Naunyn-Schmiedeberg's Arch Pharmacol (1992) 345:594–597

Naunyn-Schmiedeberg's
Archives of
Pharmacology
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Sucrose promotes the functional activity of blood vessels after cryopreservation in DMSO-containing fetal calf serum

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Received June 14, 1991/Accepted January 6, 1992

Summary. The cryoprotective effect of sucrose has been investigated using 3 different vascular smooth muscle preparations, namely canine saphenous veins and arteries and porcine circumflex coronary arteries following storage in liquid nitrogen (at -196°C). Contractile responses to noradrenaline, 5-HT, prostaglandin $F_{2\alpha}$ and KCl and relaxant responses to substance P and 5-HT were determined on fresh tissues and after cryostorage in fetal calf serum (FCS) containing either 1.8 M dimethyl sulfoxide (DMSO), or 0.1 M sucrose or both agents combined. The data demonstrate that the addition of sucrose to the DMSO-containing cryomedium promotes the preservation of both contractile and relaxant activity of cryostored blood vessels, though sucrose alone did not confer any noticeable protection.

Key words: Cryopreservation – Blood vessels – DMSO – Sucrose

Introduction

Cryopreservation has become an important tool for the storage of animal and human tissues in pharmacological research. If immersed in FCS (fetal calf serum) containing DMSO (dimethyl sulfoxide), mammalian tissues such as vascular and airways smooth muscle can be stored in liquid nitrogen indefinitely. With the exception of some reduction in the absolute contractile force, after thawing both biochemical and functional activities of the cryopreserved tissues are on the whole comparable to data determined on fresh tissues (Müller-Schweinitzer and Tapparelli 1986; Müller-Schweinitzer et al. 1986; Ebeigbe et al. 1988; Müller-Schweinitzer 1988; Thompson et al. 1989; Ku et al. 1990; Schoeffter and Müller-Schweinitzer 1990; Ellis and Müller-Schweinitzer 1991). DMSO is a permeating cryoprotectant which reduces the formation of ice crystals in both intra- and extracellular space, thereby protecting the mammalian cell from cryoinjury (Mazur 1977; Brockbank 1989). We now present evidence that the addition of the non-permeating cryoprotectant sucrose to the DMSO-containing cryomedium may further improve the preservation of the functional activity of vascular tissues during the freezing/thawing process.

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Materials and methods

Tissue preparation and storage method. Saphenous veins and arteries were obtained from beagle dogs of either sex (7–13 kg), killed by i. v. injection of pentobarbitone (50 mg/kg) and exsanguination from the femoral arteries. Left circumflex coronary arteries were removed from pig hearts, obtained from a local slaugther house within 30 min of death. The vessels were carefully removed, cut into segments (about 2 cm) and divided into 4 groups. Group 1 consisted of 'fresh vessels' which were used immediately for organ bath studies. The remaining segments were placed in 2 ml Liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with fetal calf serum (FCS) containing either 1.8 M dimethyl sulfoxide (DMSO, Group 2) or 0.1 M sucrose (Group 3) or both agents combined (Group 4) as cryoprotecting agents. The ampoules were then placed in a polystyrol box ($11 \times 11 \times 22$ cm) within 60 min and slowly frozen at a mean cooling rate of about 0.6°C/min in a freezer maintained at -70°C. After 3–20 h the ampoules were transferred into liquid nitrogen (-196°C) where they were stored for 4 to 9 weeks until investigated. On removal from the liquid nitrogen, the tissues were exposed for 10–30 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 (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) gassed with 5% CO_2 in oxygen at 37°C.

Organ bath studies. The vessels were cleaned of loose connective tissue and cut into rings (2–3 mm). Stainless steel hooks were inserted through the lumen, allowing them to be suspended for isometric tension recording in 10 ml organ baths containing Krebs-Henseleit solution at 37°C and gassed continuously with 5% CO_2 in oxygen. Changes in the tone of the preparations were recorded isometrically with electromechanical transducers (Statham model UC3) and a potentiometric recorder.

