Levosimendan has a prolonged antispasmodic effect in isolated human radial artery bypass grafts and inhibits thrombin-induced aggregation of human platelets *in vitro*

Ph. D. Dissertation

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List of abbreviations

5-HT: 5-hydroxytryptamine

ACC/AHA: American College of Cardiology and the American Heart Association

Ach: acetylcholine

ADP: adenosine diphosphate

Akt: protein kinase B

BK_{Ca}: large conductance calcium-activated potassium

CABG: coronary artery bypass graft surgery

CAD: coronary artery disease

ERK: extracellular signal-regulated kinase

IC: inhibitory concentration

IMA: internal mammary artery

K_{ATP}: ATP sensitive

KCl: potassium chloride

KH: Krebs-Henseleit

K_V: voltage sensitive

LAD: left anterior descending

LIMA: left internal mammary artery

mN: milliNewton

NA: noradrenaline

Na-citrate: sodium-citrate

PDE: phosphodiesterase

PPP: platelet poor plasma

PRP: platelet rich plasma

RA: radial artery

S.E.M.: standard error of the mean

SVG: saphenous vein graft

WP: washed platelet

Summary

We have shown that contractions of the human RA grafts are larger than those of the IMA grafts in vitro. This finding supports the higher incidence of severe vasospasm of RA during CABG. We have demonstrated that the 5-HT-induced contractions of human RA graft segments pre-incubated in 0.9% NaCl solution were stable for 120 minutes. Levosimendan, in a therapeutic concentration (0.16 μmol/L), had a prolonged effect (>90 minutes) on the 5-HT-induced tone of the human RA segment. Levosimendan also effectively decreased NA-induced contractions. The colloidal Biseko® solution is effective against both NA- and 5-HT-induced contractions in human RA graft segments. The effect of Biseko® solution on 5-HT-induced contractions lasts for only 45 minutes. The maximal contractile and vasodilating capacities as well as the endothelium-dependent relaxation of RA segments pre-incubated in levosimendan solution were comparable to controls, suggesting that the inodilator does not deteriorate the function of the graft. BK_{Ca} channels do not account for the prolonged effect of levosimendan in reducing contraction of RA graft segments. Therapeutic concentrations of levosimendan (≤ 0.2 μmol/L) reduced thrombin-induced platelet aggregation in vitro. The platelet inhibitory effect of levosimendan is markedly decreased by albumin and significantly enhanced upon increasing the time of pre-incubation. These findings may render the inodilator drug, levosimendan, effective in preventing the spasm of the RA and the thrombotic occlusion of the graft during the intraoperative phase of CABG.

Publications relating to the dissertation:

1. **Ambrus N**, Szolnoky J, Pollesello P, Kun A, Varró A, Papp JG, Pataricza J. Prolonged antispasmodic effect in isolated radial artery graft and pronounced platelet inhibition induced by the inodilator drug, levosimendan. Basic Clin Pharmacol Toxicol. 2012; 110(3):269-74.
IF: 2.371

2. Szolnoky J, **Ambrus N**, Szabó-Biczók A, Bogáts G, Papp JG, Varró A, Pataricza J. Biseko colloidal solution diminishes the vasoreactivity of human isolated radial arteries. Eur J Cardiothorac Surg. 2009; 36(1):143-7.
IF: 2.397

3. Szolnoky J, **Ambrus N**, Szabó-Biczók A, Bogáts G, Papp JG, Varró A, Pataricza J. Kolloid és krisztalloid oldatok hatása human arteria radialis bypass graftok tónusán in vitro. Cardiologia Hungarica 2009; 39 : 135-9.
IF: 0

Other publication:

4. Pataricza J, Krassói I, **Ambrus N**, Bitay M, Varró A, Papp JG. Interspecies differences and extracellular calcium dependence in the vasorelaxing effect of cromakalim in isolated human, porcine, and canine coronary arteries. J Cardiovasc Pharmacol Ther. 2010; 15(3):289-95.
IF: 1.969

Abstracts:

1. **Ambrus N**, Szolnoky J, Pollesello P, Varró A, Papp JG, Pataricza J. A levosimendan relaxálja a szerotoninnal kontrahált humán artéria radiális graftot in vitro. (Levosimendan relaxes human radial artery grafts precontracted with 5-hydroxytryptamine in vitro.) A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése és a Magyar Kísérletes és Klinikai Farmakológiai Társaság (MFT) II. közös tudományos konferenciája, Szeged. Abstract Book 145, 2010.

2. **Ambrus N**, Szolnoky J, Pollesello P, Varró A, Papp JGy, Pataricza J. A Levosimendan hatás kumulációja izolált arteria radialis graftok esetén. (Cumulation of the effect of levosimendan in isolated radial artery bypass grafts.) Cardiol. Hung. 40. Suppl.G. G55, 2010.

3. **Ambrus N**, Pataricza J, Krassói I, Márton Z, Bitay M, Varró A, Papp JGy. Endothelium in health and disease; species difference in calcium dependency of cromakalim induced relaxation in porcine and human coronary arteries. Basic Clin Pharmacol Toxicol. 107:(Suppl.1). 175, 2010.

4. **Ambrus N**, Szolnoky J, Szabó-Biczók A, Bogáts G, Papp JG, Varró A, Pataricza J. A kolloidális Biseko® oldat csökkenti az arteria radialis kontrakciós képességét in vitro. (Colloidal Biseko® solution decreases the contractility of radial artery in vitro.) Magyar Élettani Társaság LXXIII. Vándorgyűlése, Budapest. Abstract Book 191, 2009.

5. **Ambrus N**, Szolnoky J, Pollesello P, Varró A, Papp JG, Pataricza J. Long-lasting effect of Levosimendan against 5-hydroxytryptamine-induced contraction in isolated radial artery bypass grafts. Heart Failure 2009, Nice, France. Abstract book 161, 2009.

6. **Ambrus N**, Szolnoky J, Szabó-Biczók A, Bogáts G, Papp JGy, Varró A, Pataricza J. Tároló oldatok hatása az arteria radialis funkciójának megőrzésében in vitro. (Effect of storage solutions in the preservation of radial artery function in vitro.) Cardiol. Hung. 39. Suppl.A. A64, 2009.

7. Szolnoky J, Pataricza J, **Ambrus N**, Krassói I, Szabó B. A, Bogáts G. Különböző tárolóoldatok arteria radialis graftok in vitro vazoreaktivitására gyakorolt hatása. (Effect of various storage solutions in the vasoreactiviy of radial artery grafts in vitro.) Cardiol. Hung. 37. Suppl.D. D16-D17, 2007.

8. **Ambrus N**, Szolnoky J, Krassói I, Pataricza J. Perioperative preservation of graft function during CABG surgery with colloidal 'Biseko' in vitro. Endothelium: The Determinant of Cardiovascular Health and Disease, International Workshop of The Physiological Society, Krakow, Poland. Abstract book 26, 2007.

TABLE OF CONTENTS

1. Introduction

Cardiovascular mortality has a major impact on life expectancy worldwide. In the developed countries, including Hungary, cardiovascular diseases are the leading cause of mortality, representing 50-60% of total mortality. From 1980 to 2009, cardiovascular mortality rate gradually decreased, but still plays an essential role in the development of disability and decreased quality of life. Approximately 50% of cardiovascular mortality is caused by ischemic heart diseases. In 2008, in 32 countries in the European Union, Hungary had the 20th position regarding cardiovascular mortality and Hungary was the 25th on the list of mortality caused by ischemic heart diseases [1].

The primary cause of ischemic heart diseases is atherosclerosis. Atherosclerosis of the coronary arteries is a slow, gradual process, which stays undiscovered until significant stenosis develops in the lumen, or the lumen becomes suddenly occluded. Local inflammation of the atherosclerotic plaque, collagen exposure, or the movements of the vessel may lead to the rupture of the plaque, instability, or thrombus development causing partial or total occlusion. Depending on the dynamics of lesion development, symptoms may vary from angina to myocardial infarction.

1.1. Coronary artery bypass graft surgery (CABG)

Myocardial revascularization has played an essential role in the treatment of coronary artery diseases (CADs) for almost half a century. CABG has been used in clinical practice since 1960s. In CABG, bypass grafts are placed to the coronary vessel beyond the culprit lesion(s), providing extra sources of nutrient blood flow to the myocardium and offering protection against the consequences of further proximal obstructive lesions [2].

1.1.1. Indications of CABG

The indications of CABG in accordance with the guidelines of the American College of Cardiology and the American Heart Association (ACC/AHA) [3]:

Class I

- Left main CAD ≥50%

- ≥70% diameter narrowing in both the proximal left anterior descending (LAD) coronary artery and the left circumflex artery
- Triple-vessel CAD, particularly in the setting of impaired left ventricular ejection fraction

Class II

- Proximal LAD coronary artery stenosis (with accompanying impaired left ventricular ejection fraction, it becomes a class I indication)
- One-vessel or two-vessel CAD that does not involve the proximal LAD coronary artery if a moderate area of viable myocardium is at risk

Class III

- One-vessel or two-vessel CAD not involving the proximal LAD coronary artery
- One-vessel or two-vessel CAD not involving the proximal LAD coronary artery with only a small area of viable myocardium

1.1.2. Coronary artery bypass grafts

The long-term benefit of CABG is maximized with the use of multiple arterial grafts [4]. Available grafts include internal thoracic, radial, and gastro-epiploic arteries and the saphenous vein. The internal thoracic and the gastro-epiploic arteries can be used as free grafts, with the aorta or another graft as inflow. The side-to-side anastomosis used in arterial and venous grafting eliminates an aortic anastomosis, decreases the amount of graft required, and increases total graft flow. The latter factor contributes to a higher patency rate.

In 1953, William Mustard performed the first direct surgical approach to the coronary circulation: a carotid-to-coronary bypass in a patient in Toronto. In 1962, the first surgical myocardial revascularization procedure, the patch graft technique, was performed to repair an obstruction of the left main coronary artery [5]. Subsequently, saphenous vein graft (SVG) interposition became the dominant approach. The diameter of the SVGs are 2-3 times larger compared to the diameter of the coronary arteries, therefore, blood flow of the venous grafts are hemodinamically worse than that of the coronary arteries. Additionally, histological characteristics of the veins do not make the vessel suitable for permanently elevated pressure. Use of SVGs are limited as their patency rates are low, 1 year after the

CABG, the patency rates of the venous grafts are 10-15% [6,7]. 10 years after the revascularization, 50% of the SVGs showed significant intimal hyperplasia, advanced atherosclerosis, or occlusion [7,8].

Subsequently, advantages of using the left internal mammary artery (LIMA) were demonstrated [9]. The LIMA showed better long-term patency rates and improved late survival compared to SVGs [9]. Internal mammary artery (IMA) is an elastic artery located near the heart, its internal diameter is similar to that of the coronary arteries. Partial or total IMA skeletonization increases its length and possibility of use. These techniques may allow a complete revascularization. Use of bilateral IMA is associated with higher postoperative sternal dehiscence and increased rate of mediastinitis in obese and possibly diabetic patients [10]. 10 years patency rate of the IMA is approximately 90% [6,11]; event-free long-term survival, reduced risk of recurrent angina or MI, and reduced need for re-operation correlate well with the extensive use of arterial grafts [12-14]. Despite these facts, use of bilateral IMA is technically more complicated compared to the use of the SVGs, thus, saphenous veins are still commonly used grafts.

