

High-Yield Method for Isolation and Culture of Endothelial Cells from Rat Coronary Blood Vessels Suitable for Analysis of Intracellular Calcium and Nitric Oxide Biosynthetic Pathways

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ABSTRACT

We describe here a method for isolating endothelial cells from rat heart blood vessels by means of coronary microperfusion with collagenase. This method makes it possible to obtain high amounts of endothelial cells in culture which retain the functional properties of their *in vivo* counterparts, including the ability to uptake fluorescently-labeled acetylated low-density lipoproteins and to respond to vasoactive agents by modulating intracellular calcium and by upregulating intrinsic nitric oxide generation. The main advantages of our technique are: (i) good reproducibility, (ii) accurate sterility that can be maintained throughout the isolation procedure and (iii) high yield of pure endothelial cells, mainly due to microperfusion and temperature-controlled incubation with collagenase which allow an optimal distribution of this enzyme within the coronary vascular bed.

INTRODUCTION

Endothelial cells play a crucial role in maintaining cardiovascular homeostasis. Their functions have been extensively studied by means of *in vitro* cultures, which actually represent an essential tool for the study of specific cellular and molecular mechanisms involved in cardiovascular physiology and pathophysiology. These studies often demand large numbers of cultured endothelial cells, thus justifying the interest in developing reliable and reproducible methods for isolation and culture of endothelial cells that could retain the functional properties of their *in vivo* counterparts. Isolation of organ-specific endothelial cells from medium- and small-sized blood vessels, such as coronary endothelial cells, cannot be performed as described for large blood vessels, including

human umbilical cords (1), human iliac vessels (2), bovine vena cava (3), rabbit and bovine pulmonary artery (4,5). Several studies have described the isolation of endothelial cells from the heart (6-9). However, the methods described in these reports mostly have limitations represented by the need for complex equipment and/or manipulation and by a low cellular yield.

In a recent study we set up a method to obtain endothelial cell cultures from coronary blood vessels of rat hearts (10): in this article we report on the technique used. Compared with the currently employed methods, our technique has the advantages of a good reproducibility, an accurate sterility that can be maintained throughout the isolation procedure and a high yield of pure endothelial cells potentially able to maintain functional properties intrinsic to the endothelium *in situ*. To address this point, we have analyzed the ability of the isolated cells to uptake fluorescently-labeled acetylated low density lipoproteins (LDL), a property deemed specific of coronary endothelial cells (6), and to modulate intracellular Ca²⁺ levels and nitric oxide (NO) generation in response to vasoactive substances, endothelial NO being involved in relaxation of the vascular wall by an autocrine/paracrine mechanism devoted to a moment-to-moment regulation of the vascular tone (11).

MATERIALS AND METHODS

Coronary endothelial cell isolation and culture

Rat coronary endothelial (RCE) cells were isolated from hearts of Wistar rats, 250-300 g b.w. The animals were injected i.p. with heparin (300 mg/kg b.w.) dissolved in 500 µl of sterile phosphate-buffered saline (PBS) and, 15-20 minutes later, they were anesthetized with ketamine/xylazine (1 ml/kg b.w.). After 15 minutes, under careful sterile conditions, the heart was

