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Changes in the NMR Metabolic Profile of Human Microglial Cells Exposed to Lipopolysaccharide or Morphine

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Abstract

Microglial cells play a major role in host defense of the central nervous system. Once activated, several functional properties are up-regulated including migration, phagocytosis, and secretion of inflammatory mediators such as cytokines and chemokines. Little, if anything, is known about the metabolic changes that occur during the activation process. High-resolution ^1H nuclear magnetic

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resonance spectra obtained from perchloric acid extracts of human microglial cell cultures exposed to lipopolysaccharide (LPS) or morphine were used to both identify and quantify the metabolites. We found that human microglia exposed to LPS had increased concentrations of glutamate and lactate, whereas the cells exposed to morphine had decreased concentrations in creatinine, taurine, and thymine. Glutamate and creatinine were the key metabolites differentiating between the two stimuli. These results are discussed in terms of activation and differences in the inflammatory response of human microglial cells to LPS and morphine.

Keywords

microglia; metabolomics; NMR; LPS; morphine; activation

Introduction

Ramified microglial cells of the central nervous system (CNS) are referred to as resting or quiescent, whereas microglia activated by xenobiotics or injury contract and convert into an amoeboid, macrophage-like morphology. Microglia are resident immune cells of the CNS and, as such, perform functions similar to those of other tissue macrophages. Since the classification of microglia as a separate cell population in the 1930s, they have been studied in great detail (Rock et al. 2004, Rock and Peterson 2006). They constitute the first line of defense against invading pathogens and are “first responders” to a variety of brain injuries (van Rossum and Hanisch 2004; Vilhardt 2005). While it was widely accepted that they are in a quiescent state in the healthy brain (Werb and Chin 1983), it has been recently shown that even in this “quiescent” state they play an active role in surveillance of the brain parenchyma (Nimmerjahn et al. 2005). Indeed, microglial cells undergo comprehensive morphologic changes and are constantly surveying and sampling their environment by engaging in a protrusive activity, in order to monitor and control their environment as well as collect debris and/or metabolic products (Nimmerjahn et al. 2005). The motility of microglial cells is associated with the protein actin (Capani et al. 2001) with inhibitors of actin polymerization affecting their migration (Nolte et al. 1996). In the case of injury or presentation of a xenobiotic stimulus (e.g., the Gram-negative bacterial cell wall constituent lipopolysaccharide, LPS), microglial cells switch from random to targeted movement toward an injury or stimulus (Horvath and Deleo 2009). Thus, microglial cells are constantly dynamic not only when they are activated but also when they are “quiescent” or “resting” (Polazzi and Contestabile 2002).

Microglial cells are the predominant immunocyte in the CNS (Stoll and Jander 1999) where it is estimated they are present in numbers equal to neurons. Once activated, they acquire new functions, which include migration, phagocytosis, and secretion of inflammatory mediators such as cytokines and chemokines. These responses to insults or stimulation are specific and involve different cell signaling pathways and mediators (Baker and Manuelidis 2003). The activation process involves changes in cell phenotype and gene expression, including de novo expression of major histocompatibility complex (MHC) class I and II antigens, cell adhesion molecules, cytokines such as tumor necrosis factor (TNF- α) and interleukin-1 β (IL-1 β), and free radicals. When microglial cells are activated, they are able to

kill neurons in co-culture (Chao et al. 1996) and may thereby inflict damage to the CNS. Thus, activated microglia have been considered a potential neuropharmacologic target in certain CNS infections and neuroinflammatory/neurodegenerative diseases (Rock and Peterson 2006). The mechanism by which this so-called dark side of microglia is expressed involves the release of inflammatory mediators and neurotransmitters such as glutamate (Bal-Price and Brown 2001; Peterson et al. 1994). In vitro studies have demonstrated that when microglia are activated by LPS, they produce robust quantities of cytokines (Chao et al. 1992, 1995b; Peterson et al. 1995; Sheng et al. 1995), free radicals (Chao et al. 1995a), and inflammatory mediators that contribute both to brain defense and damage (Rock et al. 2004; Rock and Peterson 2006).

Recent research in the chronic pain and opioid tolerance literature has suggested that opioids can also activate rodent microglial cells (Horvath and DeLeo 2009; Hutchinson et al. 2008). Whereas the former study implicated opioid receptors in triggering migration in vitro, the latter study found that this activation process is mediated by non-opioid receptors in vivo, i.e., the target of LPS, toll-like receptor (TLR) 4. Although the literature is replete with studies of brain microglial cell activation by LPS, little attention has been paid to the response of these cells to opiates such as morphine. Research in our laboratory has shown that human microglia possess functional mu-opioid receptors (MORs), the stimulation of which can inhibit their migratory activity (Chao et al. 1994, 1997; Hu et al. 2000). Because of this lack of consensus across laboratories or species (pure vs. mixed, rodent vs. human, in situ vs. in vitro), we compared the effect of morphine on these primary cultures of human microglial cells with that of LPS.

