Video Article

Induction of an Inflammatory Response in Primary Hepatocyte Cultures from Mice

Brian Czaya*[1,2], Saurav Singh*[1,2], Christopher Yanucil[1,2], Karla Schramm[1,2], Christian Faul[1,2], Alexander Grabner[1]

[1]Katz Family Drug Discovery Center and Division of Nephrology and Hypertension, Department of Medicine, University of Miami Leonard M. Miller School of Medicine

[2]Department of Cell Biology and Anatomy, University of Miami Leonard M. Miller School of Medicine

*These authors contributed equally

Correspondence to: Alexander Grabner at alexander.grabner@duke.edu

URL: https://www.jove.com/video/55319
DOI: doi:10.3791/55319

Keywords: Immunology, Issue 121, Liver, Hepatocytes, Primary cultured cells, Inflammation, Interleukin-6, FGF23, CRP, LPS, Chronic Kidney Disease

Date Published: 3/10/2017

Citation: Czaya, B., Singh, S., Yanucil, C., Schramm, K., Faul, C., Grabner, A. Induction of an Inflammatory Response in Primary Hepatocyte Cultures from Mice. *J. Vis. Exp.* (121), e55319, doi:10.3791/55319 (2017).

Abstract

The liver plays a decisive role in the regulation of systemic inflammation. In chronic kidney disease in particular, the liver reacts in response to the uremic milieu, oxidative stress, endotoxemia and the decreased clearance of circulating proinflammatory cytokines by producing a large number of acute-phase reactants. Experimental tools to study inflammation and the underlying role of hepatocytes are crucial to understand the regulation and contribution of hepatic cytokines to a systemic acute phase response and a prolonged pro-inflammatory scenario, especially in an intricate setting such as chronic kidney disease. Since studying complex mechanisms of inflammation *in vivo* remains challenging, resource-intensive and usually requires the usage of transgenic animals, primary isolated hepatocytes provide a robust tool to gain mechanistic insights into the hepatic acute-phase response. Since this *in vitro* technique features moderate costs, high reproducibility and common technical knowledge, primary isolated hepatocytes can also be easily used as a screening approach. Here, we describe an enzymatic-based method to isolate primary murine hepatocytes, and we describe the assessment of an inflammatory response in these cells using ELISA and quantitative real-time PCR.

Video Link

The video component of this article can be found at https://www.jove.com/video/55319/

Introduction

Chronic kidney disease (CKD) can be defined as a state of acute and chronic inflammation[1]. In patients with CKD, serum levels of the phosphaturic hormone fibroblast growth factor 23 (FGF23) progressively rise in order to maintain serum phosphate homeostasis[2]. Increased serum FGF23 levels are independently associated with cardiovascular morbidity and mortality among patients who are beginning hemodialysis treatment[3,4]. Furthermore, several clinical studies have shown a strong correlation between elevated FGF23 levels and serum levels of C-reactive protein (CRP), Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα)[5,6]. Moreover, in an experimental study, we have recently demonstrated that FGF23 can directly target hepatocytes and cause an inflammatory response by increasing CRP and IL-6 production in the liver[7]. Hence, FGF23 might act as a circulating factor that contributes to systemic inflammation in CKD.

In the early 70's, primary hepatocytes were isolated and studied for the first time[8]. Since then primary cultured hepatic cells have been extensively used to examine metabolic processing, hormonal function, drug metabolism and toxicity as well as immunity and inflammatory responses[9,10]. Previous protocols have mainly described the enzymatic isolation of primary hepatocytes from human liver tissue[11,12]. While an excellent model, this leaves out the ability to study how genetic manipulation affects complex hepatic signaling mechanisms as well as functional consequences upon different types of stimuli. In the following, we describe the isolation of murine primary hepatocytes. Notably, the effect of several mediators of the hepatic acute-phase response, such as lipopolysaccharide (LPS), IL-6 and FGF23 can be analyzed in an easy, fast and reproducible manner[13].

Herein, we present a protocol for the enzymatic isolation of hepatocytes from adult mice, and we demonstrate that established inducers of inflammation, such as LPS and IL-6, as well as novel inflammatory mediators such as FGF23, can directly stimulate expression and secretion of inflammatory cytokines, such as CRP and IL-6 in cultured hepatocytes.

Protocol

All animal protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Miami Miller School of Medicine.

1. Preparation

1. Preheat liver perfusion media and liver digest media in a water bath at 37 °C.
2. Set up a perfusion pump (30 mL/min). Carefully prefill the tubing system of the pump with liver perfusion media. Avoid any air bubbles in the system and prepare the stereotactic microscope.
3. Coat cell culture dishes using collagen type I according to the manufacturer's protocol.

