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Freezing without surrounding cryomedium preserves the endothelium and its function in human internal mammary arteries ☆

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Abstract

Purpose: Cryopreserved human blood vessels may become important tools in bypass surgery. Optimal cryopreservation of an arterial graft should, therefore, preserve both histological and physiological characteristics of smooth muscle and endothelium comparable to the unfrozen artery.

Methods: Rings from human internal mammary arteries (IMA) were investigated in vitro either unfrozen or after immersion into a cryomedium (RPMI 1640 containing 1.8 M Me_2SO and 0.1 M sucrose) and cryostorage with and without surrounding medium.

Results: In unfrozen IMA, neither contractile responses to noradrenaline (NA) nor endothelium-dependent relaxant responses to acetylcholine (ACH) was modified after exposure of the IMA to cryomedium or during activation of protein kinase C by phorbol-12,13-dibutyrate (PDBu). Exposure to cryomedium with gradually increasing Me_2SO content before starting the cooling process did not improve the post-thaw functional activity of the artery. Optimal post-thaw recovery of contractile responses to NA and $PGF_{2\alpha}$ was observed after freezing at a speed of −1.2 and −3 °C/min in arteries stored with and without surrounding cryomedium. Compared to unfrozen controls, the ACH-induced endothelium-dependent relaxation during active tone induced by 3 μM $PGF_{2\alpha}$ reached 16 and 56% after freezing with and without surrounding medium. All functional data were reflected by electron microscopy images showing considerably better preservation of the endothelial layer after freezing without medium.

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Conclusion: Freezing of human arteries at a mean cooling rate of −3 °C/min and storage without surrounding medium offers the prospect of optimal preservation of both smooth muscle and endothelial function in cryopreserved human IMA.

Keywords: Human internal mammary arteries; Endothelial function; In vitro; Phorbol-12,13-dibutyrate; Freezing speed; Cryopreservation; Dimethyl sulfoxide; Scanning electron microscopy

Cryopreserved human blood vessels are interesting topics of pharmacological research [13,14,16], and may become important tools in bypass surgery and peripheral vascular reconstruction in patients without sufficient autologous graft material [3,6]. Optimal cryopreservation of an arterial graft should preserve, therefore, both histological and physiological characteristic comparable to the fresh artery. However, after thawing of cryostored human arteries the compliance of these vessels is diminished [13,14] while the sensitivity to contractile stimuli is often enhanced as indicated by a leftward shift of the concentration–response curves to various agonists [17–19]. In previous experiments on human internal mammary arteries, evidence has been presented that cryopreservation facilitates contractile responses by activation of protein kinase C (PKC) which seems to be associated with increased Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels and it has been shown that these changes were indeed related to the freezing and not simply a consequence of exposure to the hypertonic cryomedium [18,19].

The main problem with cryopreserved blood vessels, however, seems to be the reduction of endothelial function observed in various frozen–thawed human arteries [9,10,17,18,26,29]. In addition to its antithrombotic properties, the endothelium plays a central role in regulating smooth muscle tone by the release of vasoactive substances. Hence, preservation of the endothelium and its function during the freezing–thawing procedures plays a crucial role in the mid- and long-term viability of arterial grafts. The recovery of endothelial cells may be influenced by the presence or absence of medium during the freezing–thawing process. First evidence for this has been produced by Fong et al. [5] showing that rabbit cornea was less damaged when frozen without medium. This observation has been confirmed recently in vascular tissue. Using scanning electron microscopy and biochemical assays to measure mitochondrial activity and oxygen consumption of endothelial cell layer in pieces of porcine aorta, it has been shown that the endothelial layer is better preserved when frozen in the absence rather than being surrounded by medium [2].

The aim of the present study was to evaluate the effect of the presence and absence of the cryomedium during cryostorage on both the post-thaw functional activity of the arterial smooth muscle and on the preservation of the endothelium and its function in human internal mammary arteries.

Methods and materials

Harvesting procedure

Internal mammary arteries (IMA, 10–20 mm long, inner diameter ~1–2 mm) were obtained during bypass surgery or excised from multiple organ donors after obtaining informed consent from the patient and permission from the local ethical committee. Arteries from a total of 81 patients (68 male, 13 female) with a mean age of 65 (range 39–82) years were used. After explantation, the preparations were placed in modified Krebs–Henseleit (KH) solution (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.25, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, and EDTA 0.03) at room temperature and transported to the laboratory. The vessels were cleaned of adjacent connective tissue, cut into rings (about 2–3 mm long), and used either immediately unfrozen in organ bath experiments or

randomly distributed to the different study groups for cryopreservation.

Cryopreservation

Cryopreservation in medium

Arterial rings were suspended in 2 ml liquid nitrogen storage vials (Life Technologies AG, Basel, Switzerland) filled with 1.6 ml of RPMI 1640 culture medium containing 1.8 M dimethyl sulfoxide (Me_2SO) and 0.1 M sucrose [15]. After equilibration with the cryomedium at room temperature, the samples were placed in a polystyrol box to be slowly frozen in a freezer maintained at −70 °C. Under these conditions, a mean cooling speed of about 1.2 °C/min was achieved when the vials were placed in a polystyrol box of 10 mm wall thickness whereas in a polystyrol box with 45 mm wall thickness the cooling speed was −0.6 °C/min. About 2 h later, the vials were transferred into liquid nitrogen and stored there at −196 °C until use.