excised and promptly placed in a becker containing cold (4° C) Krebs-Henseleit solution without CaCl₂, pH 7.4, and quickly transferred in a sterile laminar flow hood. All subsequent work was performed in the same hood using sterile tools and materials. The aorta was cannulated with a steel cannula secured with a surgical suture and connected by a plastic tube to a 50-ml glass syringe attached to a microperfusion device (mod. 1830, Braun, Melsungen, Germany, perfusion rate varying from 0.075 to 5 ml/min). Using proper bearings, the heart was hung by its tubing in the inner chamber of a 5-ml glass organ bath, maintained at a controlled temperature of 37° C by a close circuit water thermostatic pump (Ultra-Thermostat NB-35 667, Colara, Germany). To remove all blood components trapped within the coronary circulation, the heart was perfused with 50 ml of Krebs-Henseleit solution without CaCl₂, pH 7.4, prewarmed at 37° C and gassed with a mixture of 95% O₂ - 5% CO₂, at a 5 ml/min perfusion rate. During this step, correct cannulation of the aorta can be assessed by controlling heart beating, a marker of heart viability, as well as by rapid washout of residual blood and by tissue stiffness. After washout, the tube was connected to another 50-ml syringe applied to the microperfusor and containing 0.1 % type I collagenase in 25 ml of Krebs-Henseleit solution added with 100 µM CaCl₂ and 0.1% BSA, pH 7.4, prewarmed at 37°C and gassed with a mixture of 95% O₂ - 5% CO₂. The heart was perfused for 15 min at a 1.5 ml/min perfusion rate at a constant temperature of 37° C to allow digestion of the coronary blood vessel components. Then, the heart was removed from the cannula, cleared from atria, atrioventricular septa and subepicardial fat tissue and placed in a becker containing 50 ml of harvesting solution (Krebs-Henseleit solution containing 100 µM CaCl₂ and 1% BSA, pH 7.4) prewarmed at 37°C. The heart was minced with sharp-pointed scissors and the tissue suspension was filtered with a 70 µm-mesh nylon cell strainer, collected into a 50 ml conical polypropylene tube and centrifuged at 100 g for 3 min to separate cardiomyocytes. The supernatant was recovered and centrifuged at 550 g for further 10 min. The pelleted cells were resuspended in a solution of trypsin (enzyme activity: 1500 BAEE units/mg), 25 mg in 50 ml PBS, for 20 min at 37°C and then centrifuged at 550 g for 10 min. The pellet was resuspended in growth medium (M199 medium containing 10% fetal calf serum, 10% newborn calf serum, 250 IU/ml penicillin, 0.626 µg/ml amphotericin and 250 µg/ml streptomycin) and plated into a 25 cm² flask at a density of 8,000-10,000 cells/cm². After 4 h, the cells were washed twice with PBS, added with 3 ml of growth medium and cultured until subconfluence, which was usually reached upon 5-6 days of culture.

Coronary endothelial cell characterization

To characterize RCE cells, we currently analyze their immunocytochemical pattern at the 1st culture passage using a panel of antibodies against cytokeratin, desmin, vimentin, endogenous peroxidase, α -smooth muscle actin and von Willebrand factor (vWF). To achieve a functional

characterization of the cells, we also assessed their ability to uptake fluorescein isothiocyanate (DiI)-labeled acetylated LDL (acetylated LDLs 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate complex, Biochemical Technologies Inc., Stoughton, MA, USA).

Cells plated on glass coverslips till confluence were incubated overnight in cell culture medium containing 200 µg/ml of DiI-ac-LDLs. Controls were obtained by parallel incubation with DiI-ac- LDLs solvent alone. After extensive washing, cells were fixed using 3% formaldehyde at room temperature for 20 min. Nuclei were stained with bisbenzimidazole (1 µg/ml for 2 min). Cells were analysed using an inverted epifluorescence microscope at two excitation wavelengths: 360 nm excitation for bisbenzimidazole and 550 nm excitation for DiI-ac-LDLs.

Simultaneous determination of intracellular NO generation assay with DAF-2/DA and intracellular calcium with fura-2AM

