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# EXPERIMENTAL STUDIES FOR THE EVALUATION OF ISCHEMIC PRECONDITIONING MECHANISMS IN RAT HEART

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**ABSTRACT.** Ischemic preconditioning (IPC) induced by administration of brief episodes of ischemia-reperfusion represents a protective mechanism of the heart against prolonged episodes of ischemia. The mechanism of ischemic preconditioning is far from being elucidated; there are hypotheses involving the activation of the family of protein kinase C (PKC) and also the opening of the KATP channels. Our study was designed to investigate the involvement of KATP channels and PCK activation in the mechanism of ischemic preconditioning of rat heart perfused in Langendorff system using activators of PKC (1,2-dioctanoil-sn-glycerol -DOG) and KATP channels (cromakalim) and inhibitors of PKC (cheleritrine-CHE) and of KATP channels (glibenclamide-GLY). There was an improvement of LVDP in ischemic-preconditioned hearts also a reduction of infarct size vs. controls; using the activators and inhibitors of KATP channels and PCK, it has been demonstrated that these two are involved in the pathway of the ischemia-reperfusion mechanism.

**Keywords:** ischemic preconditioning, ischemia-reperfusion, infarct size, Protein kinase C, Katp channels

## INTRODUCTION

Ischemic preconditioning is an adaptive mechanism of cardiomyocyte resistant to sustained ischemic episodes after applying repeated cycles of ischemia-reperfusion, the mechanism is theoretically present in many species of animals, but research studies in this field failed to completely clarify the problem. The signaling involved in IPC is complex, and implicates a number of known survival signaling pathways such as PI3K/AKT, ERK1/2, JAK/STAT, p38, and PKC. Classic ischemic preconditioning, originally described by Murry et al, (1986) involves short sub lethal ischemia-reperfusion periods prior to a lethal ischemic injury. Paradoxically, this initiates redox signaling sufficient to generate ROS, which can act in combination with other effectors of IPC, to signaling survival (Baines et al., 1997). However, the ROS generated by this stimulus is not damaging unlike that generated by prolonged lethal ischemia (Baines et al., 1997). The minimal stress of the IPC stimulus can trigger the release of ligands such as adenosine, opioids and bradykinin (Gross et al, 2006; Cohen et al, 2008). When these ligands bind to their GCPR, a signal for survival is promoted; for example perfusion of adenosine or an adenosine receptor agonist prior to lethal ischemia confers a level of protection equal to that of IPC (Liu et al., 1991). Furthermore, in isolated rabbit hearts perfusion with the adenosine receptor blocker, 8-sulfophenyl theophylline (8SPT) can abolish the protection conferred by IPC (Liu et al., 1991). The balance of pro survival and pro apoptotic cellular factors contributes to homeostasis. Therefore, survival from a cellular insult, such as ischemia-reperfusion injury may be achieved by modulating these pathways in order to confer cardioprotection.

Cardioprotection using IPC has been shown to trigger opening of the K+ATP channels (Hausenloy et al., 2002), however, their role in IPC is still debated (Ferdinandy et al., 2007). It has been hypothesized that opening of the K+ATP channels is associated with a delay to mitochondrial permeability transition pore (mPTP) opening however, the precise mechanism is unknown (Gross et al., 2008). Preconditioning can be induced pharmacologically without sub lethal ischemia-reperfusion stimuli. The presence of pharmacological cardioprotective agents administered prior to lethal ischemia or at the onset of the lethal reperfusion can confer protection against ischemia–reperfusion injury. Such agents are known as preconditioning and post conditioning mimetics respectively. Activators of PI3K/AKT such as adenosine, bradykinin, opioids and also insulin (a tyrosine receptor ligand) confer protection against lethal ischemia-reperfusion injury; mimicking IPC (Maddock et al., 2002; Bell et al., 2003; Gross et al., 2006). Other drugs targeting specific proteins can salvage tissue from ischemia-reperfusion, for example, the presence of diazoxide, a mitochondrial $K^+$-ATP channel opener. In addition this cardioprotective agent can activate the PKC isoform PKCε. Diazoxide is reported to instigate PKCε translocation from the cytosol to the mitochondria; where in the presence of hypoxia it can prevent Ca2+ accumulation, membrane depolarization and cytochrome c release (Kim et al., 2006). The mechanism of PKC activation during ischemic preconditioning is also poorly known, a number of potential mediators of PKC activation has

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been described in experimental studies carried out on myocardium from rat, dog, rabbit and man among these mediators include: Nitric Oxide (NO), Reactive Oxygen Species (ROS), Diacylglycerol (DAG) (Tong et al., 2000)

