Functional recovery of human mesenteric and coronary arteries after cryopreservation at −196° C in a serum-free medium

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Purpose: Long-term patency of cryopreserved vascular grafts is determined by maintained cellular and tissue viability, which implies preservation of various biochemical, smooth muscle, and endothelial functions. Therefore, it was investigated whether the presence of fetal calf serum (FCS) in the cryomedium improves the postthaw contractile and endothelial function of human arteries.

Methods: Rings from human mesenteric (HMA) and left circumflex coronary arteries (HCA) obtained from organ donors were randomized into three groups and studied either unfrozen or after storage for 3 to 6 weeks at −196° C while suspended in Krebs-Henseleit solution without or with 20% FCS as the vehicles and 1.8 mol/L dimethyl sulfoxide and 0.1 mol/L sucrose as cryoprotecting agents. The samples were slowly frozen to −70° C and then stored in liquid nitrogen. Before use, the tissues were thawed within 3 minutes in a 40° C water bath.

Results: After thawing the sensitivity to various agonists and maximal responses to the endothelium-independent relaxing agent sodium nitroprusside were unchanged. However, after cryopreservation of HMA was performed without and with FCS, maximal contractile responses to noradrenaline were significantly reduced to 10.1 ± 0.7 gm and 9.9 ± 0.9 gm compared with 13.3 ± 0.6 gm in unfrozen HMA (mean ± SEM, n = 15). After cryopreservation of HCA was performed without and with FCS, maximal contractile responses to prostaglandin $F_{2\alpha}$ (6.9 ± 0.4 gm in unfrozen HCA) were significantly reduced to 4.3 ± 0.3 gm and 3.8 ± 0.2 gm (mean ± SEM, n = 6). In both types of arteries cryopreservation also attenuated significantly the endothelium-dependent relaxant responses to bradykinin during U46619 (10 nmol/L)-induced tone. In HMA the maximal bradykinin-induced relaxation (85% ± 4%) was significantly diminished to 29% ± 7% and 38% ± 9% after cryopreservation without and with FCS (mean ± SEM, n = 6). In HCA maximal bradykinin-induced relaxation (88% ± 4%) was significantly diminished to 26% ± 10% and 36% ± 11% after cryopreservation without and with FCS (mean ± SEM, n = 6). This result was reflected by a marked endothelial denudation in all groups of cryopreserved arteries. Neither functional nor morphologic preservation of the endothelial cell lining was significantly improved by FCS supplementation of the cryomedium.

Conclusions: Cryopreservation diminished contractile and endothelium-dependent relaxant responses of human arteries. The presence of FCS in the cryomedium did not modify these changes. (J Vasc Surg 1997;25:743-50.)

Cryopreserved human blood vessels are being used in patients requiring peripheral or coronary bypass grafting[1-4] and for various pharmacologic investigations in drug development.[5,6] Ideally for both purposes preservation of all vascular cell types should be guaranteed. Although during the last few years the technique of cryopreservation of isolated tissues in liquid nitrogen (at −196° C) has been improved

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and shown to be effective in preserving various biochemical and smooth muscle cell functions, significant preservation of the endothelial cell lining during the freezing/thawing procedure is still unsatisfactory. Today two types of blood vessels, the saphenous vein and the internal mammary artery, are the most commonly used autogenous grafts in reconstructive peripheral vascular and coronary bypass surgery. However, the patency rates of coronary bypass vein grafts may be limited by complications such as vasospasms,[7-10] and the evidence suggests that especially in coronary revascularization maintained endothelial function is one of the most important determinants of patency rates of the graft material.[11-13] Arteries generally exhibit not only different response profiles to endogenous vasoactive substances but also a more pronounced production of endothelium-derived relaxing factor(s) than do veins.[12-16] Therefore, cryopreserved arteries might be potential alternatives when autogenous conduits are not available. Comparative studies on canine coronary arteries revealed significant improvement of the postthaw endothelium-dependent relaxant responses when a serum-supplemented cryomedium was used.[17] In contrast, no improvement of the postthaw endothelial function could be demonstrated with porcine coronary arteries after cryostorage in a cryomedium containing 20% fetal calf serum (FCS),[5,6] indicating the existence of considerable species differences.