Because little, if anything, is known about the metabolic changes that occur during the activation process of microglia, we set out in the present study to characterize the metabolomic profile of human microglia following activation by LPS, and for comparison, cells exposed to morphine. To elucidate this information, we used ¹H nuclear magnetic resonance (NMR) spectroscopy to both identify and map the metabolic changes, which could potentially be used as biomarkers for the “activated” state. Applied in vivo, this non-invasive diagnostic tool may have potential application in several neuroinflammatory and neurodegenerative diseases not only for early detection and screening but also for monitoring treatment with pharmaceuticals.

Materials and methods

Chemicals

The following reagents were purchased from the indicated sources: Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), penicillin, streptomycin, trypsin, LPS, perchloric acid; potassium hydroxide (KOH), and D₂O (Sigma-Aldrich, St. Louis, MO, USA); K-Blue substrate (Neogen, Lexington, KY, USA); heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA); morphine sulfate (supplied by the National Institute on Drug Abuse, Bethesda, MD, USA); rabbit anti-glial fibrillary acidic protein (GFAP, an astrocyte marker) and mouse anti-CD68 (a microglial cell marker) antibodies (DAKO, Carpinteria, CA, USA); human recombinant IL-1 β TNF- α , CXCL10 and CCL2, and anti-IL-1 β and -TNF- α antibodies (R&D Systems,

Minneapolis, MN, USA); anti-CXCL10 and -CCL2 antibodies (BD Biosciences-Pharmingen, San Diego, CA, USA).

Microglial cell cultures

Primary human microglial cell cultures were prepared as previously described (Chao et al. 1997; Peterson et al. 1997). Human fetal brain tissues were obtained from aborted fetuses under a protocol approved by the Institutional Review Board of the University of Minnesota. Briefly, brain tissues from 16–22-week aborted fetuses were obtained at the time of elective termination of intrauterine pregnancy from otherwise normally healthy individuals. Cells were dissociated by trypsinization (0.25%) for 30 min and then plated into 75-cm² tissue culture flasks in DMEM containing 6% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). After 14 days in culture with weekly medium change, floating microglial cells were collected, centrifuged, and reseeded in a 60-mm Petri dish at a density of 3×10^6 cells with fresh media. The cultures were washed 1 h after seeding to remove non-adherent cells. Purified microglia are composed of a cell population of which >99% stain with anti-CD68 antibody (a human macrophage marker) and <1% stain with anti-GFAP antibody (an astrocyte marker).

LPS and morphine exposure

Microglial cells were plated in 60-mm Petri dishes with fresh media and exposed to LPS (1 µg/mL) or morphine (3 µM) or saline (controls) for 24 h. At the end of the exposure time, culture supernatants were collected and stored at –80°C for cytokine and chemokine analysis.

Perchloric acid cell extraction

Cells were washed three times with PBS followed by addition of 200 µL of ice-cold 12% perchloric acid. Cells were then gently scraped with rubber scrapers, collected, and sonicated on ice twice for 30 s each (Fisher Scientific, Hanover Park, IL, USA). The sonicated lysates were centrifuged at 3,000×g for 20 min at 4°C. The supernatants were collected into cold tubes and the pellets were stored at –20°C for protein concentration measurement. The supernatants were neutralized with ice-cold 3 M KOH followed by centrifugation at 3,000×g for 20 min at 4°C. The supernatants were collected, lyophilized, and stored at –80°C.

Cytokine and chemokine determination

Supernatants of microglial cell cultures after treatment were harvested for enzyme-linked immunosorbent assay (ELISA) as previously described (Sheng et al. 2005). Briefly, ELISA plates (96-well) were coated with mouse anti-human IL-1β or TNF-α, and rat anti-human CXCL10 or CCL2 antibodies (1–2 µg/mL) overnight at 4°C. The plates were washed with PBS with Tween 20 and blocked with 1% BSA in PBS for 1 h at 37°C. After washing, culture supernatants and a series of dilutions of IL-1β, TNF-α, CXCL10 or CCL2 (as standards) were added to wells for 2 h at 37°C. Following washing, detection antibodies [goat anti-human IL-1β, TNF-α (2 µg/mL), CXCL10 or CCL2 antibodies (1 µg/mL)] were added for 90 min at 37°C followed by donkey anti-goat IgG horseradish peroxidase

conjugate (1:10,000) for 45 min. A chromogen substrate K-Blue was added at room temperature for color development, which was stopped with 1 M H₂SO₄. The plates were read at 450 nm to generate standard concentration curves for IL-1 β , TNF- α , and CXCL10 or CCL2. The detection sensitivity of the ELISA assay for IL-1 β and TNF- α was 3 and 10 pg/mL, respectively, and 30 pg/mL for CXCL10 and CCL2.

