Video Article

Isolation and Culture of Adult Mouse Cardiomyocytes for Cell Signaling and *in vitro* Cardiac Hypertrophy

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URL: https://www.jove.com/video/51357
DOI: doi:10.3791/51357

Keywords: Basic Protocol, Issue 87, adult mouse cardiomyocytes, collagenase, isolation, primary cell culture

Date Published: 5/21/2014

Citation: Li, D., Wu, J., Bai, Y., Zhao, X., Liu, L. Isolation and Culture of Adult Mouse Cardiomyocytes for Cell Signaling and *in vitro* Cardiac Hypertrophy. *J. Vis. Exp.* (87), e51357, doi:10.3791/51357 (2014).

Abstract

Technological advances have made genetically modified mice, including transgenic and gene knockout mice, an essential tool in many research fields. Adult cardiomyocytes are widely accepted as a good model for cardiac cellular physiology and pathophysiology, as well as for pharmaceutical intervention. Genetically modified mice preclude the need for complicated cardiomyocyte infection processes to generate the desired genotype, which are inefficient due to cardiomyocytes' terminal differentiation. Isolation and culture of high quantity and quality functional cardiomyocytes will dramatically benefit cardiovascular research and provide an important tool for cell signaling transduction research and drug development. Here, we describe a well-established method for isolation of adult mouse cardiomyocytes that can be implemented with little training. The mouse heart is excised and cannulated to an isolated heart system, then perfused with a calcium-free and high potassium buffer followed by type II collagenase digestion in Langendorff retrograde perfusion mode. This protocol yields a consistent result for the collection of functional adult mouse cardiomyocytes from a variety of genetically modified mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/51357/

Introduction

Cardiomyocytes are not proliferative. There are some atrial cardiomyocyte cell lines, like HL-1 and AT-1 cells derived from mouse atrial tumors; however, there are no adult ventricular cardiomyocyte cell lines available for research. Primary cell cultures of adult mouse cardiomyocytes provide a powerful model for heart research at the cellular and molecular levels. To date, they have been used extensively for biochemical, physiological, and pharmacological research[1]. Additionally, the frequent use of genetically modified mice has necessitated effective methods of cardiomyocyte isolation. Pure culture allows for conditions free from interaction with other organs and the systemic circulation, such as through endogenous neurohormonal and hormone-like factors[2,3]. However, successful isolation of cardiomyocytes can be challenging.

The protocol we introduce herein is based on our experiences with adult rat cardiomyocytes and the method described by O'Connell *et al*[4,5]. Especially in 2007[5], they described detailed techniques from buffer preparations to cell culture and functional assay. Since mouse myocytes are highly susceptible to contracture compared to the rat cardiomyocytes, the buffers or media used for the perfusion, digestion, Ca^{2+} toleration, plating and culture in the mouse protocol are supplemented with a nonspecific excitation-contraction coupling inhibitor, 2,3-Butanedione monoxime (BDM) to inhibit their spontaneous contraction, hence the viability and yield of rod-shaped myocytes improve significantly. In the protocol introduced here, the myocytes are separated in a high potassium buffer from the isolated heart by modified Langendorff perfusion with type II collagenase. Collagenase II effectively breaks down the intercellular matrix and releases cells. The perfusion solution also keeps cellular metabolism at a low level[6]. In addition, the isolated heart system from Harvard Apparatus we used here is well-designed for precise temperature and constant pressure control[7]. This approach provides highly reproducible preparations and uniform populations of single cell type, which can be used in overnight culture for measuring signaling proteins or 2-3 day culture for cardiac hypertrophic assays.

Protocol

All research on mice was done according to procedures and guidelines of the National Institutes of Health, and the protocols were approved by the Institutional Animal Care and Use Committee of the University of Toledo, College of Medicine and Life Sciences.

1. Perfusion System Preparation

1. The day before isolation, fill the entire perfusion system (including reservoirs) with a solution of fast alkaline residue-free detergent. Allow the solution to soak into the system for a few hours or overnight.
2. The next morning, adjust the thermocycler temperature to 37 °C and allow the solution to warm up. Remove the detergent solution and flush the system thoroughly with sterilized distilled water. Do not forget all the side branches.
3. Fill the system with perfusion buffer via a constant peristaltic pump and draw any air bubbles out from the aorta chamber (bubble trap) with a side-mounted syringe to protect the heart from coronary occlusion. The flow rate of the peristaltic pump should be checked routinely.