Table 1. Maximal responses to KCl and various agonists of blood vessels after cryopreservation at −196°C in fetal calf serum containing 1.8 M DMSO without and with 0.1 M sucrose. The effects are expressed as a percentage of the maximum response of unfrozen tissues

	Frozen without sucrose	Frozen with 0.1 M sucrose
Saphenous vein		
KCl (60 mM)	22 ± 3 (12)	27 ± 2 (12)
Noradrenaline	46 ± 6 (21)	73 ± 5 (21)**
5-HT	28 ± 5 (21)	52 ± 4 (21)**
Saphenous artery		
KCl (60 mM)	23 ± 3 (15)	23 ± 3 (15)
Noradrenaline	46 ± 5 (25)	54 ± 2 (25)
5-HT	10 ± 4 (10)	23 ± 4 (10)*
Coronary artery		
KCl (60mM)	42 ± 5 (9)	58 ± 7 (9)
$PGF_{2\alpha}$	62 ± 7 (9)	81 ± 9 (9)
Substance P	67 ± 4 (11)	86 ± 2 (12)**
5-HT	46 ± 7 (10)	45 ± 7 (10)

Values are means ± SEM (*n*). Differences between both groups significant at * $P < 0.05$, ** $P < 0.001$

At the beginning of the experiments, rings from canine saphenous veins and arteries were stretched to an initial tension of about 1.5−2 g and then allowed to equilibrate for 2 to 3 h in the bathing medium which was changed every 15 min. During this time the preparations were stimulated twice with 10 µM noradrenaline and the resting tension was adjusted to 1 g. Rings from porcine left circumflex coronary arteries were investigated at a passive preload of 4 g. Concentration-response curves for agonists were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. When relaxing agents were investigated on pig coronary arteries, the rings were precontracted by 3 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, a concentration producing about 80% of the maximal response). Contractile responses to 5-HT were determined in the presence of 30 µM cocaine, added 20 min previously. Relaxant responses to 5-HT were determined in the presence of 10 µM ketanserin, added 20 min before $PGF_{2\alpha}$. At the end of these concentration-response curves 300 µM papaverine was added to induce complete relaxation. This effect was taken as 100% relaxation. Contractile responses were expressed in mg.

Analysis of data. Concentration-effect curves were analyzed with a linear computer program and pD_2 values (negative logarithm of the molar concentration producing 50% of maximal response) were derived from this analysis. Values indicated in text, tables and figures represent means ± SEM. Statistical analysis of data was performed by the unpaired *t*-test with a *P* value < 0.05 considered to be significant.

Drugs. The following pharmacological agents were used: substance P (Bachem, Bubendorf, Switzerland), (−)-noradrenaline hydrogen tartrate (Fluka, Buchs, Switzerland), ketanserin (Janssen, Beerse, Belgium), cocaine hydrochloride (Siegfried, Zofingen, Switzerland), 5-hydroxytryptamine creatinine sulfate (5-HT), papaverine hydrochloride, sucrose (Sigma, Munich, FRG), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Upjohn, Crawley, UK). Samples of 0.2 mM substance P, dissolved in 17 mM acetic acid were stored at −20°C. $PGF_{2\alpha}$ (1 mM) was dissolved in 50% (w/v) ethanol and kept at 4°C. All other substances were freshly prepared at 10 mM and diluted in 0.9% (w/v) NaCl solution before use.

Results

When unfrozen vascular tissues were exposed for 20 min at 37°C to 1.8 M DMSO alone or with 0.1 M sucrose followed by washout, with all three vascular tissues contractile and relaxant responses to the investigated agonists were unchanged (not illustrated). Vessels which had been frozen in FCS containing 0.1 M sucrose without DMSO developed nearly no contractile response. Vascular tissues which had been frozen while being suspended in FCS containing 1.8 M DMSO responded to both contractile and relaxant agonists yet the effects were generally reduced as compared to that observed with unfrozen

