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Activated Rho/Rho kinase and modified calcium sensitivity in cryopreserved human saphenous veins ☆

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ABSTRACT

Background: We have shown previously that cryopreservation of human internal mammary arteries activates protein kinase C and enhances intracellular Ca^{2+} $[Ca^{2+}]_i$. We now present evidence that in human saphenous veins (HSV) cryoinjury is associated with activation of the Rho/Rho kinase signaling pathways and enhanced $[Ca^{2+}]_i$.

Methods: HSV were investigated in vitro either unfrozen within 12 h after removal or after storage at −196 °C in a cryomedium containing 1.8 M dimethyl sulfoxide and 0.1 M sucrose as cryoprotectant additives.

Results: Cryostorage diminished responses to receptor-mediated contractile agonists such as noradrenaline, 5-HT and endothelin-1 by up to 30% whereas responses to KCl were attenuated by about 50%. Concentration–response curves for $CaCl_2$ on unfrozen and cryopreserved HSV revealed similar inhibitory activities of both blocking 1,4-dihydropyridine derivatives nifedipine and the (−)-(*R*) enantiomer of SDZ 202-791 whereas the Ca^{2+} channel activating (+)-(*S*) enantiomer of SDZ 202-791 was 10 times less effective at enhancing contractions to $CaCl_2$ when tested after cryostorage. These functional effects were reflected by changes in $[Ca^{2+}]_i$ as demonstrated by fluorescence of Fluo-3AM loaded veins. The diminished activity of (+)-(*S*) SDZ 202-791 in cryopreserved HSV was reversed partially when the potassium channel opener pinacidil (1 μM) was present during the freezing/thawing process. Blockade of Rho kinase by HA-1077 proved to be significantly more effective at attenuating contractile responses to both endothelin-1 and KCl after cryostorage.

Conclusions: Data suggested that cryopreservation modified $[Ca^{2+}]_i$ of venous smooth muscle cells (1) through depolarization-induced changes in Ca^{2+} influx and (2) through activation of Rho kinase signaling pathways.

Banked human saphenous veins have been proposed as a low-cost alternative to synthetic prostheses for coronary bypass grafting and for peripheral vascular reconstruction [3]. In addition, cryopreservation has become an important tool for storage of human vascular tissues in pharmacological research [20,23]. Cryopreservation and storage at −196 °C of blood vessels from various species preserves important biochemical and functional properties reasonably well [20]. Moreover, cryopreservation allows long-term banking of viable human saphenous veins for many years [26] with only minor changes in post-thaw functional activity. The most consistent change observed with all types of blood vessels studied so far, is a reduction of post-thaw contractile force to both receptor stimulating agonists and depolarization by high extracellular potassium concentration. To prevent cryoinjury, i.e., to improve the cryopreservation procedure it seems important to understand the effects of cryopreservation on transmembrane signaling pathways in human vascular tissue, if banks of these vessels are to be established to preserve tissue for future bypass grafting or pharmacological research.

When living mammalian cells are frozen without any cryoprotective additive the formation of ice crystals generally induces severe cell damage and only few if any cells survive. This may be prevented or at least minimized by use of cryoprotectants such as the permeating dimethyl sulfoxide (Me_2SO) and non-permeating cryoprotectant agents such as sucrose during the cryopreserva-

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tion process [22]. Previous experiments have shown that exposure of human saphenous veins to cryomedium containing Me_2SO and sucrose without cooling to subzero temperatures did not modify contractile responses [26], indicating that the observed post-thaw functional changes were indeed due to injury occurring during the freezing/thawing process.

During the freezing/thawing process cells within a matrix are subject to additional physical factors such as cell–cell and cell–matrix interactions that may be disturbed and exacerbate freezing injury [29]. Recently McGregor et al. [19] investigated the adaption of in vitro perfused unfrozen human saphenous veins to circumferential deformations imposed by simulated arterial perfusion and suggested a role for Rho kinase-dependent pathways in this process. Rho proteins have been recognized as major regulators of smooth muscle cell contraction. By modulating the level of phosphorylation of the myosin light chain of myosin II the Rho/Rho kinase pathway contributes to agonist-induced Ca^{2+} sensitization in smooth muscle contraction. Several compounds have been reported to inhibit the activity of Rho kinase. To evaluate the contributory role of Rho kinase in the cryoinjury of venous smooth muscle in the present study the Rho kinase inhibitor HA-1077 [28] was used.

Previous studies have demonstrated that cryopreservation of human internal mammary arteries besides activating protein kinase C leads to increased intracellular Ca^{2+} $[Ca^{2+}]_i$ in arterial smooth muscle cells [21,27]. Because of markedly reduced contractile responses to high potassium chloride of cryopreserved veins [26] changes in Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCs) were investigated as well. Two enantiomers of the 1,4-dihydropyridine SDZ 202-791 [11] were employed. The (−)-(*R*) enantiomer of SDZ 202-791 is acting as Ca^{2+} channel antagonist whereas the (+)-(*S*) enantiomer of SDZ 202-791 acts as activator, i.e., it enhances the Ca^{2+} influx through VOCs.

