Published in final edited form as:
Anal Biochem. 2015 January 15; 469: 34–42. doi:10.1016/j.ab.2014.10.004.

Method for Measuring Lipid Mediators, Proteins, and mRNAs from a Single Tissue Specimen

Jessica A. Cottrell[a,1,2], **Hsuan-Ni Lin**[b,1], and **J. Patrick O'Connor**[a,b,*]

[a]Department of Biochemistry & Molecular Biology New Jersey Medical School, the State University of New Jersey 185 South Orange Avenue Newark, NJ 07103

[b]Department of Biochemistry & Molecular Biology Graduate School of Biomedical Sciences Rutgers, the State University of New Jersey 185 South Orange Avenue Newark, NJ 07103

Abstract

This paper describes a new method for extracting RNA, protein, and lipid mediators from a single tissue specimen. Specifically, mouse bone fracture callus specimens were extracted into a single solution which was processed using three different procedures to measure mRNA levels by RTqPCR, cytokines and growth factors using an xMAP method, and lipid mediators by LC-MS/MS. This method has several advantages because it decreases the number of animals necessary for experimentation, allows the division of the sample from a homogenous mixture which reduces sample variability, and utilizes a solution that protects the integrity of the macromolecules during storage.

Introduction

Bone fractures normally heal by tissue regeneration, which occurs through a temporally and spatially coordinated process [1, 2]. Fractures typically disrupt blood circulation causing hematoma formation and localized tissue hypoxia. Inflammation quickly follows and lipid mediators, growth factors, and cytokines released at the fracture site promote proliferation and chemotaxis of cells to the site. Mesenchymal cells that have proliferated or migrated to the fracture site differentiate into chondrocytes to form a cartilaginous callus around the fracture. Bone replaces the cartilage by endochondral ossification to bridge the fracture. The newly formed bone is remodeled to increase mechanical strength and restore the shape of the bone. While the histological processes that occur during fracture healing are well described, the signaling events that control this tissue regeneration process are poorly understood.

*To whom correspondence should be addressed. J. Patrick O'Connor, Ph.D. Rutgers-NJMS; Biochemistry Medical Sciences Building, room E-659 185 S. Orange Ave. Newark, NJ 07103 oconnojp@rutgers.edu Tel: 973-972-5011.
[1]These authors contributed equally to this research.
[2]Present address: Seton Hall University, Department of Biological Sciences, 400 South Orange Avenue, South Orange, NJ 07079

Previous studies showed that cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO) activity regulate bone fracture healing. Pharmacological inhibition or genetic ablation of COX-2 impairs healing [3, 4], while inhibition or ablation of 5-LO accelerates healing [5, 6]. COX-2 and 5-LO catalyze the synthesis of prostaglandins and leukotrienes, respectively, which are lipid mediators that regulate inflammation and other processes, including tissue regeneration [7-10]. Measuring the types and levels of lipid mediator during fracture healing is necessary to understand how COX-2 and 5-LO regulate this tissue regeneration process. This is complicated by the altered dynamics of fracture healing caused by loss of COX-2 or 5-LO activity. Thus, levels of each lipid mediator must be correlated to other cellular processes occurring at that time in the fracture callus in order to understand the role each lipid mediator has during fracture healing. Ideally, lipid mediators would be correlated to protein and mRNA markers of established physiological and cellular processes in order to understand how bone regeneration is regulated by COX-2 or 5-LO. Generally, measurement of mRNA, protein, or lipid mediators is performed with multiple tissue samples prepared separately using extraction methods appropriate for each target molecule [6, 11-14]. The spatial complexity of a fracture callus precludes using this approach since dividing the callus into portions would yield tissue samples with different cellular compositions unless specialized methods such as laser capture micro-dissection were employed [2, 15]. Alternatively, a fracture callus could be used to measure one type of target molecule [6, 16-18]. However, this approach would require using significantly more animals to measure different types of target molecules and would introduce another level of variability into the analysis as levels of the different target molecule types could not be compared from the same specimen.

To overcome these limitations, we developed methods for isolating lipid mediators, proteins, and RNA from the same tissue specimen. The method relies upon RNA*later* solution (Ambion, Inc., Austin, TX) to preserve RNA during callus extract preparation and modification of existing methods to measure mRNA, proteins, and lipid mediators from callus extract aliquots using RTqPCR, xMAP, and LC-MS/MS methods, respectively [19].

Materials and Methods

Animal Model

Female ICR mice (Taconic Farms, Germantown, NY) weighing 28.7 ± 2.3 g (mean ± standard deviation) were used in this study. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine (0.1 and 0.01 mg/g body weight, respectively). A closed, diaphyseal fracture was created in the right femur using a custom-made, three-point impactor (BBC Specialty Automotive Center, Linden, NJ) as described previously except the mice were allowed to recover for 7 days between insertion of the intramedullary pin used to stabilize the fracture and production of the fracture [20]. All experimental procedures were approved by the New Jersey Medical School Institutional Animal Care and Use Committee.

Tissue Collection

On day 4 post fracture, mice were injected intraperitoneally with 0.1 ml of 0.5 mg/ml heparin (USB Corp., Cleveland, OH) 30 minutes before euthanization to prevent post-mortem blood clotting. Femurs were resected with surrounding muscle and callus left intact. The proximal and distal epiphyses were removed. The final length of femur diaphysis and surrounding tissue was approximately 1cm and weighed 0.72 ± 0.10 g. The femur was divided at the fracture site to facilitate subsequent tissue homogenization, flash frozen in liquid nitrogen, and stored at −80°C.