Cryopreservation in air

Arterial rings were equilibrated for 10 min with the cryomedium (RPMI 1640 culture medium containing 1.8 M Me_2SO and 0.1 M sucrose) at room temperature, quickly blotted on a paper tissue to remove surplus medium, and placed into an empty 2 ml cryotube. In these experiments, a mean cooling speed of about −1.2 °C/min was achieved by placing the cryotubes into a polystyrol box with a wall thickness of 45 mm whereas a polystyrol box with 10 mm wall thickness gave a mean cooling speed of −3 °C/min when placed into a freezer maintained at −70 °C. To achieve a cooling rate of −15 °C/min, the vials were covered with dry ice. About 2 h later, the vials were transferred into the vapor phase of liquid nitrogen and stored there at about −190 °C until use.

Gradual increase of Me_2SO content

In some experiments, human IMA were incubated in cryomedia containing gradually increasing concentrations of Me_2SO thereby avoiding any rise in temperature and increased toxic effect of Me_2SO which occur when neat Me_2SO is dropped directly into the solution. In these experiments, the arterial rings were placed either directly into a medium containing 1.8 M Me_2SO (1-step) or the samples were first placed for 7.5 min into medium containing 0.9 M Me_2SO before being transferred for further 7.5 min into 1.8 M Me_2SO cryomedium (2-step) or the samples were placed at 5 min intervals into cryomedia containing 0.6, 1.2, and 1.8 M Me_2SO (3-step). For all samples, the total time of exposure to Me_2SO-containing cryomedium was 15 min.

Thawing

Before use, the vials containing samples frozen in surrounding cryomedium were first placed for 10 min on dry ice before being exposed for 6 min to room temperature and thawed within 4 min in a 40 °C water bath, resulting in a mean thawing rate of 15 °C/min [18]. Thereafter, the arterial rings were placed for 1 min in KH solution containing 50% cryomedium before being transferred into a dish containing 10 ml of KH solution at room temperature to washout the cryoprotecting agents. The samples cryostored in air, i.e., without surrounding cryomedium, were thawed by exposure of the vials for 10 min to room temperature and then suspended for 1 min in KH solution containing 50% cryomedium before being transferred into KH solution. Under these conditions, the mean thawing rate was about 50 °C/min.

Organ bath studies

After thawing, the arterial rings were mounted between two hooks of stainless steel wire (diameter 0.15 mm) and suspended in 10 ml organ baths containing KH solution at 37 °C, gassed continuously with 5% CO_2 in oxygen. Changes in the tone of the preparations were recorded isometrically under a resting tension of 1 g with electromechanical transducers (Statham model UC 3, Gould, Oxnard, CA, USA) and a potentiometric recorder. At the beginning of the experiments, the rings were stretched to an initial tension of about 1.5 g, allowed to relax, and equilibrate for about 90 min in the bathing medium. During this time, the baseline tension of the rings was readjusted to 1 g if required and the vascular preparations were

challenged once with noradrenaline (NA, 1 μM). When the NA effect was leveling off, the presence of functional endothelial cells was tested by the addition of 1 μM acetylcholine (ACH). Concentration–response curves for agonists were determined by cumulative additions of drug, the concentration being increased when the maximum effect had been produced by the previous concentration. When endothelium-dependent responses to ACH were investigated, only arterial rings with reliable endothelial function (i.e., if ACH relaxed the unfrozen control rings by more than 50%) were employed. In these experiments, active tone of the arteries was induced by 3 μM $PGF_{2\alpha}$ which elicited a long-lasting stable contraction of the tissue of about 60% of its maximum. After the concentration–response curves for ACH had been completed, sodium nitroprusside (100 μM) was added to induce complete relaxation of the rings and to ascertain the ability of the smooth muscle to relax in response to direct activation of soluble guanylate cyclase. Since after cryostorage, contractile responses to many agonists are diminished, it was decided to express all relaxant responses to ACH in grams and as percentages.

Temperature measurements

Temperature courses were measured with type du3s of Ellab a-s (Ellab Instruments, Copenhagen, Denmark). Temperature changes were recorded digitally every 15 s.

Scanning electron microscopy

For scanning electron microscopy, 12 arterial rings from four patients were carefully removed from the organ holders after the in vitro experiments were completed, fixed at 4 °C for 24 h in 2.5% PBS-buffered glutaraldehyde (pH 7.4, 300 mOsmol), and then stored in PBS buffer. The rings were cut longitudinally with a razor blade, dehydrated, and critical-point dried with liquid CO_2. The specimens were blinded and mounted on stubs with conductive silver paste, sputtered with 20 A gold in argon atmosphere, and examined in a Philips ESEM XL 30 scanning electron microscope at 20 kV.