The evidence suggested that in cryopreserved human saphenous veins in addition to enhanced $[Ca^{2+}]_i$, activated Rho/Rho kinase signaling pathways seem to be critical to the post-thaw functional activity. To our knowledge this is the first study presenting evidence for a contributory role of Rho kinase activation in the process of cryoinjury in human saphenous veins.

Materials and methods

Tissue preparation

Samples of human saphenous veins were obtained during bypass surgery, surgical excision of varicose veins or multiple organ procurement. Only undistended venous segments without macroscopic changes and without perfusion of any vasodilating substance by the surgeon were employed. Veins from a total of 87 patients (67 male, 20 female) with a mean age of 63 (range 25–79) years were used. After explantation, the veins were placed in cold RPMI 1640 culture medium or Krebs–Henseleit (KH) solution (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 transported to the laboratory. The veins were cleaned from connective tissue and cut into segments of about 20 mm or into rings of about 2–2.5 mm length to be investigated either unfrozen within 12 h or after cryopreservation in organ bath studies.

Cryopreservation

Venous samples were placed in 2 ml Liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with 1.6 ml vehicle solution (RPMI 1640 culture medium) containing 1.8 M dimethyl sulfoxide (Me_2SO) and 0.1 M sucrose as cryoprotecting agents [22]. After equilibration for 10–20 min at room temperature with the cryomedium the samples were placed into a polystyrene box (11 × 11 × 22 cm) and slowly frozen at a mean cooling rate of about −0.6 °C/min in a freezer maintained at −70 °C. After about 2 h the vials were transferred into liquid nitrogen at −196 °C. Before use the samples were placed for 10 min on dry ice and then exposed for 6 min to room temperature before being thawed within 4 min in a 37 °C water bath giving a mean thawing rate of about 15 °C/min [26]. Thereafter the venous samples were placed for about 2 min in KH solution containing 50% cryomedium before being rinsed in a dish containing KH solution at room temperature, cut into rings (2–2.5 mm in width) and suspended in 10 ml organ baths for isometric tension recording. When the effect of the potassium channel opener pinacidil was tested, veins were exposed for 20 min to KH solution at 37 °C containing pinacidil (0.1–10 μM) before being equilibrated for 10 min in pinacidil-containing cryomedium and frozen as described above.

Organ bath studies

Venous rings were mounted between two hooks of stainless steel wire (diameter 0.5 mm) and suspended in 10 ml organ baths containing KH solution at 37 °C, gassed continuously with 5% CO_2 in oxygen. Under a resting tension of 1 g changes in tone were recorded isometrically 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 samples were exposed once to noradrenaline (1 μM) and the baseline tension of the rings was readjusted to 1 g if required. Contractile response curves to agonists were determined by cumulative additions, the concentration in the organ bath being increased when the maximum effect had been produced by the previous concentration. Responses to contractile agonists were expressed in grams or as percentage of the maximum of a normalized preceding control effect. Inhibitors were added to the organ baths 15 min before the agonist under investigation, one ring from the same vein serving as control preparation. Each ring was exposed only once to an antagonist.

Contractile responses to calcium chloride were investigated as described previously [7,25]. Briefly, after equilibration as described above, the KH solution in the organ baths was replaced by a calcium free solution containing 60 mM potassium chloride in equimolar exchange for sodium chloride and 50 mM Trizma instead of $NaHCO_3$ buffer, gassed with O_2. In these experiments any residual extracellular calcium was removed by 15 min exposure to EGTA (100 μM) followed by washout and neuronal uptake mechanisms were blocked by cocaine (30 μM) added 20 min before the first $CaCl_2$ concentration. Concentration–response curves to $CaCl_2$ were determined by cumulative additions. All contractile responses were expressed as percentages of a normalized preceding contractile response to 3 mM $CaCl_2$. The effects of 1,4-dihydropyridine derivatives were tested in organ baths protected from light.

$^{45}Ca^{2+}$ Uptake

Tissue $^{45}Ca^{2+}$ uptake was determined according to the method described by Van Breemen [35]. Briefly, after equilibration for 60 min in KH solution at 37 °C venous rings were placed for 120 min into KH solution containing 2 μCi $^{45}Ca^{2+}$ ml^{-1} (New England Nuclear, Boston, MA) in addition to the nonradioactive calcium, to label exchangeable cellular calcium stores. This was followed by addition of the 1,4-dihydropyridine under investigation to the labeling medium. After 15 min the rings were transferred for 60 min to a solution of the same composition but containing 60 mM KCl in equimolar exchange for NaCl in addition to cocaine (30 μM) and indomethacin (0.3 μM) before being