Tissue Homogenization

Each femur was homogenized in 3 mls of pre-chilled RNA*later* that had been spiked with deuterated eicosanoids to act as internal standards. The deuterated eicosanoids were purchased from Cayman Chemicals (Ann Arbor, MI), dissolved in methanol to 500 ng/ml each, and added to a final concentration of 6.67 ng/ml which would ultimately yield a maximum of 2.67 ng of each deuterated eicosanoid per LC-MS/MS injection (see below). Callus extracts were made with a Precellys 24 Dual Tissue Homogenizer (Bertin Technologies, Washington D.C., MD) using 7 ml tubes containing 2.8 mm diameter zirconium oxide beads. The callus was pulverized twice at 6,500 rpm for 15 seconds. To reduce overheating of the callus extract, samples were placed on dry ice for 1 minute before pulverization and were cooled on ice between pulverization steps. After homogenization, 2 mls of the callus extract was immediately processed for eicosanoid analysis and the remainder was stored at −20°C for protein and RNA analysis.

Protein Extraction

Aliquots of the callus extract were dialyzed to remove RNA*later* and solubilize precipitated protein. Protease inhibitors (Sigma-Aldrich P2714 Protease Inhibitor Cocktail) were added to a 0.4 ml aliquot of the callus extract which was then transferred to a dialysis apparatus (Tube-O-DIALYZER, 1kDa Micro, GBiosciences, MO) and dialyzed against M-PER (Mammalian Protein Extraction Reagent, Thermo Fisher Scientific, IL) for 24 hours at 4°C. After the dialysis, insoluble material was removed from the samples by centrifugation (14,000 rpm for 10 minute at 4°C). To remove additional debris, the supernatant was collected and filtered through a 0.2 μm membrane by centrifugation (Nanosep, PALL, Port Washington, NY) at 14,000 rpm and 4°C until all liquid passed through the filter cartridge. The clarified extract was stored at −20°C. Aliquots of the clarified extract were used to measure total protein content using the BCA assay (BCA Protein Assay Reagent, Thermo Fisher Scientific, IL) and to measure target protein amounts using an xMAP method as described below [21, 22]. Protein extract quality was assessed by electrophoresis using 8% polyacrylamide bis-Tris gels in which 15 μg of extract protein was separated in each lane. Protein was detected by Coomassie Blue staining. Precision Plus Protein Standards (BIO-RAD, Berkeley, CA) were used to estimate protein sizes.

xMAP Assay

Growth factors and inflammation-related cytokines were measured in the clarified extracts using a Luminex 100 Multiplexing Instrument (Luminex Corp., Austin, TX) with a

Milliplex Map Mouse Cytokine/Chemokine Panel (Millipore Corporation, MA). Thirty two target proteins were analyzed (**Table 1**). Each clarified extract was measured in duplicate using 25 μl of clarified extract for each assay. Mean fluorescence intensity for each target protein was compared to a standard curve developed using standards contained within the Milliplex Map Mouse Cytokine/Chemokine Panel reagents to determine the amount of each target protein within the clarified extract aliquot. The amount of each target protein was then normalized to the total protein amount used in each assay to yield pg of target protein per mg of soluble protein.

Total RNA Purification

Total RNA was prepared from callus extract aliquots using modified TRIzol extraction and differential filter binding methods in order to eliminate RNA*later* from the purified RNA preparation [13]. One hundred μ ls of callus extract was combined with 4 volumes of TRIzol and 4 volumes of water. After mixing, another 6 volumes of TRIzol was added which was mixed gently on a shaker for 30 minutes at 4°C. The aqueous phase was separated by centrifugation (12,000 rpm for 10 mins at room temperature), collected into a new tube, and processed by differential filter binding and elution (RNeasy; Qiagen, Valencia, CA) as follows. The aqueous layer from the TRIzol extraction was mixed with 4 volumes of RLT buffer containing β-mercaptoethanol and 3.5 volumes of methanol. This solution was applied to an RNeasy mini-column. Afterwards, the column was washed with two volumes of RW1 buffer and two volumes of RPE buffer. The RNA was eluted with two washes of 25 μl water. RNA concentration was determined spectrophotometrically with a NanoDrop 1000 (Wilmington, DE). RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining. Only RNA samples with acceptable characteristics, including intact 16S and 28S ribosomal RNA, were used for further analysis.

mRNA Quantification

The reverse transcription-quantitative polymerase chain reaction (RTqPCR) method was used to measure target mRNA levels [23, 24]. cDNA was made from 0.5 μg aliquots of total RNA as described previously with use of an oligo$(dT)^{20}$ primer, RNase inhibitor (RNAaseOUT; Life Technologies, Grand Island, NY), and MMLV reverse transcriptase (New England BioLabs, Beverly, Massachusetts) [5]. Reactions proceeded for one hour at 42°C and were stopped by incubation at 92°C for ten minutes. cDNA was stored at −20°C until use. Quantitative polymerase chain reaction (qPCR) was performed with Absolute QPCR SYBR Low Rox Mix (Thermo Scientific, Lafayette, CO) and an Applied Biosystems 7500 Real-Time PCR System (Foster City, California). Reactions contained an aliquot of cDNA corresponding to 20 ng of total RNA in a 20 μ l reaction volume. The annealing temperature for the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; forward: 5'-CCTGTGACTT CAACAGCAAC TCC-3', reverse: 5'-CCACCACCCT GTTGCTGTAG CC-3'), Type II collagen (*Col2a1*; forward: 5'-TGGGTGTTCT ATTTATTTAT TGTCTTCCT-3', reverse: 5'-GCGTTGGACT CACACCAGTTAGT-3') and aggrecan (*Acan*; forward: 5'-CATGAGAGAG GCGAATGGAA-3', reverse: 5'-TGATCTCGTA GCGATCTTTC TTCT-3') mRNA primers was 60°C. Amplified DNA was measured for 40 cycles of polymerase chain reaction (PCR) as SYBR green fluorescence. Target mRNAs were assayed in triplicate for each cDNA preparation. Threshold cycle values (Ct) were

determined using the supplied Applied Biosystems software and were manually reviewed for accuracy.