Drugs

The following drugs were used: (−)-noradrenaline hydrogen tartrate, PDBu (phorbol-12,13-dibutyrate), sodium nitroprusside, dimethyl sulfoxide (Me_2SO), sucrose (Fluka, Buchs, Switzerland), acetylcholine chloride (Dispersa A.G., Hettlingen, Switzerland), RPMI-1640 culture medium without L-glutamine (Invitrogen Life Technologies, Switzerland), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Dinolytic, Upjohn, Crawley, UK). PDBu was dissolved in Me_2SO to give a 0.1 mM solution containing 1% Me_2SO. Samples of this solution were stored at −20 °C until use. Serial dilutions were performed with physiologic NaCl solution.

Data analysis

Concentration–response curves were analyzed with a computer program in RS/1 (BBN Software Products Corporation, Cambridge, MA, USA) and E_{max} (maximal effects) and pD_2 values (negative logarithm of the molar concentration of the agonists producing 50% of E_{max}) were derived from this analysis. Where appropriate, one-way analysis of variance (ANOVA) was performed, followed by the Bonferroni-corrected t test to assign differences to individual between-group comparisons when overall significance ($P < 0.05$) was attained. Data are presented as means ± SEM.

Results

Experiments on unfrozen IMA

Exposure to cryomedium without freezing

In a first series of experiments, rings from human IMA were exposed for up to 60 min to the cryomedium containing 1.8 M Me_2SO plus 0.1 M sucrose at room temperature without freezing. Following washout of these cryoprotectants neither contractile responses to NA (Fig. 1A) nor those to $PGF_{2\alpha}$ (3 μM) and endothelium-dependent relaxant responses to ACH (Fig. 1B) were significantly modified compared to time-matched controls.

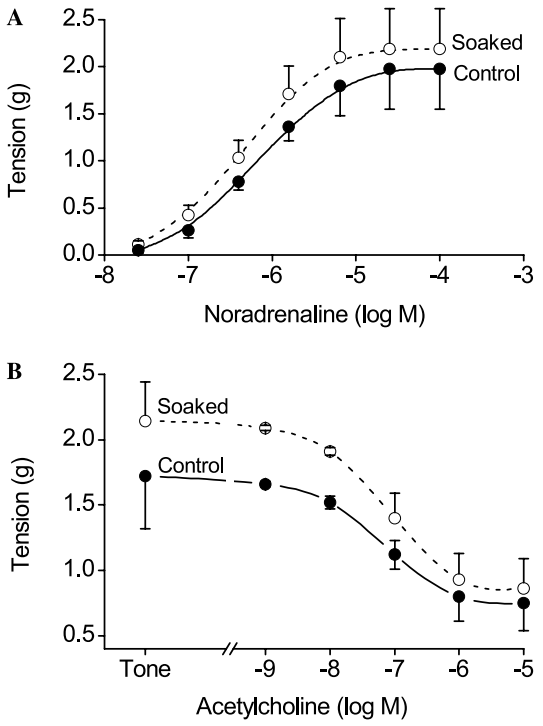

Fig. 1. Cumulative concentration–response curves on rings from unfrozen human IMA without (●) and after exposure to cryomedium (○, RPMI containing 1.8 M Me_2SO and 0.1 M sucrose) for 10 min at room temperature for NA (A) and for ACH (B) during active tone induced by 3 μM $PGF_{2\alpha}$. All responses are expressed in g, the bars represent means ± SEM, for each point $n = 6$.

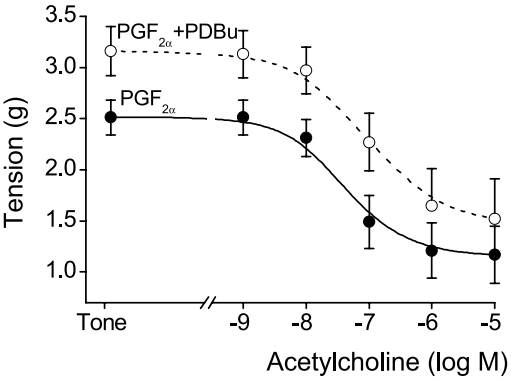

Fig. 2. Cumulative concentration–response curves for ACH on rings from unfrozen human IMA without and during activation of protein kinase C by phorbol ester (PDBu). Active tone was induced by 3 μM $PGF_{2\alpha}$ (●) and by the combined action of 1 μM $PGF_{2\alpha}$ plus 40 nM PDBu (○). Responses are expressed in grams, the bars represent means ± SEM, for each point $n = 5$.

Endothelium-dependent relaxation during activation of protein kinase C

To investigate whether relaxations mediated through endothelium-derived relaxing factors (EDRF) would be inhibited by activation of protein kinase C (PKC), concentration–responses to ACH were determined on rings from IMA during activation of PKC by phorbol-12,13-dibutyrate (PDBu). In these experiments, active tone was induced by $PGF_{2\alpha}$ (3 μM) alone or by the combined action of 1 μM $PGF_{2\alpha}$ plus 40 nM PDBu (Fig. 2). Though the tone induced by the combined action of $PGF_{2\alpha}$ and PDBu (3.2 ± 0.2 g) was significantly stronger than that induced by 3 μM of $PGF_{2\alpha}$ alone (2.5 ± 0.2 g, $P < 0.05$), ACH relaxed rings from both PDBu-treated and control tissues with similar efficacy (1.6 ± 0.4 g vs. 1.3 ± 0.2 g) and potency as indicated by the calculated apparent pD_2 values (7.15 ± 0.16 vs. 7.41 ± 0.07).