2. Liver Recovery

1. **Anesthetize the donor mouse using oxygen/isoflurane inhalation (isoflurane 4%/2 L/min oxygen).**
    1. Maintain anesthesia by lowering isoflurane to 2-2.5% or by injecting ketamine (100 mg/kg of body weight intraperitoneally) and xylazine (10 mg/kg of body weight intraperitoneally). Continuous isoflurane anesthesia is preferred since ketamine lowers blood pressure and can cause hepatotoxicity.
2. Place the anesthetized mouse on an absorbent pad. Fix the mouse's extremities with adhesive tape.
3. Shave and disinfect the abdomen of the mouse and perform a ventral laparotomy from the pubis to the cranial border of the liver using surgical scissors with sharp/blunt tips. Incise the abdominal wall to both sides, caudal of the diaphragm.
4. Expose the inferior vena cava (IVC) by carefully moving the intestine to the right side. Carefully dissect fat tissue around the IVC using sterile cotton tip swabs and angled tip forceps.
5. Incise the suprahepatic diaphragm and open the thoracic cavity by two lateral incisions using fine surgical scissors with sharp/sharp tips. Ligate the thoracic IVC using surgical silk (5/0).
6. Cannulate the infrarenal IVC using a shielded i.v. catheter, carefully remove the needle, connect the perfusion pump and start perfusing the liver. If performed properly, the liver should immediately begin to blanch and swell.
7. Transect the portal vein allowing suitable drainage of blood and perfusion media using fine surgical scissors.
8. Perfuse the liver with approximately 30 mL of liver perfusion media, and then switch to liver digest media (30 mL). Turn off the perfusion pump and remove the cannula once perfusion is complete.
9. Gently expose the central region of the liver and locate the connecting tissue linking the liver lobes. Grab the central connecting fibers and carefully dissect all tissues holding the liver in place and gently remove the liver.
10. Immediately transfer the liver into a 50 mL polypropylene conical tube containing 15 mL of liver digest media.
    NOTE: There is no specific time-line for this step. After perfusion, the liver remains in digest media for the transport to a sterile cell culture hood. All subsequent steps should be performed in a sterile environment.

3. Isolation and Treatment of Cells

1. Within a cell culture hood, transfer the liver from the 50 mL polypropylene conical tube containing digestion media into a sterile tissue culture dish.
2. Using sterile forceps, gently tear the four lobes of the liver. If performed properly, digestion medium should become turbid due to isolating hepatocytes from the liver.
3. Using a 25 mL sterile pipette tip, filter the turbid digestion medium through a 70 µm nylon cell strainer into a new 50 mL polypropylene conical tube to remove undigested tissue particles and cell debris.
4. Repeat steps 3.2 and 3.3 with sterile phosphate-buffered saline (PBS). This assists with removing any residual hepatocytes from the liver.
5. Centrifuge at 50 x g for 2 min at 4 °C. Aspirate the supernatant containing excess cell debris and gently re-suspend cells in 25 mL cold 1x Williams' Medium E.
6. Using a 25 mL sterile pipette tip, filter the re-suspension through a new 70 µm nylon cell strainer into a 50 mL polypropylene conical tube to achieve a cleaner suspension.
7. Repeat steps 3.5 and 3.6 for three additional washes using cold Williams' Medium E 1x to obtain a clearer cell suspension.
8. After the final washing step, aspirate supernatant to remove excess cell debris. Re-suspend and filter cells in 25 mL warm Williams' Media E 1x that contains primary hepatocyte thawing and plating supplements through a new 70 µm nylon cell strainer into a 50 mL polypropylene conical tube.
9. Count cells within the 25 mL cell suspension using a hemocytometer. On average expect 15 - 20 million cells per adult liver. Determine cell viability using trypan blue staining.
10. Plate cells in 2 mL of Williams' Media E 1x that contains primary hepatocyte thawing and plating supplements on a collagen coated 6-well cell culture cluster (9×10^5 cells per well).
11. After 4 h, change media to Williams' Media E 1x that contains primary hepatocyte maintenance supplements.
12. After 24 h, serum starve cells with Dulbecco's modified Eagle medium (DMEM) media 1x for 6 h.
13. Treat cells with PBS as vehicle, FGF23 (25 ng/mL), LPS (100 µg/mL) or IL-6 (50 ng/mL) in serum-free medium and incubate for 24 h.