The gastro-epiploic artery is primarily used for the revascularization of the posterior and lateral wall of the heart as an alternative solution. The use of the gastro-epiploic artery is limited by the small diameter of the artery and the risk of ileus at the donor site.

Using radial artery (RA) increases the number of arterial anastomoses beyond the use of both IMAs. The RA was first used as a conduit graft for CABG in 1973 [15], but was set aside for having high occlusion and spasm rate. Early graft failure was caused by endothelial damage during harvesting, and subsequent intimal hyperplasia or spasm [16,17]. However, long-term patency of this graft achieved by novel techniques regarding graft preparation and prevention of perioperative vasospasm supported its reappraisal [18,19]. Currently, the prevalence of vasospasm in case of RA is 5 to 10% [20-24].

The RA is a superficial artery, harvesting of this vessel does not impair the function of the forearm or hand in case of proper palmar collateral circulation. RA is a muscular artery sensitive to ischemia. It is known that RA has a higher receptor-mediated contractility (endothelin-1, angiotensin II [25], 5-hydroxytryptamine [5-HT] [26]) but similar endothelial function compared to the IMA.

In order to reduce the incidence of spasm, preparation of the RA is atraumatic, the RA grafts are usually pre-incubated in crystalloid or colloidal solutions for 30-45 minutes before the surgical implantation, and the patients are given a vasodilator infusion during the surgery. Pharmacological dilatation is used instead of mechanical dilatation. At 5 years, patency rates of RA are superior to that of the SVGs but inferior to that of the IMA. This patency is strongly related to target vessel size and stenosis severity.

1.1.3. Pre-incubation solutions for preserving the function of RA bypass grafts

Several pre-incubation solutions have been used to reduce RA graft vasospasm. However, the issue of an optimal solution for pre-incubating RA graft segments has not been resolved. Pre-incubation of RA graft segments in heparinized whole blood increased endothelium-dependent relaxation to acetylcholine (Ach) [27], but vasoconstriction of blood-stored RA grafts was also enhanced [28]. Papaverine has been widely used for the prevention of RA vasospasm, however, it was shown to impair endothelial function [29]. We have recently shown that a colloidal solution (Biseko® solution) without the addition of a vasodilator substance was able to diminish contractions to 5-HT while the RA graft retained its maximal vasoconstrictive and vasodilating capacities [30]. Vasodilators other than papaverine have also been used in pre-incubation solutions to minimize perioperative spasm of RA grafts. Phenoxybenzamine, despite its long-lasting effect, was found to be effective only in preventing α-adrenergic-mediated vasoconstriction [24], calcium antagonists were found to be relatively ineffective against receptor mediated contractions [31,32] and nitrates showed tolerance [33].

Levosimendan – an inodilator drug in the therapy of heart failure – opposed 5-HT induced contraction of the isolated RA grafts [34] suggesting that the drug is able to decrease perioperative spasm following pre-incubation of the graft before implantation. Whether this suppression of contractile capacity persists after removal of the inodilator drug following an in vitro pre-incubation of the isolated graft remained to be established. Recently, a possible long-lasting effect has been suggested by Tritapepe et al. [35], who presented a preconditioning effect of levosimendan upon the hemodynamic parameters following a single intravenous injection of the inodilator. They found an enhanced cardiac index and a decreased

peripheral resistance in patients with CABG even 48 hours after pretreatment with levosimendan. Zhang et al. [36] have shown the presence and electrophysiological function of large conductance calcium-activated potassium (BK_{Ca}) channels in human isolated RA smooth muscle samples. It is known that levosimendan has a BK_{Ca} channel activator effect on porcine coronary arteries [37] and human internal mammary arteries [38].

Graft failure during bypass surgery is not solely the consequence of vasospasm but may occur following a thrombotic occlusion. Platelet aggregation is the primary event in the pathomechanism of arterial thrombosis and several agonists or their combinations are thought to be responsible for the initiation of thrombus formation. Kaptan et al. [39] have demonstrated that therapeutically relevant concentrations of levosimendan inhibited adenosine diphosphate (ADP)- and collagen-induced platelet aggregations. Among the platelet agonists, thrombin plays a central role in blood coagulation and induces platelet aggregation being largely independent of ADP as well as the cyclooxygenase enzyme pathways in platelets [40,41]. A drug that inhibits thrombin-induced platelet aggregation would be effective in those thromboembolic complications that are resistant to the conventional therapy of acetylsalicylic acid and ADP-receptor antagonists.

Low albumin concentration in the blood is relatively frequent in heart failure [42]. Levosimendan strongly binds to plasma proteins [43], therefore, hypoalbuminemia may profoundly enhance free plasma concentration of the drug. The magnitude of the change in concentration of albumin that affects the anti-aggregatory effect of the inodilator has not been investigated yet.

The aims of the present study:

1/ to compare the contractile capacity of isolated IMA and RA bypass grafts induced by different contractile agents,

2/ to explore whether levosimendan retains its vasodilator/antispasmodic capacity following in vitro pre-incubation of RA segments and

3/ to investigate the possible effect of the drug on endothelium dependent and independent relaxations of the graft after the removal of levosimendan from the isolated organ bath,

4/ to measure whether the vasorelaxing effect of levosimendan is due to BK_{Ca} channel activation,

5/ to examine the possible platelet inhibitory effect of levosimendan against the platelet agonist, thrombin, in human washed platelets (WPs) and in platelet rich plasma (PRP) in vitro,

6/ to study the modulation of the effect of the inodilator in the absence and in the presence of albumin.

2. Patients and methods

2.1. Patients

2.1.1. Comparing the contractions of IMA and RA bypass grafts

Forty two patients undergoing elective CABG were involved in the study comparing the contractions of IMA and RA bypass grafts. The characteristics of patients can be seen in Table 1.

Table 1. Characteristics of patients participating in the study comparing contractions of internal mammary artery and radial artery bypass grafts

Characteristics	Internal mammary artery	Radial artery
Number of patients	18	24
Sex	15 males, 3 females	22 males, 2 females
Age (year)	65.1 ± 2.1	63.0 ± 1.9
Diseases		
Hypertension	18 (100%)	22 (92%)
Hypercholesterolemia	15 (83%)	17 (71%)
Diabetes mellitus	3 (17%)	5 (21%)

(%) represents the percent occurrence of the diseases within the group

For the measurement of isometric contractions of IMA and RA grafts, discarded segments of IMA or RA from artery of patients undergoing CABG performed on the beating heart were used. Patients gave informed consent to accept the aim and protocol of investigation. RA from the non-dominant forearm of patients was used in this study. Modified Allen test using the standard Allen test [44] together with Doppler test [45] on the hand was used to determine the eligibility of the patients. The positive Allen test provided evidence for an acceptable blood flow from the ulnar artery to the palmar circulation after a short-term compression of the RA. The patients with negative results were excluded. Additional inclusion criteria were the

following: the diameter of the RA should be at least 2 mm, no calcification or intima-media thickening in the RA, lack of anatomical variation and at least 20% increase in the peak systolic velocity in the ulnar artery. The RA was used as aortocoronary bypass graft to the right coronary artery or to the obtuse marginal branch of the circumflex coronary artery. Before operation, the diameter of RA had been measured with ultrasonic technique (Logiq 7, linear head, GE Healthcare, Wisconsin, USA).

2.1.2. Effect of 5% human albumin and Biseko® pre-incubatoin solutions on noradrenaline (NA)-induced contraction of RA segments

In case of fourteen patients, the effect of 5% human albumin or Biseko® solution was measured on the noradrenaline-induced contraction of RA segments. The characteristics of these patients are shown in Table 2.

Table 2. Characteristics of patients undergoing coronary revascularization using radial artery bypass grafts in the study comparing pre-incubation with human albumin or Biseko® solution on noradrenaline induced contractions.

Characteristics	Radial artery incubated in 5% human albumin or Biseko® solution
Number of patients	14
Sex	11 males, 3 females
Age (year)	60.1 ± 2.2
Diseases	
Hypertension	14 (100%)
Hypercholesterolemia	13 (93%)
Diabetes mellitus	2 (14%)

(%) represents the percent occurrence of the diseases within the group

2.1.3. Prolonged effect of different pre-incubation solutions on the contractile and vasorelaxing properties of RA segments

In a third group of patients, the prolonged effect of different pre-incubation solutions (0.9% NaCl, levosimendan, Bretschneider, 5% human albumin, or Biseko® solution) on the contractile and vasorelaxing properties of RA segments was investigated. The characteristics of patients are listed in Table 3.

Table 3. Characteristics of patients undergoing coronary revascularization using radial artery bypass grafts in the study comparing the possible prolonged effect of pre-incubation with 0.9% NaCl, levosimendan, Bretschneider, 5% human albumin, or Biseko® solution.

Characteristics	Radial artery incubated in 0.9% NaCl or levosimendan solution	Radial artery incubated in Bretschneider solution	Radial artery incubated in 5% human albumin or Biseko® solution
Number of patients	7	12	14
Sex	6 males, 1 females	10 males, 2 females	11 males, 3 females
Age (year)	58.1 ± 2.0	58.8 ± 2.7	57.4 ± 2.6
Diseases			
Hypertension	5 (71%)	10 (83%)	12 (86%)
Hypercholesterolemia	4 (57%)	7 (58%)	9 (64%)
Diabetes mellitus	2 (29%)	3 (25%)	3 (21%)

% represents the percent occurrence of the disease within the group

2.1.4. Sampling for measurement of platelet aggregation

For the measurement of platelet aggregation, blood was obtained from 14 healthy volunteers (mean age was 31 years, between 25 and 40 years). No drugs had been taken by these patients within 10 days before the investigation. The investigation received the approval of the local institutional review board (Human Investigation Review Board, University of Szeged, Hungary). Patients gave informed consent to accept the aim and protocol of investigation.

2.2. Preparation of IMA and RA segments for in vitro testing

Arterial samples were prepared atraumatically with an Ultracision Harmonic Scalpel (Ethicon Endo-Surgery, Ohio, USA). Once harvesting of the arterial tissue samples had been started, low dose nifedipine (0.2-0.4 mg/h) was given in intravenous infusion to prevent early vasospasm. An in situ IMA graft was used during the CABG and we prepared a 6 mm long segment of IMA at the bifurcation for the in vitro study. 6 mm long segments of RA were obtained at the origin of the brachial artery (proximal part). The IMA or RA was then carefully dissected and cleaned from the surrounding connective tissue. The segment of the artery was cut into two 3-mm long rings and submerged to a storage solution for 45 minutes.