Experiments conducted on rat myocardium showed that there are several isoforms of PKC which have been divided into three categories: classic, new and unusual, the difference between them being on the need of the presence of different activators (Kawamura et al., 1998). The classic PKC group contains PKC-α which requires for activation the presence of Ca2+, phosphatidylserine, DAG; the new PKC group contains PKC δ and PKC-ε, their activation is done in the presence of DAG and phosphatidylserine; the group of atypical PKC includes PKC-ζ which require for activation only the presence of phosphatidylserine (Nishizuka, 1992). A number of studies have evaluated the role of KATP channels in ischemic preconditioning mechanism (C.R. Revnic et al., 2009); there were described two types of KATP channels involved: sarcoplasmic (sarc-KATP) and mitochondrial (mito-KATP). K+ channels are a group of ionic channels responsible for physiological effects upon cellular processes: i.e. the drugs which open this type of channels have a great clinical potential. K+ channels are complex protein systems bound on cell membrane, with regulatory functions such as modulation of resting membrane potential and the length of action potential in neurons, myocyte and endocrine cells. The efflux of K+ ions as a consequence of channels opening leads to hyperpolarization or repolarization of membrane potential. This effect leads to prevention or reversing membrane depolarization activity and a decrease in cellular excitability. The term “K+ channels opener” has been associated with a group of different chemical substances (cromakalim, pinacidil, nicorandil) which produce an K+ ionic efflux through K+ channels ATP (K+ ATP) dependent. Since their discovery (Noma A., 1983) and their characterization (Gross G.J. et al, 1992), K+ATP channels have been considered of having an important role in myocardial recovery after an ischemic aggression. Noma has suggested that these channels may have an endogenous cardioprotective effect. The major electrophysiological effect observed on cardiac myocytes, as a result of K+ channels opening during ischemia is rapid. A decrease of ventricle action potential leads to a quick loss of electrical and mechanical activity and to a reduction in Ca entry within myocytes (Cole WC et al., 1991). Relative recent studies suggest that the activity of K+ATP and/or endogenous liberation of adenosine during short intervals of ischemia are basic components of these protection mechanisms. There are data which underlines the fact that these channels opening determines a benefit in case of stunning (Auchmpach J.A. et al. 1992), of stroke (Cole WC et al., 1991) and even mimics ischemic preconditioning (Toombs C.F. et al, 1993). Cardiac electrophysiological events imply cooperation of different K+ currents, offering properties which differentiate them from K+ currents from other tissues. A series of antiarrhythmic substances have the ability to lengthen the action potential in cardiac muscle, as a consequence of K+ channels blocking and delay of repolarization. Kantor et al (1987) have found that 1μM glibenclamid reduces the loss of K+ from the rat heart in the first 5 min after ischemia and avoids completely the development of ventricular fibrillation in these hearts. Also, glibenclamide inhibits the shortening of cardiac potential action induced by cromakalim, a well-known K+ channels opener (Sanguinetti M.C. et al, 1988). Protein kinase C (PKC) is a component of many intracellular signaling pathways (Bell RM. et al, 1991). It has been suggested that KATP, phosphorylation, following protein kinase C activation via diacylglycerol, leads to closing of K+ channels (Malaisse W.J. et al, 1982). Cardyomyocite ischemia involves sarc-KATP opening, leading to an increased influx of K+ and shortening the action potential with increased risk of arrhythmias. (Garaliene, 2001)

***Aims of study***

Evaluation of cardioprotection provided by ischemic preconditioning on rat myocardium by assessing the recovery of contractile function post-ischemia (LVDP), the impact upon final size of infarction as a golden standard and evaluation of the incidence of reperfusion arrhythmias. Evaluation of PKC role in ischemic rat myocardium and demonstration of its involvement in the path of ischemic preconditioning by using PKC activators (DOG) and inhibitors (CHE) with a decrease in infarct size as an end point. Evaluation of the role of KATP channels in the ischemic preconditioning mechanism by administration of a KATP channel opener (Cromakalim) or by inhibiting the opening of KATP channels with glibenclamide.

## MATERIALS AND METHODS

***The principle of retrograde myocardial perfusion in Langendorff system***

Experimental studies using isolated and perfused rat myocardium in Langendorff retrograde perfusion system are commonly performed in research laboratories to study the physiological, pharmacological, morphological and biochemical aspects of ischemia reperfusion injury as well as to evaluate the ventricular performance, the metabolic parameters and the coronary flow parameters in different experimental settings. The system was first imagined and created by Oscar Langerdorff in 1897 inquiries were made starting from Cyon Eilas Carl Ludwig Institute of Physiology in Leipzig Germany in 1866, the originally proposed Langerdorff method adapted with small changes over the time (Skrzypiec-Spring et al., 2007). The principle of the method is as follows: isolated myocardium is perfused by retrograde flow from the aorta in which a cannula system is inserted to vehiculate the oxygenated Langerdorff


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reperfusion infusion liquid. Myocardium is perfused in diastole when aortic valve closure occurs and infusion liquid enters the coronary system, later it leaves the coronary sinus, arrives into the right atrium then passes the right ventricle and leaves the heart through the pulmonary artery. The advantage of isolated rat myocardial perfusion study in Langerdorff system for experimental models compared with ischemia-reperfusion studies performed on living rats is that the extracardiac mechanisms of neuro-hormonal control are abolished and we can better study some of the myocardial properties. Using this system has been demonstrated the role of various factors upon the electrical activity and myocardial contractile function: temperature, oxygen, calcium ions, adrenaline, acetylcholine and the evaluation of myocardial reperfusion lesions and the preparation for cardiac transplantation (Skrzypiec-Spring et al., 2007).

***Description of retrograde perfusion system Langerdorff***

For this study, we used Langendorff apparatus ML870B2 (production AD Instruments) and time recording software PowerLab systems for monitoring and analyzing the following parameters: heart rate, ECG, coronary flow, left ventricular developed pressure, left ventricular systolic pressure, end diastolic left ventricular pressure and temperature.

For all these measurements there are sensors connected to CPU PowerLab/8SP. Ensuring a constant coronary flow perfusion is made using the Minipuls ™ 3 pump and the pump control unit (STH Pump Controller); the system used by us having the advantage over the classical system that glass containers for ensuring the coronary perfusion were eliminated, there is a better control of temperature during reperfusion with different solutions can be made because the tank is divided in different compartments.

***Retrograde perfusion buffers used in the Langerdorff system***

The infusion fluid used in our experiments was the standard solution Krebs Hanseleit in which were made some changes in its composition, its final content was: NaCl (118 mM), KCl (4.7 mM) , $CaCl_2$ (1.5 mM), $MgSO_4$ (1.66 mM), $NaHCO_3$ (24.88 mM), $KH_2PO_4$ (1.18 mM), glucose (5.55 mM), Na-pyruvate (2 mM) and bovine serum albumin (0.1% w / v). Infused buffer was freshly prepared and filtered using 0.45 nm hydrophobic microfiltration membrane based on a polyether sulphone polymer (Sartorius AG, Gottingen).