2. Preparation of Buffers, Culture Media, and Dishes

1. Perfusion Buffer: Make 500 ml of 1x perfusion buffer prior to use. This calcium-free buffer contains 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 10 mM Na-HEPES, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 0.032 mM phenol red, 30 mM taurine, 10 mM BDM, and 5.5 mM glucose. Adjust the pH to 7.0 with 2 M HCl and filter through a 0.2 µm filter, store at 4 °C.
   Note: a) The perfusion solution should be prepared on the same day when it is used. In some cases, it is acceptable to store the solution for less than 3 days at 4 °C. b) This buffer has a high potassium concentration up to 15.3 mM, which can keep the cardiomyocytes quiescent, reduce ATP consumption and increase Ca^{2+}-tolerant ability.
2. Digestion Buffer (300 U/ml, 25-50 ml/heart): prepare just prior to perfusion. Weigh 15,000 U of total activity of type II collagenase, dissolve in 50 ml perfusion buffer in culture hood. Add 25 µl of 100 mM $CaCl_2$ (stock solution, filter sterilized) to make the final calcium concentration 50 µM. Warm the buffer to 37 °C when ready for isolation. Unlike trypsin, collagenase works best in the presence of 50-100 µM Ca^{2+}.
3. Stop Buffer (50 ml/heart): Operate in culture hood. Mix 45 ml of perfusion buffer with 5 ml sterile FBS.
   1. Ca^{2+} solution I (12.5 µM Ca^{2+}): Add 2.5 µl of 100 mM $CaCl_2$ to 20 ml of stop buffer and mix.
   2. Ca^{2+} solution II (100 µM Ca^{2+}): Add 10 µl of 100 mM $CaCl_2$ to 10 ml of stop buffer and mix.
   3. Ca^{2+} solution III (400 µM Ca^{2+}): Add 40 µl of 100 mM $CaCl_2$ to 10 ml of stop buffer and mix.
   4. Ca^{2+} solution IV (900 µM Ca^{2+}): Add 90 µl of 100 mM $CaCl_2$ to 10 ml of stop buffer and mix.
4. Plating medium (50 ml/heart): Transfer 43 ml of MEM containing 2 mM L-glutamine and 1.26 mM $CaCl_2$ into a sterile 50-ml conical tube in culture hood and add 5 ml of FBS, 1 ml of BDM stock solution (500 mM, filter sterilized), and 0.5 ml penicillin/streptomycin (10,000 U/ml). Mix and equilibrate for 2-3 hr prior to myocyte isolation in 2% CO_2, 37 °C incubator with the caps loose. Right before myocyte plating, add 0.5 ml of 200 mM ATP (pH 7.2, stock solution, filter sterilized) into the medium.
5. Culture medium (50 ml/heart): Transfer 48 ml of MEM containing 2 mM L-glutamine and 1.26 mM $CaCl_2$ into a sterile 50-ml conical tube in culture hood and add 1 ml of BDM stock solution (500 mM, filter sterilized), and 0.5 ml penicillin/streptomycin (10,000 U/ml). Mix and equilibrate in 2% CO_2, 37 °C incubator prior to myocyte isolation. Prior to use, add 0.5 ml of 100 mg/ml BSA (filter sterilized) into the medium.
   Note: Using a 2% CO_2 incubator facilitates maintaining the culture medium pH at 6.9-7.0, which helps the myoctyes maintain their rod-shaped morphology up to 24 hr.
6. Laminin (1-2 µg/cm^2)-coated dishes or plates: Transfer 200 µl of 1 mg/ml natural mouse laminin to 20 ml of sterilized PBS (w/o calcium and magnesium) and mix. Add 1 ml to 35-mm dishes, 2.5 ml to 60-mm dishes, or 5 ml to 100-mm dishes, shake to evenly cover the surfaces, and place in 2% CO_2, 37 °C incubator until myocyte plating.
   Note: If the dishes or plates are not used on the day coated, they can be sealed with Parafilm and stored at 4°C overnight (Slow Coating). Sealing is required to prevent evaporation and contamination. Coated plates can be kept at 4 °C for up to 1 week.

