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EVIDENCE FOR PRESERVATION OF EPITHELIAL FUNCTION IN CRYOPRESERVED PORCINE AND HUMAN BRONCHI

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SUMMARY

Porcine and human bronchi have been investigated *in vitro* without or after storage at −196 °C in Krebs–Henseleit solution containing 2·0 M dimethyl sulphoxide and 0·1 M sucrose as cryoprotectants. In bronchi from both species maximal post-thaw contractile responses to acetylcholine (ACh) were reduced by about 25–30 % compared to unfrozen bronchi. To assess the viability of bronchi and endothelium-denuded rat aortic strips, was employed. The release of an epithelium-derived inhibitory factor (EpDIF) was induced by ACh and assessed in terms of concentration-dependent relaxation of the endothelium-denuded rat aortae. Following removal of bronchial epithelium, ACh failed to elicit any relaxation of rat aorta. With cryopreserved bronchi from both pig and human about 5 and 30 times higher concentrations of ACh, respectively, were required to elicit the same relaxant response as with unfrozen bronchi. The results suggest that after the freezing–thawing process both smooth muscle and epithelial function is largely preserved and provide support for the use of cryopreservation for storage of airway preparations for pharmacological studies.

INTRODUCTION

Various tissue preparations meant for experimental studies but which cannot be immediately used, are commonly stored at 4 °C in a refrigerator. This, however, only allows storage for a few days, whereas cryopreservation in liquid nitrogen allows storage of blood vessels indefinitely and a wide variety of functional activities are well preserved in these tissues (Müller-Schweinitzer, 1992, 1994*a*, *b*). In addition, it has been shown that the same technique also preserves the functional activity of human and porcine airway smooth muscle (Johnson, McKay, Alouan, Armour & Black, 1993; Müller-Schweinitzer, Hasse & Swoboda, 1993*a*, *b*). The aim of the present study was to investigate whether storage at −196 °C sufficiently preserves the epithelial function of bronchial tissue, i.e. the release of epithelium-derived inhibitory factor (EpDIF) in response to acetylcholine.

Among the numerous studies aimed at elucidating the physiological role of the epithelium, coaxial bioassay experiments, employing bronchial tissues as donor of EpDIF and endothelium-denuded arterial segments as bioassay tissues, have received considerable attention (Ilhan & Sahin, 1986; Fernandes, Paterson & Goldie, 1989; Fernandes, Preuss, Paterson & Goldie, 1990). Using this assay, we now present evidence that the cryopreservation technique preserves the viability, in particular the integrity of the epithelial function, of bronchial preparations from both pig and human.

METHODS

Tissue preparation

Rats were killed by decapitation, the aortae carefully dissected, placed in Krebs–Henseleit solution (composition, mM: NaCl, 118; KCl, 4·7; $MgSO_2$, 1·2; $CaCl_2$, 1·2; KH_2PO, 1·2; $NaHCO_3$, 25; glucose, 11; EDTA, 0·03) and cleaned of loose connective tissues. The aortae were cut into two segments and cryostored as described below. Porcine lungs were obtained from a local slaughterhouse within 30 min of death. Specimens of human lung were obtained from surgery for lung carcinoma (8 patients, aged between 50 and 66 years) and used within 3 days after removal. Bronchi (2–4 mm in diameter) were carefully removed, avoiding any touching of the inner surface of the bronchi. The segments were distributed into two groups. The samples of group 1, 'unfrozen bronchi', were studied fresh, while the samples of group 2 were frozen as described below.

Cryopreservation

For cryopreservation, tissues were placed in 2 ml liquid nitrogen storage ampoules (Gibco AG, Basel, Switzerland) containing either Krebs–Henseleit solution (bronchi) or 50% fetal calf serum in Krebs–Henseleit solution (rat aorta) as vehicles for the cryoprotecting agents, dimethyl sulphoxide (DMSO, 2·0 M) and sucrose (0·1 M). Following an equilibration time of about 30–60 min at room temperature, the ampoules were placed in a polystyrol box (11 × 11 × 22 cm) and slowly frozen at a mean cooling rate of about 0·6 °C/min in a freezer maintained at −70 °C. After 3–15 h the ampoules were transferred into liquid nitrogen (−196 °C), where they were stored for 4–5 weeks until use. Before use, the tissues were exposed for 10–20 min to −70 °C and then thawed within 2·5 min by placing the ampoules in a 37 °C water bath. Thereafter the preparations were rinsed in a dish, containing Krebs–Henseleit solution at 37 °C.

