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***In vivo* microdialysis sampling of adipokines CCL2, IL-6, and leptin in the mammary fat pad of adult female rats**

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Abstract

Adipocytes from white adipose tissue secrete cytokines and other bioactive proteins which are collectively termed adipokines. Adiposity has been linked with increased breast cancer risk as adipokines secreted by adipocytes significantly affect epithelial cells from which breast cancer arises. Measurement of extracellular adipokine concentrations that would be involved in signaling through mammary tissue is therefore of importance. In this work, microdialysis sampling was used to collect adipokines from the interstitial space of the mammary fat pad of female rats under isoflurane anesthesia. The adipokines CCL2 (MCP-1), leptin and IL-6 were quantified from dialysate samples and compared to total tissue concentrations surrounding the implanted probes. After three hours of microdialysis sampling at 1 $\mu\text{L min}^{-1}$, the respective median values for these adipokines in dialysate samples were approximately 175 pg mL^{-1} , 150 pg mL^{-1} and 750 pg mL^{-1} . Adipokine protein levels from dialysates were an order of magnitude lower than levels obtained directly from mammary tissue. However, the adipokine concentrations between excised tissue surrounding the microdialysis sampling probes and control tissue without implants did not differ. This work demonstrates the utility of microdialysis sampling to quantify mammary gland adipokine levels, with relevance to understanding mammary physiology.

Keywords

Adipokine; Microdialysis; Female Rat; Mammary Fat Pad

Introduction

Adiposity has been linked with increased breast cancer risk.¹ By volume, breast tissue contains approximately 90% adipocytes that comprise the white adipose tissue and 10% epithelial cells. Adipocytes within the white adipose tissue secrete signaling proteins which are collectively called adipokines.^{2, 3} Many different adipokines exist including CCL2, IL-6, IL-8, resistin, TNF- α , and VEGF.⁴ Cancer is believed to start from the breast epithelial cells and growth factors and cytokines secreted by adipocytes are suspected to significantly

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contribute to breast cancer progression.⁵⁻⁷ CCL2, a 13 kDa monomer that forms a dimer to be biologically active, plays a potent role in macrophage recruitment at the site of injury or infection.⁸ IL-6 is a 20.8 kDa pleiotropic cytokine exhibiting both pro- and anti-inflammatory properties, depending on cell context.⁹ The primary function of IL-6 is the initiation of acute phase response during inflammation and infection.^{10, 11} IL-6 activates multiple signaling pathways to control cellular proliferation and apoptosis. Upregulation of IL-6 and CCL2 in breast cancer promotes inflammation which in turn is linked to tumor progression. Leptin is a 16 kDa non-glycosylated protein hormone secreted mainly by adipose tissues in proportion to body mass index and is essential for normal mammary gland development.¹² Leptin expression occurs in normal mammary tissues, in breast cancer cell lines, and in tumors.¹³

Microdialysis is a well-established diffusion-based *in vivo* sampling technique that has been used for chemical sampling from extracellular fluid space in the fields of neuroscience, drug metabolism and pharmaceuticals.^{14, 15} A microdialysis probe is made up of a semipermeable dialysis membrane with a defined molecular weight cut-off (MWCO) and inlet and outlet tubing. A perfusion fluid that matches extracellular fluid in ionic strength and composition is pumped (at flow rates ~ 0.5 μL to 2 $\mu\text{L min}^{-1}$) through the inlet and dialysate containing the diffusible solutes is collected from the outlet. Relative Recovery (*RR*) or extraction efficiency (*EE*) can be defined as the ratio, $EE = C_{\text{outlet}}/C_{\infty ECF}$ where C_{outlet} is the concentration of the analyte in the outlet and $C_{\infty ECF}$ is the concentration of the analyte in the tissue extracellular fluid at far-field distance from the implanted probe.¹⁶ Since microdialysis sampling is a diffusion-based separation process, the collected solute concentrations from any tissue will be a function of the mass transport properties for the solute through the tissue, dialysis membrane, and the dialysate.¹⁷ Analytes with smaller diffusion coefficients such as the adipokine proteins will generally exhibit *EE* values of roughly 10% or less.¹⁵

Microdialysis sampling has been employed for the sampling of large protein molecules such as cytokines and adipokines.¹⁸⁻²⁴ The microdialysis sampling technique has several merits, the most important being the ability to provide continuous sampling of the extracellular fluid space within tissues. The microdialysis sampling procedure is considered to be minimally invasive due to small size of the probe (500 μm), thus causing less tissue disruption when implanted. Dialysate samples obtained from the device are analytically clean, requiring no further sample clean up and can be directly subjected to immunoassays or other quantitation methods.