3. Mouse Heart Removal and Cannulation

1. Soak the scissors and forceps in 70% ethanol for 30 min and let them dry.
2. Weigh the mouse to the nearest 0.1 gram. Record its body weight, strain, sex, and date of birth.
3. Inject 200 µl heparin (100 IU/mouse) i.p. 10 min prior to anesthesia to prevent coagulation of blood in the coronary arteries. Anesthetize the mouse with 200 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine by i.p. injection and wait for 5-10 min until the mouse stops responding to tail/toe pinches.
4. Secure the mouse in the supine position by gently fixing the forepaws and hindpaws to a pinnable work surface (*i.e.* styrofoam) on an animal surgery tray or table bench near the perfusion system.
5. Wipe the chest and abdomen with 70% ethanol. Make a midline skin incision from mid abdomen to the diaphragm with a surgical scissor.
6. Cut the diaphragm with another surgical scissor and hold the sternum with curved serrated forceps. Cut bilaterally and retroflect thoracic cage to expose the heart.
7. Lift the heart slightly using fine curved serrated and atraumatic forceps and dissect the heart out of the thoracic cavity as close as possible to the dorsal thoracic wall.
8. Transfer the heart to a 100-mm dish containing cold perfusion buffer. Carefully trim away the connective tissues such as lungs, thymus, and bronchi.
9. Identify the aorta and its cranial branches, which are usually hidden by thymus and fat pad. Cut the aorta below its first branch[13], grasp the aortic wall, lift the heart, and slightly slip it on to the perfusion fluid-filled aortic cannula (1.0 mm OD stainless steel cannula with blunt/flat tip and notches 1 and 2 mm above the tip; or a 22 G reusable feeding needle) using two ultra fine-tip forceps.
   Note: a) The perfusion fluid should be dripping during heart cannulation to prevent gas bubbles from entering the coronary arteries; b) Keep the tip of the cannula just above the aortic valve.
10. Clamp the aorta to the cannula and ligate the aorta to the cannula with 6-0 surgical silk. The whole cannulation should take less than 120 sec.

4. Heart Perfusion and Digestion

1. Perfuse the heart with calcium-free perfusion buffer at flow rate of 4 ml/min about 4-5 min until the effluents become clear, then switch to digestion buffer containing 50 µM $CaCl_2$ and perfuse for 3.5-20 min depending on mouse strain, perfusion pressure and collagenase activity. If applicable, increase the aorta pressure to 70-80 mmHg when digesting. When necessary, enzyme perfusate can be recirculated for digestion. Typically, 300 U/ml enzyme buffer will digest a heart in 10-12 min at a flow rate of 4 ml/min; If applying afterload pressure at 70 mmHg, 300 U/ml will take only 3.5-5.5 min at a flow rate of 4 ml/min.
2. Stop digestion when the heart becomes slightly pale and flaccid. It should be spongy when gently pinched.