Experiments on cryostored IMA

Effect of gradual increase of Me_2SO on contractile responses to NA

In these experiments, arterial rings were placed either directly into the cryomedium or transferred into vials containing medium with gradually higher concentrations of Me_2SO. However, compared to the conventional method, pre-freezing exposure to cryomedia containing gradually increasing concentrations of Me_2SO failed to improve the post-thaw contractile responses to NA (Fig. 3).

Freezing at different cooling rates

In this series of experiments, the arterial samples were placed directly into the cryomedium and equilibrated with the cryomedium for 10–20 min at room temperature before starting the freezing process using different cooling rates to −70 °C followed by storage in liquid nitrogen. After thawing, concentration–response curves to NA revealed that maximal preservation of the contractile function was obtained when arteries were frozen at a mean cooling speed of −1.2 °C/min

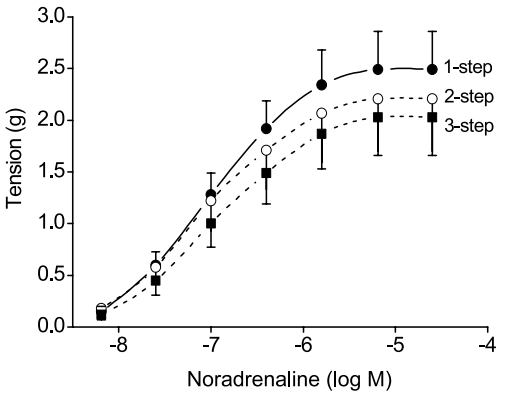

Fig. 3. Cumulative concentration–response curves for NA on human IMA following gradual increase of Me_2SO concentration. Arteries were either placed directly into medium containing 1.8 M Me_2SO (1-step, ●), or arteries were first exposed for 7.5 min to cryomedium containing 0.9 M Me_2SO before being transferred for further 7.5 min into medium containing 1.8 M Me_2SO (2-step, ○), or arteries were exposed at 5 min intervals to media containing 0.45, 0.9, and 1.8 M Me_2SO (3-step, ■). All samples were exposed for totally 15 min to Me_2SO before the cooling process was started. The bars represent means ± SEM, for each point $n = 7$.

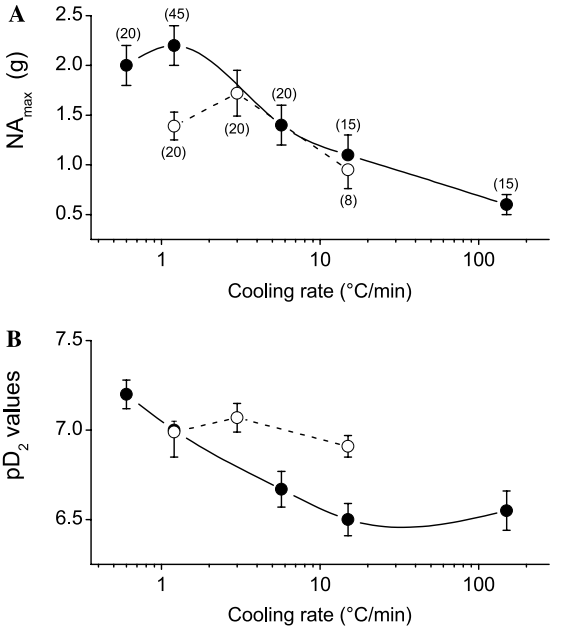

Fig. 4. Parameters calculated for (A) efficacy and (B) potency of NA on human IMA following cryopreservation in medium (●) and in air (○) at different cooling rates. All samples were equilibrated with the cryomedium for 10 min before starting the cooling process. The bars represent means ± SEM, number of determination are given in the figure.

whereas both contractile force (Fig. 4A) and pD_2 values for NA (Fig. 4B) were progressively reduced when faster cooling rates where applied. Removal of the surplus cryomedium before starting the cooling process, i.e., cryostorage of the arteries without surrounding medium, diminished the contractile responses to NA significantly when cooling was performed at −1.2 °C/min. Under these conditions, maximal responses to NA were 2.19 ± 0.18 g ($n = 45$) if frozen in medium and 1.39 ± 0.14 g ($n = 20$, $P < 0.05$) if frozen in air. When arteries were frozen without surrounding cryomedium, maximal preservation of the NA-induced contraction (1.72 ± 0.23 g, $n = 20$) was observed when a mean cooling speed of −3 °C/min was applied and this contractile effect was not different from the mean contractile NA response obtained in the group frozen at a speed of −1.2 °C/min while suspended in medium.

