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Effect of short-term organoid culture on the pharmaco-mechanical properties of rat extra- and intrapulmonary arteries

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1 Organoid cultured explants from differentiated tissues have gained renewed interest in the undertaking of physiological and pharmacological studies. In the work herein, we examined the pharmaco-mechanical properties of an *in vitro* model consisting of organoid cultured rings derived from rat extra- and intrapulmonary arteries, over a period of 4 days in culture.

2 Mechanical changes were quantified using isometric tension measurements on both fresh and cultured pulmonary arterial tissues, with experiments performed in the presence or absence of 10% foetal calf serum. Conventional histochemical and immunofluorescent stainings were also performed to assess tissue structure integrity and apoptosis.

3 The explants developed spontaneous rhythmic contractions (SRC) in approximately half of the vessels. SRC amplitude and time course were modified by conditions and agents acting on membrane potential (high-potassium solutions – levcromakalim, a potassium channel opener), while nitrendipine, an L-type calcium channel blocker, suppressed SRC.

4 Cultured explants also developed a hyper-reactivity to high potassium challenges (10–40 mM). Whereas contraction to serotonin (5-HT) was enhanced in intrapulmonary arteries, contraction to endothelin-1 remained unchanged after 4 days of culture. Serum did not alter contractile properties during the culture period.

5 Endothelial-dependent relaxation was maintained in response to A23187 500 μM, but was abolished in response to 10 μM carbamylcholine.

6 Histological and immuno-histological analyses revealed the absence of hypertrophied vascular wall or apoptosis.

7 In conclusion, the contractile phenotype as well as tissue structure integrity of organoid explants remain essentially intact during short-term culture, making this model suitable for pharmaco-genomic studies.

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Abbreviations: CCRC, cumulative concentration–response curve; DMSO, dimethyl sulphoxide; ET-1, endothelin-1; FCS, foetal calf serum; IPA1, intrapulmonary artery of the first order; IPA2, intrapulmonary artery of the second order; ITS, insulin–transferrin–selenium; KH, Krebs–Henseleit; MPA, main pulmonary artery; PAH, pulmonary arterial hypertension; PASMC, pulmonary arterial smooth muscle cell; siRNA, small interference RNA; SMC, smooth muscle cell; SRC, spontaneous rhythmic contractions; T_{max}, maximum tension

Introduction

Pulmonary arterial hypertension (PAH) represents the major disease of the pulmonary vascular bed and is characterized by proliferation and migration of smooth muscle cells (SMC), remodelling of the extracellular matrix and endothelial cell injury (Farber & Loscalzo, 2004). This disease results in a progressive increase in pulmonary vascular resistance and, ultimately, in right ventricular failure and death.

Pulmonary artery smooth muscle cell (PASMC) culture has been extensively used to study proliferation phenotype. Culture of vascular SMC including PASMC causes rapid modulation from the contractile to the synthetic or proliferative phenotype (Thyberg, 1996). This phenomenon involves a decrease in contractile and cytoskeletal proteins, as well as changes in the activity and number of ionic channels and receptors (Owens, 1995; Thyberg, 1996). In proliferating PASMC potassium conductance is decreased, thus inducing membrane depolarization and an increase in intracellular calcium (Platoshyn *et al*., 2000; Cui *et al*., 2002), whereas voltage-gated sodium channels are expressed (Jo *et al*., 2004). Voltage-gated calcium channel activity tends to decrease, while store-operated calcium entry is upregulated (Yuan *et al*., 1993; Golovina *et al*., 2001; Sweeney *et al*., 2002).

Aside from isolated SMCs, there is considerable interest for a more integrated model allowing to study cells within

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their microenvironment (extracellular matrix, adventitia, endothelium). Human tissue-engineered blood vessels have thus been developed (L'Heureux *et al*., 2001) for vascular biology research, since this circumvents the difficulties in obtaining native human blood vessels. Three cell types are first cultured separately (endothelial, SMCs and fibroblasts) and then used to reconstruct an equivalent vessel. In these latter vessels, L-type calcium channels are either absent or nonfunctional, while receptor subtypes such as purinergic receptors differ from native tissue, although vasoconstriction is maintained (Bo *et al*., 1998; L'Heureux *et al*., 2001). This interesting model remains limited, however, to the aforementioned three cell types and alternative models, derived from organoid culture of fresh vessel rings, have now been developed. Such integrated models have proven useful in analysing remodelling of native or hypertrophied rat pulmonary arteries (Cowan *et al*., 1999). Cultured rabbit pulmonary arteries have also been used to investigate the effect of chronic hypoxia (Murata *et al*., 2001) directly on vascular tissue. Hypobaric chronic hypoxia (380 mmHg – 10% O_2) is known to induce PAH in 3 weeks with pulmonary artery remodelling and right ventricular hypertrophy in rats (Bonnet *et al*., 2001a, b). Normobaric chronic hypoxia induced by flushing of an hypoxic gas mixture (3% O_2) during 3 days has also been previously used to study the effect of chronic hypoxia on cultured vascular SMC (Platoshyn *et al*., 2001). In this respect, since pulmonary arteries from patients with pulmonary hypertension are very rarely available, organoid culture of human pulmonary artery rings would be of major interest. Hence, the effect of chronic hypoxia could be tested directly on nonhypertrophied pulmonary arteries. Moreover, human tissue maintained *in vitro* would allow alternative experiments to be performed in order to address complementary issues. In this context, recent development of small interference RNA (siRNA) to selectively extinguish protein expression is a powerful technique to study pulmonary vascular pharmacology (Lin *et al*., 2004). Unfortunately, the use of siRNA requires 2–4 days of culture and most studies have been performed on cultured cells (Lin *et al*., 2004; Yu *et al*., 2004). Development of siRNA use in organoid cultured vessels would be of value to study pulmonary vascular reactivity in both health and disease. Indeed, organoid culture of intact vascular tissue has been shown to preserve the complex three-dimensional organization and contractile properties, although some cellular changes have been observed, including downregulation of L-type calcium channels and upregulation of intracellular calcium stores and store-operated calcium entry (Dreja & Hellstrand, 1999; Dreja *et al*., 2001; Bergdahl *et al*., 2005). Moreover, mRNA for the serotonin receptor 5-HT 2A and endothelin-1 (ET-1) receptor ET-B are increased, whereas angiotensin II receptor AT-1 mRNA is decreased (Moller *et al*., 2002; Luo *et al*., 2004). Endothelial integrity may also be disturbed and the presence of serum may slightly modify vascular properties in some instances of long-term culture (De Mey *et al*., 1989; Lindqvist *et al*., 1999; Bakker *et al*., 2000; Kunichika *et al*., 2004).