5. Cells dissociation and Calcium reintroduction

1. Pull the aorta from the cannula with 70% ethanol-soaked forceps and put the heart into a sterile 60-mm dish containing 2.5 ml digestion buffer. Move into the culture hood using sterile supplies, staying mindful of sterile techniques for this and follow-up steps.
2. Remove aorta, atria, and great vessels with fine surgical scissors. Gently tease the ventricle into 10-12 small pieces with two fine-tip forceps.
3. Gently pipette the heart pieces and cells up and down ~10x with a 10-ml transfer pipette and then transfer to a 15-ml polypropylene conical tube. Rinse the dish with 7.5 ml calcium solution I containing 12.5 µM $CaCl_2$ and transfer this into the tube, resulting in a final volume of 10 ml.
4. Gently pipette the cell suspension up and down using a fine-tip transfer pipette to disperse the large pieces of heart tissue.
5. Then centrifuge for 3 min at 20 x g to separate out small non-myocyte cells such as endothelial cells and fibroblasts.
6. Aspirate off the supernatant and resuspend the myocyte pellet in 10 ml calcium solution I with a supplement of 100 µl of 200 mM ATP (pH 7.2, stock solution, filter sterilized). Leave the tube in the hood for 3-5 min.
7. Transfer duplicate 10 µl aliquots to a hemacytometer to count rod-shaped and round myocytes. Calculate the total number of myocytes and calculate the percentage of rod-shaped myocytes.
8. Centrifuge the myocytes for 3 min at 20 x g. Remove the supernatant and resuspend the pellets in 10 ml calcium solution II containing 100 µM $CaCl_2$. Disperse the myocytes with the fine-tip transfer pipette by pipetting up and down.
9. Repeat the above step (5.8) using calcium solution III (400 µM $CaCl_2$) and IV (900 µM $CaCl_2$), respectively.
10. For mouse myocyte primary culture, resuspend the final myocyte pellet in 5 ml of plating medium. Count the total number of myocytes and calculate the percentage of rod-shaped myocytes.

6. Cell Culture

1. Adjust the concentration of rod-shaped myocytes to 25,000 rod-shaped myocytes/ml in a 50-ml tube. Gently pipette the myocytes using a 10-ml pipette to suspend them well. Avoid cell sedimentation in the pipette and tube to ensure equal cell density for each dish or plate.
2. After aspirating off the laminin coating solution, plate the myocytes into those dishes or plates. Gently slide the dishes or plates forward and backward and side-to-side in a cross-like pattern three to four times on the surface of the culture hood.
3. Place the dishes or plates in a 2% CO_2 incubator at 37 °C. Incubate for 1-3 hr to allow myocyte attachment at a rate of about 80%.
4. After attachment, gently aspirate off the medium containing unattached myocytes and cell debris. Gently add culture medium to the sides of the dishes or plates. Perform this medium change one by one. Immediately return the dishes or plates to the incubator.
5. After O/N culture, the myocytes can be changed to the same medium **without BDM** before studies of short term cell signaling. Keep the BDM in long term culture for hypertrophic or apoptosis assays.
   Note: BDM (2,3-butanedione monoxime) can inhibit the myosin-ATPase and prevent contraction. Its inhibition of myosin-based contraction is readily reversible. It is reported that BDM may have some potential side effects, such as acting as a non-specific phosphatase, increasing calcium release from SR, and alteration of free calcium concentration and cellular electrical properties, so before the treatment, the BDM should be removed. Moreover, in some cases, a specific myosin II inhibitor Blebbistatin could be an alternative drug to inhibit cardiomyocyte contraction[8,9]. However, in our cases, BDM is better than Blebbistatin for the sake of the quality and quantity of the cardiomyocytes.

Representative Results

1. Successful Isolation Quantification

Two criteria are used to quantify the success of the isolation: first, the total number of cardiomyocytes isolated, and second, the ratio of rod-shaped calcium-tolerant to round non-tolerant myocytes. Generally this protocol takes around 75-90 min from heart removal to myocyte plating and yields around 1 million rod-shaped cardiomyocytes (70-90% of total myocytes harvested) from one adult mouse heart. This may vary with mouse body weight and strain. Typically, 0.7-1.0 million rod-shaped myocytes can be collected from a ~25 g C57BL/6J mouse, 1.2-1.6 million rod-shaped myocytes from a ~35 g black Swiss mouse, and 0.7-1.2 million rod-shaped myocytes can be collected from a ~30 g Na/K-ATPase heterozygous ($\alpha1^{S/R}$)[10] or ~22 g cardiac specific NCX-KO mouse[11]. Isolated myocytes normally have a distinct rod-shape with rectangular ends and clear cross-striations, shown in **Figure 1**.

2. Cell Function Identification

Our previous data have clearly shown that in cultured neonatal rat cardiomyocytes, 100 µM ouabain can activate PI_3K-dependent Akt phosphorylation and induce hypertrophy [12]. To further confirm these results in adult mouse cardiomyocytes, adult C57BL/6J mouse cardiomyocytes were cultured and treated with ouabain. **Figures 2** and **3** clearly show that ouabain can increase Akt phosphorylation in a dose-dependent manner and induce [^{3}H]-leucine incorporation during protein synthesis.