Endothelial function after removal of surplus cryomedium

In this series of experiments, arterial rings were exposed for 10 min to the cryomedium before being slowly frozen at different cooling rates (i.e., at −1.2, −3, and −15 °C/min) to −70 °C and cryostored in the vapor phase of liquid nitrogen without surrounding cryomedium (Fig. 5). As observed with the contractile responses to NA, the degree of active tone induced by $PGF_{2\alpha}$ was also modified by the freezing speed. Again, maximal active tone was obtained in arteries which had been frozen at a speed of −3 °C/min to −70 °C before being stored in the vapor phase of liquid nitrogen and this effect was not significantly different from that obtained with unfrozen controls. Furthermore, the endothelium-dependent relaxant responses to ACH, though significantly weaker than in unfrozen controls, were best preserved in the group of arteries which had been frozen at a speed of −3 °C/min compared to the other two cryostored groups. While the calculated apparent pD_2 values indicated, that after cryopreservation in air at a cooling rate of −1.2 and −3 °C/min the potency of ACH was unchanged, the

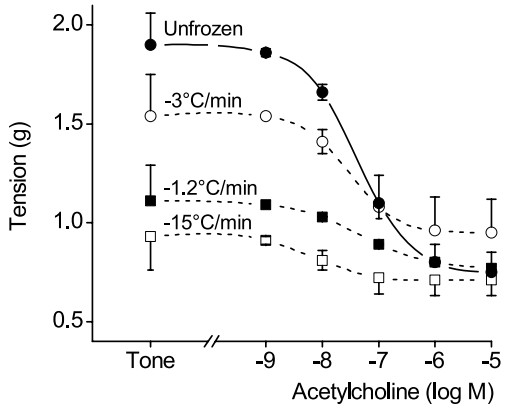

Fig. 5. Cumulative concentration–response curves for ACH during stimulation with 3 μM $PGF_{2\alpha}$ on human IMA following freezing at different cooling rates and cryopreservation without surrounding medium. The samples were equilibrated with the cryomedium for 10 min at room temperature before starting the cooling process at −1.2 °C/min (■- - -■, $n = 9$), −3.0 °C/ min (○- - -○, $n = 9$), and −15 °C/min (□- - -□, $n = 7$). In these experiments, the samples were stored in the vapor phase of liquid nitrogen (−190 °C), the bars represent means ± SEM.

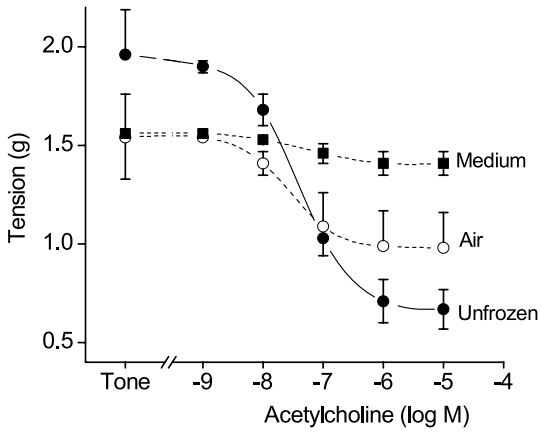

Fig. 6. Cumulative concentration–response curves on human IMA for ACH during stimulation with 3 μM $PGF_{2\alpha}$ after cryopreservation in medium and in air. Before starting the cooling process, all samples were equilibrated with the cryomedium for 10 min at room temperature. Responses to ACH were expressed in grams, for each point $n = 9$, the bars represent means ± SEM.

group of arteries which had been frozen at a speed of −15 °C/min appeared to be four times more sensitive to stimulation by ACH than the other groups. This phenomenon, however, might have been artificial and due to the extremely weak active tone, as the ACH curve, though starting at the same concentration range, reached its maximum already at 2 log units lower concentrations than in the other groups (Fig. 5, Table 1). Direct comparison of the post-thaw endothelium-dependent responses to ACH after cryostorage in air to those after cryostorage in medium is illustrated in Fig. 6.

Arterial rings from the same patients were randomly divided into three groups to be investigated either unfrozen, frozen at a speed of −3 °C/min, and stored without surrounding medium or frozen at a speed of −1.2 °C/min while surrounded by cryomedium. Compared to that observed with unfrozen arteries cryopreservation attenuated the active tone produced by $PGF_{2\alpha}$ in both cryopreserved groups to the same extent. However, removal of the surplus medium before starting the cooling process resulted in a more than 3-fold improvement of the endothelium-dependent relaxation to ACH (Table 2).

Table 1
Parameters calculated for PGF2α-induced active tone and ACH-mediated relaxation in human IMA after freezing at different cooling rates and thawing at room temperature

	n	$PGF_{2\alpha}$-induced active tone (g)	ACH-induced relaxation (g)	ACH-induced relaxation (% of tone)	pD_2 values for ACH
Unfrozen	20	1.90 ± 0.16	1.20 ± 0.09	66 ± 2	7.35 ± 0.08
Cooling rate					
−1.2 °C/min	9	1.05 ± 0.18*	0.32 ± 0.11**	31 ± 7**	7.37 ± 0.23
−3 °C/min	9	1.54 ± 0.21	0.56 ± 0.18*	38 ± 11*	7.31 ± 0.11
−15 °C/min	7	0.93 ± 0.17*	0.22 ± 0.08**	24 ± 6**	7.98 ± 0.10*

Data are presented as means ± SEM.
Difference from unfrozen controls significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.