Lipid Mediator Extraction

Eicosanoids and other lipid mediators were extracted from 2 mls of each callus extract. The callus extract was mixed with 9 volumes of ice cold distilled water and insoluble material was removed by centrifugation (14,000 rpm at 4°C for 10 mins using a Sorvall SS-34 rotor). The supernatant was collected and applied to an OASIS HLB 3cc (60mg) Extraction Cartridge (Waters, Milford, MA) which had been previously rinsed with 3 mls of methanol and then equilibrated with 3 mls of water. After sample loading, the cartridge was washed sequentially with 3 mls each of distilled water, 5% methanol, 50% methanol with 0.2% acetic acid, and 50% methanol. After the washes, the cartridge was vacuum dried to remove residual liquid. Eicosanoids were eluted from the cartridge with 1 ml of 97% methyl formate followed by 1 ml of 100% methanol. The combined eluates were dried using a vacuum concentrator (Vacufuge, Eppendorf, Hamburg, Germany).

Mass Spectrometry for Lipid Mediators

Eicosanoids and related lipid mediators were detected by LC-MS/MS and quantified using standard curves. The LC-MS/MS system used included a FAMOS autosampler (LC Packings, San Francisco, CA), an Agilent 1100 degasser and binary pump (Agilent Technologies, Inc., Santa Clara, CA), and an AB SCIEX Qtrap 2000 (AB SCIEX, Redwood City, CA). Eicosanoid samples and standards were separated by reverse-phase liquid chromatography using a Kinetex C18-column (100 × 2.10 mm, 2.6 micron; Phenomenex Inc., Torrance, CA) at room temperature and a flow rate of 300 μl/min. The column eluate was introduced into the Qtrap 2000 using the electro-spray ion source in negative ion mode or in positive ion mode after charge-reversal derivation as described below.

For negative ion mode detection, the dried eluate after SPE purification was reconstituted in 200 μ l of 50% methanol for LC-MS/MS analysis. Injection volume was 50 μ l. For all separations, solvent A was 1% methanol diluted in water and solvent B was 100% methanol. The Kinetex C18-column was developed as outlined in **Table 2**. The LC eluate was introduced into the Qtrap 2000 using the electro-spray ion source in negative ion mode. Eicosanoids were detected using multiple reaction monitoring (MRM) by measuring the ion pair transition from Q1 to Q3 with parameters listed in **Table 3**. PGD^2 and PGE^2 were distinguished by column retention time and co-elution with their cognate deuterated internal standards. The following Qtrap parameters were used: CUR = 20 psi, CAD = 10, IS = −4500 V, TEM = 500°C, GS1 = 50 psi, GS2 = 80 psi, IHE=ON. Lipid standards were dried, reconstituted in 50% methanol, then separated and detected as described above.

For quantification in positive ion mode, eicosanoids and related lipid mediators in the dried elutes were converted into positively charged molecules by attaching N-(4-aminomethylphenyl)pyridinium through an amide bond at the carboxylic acid of each lipid as described previously [25]. This was accomplished using an AMP+ Mass Spectrometry Kit (Cayman Chemicals) following the manufacturer's instructions except hydroxybenzotriazole was replaced with 20 mM 1-hydroxy-7-azabenzotriazole (Santa Cruz,

Biotechnology, Inc., Dallas, TX) to prevent ion suppression of some eicosanoids. The AMP + derived samples were diluted to a final volume of 500 μ l with water prior to LC-MS/MS analysis. Lipid standards (Cayman Chemicals) were dried and then derived as described above. The derivatized eicosanoid samples and standards were separated by reverse-phase liquid chromatography using a Kinetex C18-column (100 × 2.10 mm, 2.6 micron) at room temperature and a flow rate of 300 μl/min. Injection volume was 100 μ l. For all separations, solvent A was 1% acetic acid diluted in water and solvent B was 1% acetic acid in acetonitrile. The Kinetex C18-column was developed as outlined in **Table 2**. The LC eluate was introduced into the Qtrap 2000 using the electro-spray ion source in positive ion mode. Eicosanoids were detected using multiple reaction monitoring (MRM) by measuring the ion pair transition from Q1 to Q3 with parameters listed in **Table 3**. PGD^2 and PGE^2 were also distinguished by column retention time and co-elution with their cognate deuterated internal standards. The following Qtrap parameters were used: CUR = 40 psi, CAD = 5, IS = 5500 V, TEM = 400°C, GS1 = 50 psi, GS2 = 80 psi, IHE=ON.