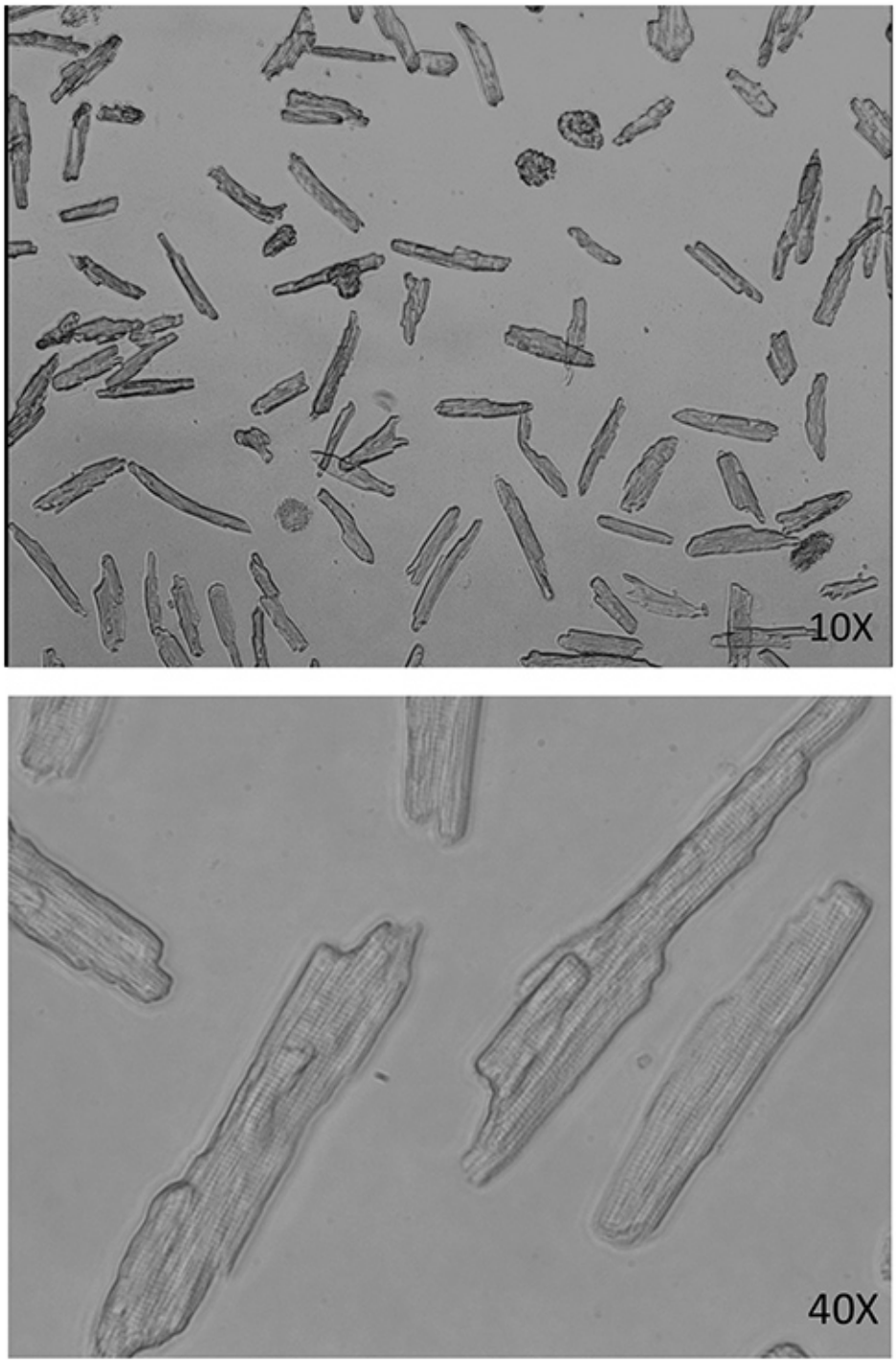

Figure 1. Normal rod-shaped cardiomyocytes from Black Swiss mouse after overnight culture. Myocytes were photographed under phase contrast microscopy using photo software.

