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Organ culture: a new model for vascular endothelium dysfunction

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Abstract

Background: Endothelium dysfunction is believed to play a role in the development of cardiovascular disease. The aim of the present study was to evaluate the suitability of organ culture as a model for endothelium dysfunction.

Methods: The isometric tension was recorded in isolated segments of the rat mesenteric artery branch, before and after organ culture for 20 h. Vasodilatation was expressed as % of preconstriction with U46619. The acetylcholine (ACh) induced nitric oxide (NO) mediated dilatation was studied in the presence of 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. Endothelium-derived hyperpolarising factor (EDHF) was studied in the presence of 0.1 mM L-NOARG and indomethacin. Prostaglandins were studied in the presence of L-NOARG, charybdotoxin and apamin.

Results: The ACh-induced NO and prostaglandin-mediated dilatations decreased significantly during organ culture (NO: 84% in control and 36% in cultured; prostaglandins: 48% in control and 16% in cultured). Notably, the total ACh-dilatation was not changed. This might be explained by the finding that EDHF alone stimulated a full dilatation even after organ culture (83% in control and 80% in cultured). EDHF may thereby compensate for the loss in NO and prostaglandin-mediated dilatation. Dilatations induced by forskolin or sodium nitroprusside did not change after organ culture, indicating intact smooth muscle cell function.

Conclusions: Organ culture induces a loss in NO and prostaglandin-mediated dilatation, which is compensated for by EDHF. This shift in mediator profile resembles that in endothelium dysfunction. Organ culture provides an easily accessible model where the molecular changes that take place, when endothelium dysfunction is developed, can be examined over time.

Background

Endothelium dysfunction is developed in cardiovascular diseases such as arteriosclerosis, diabetes, congestive heart failure, coronary artery disease, stroke and hypertension (de Meyer & Herman 2000). In conducting arteries, the response to endothelium-dependent dilators declines when endothelium dysfunction is developed mainly due to a decrease in nitric oxide (NO) release. In resistance arteries, endothelium-derived hyperpolarising factor (EDHF) is more abundant and may compensate for the loss in NO production [1–3].

Acetylcholine (ACh) is commonly used to assess endothelium-dependent dilatation. The dilatory mediators that

are released by ACh, have so far mainly been characterised as NO, prostaglandins and EDHF. NO is produced by nitric oxide synthase in endothelial cells and dilates smooth muscle cells by activating guanylate cyclase [4]. NO production can be inhibited with the L-arginine analogue L-N^G-nitroarginine (L-NOARG). Dilatory prostaglandins are produced by cyclo-oxygenase from arachidonic acid in endothelial cells and relaxes smooth muscle cells by activating adenylate cyclase [5]. Prostaglandin formation can be inhibited with indomethacin, a cyclo-oxygenase inhibitor. EDHF is an endothelium-derived mediator, distinct from NO and prostaglandins, which hyperpolarises and relaxes smooth muscle cells. Both its dilatory and hyperpolarising effect can be antagonised by a combination of the potassium channel inhibitors, charybdotoxin and apamin, in the rat mesenteric artery [6,7].

Organ culture of intact blood vessel segments has been suggested as a model for the phenotypic changes in the smooth muscle cells that occur during the development of cardiovascular disease [8]. One day of organ culture induces an upregulation of contractile endothelin type B receptors on smooth muscle cells [9], thereby mimicking atherosclerosis [10] and coronary artery disease [11]. Organ culture also induces downregulation of the angiotensin II receptor contractility (unpublished data), reflecting the phenotypic changes in heart failure and hypertension [12]. Serotonin type 1B and 1D receptors are upregulated after organ culture in rat cerebral arteries [13], which resembles the alterations in smooth muscle cell function after subarachnoidal haemorrhage [14,15]. So far, this model for vascular disease has only been applied to study the phenotypic changes of smooth muscle cells. In the present work, the phenotypic changes of the endothelium after organ culture were examined for the first time.