The purpose of this study was therefore to investigate whether a serum-supplemented cryomedium could improve the preservation of arterial endothelial cell lining along with its function in arteries of human origin.

MATERIAL AND METHODS

Tissue preparation. Samples of human mesenteric and circumflex coronary arteries were taken from five organ donors (three male, two female; 18 to 65 years old; mean age, 37.4 ± 8.5 years) after permission was obtained from the local ethical committee. The tissues were placed in Krebs-Henseleit (KH) solution (composition mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, ethylenediamine tetraacetic acid 0.03) at room temperature, pH 7.4, and transported to the laboratory. Within 12 hours after explantation coronary and mesenteric arteries (outer diameter ≈0.8 to 1 mm) were removed, cleaned of surrounding tissue, cut into rings (approximately 2 mm long), and randomized into three groups for investigations in vitro. The samples of "unfrozen arteries," group 1, were used immediately or after storage overnight at 4° C for organ bath studies. The arteries of the other two groups were frozen as described in the following text.

Cryopreservation. For cryopreservation the tissues were placed in 2 ml liquid nitrogen storage ampoules (Gibco AG, Basel, Switzerland) containing KH solution at room temperature either without (group 2) or with 20% FCS (group 3) as the vehicles and 1.8 mol/L dimethyl sulfoxide (Me_2SO) and 0.1 mol/L sucrose as the cryoprotecting agents. Within 10 minutes afterwards the ampoules were placed in a polystyrene box (11 × 11 × 22 cm) and were slowly frozen at a mean cooling rate of approximately 1.3° C/min in a freezer maintained at −70° C. After 2 to 3 hours the ampoules were transferred into liquid nitrogen (−196° C), where they were stored for 3 to 6 weeks until use. Before use the tissues were thawed rapidly within 3 minutes by placing the ampoules in a 40° C water bath. Thereafter the preparations were rinsed in a dish containing KH solution at room temperature and suspended in 10 ml organ baths for isometric tension recording.

Organ bath studies. The arterial rings were mounted between two hooks of stainless steel wire (diameter, 0.15 mm), suspended in 10 ml organ baths containing KH solution at 37° C, pH 7.4, and gassed continuously with 5% CO_2 in oxygen. The tension of the rings was recorded isometrically under a resting tension of 1 gm with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. At the beginning of the experiments the rings were stretched to an initial tension of 2 gm and allowed to relax and equilibrate for approximately 3 hours in the bathing medium, which was changed every 15 minutes. During this time the mesenteric and coronary arteries were challenged once with a submaximal concentration of noradrenaline (1 μmol/L) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μmol/L) respectively, and the preload of the rings was repeatedly readjusted until a stable baseline tension of 1 gm was achieved. Concentration-response curves for agonists were determined by cumulative additions, the concentration being increased when the maximum effect had been produced by the previous concentration. In some experiments indomethacin (1 μmol/L) was added 20 minutes before the first administration of the agonist to block endogenous prostaglandin synthesis. When relaxant responses were investigated, active tone was induced by addition of 10 μmol/L of the thromboxane analog U46619 (9,11-dideoxy-11α, 9α-epoxymethano-$PGF_{2\alpha}$), a concentration producing approximately 50% of maximal response. After these concentration-response curves were completed, sodium nitroprusside (SNP, 100 μmol/L) was added to induce com-