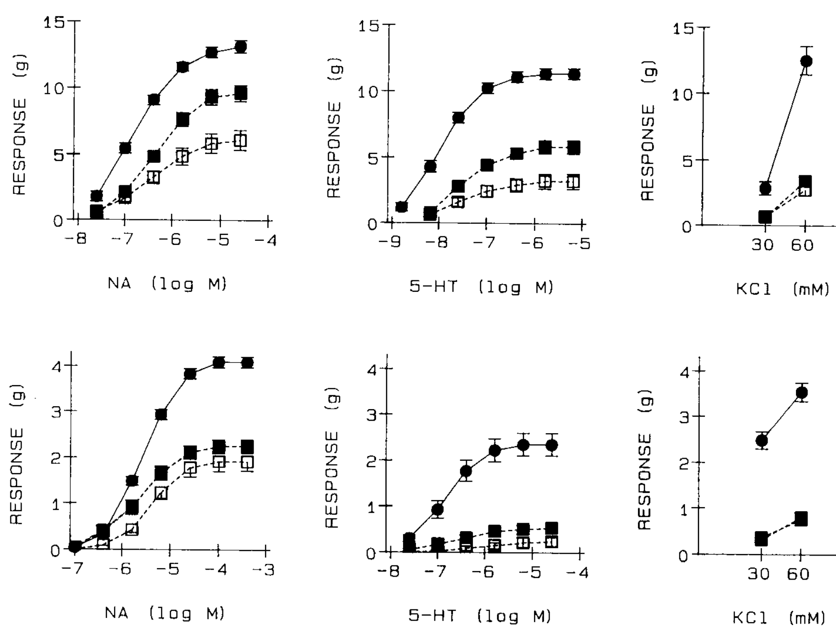

Fig. 1. Cumulative concentration-response curves in ring preparations from blood vessels after cryopreservation at −196°C in FCS containing 1.8 M DMSO without (□) and with 0.1 M sucrose (■) and on unfrozen preparations (●). *Upper traces:* Contractile responses of rings from canine saphenous veins to noradrenaline (NA, *left*), 5-HT (*middle*) and KCl (*right*). For each point *n* = 12−21. *Lower traces:* Contractile responses of rings from canine saphenous arteries to noradrenaline (NA, *left*), 5-HT (*middle*) and KCl (*right*). For each point *n* = 10−25. The effects are expressed in g, the *bars* represent means ± SEM

Table 2. pD_2 values calculated in ring preparations from unfrozen blood vessels and from tissues that had been cryopreserved at −196°C while being immersed in FCS containing 1.8 M DMSO without and with 0.1 M sucrose

	Unfrozen blood vessels	Frozen without sucrose	Frozen with 0.1 M sucrose
Saphenous vein			
Noradrenaline	6.84 ± 0.08 (18)	6.43 ± 0.04 (21)**	6.41 ± 0.05 (21)**
5-HT	7.97 ± 0.04 (18)	7.45 ± 0.06 (21)**	7.56 ± 0.06 (21)**
Saphenous artery			
Noradrenaline	5.57 ± 0.04 (25)	5.39 ± 0.02 (25)**	5.60 ± 0.08 (25)
5-HT	6.77 ± 0.08 (15)	6.51 ± 0.09 (10)*	6.51 ± 0.12 (10)*
Coronary artery			
$PGF_{2\alpha}$	5.72 ± 0.05 (14)	5.35 ± 0.10 (9)**	5.41 ± 0.09 (9)*
Substance P	9.16 ± 0.09 (19)	8.78 ± 0.13 (11)*	8.73 ± 0.10 (12)*
5-HT	7.07 ± 0.12 (20)	7.15 ± 0.20 (10)	6.86 ± 0.06 (10)

Values are means ± SEM (*n*). Differences from those determined in unfrozen tissues significant at * $P < 0.05$ and ** $P < 0.001$

tissues. The addition of 0.1 M sucrose to the DMSO-containing cryomedium improved in part the viability of the preparations after the freezing/thawing process.

Compared to those observed with *canine saphenous veins* which had been frozen in FCS containing 1.8 M DMSO without sucrose, the venoconstrictor responses to both noradrenaline and 5-HT were significantly ($P < 0.001$) improved when 0.1 M sucrose had been added to the cryomedium. Under these conditions the maximal effect of noradrenaline was 73% and that of 5-HT was 46% when compared to that produced with unfrozen veins. In contrast, contractile responses to depolarization by 60 mM KCl were not significantly improved by the addition of sucrose to the DMSO-containing cryomedium (Table 1, Fig. 1, top). The calculated pD_2 values for noradrenaline and 5-HT, though lower than those calculated in unfrozen veins, were similar in both groups of frozen/thawed veins (Table 2).

In *canine saphenous arteries* the addition of sucrose to the DMSO-containing cryomedium did not change contractions induced by depolarization or noradrenaline. The maximal contractile response to 5-HT was significantly ($P < 0.05$) enhanced when sucrose was present in the DMSO-containing cryomedium, but reached still only 23% of that produced by unfrozen tissues (Table 1), Fig. 1, bottom).

In *porcine circumflex coronary arteries* the contractile responses to both $PGF_{2\alpha}$ and depolarization were slightly but not significantly enhanced when sucrose had been added to the DMSO-containing cryomedium. In these experiments, however, the maximal response to $PGF_{2\alpha}$ reached 81% of that produced by unfrozen arteries which was statistically similar. The addition of sucrose to the DMSO-containing cryomedium induced also a significant ($P < 0.001$) enhancement of the endothelium-dependent relaxant responses to substance P (Gulati etal. 1987), being 86% of that produced with unfrozen arteries, while the endothelium-dependent relaxant responses to 5-HT (Cocks and Angus 1983; Table 1, Fig. 2) were unchanged.