Endothelium dysfunction contributes to the diminished peripheral blood perfusion in cardiovascular disease. In order to explore novel therapeutic targets, the underlying mechanism of endothelium dysfunction needs to be examined. Difficulties exist due to lack of an easily accessible experimental model in which it is feasible to follow the development of endothelium dysfunction and thereby delineate the mechanisms of action. The aim of the present study is to evaluate the suitability of organ culture as a model for endothelium dysfunction.

Methods

Tissue preparation

Female Sprague-Dawley weighing 200 g were anaesthetised by inhalation of CO_2, after which they were killed with a cardiac cut. The superior mesenteric artery was removed gently and immersed in cold oxygenated buffer solution (for composition, se below) and dissected free of adhering tissue under a microscope. In experiments where endothelium denudation was required this was performed by perfusion of the vessel for 5 sec with 0.1% Triton X-100 followed by another 10 sec with a physiologic buffer solution (for composition, see below). The vessels were then cut into 1 mm long cylindrical segments and divided into two groups; one that was kept in a 8°C refrigerator (control segments) and the other in organ culture for 20 h (cultured segments). The segments for organ culture were placed in a 48 well plate, one segment in each well, containing 750 µl Dulbecco's modified Eagle's medium (DMEM) and incubated at 37°C in humified 5 % CO_2 in air. The DMEM (4500 mg/L D-glucose) was serum-free and contained sodium pyruvate (110 µg/L) and L-glutamine (584 mg/L), and was supplemented with penicillin (199 U/mL) and streptomycin (100 µg/mL). For further method details, see Adner et al. [9].

Experimental set-up

Each vessel segment was mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer (FT03C) for continuous recording of the isometric tension, and the other to a displacement device [16]. The position of the holder could be changed by means of a movable unit allowing fine adjustments of the vascular resting tension by varying the distance between the metal prongs. The mounted artery segments were immersed in temperature controlled (37°C) tissue baths containing a bicarbonate based buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. The solution was continuously gassed with 5 % CO_2 in O_2 resulting in a pH of 7.4. Four control and four cultured vessel segments were studied at the same time in separate tissue baths. The artery segments were allowed to stabilise at a resting tension of 2 mN for 1 h before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a K^+-rich (60 mM) buffer solution in which NaCl was exchanged for an equimolar concentration of KCl (for composition, see above). When two reproducible contractions had been achieved the vessels were used for further experiments.

Preconstrictors

The efficacy and durability of the contraction elicited by 9,11-Dideoxy-11alpha,9alpha-epoxymethanoprostaglandinF_{2alpha} (U46619), noradrenaline and phenylephrine was monitored when the maximum contraction (E_{max}) was first obtained, and 10 min thereafter. The decrease in contraction after 10 min was expressed as "percent decrease of E_{max}".

Endothelium dependent dilatation

Dilatory responses were assessed by cumulative addition of ACh (0.1 nM – 0.1 mM) in arteries with intact endothe-

lium, precontracted with U46619. The concentration of U46619 was titrated to result in a submaximal precontraction, amounting to 70 % of maximum. The NO-mediated dilatation was studied after inhibiting prostaglandins with 10 μM indomethacin and EDHF with 50 nM charybdotoxin and 1 μM apamin. EDHF was studied in the presence of the NO synthetas inhibitor L-NOARG (0.1 mM) and indomethacin. Prostaglandins were studied in the presence of L-NOARG, charybdotoxin and apamin.

Smooth muscle cell dilatory responses

Endothelium removal was checked by monitoring dilatory responses to ACh after precontraction with U46619. Abolished relaxation indicated a properly removed endothelium. The smooth muscle function was thereafter evaluated by cumulative addition of the NO-donor sodium nitropruside (SNP, 0.01 nM – 1 μM) and the adenylate cyclase activator forskolin (0.01 nM – 10 μM).

Ethics

The protocol was approved by the Ethical Committee of University Hospital (Lund, Sweden).

Drugs

All drugs were purchased from Sigma-Aldrich, USA and dissolved in 0.9% saline.