Lipid Mediator Data Analysis

Eicosanoids and other lipid mediators were quantified using a standard curve for each lipid with Analyst Quantification Software (AB SCIEX). LC-MS/MS chromatogram peak areas were determined for each lipid using the Analyst Software. Each peak was manually reviewed for accuracy. For each lipid standard, 6 values ranging from 0 to 4 ng were analyzed in triplicate and the peak areas were fitted to a quadratic standard curve. These standard curves were used to calculate the amount of each eicosanoid or other lipid mediator present in each injected sample. The amount of recovered deuterated eicosanoid that had been spiked into the callus extract was used to determine the percent yield for each eicosanoid in each sample and data were corrected based upon the yields. Quality control standards and blanks were used periodically throughout the analysis to ensure accuracy. The statistical analysis was performed using SigmaPlot 12.5 software (Systat Software Inc., Chicago, IL).

Results and Discussion

An outline of the optimized procedures for extract preparation and processing to measure protein, mRNA, and lipid mediators can be found in the Supplemental Materials.

Callus Extract Preparation

Because fracture calluses contain bone, a robust tissue homogenization process is necessary to prepare callus extracts. We chose to use a bead pulverization method using the Precellys 24 Dual Tissue Homogenizer to promote reproducibility in future experiments. Pulverization was performed using different Precellys parameters to optimize callus homogenization. Lower rpm was used initially to prevent an increase in extract temperature but resulted in incomplete callus breakage. Pulverization was also tried in 2 ml rather than 7 ml tubes but resulted in excessive foam formation and increased extract temperature. Pulverization with metal rather than zirconium oxide beads caused extract temperature to increase above 40°C. Pulverizing the callus in 3 ml of buffer with 2 cycles at 6500 rpm for 15 seconds and with

cooling before and after each cycle gave complete callus disruption without extract temperature rising above room temperature.

Common methods for extracting RNA from tissues using phenol or guanidinium thiocyanate are not compatible with obtaining proteins in their native state [14]. Thus, we had to develop a procedure that would allow for callus disruption into a buffer that would be compatible with downstream mRNA, native protein, and lipid mediator isolation. We chose to test for native protein isolation using RNA*later* as the extract buffer because (a) RNA*later* should preserve the RNA, (b) most proteins precipitated by the ammonium sulfate in RNA*later* should be solubilized by dialysis, and (c) we did not expect the RNA*later* to affect the lipid mediators (see below). Extract preparation in RNA*later* was compared to M-PER buffer (Mammalian-Protein Extraction Reagent, Pierce Biotechnology, Rockford, IL) which was developed for isolating native proteins from mammalian cells and tissues. Four processing methods were tested: (LN-MPER) callus flash-frozen in liquid nitrogen and pulverized in M-PER reagent, (LN-RL) callus flash-frozen in liquid nitrogen and pulverized in RNA*later*, (RL-MPER) callus stored in RNA*later* and pulverized in M-PER reagent, and (RL-RL) callus stored in RNA*later* and pulverized in RNA*later*. All samples were stored at −80°C before pulverization. We found that calluses stored in RNA*later* were more difficult to pulverize. Following pulverization, aliquots of each callus extract that had been stored in or pulverized in RNA*later* were dialyzed against M-PER for 1 hour, clarified by centrifugation, and soluble protein measured (**Figure 1A**). The samples pulverized in RNA*later* had less protein (0.2 mg/ml) than those pulverized in M-PER, suggesting that the 1 hour dialysis was not sufficient. When dialysis was extended to 24 hours, soluble protein yield substantially increased for the LN-RL (2.2 mg/ml) and RL-RL (2.0 mg/ml) extract methods though yields were less than extracts prepared with M-PER (LN-MPER, 5.0 mg/ml and RL-MPER, 5.3 mg/ml) (**Figure 1A**).

Protein composition of extracts dialyzed for 24 hours and the LN-MPER extract were determined by SDS-PAGE (**Figure 1B**). The RL-MPER extract appeared to have the largest number of different proteins, particularly those greater than 100 kDa with a very abundant protein of approximately 220 kDa. For proteins below 100 kDa, the protein composition between extraction methods appeared to be similar except for increased abundance of an approximately 42 kDa protein in the LN-MPER and RL-MPER extracts and reduced amounts of an approximately 35 kDa protein in the LN-MPER extract (**Figure 1B**). Since cytokines and growth factors are generally below 100 kDa in molecular weight, all extraction methods appeared to be suitable for protein extract preparation.

Since RNA*later* precipitates protein, we also collected precipitated protein directly from the LN-RL and RL-RL crude extracts by centrifugation using a Beckman TL-100 Ultracentrifuge and TLA100 rotor (80,000 rpm for 30 mins at 4°C). Protein in the pellet and supernatant were determined. Ninety percent of the total protein was found in the precipitated fraction. Resuspending the precipitated protein collected by centrifugation of the RL-RL or LN-RL extracts in MPER buffer produced amounts of soluble protein similar to directly dialyzing the crude extracts (data not shown) and so collecting the precipitated protein by centrifugation was omitted.