4. Isolation of RNA

1. Prepare RNA using a commercial spin column kit according to the manufacturer's instructions.

5. Generating cDNA from isolated RNA by Reverse Transcription

1. Add 200 ng of isolated RNA sample to a PCR tube.
2. Add 14 µL of endonuclease-free H_2O to PCR tube from step 5.1.
3. Add 4 µL of reverse transcriptase to PCR tube from step 5.1.
4. Re-suspend contents gently to make mixture homogenous; centrifuge gently.

5. Place PCR tube containing the mixture into a thermocycler. Run thermocycler for reverse transcription: 1 cycle of 25 °C (5 min), 42 °C (30 min), 85 °C (5 min) and then hold at 4 °C. The final product will be the desired cDNA.

6. Analysis of Cells by Quantitative Real-time PCR (qPCR)

1. **Prepare the 96-well plate.**
    1. Place all components of qPCR reaction on ice: 1 µL cDNA, .25 µM forward primer, .25 µM reverse primer, 5 µL SYBR Green, 3.5 µL PCR grade water to a total volume of 10 µL. Using SYBR Green, prepare master mix to run all samples in triplicate.
    2. Once the master mix is complete, vortex to make homogenous. Aliquot 9 µL of qPCR master mix into each well of a 96-wll plate. After, carefully add 1 µL of cDNA into each well. Total reaction volume will become 10 µL.
    3. After loading is complete, seal 96-well plate with optical adhesive film and briefly centrifuge 96-well plate (50 x g for 1 min).
2. **Run samples in a Real Time-PCR instrument. Place sealed 96-well plate into Real Time-PCR instrument and run samples as per instrument manufacturer recommendations. Examples of standard and fast cycles are included below.**
    1. For standard cycling parameters, use the following: initial denaturation at 94 °C for 120 s, then 40 cycles of 94 °C for 15 s, 60 °C for 60 s, and then optionally hold at 4 °C.
    2. For fast cycling parameters: use the following: initial denaturation at 94 °C for 30 s, then 40 cycles of 95 °C for 5 s, 58 °C for 15 s, and then 72 °C for 10 s.
3. Refer to instrument manual for guidance on how to analyze data.

7. Analysis of Cell Supernatants by ELISA

NOTE: All steps are performed according to manufacturer's protocol.

1. **Sample Preparation**
    1. Dispense 398 µL of diluent into separate tubes. Pipette 2 µL of the cell supernatant sample into the 398 µL diluent tube. This will provide a dilution fold of 200.
    2. Repeat step 7.1.1 for each sample to be tested.
2. **Procedure**
    1. Pipette 100 µL of 200-fold diluted sample and a standard into the wells of a 96-well plate.
    2. Incubate the 96-well plate at 25 °C for 45 min on a microplate shaker at 150 rpm.
    3. Wash the wells 5 times with 1x wash buffer solution. Once the last wash is completed, remove all residual wash solution with absorbent paper.
    4. Add 500 µL of HRP-conjugate reagent into each well.
    5. Repeat steps 7.2.2 - 7.2.3.
    6. Pipette 100 µL of tetramethylbenzidine (TMB) reagent into each well of 96-well plate.
    7. Gently mix 96-well plate at 25 °C for 20 min on a microplate shaker at 150 rpm. Stop the reaction by directly adding 100 µL of stop solution to each well and mix gently. Once the stop solution is added the color should turn yellow. This informs that the stop solution has been correctly mixed.
    8. Using a microtiter plate reader, determine the optical density at 450 nm within the first 5 min.
3. **Calculation of Results**
    1. Calculate a table of average absorbance values (A_{450}) for each sample and each standard.
    2. Assemble a standard curve by plotting the A_{450} mean of each standard against its standard concentration in ng/mL on a linear graph. Allow the x-axis to represent the concentration and the y-axis to represent A_{450} values.
    3. Utilizing the mean A_{450} from each diluted sample, determine the concentration of the desired protein in ng/mL from the standard curve.
    4. Multiply the sample concentration by the dilution factor. This will allow for determining the actual concentration of the desired protein in the cell supernatant sample.
    5. Plot the constructed table from samples into a line graph. If OD_{450} values fall outside of the standard curve, samples should be diluted appropriately and re-tested.
    6. Normalize results to 500,000 cells.

Representative Results

Histology

Representative light microscopy images of primary isolated and cultured cells are depicted in **Figure 1A**. Immunocytochemical analysis demonstrates that isolated hepatocytes highly express albumin (red) as well as fibroblast growth factor receptor 4 (FGFR4) (green). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (**Figure 1B**).

