Positive Inotropic Effects of Levosimendan are Modulated by KATP Channels in Isolated Human Atrial Trabeculae

Levosimendanın Pozitif İnotropik Etkileri İzole İnsan Atriyal Trabekülasında KATP Kanalları Tarafından Modüle Edilir

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Geliş Tarihi/*Received:* 10.11.2009
Kabul Tarihi/*Accepted:* 17.05.2010

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ABSTRACT Objective: The aim of this study was to investigate whether potassium channel blocking agents were able to modulate positive inotropic effect of levosimendan in isolated human atrial trabecular muscles or not. **Material and Methods:** The right atrial appendage samples (1 cm^2, 500-1000 mg) were removed and immersed in preoxygenated and modified Tyrode's solution. Preparations were used to investigate the concentration-effect relation of levosimendan (10-9 to 10-4 M) alone or in the presence of Ca2+-dependent potassium channel blocker 4-aminopyridine (4-AP: 500 μM), ATP-dependent potassium channel blocker glibenclamide (1 μM) or mitochondrial ATP-dependent potassium channel blocker 5-hydroxydecanoate (5-HD: 300 μM) on percent developed tension (%DT). **Results:** Levosimendan produced concentration-dependent increments in %DT. Both the sensitivity (pD2) and maximum response (Emax) of human atrial trabeculae to levosimendan (7.31 ± 0.02 and 29.2 ± 1.1 mN, respectively) significantly and similarly reduced in presence of glibenclamide (5.83 ± 0.04 and 17.4 ± 1.61 mN) and 5-HD (6.14 ± 0.05 and 18.5 ± 3.1 mN). However, 4-AP did not cause a significant alteration in sensitivity (pD2= 6.77 ± 0.05) or Emax (27.6 ± 2.0 mN) to levosimendan. **Conclusion:** Both sarcolemmal and mitochondrial ATP-dependent potassium channels are implicated to modulate positive inotropic effect of levosimendan in human atrial trabeculae.

Key Words: Simendan; potassium channels; cardiotonic agents

ÖZET Amaç: Bu çalışmanın amacı izole insan atriyal trabeküler kasında potasyum kanal bloke edici ajanların levosimendanın pozitif inotropik etkilerini modüle edip edemediğini araştırmaktır. **Gereç ve Yöntemler:** Sağ atriyal apendiks örnekleri (1 cm^2, 500-1.000 mg) alındı ve önceden oksijenize edilmiş modifiye Tyrode solüsyonuna konularak organ banyosuna asıldı. Levosimendanın konsantrasyon-etki ilişkisi tek başına (10-9 ile 10-4 M), Ca2 -bağımlı potasyum kanal blokörü 4-aminopiridin (4-AP: 500 μM), ATP-bağımlı potasyum kanal blokörü glibenklamid (1μM) veya mitokondrial ATP-bağımlı potasyum kanal blokörü 5-hidroksidekonat (5-HD: 300 μM) ile birlikte oluşan kasılma gücü araştırılmak amacıyla preparatlar hazırlandı. **Bulgular:** Levosimendan % kasılma gücünde konsantrasyon-bağımlı artışa neden oldu. İnsan atriyal trabekülasının levosimendan için hem sensitivitesi (PD2) hem de maksimum yanıtı (Emax), glibenklamid (5.83 ± 0.04 ve 17.4 ± 1.61 mN) ve 5-HD (6.14 ± 0.05 ve 18.5 ± 3.1 mN) varlığında benzer düzeylerde anlamlı derecede azaldı (sırasıyla 7.31 ± 0.02 ve 29.2 ± 1.1 MN). Ancak 4-AP varlığında levosimendan hassasiyetinde (PD2= 6.77 ± 0.05) veya Emax değerinde (27,6 ± 2,0 mN) anlamlı bir değişiklik olmadı. **Sonuç:** Her iki sarkolemmal ve mitokondrial ATP-bağımlı potasyum kanallarının insan atriyal trabekülasında levosimendanın pozitif inotropik etkisini modüle ettiğine işaret edilmektedir.