xMAP Analysis

Callus extracts prepared using the different pulverization and dialysis procedures were assayed for cytokine and growth factor levels to determine which method gave the best overall yield of potential target proteins. LN-RL, RL-MPER, RL-RL samples dialyzed for 1 or 24 hours were compared to LN-MPER samples using an xMAP assay for 32 different target proteins (Milliplex Map Mouse Cytokine/Chemokine Panel). The majority of target protein readings (mean fluorescence intensity) from the LN-RL, RL-MPER, or RL-RL which had been dialyzed for 1 hour failed or fell below the detectable limits of the assay (data not shown). When dialysis time was increased to 24 hours, cytokines could be readily detected in the LN-RL, RL-MPER, and RL-RL samples (**Table 1** and **Figure 2**). Cytokine levels in the RL-MPER samples were comparable to those in the LN-MPER samples, except IL-1β, which was significantly lower in the RL-MPER samples (**Figure 2**). In RL-RL samples, eotaxin levels were significantly higher while MIP-1α level were significantly lower when compared to LN-MPER (**Figure 2**). Cytokines IL-3, IL-4, IL-13, LIX, and RANTES were under the detectable limit in all samples (**Table 1**). For the remaining 27 cytokines, the number of reactions that failed or were below the detection limit was determined from 2 replicate assays of 2 samples for each callus extract preparation method with 24 hour dialysis (**Table 1**). Cytokine and growth factor measurements for 9 of the LN-MPER, 10 of the LN-RL, 11 of the RL-MPER, and 16 of the RL-RL samples failed or were below the detection limit (**Table 1**). Although the RL-RL samples were dialyzed against M-PER buffer to remove RNA*later*, improper solvent flow through the Luminex 100 assay plate resulted in variable or undetectable target protein levels in the RL-RL samples. Development of xMAP methods that use magnetic particles may permit use of the RL-RL callus extract preparation method but this was not tested. Based upon these results, we chose to use the LN-RL callus extract preparation method because callus pulverization was more easily accomplished, extract prepared in RNA*later* should stabilize RNA, soluble protein yield was sufficient for measurement of target proteins, xMAP results were similar to the LN-MPER method (which is comparable to a standard protein extraction method), and the RNA*later* is compatible with lipid mediator extraction (see below).

RNA Extraction

After successful detection and quantitation of proteins in LN-RL callus extracts, we developed a procedure for isolation of total RNA from LN-RL callus extracts. We had previously found that sequentially performing a phenol-quanidinium extraction (TRIzol) followed by differential filter binding (RNeasy) was necessary to provide high quality total RNA from fracture callus specimens, likely because of the large amount of extracellular proteins present in these samples [13]. However, the large amount of ammonium sulfate in the RNA*later* solution necessitated modification of the previously used TRIzol extraction and RNeasy filter binding purification method. The large salt concentration in the RNA*later* solution enabled complete solvation of TRIzol which prevented separation of aqueous and organic phases after chloroform addition. This problem was overcome by diluting the LN-RL callus extract aliquot with 4 volumes of water before mixing in 10 volumes of TRIzol. After mixing, chloroform was added to 10%, mixed vigorously, and phases separated by centrifugation at 4°C at 12,000 rpm for 10 mins. The aqueous phase was collected for final

RNA purification by differential filter binding using RNeasy mini-columns as follows. Buffer RLT was prepared as described by the manufacturer except ethanol was replaced with methanol. Use of ethanol in the RLT buffer caused salt precipitation, which reduced RNA yield and purity. The RNeasy column was then washed with 6 mls of RW1 wash buffer and with 6 mls RPE buffer. These additional washes were necessary to prevent salt contamination of the RNA. Total RNA was then eluted from the RNeasy column with water.

Total RNA was prepared from 3 fracture callus extracts collected 4 days after fracture. RNA was quantified by UV absorbance, checked for quality by agarose gel electrophoresis (not shown), and analyzed for GAPDH, Type II collagen (*Col2a1*), and aggrecan (*Acan*) mRNA levels by RT-qPCR (**Table 4**). The amount of contaminating genomic DNA was not determined. The average A^{260}/A^{280} ratio for the purified RNA was 1.84 ± 0.15. RTqPCR detected GAPDH, Type II collagen, and aggrecan mRNA in all callus extract RNA preparations showing that the modified RNA isolation procedure was successful (**Table 4**). To assess RNA stability using the different extraction methods, total RNA was extracted from mouse liver using the 4 different extraction methods and the crude extracts were incubated at room temperature (data not shown). Intact total RNA was evident using all extraction methods when RNA was immediately extracted after pulverization. RNA yields from the RL-RL extract were low. Within 30 minutes, significant RNA degradation was evident in the LN-MPER and RL-MPER extracts, while no RNA degradation was evident after 3 hours in the LN-RL extract.

Lipid Mediator Extraction

Before using RNA*later* for callus extract preparation, we compared recovery of PGE^2, $PGF^{2\alpha}$, 6-keto-$PGF^{1\alpha}$ (the initial breakdown product of PGI^2), and TXB^2 (the initial breakdown product of thromboxane A^2) from RNA*later*, M-PER, and water. Prostaglandins were added to water, M-PER, or RNA*later* and then recovered by solid-phase extraction (SPE) using Oasis HLB columns. The recovered prostaglandins were analyzed by LC-MS/MS in negative ion mode and compared to the starting amount of each prostaglandin as a percentage (**Table 5)**. The best recovery of the tested prostaglandins occurred with RNA*later* which likely reflects enhanced hydrophobic interaction between the prostaglandins and the SPE matrix promoted by the high salt concentration of RNA*later*. Other SPE matrices were tested including C18, weak cation exchanger, weak anion exchanger, and hydrophobic polydivinylbenzene matrices but the Oasis HLB matrix gave the best prostaglandin yield and was used in all subsequent experiments (data not shown).

