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Maintenance of functional activity of human pulmonary arteries after cryopreservation

P. Ellis & [1]E. Müller-Schweinitzer

Pharmacological Research, Sandoz Pharma AG, 4002 Basel, Switzerland

1 Human intrapulmonary arteries have been investigated *in vitro* in fresh tissue or after storage at −190°C in foetal calf serum containing 1.8 M dimethyl sulphoxide.

2 After cryopreservation of the arteries, maximal contractile force was reduced to 76%. This was assessed by the responses (in g) to 10 nM of the thromboxane analogue, U 46619.

3 Constricting agonists such as noradrenaline, 5-hydroxytryptamine, histamine and U 46619 stimulated fresh and frozen/thawed arteries producing pD_2 values similar to the respective values determined on fresh tissues.

4 Endothelium-independent relaxant responses of U 46619-precontracted arteries to prostacyclin (PGI_2), aminophylline and papaverine were generally unchanged after storage. The same was true for relaxant response to the potassium channel activator P-1075 whereas the pD_2 values for SDZ PCO 400, RP 49356 and cromakalim were somewhat diminished.

5 Nevertheless, a significant correlation was obtained when the apparent pD_2 values for all agonists on fresh and frozen/thawed tissues were compared ($P < 0.001$).

6 The evidence suggests that after cryopreservation of human intrapulmonary arteries at −190°C, mechanisms of both contraction and relaxation are well-maintained.

Keywords: Human pulmonary arteries; cryopreservation; K^+ channel activators

Introduction

Animal tissue has been shown in many cases to be of use in human pharmacology and is widely used. Naturally, though, if human tissues were made available, the results obtained from experiments would be the most accurate and predictable for human pharmacology. Human tissue can be obtained from surgery or during autopsy. However, not all human tissue so obtained can be used as it may be diseased when healthy tissue is required or it may be damaged during surgery, by ischaemic periods during and after surgery, by patient age, medication or anaesthesia. Therefore, besides obtaining tissue, the main problems are the diversity of the tissue types available and the irregularity of supply. Once removed from the patient, the tissue has a very short life span and so experiments should start as soon as possible, if not immediately, but this is not always convenient. These factors make studies with human tissue difficult to conduct. Hence the advantage of a simple and reliable method of storing human tissues in such a way as to preserve their functional attributes, is readily apparent. Storage in such a way would also mean that tissue need not all be used at once and several days of experiments could be carried out from the same tissue divided into samples.

Various cryoprotective agents and freezing methods have been tested taking the maximal contractile force of canine saphenous vein strips as a parameter for the maintenance of cell integrity during the freezing-thawing procedure. In those studies the best recovery was obtained when venous segments were immersed in foetal calf serum containing 1.8 M dimethyl sulphoxide (DMSO), slowly frozen to −70°C and then stored at −190°C (Müller-Schweinitzer & Tapparelli, 1987; Müller-Schweinitzer, 1988). Validation studies for this method of cryopreservation have been performed with various blood vessels from different animals. These showed that in addition to functional responses to different agonists this cryopreservation method may also sustain mitochondrial enzyme activity and endogenous prostaglandin synthesis (Müller-Schweinitzer & Tapparelli, 1986), adrenergic nerve function (Müller-Schweinitzer & Tapparelli, 1986; 1987; Thompson *et al.*, 1989), calcium uptake mechanisms (Ebeigbe *et al.*, 1988) and endothelial function (Thompson *et al.*, 1989; Schoeffter & Müller-Schweinitzer, 1990). Moreover, the same method has been applied successfully to preserve specimens of human saphenous veins (Müller-Schweinitzer *et al.*, 1986) and bronchi obtained from surgery and samples of human basilar and cerebral arteries obtained during autopsy (Müller-Schweinitzer, 1988). In all these studies a very good correlation between the affinity parameters for various agonists and antagonists on fresh and frozen/thawed vessels was obtained, indicating the usefulness of this method for the storage of human material for pharmacological studies.