To the best of our knowledge, the effect of culture on pulmonary arterial rings has not been examined to date from a functional and structural point of view. We thus investigated the effect of 4 days of culture, with or without foetal calf serum (FCS), on the contractile properties and morphology of organoid rings from rat extra- and intrapulmonary arteries.

Methods

Tissue preparation and organ culture

Male Wistar rats (250–300 g) were stunned and then killed by cervical dislocation according to the guidelines issued by our Local Animal Care Ethics Committee ('Comité d'Éthique Régional d'Aquitaine'). The entire heart–lung preparation was rapidly removed and rinsed in culture medium (D-MEM–HEPES supplemented with 1% penicillin–streptomycin, 1% Na pyruvate, 1% non essential amino acids) for organ culture or in Krebs–Henseleit (KH) solution for fresh tissue experiments. KH solution contained (in mM): 118.4 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 D-glucose, bubbled with a 95% O_2–5% CO_2 gas mixture, pH 7.4. For fresh tissue experiments, main and intra-pulmonary arteries of the first and second order (MPA, IPA1 and IPA2, respectively) from the left lung were dissected free from surrounding connective tissues in KH solution. For organ culture, the same arteries were rinsed twice in fresh culture medium and dissection was performed under sterile conditions in culture medium. Pulmonary arteries were then divided into short tubular segments (1.5–2.5 mm) and pulmonary tissues were either used directly after dissection (as fresh tissues) or placed into individual wells of 12-well culture plates containing culture medium. The culture medium was either serum-free or enriched with 10% FCS and changed every other day. Serum-free culture medium was supplemented with 1% insulin–transferrin–selenium (ITS) (as previously described) (Berger *et al*., 2001). Organ culture plates were placed in a humidified incubator at 37°C under 5% CO_2 in air. The organoid rings were maintained in culture for up to 4 days.

Isometric tension measurements

The mechanical effects of a variety of agonists were measured on main and intrapulmonary artery rings as reported previously (Bonnet *et al*., 2001a; Pauvert *et al*., 2004). In brief, KH solution, bubbled with carbogen (95% O_2, 5% CO_2), was used. Passive and active mechanical properties were assessed using organ bath and transducer systems (EMKA Technologie, Paris, France), coupled to IOX software (EMKA Technologie, Paris, France) in order to facilitate data acquisition and analysis. As determined in preliminary experiments, tissues were set at optimal length by equilibration against passive loads of 1, 0.8 and 0.2 g for MPA, IPA1 and IPA2 rings, respectively. At the outset of each experiment, K^+-rich (80 mM) solution, obtained by substituting an equimolar amount of KCl for NaCl from KH solution, was applied in order to obtain a reference contraction used to normalize subsequent contractile responses. Contractile properties were tested by constructing a cumulative concentration–response curve (CCRC) to various agonists for each ring. Endothelial function was tested by relaxation with 10 μM carbamylcholine of 1 μM phenylephrine-induced preconstricted pulmonary arterial rings. All experiments were performed at 37°C.

Histology

Rat main and intrapulmonary arteries were excised and fixed in 5% formaldehyde solution for 24 h. After proper fixation,

tissues were processed and embedded in paraffin. Multiple sections from each block were prepared at 5 μm thickness and stained with haematoxylin-eosin for light microscopy examination.

Immunohistochemistry

Serial thin sections (5 μm) from MPA and IPA were deparaffinized in toluene (4 × 5 min) and re-hydrated stepwise in graded ethanol solutions (from 100% down to 40%), washed in PBS for 5 min and blocked with 50 mM ammonium chloride in PBS for 20 min, and then washed twice in PBS (5 min). Sections were permeabilized in Triton-X100 (0.2%) for 10 min under gentle stirring, washed twice for 5 min in PBS and blocked with 0.5% BSA for 45 min. Incubation with the primary antibody (monoclonal anti-α smooth muscle actin from mouse) was conducted at 1/400 dilution for 2 h, followed by two 5-min rinses in PBS. The following steps were performed in the dark. Sections were incubated with the secondary antibody, a goat anti-mouse IgG – coupled to Alexa Fluor 488, for 1 h, followed by two 5-min washes in PBS. DAPI counterstaining was performed for 3 min (1/1000) prior to final rinse in PBS and mounting with Vectashield. All sections were observed at various magnifications on a Nikon Eclipse TE300, equipped with a UV lamp and filters.

In situ *detection and quantification of tissue apoptosis*

Detection of apoptotic cells in the pulmonary arterial tissues was assayed using TUNEL methodology, as described in the procedure included with the ApopTag *in situ* apoptosis S7110 detection kit obtained from Chemicon International (Temecula, CA, U.S.A.). Following DAPI nuclear counterstaining, pulmonary arterial cells were considered as definitely apoptotic when observed to be both peroxidase positive (TUNEL) and DAPI negative, using an ultraviolet filter ($\lambda \leqslant 280$ nm).

Drugs and chemical reagents

A 23187, carbamylcholine, ET-1, 5-hydroxytryptamine (5-HT), ITS, levcromakalim, Na pyruvate, nitrendipine, non essential amino acids and phenylephrine (Phe) were purchased from Sigma (Saint Quentin Fallavier, France). D-MEM–HEPES, FCS and penicillin–streptomycin were purchased from Gibco (Invitrogen Corporation, Cergy Pontoise, France).