Quantitative real-time PCR

Primary isolated hepatocytes were treated with FGF23 (25 ng/ml) LPS (100 µg/mL) or IL-6 (50 ng/mL) for 24 h and mRNA levels of CRP and IL-6 were analyzed by quantitative real-time PCR. FGF23, LPS and IL-6 significantly increased expression of CRP and IL-6. (**Figure 2A**)

ELISA

Cell culture supernatants from isolated primary hepatocytes were analyzed by ELISA for CRP levels. In line with our quantitative real-time PCR analysis, LPS, IL-6 as well as FGF23 treatments significantly increased CRP levels in cell supernatants when compared to PBS-treated cells. (**Figure 2B**)

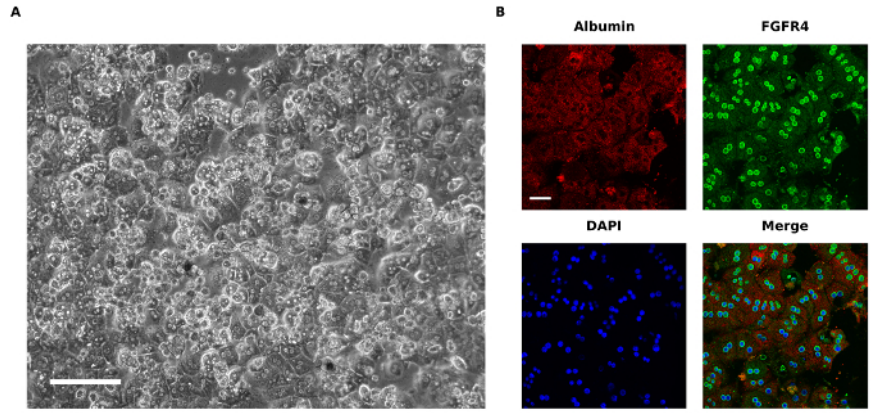

Figure 1: (**A**) Phase contrast microscopic image of isolated murine primary hepatocytes. 24 hours after plating, cells exhibit a hexagon-like shape and often appear bi-nucleated. (Scale bar = 100 µm); (**B**) Immunofluorescence microscopic analysis reveals that the majority of isolated cells express albumin (red), a hepatocyte-specific marker. Cells also highly express FGFR4 (green). DAPI stains nuclei. Original magnification 40X. (Scale bar = 50 µm). Please click here to view a larger version of this figure.

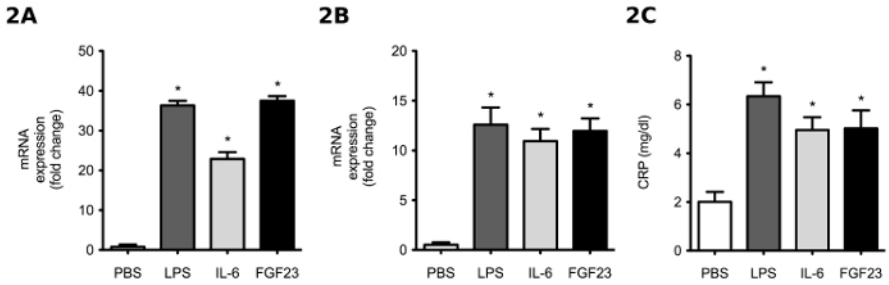

Figure 2: Quantitative Real-time PCR Analysis of Isolated Murine Primary Hepatocytes to Determine CRP and IL-6 Expression. (**A**) LPS, IL-6 and FGF23 treatments significantly increase mRNA levels of CRP when compared to PBS-treated cells. (values are expressed as fold change ± SEM; p <0.01; n = 3 independent isolations). (**B**) Cells treated with LPS, IL-6 or FGF23 also show a significant increase in mRNA levels of IL-6 when compared to PBS treatments. (values are expressed as fold change ± SEM; p <0.001; n = 3 independent isolations) (**C**) Quantification of CRP protein levels in supernatants from isolated murine primary hepatocytes by enzyme-linked immunosorbent assay (ELISA). LPS, IL-6 and FGF23 significantly increase CRP protein levels when compared to PBS treatments. Values represent CRP concentrations in mg/dl per 500,000 cells. (p <0.05, n = 3 independent isolations). Please click here to view a larger version of this figure.

Discussion

Isolating primary hepatocytes from mice is a fast, inexpensive and reliable tool to study inflammatory responses *ex vivo.* If performed correctly, results can be easily generated and reproduced in a timely and cost-efficient manner. The following points should be carefully assessed in order to ensure a successful isolation.

