, microglial glutamate uptake would induce a similar stimulatory effect to provide further lactate to neurons and also rendering abundant 2-oxoglutarate to ensure the Krebs cycle activity.

In microglia, glutamate deamination renders 2-oxoglutarate and ammonium that, combined with CO₂ released from the Krebs cycle, forms carbamic acid, a precursor of the pyrimidine bases necessary for nucleic acid synthesis and the increased gene expression. As in astrocytes (Gradinaru et al., 2012), carbamic acid direct clearance and elimination as a stable complex with glucuronides is also possible through a detoxification process (Hipkiss, 2010; Schafer et al., 2013).

In necrotic brain tissue, a net glutamine output of the glutamate/glutamine cycle caused by a reduced neuronal glutaminase activity (Ramonet et al., 2004a) increases the microglial formation of 2-oxoglutarate and ammonium and the generation of superoxide ions. After reaction with NO, superoxide ions form the highly reactive peroxynitrite products, associated with a cytotoxic microglia phenotype and a progressive microglia dysfunction (Svoboda and Kerschbaum, 2009). If this is so, microglial ammonium concentration would represent one of the contextual factors influencing the microglial net effects on neuronal survival (Luo and Chen, 2012; see Fig. 5).

The PPP oxidative branch was also increased in activated microglia, as shown by the increased G6PDH activity that supports G6P oxidation and NADPH formation. In neurons, NO facilitates a fine tuning of G6P’s destination to glycolysis or PPP that potentiates glutathione reduction against oxidative damage. Our data indicate

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**Fig. 4. Effect of stimulation with IL-4 and inhibition of iNOS with NLA on BV-2 cell metabolism (data are normalized for cell number).**

**A:** Glucose consumption was reduced 30% by IL-4 (\( *P < 0.001 \) vs. control) in control BV2 cells, and NLA reduced glucose consumption by 18% when applied to activated cells (\( \#P = 0.02 \), LPS + IFN + NLA vs. LPS + IFN). **B:** Lactate release was reduced 44% by IL-4 (\( *P < 0.001 \) vs. control) and NLA reduced this lactate production by 38% in activated cells (\( \#P = 0.001 \), LPS + IFN + NLA vs. LPS + IFN). **C:** IL-4 and inhibition of iNOS with NLA did not modified G6PDH activity.
that NO is also directly involved in the metabolic reprogramming of microglia, together with a significant contribution of the phagosomal NADPH-oxidase to ROS generation and oxidative stress damage from G6P (Wu et al., 2006; Miller et al., 2007). Furthermore, the juxtaposition of phagosomes and mitochondria recently proposed for microglia (Rodriguez, 2013) should potentiate mitochondrial ROS formation and energy production (Murphy, 2009; Dikalov et al., 2012; see Fig. 5).

In conclusion, NO appears to play a key role in the adaptation of activated microglia to the energy commitment necessary to ensure its adoption of a neurotrophic/cytotoxic phenotype. Under these conditions, the coordinated response of the four-part synapse, in which lactate is shuttling to neurons not only from astrocytes but also from microglia, has also to include the rapid glutamate removal by astrocytes and microglia. Thus, with brain damage, a new player should be included in the ANLS initially proposed by Pelerin et al. (1994), transforming it into a microglia–astrocyte–neuron lactate shuttle. Further studies are needed to determine how reduction of glutaminase activity following neuronal damage and the consequent increased ammonium output influence the microglial response of increased oxidative damage.

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Fig. 5. Proposed microglia–astrocyte–neuron lactate shuttle (MANLS). After microglial activation with LPS + IFN, glucose is mostly oxidized by astrocytes and microglia and converted into lactate that is taken up by neurons for its complete oxidation. Glutamine and glutamate synaptic removal by microglia fuels the tricarboxylic acid cycle to maintain mitochondrial activity and ATP generation. Initially, the resulting ammonium facilitates carbamic acid synthesis for pyrimidine formation and can also be cleared as carbamic acid glucuronides. Dashed arrows, type 1 represents glutamine and glutamate cycle; type 2 represents glucose metabolism; type 3 represents nondirect metabolic connections; type 4 represents ammonia uptake. Glc, glucose; Glu, glutamate; Gln, glutamine; Pyr, pyruvate; Lac, lactate; G6P, glucose-6-phosphate; G3P, glucose-3-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; KG, alpha ketoglutarate. NADPH, nicotinamide adenine dinucleotide phosphate; NTs, nucleotides; ROS, reactive oxygen species. TCA, tricarboxylic acid cycle; CbA, carbamic acid; CbA-GC, glucuronic acid synthesis from carbamic acid. NH4+, ammonium; CO2, carbon dioxide.
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