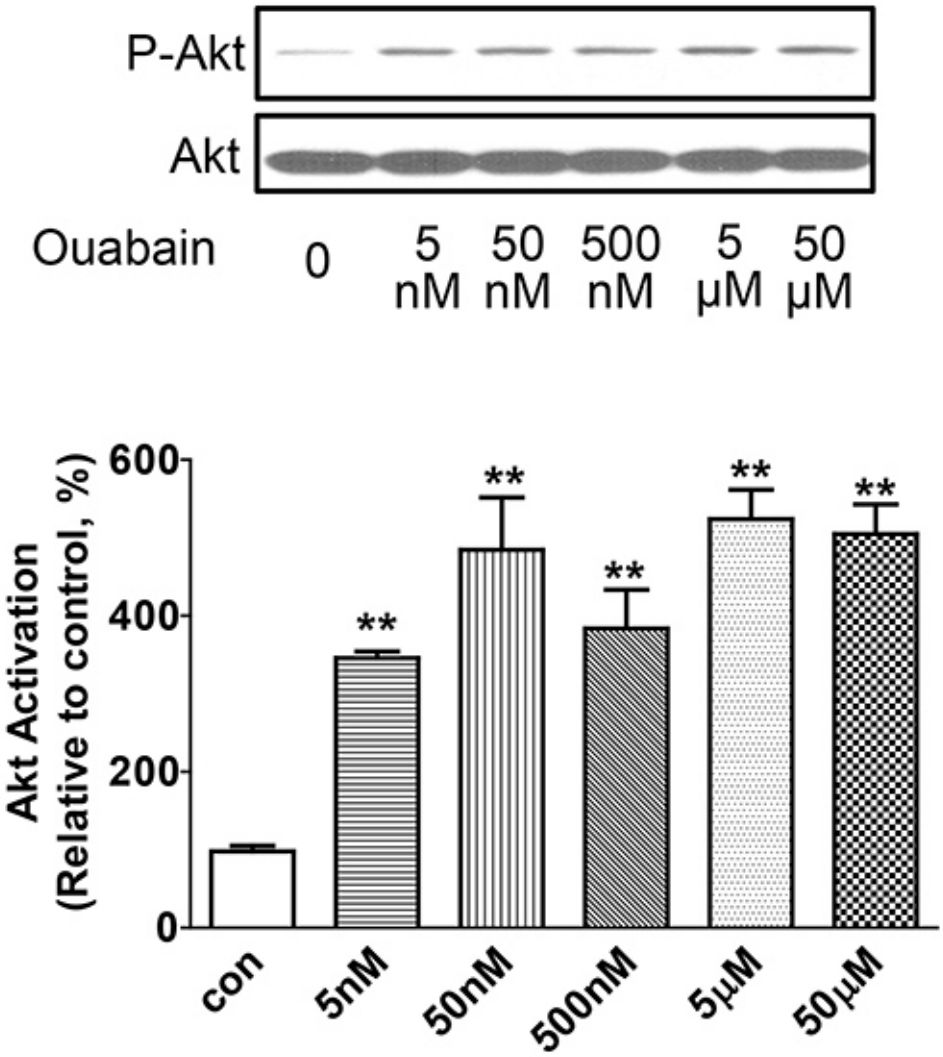

Figure 2. Activation of Akt by ouabain in cultured adult C57BL/6J mouse cardiomyocytes. Myocytes were exposed to 0-50 μM ouabain for 5 min, and lysates were assayed for phosphorylated (Ser^{473}) Akt (p-Akt) and Akt by western blots. Activation was quantified as ratio of phosphorylated to total Akt (n=5-10). *P < 0.05 vs. control, **P < 0.01 vs. control.