Table I. Parameters calculated for the contractile and relaxant responses of human mesenteric arteries in vitro

	n	*Fresh artery*	*Frozen in KH solution*	*Frozen in KH +20% FCS*
Potency (pD_2 values)				
Noradrenaline	15	6.06 ± 0.03	6.31 ± 0.06	6.20 ± 0.09
$PGF_{2\alpha}$	6	5.78 ± 0.13	5.82 ± 0.07	5.66 ± 0.07
$PGF_{2\alpha}$-Indomethacin	6	6.27 ± 0.13	6.12 ± 0.11	5.98 ± 0.12
Sodium nitroprusside	7	6.72 ± 0.11	6.97 ± 0.22	7.07 ± 0.16
Bradykinin	6	8.72 ± 0.18	7.95 ± 0.26*	7.88 ± 0.28*
Substance P	6	9.12 ± 0.11	9.09 ± 0.25	8.99 ± 0.22
Efficacy (E_{max})				
Noradrenaline (gm)	15	13.3 ± 0.6	10.1 ± 0.7*	9.9 ± 0.9*
$PGF_{2\alpha}$ (gm)	6	8.8 ± 1.6	8.7 ± 1.6	8.1 ± 0.7
$PGF_{2\alpha}$-Indomethacin (gm)	6	11.8 ± 1.7	6.7 ± 1.4*	8.6 ± 0.5
U46619, 10 nmol/L (gm)	19	3.7 ± 0.6	5.1 ± 0.8	3.9 ± 0.8
Sodium nitroprusside (%)	7	−50 ± 3	−65 ± 9	−62 ± 7
Bradykinin (% SNP)	6	−85 ± 4	−29 ± 7*	−38 ± 9*
Substance P (% SNP)	6	−70 ± 4	−15 ± 4*	−16 ± 4*

Data are presented as mean ± SEM.
*Significant difference against values in fresh arteries.

plete relaxation of the arterial rings. This effect was taken as 100% relaxation. On each arterial ring only one contracting agonist was tested followed by investigation of one relaxant compound.

Histologic examinations. After each functional study was completed, the rings were carefully removed from the hooks, fixed in 10% formalin, dehydrated, and embedded in paraffin. Each arterial sample was divided into two segments, and cross-sections were investigated. Five micrometer-thick paraffin sections were cut and stained with hematoxylin-eosin (HE) for semiquantitative assessment by light microscopy. On the basis of endothelial cells lining the inner surface of the examined arterial rings, the degree of endothelial denudation was graded on a scale of 0 (endothelial cells completely lacking) to 4 (endothelial cell lining completely preserved).

Drugs. The following pharmacologic agents were used: bradykinin triacetate, indomethacin, noradrenaline hydrochloride, U46619 (9,11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$), sodium nitroprusside (Sigma, Munich, Germany), Substance P acetate (Bachem, Bubendorf, Switzerland), and $PGF_{2\alpha}$ (Dinolytic, Provet, Lyssach, Switzerland). A stock solution (10 mmol/L) of indomethacin was prepared with 0.1 mol/L NaOH solution. U46619 was dissolved in ethanol and diluted to give a 1 mmol/L solution containing 60% ethanol. Further dilutions were performed in 5% glucose or normal saline solution. Samples of U46619 (1 μmol/L) and indomethacin (0.1 mmol/L) were stored at −20° C until use. All other compounds were dissolved in saline solution just before use.

Data analysis. Concentration-response curves were analyzed with a computer program in RS/1 (BBN Software Products Corporation, Cambridge, Mass.), and E_{max} (maximal effects) and pD_2 values (negative logarithm of the molar agonist concentration producing 50% of E_{max}) were derived from this analysis. 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 mean ± SEM. In each series of experiments arteries from two organ donors were used. The n values in the Figure legends and Tables refer to the number of arterial rings used.

RESULTS

Functional investigations

Mesenteric arteries. After thawing contractile responses to noradrenaline of cryopreserved human mesenteric arteries occurred within the same concentration ranges as in unfrozen tissues; however, in both groups of cryopreserved arteries maximal contractile responses to the amine proved to be significantly diminished by 25% (Table I, Fig. 1). In contrast, contractile responses to 10 nmol/L U46619 (a concentration producing approximately 50% of maximal effect) appeared unchanged when tested after cryopreservation (Table I). The same was true for $PGF_{2\alpha}$. After cryopreservation pD_2 values and maximal responses to $PGF_{2\alpha}$ appeared to be similar to those obtained with unfrozen control tissues (Table I, Fig. 2, *top*). However, after blockade of prosta-