We noticed that some of the salt from the RNA*later* also eluted with the prostaglandins from the Oasis HLB column. Consequently, SPE column loading and washing steps were optimized to reduce ammonium sulfate carry-over while maintaining prostaglandin recovery. The RNA*later* and prostaglandin mixture was diluted with 9 volumes of water immediately before applying to the Oasis HLB column. The SPE column was then thoroughly washed sequentially using 1 ml each of water, 5% methanol, 50% methanol with 0.1% acetic acid, and 50% methanol. Washes using methanol concentrations greater than 50% decreased prostaglandin yield. Similarly, excessive washing with 50% methanol solutions also reduced the overall yield of prostaglandins (data not shown). Lipids were

eluted from the SPE column with 1 ml methyl formate followed by 1 ml of methanol. The eluates were combined and dried in a vacuum centrifuge. Sample heating during drying decreased overall lipid yield and was avoided. Dried samples were stored at −20°C until analysis.

LC-MS/MS Analysis of Callus Extract Lipid Mediators

To initial optimize LC methods, eicosanoid standards were detected using negative ion mode, electrospray ionization. A variety of solvents, gradients, and columns were tested based upon prior work [25-35]. We found that a Kinetex C18 (100 × 2.1 mm) column developed using a multi-step gradient with water and acetonitrile, each containing 1% acetic acid, gave satisfactory separation of the target eicosanoids in our system. We also found that the eicosanoids were more stable in 1% acetic acid than in 1% formic acid (data not shown). Initial experiments using lipids prepared from callus extracts found that the AB SCIEX Qtrap 2000 had sufficient sensitivity to detect only the most abundant prostaglandins in negative ion mode. For instance, when all of the lipids prepared from 2 mls of callus extract were used in a single separation, only PGE^2 and TXB^2 could be readily detected. Since our goal is to survey lipid mediators present during fracture healing, we sought methods to increase the sensitivity of the LC-MS/MS assay. Mass spectrometers with better ionization devices and ion detectors with much greater sensitivity than the Qtrap 2000 could obviate the necessity for the following procedures.

Multiple methods have been developed to derivatize prostaglandins and other fatty acids in order to enhance detection of these lipids. Most derivation methods target the carboxyl group of the prostaglandins or other fatty acids in order to add a moiety for fluorescence detection or to convert the carboxyl group into an amine derivative that will promote positive ion formation for detection by mass spectrometry [25, 30-38]. Since formation of positive ions by electrospray ionization is more efficient than formation of negative ions, we chose to use a charge reversal derivatization method on the callus extract lipid preparations in order to enhance sensitivity. The method developed by Bollinger et al. adds N-(4-aminomethylphenyl)pyridinium (AMP) at the carboxylic acid of fatty acids through an amide linkage [25]. The AMP moiety is resistant to fragmentation in the mass spectrometer and so provides a constant increase of 168 amu to each prostaglandin. We also modified the AMP derivation method by using 1-hydroxy-7-azabenzotriazole instead of hydroxybenzotriazole in the derivation reaction because use of hydroxybenzotriazole caused ion suppression of LTB^4 (data not shown and personal communication from James Bollinger and Michael Gelb, U. of Washington, Seattle, WA).

Using the optimized methods for callus extraction, lipid mediator purification, and derivation, we assayed for 10 different eicosanoids and their deuterated standards in the lipids purified from 2 mls of callus extract. Characteristic MS/MS ion pairs and eicosanoid peak retention times were used to identify each lipid mediator (**Figure 3**). Only eicosanoid MS/MS curves with peak values 10X greater than baseline were used for quantification. Consequently, identification and quantification of the eicosanoids was highly reproducible. The limit of quantification for each eicosanoid are noted into **Table 6**. Average percent recovery of the deuterated standards ranged from 9.5% for 6-keto-PGF1alpha to 62% for

5(S)-HETE (**Table 6**). We were able to detect and quantify all 10 eicosanoids in the day 4 fracture callus extracts (**Table 6**).

Conclusions

For this project, our goal was to isolate lipid mediators, protein, and RNA from the same bone tissue sample. A variety of methods exist for extracting proteins [39-41], lipid mediators [6, 18], and RNA [13, 42] from bone. Unfortunately, these methods typically employ different initial extraction methods. Using the same initial extraction procedure as described here, eliminates the need to divide the initial tissue sample for each separate extraction procedure or use tissues from multiple animals in order to measure all 3 molecular classes. This method successfully extracted lipid mediators, proteins, and RNA simultaneously from a single bone callus tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by Award Number R01DE019926 from the National Institute of Dental & Craniofacial Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Dental & Craniofacial Research or the National Institutes of Health.

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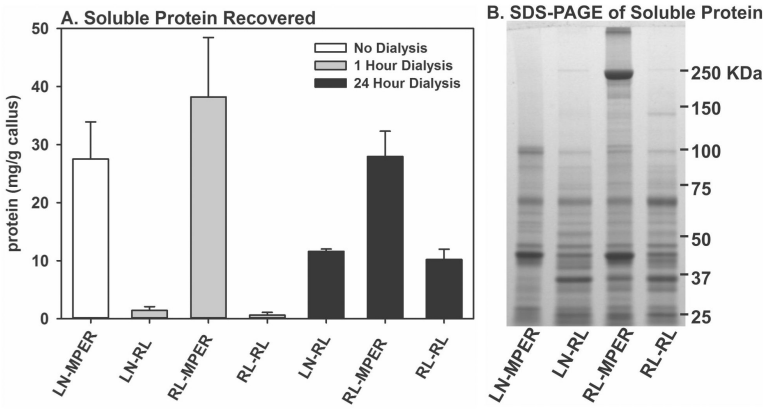

Figure 1. Callus Extract Protein Yields and Quality

Panel A: Soluble protein levels were measure in callus extracts after 1 hour or 24 hours of dialysis to remove RNA*later*. Samples sizes were LN-MPER = 12 (not dialyzed); LN-RL 1 hour = 8; RL-MPER 1 hour = 4; RL-RL 1 hour = 4; LN-RL 24 hour = 3; RL-MPER 24 hours = 3; RL-RL 24 hours = 2. Error bars represent standard errors of the mean. Panel B: Aliquots of soluble protein from extracts prepared using each method were separated by SDS-PAGE and detected by Coomassie Blue staining. Positions for protein standards are shown.