¹H NMR analysis

Lyophilized extracts were reconstituted in 50 mM phosphate buffer pH7.4 made in D₂O (Sigma-Aldrich). Trimethylsilyl-tetradecuteriosodium propionate (TSP) was added as an internal standard for metabolite concentrations and as a chemical shift reference. The pH was adjusted with either DCl (35%) or NaOD (30%) (Sigma-Aldrich). NMR experiments were performed at 25°C on a Bruker Avance 700 MHz spectrometer. Spectra were acquired using a 90° pulse every 6 s with 63,022 data points and an acquisition time of 3 s. The residual water peak was suppressed using a presaturation pulse of 1.5 s. Collection of 1,024 scans was performed on each sample. Resonance assignments were done using Chenomx software (Edmonton, Canada). Spectra were uploaded into the software and then Fourier-transformed with line broadening of 1 Hz. Metabolite assignment was done according to the chemical shifts and pattern of coupling constants and searched against the Chenomx library. Metabolite concentrations were determined after a baseline correction, using TSP as an internal standard.

Total protein concentration

The pellets collected from the PCA extraction were dissolved in 200 μ L NaOH (2 M) and the protein concentration was determined using the Lowry method (Lowry et al. 1951).

Data analysis and statistics

Metabolite concentrations are expressed as millimoles per milligram of total protein and were further normalized to the concentrations of phosphorylcholine for each sample. The ratios of the metabolite concentration in stimulated cells and their corresponding controls were determined. Student's *t* test and decision tree analysis were used to analyze the data along with receiver operating characteristic (ROC), which is a metric for comparing predicted and actual target values in a classification model. It is useful for organizing classifiers and visualizing their performance, by plotting the true positives rate vs. the false positives rate. ROC helps gain insight into the ability of the model to accurately predict the classes (Fawcett 2006). ROC area, also called area under the curve, is an estimate (probability) of the accuracy of the test. The significance of the ROC area was tested using the Wilcoxon rank sum test (Hollander and Wolfe 1999).

Results

Assignment of resonances in the NMR spectra

¹H NMR spectra of whole-cell extracts from human microglial cells, both control and after 24 h exposure to LPS or morphine, were collected and analyzed in order to characterize the metabolomic responses to these two stimuli. Spectral assignment to specific metabolites was based upon chemical shifts and coupling constant patterns compared to a reference in the

Chenomx library. A typical high-resolution spectrum obtained from a PCA extract of microglial cells is shown in Fig. 1. Hundreds of resonances were detected as depicted by the peak picking lines at the top of the spectrum. We were able to reliably assign several metabolites across the 14 samples analyzed: acetate, adenine, alanine, aspartate, beta-alanine, choline, creatinine, ethanolamine, glutamate, lactate, myo-inositol, phosphoethanolamine, pyruvate, taurine, thymine, valine, xanthine, and phosphorylcholine. The quantification of these metabolites was done using TSP as an internal standard. The concentrations were expressed as millimoles per milligram of total protein in the cell extract pellet; these concentrations were then normalized to that of phosphorylcholine as this latter was unchanged between treated and control cells.

Metabolomic responses to LPS

The quantification of the metabolites after exposure of human microglial cells to LPS (1 $\mu\text{g/mL}$) for 24 h revealed significant changes in the concentrations of glutamate and lactate and trends to changes for pyruvate and beta-alanine (Fig. 2 and Fig. 6S_suppl.). Concentrations of glutamate and lactate were highly up-regulated ($p < 0.05$) with an increase in their concentrations of 67% and 45%, respectively, in LPS-stimulated microglial cells compared to unstimulated controls. There was also a trend to significance for changes in pyruvate and beta-alanine concentrations ($p < 0.1$) in the LPS-stimulated human microglial cells compared to the controls, the former increasing by 66% and the latter decreasing by 27% in LPS-treated microglia.