To clarify whether RCE cells may retain their functional property to upregulate intracellular NO ([NO]i) production and to modulate intracellular Ca²⁺ ([Ca²⁺]i) in response to vasoactive substances (10), we used a microscopic image analysis system as described previously (12). Briefly, RCE cells at the 1st culture passage were grown to subconfluence on round coverslips. We loaded the cells simultaneously with the NO-sensitive fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2/DA, 10 µmol/l, Calbiochem, La Jolla, CA, USA), the Ca²⁺-sensitive probe fura-2AM (4 µmol/l) and Pluronic F (0.02%, Molecular Probes, Eugene, OR, USA) in HEPES-bicarbonate buffer containing (mmol/l): NaCl 140, KCl 2.9, NaH₂PO₄ 0.5, NaHCO₃ 12, MgCl₂ 0.9, HEPES 10, CaCl₂ 1, glucose 10, L-arginine 0.1, adjusting pH to 7.4 with NaOH 1 N, for 45 min at 37° C. Both fluorescent probes were dissolved in DMSO in a ratio of 1:1000 of total buffer. Upon loading, we used the cells as soon as possible since DAF-2/DA, which becomes fluorescent upon oxidation by NO, tends to compartmentalise into cytosolic organelles and to diffuse back in the external medium. In some experiments, cells were loaded in the presence of 10 µmol/l L-NAME, a widely used NO synthase competitive inhibitor. [NO]i was determined by analysing the fluorescence emission at a 510 nm wavelength on excitation at a 490 nm wavelength typical for DAF-2/DA. [Ca²⁺]i dynamic was evaluated using a ratiometric method (excitation 340 nm/380 nm, emission 510 nm typical for fura-2AM) as previously described (10). For each experimental group, we performed fluorescence analysis of [NO]i and [Ca²⁺]i on at least 20 cells in the same microscopic field, examined with a 20x objective under a fluorescence inverted microscope equipped with a 75 Watt UV-Xenon lamp (Nikon Diaphot, Tokyo, Japan). Consecutive images, obtained with the noted excitation and emission wavelengths, were viewed with a charge coupled device (CCD) video camera (Photonic Science, UK), digitized by an analogical/digital converter (resolution: 256x256 pixels, Flashbus FBG32) and analyzed on-line using

an image analysis Microsoft Windows-based software (Autolab, RCS, Florence, Italy). Images were obtained every 3 sec after subtraction of the background. A noise/signal ratio of 0.5 was considered as the lowest detectable limit for both $[\text{NO}]_i$ and $[\text{Ca}^{2+}]_i$. After measurements of $[\text{NO}]_i$ and $[\text{Ca}^{2+}]_i$ in basal conditions, the agonists angiotensin II ($1 \mu\text{mol/l}$) was added directly to the RCE cell cultures, and the time course of $[\text{NO}]_i$ and $[\text{Ca}^{2+}]_i$ changes induced by the agonist was analyzed for at least 15 min. Calibration curves for $[\text{Ca}^{2+}]_i$ were performed using ionomycin and ethylene-bis-(oxyethylenitrilo)-tetra acetic acid (EGTA) and assuming a dissociation constant for fura 2-AM of 224 nmol/l . Calibration curves for $[\text{NO}]_i$ were performed using the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, Tocris Cookson, Ltd., Bristol, UK). The experimental data were exported as ASCII file format and graphically elaborated with MicroCal Origin 2.8 software (OriginLab, Northhampton, MA). The reported values are the mean (\pm s.e.m.) of three separate RCE cell isolations.

Reagents

Unless otherwise specified, all the reagents used were from Sigma (St Louis, MO, USA). Sera for cell culture were from Gibco BRL (Paisley, Scotland). Cell culture plastic ware was from either Costar (Corning Costar Co., Costar Italia, Milan, Italy) or Falcon (Becton Dickinson Europe, Meylan, France).

RESULTS AND DISCUSSION

During microperfusion with collagenase solution, the hearts hung in the inner chamber of 5 ml organ bath at a controlled temperature of 37°C should stop beating and change their color from deep red to dark pink, thus indicating successful perfusion of the whole cardiac wall. Upon removal of the hearts from the chamber and clearing from atria and atrioventricular septa, enzymatic digestion of cardiac stroma can be also inferred by softness of the ventricular walls at mincing with scissors. If digestion was properly carried out, the harvesting solution in which hearts are minced quickly becomes turbid due to detachment and suspension of individual cells. When the tissue suspension is filtered through $70 \mu\text{m}$ -mesh cell strained, a loose pellet should already be visible at the bottom of 50 ml conical tube before centrifugation. After the first, low-speed centrifugation, cells in the pellet should be mostly cardiomyocytes, which can be easily recognized under an inverted phase contrast microscope based on cross-banding of myofibrils within cytoplasm (Fig. 1).