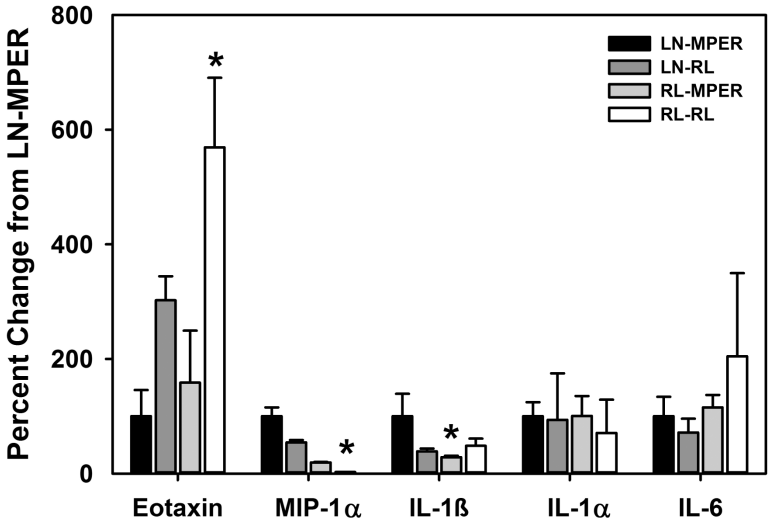

Figure 2. Variation in Cytokine Recovery using Different Extraction Methods

Selected cytokine protein levels from LN-MPER, LN-RL, RL-MPER, and RL-RL samples were normalized against the mean value for the LN-MPER samples as the percent change. These data are shown as mean values (± SEM). Significant differences were determined using One Way ANOVA or Kruskall Wallis tests. Post-hoc tests was used to determine the differences between groups. Dunnett post-hoc method was used when sample sizes were the same and Dunn's post-hoc method was used when sample sizes were different in each group. Differences were considered to be significant at $P < 0.05$. The statistical analysis was performed using SigmaPlot 12.5 software (Systat Software Inc.).

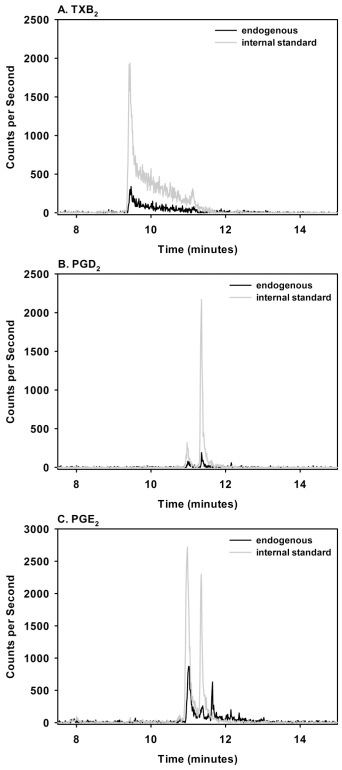

Figure 3. Representative Chromatograms from LC-MS/MS Measurements

Lipids extracted from a mouse femur fracture callus frozen in liquid nitrogen and pulverized in RNAlater were derivatized and then separated on a reverse phase column. Endogenous eicosanoids (black lines) and deuterated internal standards (gray lines) were detected by MS/MS. Shown are chromatograms for TXB^2 (A), PGD^2 (B), and PGE^2 (C) from a single fracture callus.

Table 1

Extract Preparation Effects on Target Protein Yield

Target Protein	pg per mg soluble protein Each sample was measured twice. Single values = mean from 2 samples. B = sample values below detection limit. F = sample failed in assay.			
	LN-MPER	LN-RL	RL-MPER	RL-RL
Eotaxin	29.0	87.7	46.0	165.2
G-CSF	1.9	9.6	2.5	7.1
GM-CSF	2.6	4.7	2.4	5.2
IFN-γ	B, B	2, B	B, B	B, B
Il-1α	1.6	2.8, B	1.6	2, B
IL-1β	16.7	6.5	4.8	8.1
IL-2	B, B	1.6	0.8	1.8, B
IL-3	B, B	B, B	B, B	B, B
IL-4	B, B	B, B	B, B	B, B
IL-5	1.1	B, B	B, B	B, B
IL-6	8.8	6.3	10.2	18.1
IL-7	1.2	2, B	1, B	4.2, B
IL-9	B, B	B, B	B, B	100, B
IL-10	4.4	7.0	2.7	4.9
IL-12 (p40)	3.2, B	11.6	0.6	2.1
IL-12 (p70)	3.4	2.8	B, B	B, B
IL-13	B, B	B, B	B, B	B, B
IL-15	2.6	2.3	1.3	2.0
IL-17	4.6, B	B, B	1.2	3.2, B
IP-10	30.7	73.0	35.5	140.1
KC	6.9	7.9	3.1	15.6
LIF	1.9	1.9	1.9	4.3
LIX	B, B	B, B	B, B	B, B
MCP-1	9.8	7.8	2.2	15.0
M-CSF	3.5	4.4	1.8	4.2
MIG	6.7	33.0	126.9	65.2
MIP-1α	7.5	4.1	1.5	B, B
MIP-1β	1.4	1.8	0.6	1.4, B
MIP-2	4.9	8.0	1.9	1.9
RANTES	B, B	B, B	B, B	B, B
TNF-α	0.6, F	1.2, F	B, B	B, B
VEGF	3.1	4.2	1.0	4.9