Anahtar Kelimeler: Simendan; potasyum kanalları; kardiyotonik ajan

Turkiye Klinikleri J Med Sci 2010;30(5):1476-81

Levosimendan is a newly proposed inotropic drug (Ca^{2+} sensitizer) that improve sensitivity of cardio-myofilaments to Ca^{2+} by selectively binding to troponin C.[1] Myocardial contractility is accordingly enhanced without increasing intracellular Ca^{2+} or stimulating ß-adrenoreceptors, and phosphodiesterases are not substantially inhibited so that favorable clinical effects can be exerted by levosimendan without increasing myocardial oxygen consumption or arrhythmias in contrast to catecholamines.[2,3] Thus this drug is recommended in acute and decompensated heart failure treatment thank to its with positive inotropic and anti-stunning effects mediated by calcium sensitization of the contractile proteins.[4,5]

Besides increasing the strength of cardiac contractions, levosimendan induces coronary and peripheral vasodilatation through the opening of sarcolemmal ATP-dependent K^+ channels,[6,7] an effect reported also in rat atrial and ventricular myocytes.[8,9] Mitochondrial ATP-dependent K^+ channels in preparations of rat liver and heart are opened by levosimendan, as well.[10-12]

Since opening of mitochondrial ATP-dependent K^+ channels through transmembrane K^+ flux mainly regulates mitochondrial matrix volume[13] and may interfere with fine-tune processes intimately connected to contractility,[10,13-16] we investigated whether potassium channel blocking agents were able to modulate positive inotropic effect of levosimendan on isolated human atrial trabeculae contracting isometrically *in vitro or not* , as a model to study both positive and negative inotropic effects of drugs.[17,18]

MATERIAL AND METHODS

SUBJECTS AND PATIENTS

Informed consent was obtained from 22 adult patients who underwent elective open heart surgery for coronary artery disease or rheumatic valve lesions. These patients were routinely taking cardioactive drugs according to standard prescriptions. Ethical considerations prevented us from discontinuing the drugs >24 h before surgery. Exclusion criteria were arrhythmias, congestive heart failure, dilated heart and administration of antiarrhythmic or oral hypoglycemic medication.

PREPARATION

Premedication consisting of midazolam (0.03 mg/kg) was administered intravenously 10 min before induction of anesthesia using target controlled infusion (TCI) of propofol (site effect 1.6 mg/ml), sufentanil (0.35-0.5 mg/kg) and rocuronium (0.6 mg/kg). Following tracheal intubation, the lungs were mechanically ventilated with an oxygen/air mixture (FiO_2 ~ 40%). The maintenance of anesthesia was obtained with propofol in TCI, sufentanil (0.35 mg/kg/h) and supplemental boluses of rocuronium.

Cardiac surgery was performed during cardiopulmonary bypass. Samples of right atrial appendages were obtained during cannulation for cardiac surgery. The methods to obtain human atrial trabeculae working isometrically *in vitro* have been previously described in detail.[17-19] Briefly, a sample of the right atrial appendage (1 cm^2, 500-1000 mg) was removed and immersed in preoxygenated and modified Tyrode's solution (in mM: NaCl, 120; KCl, 4; $CaCl_2$, 2.7; $MgCl_2$, 1.1; $NaHCO_3$, 25.7; Na H_2PO_4, 1.8; and glucose, 11) at 22 ºC. The time between excision and beginning of laboratory processing was 1-5 min. The sample was gently pinned down in a chamber with oxygenated modified Tyrode's solution gassed with a 95% O_2 and 5% CO_2 mixture, leading to pO_2 of, 640±20 mmHg, measured at 755 mm Hg barometric pressure and with a pH 7.4 ± 0.1. The sample was cut into two to three pieces containing free-running trabeculae (pectinate muscle); macroscopically damaged tissue was discarded. In the organ bath, the preparation was warmed gradually (circulating thermostat-regulated bath) over a period of 60 min up to 37ºC (continuously monitored). The base of the pectinate muscle was fixed to the chamber floor with fine stainless steel pins, and the opposite end was connected to a pre-calibrated force transducer via a stainless steel hook. The muscle was stimulated by 1-ms square pulses delivered from an orthorhythmic stimulator at 2 mA after the diastolic

threshold intensity was measured (between 0.5 and 1.5 mA) via a bipolar Teflon-coated 99.99 % silver-wire electrode (0.375 mm in diameter) placed on the muscle surface. The chamber received incoming oxygenated solution at 5 ml/min by a single headed peristaltic pump. The preparation was made to contract isometrically and stretched to the peak of its length-tension curve (L_{max})(11.3 ± 0.9 mN). Muscle length remained at L_{max} throughout the experiment. Baseline force development at L_{max} in oxygenated and thermostatically controlled Tyrode's solution was obtained after stabilization (60 min) at 1.000-ms (1 Hz) cycle length. The same stimulation rate was continued during the study. The data were monitored on a digital memory oscilloscope (Tektronix 2230, Tektronix Inc., Beaverton), digitized at a sampling frequency of 8 k Hz and stored on computer. The software automatically measured resting tension (or preload, in mg) and developed tension (DT in mg). After completion of the study, the muscle was dried, the base used to fix it to the chamber floor was cut off and the actively contracting portion weighed on a precision scale.

EXPERIMENTAL PROTOCOLS

To determine time-dependent loss in contractility, spontaneous DT changes of all 22 atrial trabeculae were followed up with frequent data acquisitions, to 60 min after stabilization, during superfusion with modified and gassed Tyrode's solution at 37 ºC.