2.3. Isometric tension measurement

Two 3 mm segments of human IMA as well as that of the human RA were mounted in parallel on stainless-steal hooks and placed into organ chambers containing 2 mL Krebs-Henseleit (KH) solution maintained at 37°C. The solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 at pH 7.4. One of the hooks was anchored and the other one was connected to a force-displacement transducer (Isometric transducer, Type F30, Hugo Sachs Elektronic, March-Hugstetten, Germany) to measure changes in isometric tension as described previously [37]. Vessel rings were subjected to 20 milliNewton (mN) tension and equilibrated for 45 minutes. During this period, the tension was continuously readjusted to the above value of stretch. The assessment of the optimized value for resting (basal) tone has been presented formerly [34]. The scheme of the organ bath is depicted in Figure 1.

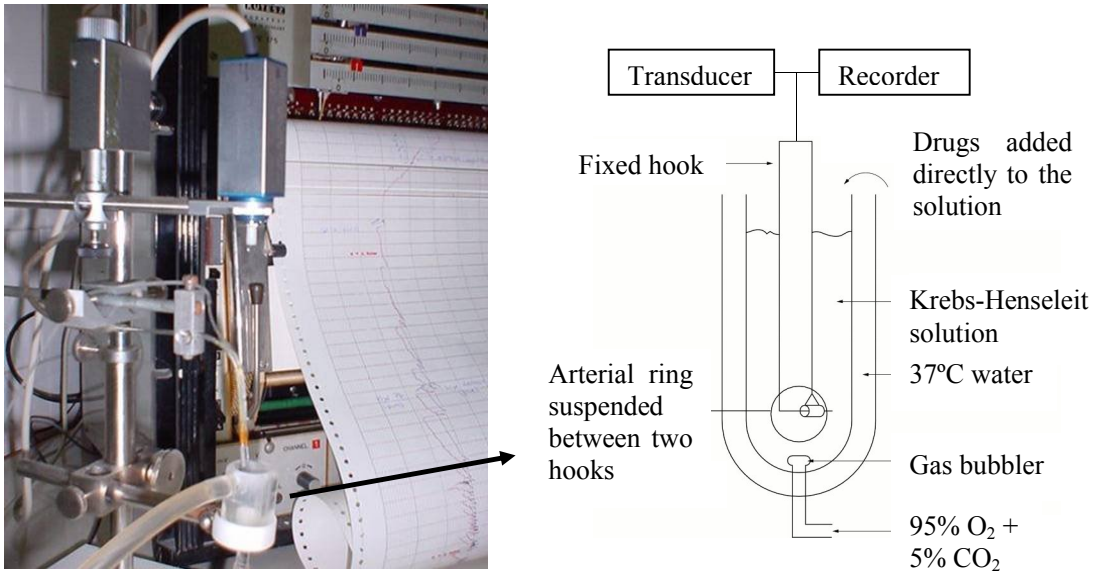

Figure 1. Scheme of an organ bath for measuring isometric tension of isolated radial artery.

2.4. Protocols of investigations with human arterial segments

2.4.1. Protocol 1

For the comparison of the contractions of human IMA and RA, two 3 mm segments of IMA or RA were incubated in 0.9% NaCl for 45 minutes (Figure 2). At zero (0) minute, the storage solution was washed out and replaced with KH solution. Two rings of IMA or RA graft segments were mounted in parallel on stainless-steal hooks and placed into organ chambers containing 2 mL KH solution maintained at 37°C to measure isometric tensions as described in isometric tension measurement (2.3.). Following the equilibration period, contractions were induced by either receptor mediated contractile agents (10 µmol/L NA and 0.31 µmol/L 5-HT) or receptor independent contractile agents (5 and 80 mmol/L potassium chloride [KCl]).

Protocol 1.

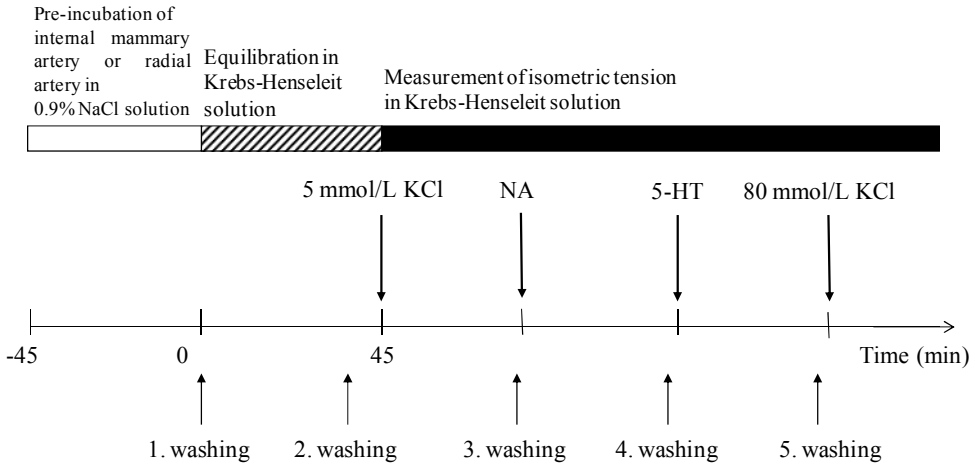

Figure 2. Protocol 1: An internal mammary artery ring (3 mm) or a radial artery ring (3 mm) was pre-incubated in 0.9% NaCl solution for 45 minutes as described in the methods. At 0 time, the storage solution was washed out and replaced with Krebs-Henseleit solution. After the 2nd washing, contractions were induced by 10 mmol/L potassium chloride (KCl), 10 µmol/L noradrenaline (NA), 0.31 µmol/L 5-hydroxytryptamine (5-HT), and 80 mmol/L KCl (representing maximal contractions). The arterial rings were washed before the administration of each contractile agent.

2.4.2. Protocol 2

Figure 3 shows the comparison of the effect of pre-incubation in 5% human albumin solution and Biseko® solution on the NA-induced contractions of human radial arteries. Two 3 mm RA segments were incubated in 5% human albumin or Biseko® solution for 45 minutes. At zero (0) minute, the storage solution was washed out and replaced with KH solution. Two rings of RA graft segments were mounted in parallel on stainless-steal hooks and placed into organ chambers containing 2 mL KH solution maintained at 37°C to measure isometric tensions as described in isometric tension measurement (2.3.). Following the equilibration period, contractions were induced by cumulative administration of 1-100 µmol/L NA.

Protocol 2.

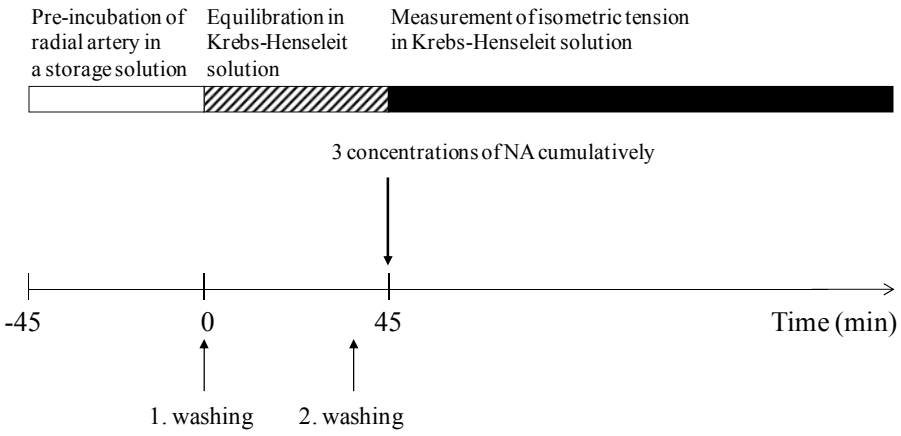

Figure 3. Protocol 2: A radial artery ring (3 mm) was pre-incubated in 5% human albumin or Biseko® storage solutionfor 45 minutes as described in the methods. At 0 time, the storage solution was washed out and replaced with Krebs-Henseleit solution. After the 2nd washing, contractions were induced by cumulative administration of 1-100 µmol/L noradrenaline (NA).

2.4.3. Protocol 3

To compare the prolonged effect of different storage solutions on the contractile and vasorelaxing properties of human RA segments (Figure 4). Two 3 mm segments of human RA were incubated in one of the following storage solutions: 0.16 μmol/L levosimendan, 0.9% NaCl, Bretschneider, 5% albumin, or Biseko® solution for 45 minutes. Pre-incubation in one of the above storage solutions and the equilibration period were the same as in Protocol 2 (2.4.2.) Following the equilibration period, contractions were induced by three consecutive administrations of 5-HT (0.31 μmol/L) 45, 90 and 120 minutes after removing the pre-incubation solution from the organ baths. In csase of 0.16 μmol/L levosimendan and 0.9% NaCl solutions, at the end of the investigation, endothelium dependent relaxation was evaluated by Ach (1.0 μmol/L), maximal contraction was measured by 80 mmol/L KCl, and maximal relaxation was tested with papaverine (100 μmol/L).

Protocol 3.

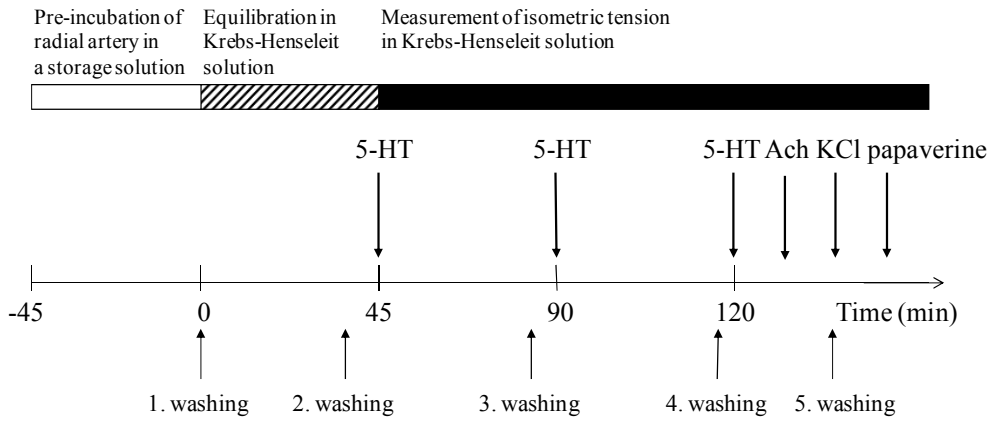

Figure 4. Protocol for measurements of isometric tension. Protocol 3: A radial artery ring (3 mm) was pre-incubated in one of the following storage solutions for 45 minutes as described in the methods: 0.16 μmol/L levosimendan, 0.9% NaCl, Bretschneider, 5% albumin or Biseko® solution. At 0 time, the storage solution was washed out and replaced with Krebs-Henseleit solution. After the 2nd washing, 5-hydroxytryptamine (5-HT, 0.31 μmol/L) was added to induce contraction. This procedure was repeated at 90. and 120. minutes. The endothelium-dependent relaxant, acetylcholine (Ach, 1.0 μmol/L) was administered after the third 5-HT-induced contraction had been completed. Following the 5th washing procedure,

contraction was induced by potassium chloride (KCl, 80 mmol/L). At the maximum of KCl-induced tone, the endothelium-independent relaxation was induced with papaverine (100 μmol/L). When the storage solution contained levosimendan (0.16 μmol/L), another radial artery ring – prepared from the same patient – was investigated in parallel using the same protocol except that instead of levosimendan, the solvent of the inodilator (8 μL) in sodium chloride (NaCl, 0.9%) was used as storage solution (time-matched protocol).