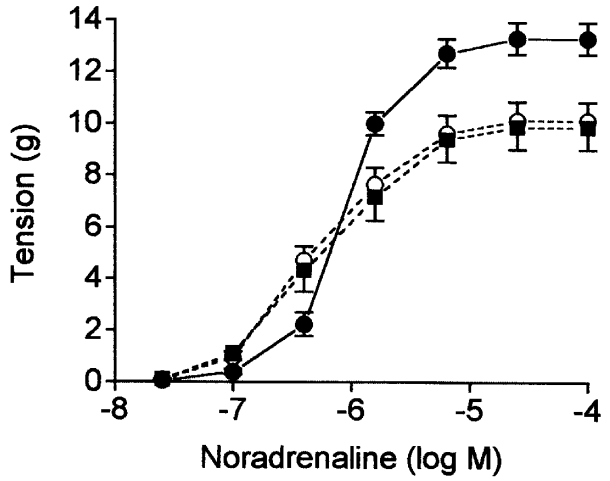

Fig. 1. Cumulative concentration-response curves to noradrenaline on rings from human mesenteric arteries unfrozen *(solid circles)* and after cryopreservation in KH solution without *(empty circles)* and with 20% fetal calf serum *(solid squares)*. Points represent means from 15 individual experiments; *bars* represent mean values ± SEM.

glandin synthetase by 1 μmol/L indomethacin was performed, the postthaw maximal responses to $PGF_{2\alpha}$ were reduced by 43% and 27% in mesenteric arteries, which had been frozen without and with FCS, respectively (Table I, Fig. 2, *bottom*).

Sodium nitroprusside was used to investigate endothelium-independent relaxant responses of arterial rings during active tone induced by the addition of 10 nmol/L U46619. In this series of experiments the effects were expressed as percentages of the active tone induced by U46619 in addition to the existing passive preload. After cryopreservation the relaxant effects of sodium nitroprusside appeared to be rather enhanced compared with the effects obtained with unfrozen control preparations; the differences were, however, not significant (Table I, Fig. 3, *top*). To investigate endothelium-dependent relaxant responses, bradykinin and substance P were used. Compared with unfrozen control rings the relaxant effects of both compounds were markedly diminished when investigated after thawing of cryostored arteries (Table I). Taking the effects on unfrozen control tissues as 100%, bradykinin relaxed precontracted rings of mesenteric arteries that had been frozen in media without and with FCS by 34% ± 8% and 45% ± 11%, respectively (Fig. 3, *middle*). The same was true for the effects of substance P, which relaxed the arterial rings by 21% ± 6% and 23% ± 6% after cryostorage without and with FCS, respectively (Fig. 3, *bottom*).