In oxygenated Tyrode's solution, preparations were used to investigate the concentration-effect relation of levosimendan on %DT and in the presence of 4-aminopyridine (4-AP) (Ca^{2+}-dependent potassium channel blocker, 500 mM) or glibenclamide (sarcolemmal and a specific mitochondrial ATP-dependent potassium channel blocker, 1 mM) or 5-hydroxydecanoate (5-HD) (specific mitochondrial ATP-dependent potassium channel blocker, 300 mM).[18] Levosimendan (Abbott, Roma,Italy) was superfused for 15 min at incremental concentrations, from 10^{-9} to 10^{-4} M. Potassium channel blockers were added to superfusion solution 15 min prior to the beginning of experiment and continued thereafter. The effects of 4-AP (500 mM), glibenclamide (1 mM) and 5-HD (300 mM) on basal DT were also studied. Levosimendan was obtained from Abbott (Roma). 4-AP, 5-HD and glibenclamide were purchased from Sigma Chemical (St.Louuis, Mo.) 4-AP and 5-HD were dissolved in the distilled water. Glibenclamide was dissolved in distilled water containing 20% ethanol and 20% dimethylsulphoxide. Incubation with ethanol and dimethylsulphoxide had no significant effects on the on developed tension in isolated human atrial trabeculae (Data not shown).

Statistical analysis

The contractile responses were expressed as mN of tension and percent changes were calculated, considering those obtained at 60 min after stabilization as the baseline. Data in table and figure are expressed as mean ± standard error of the mean (SEM), and n indicates the number of human atrial preparations. The logarithm of the concentration of drugs, which elicited a 50 % maximal response, was designed as the EC_{50}. These values were determined by regression analysis of the linear portions of the log concentration-response curves. Sensitivity was expressed as pD_2 (-log EC_{50}). Statistical analysis of the results was performed using Student's t test (SPSS for windows, Ver. 11.0, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

RESULTS

PATIENT'S CHARACTERISTICS

Of the 22 patients who underwent elective open heart surgery, 77% had coronary artery disease and 23% rheumatic valve lesions. There were 86% men and 14% women, who ranged from 43 to 75 years of age (64 ± 2).

EFFECT OF LEVOSIMENDAN ON DEVELOPED TENSION (DT) IN ISOLATED HUMAN ATRIAL TRABECULAE

Superfusion of glibenclamide (1 mM), 4-AP (500 mM), or 5-HD (300 mM) for 15 min did not cause significant alterations in baseline DT. Baseline DT in levosimendan (n= 9), levosimendan + glibenclamide (n= 5), levosimendan + 5-HD (n= 5) and

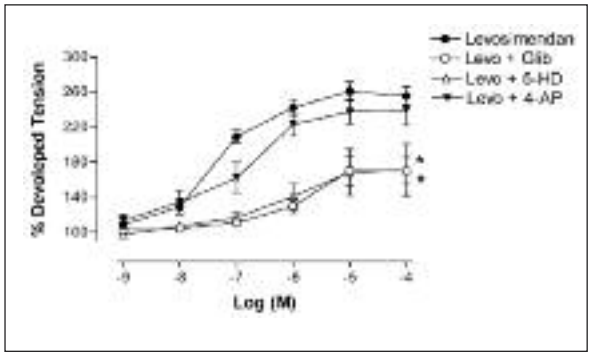

FIGURE 1: The concentration-response curves for levosimendan alone (n=9) and in the presence of 1 μM glibenclamide (Glib, n=5), 300 μM 5-hydroxydeconate (5-HD, n=5), or 500 μM 4-amynopyridine (4-AP, n=3) on developed tension (percentage of basal tension) in human atrial trabeculae. *: P< 0.05, as compared with levosimendan.

levosimendan + 4-AP (n= 3) preparations were 11.2 ± 0.9, 10.2 ± 1.8, 10.8 ± 2.2 and 11.5 ± 1.4 mN, respectively (Table 1).

Levosimendan (10^{-9}-10^{-4} M) produced concentration-dependent increments in baseline DT. There were significant reductions in both sensitivity and maximum response of human atrial trabeculae to levosimendan in presence of glibenclamide and 5-HD. However, 4-AP did not cause significant alterations in sensitivity and maximum response to levosimendan (Figure 1, Table 1).