Contractile responses to acetylcholine (ACh)

Bronchial rings (2–3 mm long) were mounted between two hooks of stainless-steel wire (diameter 0·15 mm) and suspended in 10 ml organ baths containing Krebs–Henseleit solution at 37 °C, gassed continuously with 5% CO_2 in oxygen. The tension of the rings was recorded isometrically under a resting tension of 1 g with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The tissues were allowed to equilibrate for about 2–3 h in the bathing medium. During this time the preparations were challenged twice with 10 μM ACh and the baseline tension of the rings was readjusted to 1 g if required. Concentration–response curves for ACh were determined by cumulative additions, each being added when the maximum effect had been produced by the previous concentration.

Coaxial bioassay

The luminal surfaces of the aortic segments were rubbed gently with a pair of forceps to remove the endothelium and the segments were cut into helical strips and suspended in 10 ml organ baths. Before setting up the coaxial preparation, verification of the aortic de-endothelialization procedure was ascertained by a lack of ACh-induced relaxation of noradrenaline-induced contraction. The coaxial bioassay set-up consisted of an aortic strip inserted into the lumen of a bronchus and suspended vertically between two hooks of stainless-steel wire in 10 ml organ baths containing Krebs–Henseleit solution at 37 °C. The medium was gassed continuously with 5% CO_2 in oxygen. Changes in the tone of the aortic strips were recorded isometrically under a resting tension of 1 g with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The tissues were allowed to equilibrate for 1 h, during which the resting tension was readjusted if necessary. The aortic strips were made to contract in response to 1 μM noradrenaline, which induced a submaximal contractile response. When the contractions were stable, concentration–response tests for ACh were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. To exclude the influence of prostaglandins, indomethacin (1 μM) was added to the bathing medium 20 min prior to noradrenaline stimulation and maintained throughout the experiment. At the end of the ACh responses, papaverine (300 μM) was applied, to elicit complete relaxation of the aortic strips. This response was taken as 100% relaxation and a reference for estimating the ACh-induced relaxation. In some experiments, responses to ACh were examined in coaxial preparations in which the epithelial lining of the bronchi had been removed by rubbing.

Statistics

Values are presented as means ± S.E.M. Contractile responses to ACh were analysed with a linear computer program in RS/1 (BBN Software products Corporation, Cambridge, MA, USA) and E_{max} (maximal effects) and $-\log EC_{50}$ values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Data obtained in the co-axial bioassay were analysed using the procedure, Fit Function in RS/1 according to the equation $f(x) = A/(1+B/X)$, where $f(x)$ is the fraction of receptors activated by the agonist concentration X; A represents the maximum response and B represents EC_{50} values.

Drugs

The following drugs were used: acetylcholine chloride (Acetylcholinum ophthalmicum, Dispersa, Hettlingen, Switzerland), (−)-noradrenaline hydrochloride (Fluka, Buchs, Switzerland), papaverine hydrochloride and indomethacin (Sigma, Munich, FRG).

RESULTS

When added to the organ bath ACh (0·1–1000 μM) induced concentration-dependent contractions of the bronchial rings. After cryopreservation, the maximal contractile