Metabolomic responses to morphine

Morphine induced a significant down-regulation of thymine, creatinine, and taurine (Fig. 2 and Fig. 7S_suppl.). Creatinine concentration was significantly ($p < 0.01$) decreased by 30% in microglial cells exposed to morphine compared to controls. Taurine and thymine concentrations were also significantly ($p < 0.05$) reduced by 11% and 46%, respectively, when compared to controls. The beta-alanine concentration was unchanged. Morphine had no effect on any of the other identified metabolites.

Production of cytokines and chemokines by stimulated microglia

Media from the same microglial cells processed for the metabolomic analysis above were analyzed to determine cytokine and chemokine release. Consistent with our previous studies (Chao et al. 1992, 1995b), exposure of microglial cells to LPS resulted in the robust production of cytokines (IL-1 β and TNF- α) and chemokines (CXCL10 and CCL2) (Figs. 3 and 4). On the other hand, morphine itself (3 μM) induced no significant changes in secreted cytokines and chemokines after 24-h stimulation. Thus, unlike LPS, morphine did not activate human microglial cells as defined by production of inflammatory mediators and, if anything, morphine suppressed production of certain metabolites when compared to unstimulated control cells.

Data analysis and statistics

Decision tree learning is a method commonly used in data mining and machine learning to find one or more variables that can distinguish between two groups (i.e., LPS and morphine

treatment). We built decision trees in the machine-learning package Weka (Witten and Eibe 2005) and evaluated the resulting classifiers using 10-fold cross-validation. As shown in Fig. 5, we found that glutamate and creatinine could be used to distinguish between the two treatments, LPS vs. morphine. Creatinine had a critical ratio of 0.78 and a precision (percentage of items correctly assigned to a class) of 100% for morphine treatment and 78% for LPS treatment (Fig. 5, left panel). The recall percentage (percentage of items of a particular class correctly assigned to that class) was 71% for morphine and 100% for LPS. The ROC area for this analysis was 0.79, indicating good separation power for creatinine between LPS and morphine. The Wilcoxon rank sum test on the ROC area for creatinine yielded a significant p value of 0.0105.

Glutamate could also be used to distinguish the two groups with a critical ratio of 1.18 as shown in Fig. 5 (right panel). The precision was 83% for LPS and 75% for morphine, while the recall was 86% for morphine and 71% for LPS. The ROC area for glutamate was 0.68, indicating a fair-to-good separation power for glutamate between LPS and morphine exposure. The Wilcoxon rank sum test on the ROC area for glutamate trended to significance ($p < 0.1$).

Discussion

These ^1H NMR spectroscopy studies demonstrate for the first time to our knowledge that activation of human microglial cells by LPS is associated with metabolic changes in the cells including increased glutamate production, which is consistent with previous data showing increased glutamate release by rodent microglia following activation by LPS (Yawata et al. 2008). Glutamate plays a key role as a neurotransmitter in the CNS, acting as the principle excitatory neurotransmitter, which in excess can cause excitotoxicity (Bal-Price and Brown 2001). Glutamate has been shown to be released by activated microglia and to be responsible for the neuritic beading observed following microglial activation; this effect is mediated by activation of the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype and may predict the neuronal death caused by activated microglia (Liang et al. 2008; Takeuchi et al. 2005).

We also found that lactate levels in the LPS-activated microglial cells were 45% higher when compared to the controls. An increase in lactate levels has been associated with macrophage or microglial activation (Bal-Price and Brown 2001), as well as with the production of NO (Xiang et al. 2004). Lactate is an end product of anaerobic cellular metabolism, and increases in lactate are seen in pathologies that involve anaerobic conditions. Significant increases in lactate levels were found in the hypothalamus, striatum, thalamus, and frontal cortex in brains of rats after LPS administration (Chang et al. 2005). The results of the current study suggest that some of this increase may be attributable to microglial cell activation. Correlated increases in lactate are possibly a secondary effect of the increase in glutamate as can be seen in the Fig. 8S (supplemental), where its metabolic pathways are linked to that of glutamate.

Whereas glutamate levels were high in LPS-activated microglia, no significant changes were evident in glutamate concentrations in cells exposed to morphine. Indeed, decision tree

analysis demonstrated glutamate to be a key metabolite differentiating microglia treated with LPS from those treated with morphine (Fig. 5). In fact, none of the changes seen with morphine were detected in the LPS-activated microglial cells. However, morphine induced a decrease in taurine, creatinine, and thymine levels. Creatinine was a key metabolite distinguishing morphine from LPS exposure. Creatinine's involvement in high energy oxidative metabolism (Miller 1991) together with the decrease in thymine suggests the possibility that morphine exposure leads to increased DNA synthesis. Indeed, the thymine pathway (Fig. 10S, supplemental) affected by morphine involves thymidine phosphorylase, dihydrouracil dehydrogenase, cytosine deaminase, and dihydropyrimidine dehydrogenase, all of which are involved in pyrimidine and beta-alanine metabolism.