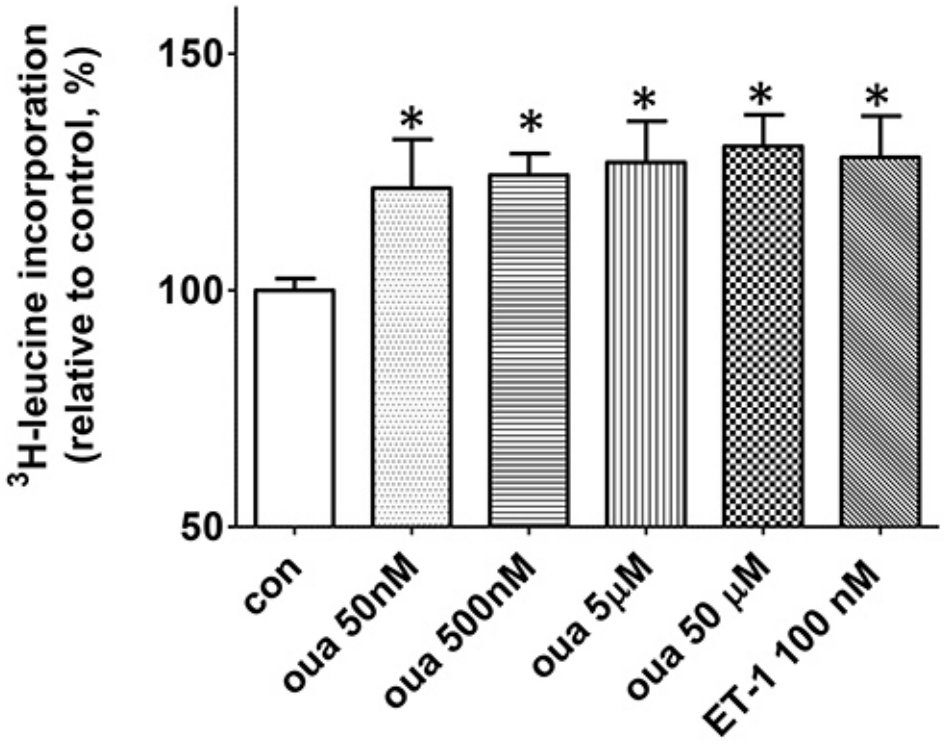

Figure 3. Ouabain stimulates protein synthesis in cultured adult C57BL/6J mouse cardiomyocytes. After 4 hr serum deprivation, myocytes were treated with the indicated conditions in the presence or absence of ouabain or 100 nM ET-1 (positive control) together with [^{3}H]-leucine (1 μCi/ml) for 12 hr. Cellular lysates were precipitated with 5% TCA and resuspended in 0.2 M NaOH/0.1% SDS, and the radioactivity was counted in a scintillation counter. The protein synthesis per sample was calculated to cpm/mg protein and normalized as compared to the control.

Solution/Buffer	Perfusion Buffer	Digestion Buffer	Stop buffer	Ca^{2+} Solution I	Ca^{2+} Solution II	Ca^{2+} Solution III	Ca^{2+} Solution IV
NaCl, mM	113	113	113	113	113	113	113
KCl, mM	4.7	4.7	4.7	4.7	4.7	4.7	4.7
KH_2PO_4, mM	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Na_2HPO_4, mM	0.6	0.6	0.6	0.6	0.6	0.6	0.6
MgSO4, mM	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Na-HEPES, mM	10	10	10	10	10	10	10
$NaHCO_3$, mM	12	12	12	12	12	12	12
$KHCO_3$, mM	10	10	10	10	10	10	10
Phenol red, μM	32	32	32	32	32	32	32
Taurine, mM	30	30	30	30	30	30	30
BDM, mM	10	10	10	10	10	10	10
Glucose, mM	5.5	5.5	5.5	5.5	5.5	5.5	5.5
FBS, % (v/v)	0	0	10	10	10	10	10
Collagenase II, mg/ml	0	1	0	0	0	0	0
$CaCl_2$, μM	0	50	0	12.5	100	400	900

Table 1. Solutions/buffers for adult mouse cardiomyocytes isolation.

Name	Company	Catalog #	Preparation	Storage
Fetal Bovine Serum (FBS)	Atlanta Biolgoicals	S11550	N/A	25 ml aliquots in sterile 50 ml tubes at -20 °C.

