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Antibody-Enhanced Microdialysis Collection of CCL2 from Rat Brain

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Abstract

Chemokine(C-C motif) Ligand 2 (CCL2 or MCP-1) is a signaling protein that is released under various conditions. In this study we demonstrate the first microdialysis collection of CCL2 from rat brain tissue using antibody-enhanced microdialysis. A monoclonal antibody to CCL2 was included in the dialysis perfusion fluid as an affinity agent to enhance the recovery of CCL2 both in vitro and in vivo. In vitro it was found that the use of antibody affinity agent increases the relative recovery of CCL2 from $9.6\% \pm 3.4\%$ to $37.5\% \pm 10.2\%$ and $64.8\% \pm 11.7\%$ ($n=10$) at flow rates of $2 \mu\text{L}/\text{min}$ and $1 \mu\text{L}/\text{min}$, respectively. Following the in vitro observation, CCL2 was collected from rat brain with microdialysis sampling using both control and antibody-included perfusion fluids. The in vivo data showed that relative recovery was increased at all but the first time point. This shows that the use of free antibody in the perfusion fluid increases the relative recovery of CCL2 and this enhanced microdialysis method may be applicable to other cytokines.

Keywords

Microdialysis sampling; Antibody; CCL2/MCP-1; Enhanced Relative Recovery; Brain; Rat

Introduction

Chemokines (*chemoattractant cytokines*) are proteins released from immune cells that serve to chemically guide cells. The presence of cytokines, cytokine receptors, and the role of the immune system in the brain has been extensively described (Bajetto et al., 2001; Quan and Herkenham, 2002; Ransohoff and Benveniste, 2006; Watkins and Maier, 2005).

Chemokine(C-C motif) Ligand 2(CCL2)/ monocyte chemoattractant protein-1(MCP-1) is a 13 kDa chemokine that is physiologically active as its 26 kDa dimer. CCL2 serves to recruit monocytes/macrophages and has been actively studied in various immunological contexts (Daly and Rollins, 2003; Deshmane et al., 2009).

There has been significant interest in the functional roles of chemokines and their intracellular communication networks within the brain (Adler et al., 2005; Adler and Rogers, 2005). CCL2 has been shown to be expressed by microglia (Ambrosini and Aloisi, 2004). Additionally CCL2 recruits monocytes across the blood brain barrier (Chui and Dorovini-Zis, 2010). These observations have lead researchers to investigate the role of CCL2 in various neuroinflammatory states (Conductier et al., 2010), including, but not limited to,

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alcoholism (He and Crews, 2008), HIV-dementia (Dhillon et al., 2008), infarction (Kuriyama et al., 2009), and ischemia (Schilling et al., 2009).

A variety of different experimental approaches have been applied to elucidate the presence of cytokines in the central nervous system. These methods include: 1) mRNA analysis via *in situ* hybridization or Northern blot analysis (Shen et al., 2005); 2) radioimmunoassay or ELISA for protein content; 3) radiolabeled cytokine studies in brain slices using autoradiography to map out receptor sites; 4) immunohistochemistry; 5) different imaging methods to map receptors (Signore et al., 2010); and 6) different cell lines exposed to different permutations of cytokines to release neuropeptides or exposure of neuropeptides to release cytokines. While all these measurement methods serve their purpose to map out the different cytokine locations, none of these methods allows for real-time *in vivo* cytokine collection, which is of great interest to numerous neuroscience researchers.

The difficulty with all of these different techniques is that either they do not provide actual protein concentrations especially from the extracellular space or the animal has to be sacrificed to obtain protein content. For example, mRNA expression does not always equate to actual protein concentrations (Greenbaum et al., 2002; Maier et al., 2009).

Immunohistochemical analyses do not allow for measurements of concentrations over time within the same animal. For these reasons, there has been interest in collecting cytokines from the brain using microdialysis sampling. Microdialysis has been used to collect chemokines and cytokines in the brain, but these studies have been predominantly in humans since the length of the membranes is typically 10 mm vs. the 1 to 4 mm range used for rodents (Helmy et al., 2009; Maurer et al., 2008; Møllergaard et al., 2008).

Cytokines have been collected from rat brain under traumatic injury conditions (Woodroffe et al., 1991). The difficulty with collecting cytokines is their low basal (pg/mL) concentrations combined with low recovery through the microdialysis probe. We have typically collected cytokines with relative recoveries ($RR = C_{\text{dialysate}}/C_{\text{sample}}$) between 5 and 10% for 10 mm 100 kDa MWCO membranes (Ao and Stenken, 2006). For this reason, we have been using either free antibodies (Fletcher and Stenken, 2008) or antibodies immobilized to polymeric beads used in the typical Luminex assay for improvement of protein recovery into microdialysis sampling probes (Duo et al., 2006).

In this work, we used affinity microdialysis with a monoclonal (detection) antibody specific for CCL2. This antibody was included in the microdialysis sampling perfusion fluid allowing the collected dialysate to be quantified using a standard ELISA. To our knowledge, this is the first demonstration of *in vivo* microdialysis sampling with antibody enhancement of CCL2 collection.