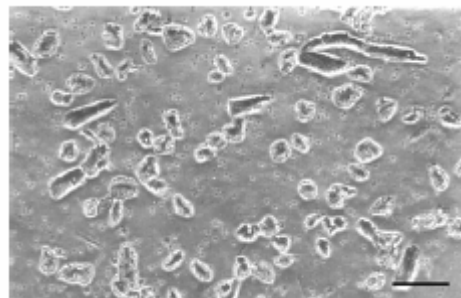


Fig. 1: Phase-contrast microscopy of cardiomyocytes and tissue debris found in the pellet upon the first, low-speed centrifugation. Bar = $100 \mu\text{m}$

Many viable cardiomyocytes, characterized by typical rod-like shape and rhythmic contractions, can be usually seen. Upon trypsin digestion of the supernatant and subsequent seeding in growth medium into 25 cm^2 flasks for 4 hours, most RCE cells appear as small-sized, round-shaped bodies adherent to the flask floor (Fig. 2).

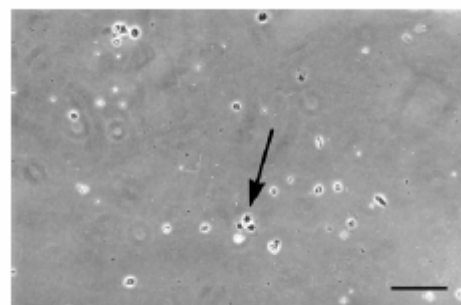


Fig. 2: Phase-contrast microscopy of putative RCE cells adherent to the floor of a 25 cm^2 flask 4 h after seeding. Most of them appear as small-sized, round-shaped bodies. A few cells already show a more flattened shape (arrow). Bar = $100 \mu\text{m}$

In our experience, there are no differences in RCE cell yield between untreated flasks and gelatin-coated flasks, at variance with HUVEC isolation which requires gelatin-pretreated plastic ware (1). Depending on the overall number of viable cells recovered, scanty RCE cells may already show a flattened shape. Flattened RCE cells, often grouped together in epithelial-like clusters, usually appear in the culture from day 1 on (Fig. 3) and reach subconfluence in 4-5 days (Fig. 4).

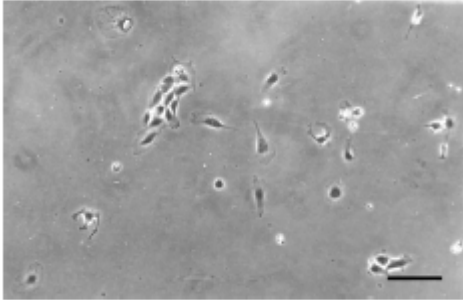


Fig. 3: Phase-contrast microscopy of putative RCE cells upon 3 days culture. They appear as flattened cells grouped together in an epithelial-like fashion. Bar = 100 μm

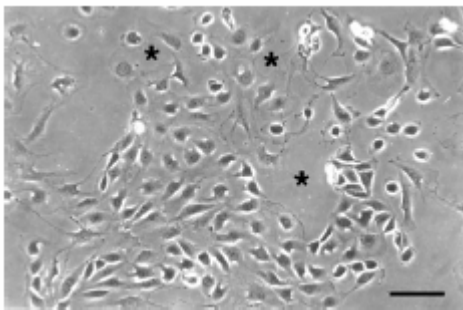


Fig. 4: Phase-contrast microscopy of subconfluent RCE cells upon 5 days culture. They appear as an almost continuous layer of epithelial-like cells. Some of them are seen surrounding open spaces, so-called pseudolumina (asterisks). Bar = 100 μm

In general, the younger the donor rat (best 200-250 g body weight), the higher the amount of viable RCE cells that can be obtained. Possibly due to the secretion of autocrine growth factors by RCE cells, it is advisable to put a reduced amount of growth medium in the flask and not to change it for a couple of days, in order to sustain RCE cell proliferation. Indeed, centrifugation of the tissue suspension yields a mixed cell population including endothelial cells, pericytes and fibroblasts. Upon trypsin digestion, 4-hour seeding and washing, adherent cells are mostly endothelial cells, but scattered cells of the other types may be also present. Nonetheless, in the specific culture conditions used, endothelial cells have a marked growth advantage which lead them to almost completely overwhelm the other cell types. Immunocytochemical characterization of cells at the first culture passage showed a negative staining for cytokeratin, endogenous peroxidase, α -smooth muscle actin and desmin, whereas vimentin and vWF stainings were weakly positive (data not shown). This latter finding may account for their capillary origin since, according to literature (13), vWF is weakly expressed by microvascular endothelial cells. Moreover, the cells took up fluorescently labelled acetylated LDL (Fig. 5), a specific marker for rat coronary endothelial cells (6).