***Heart preparation for the Langerdorff system***

Rats were injected intraperitoneally (i.p.) with anticoagulant (500 IU heparin) and anesthetized by ip injection of 50-80 mg / kg pentobarbital. After anesthesia took effect, tracheotomy was performed and a cannula connected to an artificial ventilator was introduced in the tracheae, artificial ventilator is set at a respiratory frequency of 35 cycles / min and an amount of 5ml Tidall volume. Thoracotomy was performed and subsequently underwent surgical excision of the heart, which was transferred to a Krebs buffer solution Hanseleit maintained at a temperature of 4°C. The next step was mounting the heart in the perfusion system after attaching Langerdorff retrograde perfusion cannula at the ascending aorta, the heart that receive Hanseleit modified Krebs buffer solution, the device was previously set to a constant flow rate (9.7 ± 0.5 ml / min.) the perfused liquid having a concentration of 95% $O_2$, 5% $CO_2$, pH 7.4 and a temperature-controlled value of 37 degrees Celsius. To achieve cardiac ischemia the left anterior descending coronary artery (LAD) ligation was performed using a silk thread, performing reperfusion by releasing the ligature. Measuring heart function in the Langendorff perfused myocardium Hemodynamic parameters were measured to ensure fully functional hearts were used in all experiments and only healthy hearts were included. Coronary flow was recorded by measuring the volume of perfusion buffer per minute required to maintain the set perfusion pressure. This closed circuit included a pressure transducer, attached to a fluid filled tube which was connected to the Langendorff apparatus (just above the heart attachment point), which provided a feedback into a pump controller (AD Instruments, UK). This adjusts flow of perfusion buffer to achieve the fixed perfusion pressure, which was set between 80-100mmHg. The status of the heart, was measured using a pressure transducer connected to a water filled balloon (roughly 4 mm x 3mm) inserted in the left ventricle and inflated to 5 –10 mmHg. The balloon detects the left ventricular end diastolic pressure (LVEDP) and left ventricular peak pressure (LVPP, the maximum pressure in systole). Left ventricular developed pressure (LVDP) is a measure of the force of contraction which was calculated by subtracting LVEDP from LVPP. Heart rate (HR) was calculated based on the number of systole-diastole cycles per minute. The rate pressure product (RPP) is an arbitrary measurement of heart function, which was obtained by multiplying HR by LVEP. The temperature of the circulating perfusion buffer was adjusted in order to maintain the myocardium at 37±0.5°C. A desk lamp and a heated glass water jacket were additionally used to assist in controlling any smaller temperature fluctuations of the heart. The temperature was measured by placing a thermometer probe (T type) connected to a thermo pod and coupler (AD Instruments, UK) at the heart. These hemodynamic parameters were recorded during a stabilization period to ensure the hearts were functioning well. In order to measure myocardial contractile function, we used a latex balloon inserted into the left ventricle through an incision in the left atrium and transmitral crossing the balloon was attached to a polypropylene tube containing a pressure sensor at the end. Balloon inflation was performed with 50% methanol to create a diastolic pressure of 5 to 6 mmHg and hemodynamic parameters determined were as following:


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a) Left ventricle systolic pressure (LVSP),
b) Left ventricle end diastolic pressure (LVEDP)
c) Heart rate (HR)
d) Pressure of the coronary perfusate (PCP)

Plus, LVDP is defined as LVSP – LVEDP, and the coronary vascular resistance (CVR) is calculated by the fraction between the perfusion pressure and the coronary flow.

***Method of assessing infarct size***

Methods of assessing the size of myocardial infarction have evolved over time, initially using indirect methods were used markers of myocardial necrosis (CK, Troponin) but because these methods don’t have high accuracy, new methods have been found using various dyes to determine the risk area and infarct size. In our experiment we used Evans blue solution and the trimethyl tetrazolium chloride 1%. Method`s principle: the assessment of risk area was made by injecting Evans blue solution (0.2%) in the left artery after the LAD was previously occluded by ligation, the ischemic zone remaining unstained. After applying cycles of ischemia-reperfusion, hearts were disconnected from the Langerdorff retrograde perfusion system, were frozen by placing them in a refrigerator at -20°C for 2 hours, then sections of 2 mm thick were made from the apex to the base of the heart. Results sections were treated with solution of trimetiltetrazolium 1% (Sigma-Aldrich) and a phosphate buffer solution at pH 7.4 for 15-20 minutes at a temperature of 37°C. The tetrazolium salt powder was diluted in a phosphate buffer solution with pH 7.4 which were used for the preparation of two other solutions: a solution obtained from dilution of 14.2 g of $NaH_2PO_4$ (0.1 M) in one liter of distilled water, the final solution with a low pH and a second solution obtained from the dilution of 12 grams of $Na_2HPO_4$ (0.1M) in one liter of distilled water, the resulting solution having a high pH. To obtain the solution with pH 7.4 phosphate buffer the two final solutions obtained were combined in these proportions: 22.6% $NaH_2PO_4$ solution with low pH and 77.4% $Na_2HPO_4$ solution with high pH. Staining technique of the myocardial sections with the trimetiltetrazolium chloride has the following principle: viable myocardium is identified by the interaction of dehydrogenases with tetrazolium salt resulting in a scarlet-coloured pigment (formasan), in exchange, the reaction is absent in nonviable myocardium (Klein H. et al., 1981). After incubation of sections in trimetiltetrazolium solution, sections were transferred into a bath solution formaldehyde 10% were kept for one day, the final result showing the contrast between the dye fixing area (coloured in red) and the nonviable myocardial area (coloured in white-gray). The next day sections were arranged on a glass slide and pressed uniform using a second glass slide placed over the sections, the distance between the two blades of 2 mm was achieved by using spacers. Measuring the size of myocardial infarction in rat hearts was performed using infarct area of each section which has been multiplied by 2 mm (the thick of the section) resulting the total volume of the myocardial infarction; final infarct size was estimated as a percentage of risk area. To determine myocardial area on sections computerized planimetry has been used, each section was analyzed separately by drawing the area of infarction using an electronic tablet (Genius G-PEN RS M609X, 9x6 Multimedia Tablet) connected to the computer.