2. Methods

2.1 In Vitro

Microdialysis probes (CMA 20 PES, 10 mm length, 100 kDa MWCO) were immersed into a 5 mL solution of 2 ng/mL CCL2 (Preprotech, Rocky Hill, NJ) in 10 mM PBS. The solution was heated to 35°C and stirred. An aECF perfusion fluid (153.3mM Na⁺, 4.3mM K⁺, 0.41 mM Mg²⁺, 0.71 mM Ca²⁺, 139.4 mM Cl⁻, pH 7.4) was used as a control. The aECF is an artificial extracellular fluid described by McNay and Sherwin (McNay and Sherwin, 2004). For antibody-included perfusions the aECF fluid was supplemented with the detection antibody from a BD Opt EIA kit (BD Biosciences, San Diego, CA) to a dilution of 1:500 from the provided material. The protein concentration for the antibody in solution is not provided and is considered proprietary by the manufacturer. Samples were obtained every

30 minutes during a 180 min time period. Samples were stored at -20°C and were quantified with BD optEIA MCP-1 Kit the following day.

2.2 In vivo

2.2.1 Surgery—Male Sprague-Dawley rats were purchased from Harlan Laboratories, Inc (Madison, WI). Rats were housed in an environmentally-controlled facility with a 12-hour on/off light cycle and *ad libitum* access to food and water. Six rats (275-350 g) were assigned to either a control or antibody-included perfusion regime. Isoflurane (5%) was administered at a flow rate of 0.8 L/min by vaporizer. The rat was secured to the stereotaxic unit and anesthesia was maintained by 1-2% isoflurane at 0.5 - 0.8 L/min as needed. Coordinates of 4.6 mm anterior to bregma, +0.5mm lateral, and +0.5 mm dorsal along the horizontal zero plane (ear bars) were used. After sampling, the probe was removed and the rat was euthanized by CO₂. All protocols were approved by the University of Arkansas IACUC.

2.2.2 Microdialysis—CMA 12 PES (4 mm, 100 kDa MWCO, CMA Microdialysis, Inc., North Chelmsford, MA) probes were inserted into the brain. The aECF perfusion fluid was used in one set of animals as a control (n=3). The aECF with antibody (BD Opt EIA detection antibody dilution of 1:500) was used in a second set of animals (n=3). After an initial 5 minute flush at 5 µL/min, a flow rate of 2 µL/min was used to collect dialysates, and samples were collected every 30 minutes for 3 hours. Samples were stored on ice, and kept frozen at -20°C, no longer than three days, until quantified using ELISA. We have previously demonstrated that samples remain stable for at least one week under these conditions.

2.3. ELISA

Samples were then quantified with BD optEIA Rat MCP-1 ELISA kit (BD Biosciences, San Diego, CA). To meet kit volume specifications, assay diluent (50 µL) from the BD optEIA kit was added to dialysate samples (50 µL) to a final sample volume of 100µL. A comparison of standards that included the 1:500 dilution of detection antibody to control standards was performed. Samples that included the diluted antibody were treated the same way as samples without antibody or standards. This included the required step of adding the detection antibody to all samples during the sample preparation steps involved with the ELISA. The UV absorbance was measured with a Tecan infinite M200 plate reader (Tecan, Research Triangle Park, NC).

2.4 Statistics

Antibody-enhanced values were compared to control values at each time point with a paired Students t-test at the 95% confidence level using Microsoft Excel[®]. ANCOVA was performed using JMP8[®] software (SAS Institute, Cary, NC).

3.0 Results

3.1. Calibration Curve Statistics

The calibration curves were compared using an Analysis of Covariance (ANCOVA). There was no significant difference found between the slopes of the calibration curves (normalized to log-log plots) between samples handled using the standard ELISA protocol and antibody-inclusion protocol, p=0.44.

3.2 In Vitro Microdialysis

Antibody enhanced microdialysis in vitro showed a significant increase in the relative recovery of CCL2 vs. control for both the 1.0 and 2.0 $\mu\text{L}/\text{min}$ flow rates (Figure 2). The recovery was $37.5\% \pm 10.2\%$ and $64.8\% \pm 11.7\%$ at flow rates of 2.0 $\mu\text{L}/\text{min}$ and 1.0 $\mu\text{L}/\text{min}$, respectively ($n=10$). For the controls, the recovery values were $9.6\% \pm 3.4\%$ and $12.2\% \pm 4.1\%$ ($n=10$), respectively, noting there is not a significant difference in the values.

3.3 In Vivo Microdialysis

Antibody-included microdialysis samples had CCL2 concentrations of 500 ± 30 , 810 ± 100 , 1720 ± 200 , 1735 ± 370 , 1590 ± 300 pg/ml at time points of 30, 60, 90, 120, and 150 minutes, respectively ($n=6$). At time point 180, antibody-included sample gave a concentration value over that for the highest standard (2000 pg/mL) and thus could not be quantified. Control samples gave a collected value of 560 ± 95 , 260 ± 85 , 310 ± 75 , 480 ± 55 , 700 ± 200 , and 700 ± 195 pg/mL at time points of 30, 60, 90, 120, 150, and 180 minutes respectively ($n=6$). A significant difference was found between controls and antibody included at time points of 60, 90, 120, and 150 minutes.