The goal of the present study was to employ microdialysis sampling to collect and quantify adipokines from the mammary fat pad of young adult female rats. Rats are a commonly-used animal model for human breast cancer.²⁵⁻²⁷ While Xu *et al.* employed microdialysis sampling technique to sample osteopontin, clusterin and cystatin-c from mammary tumors in female rats, to our knowledge no reports of microdialysis sampling in normal mammary glands of rodents has been reported.²⁸ CCL2, IL-6, and leptin were selected for the study as they are important mediators of inflammation underlying mammary tumor development and progression. A critical issue with microdialysis sampling, particularly with larger molecules, is the calibration of the dialysate concentrations relative to actual tissue concentrations.²⁹

Dialysate concentrations are representative of a certain percentage of the tissue concentrations. While whole tissue extracts would also represent any cytokines stored in granulocytes, we chose to also compare dialysate protein concentrations with those extracted from whole tissue.

Materials and Methods

Microdialysis Supplies and Perfusion Fluid

CMA-20 (10 mm (length) $\times$ 0.5 mm (outer diameter, o.d.)) 100 kDa molecular weight cutoff (MWCO) polyethersulphone (PES) microdialysis probes (CMA Microdialysis, North Chelmsford, MA) were used for all experiments. The Baby Bee single channel (BASi, W. Lafayette, IN) syringe pump was used with a 1000 μ L BAS glass syringe (BASi, W. Lafayette, IN) to deliver the perfusion fluid through microdialysis probe. Perfusion fluids contained phosphate-buffered saline (PBS), containing 0.137 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄ with 6% (w/v) Dextran 70 (Sigma-Aldrich, St. Louis, MO) and 0.1% (w/v) bovine serum albumin, BSA, (Sigma-Aldrich, St. Louis, MO). Dextran was added to prevent fluid loss across the high MWCO membranes during sampling and BSA was used to reduce non-specific adsorption of adipokines to membrane materials. Perfusion fluids were prepared as needed and filter sterilized with a 0.2 μ m PES membrane filter (Whatman, Florham Park, NJ).

In Vitro Microdialysis Recovery Experiment

The calibration of the probe to determine the relative recovery of CCL2, IL-6 and leptin was conducted in vitro. Briefly, CMA 20 microdialysis probes (100 kDa MWCO and 10 mm length) were immersed into a solution containing 2 ng mL⁻¹ of each protein, with stirring at 37°C. The perfusion flow rate was set at 2 μ L min⁻¹ and dialysates were collected every hour for a total of five hours. Samples were quantified as outlined below.

Animals and Probe Implantation Procedure

Sixteen adult female Sprague-Dawley rats (Harlan Laboratories Inc., Madison, WI) weighing between 220 to 250 g were used in this study. The first set of experiments used ten rats (Set 1) while the second set had six rats (Set 2). All animals were housed in an environmentally-controlled facility with a 12-hour on/off light cycle and had *ad libitum* access to food and water. Surgical procedures followed approved protocols by the University of Arkansas Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines for the care and treatment of animals. The animals were anesthetized with 5% isoflurane (Butler Schein, Dublin, OH) (in oxygen). The body temperature was monitored using a rectal thermometer and maintained using a temperature-controlled heating pad (CMA microdialysis, USA). An 18-gauge needle was used to place the microdialysis probe in the abdominal mammary fat pad of the animal while under 2.5% O₂ inhalation. The needle was inserted from one side and the microdialysis probe was tunneled through the needle; the needle was then pushed out from the side of insertion leaving the microdialysis probe embedded in the mammary fat pad (Figure 1). Post mortem analysis was performed to confirm probe location in the mammary fat pad.