All drugs were diluted in distilled water, except for A 23187, levcromakalim and nitrendipine, which were dissolved in dimethyl sulphoxide (DMSO). The maximal concentration of DMSO used in experiments was $<0.1\%$, and had no effect on the mechanical activity of the rings.

Data analysis and statistics

Results are expressed as mean ± s.e.m.; n indicates the number of rings used. Statistical analyses were performed using unpaired Student's t-tests, as well as ANOVA for global comparisons of the curves. Values of $P<0.05$ were considered significant. Data curve fittings were performed using Origin 6 software (Microcal). Concentration–response curves to agonists were fitted to the logistic equation:

$$T = ((T_0 - T_{max})/(1 + (X/EC_{50})^p)) + T_{max}$$

where T, maximum tension (T_{max}) and T_0 are, respectively, the amplitude of tension developed and the relative maximum and minimal tensions for a given agonist concentration normalized to the 80 mM KCl responses, X is the concentration of agonist used, EC_{50} is the concentration of agonist which produces half-maximal tension and p is the slope of the curve.

Results

Spontaneous contractile activity of the explants

The mechanical activity of 4-day organoid-cultured explants from various segments of rat pulmonary arteries (MPA, IPA1 and IPA2), in the presence or absence of 10% FCS in the culture medium, was examined. Approximately half of the MPA, IPA1 and IPA2 rings developed spontaneous rhythmic contractions (SRC) in the presence of 10% FCS (Figure 1, Table 1). For all rings, amplitude and frequency of SRC were similar in the presence or absence of FCS, although the percentage of rings exhibiting SRC was decreased in the absence of FCS (ITS supplemented) (Table 1). Fresh tissues did not develop SRC at any time (data not shown). The L-type calcium channel blocker, nitrendipine 1 μM, fully abolished SRC ($n=9$ and 4 rings for MPA and IPA1, respectively). Moreover, levcromakalim 0.1–1 μM, a potassium channel opener, inhibited basal tone and SRC amplitude in a dose-dependent manner in both tissues (Table 2). These two sets of experiments demonstrate a pivotal role for membrane potential in the setting of SRC in cultured pulmonary arteries.

Contractile responses to increasing KCl concentrations

To further assess the role of membrane potential in the reactivity of cultured explants, experiments were performed to test and compare the effects of stepwise increases in KCl concentrations in both fresh and organoid cultured pulmonary arteries. Figures 2 and 3 show a significant hypersensitivity to increasing concentrations of KCl (10–40 mM). High potassium solutions increased SRC frequency and decreased SRC amplitude in cultured pulmonary arterial rings (Figure 2b). Replacing FCS by ITS reduced the hypersensitivity to high potassium solutions in IPA2 (Figure 3c).

Responsiveness to various pulmonary vasoconstrictors (ET-1 and 5-hydroxytryptamine (5-HT)) in 4-day cultured vessels

5-HT and ET-1 are two potent pulmonary arterial vasoconstrictors also involved in PAH (MacLean *et al*., 2000; Bonnet *et al*., 2001a; Guibert *et al*., 2004). Hence, the contractile responses to these two agonists were investigated in fresh tissue and in 4-day cultured MPA, IPA1 and IPA2 rings in the presence or absence of 10% FCS (Figures 4a–c and 5a–c). Contraction to increasing concentrations of 5-HT was significantly potentiated only in intrapulmonary arterial rings after 4 days of culture in the presence of 10% FCS for IPA1 and IPA2 (Figure 4b, c) and in the absence of FCS (ITS supplemented) for IPA2 (Figure 4c). In contrast, contraction to increasing concentrations of ET-1 was not modified in any of the vessel rings in the presence of 10% FCS (Figure 5a–c). When global comparisons of the curves were significantly

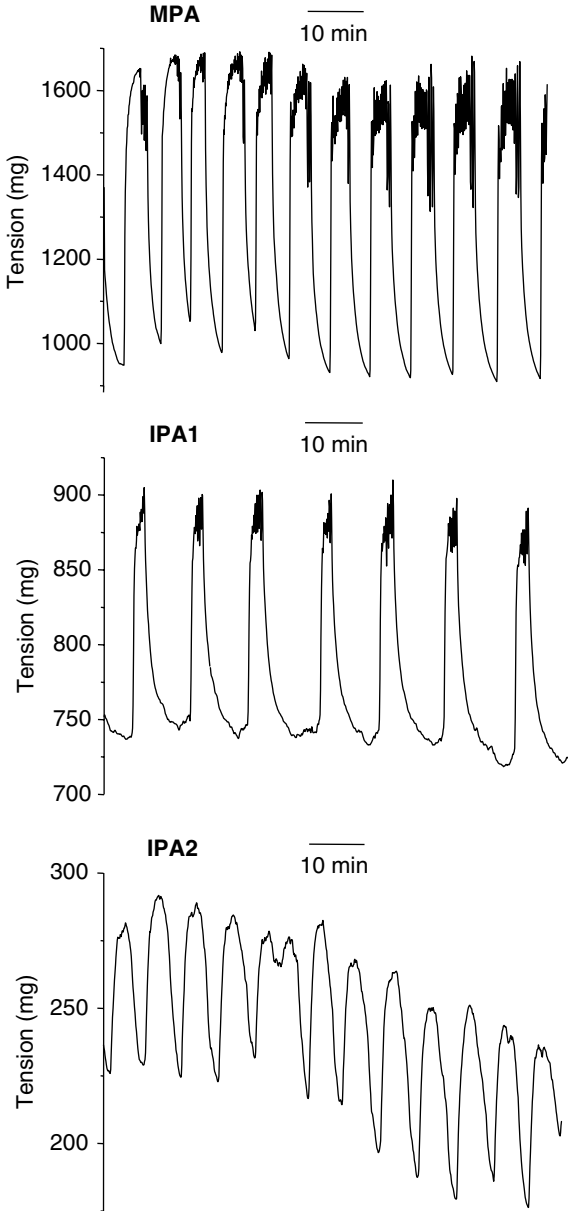

Figure 1 Organoid culture (4 days) induces spontaneous and rhythmic contractions (SRC). Representative traces showing SRC on 4-day cultured pulmonary arteries (culture medium: DMEM–HEPES, 1% penicillin–streptomycin with 10% FCS for MPA and without FCS replaced by 1% ITS for IPA1 and IPA2). The pulmonary arteries MPA, IPA1 and IPA2 were initially stretched to 1, 0.8 and 0.2 g, respectively.