The present study demonstrates that cryopreservation of small human pulmonary arteries in the presence of DMSO is not only able to preserve attributes such as contractile responses but also relaxant effects in response to potassium channel activation.

Methods

Tissue preparation and storage method

Specimens of lung segments, obtained from patients who had undergone surgery for removal of lung carcinoma, were placed in ice cold Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) and transported to the laboratory within 1–2 h after removal. Small pulmonary vessels (outer diameter ≈ 1–3 mm) were carefully removed and divided into two groups. Group 1 consisted of 'fresh vessels' which were stored in Krebs-Henseleit solution at 4°C and used within 20 h of surgery. The samples of Group 2, 'frozen/thawed vessels', were placed in 2 ml liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with foetal calf serum (FCS) containing 1.8 M dimethylsulphoxide (DMSO) as cryoprotecting agent. 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^{-1} in a freezer maintained at −70°C. After 3–20 h the ampoules were transferred

[1] Author for correspondence.

into liquid nitrogen (−190°C) where they were stored until use. Before use the tissues were exposed for 20–60 min to −70°C before being thawed within 2.5 min by placing the ampoules in a 37°C water bath. Thereafter the vessel segments were rinsed in a dish containing Krebs-Henseleit solution at 37°C, cleaned of loose connective tissue and cut into rings (about 2–3 mm in length).

Experimental procedures

The rings were mounted between two stainless steel wire hooks and suspended in 10 ml organ baths containing Krebs-Henseleit solution at 37°C, gassed continuously with 5% CO_2 in O_2. The tension of the rings was recorded isometrically under a resting tension of about 1 g with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The preparations were allowed to equilibrate for 2–3 h in the bathing medium. During this time the preparations were challenged twice with noradrenaline (1 μM) and the baseline tension of the rings was readjusted to 1 g. Concentration-response curves for agonists were established by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. The contractile response curves were normalized in terms of the percentage of maximal contraction. When relaxant responses to drugs or solvents were investigated, active tone was induced by adding 10 nM of the thromboxane analogue, U 46619. At the end of these concentration-response curves papaverine (300 μM) was added to induce complete relaxation of the vascular rings. This effect was taken as 100% relaxation.

Drugs

The following pharmacological agents were used: (−)-noradrenaline hydrogentartrate, histamine dihydrochloride (Fluka, Buchs, Switzerland), 5-hydroxytryptamine creatinine sulphate (5-HT), U 46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), papaverine hydrochloride, aminophylline (Sigma, Munich, F.R.G.), PGI_2 (Iloprost, Schering, Berlin, F.R.G.), cromakalim (Beecham Pharmaceuticals, Harlow, Essex, U.K.), P-1075 (N-tert-pentyl-N′-3-pyridyl-N″-cyanoguanidine, Leo Pharmaceuticals, Ballerup, Denmark), RP 49356 (N-methyl-2-(3-pyridyl)-tetrahydrothiopyran-2-carbothioamide-1-oxide, Rhône-Poulenc Vitry, France). SDZ PCO 400 ((−)-(3S,4**R**)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(3-oxo-cyclopent-1-enyloxy)-2H-1-benzopyran-6-carbonitrile) was synthesized at Sandoz. Both U 46619 and cromakalim were dissolved in ethanol and diluted in distilled water to give 1 mM solutions containing 60% ethanol. Both P-1075 and RP49356 were dissolved in ethanol and diluted to give 1 mM solutions containing 5% ethanol. SDZ PCO 400 was dissolved in DMSO (Merck-Schuchardt, Hohenbrunn, F.R.G.) and diluted to give a 1 mM solution containing 5% DMSO. Further dilutions were performed in 5% glucose or physiological salt solution. Aliquots of U 46619 (1 μM) were kept frozen at −20°C. All other compounds were prepared just before use.

Data analysis

Concentration-response curves were analyzed with a linear computer programme and E_{max} (maximal effects) and pD_2 values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Data are given as means ± s.e.mean. Statistical analysis was performed by the unpaired t test with a P value < 0.05 considered to be significant.