Microdialysis Sampling

Animals remained under anesthesia (2.5 % isoflurane in oxygen) throughout the sampling procedure. Prior to implantation, microdialysis probes were perfused with perfusion fluid at a flow rate of $3 \mu\text{L min}^{-1}$ for a total of 20 minutes. The tubing lines were then flushed with perfusion fluid (flow rate of $3 \mu\text{L min}^{-1}$) for 15 minutes. The flow rate was then reduced to $1 \mu\text{L min}^{-1}$ and samples were collected every hour for a total of three hours. Dialysates were collected into Eppendorf 0.5 mL protein LoBind™ tubes (Eppendorf, NY) in ice. To prevent protease degradation of adipokines, protease inhibitor cocktail (Halt™, Thermo Scientific, Rockford, IL) was added to the collection tubes at 2X final concentration. All dialysate samples were stored at -80°C until analysis was performed within 2 days of collection.

Tissue Harvest and Protein Extraction

Rats were euthanized by CO_2 asphyxiation immediately after microdialysis sampling. Mammary tissue (weighing between 0.4 to 0.5 g per rat) was harvested from right (probe implant side) and left (control side) abdominal mammary fat pad of each rat (Set 1). For Set 2, mammary tissue within ~2 mm of the region where the probe was implanted was harvested. An equivalent amount of mammary tissue was also excised from tissue with no implanted probe. Tissue samples were frozen immediately in liquid nitrogen and stored at -80°C until analysis within 2 days of tissue collection. T-PER buffer (Pierce Protein Research Products, Thermo Scientific, Rockford, IL) was used for tissue protein extraction, following the manufacturer's guidelines. Briefly, tissue samples were thawed on ice, weighed and divided into 0.25 g minced sections. T-PER buffer containing 1 X Halt Protease inhibitor cocktail was added ($4 \mu\text{L mg}^{-1}$ of tissue), and tissues were lysed by at least 5 freeze/thaw cycles, followed by homogenization using a Bullet Blender (Next Advance Inc., Averill Park, NY). Tissue homogenates supernatant was collected by centrifugation at 8000g for 15 minutes at 4°C . Total protein concentration in tissue lysates was determined using a bicinchoninic acid (BCA) assay kit (Calbiochem, San Diego, CA) as per the manufacturer's protocol.

Adipokine Quantification

The amounts of CCL2, IL-6, and leptin in $25 \mu\text{L}$ of dialysate samples were determined using the Multiplex MAP kit (Millipore, Billerica, MA) following the manufacturer's instructions. Sample concentrations (pg mL^{-1}) were determined by comparing median fluorescence intensity (MFI) against a standard curve of known protein concentrations, using a Luminex™ 100 IS analyzer (Luminex Corp, Austin, TX). The concentration range for the leptin assay was $2.4 - 10,000 \text{ pg mL}^{-1}$ and $4.88 - 5000 \text{ pg mL}^{-1}$ for the CCL2 and IL-6. In vitro dialysates samples and tissue extracts were quantified by ELISA using Leptin (Millipore, Billerica, MA), IL-6 and CCL2 (BD Pharmingen, San Diego, CA) ELISA kits.

Statistical Analysis

Origin 8 software platform (OriginLab Corp, Northampton, MA) was used for statistical analysis. The normality of the concentrations of the samples from microdialysis and protein extraction experiments was analyzed using a Shapiro-Wilk test. Data are presented as box

and whiskers plots. Comparisons between samples were made using a Kruskal-Wallis ANOVA with a Bonferroni post hoc test and were considered significant at $p < 0.05$.