Table 2

Liquid Chromatography Methods for LC-MS/MS Analysis

Step	Negative Ion Mode			Positive Ion Mode		
	Time (min)	Buffer A	Buffer B	Time (min)	Buffer A	Buffer B
0	0.0	100%	0%	0.0	100%	0%
1	1.0	100%	0%	1.0	100%	0%
2	1.1	100%	0%	2.0	78%	22%
3	5.0	20%	80%	8.0	74%	26%
4	5.1	0%	100%	8.1	55%	45%
5	10.0	0%	100%	13.1	40%	60%
6	10.1	100%	0%	14.0	0%	100%
7	14.0	100%	0%	19.0	0%	100%
8	NA			21.0	100%	0%
9	NA			25.1	100%	0%

Table 3

Mass Spectrometry Parameters for Eicosanoid Detection

Compound	Negative Ion Mode Parameters						Positive Ion Mode Parameters					
	Precursor (m/z)	Fragment (m/z)	DP	EP	CE	CXP	Precursor (m/z)	Fragment (m/z)	DP	EP	CE	CXP
PGD_2	351	189	−39	−7	−29	−3	519	239	62	11	58	2
PGD_2-d_4	355	193	−39	−7	−29	−3	523	311	62	11	58	2
PGE_2	351	189	−41	−9	−30	−2	519	239	62	11	58	2
PGE_2-d_4	355	193	−41	−9	−30	−2	523	241	62	11	58	2
$PGF_{2\alpha}$	353	193	−41	−5	−30	−2	521	239	90	5	61	2
$PGF_{2\alpha}$-d_4	357	197	−41	−5	−30	−2	525	241	90	5	61	2
6-Keto-$PGF_{1\alpha}$	369	207	−41	−5	−30	−2	537	239	90	6	74	2
6-Keto-$PGF_{1\alpha}$-d_4	373	211	−41	−5	−30	−2	541	241	90	6	74	2
TXB2	NA						537	337	72	10	56	5
TXB_2-d_4	NA						541	341	72	10	56	5
15-deoxy-Δ12,14-PGJ_2	NA						482	254	80	10	30	2
15-deoxy-Δ12,14-PGJ_2-d_4	NA						486	241	80	10	30	2
LTB_4	NA						503	323	45	10	45	3
LTB_4-d_4	NA						507	325	45	10	45	3
5(S)-HETE	NA						487	283	65	10	54	3.5
5(S)-HETE-d_8	NA						495	284	65	10	54	3.5
Lipoxin A_4	NA						519	283	110	11	55	2
Lipoxin A_4-d_5	NA						524	283	110	11	55	2
5-oxo-ETE	NA						485	239	120	10	60	0
5-oxo-ETE-d_7	NA						492	239	120	10	60	0

DP = Declustering Potential; EP = Entrance Potential; CE = Collision Energy; CXP = Collision Cell Exit Potential

Table 4

RNA Yields from 100 μl of LN-RL Callus Extract

	Sample Size	Range	Mean	Std. Dev.
RNA Yield (μg)	3	0.29-0.62	0.40	0.19
Gapdh mRNA (Ct value)	3	18.43-19.27	18.97	0.47
Col2a1 mRNA (Ct value)	3	5.96-9.47	8.04	1.84
Acan mRNA (Ct value)	3	6.14-9.73	8.31	1.91

Table 5

Prostaglandin Recovery following SPE after Dilution in Different Solvents

Prostaglandin	Percent Recovery from:		
	Water	M-PER Buffer	RNA*later*
PGE_2	60	100	100
$PGF_{2\alpha}$	38	40	100
6-keto-$PGF_{1\alpha}$	51	72	90
TXB_2	15	22	77

Table 6

Lipid Mediator Yields: LN-RL Extraction of Day 4 Fracture Calluses

Lipid Mediator	Sample Size	Deuterated Standards: Percent Recovery			Fracture Callus Eicosanoids: ng per mg protein			LOQ (ng)
		Range	Mean	SD	Range	Mean	SD	
PGD_2	7	17-42	29.50	12.82	0.72-1.11	0.91	0.16	0.115
PGE_2	7	7-49	19.00	14.27	1.41-2.54	1.89	0.43	0.110
$PGF_{2\alpha}$	7	20-39	27.00	8.52	0.35-0.84	0.58	0.18	0.120
TXB_2	7	9-36	20.57	10.29	1.00-1.78	1.44	0.28	0.100
6-keto-PGF1α	6	4-18	9.50	5.72	0.79-1.53	1.19	0.28	0.130
15-deoxy-Δ12,14-PGJ_2	3	46-78	56.67	18.48	12.29-14.11	13.22	0.91	0.265
LTB_4	6	19-100	46.00	30.59	0.0-3.7	1.62	1.60	0.100
5(S)-HETE	5	49-100	62.15	18.82	0.42-1.53	0.97	0.42	0.100
Lipoxin A_4	4	17-52	31.50	14.98	0.38-0.71	0.56	0.14	0.140
5-oxo-ETE	7	23-100	52.43	31.89	0.35-1.58	0.82	0.43	0.240