Calculations and statistics

Calculations and statistics were performed using the GraphPad Prism 3.02 software. The negative logarithm of the drug concentration that elicited 50% contraction or relaxation (pEC_{50}) was determined by fitting the data to the Hill equation. R_{max} refers to maximum relaxation calculated as percentage of the corresponding precontraction with U46619, while E_{max} refers to maximum contraction calculated as percent of the contractile capacity of 60 mM K^+. *n* denotes the number of experiments that were performed, each in a different animal. Statistical significance was accepted when $P < 0.05$, using Student's t-test. All differences referred to in the text have been statistically verified. Values are presented as means ± S.E.M.

Results

The contractile response to 60 mM K^+ did not differ between control (3.4 ± 0.5 mN, $n = 14$), and cultured vessel segments (3.6 ± 0.4 mN, $n = 14$, P = n.s.).

Preconstrictors

The efficacy of the phenylephrine and noradrenaline contractions consecutively decreased over time. 10 min after the maximum response was first obtained, the efficacy of the contraction had decreased with 40 ± 11% for phenylephrine and 36 ± 16% for noradrenaline in the cultured vessels ($n = 6$, $P < 0.05$). In the control vessels, the contraction had decreased with 30 ± 6% for phenylephrine and 21 ± 10% for noradrenaline ($n = 6$, $P < 0.05$). In contrast, the efficacy of the U46619 contraction was unchanged when maintained for 10 min (contraction decrease after 10 min = 5.4 ± 3% for control and 2.5 ± 2% for cultured vessels, $n = 6$, P = n.s.). Also, U46619 elicited a contraction of the same efficacy in control and cultured arteries (E_{max} = 5.1 ± 0.6 mN control and 5.9 ± 1.7 mN cultured, $n = 6$, P = n.s.). U46619 was therefore used as preconstrictor in the dilatory experiments.

NO-mediated dilatation

The efficacy of the NO-mediated ACh-dilatation, studied in the presence of indomethacin, charybdotoxin and apamin, was significantly decreased after organ culture, while the NO-donor SNP induced similar relaxations in control and cultured arterial segments (Table 1, Fig. 1). These results suggest that the NO-release from the endothelium is decreased while the smooth muscle cell dilatory response to NO is intact.

Prostaglandin-mediated dilatation

The prostaglandin-mediated ACh-dilatation, examined in the presence of charybdotoxin, apamin and L-NOARG, was abolished after organ culture (Table 1, Fig. 1). The adenylate cyclase stimulator, forskolin, relaxed cultured and control arterial segments with the same potency and efficacy (Table 1), indicating that the dilatory response to prostaglandins in the smooth muscle cells is intact.

EDHF-mediated and total dilatation

Even though the NO and prostaglandin-mediated ACh-dilatation was decreased, there was no difference in the total ACh-dilatation in control and cultured arteries without inhibitors (Table 1, Fig. 1). This might be explained by the finding that the ACh-induced EDHF-mediated dilatation, studied in the presence of indomethacin and L-NOARG, was capable of fully dilating the artery (R_{max} = 83 ± 3 %, $n = 10$, Table 1, Fig. 1). This response was not significantly affected by organ culture (80 ± 3 %, $n = 10$, P = n.s., Table 1, Fig. 1).

When the artery segments were endothelium-denuded or pre-treated with a combination of indomethacin, L-NOARG, charybdotoxin and apamin, all dilatory responses were abolished in both the control and the cultured vessel segments. These results indicate that no other dilatory mediator than NO, prostaglandins and EDHF was present and that the ACh-dilatation was endothelium-dependent.

Discussion

The present study demonstrates that organ culture may be a suitable model for studying the development of endothelium dysfunction. Organ culture induces a decrease in the NO and prostaglandin-mediated dilatation, while EDHF serves as a backup system that preserves the capa-