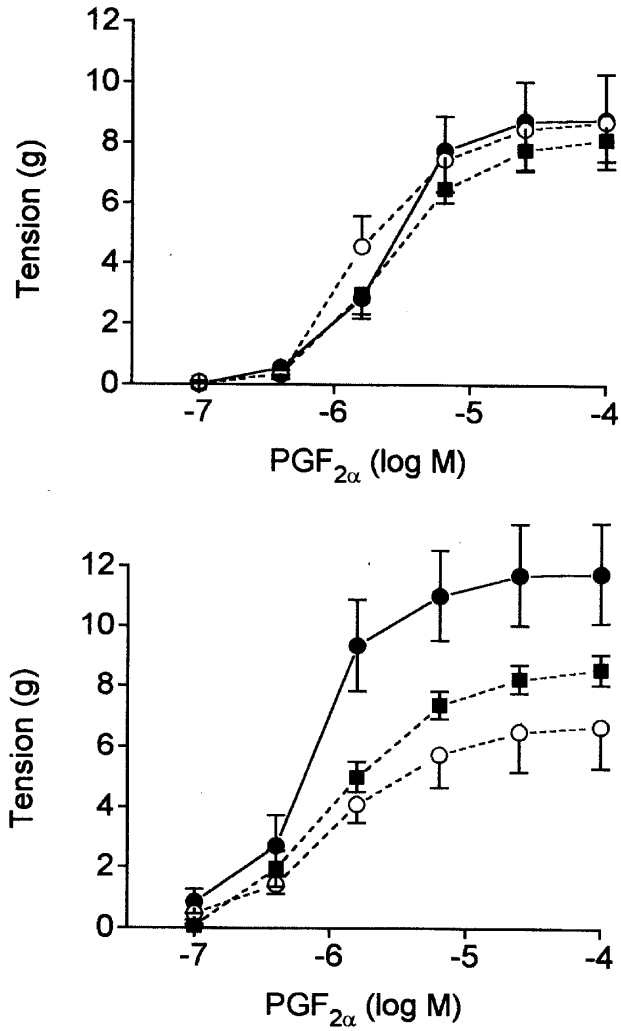

Fig. 2. Cumulative concentration-response curves on rings from human mesenteric arteries unfrozen *(solid circles)* and after cryopreservation in KH solution without *(empty circles)* and with 20% fetal calf serum *(solid squares)* for $PGF_{2\alpha}$ without *(top)* and in presence of 1 μmol/L indomethacin *(bottom)*. Effects are expressed in grams; points represent mean values from six individual experiments. *Bars* represent mean values ± SEM.

Coronary arteries. As observed with mesenteric arteries, the postthaw contractile responses of coronary arteries to U46619 (10 nmol/L) were unchanged (Table II). On the other hand, after thawing maximal responses to $PGF_{2\alpha}$ proved to be significantly diminished by 38% ± 4% and 45% ± 3% in rings cryopreserved without and with FCS, respectively, although there was no significant change in the pD_2 values (Table II, Fig. 4, *top*). Endothelium-independent relaxant responses to sodium nitroprusside occurred with unchanged maxima, but 4 and 5 times higher concentrations of the compound were

required to produce the 50% effect (Table II, Fig. 4, *middle*). In contrast, endothelium-dependent relaxant responses to bradykinin were markedly diminished by 70% ± 11% and 59% ± 13% in coronary arteries that had been frozen without and with FCS, and again significantly higher concentrations of bradykinin were required to elicit these effects (Table II, Fig. 4, *bottom*).

Morphologic examinations

A total of 30 mesenteric and 16 coronary ring segments underwent histologic examination after the in vitro experiments were terminated. Generally, cryopreservation of the arterial segments did not change the morphologic appearance of the medial layer in these tissues. However, there were considerable differences in the preservation of the endothelial lining. Semiquantitative assessment of the endothelium revealed marked endothelial denudation in rings from both mesenteric and coronary arteries that had been frozen without and with serum supplementation compared with unfrozen preparations. The endothelium of frozen/thawed coronary arteries appeared to be somewhat better preserved than that of mesenteric arteries; however, after cryopreservation generally at least 50% of the endothelium was detached or removed (Table III), and there was no improvement if arteries had been cryopreserved in a serum-supplemented medium.

DISCUSSION

The aim of this study was to investigate whether cryopreservation of human arteries in a serum-supplemented medium improves the postthaw functional recovery of smooth muscle and endothelial cells. The effectiveness of two cryomedia, KH solution without and with 20% FCS, was compared. Both media contained dimethylsulfoxide and sucrose, the combined action of which has been shown to give optimal protection against cryoinjury in various animal blood vessels.[18] Furthermore, based on the observation that prolonged exposure of canine femoral arteries to a dimethyl sulfoxide–containing medium may result in progressive reduction of the postthaw functional recovery,[19] a relatively short incubation time of only 10 minutes at room temperature was allowed to equilibrate the tissues with the cryomedia before the cooling process was started. With both media the postthaw maximal responses of human mesenteric arteries to noradrenaline, which are mediated predominantly by α_1-adrenoceptors,[20] proved to be reduced compared with responses obtained with unfrozen control tissues, but there was no significant difference between the contractile force developed by rings that had been cryopreserved without or with serum-supplementation of the medium.