Bovine serum albumin (BSA) 100 mg/ml, 100x	Sigma	A7906	5 g in 50 ml diH_2O, sterile filter through 0.22 µm sterile syringe filter	5 ml aliquots in sterile 15 ml tubes at -20 °C.
$CaCl_2$ 100 mM, 100x	Sigma	C4901	0.555 g in 50 ml diH_2O, and sterile filter through 0.22 µm sterile syringe filter	10 ml aliquots in sterile 15 ml tubes at room temperature.
Laminin 1 mg/ml, 100x	Life technologies	23017-015	N/A	200 µl aliquots in sterile 0.5 ml microcentrifuge tubes at -20 °C.
2,3-Butanedione monoxime (BDM) 500 mM, 50x	Sigma	B0753	1.01 g BDM in 20 ml diH_2O, sterilize the solution by filtering through 0.22 µm filter in hood.	store at 4 °C.
Adenosine-5'-triphosphate disodium salt (Na_2-ATP) 200 mM, 100x	Sigma	A6419	Add 5 ml diH_2O to dissolve 1 g Na_2-ATP in 50 ml centrifuge tube, then use 2 mol/l NaOH to adjust pH to 7.2 and bring the final volume to 9 ml with diH_2O. sterilize the solution through 0.22 µm syringe filter.	0.5 ml aliquots in 1.5 ml sterile microcentrifuge tubes at -20 °C.
NaCl 3.77 M, 33.3x	Sigma	S7653	66 g in 300 ml diH_2O	store at 4 °C.
KCl 470 mM, 100x	Fisher Scientific	P217	3.5 g in 100 ml diH_2O	store at 4 °C.
KH_2PO_4 60 mM, 100x	Sigma	P5379	0.82 g in 100 ml diH_2O	store at 4 °C.
Na_2HPO_4 60 mM, 100x	Sigma	S0876	0.85 g in 100 ml diH_2O	store at 4 °C.
$MgSO_4$ 120 mM, 100x	Sigma	M-1880	3 g in 100 ml diH_2O	store at 4 °C.
HEPES 1 M, 100x	Life technologies	15630-080	N/A	store at 4 °C.
$NaHCO_3$ 600 mM, 50x	Sigma	S6014	10.1 g in 200 ml diH_2O	store at 4 °C.
$KHCO_3$ 1 M, 100x	Fisher Scientific	P235	10.1 g in 100 ml diH_2O	store at 4 °C.
Phenol red 3.2 mM, 100x	Sigma	P5530	0.12 g in 100 ml diH_2O	store at room temperature.

Table 2. Stock solution preparation and storage for adult mouse cardiomyocytes isolation and culture.

Discussion

For the best preparation, the most critical steps are: 1) promptly hooking up the mouse heart to the cannula after excision; 2) appropriate heart perfusion. Also, note that water quality, perfusion temperature, pH of buffer, sterilization, chemical purity, and clean, non-contaminated tubing and chambers are also important factors. 18.2 mΩ molecular biology-grade H_2O is highly recommended for buffer preparation. The pH of 200 mM ATP stock solution should be adjusted to 7.2, a step which is easily missed.

It is essential to quickly identify the aorta and to cut just below its first branch as shown by Flynn[13]. For efficient hook-up, a suitably-sized stainless steel cannula with notches and small serrated cross clamp are recommended. After ligation, the tip of the cannula should be above the aortic valve in order to help the collagenase fully interact with heart tissue during coronary circulation. Sometimes, the cell yield seems large initially but many cells are round-shaped after the calcium toleration step. One explanation is that coagulated blood in the vessels may not be completely removed from the heart, which leads to inadequate digestion. Another possibility is over digestion of the heart. Prolonged exposure to collagenase reduces myocyte calcium-tolerance[2]. During dissociation and calcium reintroduction, it is also important to handle the cardiomyocytes as gently as possible to maintain myocyte viability.

In summary, adult cardiomyocyte culture from genetically modified mice is a powerful tool for cardiac research. However, future work should keep in mind the genetic difference between humans and rodents when interpreting results.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was supported by National Heart, Lung, and Blood Institute Grant HL-36573. We thank Mr. David Sowa for editing this manuscript.

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