*Table 1*

**Scheme of treatments individualized by groups**

| Group | Stabilization | Pre-treatment | | | Ischemia | Reperfusion |
|---|---|---|---|---|---|---|
| CONTROL (Group A) | Stabilization 20’ | 15’ perfusion | | | Ischemia 45’ | Reperfusion 120’ |
| K - 7μM (Group B) | Stabilization 20’ | K – 5’ | 10’ perfusion | | Ischemia 45’ | Reperfusion 120’ |
| GLY - 1μM (Group C) | Stabilization 20’ | GLY – 15’ | | | Ischemia 45’ | Reperfusion 120’ |
| DOG (Grpup D) | Stabilization 20’ | DOG 5’ | 10’ perfusion | | Ischemia 45’ | Reperfusion 120’ |
| CHE - 30μM (Group E) | Stabilization 20’ | CHE 15’ | | | Ischemia 45’ | Reperfusion 120’ |
| DOG + GLY (Group F) | Stabilization 20’ | DOG + GLY – 15’ | | | Ischemia 45’ | Reperfusion 120’ |
| CHE + K (Group G) | Stabilization 20’ | CHE+ K – 15’ | | | Ischemia 45’ | Reperfusion 120’ |
| Precond (Gropup H) | Stabilization 20’ | Perf-5’ | Isch-5’ | Reperf-5’ | Ischemia 45’ | Reperfusion 120’ |
| CHE+Precond (Group I) | Stabilization 20’ | CHE-5’ | Isch-5’ | Reperf-5’ | Ischemia 45’ | Reperfusion 120’ |
| GLY+Precond (Group J) | Stabilization 20’ | GLY-5’ | Isch-5’ | Reperf-5’ | Ischemia 45’ | Reperfusion 120’ |


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***Treatments***

All treatment solutions were initially dissolved in dimethyl sulfoxide (DMSO) and finally in Krebs-Henseleit solution so that final concentration of DMSO did not exceed 0.04% in order not to interfere with cardiac physiology.

- 1,2-dioctanoil-sn-glycerol (DOG) – PKC activator – solution 30μM
- cheleritrine (CHE) – PKC inhibitor solution 30μM
- cromakalim (K) – KATP –opener-solution 30μM
- glibenclamide (GLY) – KATP –blocker solution 1μM

***Study groups***

Our study has been conducted on 50 male white Wistar rats aged 10 months old, 250-320 g weight, kept in standard laboratory conditions. They have been divided into 10 groups of 5 rats each, having an individualized scheme of treatment presented in the table below.

After mounting the isolated heart in the Langerdorff retrograde perfusion system, perfusion of hearts was performed with Krebs Hanseleit solution for 20 minutes with the stabilization of the hemodynamic parameters (heart rate, coronary flow, left ventricular systolic pressure), that period is called the stabilization period. Special treatment stage is different from one group to another, this stage lasted 15 minutes and consisted of: infusion for 15 min with Krebs Hanseleit solution for the hearts from the control group (Group A), infusion solution Cromakalim (K) 30 μM for 5 minutes after infusion of Krebs Hanseleit solution for 10 minutes (group B), infusion solution of glibenclamide (Gly) 1 μM for 15 min (group C), infusion solution 1.2 dioctanoil-sn-glycerol (DOG) 30μM for 5 min followed by perfusion with Krebs Hanseleit solution for 10 min. (group D), perfusion with cheletitrine 30μM solution for 15 min (group E). There were two groups who underwent combined solutions infusions: infusion combined with DOG and Gly solutions in concentrations mentioned above for 15 minutes (group F) and infusion solutions combined with CHE and K concentrations mentioned above, with the same period of infusion and 15 minutes (group G). In three groups, ischemic preconditioning was applied using a cycle consisting of 5 minutes of ischemia followed by 5 minutes of myocardial reperfusion, two of the three groups were given infusions of solutions with CHE (groups H, I), respectively Gly for 5 min (group J) at concentrations above mentioned anterior to the preconditioning cycle.

## RESULTS

***Statistical analysis***

For data processing we used SPSS 14.0 and Microsoft Excel in Office 2003. The results were expressed as mean value ± standard deviation, for statistical significance testing we used ANOVA and Fisher test, $p \leq 0.05$. Define the role of PKC in ischemic preconditioning in rat a specific antagonist of PKC and 1,2 DOG is a specific agonist of PKC have been investigated in order to see if PC has been blocked or activated We used white Wistar rats 12 month old anesthetized and sacrificed and using beating heart Langendorff model of 45 min occlusion followed by 120 min reperfusion. Preconditioning protocol: 1 cycle of 5 min. ischemia and 5 min. reperfusion.

The differences in body weight and myocardial mass between the studied groups were not statistically significant.