Results and Discussion

In vitro relative recovery values

The average relative recovery values of leptin, IL-6 and CCL2 were found to be $11.6 \pm 1.2\%$, $3.3 \pm 0.6\%$ and $18.6 \pm 9\%$ ($n = 5$), respectively with a $2 \mu\text{L min}^{-1}$ flow rate. These relative values for IL-6 and CCL2 are within the range of values reported previously.³⁰ By contrast, the relative recovery of leptin ($\sim 12\%$) is an order of magnitude higher than previously described. Dostalova et al. reported in vitro recovery of leptin at 5.85 % (flow rate of $1 \mu\text{L/min}$) and 5.17 % (flow rate of $2 \mu\text{L min}^{-1}$), while Duo et al. reported recoveries of 4% and 2% using flow rates of $1 \mu\text{L min}^{-1}$ and $2 \mu\text{L min}^{-1}$ respectively.^{19, 31, 32} Variations in experimental conditions (37°C under stirred conditions in our studies) which maximized in vitro recovery may account for these observed differences.

Probe implantation in rat mammary fat pad

Given the anatomical structure of the mammary fat pad, the use of a linear microdialysis probe for collection would have been ideal.³³ In our initial studies, we attempted to use the CMA 31 PES 55 kDa MWCO linear probes. While this probe could be inserted into the fat pad and its membrane positioned within the fat pad itself, no adipokines were collected using these probes. Additional studies using in vitro standards demonstrated that for the 55 kDa MWCO probes, neither CCL2 (13 kDa) or leptin (16 kDa) could be recovered in quantifiable levels across this membrane at a low flow rate of $0.5 \mu\text{L min}^{-1}$. It is important to note that MWCO values for the membranes used here are based on the values used for standard kidney dialysis methods. Since MWCO values are obtained using bulk or equilibrium measurements performed under different conditions than microdialysis sampling, it is common with microdialysis sampling to see recovery rates far lower for solutes with molecular weights under the MWCO.^{34,35} We subsequently selected the CMA 20 microdialysis probes with the 100 kDa MWCO based on our previous experience for cytokine collection.

In vivo microdialysis collection and protein extraction from tissue

Two sets of rats (with ten and six animals, respectively, in Sets 1 and 2) were used to compare cytokine dialysate and extracted tissue protein concentrations. In contrast to those for CCL2 and IL-6, the multiplex assay carried out for leptin in set 1 did not result in standard curves that were appropriate for quantitative measurements. This is a common problem with these bead-based assays in that they do not behave as ruggedly and reliably as standard ELISA.³⁶ However, given the low volumes obtained using microdialysis sampling, the bead-based assays do allow for multiplexed measurements in our low μL samples. In the second set of six rats, the multiplex assay for CCL2, IL-6 and leptin yielded standard curves that subsequently permitted the comparisons between dialysate and tissue concentrations for all adipokines.

CCL2, IL-6, and leptin concentrations in the dialysates

Figure 2 depicts the dialysate concentrations obtained for CCL2 and IL-6 for the two independent experiments. The concentrations obtained for each adipokine between the two groups are comparable, indicating reproducibility of the experimental procedures. During the sample collection periods, the concentrations of both IL-6 and CCL2 gradually increased temporally during collection. By the end of the collection period (180 minutes), CCL2 concentrations increased from ~20 to 175 pg mL⁻¹ (median values) while IL-6 concentrations increased from median values of 30 to 105 pg mL⁻¹. The increase in CCL2 and IL-6 concentrations reflects the anticipated wounding response from insertion of the microdialysis probe.

Analyses by the non-parametric Kruskal-Wallis ANOVA followed by the Bonferroni post hoc test were performed on this data giving the following results. For set 1 animals, there is a significant difference in the protein concentrations at 60 min and 180 mins for both CCL2 and IL-6 ($p < 0.05$). However, there is no significant difference in the protein levels between 60 min and 120 mins and similarly between 120 mins and 180 mins for both CCL2 and IL-6. For set 2 animals, there is no significant difference between the protein levels obtained at the different time points for CCL2, IL-6 and leptin. This is likely due to the different outlier concentrations in this smaller data set. Finally and more important, there is no significant difference in CCL-2 and IL-6 concentrations at comparable time points between set 1 and set 2 animals.