The percentage of cells showing an active uptake for labeled LDL, and hence identifiable as true RCE cells, ranged between 96 and 98%.

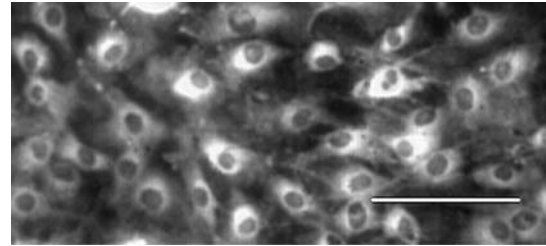


Fig. 5: Uptake of fluorescently-labeled acetylated LDL by RCE cells at the 1st culture passage. Almost all the cells are positively stained. Bar = 100 μm .

To dynamically evaluate [NO]_i generation, a typical property of endothelial cells (11), and assess simultaneously [Ca²⁺]_i dynamics, experiments were carried out with the NO-sensitive fluorescent probe DAF-2/DA, which has a fluorescence emission at a 510 nm wavelength upon excitation at a 490 nm wavelength, and the Ca²⁺-sensitive fluorescent probe fura-2AM, which has a fluorescence emission at 510 nm wavelength upon excitation at 340 and 380 nm wavelengths. Ratio intensity indicates [Ca²⁺]_i concentration and its increase accounts for [Ca²⁺]_i increase. Cells at the 1st culture passage were loaded with 10 $\mu\text{mol/l}$ DAF-2/DA and 4 $\mu\text{mol/l}$ fura-2AM for 45 min at 37° C. As expected, stimulation with angiotensin II, a vasoactive agonist linked to G α proteins, induced a rapid increase in [Ca²⁺]_i, followed by a slower increase in [NO]_i (Fig. 6).

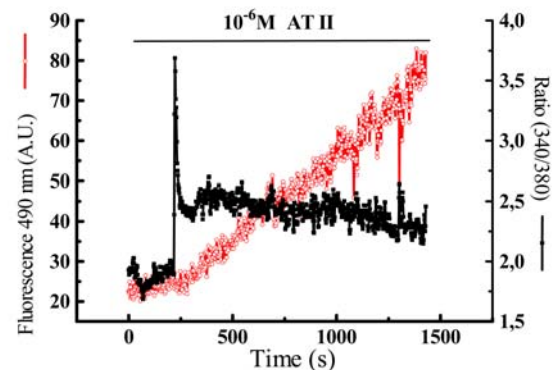


Fig. 6: Representative experiment with RCE cells loaded simultaneously with DAF-2/DA and fura-2AM showing the time-course of intracellular NO (left value axis & red curve) and Ca²⁺ (right value axis & black curve).

Of note, NO-dependent fluorescence of DAF-2/DA being due to irreversible oxidation of the probe molecule by NO, the fluorescence trend of DAF-2/DA is always positive, since it

reflects accumulation of the fluorescent product of intracellular NO and the probe. Therefore, the parameter to be evaluated is not the absolute, time-related increase in fluorescence but rather its slope, compared with that of adequate controls. This is at variance with the methods for assay of $[Ca^{2+}]_i$ based on fura 2-AM, which binds reversibly to Ca^{2+} and can reveal moment-to-moment changes of intracellular levels of this ion.