2.5. Wet weight measurement of human arteries

Following Protocol 1 (2.4.1.), the wet weights of IMA and RA segments were measured. At the end of isometric tension measurement, the excess fluid was removed by placing the 3-mm-long arterial segments on a blotting paper. Then, the arterial segments were weighed immediately using a laboratory scale (Ohaus Analytical Explorer balance, Ohaus Europe GmbH, Switzerland).

2.6. Measurement of platelet aggregation

Turbidimetric aggregometry and the increasing light transmission in stirred platelet suspensions during aggregation of platelets can be seen on Figure 5.

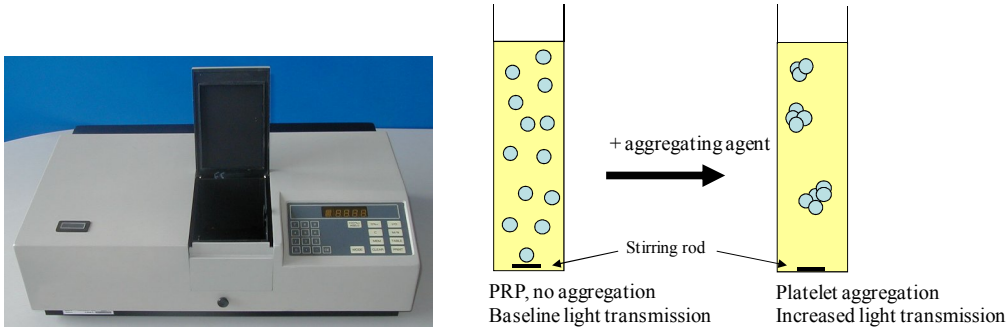

Figure 5. Measurement of platelet aggregation by turbidimetric aggregometry. A scheme of the aggregometer and a schematic figure of platelet aggregation and the direction of the changes in light transmission.

2.7. Preparation of PRP and WPs

Venous blood samples were obtained from the antecubital vein using 0.13 mol/L trisodium citrate as anticoagulant (blood to anticoagulant ratio was 9:1). PRP was prepared by centrifugation of the citrated blood at 200 g for 15 minutes (see Figure 6). PRP was removed and platelet poor plasma (PPP) was obtained following centrifugation of the supernatant at 1000 g for 10 minutes. The platelet count of PRP was adjusted to 3.0±0.3 x 1011/L with PPP using Picoscale Particle Counter (Medicor, Budapest, Hungary). Turbidimetric light transmission of PRP was measured against PPP in a modified Born aggregometer [46].

The preparation of human WPs was performed according to Blackwell et al. [47]. Citrated PRP was prepared as described above. PRP was further centrifuged at 800 g for 18 minutes. The pellet was resuspended in calcium-free KH solution without prostacyclin and centrifuged again at 800 g for 18 minutes. Finally, platelets were resuspended in calcium-free KH solution (see Figure 6). The platelet count of this washed suspension was adjusted to 3.0±0.3 x 1011/L with calcium-free KH solution as diluent. Light transmission of WPs was measured against KH solution.

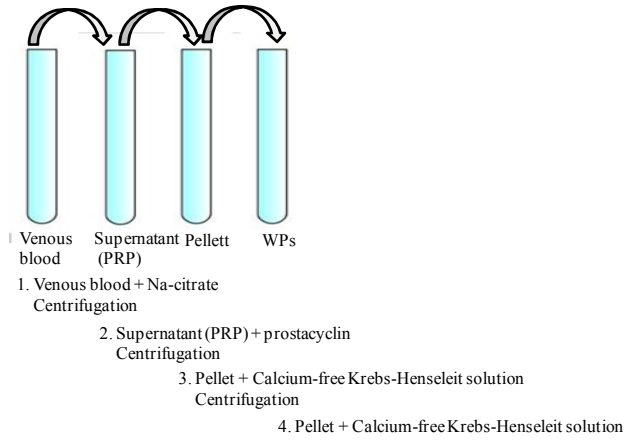

Figure 6. Preparation of platelet rich plasma (PRP) and washed platelets (WPs) from a patient's venous blood sample. 1) 10 mL blood was anticoagulated with sodium-citrate (Na-citrate, 0.13 mmol/L) and centrifuged (200 g for 15 minutes), and the supernatant (PRP) was used without further preparation. 2) In another series of experiments, PRP was further centrifuged (800 g for 18 minutes) in the presence of prostacyclin (0.8 μmol/L). 3) The pellet of the second centrifugation was resuspended in calcium-free Krebs-Henseleit solution. This was centrifuged (800 g

for 18 minutes) again. 4) Finally, the pellet (WPs) was resuspended in calcium-free Krebs-Henseleit solution.

2.8. Protocols of investigations for the measurement of platelet aggregation

2.8.1. Protocol 4

To measure the effect of levosimendan on thrombin-induced platelet aggregation, levosimendan was administered as a single concentration 2 minutes before inducing aggregation of platelets in PPR (Protocol 4A). Aggregation of PRP was induced by thrombin at 37°C (see Figure 7). The experiment was repeated with increasing concentrations of the inodilator using new platelet suspensions obtained from the same patient. The effect of levosimendan on thrombin-induced platelet aggregation was analyzed in WPs as well (Protocol 4B). In this study, levosimendan was added 2 minutes before the aggregation together with 1 mmol/L CaCl2. The experiment was repeated with increasing concentrations of the inodilator using new platelet suspensions obtained from the same patient. Aggregation of WPs was induced with thrombin.

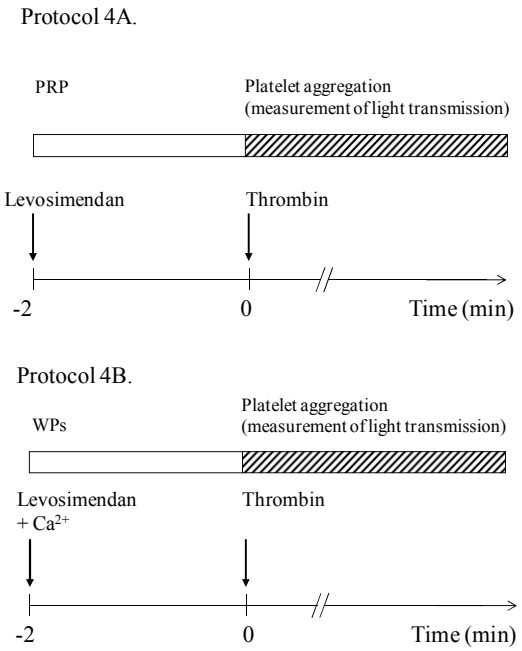

Figure 7. Protocols for measuring inhibition of platelet aggregation by levosimendan. Protocol 4A: Platelet rich plasma (PRP, 0.6 mL) was placed into a 1 ml glass cuvette, and a single concentration of levosimendan was administered

2 minutes before the induction of platelet aggregation. At 0 time, thrombin (1 IU/mL) was used to initiate platelet aggregation measured by the increase of light transmission in time. Experiments were repeated with increasing concentrations of levosimendan (0.06-0.52 μmol/L) using PRP from the same patient. Protocol 4B: Washed platelets (WPs, 0.6 mL) were placed into a 1 mL glass cuvette, and a single concentration of levosimendan and 1 mmol/L CaCl2 were administered 2 minutes before the induction of platelet aggregation. At 0 time, thrombin (0.1 IU/mL) was used to initiate platelet aggregation measured by the increase of light transmission in time. Experiments were repeated with increasing concentrations of levosimendan (0.01-0.30 μmol/L) using WPs from the same patient.

2.8.2. Protocol 5

In a separate series of experiments, WPs were pre-incubated with a single concentration of levosimendan for 15 minutes in the absence and presence of human albumin (Figure 8, Protocol 5). After pre-incubations, thrombin was added to induce platelet aggregation.

Protocol 5.

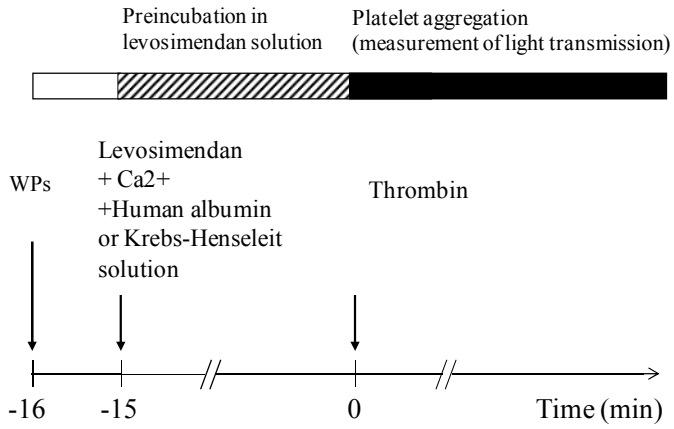

Figure 8. Protocol 5: Washed platelets (WPs, 0.6 mL) were placed into a 1 ml glass cuvette and were pre-incubated with human albumin (1.7, 3.3, and 5 g/L) or the corresponding volumes of Krebs-Henseleit solution and then 0.06 μmol/L levosimendan was added together with 1 mmol/L CaCl2. After 15 minutes, thrombin (0.1 IU/mL) was used to initiate platelet aggregation measured by the increase of light transmission in time.

2.9. Statistical analysis

Contractions and relaxations were expressed in mN. Data are presented as mean ± standard deviation or standard error of the mean (S.E.M.). For comparisons of the data, one-way analysis of variance followed by Newman-Keuls multiple range test were used. Statistical significance between two groups was tested with two-sided, paired Student's t-test, and p values less than 0.05 were considered as statistically significant.

Light transmission is an arbitrary unit, and is expressed as percent of actual control. KH solution was used as a control of WPs, and PPP was used as a control of PRP. The maximum increase in light transmission in the presence of different volumes of solvent was deduced from that obtained with the corresponding concentrations of levosimendan. Inhibition of platelet aggregation by the inodilator was expressed as percent decrease of the thrombin-induced maximal amplitude of aggregation. Results are expressed as mean ± S.E.M. For the calculation of inhibitory concentrations (ICs) of the drug, the y=(a*x)/(x+b) logistic equation was fitted to the individual concentration-response curves. The effective concentrations of levosimendan which caused 10, 25, and 50% inhibition of aggregation were defined as IC_{10}, IC_{25}, and IC_{50}, respectively. For statistical analysis, two-sided, paired Student's t-test or one-way analysis of variance followed by Newman-Keuls multiple range test (in case of experiments with albumin) were used. In all cases, a P value less than 0.05 was considered statistically significant.