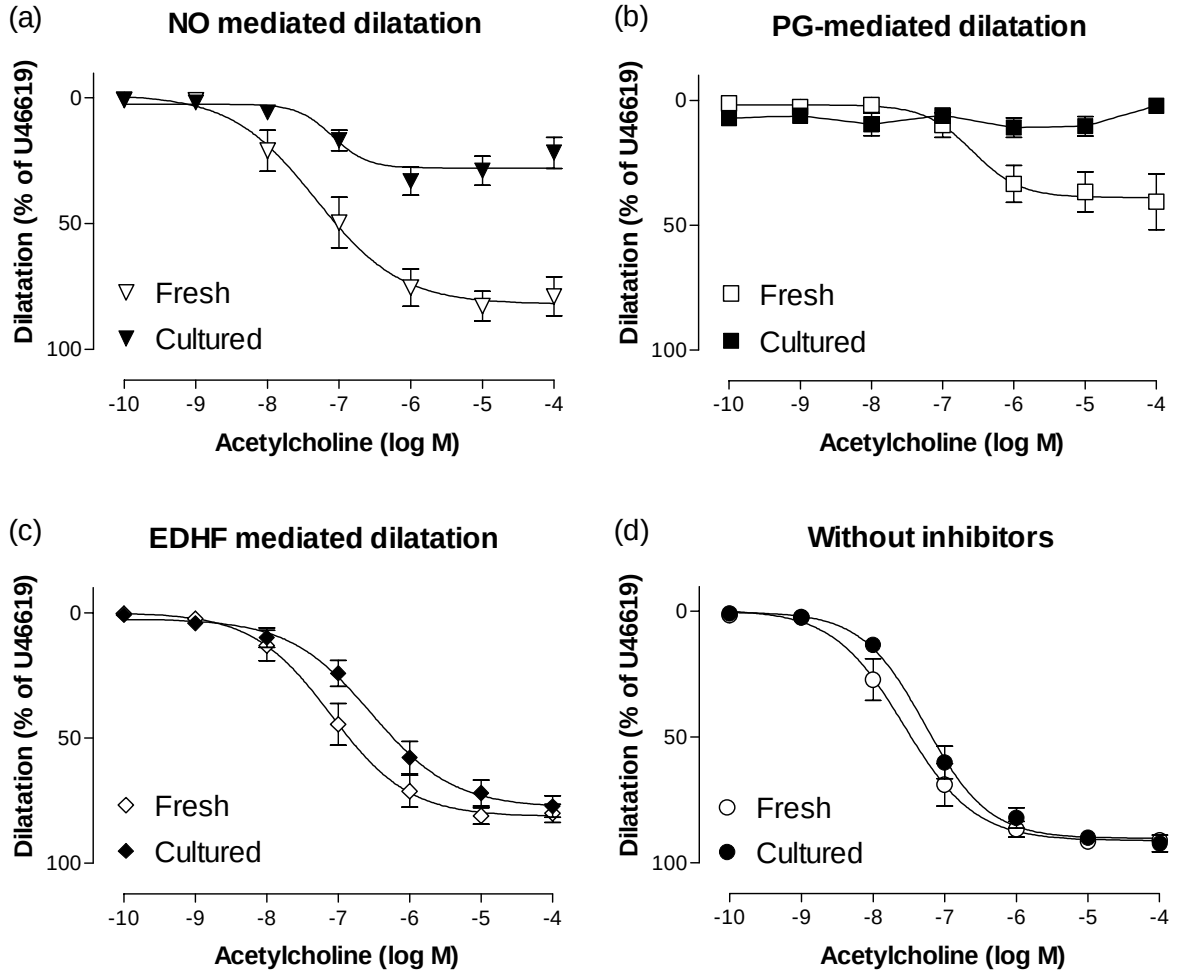

Figure 1
Organ culture induced changes in endothelium-derived dilatory mediators Concentration-dependent dilatations to ACh in the U46619-precontracted rat mesenteric artery branch, before and after 20 h of organ culture. (a) NO-mediated relaxation, studied in the presence of 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin, was decreased after culture, n = 10, $P < 0.0001$. (b) Prostaglandin-mediated relaxation, studied in the presence of charybdotoxin 50 nM apamin 1 μM and L-NOARG 0.1 mM was abolished after organ culture, n = 10, $P < 0.0001$. (c) EDHF-mediated relaxation, studied in the presence of indomethacin and L-NOARG, did not change after organ culture, n = 10, P = n.s. (d) The total dilatory response to ACh was unaltered by organ culture, n = 10, P = n.s. Data are shown as means ± S.E.M. of 10 experiments.

bility of the artery to respond to a dilator. This phenotypic change occurs in the endothelium, while the smooth muscle cell function remains intact.