Table 2
Parameters calculated for $PGF_{2\alpha}$-induced active tone and ACH-mediated relaxation on human IMA after freezing with solution and without surrounding solution in air

	$PGF_{2\alpha}$-induced active tone (g)	ACH-induced relaxation (g)	ACH-induced relaxation (% of tone)	pD_2 values for ACH
Unfrozen	1.96 ± 0.23	1.29 ± 0.10	68 ± 3	7.46 ± 0.14
Cryostored in air	$1.54 \pm 0.21^{*}$	$0.56 \pm 0.18^{*}$	$38 \pm 11^{*}$	7.31 ± 0.11
Cryostored in solution	$1.56 \pm 0.20^{*}$	$0.17 \pm 0.06^{**}$	$11 \pm 2^{**}$	$6.74 \pm 0.23^{*}$
Difference between cryostored groups	n.s.	$P = 0.058$	$P < 0.05$	$P < 0.05$

Data are presented as means ± SEM, for each group $n = 9$.
Difference from unfrozen controls significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.

Scanning electron microscopy

In control arteries, the endothelial layer was well preserved. Some spikes and blebs indicative for cell cytoskeleton damage could be noticed but with the exception of a few spots the integrity of the layer was complete (Figs. 7A and B). After exposure to the Me_2SO-containing cryomedium without freezing, the endothelial cells appeared slightly flattened, but the integrity of the layer was complete (Figs. 7C and D). Freezing without surrounding medium resulted in well-preserved endothelium with neglectable endothelial cell loss (Figs. 7E and F). When frozen in medium flaking became more apparent and exfoliation of the endothelium was significant (Figs. 7G and H). Thus, preservation of the endothelial layer was markedly better in samples cryopreserved in the absence rather than being surrounded by medium.

Discussion

The most relevant findings of the present experiments on human IMA were the observations (1) that in unfrozen human IMA the endothelium-dependent relaxation was unchanged by activation of protein kinase C (PKC) and (2) that during cryopreservation the endothelium and its function could be largely preserved if the arterial rings were only equilibrated with the cryomedium and frozen without surrounding medium.

In accordance with earlier reports [18], the present data confirmed that both contractile responses to NA and endothelium-dependent relaxations to ACH were unchanged after exposure of arterial tissues to the Me_2SO-containing cryomedium without subsequent freezing. These functional data were also in line with electron microscopy examinations showing only minimal alterations of the endothelium in unfrozen human IMA which had been exposed for 10 min to the cryomedium. Our present observations are also consistent with reports on animal tissues showing a rather low proportion of damaged endothelial cells in pig aortae [2] and only some loss of cell–cell junctions among the endothelial cells from minipig iliac arteries after incubation with a Me_2SO-containing medium without subsequent freezing [22].

In previous studies, evidence has been presented that the freezing–thawing procedure besides increasing calcium-influx into the smooth muscle cell enhances the activity of protein kinase C (PKC) in human IMA [18,19]. Experiments on various unfrozen animal arteries have shown that activation of PKC inhibits endothelium-dependent relaxations by depression of both synthesis–release and action of EDRF on smooth muscle [11,12,25]. It could have been possible, therefore, that the post-thaw loss of endothelial function of cryostored human IMA was due to an activation of PKC. However, unchanged relaxant responses to ACH of unfrozen human IMA during activation of PKC by PDBu (phorbol-12,13-dibutyrate) demonstrated that in human IMA synthesis–release and action of ERDF is not depressed by activation of PKC. Whilst these findings indicated marked species and/or organ differences, they also suggested that the diminished endothelium-dependent relaxant responses of frozen–thawed human IMA in the present study was not the consequence of PKC activation. These results suggested, therefore,