2.10. Drugs and solutions

5-HT (serotonin creatinine sulfate complex), Ach (acetylcholine chloride), papaverine (papaverine hydrochloride), thrombin from human plasma, and prostacyclin sodium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). Levosimendan (From Orion-Pharma, Espoo, Finland for the isometric tension measurement study and from American Custom Chemicals Co., San Diego, CA, USA for the measurements of platelet aggregation) was dissolved in a solution containing 6 mmol/L NaOH and 160 mmol/L $Na_2HPO_42H_2O$ and further diluted in 0.9% NaCl. 5% human albumin (50 g/L human albumin, 4 mmol/L caprylate, 4 mmol/L acetyltryptophan, 145 mmol/L sodium, 2 mmol/L potassium) and Biseko® solution (50 g/L human serum protein [31 g/L albumin, 10 g/L human

immunoglobulin], 154.85 mmol/L sodium, 4.09 mmol/L potassium, 1.99 mmol/L calcium, 0.82 mmol/L magnesium, 100.56 mmol/L chloride) were obtained from Biotest (Hungaria Kft., Törökbálint, Hungary); the salts for Bretschneider solution (15 mmol/L NaCl, 10 mmol/L KCl, 4 mmol/L $MgCl_2$, 180 mmol/L histidine, 2 mmol/L tryptophane, 30 mmol/L mannitol, 1 mmol/L potassium dihydrogen oxoglutarate), KH solution (120 mmol/L NaCl, 4.2 mmol/L KCl, 1.5 mmol/L CaCl2, 20 mmol/L $NaHCO_3$, 1.2 mmol/L $MgCl_2$, 1.1 mmol/L KH_2PO_4, 11 mmol/L glucose and 0.27 μmol/L EGTA), calcium-free KH solution (120 mmol/L NaCl, 4.2 mmol/L KCl, 20 mmol/L $NaHCO_3$, 1.2 mmol/L $MgCl_2$, 1.1 mmol/L KH_2PO_4, 11 mmol/L glucose), and the anticoagulant, trisodium citrate, were obtained from Reanal (Budapest, Hungary).

2.11. Research funding

The present work was supported by the Hungarian Scientific Research Fund (OTKA T037520 and F-61222), the Hungarian Ministry of Health (ETT T-144/2001) and the Hungarian Academy of Sciences.

3. Results

3.1. Contractions of human isolated RA segments

3.1.1. Comparison of contractions of isolated RA and IMA bypass grafts induced by different contractile agents

In our study, we have measured the contractile capacity of isolated IMA and RA bypass grafts pre-incubated in 0.9% NaCl for 45 minutes according to Protocol 1 (see Figure 2). As it can be seen in Figure 9, receptor mediated contractions evoked by 5-HT and NA were larger in RA grafts than in IMA grafts. Non-receptor mediated contractions induced by low (5 mmol/L) and high (80 mmol/L) KCl were also enhanced in the RA samples. The length of both types of grafts was the same (3 mm). The average wet weight of RA rings was 19.3 ± 2.5 mg, while that of the IMA was 9.9 ± 0.8 mg. The ratio of average contractions expressed as RA (mN): IMA (mN) were the following:

5-HT: 3.9, NA: 3.9, 80 mmol/L KCl: 3.1, 5 mmol/L KCl: 8.2.

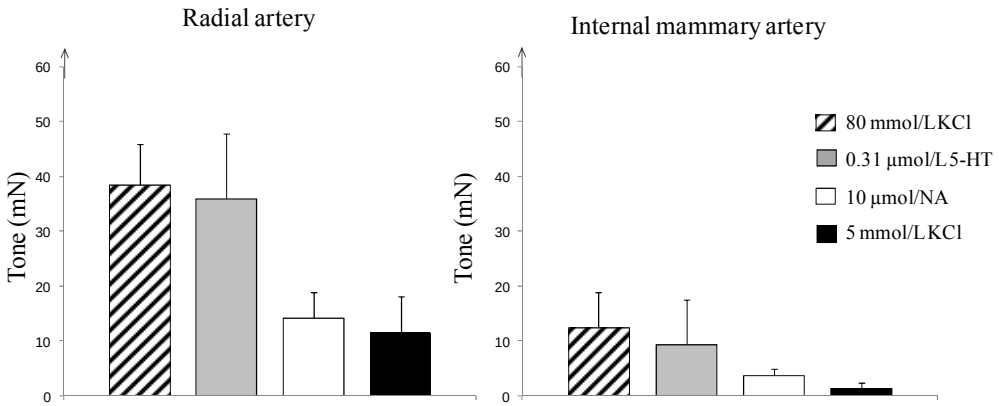

Figure 9. Contractions of isolated radial artery and internal mammary artery bypass grafts induced by different receptor mediated (5-HT: 5-hydroxytryptamine and NA: noradrenaline) and non-receptor mediated (KCl: potassium chloride) contractile agents. Radial artery samples (n=24) pre-incubated in 0.9% NaCl for 45 minutes (according to Protocol 1, see Figure 2) were used for the measurement of contractile capacity induced by various contractile agents. The same protocol was used for the internal mammary artery (n=18). Values represent mean ± standard error of the mean (S.E.M.). Tone was expressed in milliNewton (mN).

3.1.2. Contractile tensions induced by NA after pre-incubation of the grafts in 5% human albumin or Biseko® solutions

Figure 10 shows that pre-incubation in Biseko® solution decreased NA-induced contractions of RA bypass graft segments. At 100 µmol/L NA, the difference was significantly less in the RA rings incubated in Biseko® (32.9 ± 6.2 mN, n=14) than that incubated in the control colloidal solution, 5% albumin (49.2 ± 6.4 mN, n=14, p = 0.01 vs. Biseko®).

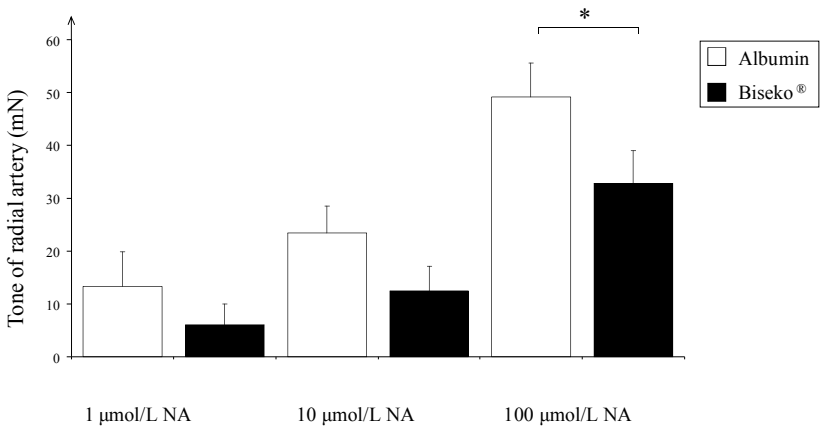

Figure 10. Isometric contractions of human isolated radial artery rings after pre-incubation with 5% human albumin or Biseko® colloidal solution. Radial artery rings were obtained from 14 individuals in both groups. Values are shown as mean ± standard deviation obtained from 7 individuals. Asterisks represent significant differences (* $p < 0.05$).

3.1.3. Contractile tensions induced by 5-HT 45, 90 and 120 minutes after pre-incubation of the grafts with levosimendan

Figure 11 shows a part of a representative tracing for the measurement of contractions of RA segments incubated in 0.16 µmol/L levosimendan solution or 0.9% NaCl. We found that the contractions were significantly decreased in case of RA rings pre-incubated in 0.16 µmol/L levosimendan solution compared to segments pre-incubated in 0.9% NaCl solution 45 and 90 minutes after the replacement of the solutions with KH solution.

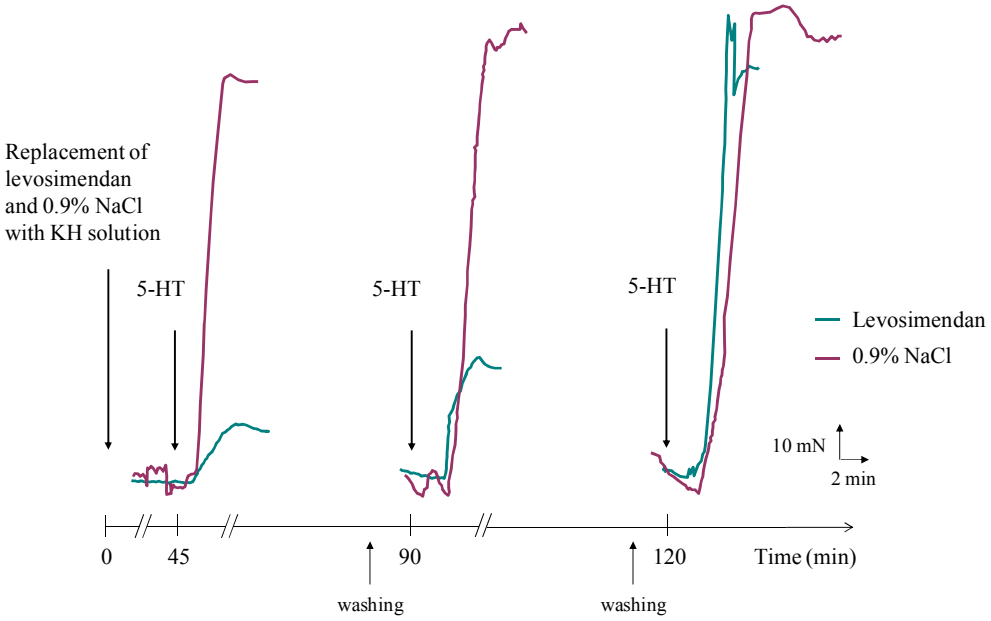

Figure 11. Part of a representative tracing for the demonstration of the prolonged effect of levosimendan. Two parallel radial artery rings from the same patient were investigated according to Protocol 1 (see Figure 2). Isometric tensions of human isolated radial artery grafts are depicted following 45 minutes pre-incubation with either 0.9% NaCl or levosimendan (0.16 µmol/L) solution. Contractions were induced by 5-hydroxytryptamine (5-HT, 0.31 µmol/L) 45, 90, and 120 minutes after the replacement of levosimendan solution with Krebs-Henseleit (KH) solution.

Contractions of RA segments incubated in 0.9% NaCl were stable in time (Figure 12). 45 and 90 minutes after the pre-incubation of the RA segments, the contractions were significantly decreased in case of RA rings pre-incubated in 0.16 μmol/L levosimendan solution compared to segments pre-incubated in 0.9% NaCl solution (0.16 μmol/L levosimendan solution = 6.0 ± 1.3 mN at 45 minutes vs. 0.9% NaCl = 40.0 ± 8.8 mN at 45 minutes, n=7 and 7, $p = 0.035$; 0.16 μmol/L levosimendan solution = 4.3 ± 3.8 mN at 90 minutes vs. 0.9% NaCl = 36.2 ± 2.6 mN at 90 minutes, n=7 and 7, $p = 0.03$). Contractions were not significantly different in the two groups at 120 minutes following the removal of the solutions (0.16 μmol/L levosimendan solution = 29.5 ± 18.0 mN at 120 minutes vs. 0.9% NaCl = 39.7 ± 3.8 mN at 120 minutes), although the RA segments obtained from more than half of the patients in the levosimendan group revealed smaller contractions compared to the control (see the large S.E.M. value of levosimendan at 120 minutes).