different, unpaired Student's *t*-tests were performed on T_{max}. For 5-HT, T_{max} were significantly increased in culture (Table 3), indicating an increase in the efficacy rather than the potency of 5-HT. These results suggest that the active as well as the passive properties of the cultured rings can be modified and yield increased reactivity to high potassium solutions and 5-HT, as compared to native tissues.

Effect of 4 days of culture on endothelial-dependent relaxation

Vessel tone is driven by a balance between vasoconstrictor and vasorelaxant signals, with vasorelaxant signals mainly originating from the endothelium. Thus, the functional integrity of the pulmonary artery endothelium was assessed after 4 days of culture. The relaxation induced by 10 μM carbamylcholine of pulmonary arterial rings preconstricted with 1 μM phenylephrine was completely abolished along with a slight contraction to carbamylcholine in all vessel rings (100%) after 4 days of culture in the presence or absence of 10% FCS (Figure 6).

To determine whether the absence of the endothelium-dependent relaxation to carbamylcholine was due to endothelial damage, the effect of a calcium ionophore, A 23187 500 μM, was tested. Accordingly, A 23187 induced similar relaxations in control and in 4-day cultured MPA and IPA1 rings (Figure 6a, b).

Histological analysis of the smooth muscle layer

Histological analysis was carried out to determine the possible effects of culture on vessel morphological integrity. Haematoxylin–eosin staining did not reveal any obvious alterations of IPA1 rings after 4 days of culture (Figure 7a, c), while immunofluorescent labelling experiments showed smooth muscle α-actin staining of fresh and 4-day cultured IPA1 rings (Figure 7b, d). There was neither hypertrophy of the vascular wall nor an increase in smooth muscle mass that could explain the observed hypersensitivity to high potassium solutions and to 5-HT.

In situ *detection and quantification of tissue apoptosis*

Figure 8b reveals the presence of DAPI nuclei staining and the absence of TUNEL-positive cells in IPA1 rings cultured during 4 days with 10% FCS. As negative controls, the apoptosis protocol was performed without digoxigenin–nucleotides or with different protein-digesting enzyme incubation times (proteinase K for 15 and 45 min) (data not shown). As a

Table 1 SRC characteristics after 4 days of culture with or without 10% FCS

	MPA		*IPA1*		*IPA2*	
	FCS	*ITS*	*FCS*	*ITS*	*FCS*	*ITS*
Amplitude (mg)	556.6±72.4	801.6±2.65	129.9±41.2	96.5±18.5	22.9±3.7	44.72±9.9*
Frequency (SRC/min)	0.145±0.01	0.121±0.02	0.135±0.01	0.120±0.01	0.122±0.004	0.118±0.02
% of rings with SRC	57.1	10.5	44.1	21	46.7	21.4
n	35	19	34	19	15	14

When removed, FCS was replaced by ITS. Amplitude is the isometric tension developed by the rings in mg. Frequency is expressed as the number of SRC per minute. *n* is the number of rings tested in each experimental condition. Data are expressed as mean±s.e.m. and all values obtained in serum-free medium (ITS supplemented) conditions are compared to those obtained in serum containing medium. *$P<0.05$.

Table 2 Effects of levcromakalim on spontaneous and rhythmic contractions

	MPA		*IPA1*	
	Basal tone (mg)	*Amplitude* (mg)	*Basal tone* (mg)	*Amplitude* (mg)
Control	1168.3±78.4	451.6±103.2	956.7±115	96.7±20.1
Levcromakalim 0.1 μM	1015.4±103.3**	156.9±66.2*	850.4±111.4	71.6±27.2
Levcromakalim 1 μM	928.2±120.8**	50±31.6**	797.2±117*	55.8±23.5
n	10		5	

The effect of levcromakalim was tested on 4-day-cultured (with or without 10% FCS) MPA and IPA1 rings. Basal tone and SRC amplitude were recorded in the absence (control) and in the presence of levcromakalim 0.1 and 1 μM, respectively. Data are mean±s.e.m. Significant inhibition of the basal tone and the amplitude of the response in the presence *versus* in the absence of levcromakalim is indicated by * and ** when $P<0.05$ and $P<0.01$, respectively. *n* represents the number of rings tested.