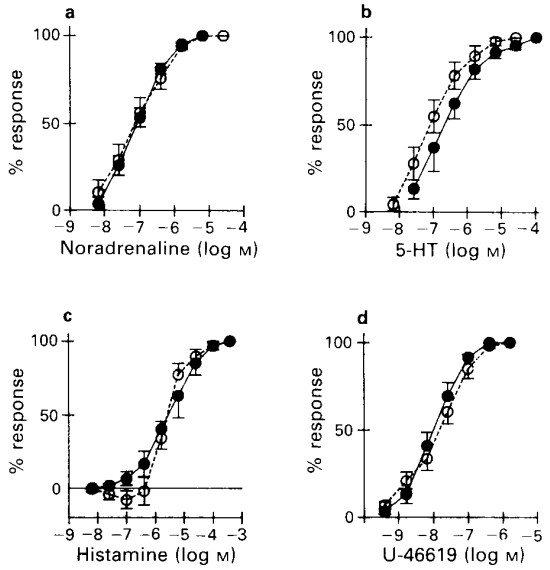

Figure 1 Cumulative concentration-response curves on rings from unfrozen (●) and frozen/thawed (○) human pulmonary arteries for contractile responses to (a) noradrenaline, (b) 5-hydroxytryptamine (5-HT), (c) histamine and (d) U 46619 expressed as percentages of the maximal effect. The bars represent s.e.mean, for each point, n = 5–8.

Results

Contractile responses

The contractile force as assessed by the response in g to 10 nM U 46619 of rings from human pulmonary arteries was diminished to 76% in preparations from frozen/thawed arteries (0.84 ± 0.05 g, n = 72) compared to that produced by fresh tissues (1.11 ± 0.08 g, n = 56, P < 0.005, means ± s.e.mean). Nevertheless all constricting agents tested, i.e., noradrenaline (Figure 1a), 5-HT (Figure 1b), histamine (Figure 1c) and the stable thromboxane analogue U 46619 (Figure 1d) stimulated the vascular preparations of both groups with similar potencies (Figure 1, Table 1). When histamine was investigated, a small relaxant response to low concentrations of the agonist was observed in tissues from 2 out of 4 patients in the group of frozen/thawed arteries (Figure 1c).

Relaxant responses

Responses to agents known to relax vascular smooth muscle were investigated on rings from fresh and frozen/thawed human pulmonary arteries which had been precontracted by a submaximal concentration (approximately EC_{50}) of 10 nM U 46619. The potassium channel activators RP 49356 (Mondot *et al.*, 1988; Figure 2a), P-1075 (Petersen *et al.*, 1978; Figure 2b) and SDZ PCO 400 (Quast *et al.*, 1990; Figure 2c) produced about 40–50% relaxation of precontracted arterial rings from both groups and the apparent pD_2 values were slightly reduced in frozen/thawed arteries (Table 1). Marked differences of both efficacy and potency between fresh and frozen/thawed arteries were observed when cromakalim (Hamilton *et al.*, 1986) was investigated (Figure 2d). This potassium channel activator was significantly more potent and more efficacious on fresh arteries than on frozen/thawed pulmonary vessels. This difference, however, might have been due to the solvent employed, reaching 1% ethanol at the highest cromakalim concentration tested. Solvent controls indicated that ethanol was a significantly more efficacious relaxant agent on fresh than on frozen/thawed arteries (Figure 2f), whereas DMSO was equieffective on arteries from both groups (Figure 2e). Like papaverine and aminophylline, PGI_2 also elicited