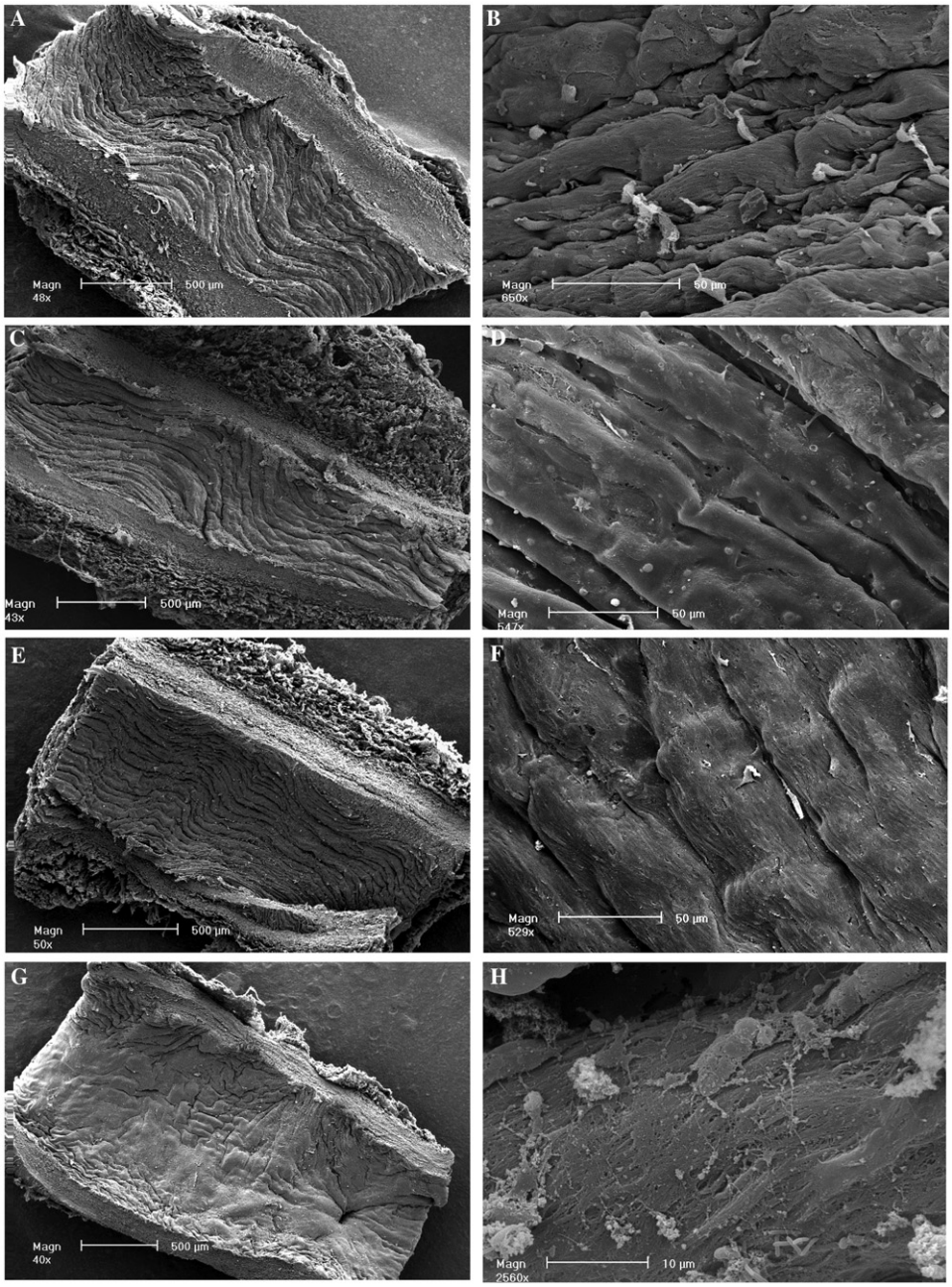

Fig. 7. Scanning electron micrographs of the endothelium of human IMAs investigated after the organ bath experiments at different magnifications (see bars). The micrographs represent typical images of an unfrozen artery without (A and B) and after exposure to the Me_2SO-containing cryomedium (C and D), and after cryopreservation without (E and F) and with surrounding cryomedium (G and H). Unfrozen arteries are well covered with endothelial cells (A,C). Some depositions of blood cells and noncellular material are seen (B). After exposure of arteries to the Me_2SO-containing cryomedium without freezing cytoskeletal alterations as indicated by spikes and blebs are seen more frequently (D). After freezing without surrounding cryomedium, the endothelial layer seemed largely well preserved (E and F) whereas freezing with surrounding cryomedium induced marked changes of the endothelium with oedema (G). Areas of de-endothelialization and exposure of the subendothelial surface were most extensive in samples which had been frozen with surrounding cryomedium (H).

that in human IMA neither exposure to the Me_2SO-containing medium nor activated PKC were involved in the mechanism(s) responsible for the post-thaw diminished EDRF effect on smooth muscle in cryostored human IMA.

To keep cell shrinkage within safe limits, eventually the cryoprotectant Me_2SO is added gradually over a period of 20–30 min. This procedure can be successful with isolated cells where permeability characteristics of the particular cells to both water and the cryoprotectant can be determined [30] and taken into consideration. Multiple cell systems such as blood vessels, however, contain many diverse types of cells and respond differently to freezing stress than isolated cells. In such complex cell systems, each cell type requires differing requirements for optimal preservation [1]. Stepwise addition of the cryoprotectant has also been applied for cryopreservation of human femoral [26] and minipig iliac arteries [22] but experimental evidence for the beneficial effect of a stepwise addition of the cryoprotectant for cryopreservation of tissues such as blood vessels is lacking. Indeed, in our present study pre-freezing exposure of the arterial rings to cryomedia containing gradually increasing Me_2SO concentrations at room temperature failed to improve the post-thaw contractile responses to NA of human IMA compared to controls which had been immersed directly into the cryomedium containing the final Me_2SO concentration. Therefore, in the present experiments all samples of human IMA were placed directly into the cryomedium.

