Intact endothelial and smooth muscle function in small resistance arteries after 48 h in vessel culture

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Bolz, Steffen-Sebastian, Susanne Pieperhoff, Cor De Wit, and Ulrich Pohl. Intact endothelial and smooth muscle function in small resistance arteries after 48 h in vessel culture. Am J Physiol Heart Circ Physiol 279: H1434–H1439, 2000.—Long-term culture of resistance vessels allows introduction of molecular biology techniques for use in microvascular research. The aim of the present study was to establish a culture protocol that preserved vascular integrity and function in microvessels for 48 h in culture. Skeletal muscle resistance arteries were excised from the hamster gracilis muscle. Segments were assigned to immediate functional tests or to vessel culture, during which segments were perfused and superfused at a transmural pressure of 45 mmHg with Leibovitz (L15) medium containing 15% fetal calf serum and antibiotics for 48 h. Cultured and freshly isolated vessels showed similar levels of spontaneous tone, myogenic responses, changes in smooth muscle intracellular calcium (Ca$^{2+}$) (fura 2), and vascular diameter (video microscopy) in response to 0.3 M norepinephrine and similar concentration-response curves for acetylcholine (endothelium dependent, $\pm$ N$^\text{G}$-nitro-L-arginine) and sodium nitroprusside (endothelium independent). Measurements of endothelial Ca$^{2+}$ revealed similar acetylcholine-induced increases in endothelial Ca$^{2+}$ in both groups. It is concluded that vascular function can be preserved while maintaining vessels in culture. Thus it is possible to utilize protocols that require long-term treatment.

endothelium-derived hyperpolarizing factor; nitric oxide; calcium measurements; long-term treatment

APPLICATION OF MOLECULAR TECHNIQUES to vascular biology has led to advances in our understanding of vascular function. The majority of these advances have involved the combination of molecular biology with culture of various vascular cell types. More recently, with increasing interest in gene manipulation as a tool for therapy or investigation, medical scientists have begun to extend molecular approaches into the intact animal. Such techniques generally involve implantation of genetically manipulated cells or transfection technology. However, difficulties are frequently encountered with these approaches. Problems have included transfection efficiency, reaching target cells, and unwanted immunological reactions (8, 10, 15–17). Thus there is a need for development of experimental models that more easily permit molecular manipulation of vascular cells in preparations that allow assessment of vascular function.

The aim of the present study was therefore to establish a culture protocol that allows the preservation of vascular integrity and function in microvessels after 48 h in culture. Previous studies employing the vessel culture technique to microvessels have failed to document retention of normal vessel function. In our study, functional tests and morphological studies were performed to compare freshly isolated and cultured small skeletal arteries, both derived from the same stem of a small skeletal muscle artery. With regard to the smooth muscle function, vessels were tested for their ability to develop spontaneous tone, their myogenic reactivity, and their response to sodium nitroprusside (SNP). Endothelial function was assessed as the vessel response to acetylcholine (ACh). It was of particular interest whether the production of the endothelial autacoids nitric oxide (NO) and the endothelium-derived hyperpolarizing factor (EDHF) could be maintained in cultured vessels. Treatment with a combination of NO synthase inhibitor (N$^\text{G}$-nitro-L-arginine; L-NNA) and indomethacin allowed us to study whether NO and EDHF were still released in cultured vessels in response to ACh.

MATERIALS AND METHODS

Surgical preparation of small skeletal arteries. Female golden syrian hamsters were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Segments of small resistance arteries (resting outer diameter 228 ± 5 μm)
were excised under sterile conditions from the gracilis muscle, cannulated with glass micropipettes, and transferred to an organ bath. The segments were cannulated and stretched to their in situ length. Transmural pressure was maintained hydrostatically at 45 mmHg throughout the culture period. To avoid bacterial contamination, the room where the arteries were kept in culture was regularly cleaned with a strong disinfectant. The transportable setups, the surface of the aluminum table on which they were positioned, and all lines connected to the culture setup, e.g., perfusor lines to supply or drain medium, were wiped with isopropyl alcohol (70%) once per day. The room temperature was kept constant at 28°C.