*Table 2*

**Basic value: – in case of PPC and LVDP the values are those from the end of stabilization. HW = weight heart; PPC = pressure of coronary perfusate; LVDP = pressure developed by the left ventricle**

| Group | N | HW (g) | CPP (mmHg) | LVDP (mmHg) |
|---|---|---|---|---|
| Control | 5 | 7.3 ± 0.3 | 49.3 ± 9.5 | 78.0 ± 1.1 |
| Cromakalim (K) | 5 | 7.4 ± 0.2 | 45.8 ± 3.7 | 80.0 ± 2.0 |
| Glibenclamide (GLY) | 5 | 7.7 ± 0.6 | 40.5 ± 5.5 | 90.0 ± 0.4 |
| 1,2-sn-dioctanoilglycerol (DOG) | 5 | 6.7 ± 0.3 | 44.7 ± 5.0 | 89.0 ± 1.1 |
| Cheliretrin (CHE) | 5 | 7.0 ± 0.5 | 43.8 ± 3.5 | 86.0 ± 0.9 |
| DOG + GLY | 5 | 7.2 ± 0.5 | 52.3 ± 1.5 | 88.0 ± 0.5 |
| CHE + K | 5 | 7.1 ± 0.8 | 49.0 ± 2.6 | 86.0 ± 0.9 |

*Table 3*

**Features of experimental groups**

| GROUPS | No. rats | B.W. (g) | Heart weight (g) |
|---|---|---|---|
| A | 5 | 302±6 | 7.3±0.3 |
| B | 5 | 300±2 | 7.4±0.2 |
| C | 5 | 310±8 | 7.7±0.6 |
| D | 5 | 296±5 | 6.7±0.3 |
| E | 5 | 298±7 | 7.0±0.5 |
| F | 5 | 304±5 | 7.2±0.5 |
| G | 5 | 312±6 | 7.1±0.8 |
| H | 5 | 303±4 | 6.9±0.4 |
| I | 5 | 294±7 | 7.1±0.5 |
| J | 5 | 297±3 | 6.8±0.3 |


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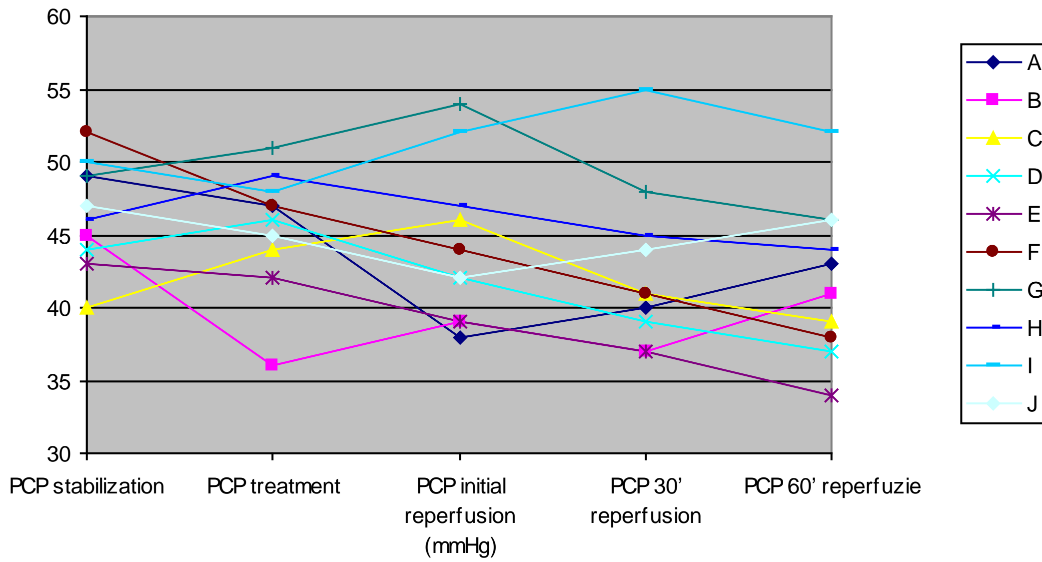


**Fig. 1** Pressure of the coronary perfusate (CPP) evolution in the studied groups

*Table 4*

**CPP results**

| Lots | PCP stabilization (mmHg) | PCP treatament (mmHg) | PCP initial reperfusion (mmHg) | PCP 30' reperfusion (mmHg) | PCP 60' reperfusion (mmHg) |
|---|---|---|---|---|---|
| A | 49.3±9.5 | 47.3±4.5 | 38.4±6.7 | 40.7±5.8 | 43.1±3.7 |
| B | 45.8±3.7 | 36.5±5.4 | 39.7±6.4 | 37.4±4.6 | 41.6±5.2 |
| C | 40.5±5.5 | 44.3±8.3 | 46.4±7.6 | 41.9±6.4 | 39.3±7.4 |
| D | 44.7±5.0 | 46.8±4.2 | 42.2±3.7 | 39.2±3.2 | 37.2±6.3 |
| E | 43.8±3.5 | 42.3±5.4 | 39.7±3.2 | 37.3±4.2 | 34.7±4.9 |
| F | 52.3±1.5 | 47.3±5.8 | 44.7±4.1 | 41.1±4.2 | 38.3±4.8 |
| G | 49.0±2.6 | 51.3±4.3 | 54.3±3.7 | 48.3±4.7 | 46.9±8.3 |
| H | 46.5±6.4 | 49.4±6.7 | 47.3±8.5 | 45.2±7.5 | 44.1±9.3 |
| I | 50.3±4.3 | 48.3±4.4 | 52.4±7.4 | 55.2±5.3 | 52.4±4.6 |
| J | 47.8±3.2 | 45.3±6.4 | 42.1±8.3 | 44.6±5.3 | 46.4±7.3 |