We also quantified the levels of selected chemokines and cytokines released by human microglial cells (the same cells analyzed metabolically above) after LPS and morphine treatment (24 h). Morphine alone induced no significant increase in the production of these inflammatory mediators, in marked contrast to the LPS-stimulated cells.

LPS is known to activate microglial cells through TLR4, upon binding of the CD14 protein (Miyake 2004). In mouse microglia, LPS up-regulates IL-1 β , inducible nitric oxide synthase (iNOS), TNF- α , IL-6, cytochrome b245 β (gp91^{phox}), IP-10 (CXCL10), MIP-2 (CXCL2), and MCP-1 (CCL2) (Yawata et al. 2008), through NF- κ B and MAPK signaling pathways (Watkins et al. 2009). High glutamate concentrations after LPS stimulation can act through the metabotropic glutamate receptor family (GRM1, GRM2, GRM3) (see Fig. 8S) that in turn regulates the NF- κ B pathway. Other proteins that could be involved in this metabolic pathway are represented in Fig. 8S (supplemental). All these proteins either regulate glutamate signaling or are regulated by glutamate.

Morphine seems to affect human brain microglial cells through a different signaling pathway than LPS; this finding contrasts sharply with findings in rodent spinal cord microglia in situ, which may respond similarly to morphine and LPS (Hutchinson et al. 2008; Watkins et al. 2009). On the other hand, in vitro exposure of rodent microglial cells to morphine triggers migration, another indicator of activation, via an action at opioid receptors (Horvath and DeLeo 2009). We found that morphine produced none of the changes caused by LPS exposure; neither elevated glutamate and lactate nor increased release of cytokines and chemokines. In fact, morphine has been shown to reduce inflammatory reactions by down-regulating NF- κ B in rodents (Walker 2003) and inhibited LPS-induced increases in the expression of iNOS, TNF- α , MIP-1 α , IL-6, IL-1 β , and gp91mRNA (Qian et al. 2007). Morphine protection against the LPS microgliosis, which occurs through inhibition of the translocation of p47^{phox}, is mediated by inhibition of the phosphorylation of p47^{phox} in an extracellular signal-regulated kinase-mediated manner (Qian et al. 2007). The metabolomic pathways that seem to have been affected by morphine, as suggested by the Ingenuity pathway analysis, are represented in Fig. 9S (supplemental). Although the mechanism, by which creatinine and taurine concentrations are reduced after morphine exposure, remains unclear, this decrease in the concentration of these two metabolites could explain how morphine can reduce the inflammatory response by altering this metabolic pathway (Fig. 9S).

Our findings, regarding metabolomic profiling of human microglia upon stimulation with LPS and morphine, show a major difference between the two stimuli. While the signaling pathways involved are not yet fully understood, morphine and LPS clearly affect human microglial cells differently, and morphine may affect human microglia differently than it does rodent microglia. Elucidating the basis for these differences could lead to increased understanding of both the role of microglial cells in pain modulation and the role played by these cells in neuroinflammatory and neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
¹H NMR (700 MHz) of perchloric acid extract of human microglia cells exposed to morphine (3 μM) for 24 h. *Ins* myo-inositol, *Tau* taurine, *Cr* creatinine, *Glu* glutamate, *Thy* thymine

Fig. 2. Normalized metabolite changes (in percent of their corresponding controls) in microglial cells after stimulation with LPS or morphine (24 h). Data (mean $\pm$ SEM) were derived from seven experiments with cells harvested from different donors. *Solid ellipses* indicate a significant change (Student's *t* test, *** $p < 0.01$, ** $p < 0.05$), *dashed ellipses* indicate a trend to significance (* $p < 0.1$)

Fig. 3. Chemokines secreted by human microglial cells after stimulations with morphine (3 μ M) or LPS (1 μ g/mL) for 24 h (** p <0.001). Data (means $\pm$ SEM) are from six separate experiments

Fig. 4. Cytokines (IL-1 β , *right panel*; TNF- α , *left panel*) secreted by human microglial cells after stimulation with LPS (1 μ g/mL) or morphine (3 μ M) for 24 h (** p <0.001). Data (means $\pm$ SEM) are from six separate experiments

Fig. 5. Decision tree of the morphine and LPS treatment of microglial cells for 24 h. Creatinine and glutamate have a critical ratio (stimulant/control) of 0.78 and 1.18, respectively. *The numbers between parentheses* represent the number of samples correctly and incorrectly classified, respectively