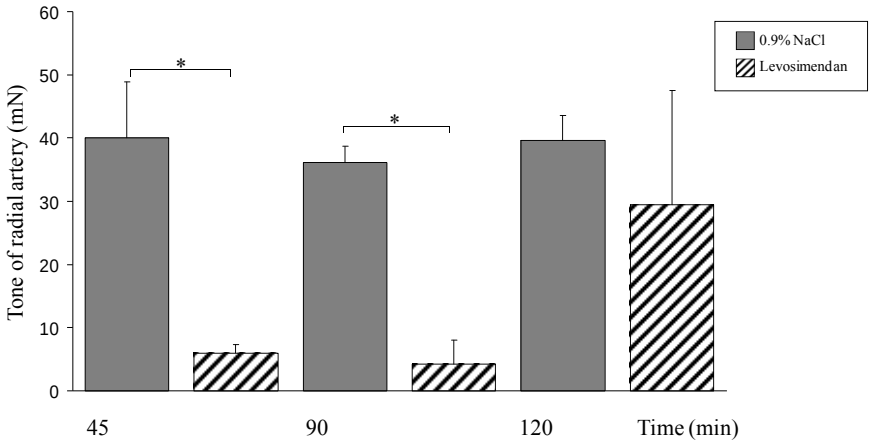

Figure 12. Isometric contractions of human isolated radial artery grafts 45, 90 and 120 minutes following the replacement of pre-incubation solutions – 0.9% NaCl or 0.16 μmol/L levosimendan solution – with Krebs-Henseleit solution. Contractions were induced by 5-hydroxytryptamine (5-HT, 0.31 μmol/L). Values are shown as mean ± standard error of the mean (S.E.M.) obtained from 7 individuals in both groups. Asterisks represent significant differences (* $p < 0.05$).

3.1.4. Contractile tensions induced by 5-HT 45, 90 and 120 minutes after pre-incubation of the grafts with different pre-incubation solutions as controls

Contractions were similar 45, 90, and 120 minutes after the removal of Bretschneider solution (33.4 ± 8.6 mN at 45 minutes, 34.1 ± 6.6 mN at 90 minutes, 41.4 ± 5.9 mN at 120 minutes n=12) and 5% albumin (37.9 ± 13.0 mN at 45 minutes; 30.6 ± 8.2 mN at 90 minutes; 35.1 ± 6.4 mN at 120 minutes, n=14). RA segments revealed significantly smaller contractions to 5-HT 45 minutes after washing out the colloidal Biseko® solution from the organ bath compared to those obtained after pre-incubation of the graft samples in 5% albumin (Biseko®= 16.3 ± 4.5 mN at 45 minutes vs. 5% albumin= 37.9 ± 13.0 mN at 45 minutes, n=14 and 14, p = 0.049).

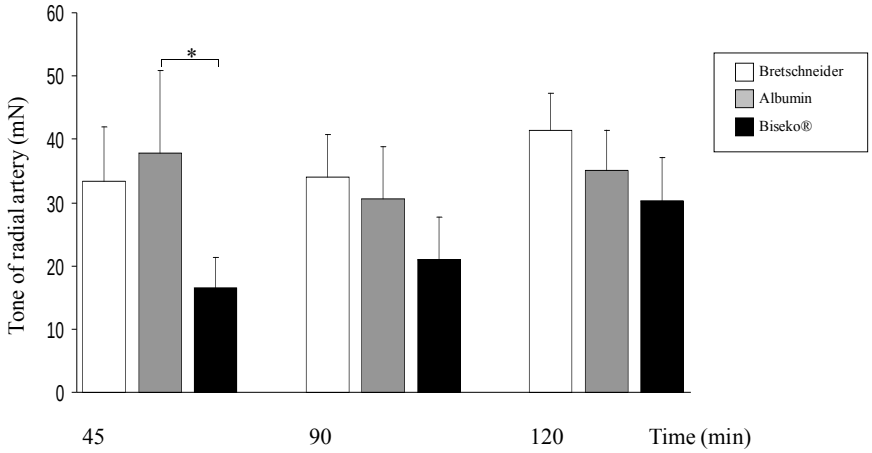

Figure 13. Isometric contractions of human isolated radial artery grafts 45, 90 and 120 minutes following the replacement of pre-incubation solutions - Bretschneider, 5% albumin or Biseko® solution - with Krebs-Henseleit solution. Contractions were induced by three consecutive administrations of 5-hydroxytryptamine (5-HT, 0.31 µmol/L). Values are shown as mean ± standard error of the mean (S.E.M.) obtained from 12 (Bretschneider) or 14 (5% albumin and Biseko®) patients. Asterisk represents significant difference (* $p < 0.05$).

3.1.5. Contractile tensions induced by KCl on RA grafts

Table 4. Maximum tensions of RA grafts obtained with 80 mmol/L potassium chloride (KCl)

	Pre-incubation solution	
Contraction (mN)	Levosimendan	0.9 % NaCl
Potassium chloride (80 mmol/L)	49.3±3.0 n=7	38.4±7.5 n=7

Contractions expressed as the maximal amplitude of the changes in tone. Values are shown as mean ± standard error of the mean (S.E.M.).
n= number of different individuals; mN= milliNewton

No significant differences were found between the reactivity of the arteries pre-incubated in 0.16 μmol/L levosimendan solution and in 0.9% NaCl.

3.2. Endothelium-dependent and independent relaxations on RA grafts

Table 5. Endothelium-dependent and independent relaxations on radial artery grafts

	Pre-incubation solution	
Endothelium dependent relaxation (mN)	Levosimendan	0.9 % NaCl
Acetylcholine (1.0 μmol/L)	-5.4±2.7 n=7	-6.5±4.6 n=7
Endothelium independent relaxation (mN)		
Papaverine (100 μmol/L)	-39.4±4.6 n=7	-48.8±10.7 n=7

Relaxations are expressed as the maximal amplitude of the changes in tone. Values are shown as mean ± standard error of the mean (S.E.M.). Minus represents a decrease of tone.
n= number of different individuals; mN= milliNewton

No significant differences in endothelium dependent relaxations were obtained among the arterial segments incubated in 0.16 μmol/L levosimendan or 0.9% NaCl solutions.

Papaverine almost completely relaxed the contractions evoked by 5-HT in the isolated RA segments. The magnitudes of the decrease of arterial tensions did not

significantly differ from each other: in 0.16 μmol/L levosimendan or 0.9% NaCl solutions.

3.3. Effect of the BK_{Ca} channel inhibitor, iberiotoxin, on levosimendan-induced relaxation in isolated human RA

We measured the isometric tension of RA segments from 4 patients undergoing CABG, and studied the inhibitory effect of BK_{Ca} channel selective potassium channel blocker, iberiotoxin, on the vasorelaxing effect of levosimendan. RA segments were pre-incubated in KH or 0.1 μmol/L iberiotoxin solution for 30 minutes. Vasorelaxing effect of levosimendan was examined in NA-precontracted (10-50 μmol/L) arterial rings. The responses of the RA segments of two patients (one of them is depicted in Figure 14) were relaxations to levosimendan without inhibition by iberiotoxin (patient No. 1 and No. 2) while another two patients (patient No. 3 and No. 4) responded with inhibitions to the BK_{Ca} channel inhibitor of inodilator induced relaxation.

The RA graft of patient No. 1 (Figure 14) revealed almost complete relaxation to the cumulative administration of levosimendan (maximal effect: 103.8% expressed of the maximum contractile effect of NA at 0.567 μmol/L levosimendan). Iberiotoxin did not considerably influence the maximal relaxation (108.4%) and even enhanced the effect of the inodilator. Patient No. 2. (57 years, male, non-smoking) responded in a similar way (maximal levosimendan relaxation of the control segment: 142.4%, maximal levosimendan of the segment pretreated with iberiotoxin: 144.2%). Figure 15 represents the response of patient No. 3, who revealed large relaxations to levosimendan (maximal levosimendan relaxation of the control segment: 148.0%). Iberiotoxin completely inhibited this relaxation (maximal levosimendan of the segment pretreated with iberiotoxin: 0.0%). The RA of patient No. 4. (59 years, male, non-smoking) also revealed a decrease of levosimendan-induced relaxation by iberiotoxin. The inodilator relaxed the artery with a maximum of 111%, and this effect was only 4.8% in the presence of the BK_{Ca} channel inhibitor.

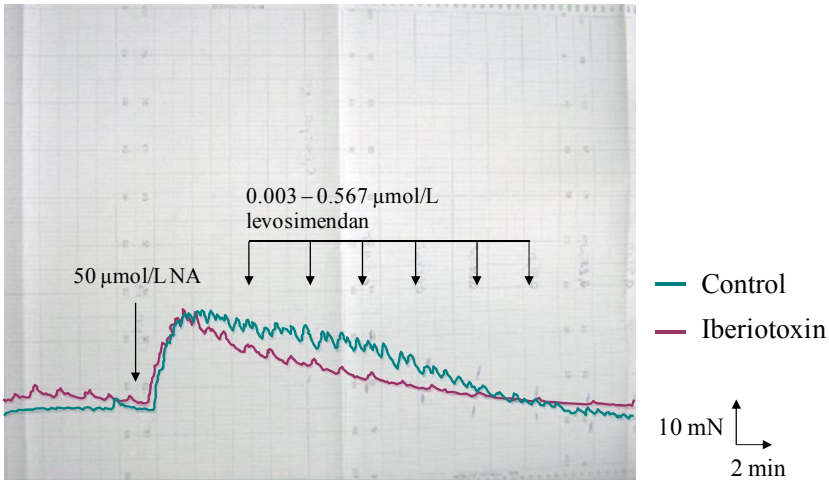

Figure 14. Original tracing for the demonstration of the lack of effect of iberiotoxin on the vasorelaxation induced by levosimendan. Isometric tension of human isolated radial artery graft segments from patient No. 1 (57 years, male, smoking for 32 years) following either pre-incubation with 0.1 µmol/L iberiotoxin or with the corresponding volume of Krebs-Henseleit solution for 30 minutes. Contractions were induced by noradrenaline (NA). After the development of NA-induced contraction, levosimendan was administered cumulatively.

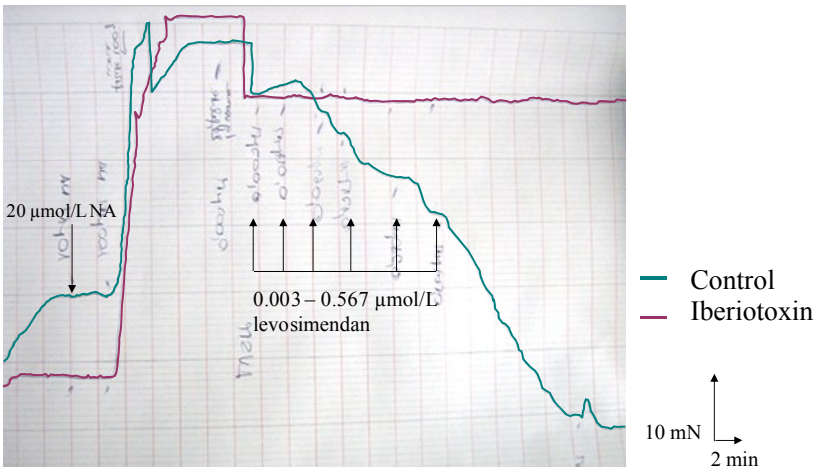

Figure 15. Original tracing for the demonstration of the effect of iberiotoxin on the vasorelaxation induced by levosimendan. Isometric tension of human isolated radial artery graft segments from patient No. 3 (75 years, female, non-smoking) following either pre-incubation with 0.1 µmol/L iberiotoxin or with the corresponding volume of Krebs-Henseleit solution for 30 minutes. Contractions were induced by noradrenaline (NA). After the development of NA-induced contraction, levosimendan was administered cumulatively.