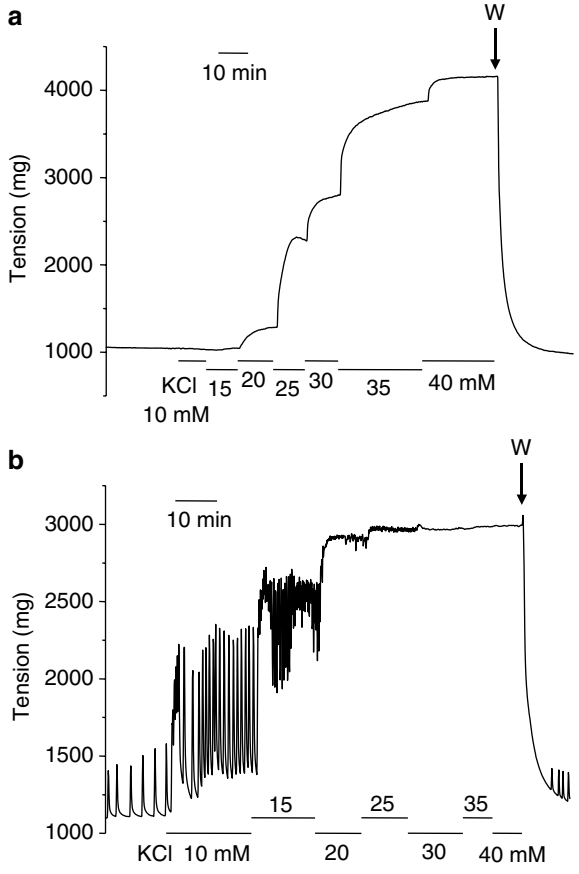

Figure 2 Effect of stepwise increases in potassium concentrations on fresh tissue and 4-day organoid cultured MPA rings. Representative trace shows the concentration-dependent mechanical effect of increasing KCl concentrations on native tissue (a) and 4-day cultured MPA rings exposed to serum-free medium (b). The resting tension was adjusted to 1 g and cumulative KCl concentrations were bath-applied. W: washout with normal physiological solutions.

positive control, the same TUNEL methodology was used and detected apoptosis on 7-day-cultured bronchus (Figure 8d).

As previously shown in immunofluorescent labelling of pulmonary artery sections (Nakamura *et al*., 1999), nonspecific staining was detected on the internal elastic lamina (Figure 8a, b). It should be noted that DAPI nuclei staining was also observed on the luminal side of the cultured vessel adjacent to the internal elastic lamina, confirming that endothelium integrity was preserved (Figure 7d and 8b). These results thus suggest that the absence of endothelium-dependent relaxation is not dependent on apoptosis or endothelial disappearance with culture, but rather rely on functional uncoupling.

Discussion and conclusions

In the present work, we have characterized an *in vitro* integrated model using rat extra- and intrapulmonary arteries for 4 days in organoid culture. Compared to control arteries, cultured pulmonary arteries developed SRC linked to membrane depolarization and L-type calcium channel activity. Pharmaco-mechanical hyper-sensitivity was observed in response to both serotonin (5-HT) and high potassium solution, whereas contraction to ET-1 remained unchanged. Unlike endothelial-dependent relaxation to A23187, relaxation to carbamylcholine disappeared. There were no observed modifications in 3D tissue structure and apoptosis was absent. Since tissue integrity and contractile properties were well preserved, this model would appear suitable for pharmacogenomic studies. To our knowledge, this is the first report on the characterization of this type of pulmonary arterial model.

In 4-day-cultured pulmonary artery rings, basal tone was characterized by the occurrence of SRC in approximately half of the vessels. These SRC were sensitive to an L-type calcium channel blocker, depolarizing solutions (high potassium solutions – Figure 2) and the potassium channel opener levcromakalim (Table 2), a compound known to induce hyperpolarization in rat and human pulmonary artery SMC (Bonnet *et al*., 2001b; Cui *et al*., 2002). In isolated rat PASMC in culture, membrane potential was shown to spontaneously depolarize to −40 mV (Yuan *et al*., 1993) comparatively to −56 mV in freshly isolated cells (Archer *et al*., 2004). In the present study, PA rings also demonstrated hyper-responsiveness to high potassium solutions predicting for basal membrane depolarization in cultured tissue in comparison to fresh tissue (Figure 3a–c). Altogether, SRC could well be explained by membrane depolarization activating L-type calcium channels balanced by calcium-activated potassium channel activity. Indeed, small changes in membrane potential (depolarization or hyperpolarization) can markedly alter the availability of Ca^{2+} and may thereby affect tension in vascular smooth muscle (Nelson *et al*., 1990).

This oscillating tone mimics that observed at the onset of chronic hypoxia-induced hypertension in rats (Bonnet *et al*., 2001b). Although the frequency of SRC was lower in the present study, they were similarly sensitive to levcromakalim and L-type calcium channel blockers. In the hypoxic model,

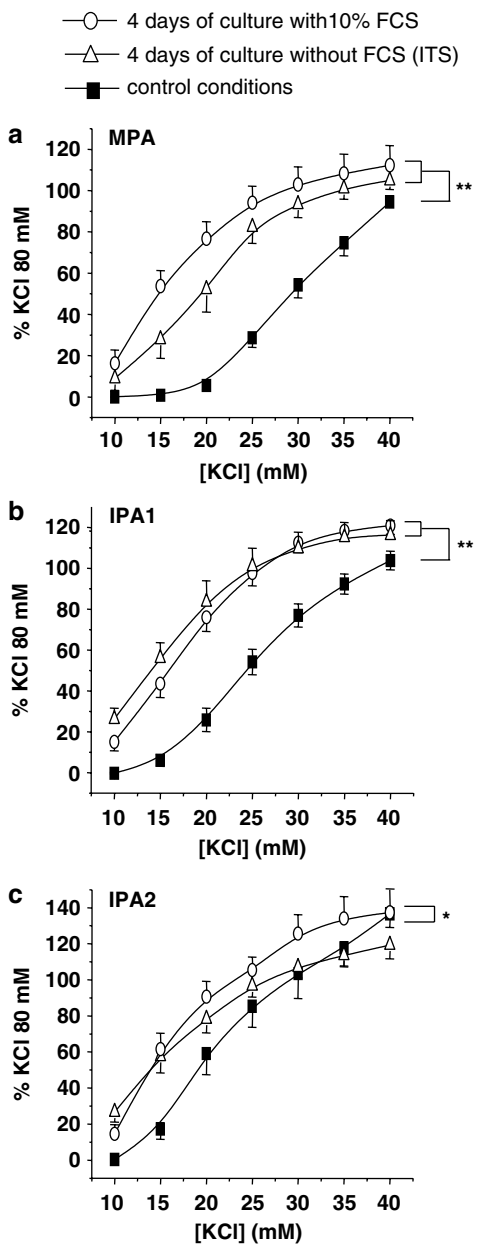

Figure 3 Effect of 4-day organoid culture with or without FCS on contractile responses to depolarizing KCl solutions (10–40 mM). Concentration–response curves to KCl (10–40 mM) are shown for MPA (a), IPA1 (b) and IPA2 (c) on control rings (■) and 4-day organoid cultured rings with or without 10% FCS (○ and △, respectively). Data are mean ± s.e.m. for 9–19 rings and are expressed as a percentage of the high potassium solution (80 mM)-induced response. $*P<0.05$ and $**P<0.01$.