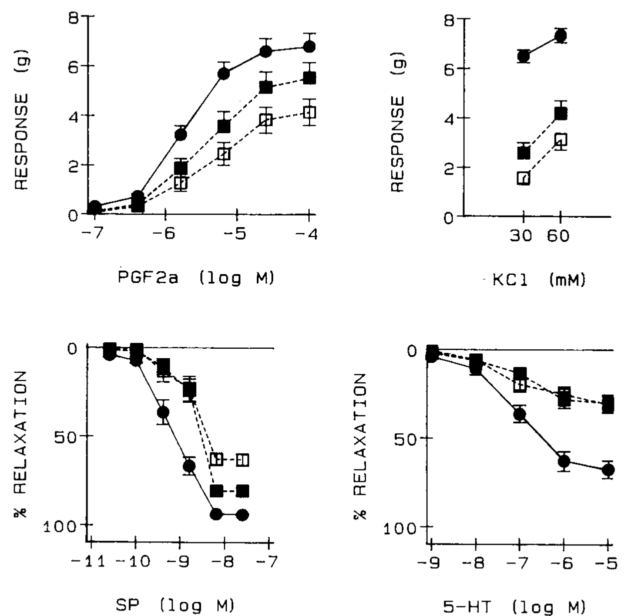

Fig. 2. *Upper traces:* Contractile responses of rings from porcine circumflex coronary arteries to $PGF_{2\alpha}$ (*left*) and HCl (*right*). The effects are expressed in g, the *bars* represent means ± SEM, for each point $n = 9$. *Lower traces:* Relaxant responses of rings from porcine circumflex coronary arteries stimulated with 3 μM $PGF_{2\alpha}$ to substance P (SP, *left*) and 5-HT (*right*). The effects are expressed as percentages of the maximal relaxant response to papaverine, the *bars* represent means ± SEM, for each point $n = 10-12$

Discussion

Cryopreservation has been shown to be a useful method for storing vascular and nonvascular tissues for subsequent pharmacological experiments (Müller-Schweinitzer 1988, 1992). However, though biochemical properties and various affinity parameters are mostly comparable to those determined in fresh tissues, up to now, the cryopresservation technique employed, provides only partial preservation of the smooth muscle contractile functions.

Freezing of living mammalian cells in physiological media without cryoprotective additives generally leads to severe cell membrane damages and only few if any cells survive (Litvan 1972; Mazur 1977; Pegg 1985). There are

two basic mechanisms of freeze injury: (1) damage of the cell membrane by the formation of intra- and extracellular ice crystals and (2) cell damage by osmotic disequilibrium due to concentrated electrolytes during freezing. It is assumed, that penetrating cryoprotectants such as DMSO, by entering the cell, replace some water, thereby limiting the formation of intracellular ice crystals. Nonpenetrating cryoprotectants such as sucrose are suggested to stabilize the cell volume by retaining more liquid water at low temperatures thereby reducing the external electrolyte concentrations (Mazur 1977; Pegg 1985; Brockbank 1989).

The present experiments provide evidence that the effect of a penetrating and a non-penetrating cryoprotectant may be additive, i.e., that protection against cryoinjury of the vascular smooth muscle can be improved by the combined action of DMSO and sucrose. The results show, however, that in both groups of cryopresserved blood vessels after thawing the contractile force of the vascular preparations was considerably reduced when compared to that produced by unfrozen tissues. Not only receptor-mediated contractions but also contractions induced by depolarization during exposure to 60 mM potassium chloride were attenuated suggesting that the diminished contractile force of frozen/thawed vessels reflects a decrease in contractile capacity of the tissues per se. In addition, exposure of unfrozen vascular tissues to DMSO and sucrose at the same concentrations as used for cryopreservation did not change the functional activity, indicating that the reduction of contractile force after cryopreservation was due to the freezing/thawing process and not induced by the cryoprotectants used.

Three different types of vascular smooth muscle have been employed. The most marked sucrose-induced improvement of the functional activity was observed in canine saphenous veins and porcine circumflex coronary arteries, whereas the improvement was considerably less in canine saphenous arteries supporting the suggestion that different cell types differ in their susceptibility to the cryoprotectant and/or to the freezing/thawing procedure. It has also been noted that close packing of cells tends to reduce their survival following cryopreservation (Pegg 1985). It is possible, therefore, that the time allowed for equilibration of the arterial tissues with the cryoprotectants (about 30 to 60 min at room temperature) was insufficient to achieve optimal protection. Nevertheless, our present data demonstrate that addition of sucrose to a DMSO-containing cryomedium may improve the functional activity of vascular smooth muscle cells after thawing.

Acknowledgement. The authors thank Mrs. M. Bretz for her skillful technical assistance.

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