The surgical incision and the cannulation of the IVC should be performed under general anesthesia and not after euthanasia. A young, inexperienced investigator will need more time in the beginning to become familiar with the anatomical features of the murine abdomen and to perform a correct cannulation. Keeping the donor animal alive until the perfusion starts, will significantly increase the viability of isolated cells. Ligation of the supra-hepatic IVC should be performed since it will significantly increase the yield of isolated hepatocytes. Alternatively, a micro-surgical clamp can be placed on the IVC. However, following thoracotomy, the animal will become deceased within a couple of minutes. Hence all subsequent steps should be performed promptly to ensure that the liver maintains sufficient blood flow until initiation of perfusion. For the cannulation, the usage of a retractable intravenous catheter is highly recommended. After cannulating the IVC, the catheter needle can be carefully retracted, which minimizes the risk of vessel perforation. The last critical step is the connection of the perfusion system to the catheter. After removing the needle from the catheter, blood should drain from the end of the catheter before connecting it to a perfusion pump. This step minimizes the risk of air-emboli, which can cause a significant reduction in quantity as well as the quality of isolated cells.

Once the perfusion is initiated, the liver should immediately and homogenously change its color from red to a pale yellow. Parts remaining red will indicate insufficient perfusion or the presence of air-emboli. Washing and filtering of isolated cells should repeatedly be performed. This will ensure efficient removal of excess cell debris. Healthy isolated hepatocytes will adhere within the first 4 h of being plated. Hence, plating media can be changed to maintenance media at this time point. After overnight incubation, cells will appear in a more hexagonal shape with a prominent nucleus. Hepatocytes can also be bi-nuclear (**Figure 1A**). Granulation or cellular blebbing is an indicator for a poor isolation. If

hepatocytes continuously not adhere properly or the cell viability remains low, the amount of digestion media used for perfusion should be altered. This should avoid over- or underdigestion of hepatic tissue.

If a higher yield of cells is required, an anterograde perfusion via the portal vein can be performed. Klaunig et al. have shown that this particular technique can increase total cell yield. Alternatively, following portal vein cannulation intermittent clasping of the IVC can be applied. This will periodically increase the pressure during the perfusion and supposedly also can increase total number of isolated cells. Perfusion time as well as perfusion rate might have to be adjusted[14]. We, however, have not performed alternative perfusion protocols.

After a first successful isolation, a hepatocyte-specific immunostaining (*e.g.* for albumin) should be performed to ensure the purity of the isolation (**Figure 1B**). 24 h after isolation, hepatocytes should be serum starved for 4 to 8 h and then used for treatments. When studying the acute phase response, cells can be treated with LPS, which is known to induce inflammation via Toll-like receptor 4 and NF-κB[15]. Pro-inflammatory cytokines, such as IL-6, mediate their effects through interleukin receptors[16]. Moreover, we have recently demonstrated that FGF23 can activate FGFR4 and the PLCγ/calcineurin/NFAT pathway and induce an inflammatory response in the liver[7,17]. To ensure sufficient transcription and translation of target genes, cells should be treated for 24 h. Isolating mRNA and consecutive analysis of gene expression using quantitative real-time PCR together with ELISA of cell supernatants provides robust information that can easily be repeated. This allows the investigation of novel pro-inflammatory mediators, its hepatic receptors and their consecutive downstream signaling mechanisms. Isolated primary cells, especially from transgenic animals will provide superior results when compared to generic cancer cell lines such as HepG2 and Hep3B cells. Due to its simplicity, this approach can also be used for screenings to investigate the effect of novel drugs. Nevertheless, studying complex pharmacological or patho-physiological mechanisms will require additional confirmative experiments, mainly in an *in vivo* setting.

We have not performed long-term studies since isolated hepatocytes tend to dedifferentiate within a couple of days. However, this can be overcome by special cell culture techniques, as reported by others[18]. These include a collagen double-gel configuration, hepatocyte spheroids, co-culture with endothelial cells, and micropatterned co-cultures with 3T3-J2 fibroblasts. Taken together, the isolation of primary murine adult hepatocytes provides a robust tool for scientists interested in metabolomics, inflammation, immunity and the hepatic response to drugs and toxins. It is characterized by simple feasibility, moderate costs and fast reproducibility.

Disclosures

The authors have no conflict of interests to declare.

Acknowledgements

This work was supported by the NIH (R01HL128714 to C.F.) and (F31DK10236101 to K.S) and the American Heart Association (C.F. and A.G.).

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