U46619 is a thromboxane A_2 receptor agonist that was used as precontracting agent for the following reasons: Firstly, organ culture has been proven to induce changes in the contractile responses to endothelin [8], serotonin [13] and angiotensin II (unpublished data), while the contractile responses to U46619 is unaltered. Secondly, U46619 possess a more durable precontraction than phenylephrine and noradrenaline. Stable experimental conditions can in this way be established.

Table 1: Vasodilator responses after organ culture

		n	pEC_{50} (-log M)	P	R_{max} (%)	*P*
ACh	Control	10	7.5 ± 0.2	} n.s.	93 ± 1	} n.s.
	Cultured	10	7.1 ± 0.1		93 ± 3	
ACh, NO-mediated	Control	10	7.2 ± 0.2	} n.s.	84 ± 6	} <0.0001
	Cultured	10	7.1 ± 0.2		36 ± 6	
ACh, prostaglandin-mediated	Control	10	6.1 ± 0.4	} n.s.	48 ± 7	} <0.001
	Cultured	10	6.6 ± 0.4		16 ± 4	
ACh, EDHF-mediated	Control	10	7.1 ± 0.2	} n.s.	83 ± 3	} n.s.
	Cultured	10	6.1 ± 0.5		80 ± 3	
Forskolin	Control	7	7.1 ± 0.2	} n.s.	95 ± 1	} n.s.
	Cultured	7	7.3 ± 0.2		97 ± 2	
SNP	Control	9	8.8 ± 0.1	} n.s.	96 ± 1	} n.s.
	Cultured	9	8.9 ± 0.2		93 ± 4	

Vasodilatation was assessed by cumulative addition of ACh, forskolin or SNP in the precontracted mesenteric artery branch, before and after organ culture for 20 h. NO-dilatation was studied in the presence of indomethacin (10 μM), charybdotoxin (50 nM) and apamin (1 μM); prostaglandin-dilatation in the presence of L-NOARG (0.1 mM), charybdotoxin and apamin; and EDHF-dilatation in the presence of indomethacin and L-NOARG. Dilatory responses are expressed as percentage of a precontraction induced by U46619. *n* denotes the number of experiments (animals). Statistical significance is expressed as *P*. Data are given as mean values ± S.E.M.

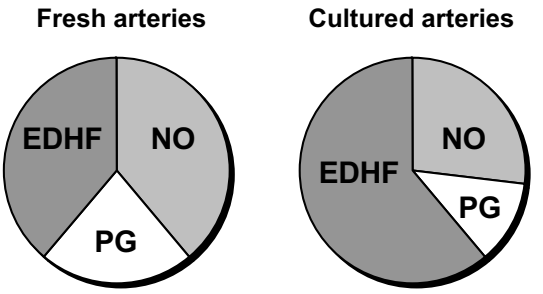

Figure 2
The relative contribution of the endothelium-derived mediators to the total dilatation Schematic illustration of the respective shares of NO, prostaglandins (PG) and EDHF in the ACh-stimulated vasodilatation, before and after organ culture, calculated as percent of the total dilatation induced by ACh, based on the maximum contraction for each mediator. In control arteries, 39 % of the dilatation was mediated by NO, 22 % by prostaglandins and 39 % by EDHF. In cultured arteries, 27 % of the dilatation was mediated by NO, 12 % by prostaglandins and 61 % by EDHF.

The NO-mediated ACh-dilatation was decreased after organ culture (Fig. 1). Equally efficient responses to the endothelium-independent NO-donor SNP were observed in cultured and control endothelium-denuded artery segments, indicating intact smooth muscle cell relaxant responsiveness. Similar changes have been observed in cardiovascular diseases like atherosclerosis and hypercholesterolemia: The NO release from the endothelium is impaired while the response to exogenous applied NO is preserved [17].