When samples were cryostored without surrounding medium, the optimal cooling speed for preservation of contractile function was about −3 °C/min. However, maximal contractile responses to NA of human IMAs which had been cryostored under these conditions were generally diminished compared to those of arteries cryostored in medium. Since in these experiments mainly arteries with no or only negligible endothelial function were employed, a modification of the contractile responses by EDRF seemed unlikely. It might be possible rather, that this phenomenon was due to the different thawing procedures applied. While with the group of arteries stored in medium thawing was performed according to the 3-step method giving a mean thawing rate of 15 °C/min [18], the group of arteries stored without medium was thawed by exposure of the vial for 10 min to room temperature resulting in a three-times faster thawing rate. Previous experiments have demonstrated that slow thawing at about 15 °C/min of human IMA which had been cryostored in surrounding medium improved the post-thaw contractile responsiveness to NA compared to rapid thawing [18]. It might be possible, therefore, that a slower thawing protocol should contribute to improve the post-thaw contractile activity after cryostorage in air as well. Further experiments are required to clarify this phenomenon.

In most publications, relaxant responses are usually expressed as percentages of a contractile effect. This method, however, may lead to false conclusions since after cryostorage contractile responses to many agonists are markedly diminished. In human IMA this is especially true for NA and KCl, the maximal contractions of which are significantly diminished after cryostorage compared to unfrozen controls [18–20]. The same applies for other human arteries such as coronary [9,10,17], mesenteric [17], and femoral arteries [26–28]. Therefore, in the present study all relaxant responses to ACH as well as those to contractile agonists were evaluated in g.

In accordance with earlier findings [18], the endothelium-dependent relaxant responses to ACH of human IMA were significantly diminished when arteries had been cryostored with Me_2SO-containing medium surrounding the sample. By contrast, there was a significant improvement of the relaxant responses to ACH when the surplus cryomedium was removed before starting the cooling process, indicating improved preservation of the endothelial function. It is not possible to say whether this improvement should be ascribed to the different thawing rates applied in both groups or to the removal of the surplus medium before freezing or merely to the fact that the samples frozen without surrounding medium were stored in the vapor phase of liquid nitrogen.

Rapidly thawed carotid arteries from rabbits show a high incidence of circumferential fractures. Moreover, the rate of fracturing is not affected by

the presence or absence of surrounding medium if rapid thawing is applied [8]. These fractures occur when during rewarming the temperature range of −150 to −100 °C is transversed and are prevented when warming to −100 °C is performed at a slow rate [23]. Similar changes may be induced by rapid thawing of minipig iliac arteries. These arteries also undergo a high incidence of fractures and microfractures, and show accumulation of liquid in the subelastica, and increased expression of wall-degradative enzymes [4]. Such changes lead to severe damages of both the extracellular matrix and the living cells, and affect the post-thaw function of the tissue. Indeed, contractile responses of human IMA to NA have been shown to be considerably better preserved when a slow thawing rate is applied [18]. It might be possible, therefore, that a slower thawing would also contribute to improve the post-thaw endothelial function. Morphologic evidence for this has been provided by microscopic examinations of the endothelial surface of frozen–thawed pig iliac arteries and by a modified TUNEL technique detecting damaged endothelial cells. In those studies, a slow thawing at 1 °C/min resulted in markedly improved morphological features of the endothelial surface and a lower proportion of damaged endothelial cell compared to rapidly thawed arteries [4,21,22].

On the other hand, though rapidly thawed, corneae from rabbits exhibit considerably less ultrastructural damage when cryopreservation is performed without medium, i.e., in air. While after freezing of the cornea in medium the endothelium is generally detached, it appears to be firmly attached to Descement's membrane after cryostorage in air [5]. In addition, electron microscopic examinations of pieces from rapidly thawed pig aorta demonstrate that the endothelial layer is better preserved after freezing in air whereas flaking becomes more apparent after freezing in medium [2]. One contributory factor to the better preservation of endothelial layers in the absence of medium might be the observation that freezing in air leads to the formation of numerous small ice crystals throughout the tissue whereas freezing of a tissue immersed in medium gives rise to fewer but much larger ice crystals which may disrupt cell-to-cell contacts [7].

Finally, it might be possible, that the improved endothelial function of IMAs which had been cryostored without medium was due merely to the fact that these samples were stored in the vapor phase of liquid nitrogen. It never can be ruled out completely that during freezing liquid nitrogen enters the cryovials and causes direct damage of the tissue. Indeed, slow freezing with and without surrounding medium followed by storage in the vapor phase of liquid nitrogen has been reported to reveal good preservation of the endothelial function after slow thawing of femoral arteries from organ donors [27,28] and of human IMA even after rapid thawing [24].

In the present study, functional data determined in organ bath experiments were confirmed by scanning electron microscopy. Electron micrographs of the IMA after cryostorage in surrounding medium revealed significant morphological alteration, i.e., flattening of the endothelial cells with loss of large numbers of cells and exfoliation of the endothelial layer, supporting the weak endothelium-dependent relaxation in response to ACH of this group. In contrast, the endothelial layer was considerably better preserved when arterial samples were frozen without medium confirming the significantly better preservation of the endothelial function after cryostorage of IMAs in air.

In summary, electron microscopy and functional data indicated that in cryopreserved human IMA the endothelial function may be considerably better preserved if the cryomedium is removed before starting the cooling process and the arteries are stored in the vapor phase of liquid nitrogen without surrounding medium.

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