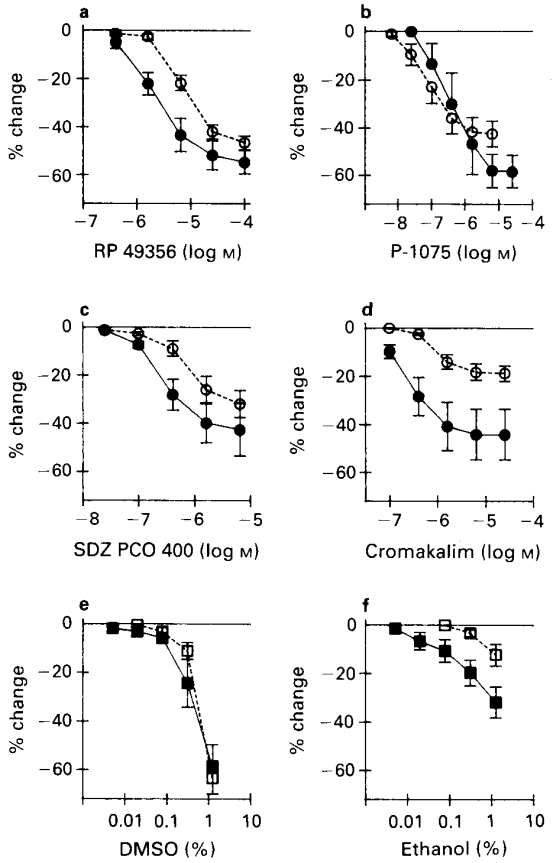

Figure 2 Cumulative concentration-response curves on rings from unfrozen (●) and frozen/thawed (○) human pulmonary arteries for relaxant responses to (a) RP 49356, (b) P-1075, (c) SDZ PCO 400, (d) cromakalim, and the solvents (e) dimethylsulphoxide (DMSO) and (f) ethanol. Active tone was induced by 10 nM U 46619 and the relaxant effects were expressed as percentages of the maximal relaxation induced by 300 μM papaverine. The bars represent s.e.mean; for each point, $n = 4$–9.

marked relaxation of the precontracted rings being the most potent relaxing agonist tested and equipotent on both fresh and frozen/thawed arteries (Table 1). A significant correlation ($r = 0.95$, $P < 0.001$) was obtained when the apparent pD_2 values for all compounds on fresh and frozen/thawed arteries were compared (Figure 3).

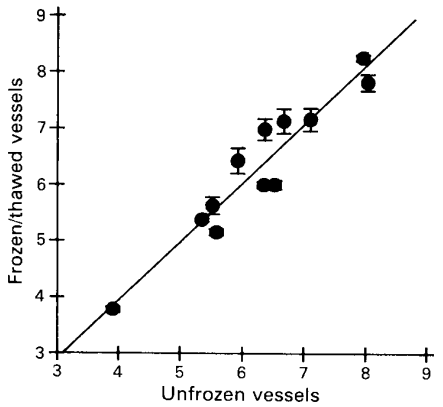

Figure 3 Correlation between pD_2 values for contractile and relaxant agonists on rings from unfrozen (abscissa scale) and frozen/thawed (ordinate scale) human pulmonary arteries. Data were compared by linear regression analysis. $y = 1.04x - 0.22$; $r = 0.95$; $P < 0.001$.

Table 1 Parameters for various contractile and relaxant effects on rings from fresh and frozen/thawed human pulmonary arteries

	Fresh arteries	*Frozen/thawed arteries*
pD_2 values for contractile agents		
Noradrenaline	7.10 ± 0.12 (8)	7.16 ± 0.21 (6)
5-HT	6.67 ± 0.21 (7)	7.13 ± 0.22 (5)
Histamine	5.52 ± 0.33 (4)	5.63 ± 0.16 (8)
U 46619	8.03 ± 0.15 (7)	7.82 ± 0.15 (7)
Carbachol	5.92 ± 0.42 (4)	6.42 ± 0.23 (6)
pD_2 values for relaxant agents		
SDZ PCO 400	6.35 ± 0.14 (6)	6.00 ± 0.06 (9)*
P-1075	6.36 ± 0.31 (4)	6.98 ± 0.20 (6)
RP 49356	5.58 ± 0.14 (6)	5.16 ± 0.06 (6)**
Cromakalim	6.52 ± 0.11 (6)	6.00 ± 0.08 (9)***
PGI_2	7.95 ± 0.24 (6)	8.26 ± 0.06 (7)
Aminophylline	3.91 ± 0.14 (3)	3.71 ± 0.08 (4)
Papaverine	5.35 ± 0.30 (3)	5.38 ± 0.03 (3)
E_{max} (%) of relaxant responses		
SDZ PCO 400	47 ± 9% (6)	37 ± 6% (9)
P-1075	58 ± 7% (4)	44 ± 5% (6)
RP 49356	55 ± 5% (6)	47 ± 3% (6)
Cromakalim	44 ± 11% (6)	19 ± 3% (9)*
PGI_2	73 ± 9% (6)	82 ± 4% (7)
Aminophylline	92 ± 3% (3)	75 ± 11% (3)
Papaverine	100 ± 0% (3)	100 ± 0% (3)