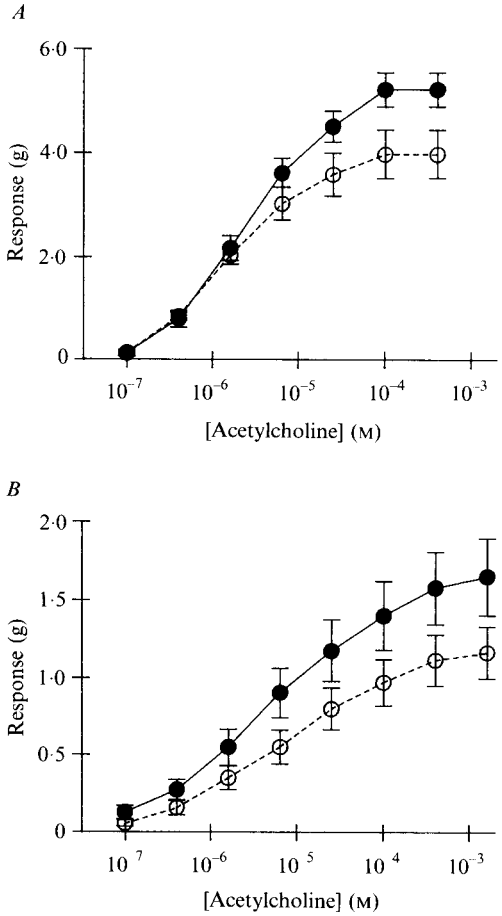

Fig. 1. Contractile responses to acetylcholine of rings from unfrozen (●) and frozen–thawed (○) bronchi from pig (*A*, for each point $n = 11$) and human (*B*, for each point $n = 12$). The effects are expressed in grams; the bars represent means ± S.E.M.

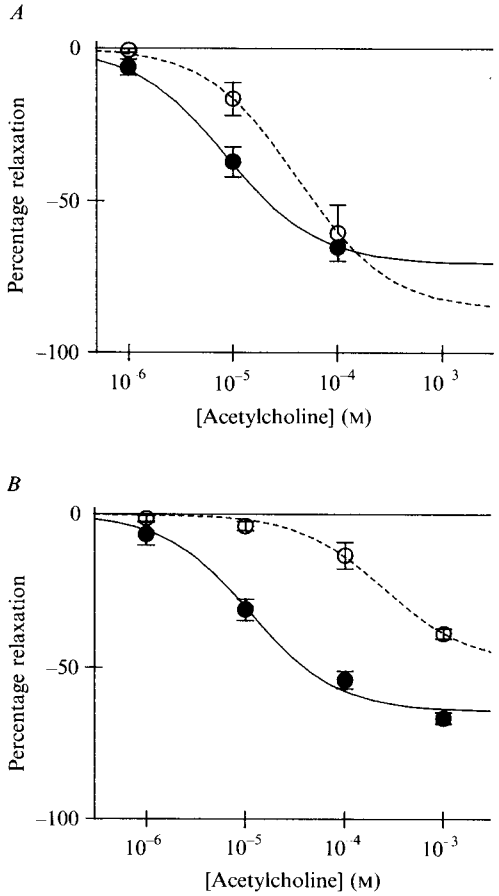

Fig. 2. Responses to acetylcholine of helical strips from endothelium-denuded rat aorta mounted coaxially within unfrozen (●) and frozen thawed (○) bronchi from pig (*A*, for each point $n = 10$) and human (*B*, for each point $n = 4$). The aortae were incubated with indomethacin (1 μM) and precontracted with noradrenaline (1 μM). The effects are expressed as percentages of the maximal relaxant responses to papaverine; the bars represent means ± S.E.M.

responses (g) to ACh of bronchi from both pigs (Fig. 1 *A*) and man (Fig. 1 *B*) were reduced by about 25 and 30%, respectively, compared to those observed in unfrozen tissues. In pig bronchi after thawing, the $-\log EC_{50}$ value for ACh (5·87 ± 0·05) was slightly but significantly higher than in the unfrozen tissues (5·63 ± 0·07, $P < 0·05$, $n = 11$), whereas in human bronchi the post-thaw $-\log EC_{50}$ value for ACh (5·11 ± 0·13) was statistically not different from that determined in fresh human bronchi (5·21 ± 0·12, $n = 12$).

In coaxial preparations with intact bronchial epithelium, the addition of ACh (1–1000 μM) resulted in concentration-dependent relaxation of the precontracted rat aorta, whereas aortic strips suspended without ($n = 10$) or in coaxial bioassays with bronchi after mechanical removal of the epithelium ($n = 6$), the addition of ACh never induced any response (data not illustrated). With frozen–thawed tissues from both species, higher ACh concentrations were required compared to unfrozen bronchi (Fig. 2). With porcine bronchi, the $-\log EC_{50}$ values for ACh relaxation by unfrozen and frozen–thawed bronchi were 5·02 ± 0·11 and 4·29 ± 0·18 ($n = 10$, $P < 0·05$), indicating that after cryopreservation, about