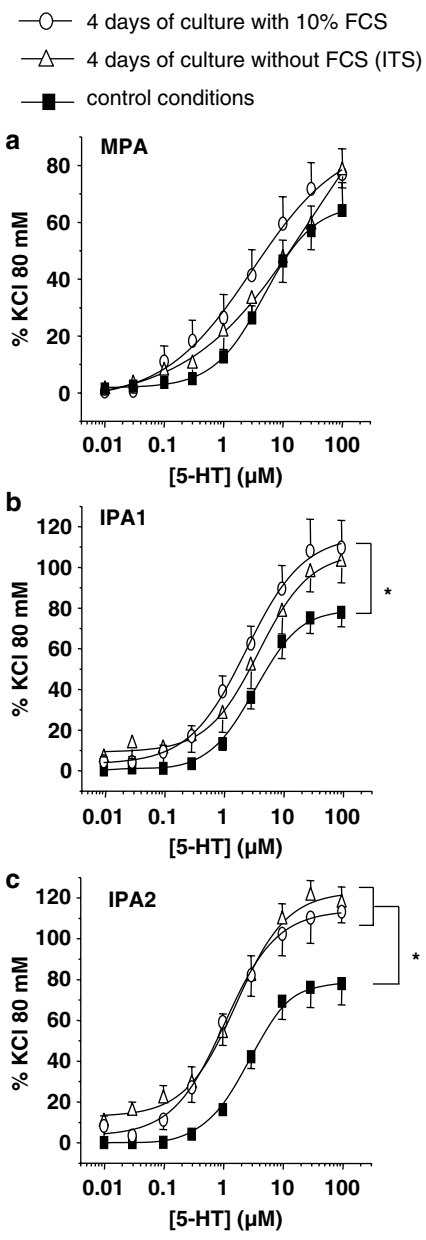

Figure 4 Effect of 4-day organoid culture with or without FCS on contractile responses to 5-HT (0.01–100 μM). Concentration–response curves to 5-HT (0.01–100 μM) are shown for MPA (a), IPA1 (b) and IPA2 (c) on control rings (■) and 4-day organoid cultured rings with or without 10% FCS (○ and △, respectively). Data are mean ± s.e.m. for 7–16 rings and are expressed as a percentage of the high potassium solution (80 mM)-induced response. $*P<0.05$.

SRC were correlated with spontaneous rhythmic membrane depolarization. Moreover, the membrane potential of rat main pulmonary arteries was depolarized from −60 mV in normoxia to −40 mV in chronic hypoxia (Bonnet *et al.*, 2001b). Interestingly, this depolarization is similar to that observed in isolated rat PASMC in culture (Yuan *et al.*, 1993). In monocrotaline-induced rat pulmonary hypertensive arteries (a PAH model close to primary pulmonary hypertension), the membrane potential was also depolarized compared to control arteries (from −60 to −40 mV) (Ito *et al.*, 2000). Moreover, in this latter study, spontaneous rhythmic depolarizations were observed, the amplitude of which was increased upon removal of the endothelium (Ito *et al.*, 2000). In this study, functional properties of the endothelium could be impaired since relaxation to carbamylcholine was abolished and, by analogy with monocrotaline-induced rat PAH, this may also contribute in explaining the presence of SRC. Oscillations, membrane depolarization, as well as the loss of endothelial-dependent relaxation to carbamylcholine suggest that the present model may be close to different hypertensive models and therefore suitable to study secondary as well as primary PAH.

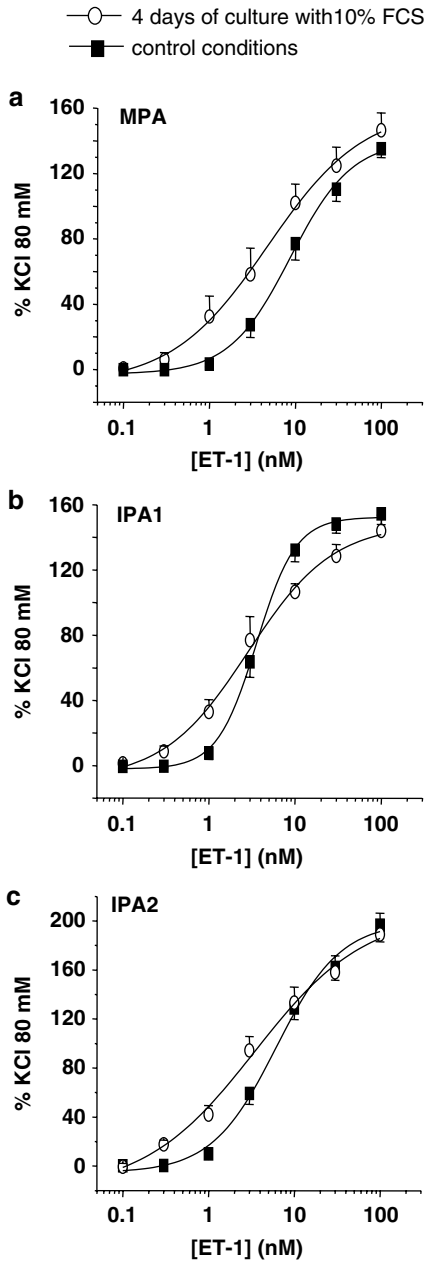

Figure 5 Effect of 4 days of culture with or without FCS on contractile responses to ET-1. Concentration–response curves to ET-1 (0.1–100 nM ET-1) are shown for MPA (a), IPA1 (b) and IPA2 (c) on control rings (■) and 4-day organoid cultured rings in the presence of 10% FCS (○). Data are mean±s.e.m. for 10–18 rings and are expressed as a percentage of the high potassium solution (80 mM)-induced response.