E_{max} = maximal relaxation expressed as percentage of maximal response to papaverine; pD_2 = negative logarithm of the molar concentration producing 50% of maximum response. Data are given as means ± s.e.mean. Number of determinations in parentheses. Differences from fresh control significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The present data confirm and extend the usefulness of cryopreservation for storing human vascular tissue for subsequent pharmacological studies. As observed in previous studies on animal (Müller-Schweinitzer, 1988, Thompson *et al.*, 1989; Schoeffter & Müller-Schweinitzer, 1990) and human vascular tissues (Müller-Schweinitzer, 1988), the maximal contractile responses of frozen/thawed human pulmonary arteries was somewhat diminished compared with fresh arteries. However, the calculated apparent pD_2 values for the contractile agents, which were in good agreement with those published by other groups (Houghton & Phillips, 1973; Boe & Simonsson, 1980; Boe *et al.*, 1980; Goldie *et al.*, 1982; Schellenberg & Foster, 1984), proved to be similar on both fresh and frozen/thawed tissues.

The relaxant responses of frozen/thawed arteries to agents acting through various endothelium-independent mechanisms were largely comparable to those produced by fresh tissues. Thus after cryopreservation the maximal relaxant responses, expressed as percentages of that induced by papaverine, to PGI_2, aminophylline and the potassium channel activators SDZ PCO 400, RP 49356 and P-1075 were not significantly different from those obtained with fresh arteries. Only with the potassium channel activator cromakalim was the difference between the relaxant efficacies in both groups of arteries significant. However, this discrepancy might have been due to the relatively high concentration of the solvent ethanol, the relaxant efficacy of which was significantly stronger on fresh than on frozen/thawed arteries. Nevertheless, with the exception of the potassium channel activators cromakalim, RP 49356 and SDZ PCO 400, the potencies in terms of apparent pD_2 values for all relaxant agonists, proved to be similar in arteries from both groups.

One interesting finding was a relaxant response to low concentrations of histamine in frozen/thawed tissues from 2 out of 4 patients. The histamine-induced relaxation in human pul-

monary arteries has been shown to be endothelium-dependent and mediated through enhanced PGI_2 production via stimulation of H_1 histamine receptors (Schellenberg *et al.*, 1986). The present observation, therefore, strongly suggested that the endothelial function was also preserved during cryo-storage. When human material is used, an endothelium-dependent relaxation can be shown only in approximately 50% of the vessels studied (Thom *et al.*, 1987). This variability may be due to various factors such as tissue handling during surgery, ischaemic periods during and after surgery, time between surgery and organ bath study, patient age and disease, pre- and perioperative medication and anaesthesia. Further experiments are planned, to investigate whether the well documented endothelial function of human pulmonary arteries (Furchgott & Zawadzki, 1980; Schellenberg & Foster, 1984; Hadházy *et al.*, 1985; Thom *et al.*, 1987; Greenberg *et al.*, 1987a,b; Dinh Xuan *et al.*, 1989a,b; Crawley *et al.*, 1990) can be maintained during the freezing/thawing process.

In conclusion, the present experiments suggest that the cryopreservation technique used, preserves both contractile and relaxant mechanisms in human intrapulmonary arteries.

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