5 times higher ACh concentrations were required to elicit the same relaxant effect as with unfrozen bronchi. With unfrozen and frozen–thawed human bronchi, the $-\log EC_{50}$ values for ACh were $4{\cdot}98 \pm 0{\cdot}12$ and $3{\cdot}46 \pm 0{\cdot}24$ ($n = 4$, $P < 0{\cdot}001$) respectively, indicating that with frozen–thawed human bronchi about 30 times higher ACh concentrations were required compared to unfrozen bronchi. In both series of experiments, the calculated $-\log EC_{50}$ values for ACh-induced EpDIF release represented concentrations which in control experiments elicited similar contractions in grams of unfrozen and cryopreserved bronchi. With both unfrozen and frozen-thawed porcine bronchi, the ACh concentrations required for 50% EpDIF-mediated relaxation of the endothelium-denuded rat aorta coincided with ACh concentrations that increased the bronchial tone by about 3·9 and 3·7 g, respectively, whereas in both groups from human bronchi, the $-\log EC_{50}$ values for EpDIF release corresponded to ACh concentrations which induced an increase in tone of human bronchi by about 1·0 and 1·1 g.

DISCUSSION

Airway reactivity to various agonists is modulated by the release of non-prostanoid spasmolytic substance(s) from the bronchial epithelium. Evidence for this comes from the observation that mechanical removal of epithelium increases the sensitivity to a variety of spasmogens of isolated airway smooth muscle from both animals (Goldie, Fernandes, Farmer & Hay, 1990) and humans (Raeburn, Hay, Farmer & Fedan, 1986; Aizawa, Miyazaki, Shigematsu & Tomooka, 1988; Fernandes *et al.* 1989, 1990). More direct evidence for the release of an epithelium-derived inhibitory factor (EpDIF) has been provided by the coaxial bioassay, consisting of an assay preparation mounted within the lumen of an airway tube segment, which was first described by Ilhan & Sahin (1986). Using this technique, both histamine and methacholine have been shown to induce EpDIF-mediated relaxation of a preconstricted endothelium-denuded rat aorta, if mounted within a human bronchial segment (Fernandes *et al.* 1989, 1990). Our present data, showing ACh-induced release of EpDIF from porcine and human bronchi, support these observations, the evidence being that ACh failed to elicit aortic relaxation when mounted with epithelium-denuded bronchial preparations.

We have recently extended the technique of cryopreservation of blood vessels to human and porcine airway tissues and reported that cryopreserved bronchi retain both contractile and relaxant functions of the smooth muscle cells (Müller-Schweinitzer, Hasse & Swoboda, 1993*a*, *b*). The present results, showing well-maintained contractile responses to ACh, with a post-thaw reduction of the contractile force by about 25 and 30% in porcine and human bronchi, respectively, add support to our previous data. The important observation in the present study, however, is that the functional integrity of the bronchial epithelium, i.e. the ability to produce EpDIF, is also largely maintained following cryopreservation. Although in our experiments with bronchi from both pig and human, the calculated EC_{50} values of ACh for EpDIF release were higher than those eliciting 50% contraction of the bronchial rings, the generation of ACh-induced EpDIF was most probably initiated by activation of muscarinic cholinoceptors (Fernandes *et al.* 1989). However, from the present experiments, it is not possible to define the location of these receptors. It may be that the release of EpDIF was simply due to shearing forces following contraction of the bronchial smooth muscle during exposure to ACh. In both series of experiments, the calculated EC_{50} values for EpDIF release represented ACh concentrations which elicited similar contractions in grams of unfrozen and cryopreserved bronchi, suggesting that it was the absolute shearing

stress that induced EpDIF release. Nevertheless, our data suggest functional integrity of the epithelium following the freezing–thawing procedure, thereby confirming histological findings in cryopreserved canine and human airway tissues, which showed normal epithelium with mucus production and well-retained ciliary activity (Wulffraat, Veerman & Stamhuis, 1985; Deschamps, Trastek, Ferguson, Martin, Colby, Pairolero & Payne, 1989).

The various advantages of the cryopreservation technique for storage of vascular tissues for subsequent pharmacological studies have been highlighted in recent reviews (Müller-Schweinitzer, 1992, 1994*a*, *b*). Except for a few reports on human airway epithelium (Raeburn *et al.* 1986; Aizawa *et al.* 1988; Fernandes *et al.* 1989, 1990), most *in vitro* studies on airway epithelial function are routinely carried out with animal tissues, largely because tissues from humans (normally excised following surgery) are not readily available for experimental studies. Thus the present results, showing maintained post-thaw smooth muscle and epithelial function, provide support for the use of the cryopreservation technique for storage of human airway preparations for experimental studies.

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