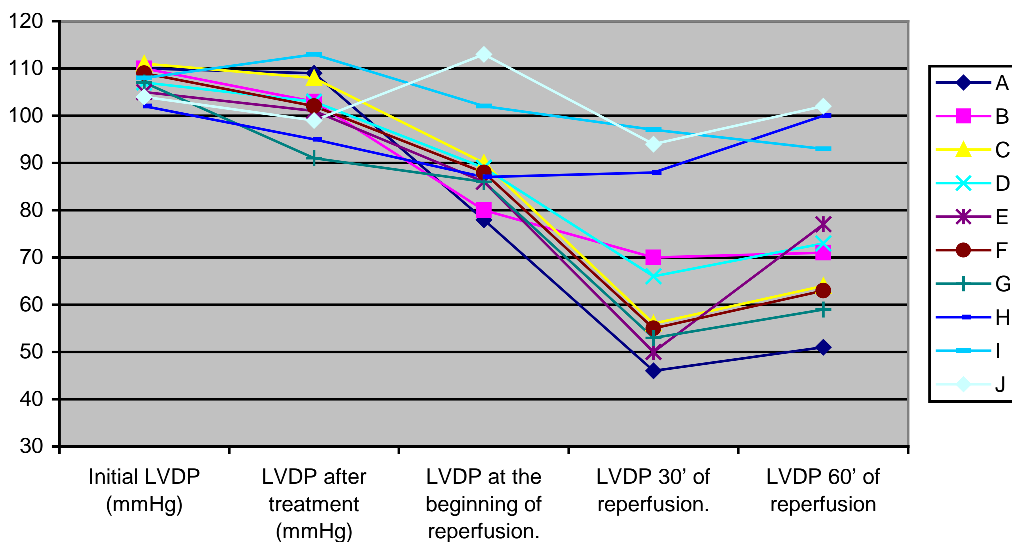


**Fig. 2** LVDP evolution in the groups studied in the stages of the study


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Table 5

**LVDP results**

| LOT | LVDP initial (mmHg) | LVDP after treatment (mmHg) | LVDP at the begining of reperfusion (mmHg) | LVDP 30' of reperfusion (mmHg) | LVDP 60' of reperfusion (mmHg) |
|---|---|---|---|---|---|
| A | 110±4 | 109±5 | 78±1 | 46±6 | 51±6 |
| B | 110±7 | 103±6 | 80±2 | 70±6 | 71±8 |
| C | 111±9 | 108±8 | 90±0.5 | 56±7 | 64±7 |
| D | 107±7 | 103±6 | 89±1 | 66±6 | 73±7 |
| E | 105±8 | 101±5 | 86±1 | 50±5 | 77±9 |
| F | 109±5 | 102±4 | 88±0.5 | 55±4 | 63±4 |
| G | 107±2 | 91±7 | 86±1 | 53±4 | 59±4 |
| H | 102±7 | 95±3 | 87±6 | 88±3 | 100±8 |
| I | 108±2 | 113±8 | 102±6 | 97±4 | 93±7 |
| J | 104±5 | 99±5 | 113±8 | 94±5 | 102±4 |

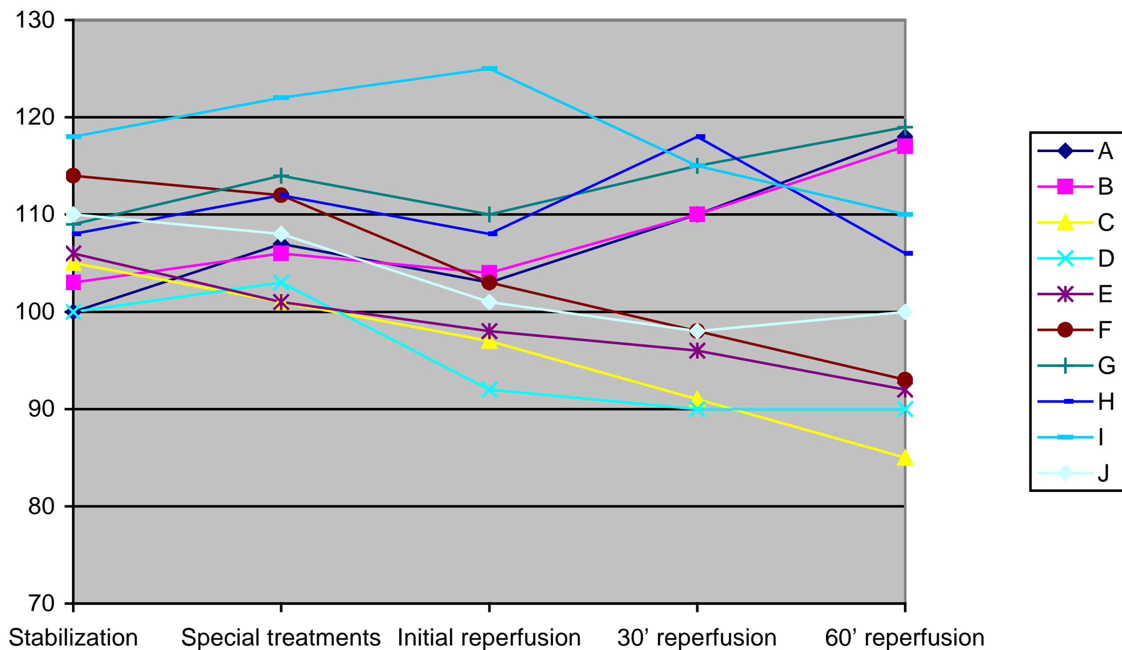


**Fig. 3** Heart rate evolution in the groups studied

Table 6

**HR results**

| Groups | Stabilization Bpm | Special treatments Bpm | Initial reperfusion Bpm | 30' reperfusion Bpm | 60' reperfusion Bpm |
|---|---|---|---|---|---|
| A | 100±3 | 107±4 | 103±8 | 110±4 | 118±5 |
| B | 103±2 | 106±4 | 104±2 | 110±7 | 117±8 |
| C | 105±3 | 101±5 | 97±4 | 91±3 | 85±3 |
| D | 100±4 | 103±7 | 92±6 | 90±2 | 90±2 |
| E | 106±3 | 101±3 | 98±6 | 96±3 | 92±8 |
| F | 114±7 | 112±8 | 103±5 | 98±6 | 93±5 |
| G | 109±4 | 114±8 | 110±4 | 115±7 | 119±3 |
| H | 108±7 | 112±5 | 108±4 | 118±9 | 106±7 |
| I | 118±5 | 122±4 | 125±8 | 115±6 | 110±8 |
| J | 110±4 | 108±6 | 101±7 | 98±4 | 100±6 |