Vessel culture. Cannulated small skeletal muscle resistance arteries were continuously perfused (1 ml/h) and superperfused (1 ml/h) with Leibovitz medium (L15) containing 20,000 U/l penicillin and 20 mg/l streptomycin. The L15 medium was developed for use in CO2-free culture systems (11) in free exchange with the atmosphere. It was supplemented with 15% heat-inactivated Cool Calf serum. The perfusion rate was calculated to apply a physiological constant wall shear stress within the range of 15–20 N/m² to the luminal surface of segments. The segments were continuously perfused with culture medium by means of a perfusor (Braun) and maintained under sterile conditions at room temperature (28°C) for 48 h. At the end of the culture period, cultured arteries were subjected to the same functional tests as freshly isolated vessels immediately after preparation. See Fig. 1 for diagrammatic representation of setup.

Calcium and diameter measurements. The segments were incubated with calcium (Ca²⁺)-sensitive dye, the acetoxy-methyl ester of fura 2 (fura 2-AM). To allow for selective measurements of smooth muscle intracellular Ca²⁺ (Ca_i²⁺), fura 2-AM was added to the organ bath, and selective loading of the endothelium (endothelial cells) could be achieved by perfusion of the segments with fura 2-AM. Ca_i²⁺ in vascular smooth muscle cells or endothelial cells was measured as described elsewhere in more detail (4). Video microscopy was performed at wavelengths >610 nm, which did not interfere with the fluorescence signal emitted by fura 2.

Before the functional tests were started, the lumen of tissue-cultured segments was cleared from culture medium by perfusion with MOPS-buffered saline. The organ chamber temperature was slowly increased to 37°C and was maintained at this level throughout the experiments. Transmural pressure during functional tests was set to 45 mmHg, unless stated otherwise (myogenic response).

Experimental protocols. All experiments were carried out in the presence of indomethacin. Smooth muscle function was tested by the ability of the segment to develop spontaneous tone in response to 45 mmHg of transmural pressure and to constrict on pressure increases from 45 to 110 mmHg (pressure-induced myogenic vasoconstriction) and by its sensitivity to norepinephrine (NE).

Endothelial function was assessed on the basis of the vessel response to ACh. ACh-induced increases in endothelial Ca²⁺ were compared in fresh and cultured segments. Subsequently, decreases in smooth muscle Ca_i²⁺ and dilations, both induced by ACh (0.01–1 μM) in NE (0.3 μM)-preconstricted vessels, were compared. We have shown previously (2) that the endothelium of these hamster skeletal muscle arteries releases NO and an as-of-yet-unidentified EDHF in response to ACh. The relative contribution of

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Fig. 1. Diagrammatic representation of the setup used for tissue culture of skeletal muscle resistance arteries. The arteriole was connected to a perfusor line and perfused with Leibovitz (L15) culture medium at a rate of 1 ml/h into a sealed reservoir (perfusion). The height of the reservoir determined the transmural pressure (45 mmHg). The culture medium in the organ bath was continuously exchanged at a rate of 1 ml/h (superfusion). The whole culture setup was kept at 28°C at ambient air conditions. Arrows indicate the direction of flow in the system.
EDHF and NO to the ACh-induced dilation was assessed by inhibition of NO production with L-NNA.

Drugs. Fura 2-AM (Molecular Probes) was dissolved in water-free dimethyl sulfoxide (DMSO) and stored as a 1 mM stock solution (1 mg fura 2-AM in 1 ml DMSO) in 10-μl aliquots. For incubation, it was diluted to its final concentration of 2 μM (2 μM fura 2-AM and 0.5% bovine serum albumin in MOPS-buffered salt solution).

ACh, NE, l-NA, L15 culture medium, and SNP were purchased from Sigma Chemical (Deisenhofen, Germany); MnCl₂ was from Merck (Darmstadt, Germany); and S-nitroso-N-acetyl-DL-penicillamine (SNAP) was from Alexis Chemicals (Grüneberg, Germany). Felodipine was a generous gift from Astra chemicals.

The MOPS-buffered salt solution (composition in mM: 145 NaCl, 4.7 KCl, 1.5 CaCl₂, 1.17 MgSO₄, 1.2 NaH₂PO₄, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS, and 5.0 glucose) was prepared daily. Drugs were stored in stock solutions and, before use, were diluted to five times that of the final concentration. To assure homogenous stimulation, one-fifth of the bath volume was exchanged when substances were added to create the final bath concentration. All concentrations given in the text refer to final bath concentrations.