Finally, we would like to point out that, in our experience, the procedure we used completely prevents bacterial contamination of the hearts and the cell preparations during isolation. In fact, most handling can be carried out in a sterile environment, at variance with previous methods based on heart perfusion by a Langendorff apparatus, which cannot be easily sterilized and placed in a sterile hood (6). Another advantage of our method over other, apparently easier ones described in the literature (9) relies on the use of microperfusion, which allows an optimal distribution of collagenase within the coronary vascular bed, with obvious benefits in terms of endothelial cell yield and reproducibility from time to time. This latter advantage allows a reliable comparison between replicate experiments, as we could verify in our previous studies with RCE cells loaded with fluorescent probes (10).

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PROTOCOLS

All the solutions and the instruments used for the isolation of RCE should be sterilized before the experiment.

1. Treat the rat with i.p. injection of heparin (Prodotti Gianni, Milan, Italy. 300 mg/kg b.w.) in 0.5 ml PBS for 15-20 minutes.
2. Anesthetize the rat by i.p. injection of ketamine/xylazine (Sigma, St. Louis, MO, USA. 1 ml/kg b.w.)
3. Using sterile tools, remove the heart and place it in a sterile becker containing sterile Krebs-Henseleit solution without CaCl₂, pH 7.4, at 4°C.

All subsequent work must be performed in a sterile laminar flow hood .

4. Insert a steel cannula into the aorta, secure it with a surgical suture and connect it to the microperfusor syringe.
5. Meanwhile, gas 50 ml of Krebs-Henseleit solution without CaCl₂, pH 7.4, with a mixture of 95% O₂ and 5% CO₂. This solution should be prewarmed at 37° C.
6. Perfuse the heart with the solution described at point 5 at a 5 ml/min perfusion rate.
7. Meanwhile, gas 25 ml of sterile Krebs-Henseleit solution containing 100 µM CaCl₂, 0.1% BSA (Sigma) and 0.1 % collagenase type I (Sigma), pH 7.4, with a mixture of 95% O₂ and 5% CO₂. This solution should be prewarmed at 37° C.
8. Perfuse the heart with the solution described at point 7 for about 15 min at a 1.5 ml/min perfusion rate. The heart becomes brown and soft and quickly stops beating.
9. Remove the heart from the cannula, clear it from atria, atrioventricular septa and subepicardial fat tissue and place it in a becker containing 50 ml of Krebs-Henseleit solution with 100 µM CaCl₂ and 1% BSA, pH 7.4, at 37°C.
10. Mince the heart with a sharp-bladed scissors. The solution becomes turbid.
11. Filter the tissue suspension with a nylon cell strainer (Falcon), 70 µm-mesh, and collect it into a 50 ml conical polypropylene tube (Falcon).
12. Centrifuge the solution at 100 g for 3 min at room temperature.
13. Recover the supernatant and centrifuge it at 550 g for 10 min at room temperature.
14. Discard the supernatant and resuspend the pellet in 50 ml PBS containing 25 mg trypsin (Sigma, enzyme activity: 1500 BAEE units/mg) for 20 min at 37°C.
15. Centrifuge the suspension at 550 g for 10 min at room temperature.
16. Discard the supernatant and resuspend the pellet in M199 culture medium (Sigma) containing 10% fetal calf serum (Gibco BRL, Paisley, Scotland), 10% newborn calf serum (Gibco), 250 U/ml penicillin, 0.626 µg/ml amphotericin and 250 µg/ml streptomycin.
17. Seed the cell suspension into a 25 cm² vented cap flask (Falcon)
18. After 4 hours, wash the cells twice with PBS and add a small volume (3 ml) of culture medium to avoid excessive dilution of autocrine growth factors produced by the RCE cells.
19. When cells are subconfluent, change the culture medium and let them grow until confluence.
20. Subcultures can be obtained upon detachment of the cells with trypsin 0.25% plus EDTA 0.03% in PBS for 2-3 min.

Krebs-Henseleit solution without CaCl₂, pH 7.4 (g/100 ml):

NaCl	0.7
KCl	0.035
MgSO ₄ ·7 H ₂ O	0.029
KH ₂ PO ₄	0.016
NaHCO ₃	0.21
Glucose	0.18
CaCl ₂ , when needed	0.0011