4.0 Discussion

In this work, we demonstrate the first usage of antibody-enhanced in vivo microdialysis sampling for the chemokine, CCL2. Pich et al. used antiserum against corticotropin-releasing factor and observed an approximate two-fold increase in their relative recovery (Pich et al., 1993). This increase is most likely caused by the binding to the antibody which serves to increase the flux of the targeted analyte into the dialysis probe. For CCL2, we observed an approximate 3.5 fold increase in the *in vitro* relative recovery. This approximate 3.5 fold increase held for the *in vivo* studies at the time points of 90, 120 and 150. This was somewhat unexpected since differences in the mass transfer properties between in vitro and in vivo experiments exist.

At the 30 minute time point, different scenarios could lead to the observed similar CCL2 concentrations. The first is that glial cells have been reported to contain MCP-1 (Hayashi et al., 1995; Magge et al., 2009). In these studies, samples were immediately collected after probe insertion. This is because the rat was under isoflurane anesthesia and after several hours it becomes tricky to maintain rodents under this anesthetic. If the probe insertion caused significant release in the CCL2 from disrupted glial cells, it is likely the concentrations will be much higher in the collected dialysate before the protein is cleared. Additionally, it is important to remember that microdialysis sampling provides a time-averaged sample (Stähle, 1992). Depending on the time average, it is possible that concentrations could be similar between both dialysis probes. Then, after the initial injury, the dialysis probe may serve as a sink to help clear out some of the initially released cytokines. At this point, cytokines may be coming from local cells surrounding the dialysis probe resulting in much lower concentrations collected into the dialysate.

Once the initial cells have started to respond to the injury by producing their own cytokines increases in concentration are observed at 90, 120, 150 minutes. We have previously observed such increases in cytokines after implantation injury in the subcutaneous tissue (Wang et al., 2007). At the 180 minute time point, the CCL2 concentration in the dialysate for the antibody-perfused probe was over the high end of the range (2000 pg/mL) and thus cannot be quantified.

Since this is a reported short communication, there is a significant amount of additional validation that would be necessary to more fully elucidate the biological signaling events that are occurring after probe insertion. Furthermore, our long-term goal is to perform these

studies in awake and freely-moving animals and the concentrations from such studies may be very different than observed here.

In mouse homogenized brain, measured concentrations of CCL2 were reported as 50 pg/mg protein and 275 pg/mg protein at time points of 90 and 180 minutes respectively after a lipopolysaccharide (LPS) injection (Thompson et al., 2008). It is our understanding that in rodents, CCL2 has only been extracted from brain tissue via the use of homogenized tissues. Although the exact amount of brain volume from which this protein amount has been derived is unknown, the homogenized tissue CCL2 concentrations are presented as a general reference to compare the concentrations.

Recently, the collection of CCL2 from human brain has been reported from head trauma patients (Helmy et al., 2011). From these patients, median CCL2 values found in dialysates were reported to be between 2500 – 2550 pg/mL. For comparison, it is important to note that human microdialysis catheters have 10 mm long membranes compared to the 4-mm membranes used in our study. Additionally, flow rates of 0.3 μ L/min were used in the Helmy et al. study vs. 2 μ L/min in this study. Smaller membrane lengths and higher perfusion fluid flow rates would lead to lower relative recovery values and lower collected CCL2 concentrations reported for this study. Here we report CCL2 concentrations in control dialysates between 260 and 700 pg/mL and antibody enhanced values as high as 1735 pg/mL throughout the experimental collection period.

There are many possible reasons for these differences between our values and those of others including the foreign body or wounding response to the probe, the difference in species, and other methodological differences between the different studies.

The optimization of the use of a detection antibody in the perfusion fluid allowed for straight-forward measurement of the samples using a standard ELISA plate and kit. For other protein targets, the method would have to be validated. This initial work presented here paves the way for trying the antibody-enhancement approach to many other soluble protein targets in the brain ECF.

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Abbreviations

CCL2	Chemokine(C-C motif) Ligand 2
MCP-1	Monocyte Chemoattractant Protein-1
MWCO	molecular weight cutoff

Research Highlights

- Chemokine, CCL2, a 26 kDa protein has been collected in rat brain using microdialysis sampling
- Antibodies in the microdialysis perfusion fluid increased CCL2 in vitro recovery 3.5 times
- In vivo recovery of CCL2 was increased using antibodies in the perfusion fluid.

Figure 1.
Graphical representation of standard microdialysis procedures vs. antibody-enhanced microdialysis

Figure 2.

Error bars represent a standard deviation of $n=10$ in $2 \mu\text{L}/\text{min}$ and control, and $n=5$ in $1 \mu\text{L}/\text{min}$. * Denotes a significant difference to the control at the 95% confidence level.

Figure 3.

Error bars represent a standard deviation were n=6. A * denotes significant difference to the control at the 95% confidence level. Antibody-Infused concentrations at time point 180 were over range with respect to the standard curve (> 2000 pg/mL).