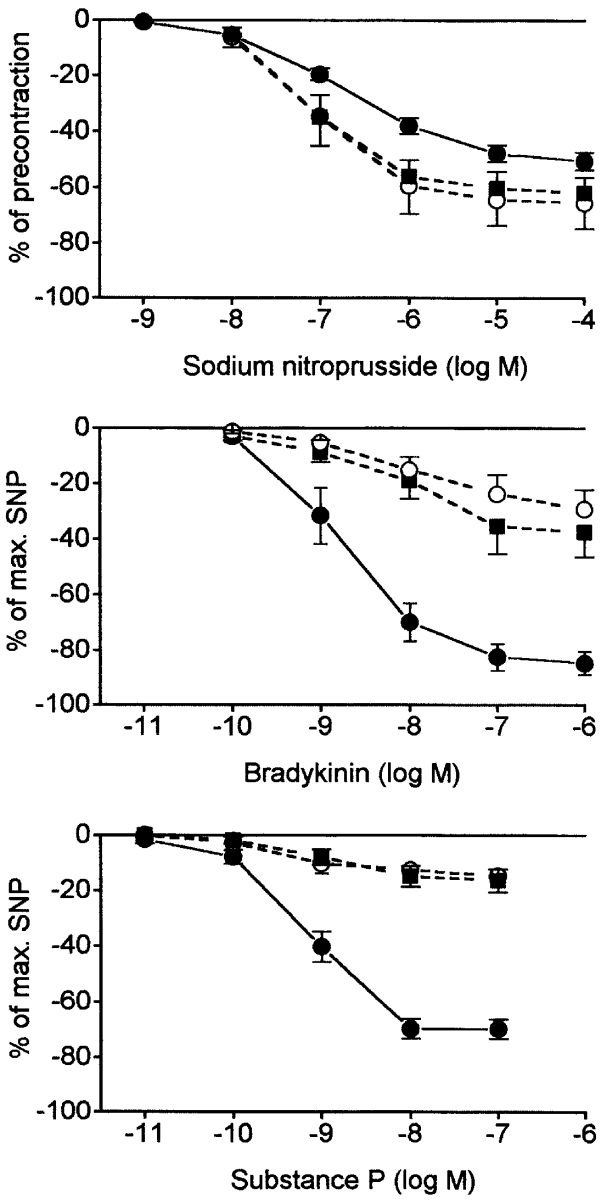

Fig. 3. Cumulative concentration-response curves on rings from human mesenteric arteries, unfrozen *(solid circles)* and after cryopreservation in KH solution without *(empty circles)* and with 20% fetal calf serum *(solid squares)* during active tone induced by U46619 (10 nmol/L) for sodium nitroprusside (*top*, for each point n = 7), for bradykinin (*middle*, for each point n = 6), and for substance P (*bottom*, for each point n = 6), expressed as percentage of maximal relaxation induced by 100 μmol/L sodium nitroprusside *(SNP)*. *Bars* represent mean values ± SEM.

It has been demonstrated that relaxant prostaglandins modulate both basal tone and receptor-mediated contractile responses of human mesenteric

Table II. Parameters calculated for the contractile and relaxant responses of human circumflex coronary arteries in vitro

	n	*Fresh artery*	*Frozen in KH solution*	*Frozen in KH +20% FCS*
Potency (pD_2 values)				
$PGF_{2\alpha}$	6	6.02 ± 0.07	5.79 ± 0.03	5.90 ± 0.03
Sodium nitroprusside	9	7.38 ± 0.09	6.76 ± 0.09*	6.71 ± 0.11*
Bradykinin	6	9.21 ± 0.18	7.66 ± 0.22*	7.74 ± 0.43*
Efficacy (E_{max})				
$PGF_{2\alpha}$ (gm)	6	6.9 ± 0.4	4.3 ± 0.3*	3.8 ± 0.2*
U46619, 10 nmol/L (gm)	15	2.2 ± 0.3	2.4 ± 0.3	2.3 ± 0.3
Sodium nitroprusside (%)	9	−60 ± 2	−64 ± 6	−62 ± 7
Bradykinin (% SNP)	6	−88 ± 4	−26 ± 10*	−36 ± 11*

Data are presented as means ± SEM.
*Significant difference against values in fresh arteries.