Statistics. All results were presented as means ± SE for n no. of experiments. NE-induced constrictions and ACh-induced dilations were either presented as absolute values or normalized for the maximal diameter of the vessel. Changes in Cai (ΔCai) were expressed as percentage of control ratio with \( \frac{R_{treat}}{R_{control}} \times 100 \) – 100, with \( R_{treat} \) being the ratio under treatment and \( R_{control} \) being the ratio under stimulation with NE. Because of potential errors associated with the measurements in an intact vessel (13), the true Cai in vascular smooth muscle cells can only be estimated. We therefore decided to use ratio changes to describe percent changes of Cai. In accordance with calibrations performed with appropriate calibration solutions in the absence of cells, the range of ratios observed here (0.4–36) fit well into the linear range of the calibration curve (42.2–1,520 nM), which is a prerequisite for calculating percent changes.

In every single vessel, a certain treatment was studied after the respective controls were performed. This protocol allowed the use of every vessel as its own control.

RESULTS

Vascular smooth muscle function. During an equilibration period of 30 min, cultured vessels (maximal diameter 211 ± 13 μm, n = 8) developed 8.4 ± 2.2% spontaneous tone, which did not significantly differ from those in freshly isolated vessels (8.1 ± 4.2%, maximal diameter 217 ± 11 μm, n = 8). NO synthase inhibition by l-NNA (30 μM, 30 min) did not significantly increase spontaneous tone. The rapid increases in Ca²⁺ and the contractions elicited by increasing transmural pressure from 45 to 110 mmHg (myogenic response) were virtually identical in both groups (increase in Ca²⁺ by 29 ± 11% in fresh vs. 29 ± 3% in cultured, and reversal of initial distension by 49 ± 14% in fresh vs. 51 ± 16% in cultured resistance arteries; see also Fig. 2). Increases in Ca²⁺ (fresh 36 ± 3%, and cultured 33 ± 2%) and subsequent constrictions (fresh 43 ± 5%, and cultured 42 ± 4%) as induced by 0.3 μM NE were also not significantly different. A concentration of 0.3 μM NE was routinely used to preconstrict the segments before application of the respective vaso-dilating agents.

As shown in Fig. 3, the dose-response curves for SNP as obtained in cultured and fresh vessels revealed no

![Fig. 2](image-url) Pressure-induced myogenic vasoconstrictions in skeletal muscle resistance arteries. A: original recordings of changes in smooth muscle intracellular calcium (Ca²⁺) after an increase in transmural pressure from 45 to 110 mmHg in a cultured (left) and a freshly isolated (right) artery. Insets: the small bar graphs represent the mean of changes in Ca²⁺ (ΔCai) after 2 min of elevated transmural pressure in 7 vessels in each group. B: corresponding changes in vascular diameter (Δdia) that have been normalized to resting diameter of the vessel. Insets: bar graphs represent the means of myogenic constriction (reversal of the pressure-induced initial distension).
differences in the responsiveness of the vascular smooth muscle to NO in both groups.

**Endothelial function.** As shown in Fig. 4, 1 μM ACh elicited an increase in endothelial Ca$^{2+}$, which is a prerequisite for the synthesis of the endothelial autacoids NO and EDHF. These increases were qualitatively and quantitatively similar in endothelial cells in fresh as well as in cultured vessels (Fig. 4). In vascular smooth muscle, ACh induced a decrease in Ca$^{2+}$ and a subsequent dilation in freshly isolated segments. Both effects were maintained in cultured vessels (Fig. 5). Comparison of dose-response curves for ACh (0.01–1 μM) obtained under control conditions (30 μM indomethacin) and in 1-NNa (30 μM)-treated vessels revealed that in fresh as well as in cultured segments, only part of the ACh-induced dilation was mediated by NO. Responses to 1 μM ACh were insensitive to treatment with indomethacin 1-NNa but were abolished by the Ca$^{2+}$-activated K$^+$-channel inhibitor charybdo toxin (1 μM), suggesting mediation by EDHF (2). Additional removal of the endothelium (perfusion of the lumen with air for 5 min) completely abolished the responses to ACh but did not affect constrictor responses to NE (n = 6, data not shown).