3.4. Effect of levosimendan on thrombin-induced aggregation in PRP and WP suspension

Figure 16 demonstrates that levosimendan concentration dependently inhibited platelet aggregation induced by thrombin in PRP and WPs. In WPs, the maximum inhibition of aggregation was found to be 93.7±2.5% (n=8) at 0.3 μmol/L concentration of the drug. The IC_{50} was calculated as 0.06±0.02 μmol/L showing that the inodilator is a very potent inhibitor against thrombin in a plasma-free crystalline solution. The concentration-response curve of PRP showed a maximum of 96.4±4.1% (n=8) inhibition of aggregation at 0.52 μmol/L levosimendan. The IC values are summarized in Table 6. All calculated IC values of the drug were significantly smaller in WPs than in PRP revealing higher potency of the drug in the absence of plasma proteins. IC_{10} value of levosimendan was in the low submicromolar range even in the protein–rich PRP (0.07±0.01 μmol/L, n=8).

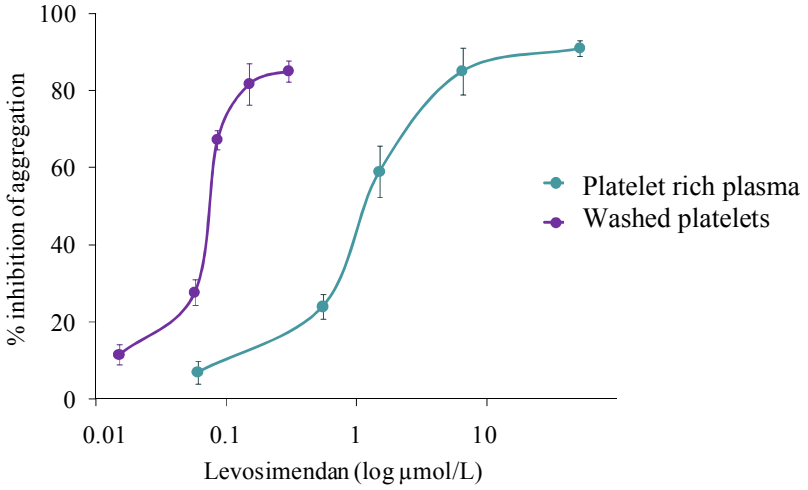

Figure 16. Inhibitory effect of levosimendan on the aggregation of washed platelets and platelet rich plasma. Results are expressed as mean±standard error of the mean (S.E.M.). Circles represent values obtained from 8 different individuals.

Table 6. Comparison of the potency values of levosimendan on platelet aggregation

Inhibitory concentrations (ICs) of levosimendan (μmol/L)			
	IC_{10}	IC_{25}	IC_{50}
Washed platelets	0.04±0.01 *	0.05±0.01 *	0.06±0.02 *
Platelet rich plasma	0.07±0.01	0.52±0.09	3.00±0.90

Aggregation agent: 1 IU/mL or 0.1 IU/mL thrombin in platelet rich plasma and washed platelets, respectively.

Results are expressed as mean±standard error of the mean (S.E.M.) and the number of experiments are 8 in each group. IC_{10}, IC_{25}, and IC_{50} are expressed as 10, 25, and 50% inhibitory concentrations of levosimendan on platelet aggregation, respectively. Asterisks represent significant differences (* $p < 0.05$).

3.5. Effect of pre-incubation with levosimendan on thrombin-induced aggregation in WPs in the presence of albumin

In order to clarify whether the presence of plasma proteins in PRP is responsible for the decreased efficiency of levosimendan, experiments were performed on WPs in the presence of human albumin. WPs were pre-incubated with the solvent of levosimendan (together with 1 mmol/L $CaCl_2$) for 15 minutes and the aggregation was induced by thrombin in the absence of human albumin (Figure 17). Under a control condition – with solvent of levosimendan – thrombin induced 98% aggregation compared to the light transmission of KH solution.

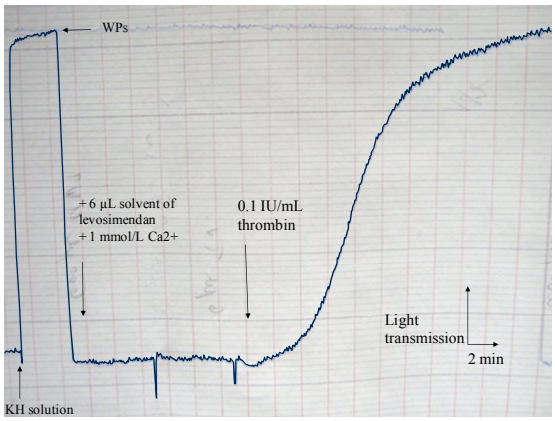

Figure 17. Original tracing for light transmission measurement of washed human platelets without treatment (control). Washed platelets (WPs) were incubated in Krebs-Henseleit (KH) solution for 15 minutes. Platelet aggregation was induced by

thrombin. Light transmission (100%) obtained with KH solution without platelets can be seen on the left side of the figure.

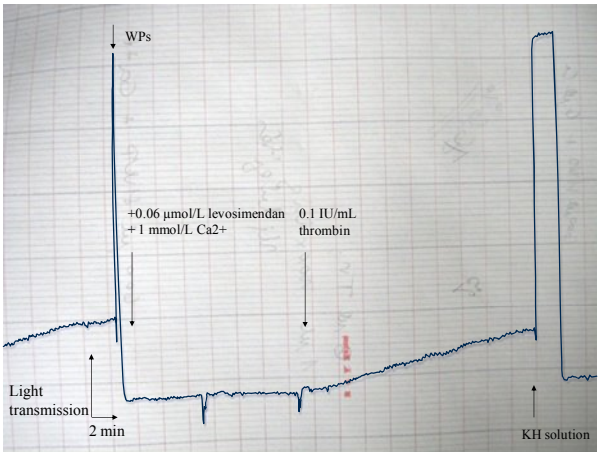

Figure 18. Original tracing for light transmission measurement of washed human platelets in the presence of levosimendan. Levosimendan and Ca^{2+} were administered to the washed platelets (WPs). Following 15 minutes pre-incubation, platelet aggregation was induced by thrombin. Pre-incubation with levosimendan decreased platelet aggregation to 23.4% (86.6% inhibition) compared to the maximum light transmission (100%) obtained with Krebs-Henseleit (KH) solution without platelets (right side of the figure).

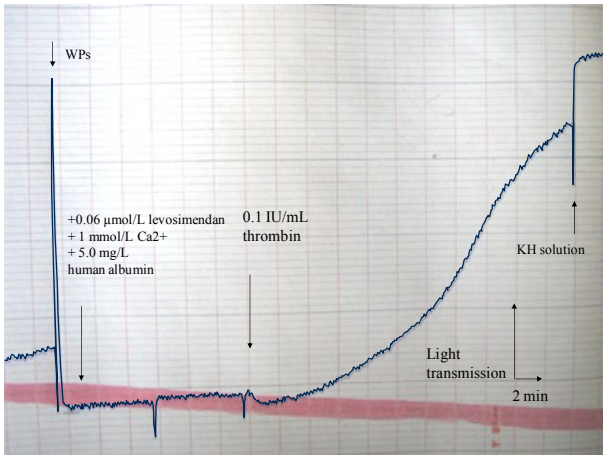

Figure 19. Original tracing for light transmission measurement of washed human platelets in the presence of levosimendan and human albumin. Levosimendan, Ca^{2+} and human albumin were administered to the washed platelets (WPs). Following 15 minutes pre-incubation, platelet aggregation was induced by thrombin. Platelet inhibitory effect of levosimendan (see Figure 18.) was decreased by albumin. Light transmission achieved 80.5% (19.5% inhibition) compared to the maximum light

transmission (100%) obtained with Krebs-Henseleit (KH) solution without platelets (right side of the figure).

When the IC_{50} concentration of the inodilator (0.06 µmol/L, see Table 6, WPs) was administered for 15 minutes instead of 2 minutes before the aggregation agonist, thrombin, the inhibition of platelet aggregation was considerably enhanced in the absence of albumin (see Figure 18). Figure 20 shows that enhancement of the incubation time from 2 minutes to 15 minutes increased the inhibitory effect of 0.06 µmol/L levosimendan to 83.6±2.7% (black column, n=6). The presence of albumin decreased the anti-aggregatory efficiency of levosimendan to about half in the presence of 5 g/L albumin (39.7±11.2%, n=6).

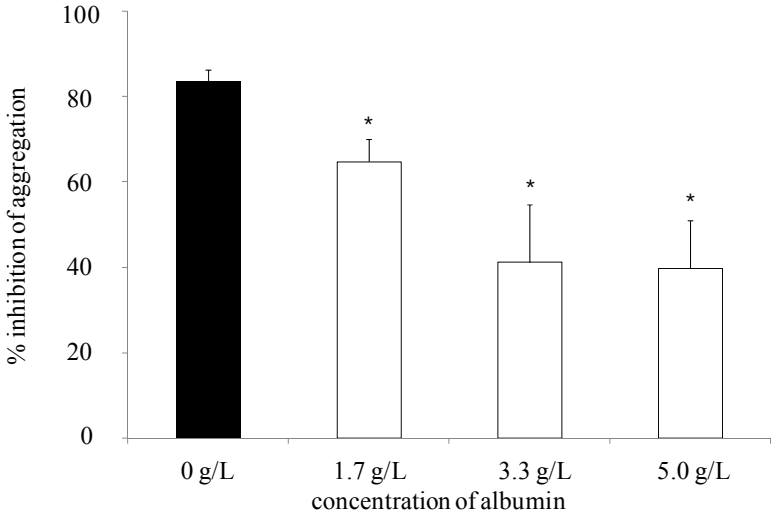

Figure 20. Effect of increasing concentrations of human albumin on levosimendan-induced inhibition of platelet aggregation. Washed platelets were pre-incubated with 0.06 µmol/L levosimendan, 1 mmol/L Ca^{2+} and increasing concentrations of albumin for 15 minutes prior to the administration of the aggregating agent, thrombin (0.1 IU/mL). Inhibition of platelet aggregation was calculated by deducing the light transmission obtained at different concentrations of albumin from that produced with Krebs-Henseleit solution (100%). Results are expressed as mean±standard error of the mean (S.E.M.). Columns represent values obtained from 6 different individuals. Asterisks represent significant differences compared to 0 g/L albumin (* $p < 0.05$).