The dialysate concentrations of leptin obtained from the 2nd set of animals did not change at the different collection points, with median values of 750 pg mL⁻¹ (Figure 3). This result is consistent with leptin, unlike that of CCL2 and IL-6, to be unresponsive to a foreign body insult. Thus, the lack of change in leptin concentrations after probe insertion suggests that dialysate leptin concentration may reflect basal levels within the extracellular fluid space in the mammary gland.

IL-6 and CCL2 concentrations in tissue extracts

The concentrations of CCL2 and IL-6 in mammary tissue extracts are shown in Figure 4. For these studies, tissues around the areas with and without implanted probes were used. No significant differences in tissue CCL2 and IL-6 concentrations were observed. Figure 5 summarizes the adipokine concentrations in tissue extracts when analyzed tissue was limited to an area within 2 mm surrounding the microdialysis implant. For CCL2, concentrations were found to be significantly higher in tissue with than without probe. For IL-6, the concentrations did not differ for tissue with and without implants. It is worth noting the concentrations of CCL2 and IL-6 in control tissue in the second experiment (Figure 5) differed significantly from those in the first set. However, one of the significant advantages of microdialysis sampling procedures is that the animals serve as their own control. Thus, despite the differences in the absolute values in control tissues between the two experiments, their relative values with respect to those for probe-implanted tissue within each experiment did not change.

Comparison of tissue vs. dialysates concentrations

During microdialysis sampling, solute concentrations obtained in the dialysates represent a fraction of the free and unbound solute concentration in tissues. The *in vitro* recovery values obtained under stirred conditions for the adipokines represent the maximum recovery that might be expected for any solute. This is because tissue properties and other processes (binding to tissue components such as glycosaminoglycans or tissue receptors) can cause an increase in mass transport resistance in a tissue which is otherwise lacking in aqueous solutions. The median IL-6 concentration in dialysates was found to be 1% of the average IL-6 concentration in probe-implanted tissues. Similarly, the median CCL2 concentration in dialysates was 3% that in probe tissue. These values are much lower than the *in vitro* recoveries of 3.3% and 18.6% for IL-6 and CCL2, respectively. Additional sources of variance between tissue and dialysate concentrations include the presence of various proteins in adipocytes that can bind cytokines or of immune cells recruited to the adipocyte microenvironment due to the implant. Further, differences in the analysis platforms (ELISA for tissue lysates and bead-based assays for dialysate) may contribute to the discrepancies, although the comparable sensitivities of these platforms have been reported previously.³⁷⁻⁴⁰

A challenge to all *in vivo* microdialysis sampling work is the issue of microdialysis probe calibration. Known differences exist between microdialysis calibration *in vitro* vs. *in vivo*. For collection of pharmaceutical solutes, different *in vivo* calibration procedures exist and require solute steady state concentrations typically achieved with drug infusions.⁴¹ For endogenous solutes such as the adipokines that may not exist at steady-state concentrations, *in vivo* calibration methods are not available to determine the true extracellular space concentration. For this reason, we chose to compare total tissue concentrations to the dialysate concentrations.

Analyte transport through any tissue is related to various tissue properties including the metabolism and uptake within the tissue, vascularity, and density of the tissue.^{42, 43} Any process that influences solute mass transport through a tissue space will affect the amount of solute that eventually is collected with microdialysis sampling.^{17, 44} Additionally chemokines and other cytokines are known to bind to the glycosaminoglycans in the tissue.⁴⁵ Solutes that bind to tissue components cannot be collected via the microdialysis sampling approach and thus this binding influences the amount of protein recovered into a microdialysis probe.

Another tissue factor that would have to be considered in longer term studies with microdialysis probes in breast tissue would be differences in tissue density. Rats in different age groups of 21, 28 and 63 days of age have exhibited differences in the tissue density.⁴⁶ To minimize this variable, we chose to use a narrow weight range among the animals used for this study.