Table III. Preservation of endothelial cells in rings from human mesenteric and circumflex coronary arteries as assessed after the in vitro experiments by light microscopy

Score	*4*	*3*	*2*	*1*	*0*	*Total*
Mesenteric artery						
Fresh artery	2	3	1			6
Frozen in KH solution			6	4	2	12
Frozen in KH + 20% FCS			2	5	5	12
Coronary artery						
Fresh artery	5	1				6
Frozen in KH solution		1	3	1		5*
Frozen in KH + 20% FCS		2	1	2		5*

Score: 4 = endothelial cell lining preserved, 1 = single cells present, 0 = endothelium not seen. * = 1 preparation in this group could not be assessed.

arteries in vitro.[21] Furthermore, biochemical experiments on human saphenous veins have shown that 70% of vortex-stimulated prostacyclin production as assessed by measurement of the 6-keto-prostaglandin $F_{2\alpha}$ release derives from the endothelium[22] and that this endothelial function is completely preserved after slow freezing in the presence of dimethyl sulfoxide.[23] In our present functional studies blockade of the endogenous prostaglandin synthesis by indomethacin enhanced responses to $PGF_{2\alpha}$ of mesenteric arteries before but not after cryopreservation. These findings supported the contention that in human mesenteric arteries under normal conditions contractile responses to $PGF_{2\alpha}$ are counteracted by endogenous relaxing prostanoids[21] and suggested that the impairment of this mechanism in frozen/thawed arteries was due to cryoinjury of the endothelial cells. The same might be true for human coronary arteries, the basal tone of which is modulated by endogenous dilator products of arachidonic acid as well.[24] Because in this study coronary arteries were investigated without cyclooxygenase inhibition by indomethacin, the postthaw contractile responses of these vessels to $PGF_{2\alpha}$ might have been rather overestimated. In similar experiments with human coronary arteries and a serum-supplemented cryomedium, Ku et al.[25,26] obtained postthaw contractile responses of only approximately 20%, whereas in our study these effects amounted to 55% of that obtained with unfrozen control tissues. The main reason for this apparent discrepancy may derive from the blockade of endogenous dilator products of the cyclooxygenase pathway by indomethacin in those experiments. Furthermore methodologic differences such as different equilibration times of the tissues with the cryomedium before starting the cooling process and storage of the samples at −75° C might also have contributed to the observed differences.

An increased accumulation of guanosine 3′,5′-cyclic monophosphate within the vascular smooth-muscle cells is the common mechanism by which substance P, bradykinin, and nitrovasodilators such as sodium nitroprusside elicit smooth muscle relaxation.[27] However, whereas sodium nitroprusside is thought to act directly through activation of soluble guanylate cyclase,[28] both substance P and bradykinin first interact with specific receptors on the vascular endothelium[29-31] to trigger nitric oxide formation,

which then activates soluble guanylate cyclase in smooth muscle cells, leading to increased intracellular concentrations of 3′,5′-cyclic monophosphate.[32] In both mesenteric and coronary vascular preparations the postthaw endothelium-dependent relaxant responses to substance P and bradykinin proved to be markedly attenuated compared with those of unfrozen control tissues, suggesting again considerable cryoinjury of the endothelium. Indeed, morphologic examinations revealed an extensive loss of the endothelial cell lining in both types of arterial rings that had been frozen in either medium, thereby excluding the possibility that the postthaw functional changes were due to a reduction of the number of endothelial receptor sites. The correlation between functional and morphologic preservation of the endothelial cell in arterial preparations is in contrast to findings with human saphenous veins, where it seems to be impossible to predict the endothelial function on the basis of microscopic examinations.[33] In both mesenteric and coronary arteries postthaw maximal endothelium-independent relaxant responses to sodium nitroprusside were unchanged. However, a slight, although not significant, shift to the left of the sodium nitroprusside curve in cryopreserved mesenteric arteries was in agreement with the development of supersensitivity to nitrovasodilators after the removal of basal release of nitric oxide synthesis in deendothelialized vascular tissue.[34] Conversely, cryopreserved human coronary arteries proved to be significantly less sensitive to sodium nitroprusside than unfrozen tissues, suggesting that the development of supersensitivity to nitrovasodilators after deendothelialization is tissue-specific. This result is also in line with the observation that glyceryl trinitrate relaxes atherosclerotic human coronary arteries with reduced potency but unimpaired efficacy.[35] Further experiments are required to elucidate the mechanism(s) of cryoinjury and to further improve the method for cryopreservation of human arteries.