4. Discussion

In the first part of our investigations, we demonstrated that RA grafts produced larger contractions than IMA grafts in vitro. For comparison, 5-HT, NA, and KCl were used as contractile agents. 5-HT derives from platelets that are known to be activated during CABG [48], and it has been demonstrated that serum NA level is also enhanced [49]. High KCl (80 mmol/L) is non-physiological and was used for detecting the maximal contractions in the present study. All types of contractions were higher in RA than in IMA. The difference in the contractile response is partly due to the higher muscle mass in RA than in IMA (RA:IMA=1.95:1). The response of RA to 5-HT and NA was larger compared to that of the IMA (RA:IMA=3.9:1, RA:IMA=3.9:1, respectively). He and Yang also found similar 3.8 times larger contractions of RA than IMA following administration of angiotensin II [25]. The receptor independent contraction with high KCl was also larger in RA than IMA, a result that has also been obtained by others [50]. In our study, contractions with high KCl showed a similar ratio (RA:IMA=3.1:1) as in case of receptor-mediated contractions suggesting that the effector pathway of contractions is more pronounced in RA than in IMA. Low KCl (4.2 mmol/L in KH solution + 5 mmol/L =9.2 mmol/L) can be considered as a physiological regulator of the arterial tone [51]. RA showed 8.2 times larger contractions than IMA to this low concentration of KCl. This augmented difference was an unexpected finding and may be due to a more effective vasodilatation by low concentration of potassium ion in IMA resembling an endothelium-derived hyperpolarizing mechanism [52]. The above results support the more frequent vasospasm of RA graft in the perioperative period of CABG [16,17,25,26].

We have shown a prolonged effect of levosimendan on the tone of isolated human RA segments. Pre-incubation of the graft with levosimendan diminished 5-HT-induced contraction, an effect that persisted for at least 90 minutes following the removal of the drug from the organ bath. The concentration of levosimendan applied during the pre-incubation procedure was 0.16 μmol/L, and this is in the range of therapeutic concentrations of the drug used to manage the human failing heart [39,53]. Levosimendan has both inotropic and vasodilating effects in this concentration [54-57]. It has been demonstrated recently that a single loading dose of the inodilator would normalize the hemodynamic conditions including total

peripheral resistance days after bypass graft surgery [35]. Active metabolite(s) with long half-life has been suggested to be responsible – at least in part – for this long-lasting effect of levosimendan in vivo [53]. However, our present observation in vitro provides evidence that the drug by itself and not its metabolite(s) is able to decrease the contractions of the RA graft.

In addition to the prolonged effect, levosimendan produced larger relaxations than it was expected from its concentration-response curve against 5-HT. Administration of the inodilator, after the contraction of the RA had been completed, resulted in an EC_{50} value of 0.3 μmol/L [31]. Based on the short half life of levosimendan (approximately 1 hour) [54], the relaxing effect was expected to decrease profoundly as late as after one and a half hour of the removal of the inodilator from the organ bath. In our study, 0.16 μmol/L levosimendan exerted 85% relaxation after 45 minutes and 88% after 90 minutes, an increasing rather than a decreasing vasorelaxing effect in time.

One possible mechanism of the prolonged effect of levosimendan is an activation of BK_{Ca} channels in the smooth muscle of RA. Therefore, we have studied the possible role of BK_{Ca} channels in the mechanism of action of levosimendan. Zhang et al. [36] have shown the presence and electrophysiological function of BK_{Ca} channels in human isolated RA smooth muscle. It is known that levosimendan has an activator effect of BK_{Ca} channels on porcine coronary arteries [37] and human IMA segments [37]. In RA samples of two patients, levosimendan-induced relaxation was markedly decreased by iberiotoxin, the selective inhibitor of BK_{Ca} channels, while in independent samples from two other patients, iberiotoxin was without any effect. BK_{Ca} channels were shown to have diminished expression and function in arteries with the increase of age [58], an explanation for the variable effect obtained in our isolated RA preparations. Thus, the heterogeneity of the function of BK_{Ca} channels does not support the consistent role of this ion channel in the prolonged effect of levosimendan in reducing contraction of RA segments. The exact mechanism of the maintained vasorelaxing effect of the inodilator remains to be investigated.

In this series of investigations, levosimendan completely relaxed NA-induced contractions. The effective concentration range of the inodilator in the above four different RA preparations was 0.003-1.14 μmol/L. This matches our previous finding obtained in 5-HT-induced contractions [34]. The exact mechanism in low

submicromolar concentrations of the inodilator on RA grafts is not known, although we cannot exclude that inhibition of phosphodiesterase (PDE) enzyme plays a role [58,59]. Other candidates – presented only at high concentration of levosimendan (10 μmol/L) – would be vascular mediators and effector signals including nitric oxide, endothelin-1, mitochondrial potassium channels, cyclicAMP, Akt, ERK, and p38 [60]. Concerning other potassium channels, ATP sensitive (K_{ATP}) and voltage sensitive (K_V) channels may also play roles[37,61].

Following the vascular investigations with levosimendan, we measured the effect of Biseko® colloidal solution – previously found to inhibit the contractions of RA grafts – against NA. Incubation in Biseko® solution rendered the RA grafts relatively resistant to contractions evoked by NA as levosimendan did it, albeit with possibly different mechanisms of action. Albumin, another colloidal solution, and Bretschneider, a cardioplegic solution did not change 5-HT-induced contractions in time. The difference between the effect of Biseko® solution and levosimendan was that the effect of the colloidal solution lasted only for 45 minutes. Pre-incubation in both Biseko® and levosimendan solutions was effective against both 5-HT- and NA-induced contractions and, in this respect, they appear to be superior to phenoxybenzamine, calcium antagonists, or nitrovasodilators. Phenoxybenzamine is highly effective in preventing α-adrenergic-mediated vasoconstriction for at least 18 hours, but its effect is limited to NA, and irreversibly suppresses the vasomotion of the conduit graft [24,61-65]. Calcium antagonists are relatively ineffective against receptor mediated contractions [31,32]. Nitrovasodilator therapy may be effective in relieving the established vasoconstriction, but may be less potent in preventing vasospasm [66]. Levosimendan is equally effective against NA, 5-HT, and thromboxane A2 analogue in conduit grafts and similar effectiveness by the drug was obtained in porcine coronary arteries partially depolarized with potassium ion [34,37,67]. These findings suggest that levosimendan is a "broad spectrum" antispasmodic agent that may serve an effective medication during pretreatment of the graft before implantation to the heart. However, it is important to note that neither pre-incubation in Biseko® solution, nor in levosimendan solution can cover the complete duration of the CABG, especially in the case of Biseko® solution.

At the end of the incubation study, the graft remained intact in the organ bath. The function of the RA grafts was not deteriorated by the treatments with levosimendan and with the different crystalloid and colloidal solutions. During the 120 minutes

duration of in vitro investigations on RA grafts, 5-HT-induced contractions remained stable in time. Following pre-incubation with levosimendan, the maximum contractile capacity, endothelial function, and maximum relaxation of the isolated RA segments were comparable to those obtained in physiologic saline (see Table 4 and 5). In this respect, levosimendan seems to be safer than papaverine that impairs endothelial function [29,61-63,68,69]. Furthermore, graft segments stored in heparinized whole blood improved endothelium-dependent relaxation to Ach [27], but these blood-stored RA grafts revealed markedly increased smooth muscle contractions [28].

The endothelium-dependent relaxation of the RA segments was small and did not exceed an average of about 20% compared to that evoked with papaverine (see Table 5). The explanation of this low endothelial function is that the diseases that determine the reactivity of the arterial wall (hypertension, hypercholesterolemia, diabetes) were found to be severe (see Table 3). This depressed endothelium was similar in the controls and in the levosimendan-treated grafts representing a poor cardiovascular status being comparable to our previous populations of patients undergoing CABG [30,31]. We suggest that the poor endothelial function was not due to a mechanical damage of the blood vessel (atraumatic ultrasound technique, no inflation of the artery) but rather represented a severely diseased population of our patients compared to other centers of cardiac surgery [70-72].

Finally, we have investigated the effect of levosimendan on platelet aggregation, known to play a role in the initiation of thrombus formation, another possible cause of intraoperative graft failure.

The important finding of the present study is that the inodilator drug was able to inhibit the aggregation of thrombin-stimulated platelets. In WPs, without plasma proteins, the calculated IC_{50} value of the drug was 0.06 μmol/L, while in PRP it was 3 μmol/L, suggesting that some of the effective concentrations of the drug in inhibiting thrombin-induced aggregation of human platelets matched the therapeutic level of the drug.

In PRP, the 10% IC of levosimendan (IC_{10}=0.07 μmol/L) is at the lower level of the in vivo range with peak concentrations between 0.12-0.2 μmol/L [43,53].

Kaptan et al. [39] found 40% inhibition of human platelet aggregation by 0.09 μmol/L concentration of the inodilator drug against ADP and collagen. However, they used longer incubation time for the drug (15 minutes) in whole

blood and lower platelet count after the preparation of PRP. Levosimendan is known to highly bind to plasma proteins (97-98%) [43]. Because PRP contains the total amount of plasma proteins, we proposed that in protein-free WPs, a much lower potency of the inodilator would have been detected. Following the removal of plasma proteins, the EC_{50} of levosimendan became 50 times lower in WPs (0.06 μmol/L). Albumin concentration dependently enhanced the anti-aggregatory efficiency of levosimendan. 5 g/L albumin, which is about 6 times less as the physiological concentration (≥ 36 g/L) decreased the effect of the drug by about half (form 83.6% to 37.9% inhibition of platelet aggregation). This result suggests that among plasma proteins, levosimendan mainly binds to albumin.

Hypoalbuminemia is relatively frequent in heart failure [73,74] being the main indication for the use of levosimendan. In about 20% of patients with acute decommpensaiton of heart failure, the concentration of albumin is decreased by an average of 3-4 g/L [75]. Under this condition, the free concentration of the inodilator and the consequent enhancement of the anti-aggregatory effect would be expected.

In the studies with albumin, we used 15 minutes pre-incubation of levosimendan with platelets similar to Kaptan et al. [39]. The IC_{50} value of the drug in WPs (0.06 μmol/L after 2 minutes of incubation) increased to more than 80% after 15 minutes pre-incubation, a similar finding obtained in RA grafts revealing larger effect of the inodilator with the increase of incubation time. These observations propose the accumulation of the drug or its effect both in the vascular tissue and in platelets.

Levosimendan decreases platelet aggregation, which is not specific for a particular receptor on platelet membranes. It decreases ADP-, collagen-, and thrombin-induced aggregations in therapeutically acceptable concentrations. Although the precise mechanism is not known at present, intracellular calcium could be the target of levosimendan, which plays a central role in thrombin-induced aggregation [76-78]. The broad-spectrum anti-platelet effect of the inodilator would render the drug effective in aspirin- and clopidogrel-insensitive conditions having a prevalence of 5.5-29.9% and 25% of patients with cardiovascular diseases, respectively [78-80].

Activation of the coagulation system and enhanced thrombin level, especially during on-pump CABG has been demonstrated [81]. Therefore levosimendan, in

addition to preventing RA spasm, may reduce the incidence of thrombus-formation during the intraoperative phase of CABG.

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