DISCUSSION

Levosimendan expectedly exhibited a potent inotropic effect in isolated human atrial trabeculae in accordance with our earlier findings.[18] It is well known that levosimendan increases Ca^{+2} sensitivity of the heart by myofibrillar Ca^{+2} sensitization via Ca^{+2}-dependent stabilization of the Ca^{+2}-bound conformation of cardiac troponin C[1,20,21] without affecting the Ca^{+2} affinity of troponin C. Phosphodiesterase inhibition may also play a role in the cardiac effect of levosimendan.[22,23] Accordingly, it has been noted that the activity of phosphodiesterases may prevent intracellular cAMP accumulation following levosimendan application in both human and guinea-pig myocardium.[24]

In this experiment, positive inotropic effects induced by levosimendan in human atrial trabeculae were significantly inhibited by both ATP-dependent potassium channel blockers glibenclamide and 5-HD, but not by Ca^{2+}-dependent potassium channel blocker 4-AP. The partial inhibition of the positive inotropic effect of levosimendan by these agents may indicate a new role for ATP-dependent potassium channels in the mechanisms whereby levosimendan induces positive inotropism in human heart. A similar interaction between levosimendan and ATP-dependent potassium channels was also observed in rat atrial and ventricular myocytes[8,9] which was attributed to its beneficial effects on ischemic myocardium and infarct size in coronary-ligated animals.[9,25,26]

It is generally considered that mitochondria regulate cardiac cell contractility by providing ATP for cellular ATPases and by participating in Ca^{2+} homeostasis, although other important roles in mitochondrial physiology such as ATP-dependent potassium channel regulation of volume may be instrumental in fine-tuning inotropism.[10,13-16] Thus mitochondrial matrix volume may control the rate of oxidation through the respiratory chain[13] or ensure an optimal spatial arrangement of intermembrane proteins involved in energy transfer between mitochondria and cytosol.[14,15] However, Kaasik et al. have recently proposed a third mechanism of regulation of cardiac contractility by mitochondrial functional state (and volume) which is independent from the ATP-generating activity of

TABLE 1: Baseline developed tension (DT, mN), maximum response (Emax, mN) and pD2 (-Log EC50) values for levosimendan (n=9), levosimendan + 4-AP (n=3), levosimendan + glibenclamide (n=5) and levosimendan + 5-HD (n=5) in human atrial trabaculae.

	Levosimendan	Levosimendan + 4-AP	Levosimendan + glibenclamide	Levosimendan + 5-HD
Baseline DT	11.2 ± 0.9	11.5 ± 1.4	10.2 ± 1.8	10.8± 2.2
Emax	29.2 ± 1.1	27.6 ± 2.0	17.4 ± 1.61*	18.5 ± 3.1*
PD_2	7.31 ± 0.02	6.77 ± 0.05	5.83 ± 0.04*	6.14 ± 0.05*

*: P < 0.05 as compared with levosimendan group.

mitochondria or Ca^{2+} homeostasis.[16] It was shown that various conditions that increase K^+ accumulation in the mitochondrial matrix such as activation of ATP- or Ca^{2+}-dependent K^+ channels induce similar increases in Ca^{2+}-dependent and independent isometric force development in rat ventricular fibers.[16] In proposing a role for mitochondrial volume as a regulator of myofibrillar function the possibility of a physical interaction of mitochondria with myofibrils participating in the beat-to-beat regulation of force during shortening and lengthening of cardiac cells was pointed out; the pathophysiological consequences of this may be far reaching when one considered that agents aimed at controlling transmembrane ionic fluxes through the mitochondrial membrane might provide valuable tools for modulating cardiac contractility.[16] Since levosimendan was shown to increase ATP-sensitive potassium flux to the mitochondrial matrix in preparations from rat liver[11] and heart,[12] it may be speculated that such a levosimendan-induced increase in potassium influx to mitochondrial matrix through ATP-dependent potassium channels might play a role in the positive inotropic effects, as observed on human atrial trabeculae inasmuch as the specific mitochondrial ATP-dependent potassium channel blocker 5-HD was partially inhibitory. However, a similar inhibition of levosimendan positive inotropic effects was also observed with the nospecific agent glibenclamide which deserves further study. Recently, Loubani et al. showed that glibenclamide abolished the protective effect of mitochondrial ATP-dependent potassium channel opening in human right atrial appendages.[27] Taken together, these data point to a critical role of mitochondrial ATP-dependent potassium channel in enabling the full development of inotropic effects of levosimendan whereas Ca^{2+}-dependent potassium channels donot seem to contributory.

In summary, levosimendan produced concentration-dependent positive inotropic responses in human atrial trabeculae which were significantly inhibited by ATP-dependent potassium channel blockers. It is concluded that positive inotropic effects of levosimendan in human atrial trabeculae are at least in part mediated by mitochondrial ATP-dependent potassium channels.

Acknowledgement

This study was supported by Akdeniz University Research Projects Unit. It was performed during the tenure of a research Fellowship (C.U.) and in partial fulfillment of the MURST Research Projects 106/2000, 16661/2003 and 23161/2004 in La Sapienza University.

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