In conclusion, these findings support the contention that cryopreservation may provide a useful technique for the storage of limited supplies of vascular tissues not only for scientific research but also for graft transplantation. The patency of autogenous grafts is definitely superior to that of any artificial graft or antigenic allograft. However, cryopreserved arteries might be potential alternatives when autogenous conduits are not available. Cryopreservation thus offers the prospect to store different types of arteries, for example, from multiple organ donors, to be readily available for human leucocyte antigen matching of donor and recipient before bypass surgery.

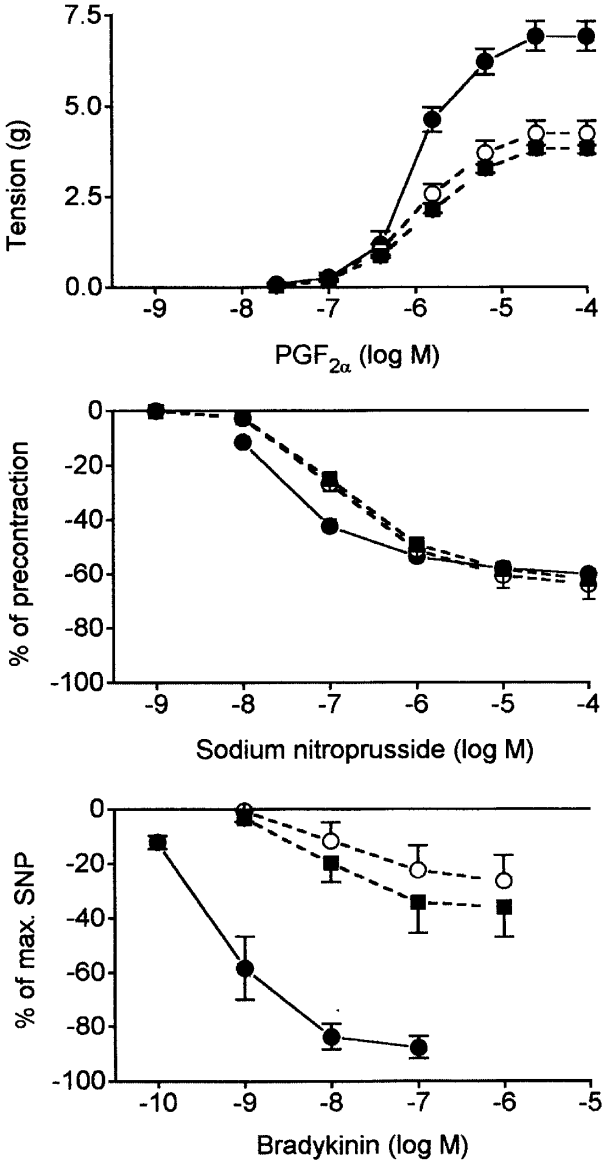

Fig. 4. Cumulative concentration-response curves on rings from human coronary arteries, unfrozen *(solid circles)* and after cryopreservation in KH solution without *(empty circles)* and with 20% fetal calf serum *(solid squares)*. Contractile responses to $PGF_{2\alpha}$ expressed in grams (*top*, for each point n = 6) and relaxant responses during active tone induced by U46619 (10 nmol/L), for sodium nitroprusside expressed as percentage of tone induced by U46619 in addition to passive preload (*middle*, for each point n = 9), and for bradykinin expressed as percentage of maximal relaxation induced by 100 μmol/L sodium nitroprusside (*SNP, bottom*, for each point n = 6); *bars* represent mean ± SEM.

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