Analyzing the evolution of LVDP and expressing the result as a percentage of the initial value, we found an improvement of LVDP recovery at 30' of reperfusion (86±4% in the group that received ischemic preconditioning (group H) compared with controls (group A) 42±3%, p<0019) and a significant recovery of LVDP at 60' of reperfusion (98 ± 5% in the group with ischemic preconditioning and 46 ± 3% in the


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control group, $p<0.010$). The investigation of the involvement of PKC in the mechanism of ischemic preconditioning was done using the administration of PKC activators (DOG) and of PKC inhibitors (cheleritrine).

When we compared the group with ischemic preconditioning (group H), there is a significant reduction of LVDP values at 30' of reperfusion, at 60' of reperfusion in the group treated with cheleritrine (CHE) (group E) (47±3% and 73±5%, $p<0.05$), LVDP recovery at 30' and 60' of reperfusion in the group that received a PKC activator (DOG) (group E) (61±4% and 68±3%) was not important when compared with the results from the group receiving simple preconditioning (group H), but is important when compared with the control group (group A) (42±3% respectively 46±3%, $p<0.05$).

After 15 minutes of reperfusion there is a significant decrease in LVDP in all studied cases. The decrease in cardiac contractility by blocking $K^+$ channels with GLY is increased by its association with CHE, an inhibitor of protein kinase C. In case of association of GLY with DOG, activator of protein kinase C, the reduction of contractile power is less. In none of the cases there was no recovery of contractile force and the returning to the basal values. (table 3 and fig.2) Blocking $K^+$ channels with GLY constantly decrease cardiac frequency, while activation of protein kinase C with DOG or opening $K^+$ channels with cromakalim, after a significant reduction of frequency, mean in the same values along the experiment. Protein kinase C activation abolishes the blocking $K^+$ channels effect by GLY in such a way that at the beginning of perfusion increases cardiac frequency above Control values, leading to its reduction in time. In these experiments, the activation of protein kinase C modifies the contractile behavior of cardiac myocytes and blocking or opening of $K^+$ channels ATP-dependent is not able to abolish this action. The traditional mechanism of pharmacological agents' action to limit lesions produced by experimental ischemia/reperfusion is related with favorable modification in oxygen supply balance. $K^+$ channels openers seem to possess a unique anti-ischemic mechanism which cannot be explained by improving the oxygen supply or reduction of its consumption, and the anti-ischemic effect is produced by a direct cardioprotective action. The sarcolemmal $K^+_{ATP}$ channels (sarco-$K^+_{ATP}$ channels) seem also to play some role under certain conditions, particularly in small animal models like mice [12, 27, 33, 38]. Activation of the sarco- $K^+_{ATP}$ channels (potassium flows out of the cell) cause membrane hyperpolarization and shorten the duration of the action potential (AP) thus reducing Ca2+ influx into the cell. Activation of the mito $K^+_{ATP}$ channels at the inner mitochondrial membrane (IMM) causes potassium to flow into the mitochondrial matrix (MM) for regulation of the mitochondrial matrix volume, supports coupling of the respiratory chain and ATP synthesis, attenuates Ca2+ entry from the cytoplasm, and inhibits apoptosis. Activation of the $K^+$-ATP channels during ischemia is thought to be due to phosphorylation by Ser/Thr-kinases PKC and/or PKG. The mPTP represents a multiprotein complex comprising the adenine nucleotide transporter (ANT) at the IMM and the voltage-dependent anion channel (VDAC or porin) at the outer mitochondrial membrane (OMM). Additional proteins may be associated with the mPTP at these contact sites between the OMM and IMM including the mitochondrial creatine-kinase (mCK), hexokinase (HK), glycerol kinase (GK), the proapoptotic Bax and the antiapoptotic Bcl2 [1, 5, 27, 38]. The mPTP and the mitoKATP channel are functionally coupled in so far as opening of the mPTP solutes exchange between the cytoplasm and the MM, Ca2+ enters the MM, ROS are generated, the proton gradient driving ATP synthesis collapses, and the energy production comes to a halt.