**DISCUSSION**

This study describes a novel vessel culture technique that permits resistance vessels to be kept functionally intact over a 48-h period. The ability to keep vessels in culture for extended periods allows application of molecular techniques previously restricted primarily to cultured cells. In addition, vessel culture permits adaptive responses of the vascular wall to be studied over longer time periods, for example, vascular wall remodeling, chronic effects of pharmacological substances, and altered gene and protein expression. The use of an isolated vessel offers advantages over intact animal models and cell culture systems. Interpretation of studies from whole animal models is often complicated by additional extrinsic variables. In comparison, information from isolated cells can be difficult to extrapolate to the functionally intact vessel. Cultured cells also experience phenotypic changes such as rearrangements of the cytoskeleton (14, 19), variations of receptor expression (18), and, in the case of vascular smooth muscle cells, the change from a “contractile” to a “synthetic” phenotype (7, 19). These changes introduce further complications to data interpretation. Thus the vessel culture technique will significantly extend our abilities to study and understand vascular function.

Vessel culture techniques have been described before, mainly for the use of larger arteries and for studies of biochemical and structural alterations of vascular smooth muscle (1, 5, 6, 9, 12). However, no data are available from these studies about preservation of endothelial vasomotor function. We now report a resistance vessel culture technique in which isolated, cannulated arterioles are kept under physiological conditions of pressure and flow and are able to maintain normal spontaneous tone as well as contractile and endothelium-dependent responsiveness. In arterioles cultured for 48 h, the myogenic response as well as the response to NE was preserved compared with freshly isolated vessels, indicating that the vascular smooth

![Fig. 3. Dilations induced by sodium nitroprusside (SNP; 0.1–100 μM) were similar in freshly isolated and cultured (48 h) vessels. The steady-state diameter after 2 min of preconstriction with norepinephrine was 132 ± 12 μm in freshly isolated segments and 137 ± 11 μm in cultured segments (means ± SE; n = 8).](image)

![Fig. 4. Endothelial increases in Ca$^{2+}$ in freshly isolated and cultured vessels after application of 1 μM acetylcholine (ACh) were similar in freshly isolated (A) and cultured (B) vessels. Original recordings in both groups were representative for Ca$^{2+}$ measurements in 3 different segments/group.](image)
muscle cells did not de-differentiate from a contractile to a synthetic phenotype, because such a transformation would have resulted in a loss of contractility (7, 19).

Furthermore, the culture conditions chosen allowed for a fully preserved endothelial vasomotor function, because the vessels showed dilatory responses to ACh that were virtually identical to those observed in freshly isolated arterioles. This is in remarkable contrast to the rapid loss of responsiveness to ACh in cultured endothelial cells. Because ACh is known to release a number of different vasoactive autacoids from the endothelium, we conducted further studies to determine whether the synthesis of each of these autacoids was preserved. In previous studies from our laboratory (2), we have shown that the endothelium-dependent dilation in response to ACh in these arterioles is due to NO and EDHF release and is preceded by an increase in endothelial cell Ca\(^{2+}\). Of these two autacoids, EDHF appeared to be the predominant mediator (2). In the present study, similar NO- and EDHF-dependent contributions to the ACh-induced vasodilation were observed for cultured vessels. Also, the changes in endothelial cell Ca\(^{2+}\) in response to ACh were equivalent. This suggests that vessel culture is not leading to changes in muscarinic receptor expression or receptor coupling to cell signaling pathways. By comparison, in endothelial cell cultures, muscarinic receptors characteristically undergo downregulation, resulting in abolition of responsiveness to ACh (20). Furthermore, our data provide no evidence for an immunological tissue reaction due to bacterial contamination. In a separate study (3), our laboratory demonstrated that the new vessel culture technique also allows for efficient transfection of cells within the vascular wall. The FITC-labeled antisense oligonucleotides against cytochrome P-450 2C8 and 2C9, both putative EDHF synthases, could not only be detected within endothelial cells after 28 h but also reduced functional effects of EDHF. This functionally demonstrates that the culture temperature of 28°C used for vessel culture does not prevent sufficient transfection of vascular cells. Similar experiments (n = 3) conducted at 37°C produced identical results with regard to endothelial and smooth muscle function.

In conclusion, the results of our studies demonstrate the feasibility of maintaining arterioles in culture for extended periods of time without loss of function. This preparation will prove useful for probing the molecular basis of blood vessel function.

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