Table 3 Effect of 5-HT on the contractile properties of IPA1 and IPA2 rings in control conditions and after 4 days of culture

	IPA1		*IPA2*	
	T_{max} (mg)	EC_{50} (μM)	T_{max} (mg)	EC_{50} (μM)
Control	78.2±7.1	5.3±1.2	78.5±10.4	2.8±0.4
10% FCS	113.7±14.9*	3.1±0.5	121.3±7.4**	2.1±1.1
ITS	ND	ND	122.9±7.6**	2.2±0.5

5-HT significantly increased contraction in 4-day-cultured IPA1 and IPA2 rings (in the presence of 10% FCS or absence (ITS supplemented)). Data are mean±s.e.m. Significant increase in T_{max} after 4 days of culture *versus* control conditions is indicated by * and ** when $P<0.05$ and $P<0.01$, respectively.

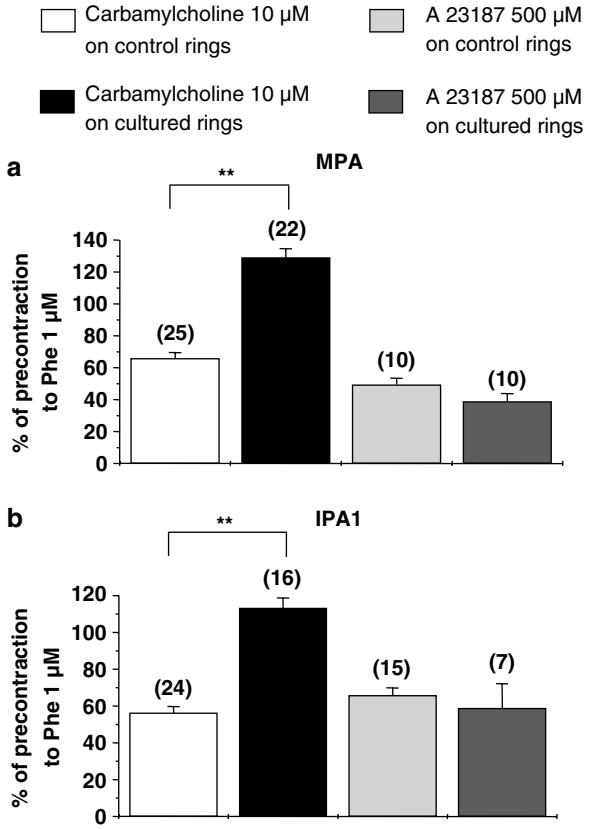

Figure 6 Effect of 4-day organoid culture on endothelium-dependent relaxation. Effect of 10 μM carbamylcholine on MPA (a) and IPA1 (b) preconstricted with phenylephrine 1 μM (Phe). Experiments were performed on native tissue (white and light grey columns) and rings cultured during 4 days in the presence of 10% FCS (black and dark grey columns). Data are mean±s.e.m. and expressed as a percentage of the precontraction to phenylephrine 1 μM. The number of rings used is indicated in brackets. **$P<0.01$.

In the present study, carbamylcholine induced a contraction rather than the expected relaxation when the endothelium is intact and functional, as in the case of fresh tissues (Figure 6). Such effect of culture has already been described in both rat systemic resistance and pulmonary arteries (Bakker *et al*., 2000; Kunichika *et al*., 2004). A loss of a muscarinic receptor subtype (e.g., M1 and M3) may account for the observed results, in agreement with observations on cultured bovine aortic endothelial cells (Tracey & Peach, 1992). Alternatively, membrane depolarization may play a role in the impairment of pulmonary endothelial function (Seiden *et al*., 2000). Indeed, a maintained depolarization induced by a depolarizing high potassium solution (25 mM) abolished the relaxation to acetylcholine in contrast to the relaxation to levcromakalim in rat pulmonary arteries. Since relaxation to A23187 was not altered by 4 days of culture (Figure 6a–b), a decrease in endothelial nitric oxide synthase levels is not a plausible hypothesis. Furthermore, DAPI nuclei-stained labelled