The prostaglandin-mediated ACh-dilatation was abolished after organ culture (Fig. 1). This effect was also endothelium-dependent since forskolin induced a similar dilatory response in control and cultured artery segments. Forskolin activates adenylate cyclase directly in the smooth muscle cells and thereby mimics the dilatory pathways that are activated by prostaglandins [5]. The smooth muscle cell response to the prostaglandins, which are released from the endothelium, is the net effect of the prostaglandins that induce relaxation and those that induce contraction. Subsequently, the decreased prostaglandin-mediated dilatation in the present study may either be due to a reduction in the release of dilatory prostaglandins or a relative increase in the release of contractile prostaglandins. Increased release of contractile prostaglandins such as prostaglandin H_2 and thromboxane A_2 has been observed in hypertension [18] and diabetes [19].

Acetylcholine induces dilatation by release of NO, prostaglandins and EDHF. No other mediator was involved since a combination of L-NOARG, indomethacin, charybdotoxin and apamin completely abolished the ACh-induced relaxant response.

Although the release of prostaglandins and NO was substantially decreased after organ culture, the maximum dilatory response to ACh was unchanged. EDHF induced a maximum dilatation of the artery even after organ culture (80% before and 83% after organ culture). It can therefore be expected that the smooth muscle cell response to EDHF is functioning normally after culture, although there is no proof of this since a K^+ channel opener was not used in the present experiments. EDHF seems to be more resistant to the phenotypic change of the endothelium and served as a backup dilatory system when NO and prostaglandins failed. After organ culture, EDHF accounted for the major part of the mediators that were released from the endothelium. The share of the total ACh-dilatation that is mediated by NO, prostaglandins and EDHF respectively is illustrated in figure 2.

After organ culture, the EDHF-mediated dilatation amounted to 80 %, NO to 84 % and prostaglandins to 16 % of preconstriction. The additive NO, prostaglandin and EDHF responses thus exceeded the total ACh-dilatation by far. Rather than functioning in an additive way, NO, prostaglandins and EDHF might provide separate systems that are capable of fully dilating the blood vessel independently. This may be a reserve mechanism, where one factor is backup for the other if affected by pathological conditions like endothelium dysfunction in congestive heart failure [1,2,20,21].

When the endothelium is malfunctioning in cardiovascular disease, EDHF compensates for the increased vascular tone elicited by a decreased NO synthesis. This compensatory mechanism has mainly been observed in small resistance arteries where EDHF is abundant [22]. Conversely, in larger conduction arteries, EDHF is virtually absent and the decreased NO release is not compensated for, resulting in a reduced response to various dilators. The present study has been performed on the rat mesenteric artery branch, which in many ways is a representative model of a peripheral resistance vessel [23]. In this artery, the NO and prostaglandin mediated dilatation was substantially decreased, while a maximum EDHF-dilatation was preserved and served as a backup dilatory system. Similar changes have been seen when endothelium dysfunction is developed in small arteries [2,3,17,20,22]. Organ culture of the rat mesenteric artery branch may therefore be a suitable model for endothelium dysfunction in resistance arteries.

Conclusions

Organ culture induces a decrease in the NO and prostaglandin-mediated dilatation, while EDHF serves as a backup system to preserve the capability of the artery to respond to a dilator. This phenotypic change occurs in the endothelium, while the smooth muscle cell function remains intact. Similar changes have been observed when endothelium dysfunction is developed in cardiovascular diseases like hypertension [1], diabetes [3], hypercholisterolemia [2] and congestive heart failure [20]. Organ culture may therefore provide an experimental model in which the development of endothelium dysfunction can be studied in detail to further delineate the mechanisms of action. Culture in the presence of the different humoral factors or intracellular messenger inhibitors may reveal important pathways that lead to the development of endothelium dysfunction. The method thereby combines the advantage of cell culturing techniques with the advantage of functional evaluation of intact blood vessels.

Authors' contributions

Rikard Alm: Performed the majority of the experiments. Helped writing the manuscript.

Lars Edvinsson: Scientific discussions. Helped writing the manuscript.

Malin Malmsjö: Conceived and designed the study. Wrote the majority of the manuscript. Helped performing the experiments.

Competing interests

None declared.

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