The placement of microdialysis probe in target tissue requires either incision with removal of enough tissue fascia or use of a guide needle. It is well known that trauma associated with microdialysis probe insertion causes some tissue damage, local blood flow perturbations, and release of inflammatory mediators.⁴⁷⁻⁴⁹ Trauma stimulates the release of IL-6, IL-8 and CCL2. In previously published data from this lab, CCL2 levels from first day implant in

subcutaneous tissue gradually increased from 20 to 135 pg/mL between 40 to 160 min while those from IL-6 increased from 20 to 950 pg/mL.⁵⁰ In human subcutaneous tissue studies using microdialysis sampling, the levels of IL-6 and IL-8 were undetectable within the first hour of probe insertion but continuously increased in the next 6 hours.^{29, 51, 52} The data presented here showing the temporal concentration increases for CCL2 and IL-6 are consistent with others.

Traditional methods of assessing adipokine expression involve using either qRT-PCR for measurement of mRNA levels or total protein analysis from tissue. While the microdialysis sampling technique is minimally invasive, there are advantages of the microdialysis sampling technique when compared the traditional methods to determine protein expression or concentrations.⁵³ Microdialysis sampling allows collection of only unbound solutes. This means all the adipokines collected within the dialysis probe were not bound to other tissue components and were released into the extracellular space and not stored within cells. The other advantage of the microdialysis technique is the possibility for reduction of total animals if you can dose the animal while the probe is implanted.

Conclusions

Microdialysis sampling techniques have been successfully applied to the collection of adipokines (CCL2, IL-6 and leptin) from adult rat mammary glands. CCL2 and IL-6 concentrations steadily increased after microdialysis probe implantation. Leptin concentrations did not differ between the sampling points indicating these levels may reflect basal concentrations and not levels induced by microdialysis probe insertion trauma. We suggest that this procedure has significant potential for application in basic studies related to further understanding the signaling biology of the mammary fat pad as targets of inflammatory and obesogenic instigators of breast cancer development and progression. Additionally, the microdialysis sampling technique could be used to collect adipokines during different variable conditions such as animals with different ages, tumors or those given chemotherapeutic agents during the sampling process.

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Figure 1.

Microdialysis sampling probe implantation into the mammary fat pad. An increasing microscopic view of the probe implantation is illustrated from left to right. Farthest right: The circle indicates the location of microdialysis probe implanted in the right abdominal mammary fat pad.

Figure 2.

CCL2 (left) and IL-6 (right) concentrations from $1 \mu\text{L min}^{-1}$ perfused microdialysis probes in two sets of female rats during a three-hour collection period following probe implantation. The “n” value is 10 for set 1 (ten animals) and 6 for set 2 (six animals) and represents the number of animals that provided detectable adipokine concentrations out of total animals used in the study. The box represents the 25-75 percentile. The line through the box represents the median, whiskers represent the fence and $\square$ represents the mean.

Figure 3.

Leptin dialysate concentrations collected ($1 \mu\text{L min}^{-1}$) from set 2 animals during the three hour collection period following probe implantation. The “n” value is 6 representing the number of animals that provided detectable adipokine concentrations out of six total animals used in the study. Box represents the 25-75 percentile. The line through the box represents the median, whiskers represent the fence and $\square$ represents the mean.

Figure 4.

CCL2 (left) and IL-6 (right) concentrations in the probe-implemented vs. control tissue lysates from animals in set 1. In this set of animals, 1 cm tissue surrounding the probe was harvested. The “n” value is 7 representing the number of animals that provided detectable adipokine concentration out of ten animals in this set. The box represents the 25-75 percentile. The line through the box represents the median, whiskers represent the fence and $\square$ represents the mean.

Figure 5.

CCL2 (left) and IL-6 (right) concentrations in probe-implanted vs. control tissue lysates from animals in set 2. In this set of animals, ~2 mm tissue surrounding the probe was harvested. Probe vs. control tissue concentrations are significantly different for CCL2 (†), but not IL-6 using a Kruskal-Wallis ANOVA with a Bonferroni post hoc test ($p < 0.05$). The “n” value is 6 representing the number of animals that provided detectable adipokine concentration out of six animals in this set. The box represents the 25-75 percentile. The line through the box represents the median, whiskers represent the fence and $\square$ represents the mean.