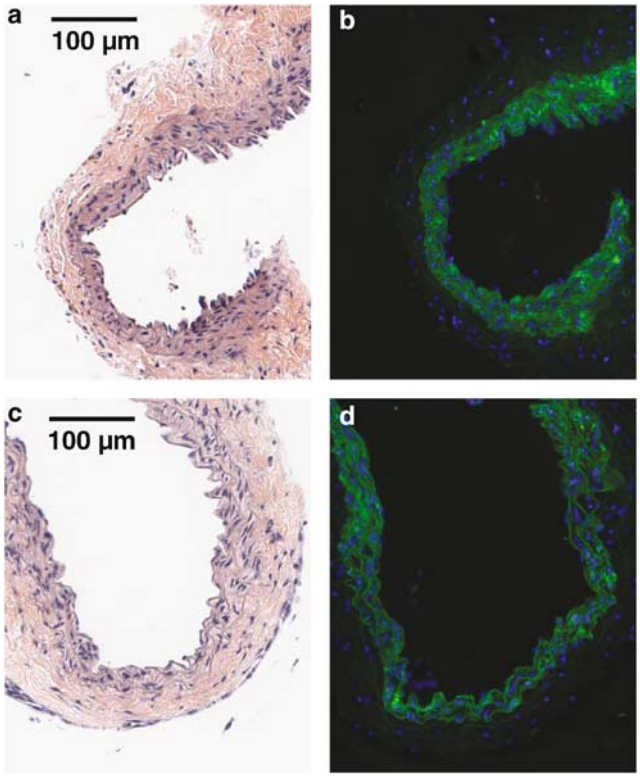

Figure 7 Light microscopy and immunofluorescent labelling of cross-sections derived from native and cultured IPA1 from the same rat. Fresh tissues (a, b) and organoid-cultured IPA1 preparations (c, d) were fixed in 5% formaldehyde and paraffin-embedded. Serial thin sections, prepared from fresh IPA1 (a, b), served as controls for conventional histochemical and immunofluorescent staining. Haematoxylin–eosin stainings are shown in (a) and (c). Smooth muscle α-actin staining (FITC, green) followed by nuclei counterstaining (DAPI, blue) are shown in (b) and (d). Organoid culture was performed for 4 days in DMEM + 10% FCS. Images are representative of experiments on multiple thin sections from two separate preparations. Scale bar = 100 μm.

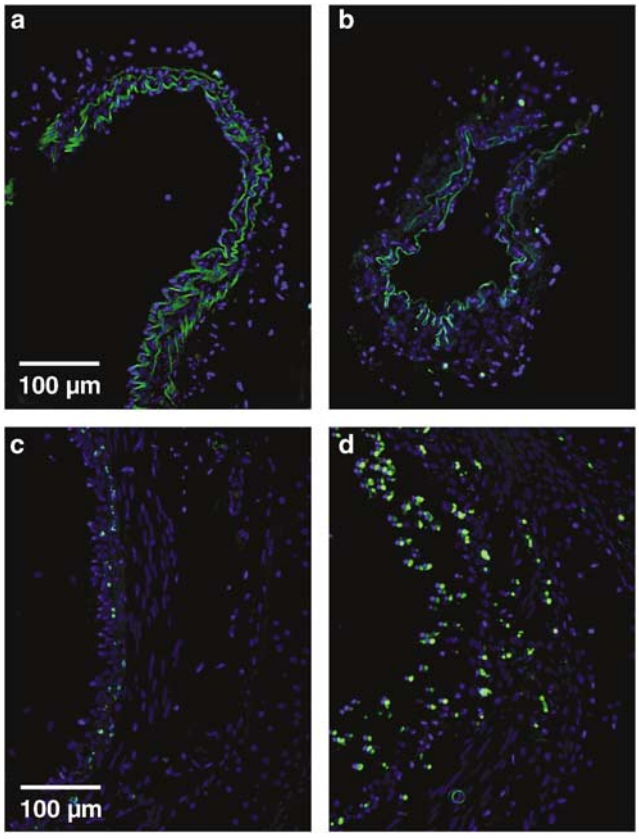

Figure 8 *In situ* detection of apoptosis on cross-sections from native and cultured bronchus and IPA1 rings. Organoid cultured preparations were fixed in 5% formaldehyde and paraffin-embedded. *In situ* detection of apoptosis (FITC, green) followed by nuclei counterstaining (DAPI, blue) were performed on native IPA1 rings (a) and native bronchus (c). The same experiment was performed on 4-day-cultured IPA1 rings (with 10% FCS) (b) and on 7-day-cultured bronchus rings (d). Images are representative of experiments on multiple thin sections from two separate preparations for IPA1 rings and five preparations for bronchus rings. Scale bar = 100 μm.

cells were observed adjacent to the internal elastic lamina (Figures 7d and 8b), suggesting the presence of viable endothelial cells.

Contraction to various agonists was either not modified (ET-1; Figure 5) or increased in IPA1 and IPA2 rings (5-HT; Figure 4). In pulmonary arteries, 5-HT induces vasodilatation and vasoconstriction by acting on receptors, respectively, located on endothelium and SMCs (Glusa & Pertz, 2000; MacLean *et al*., 2000). In the case of an altered endothelium, endothelial receptors may be uncoupled or subjected to downregulation (reduced expression), inducing a potentiated contraction to exogenous 5-HT. An alternative explanation would be an increase of 5-HT receptor expression on pulmonary artery smooth muscle, since previous studies have shown an increase in mRNA for 5-HT_{2A} receptors in organoid cultured rat mesenteric arteries (Luo *et al*., 2004). Potentiation of contraction to 5-HT does not appear to involve smooth muscle hypertrophy, since vascular wall thickness (Figure 7) and contraction to ET-1 (Figure 5) did not show any obvious (detectable) modifications. Moreover, apoptosis was absent after 4 days of culture in the presence of 10% FCS in IPA1 rings (Figure 8b), whereas apoptosis was detected in the bronchus after 7 days of culture (Figure 8d).

Some functional and phenotypical characteristics have been shown along the pulmonary vascular bed. Differences in the contractile response to acute hypoxia and in the expression of receptors, ionic channels, have also been demonstrated according to the size and/or location of the pulmonary arteries (extra- and intrapulmonary arteries) (Madden *et al*., 1992; Chootip *et al*., 2002). Such characteristics have been proven to be important in the physiology and pathophysiology of the pulmonary artery. In the present study, differences in the effect of culture between extra- and intrapulmonary arteries were minor. Similarly, regardless of the experimental protocol used in the present study, the presence or absence of serum in the culture medium did not seemingly alter the effect of culture. Therefore, this *in vitro* model of pulmonary arteries in organoid culture may be used either in the presence or absence of serum, and on both extra- and intrapulmonary arteries.

In summary, the contractile phenotype of smooth muscle is well preserved in rat pulmonary extra- and intrapulmonary arteries after 4 days of culture in the presence or absence of serum. Organoid cultured rings developed SRC, likely resulting from a slight depolarization. Endothelial functional integrity was partially affected as previously shown in primary and secondary

PAH. Since remodelling was not observed in this *in vitro* model, the effect of pressure and chronic hypoxia could be uncoupled and therefore studied separately. This may be relevant in the study of the role of these parameters in the onset of remodelling, and hyper- or hyporeactivity to agonists in secondary PAH.

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