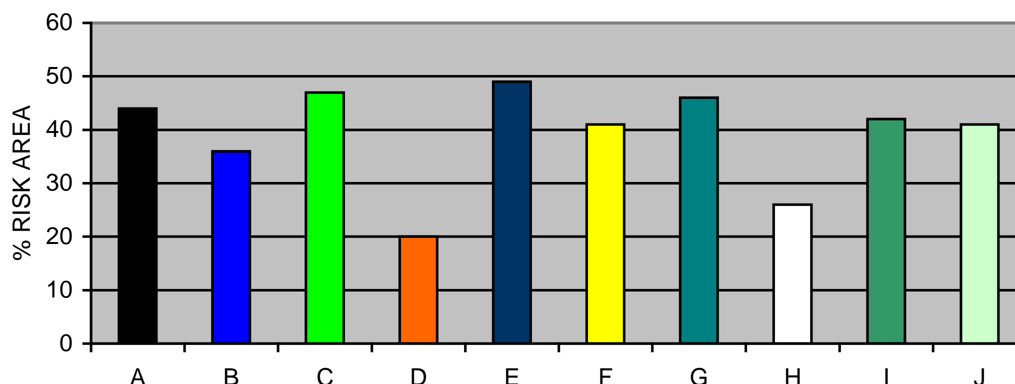


**Fig. 4** Evaluation of infarct size determined as % from risk area


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Under such conditions the ATP synthase (complex-V) was observed to hydrolyze ATP thus using up what was left as energy source. Activation of the mito- $K^+$-ATP channels favors closure of the mPTP and may support survival while irreversible opening of the mPTP irrevocably leads to death. High Ca2+ in the MM, cyclophilinD (peptidylprolyl cis-trans isomerase), and depolarization promote recently, attention has been focused on new therapies that prevent/attenuate ischemia and reperfusion injury associated with MI. Two potential strategies for protecting the heart are ischemic preconditioning (IPC) and ischemic postconditioning (IPost), which describe the cardioprotection obtained from applying transient episodes of MI and reperfusion, either before or after the index ischemic event respectively.

Comparing the final size of myocardial infarction in the studied groups, there is a statistically significant reduction in final infarct size in hearts from ischemic preconditioning group (group H) compared with the control group (group A) (26 ± 2.8% vs. 44 ± 4.6%) $p < 0.05$, and the group treated with DOG (group D) compared with the control group (group A) (20 ± 1.4% vs. 44 ± 4.6%) $p<0.01$.

Groups treated only with GLY (group C) and CHE (group E) showed only a tendency to increase the final size of myocardial infarction when compared with the control group (group A) (47 ± 3.6% and 49 ± 4.2% vs. 44 ± 4.6% ) but the result was not statistically significant ($p = 0.12$), also the group treated only with K (group B) did not show a statistically significant reduction in final size of myocardial infarction compared with control group (group A) (36 ± 3.5% vs. 44 ± 4.6% $p = 0.15$).

There is a tendency of reduction of the cardioprotective effect of ischemic preconditioning when the administration of CHE (group I) (42 ± 4.1%) or GLY (group J) (41 ± 2.7%) is associated $p<0.05$; a similar result was obtained for administration of Gly in addition to the group treated with DOG (group F) (41 ± 4.7%) $p<0.01$.

## DISCUSSIONS

Incubation with CHE has abolished protection, being no significant difference between control, preconditioned group + CHE and PPC group. CHE and DMSO vehicle had no direct influence upon infarct size. Stimulation of PKC with DOG the analogue of diacyglicerol resulted in protection and inhibition with CHE abolished protection. The discovery that the treatment with agonists of CPK and offered a similar protection degree towards MI as it has been seen after PC stimulation, and that this protection may be blocked by PKC inhibitor may be a support for the hypothesis that PKC may play a role in preconditioning. Ischemic preconditioning represents an important research area in the cardioprotection field, although the mechanism of ischemic preconditioning has been extensively studied, however, until now it is insufficient elucidated; in the present study we aimed to evaluate the involvement of $K^+_{ATP}$ and PCK in the pathway of ischemic preconditioning by using selective agonists and antagonists for PKC and $K^+_{ATP}$. Using PKC inhibitors e.g. Cheleritrine, a natural alkaloid of benzofenadrine, determines blocking of all isoforms of PKC by interacting with the catalytic domains, the final effect being the loss of cardioprotective effect of ischemic preconditioning (Herbert et al., 1990). It has been proposed that PCK plays a central role in ischemic preconditioning according with the following mechanism: an endogenous ligand as adenosine in rabbit binds to the A1 adenosine receptor at the myocyte surface which then activates phospholipase C via G protein. The activated Phospholipase C then produces the splitting of phosphatydil inositol 4,5 diphosphat and phosphatidyl choline to produce DAG and inositol 1,4,5 triphosphate. Increased levels of DAG activated then PCK in membrane determines its translocation and activation in cytosol and these activated CPK phosphorylates a second effector which is able to reduce the protective effects of preconditioning. In our study CHE, a strong antagonist has been chosen because it interacts with catalytic domain. CHE has been administered towards the end of preconditioning reperfusion protocol because as we have seen that activated PKC will be translocated at the membrane level and in that moment it could be efficiently blocked with staurosporin before preconditioning. There are experimental studies that evaluated the effect of PKC inhibitors on all PKC isoforms involved in the ischemic preconditioning (cheleritrine, staurosporine, polymyxin B studies); cheleritrine studies have shown blocking the translocation of all PKC isoforms, studies with the remaining inhibitors have shown variable results. Rat heart experiments comparing the effects of management and staurosporine cheleritrine in ischemic preconditioning have shown that there is an increased selectivity of PKC sites, staurosporine shows a higher selectivity for other types of proteinkinase (eg, PKA), in both groups noticing a reduction of cardioprotective effects of the ischemic preconditioning (Speechly-Dick et al., 1994). Diacylglycerol is an endogenous activator of PCK, in our study, we evaluated the effect of administration of an analogue of diacylglycerol: 1,2-sn-glycerol-dioctanoil (DOG) in the myocardial ischemia reperfusion. DOG has been administered 10` before ischemia in order to allow the activation of PKC before the prolonged DOG is only transitory present in membrane and is part of the physiological pathway for PKC activation. The activation of PKC with DOG has been seen to be highly specific.

DAG with 1,2 sn configuration is necessary between lipids and binding site of PKC for activation. Alfa, beta, epsilon and gamma isoforms are predominant in rat heart Short periods of ischemic preconditioning (2.5 min.), determine rapid translocation at the membrane level of subtypes delta, epsilon and gamma and of alpha Ca-dependent subtype. Prolonged ischemia 45 min leads to induction


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of delta and epsilon Ca-dependent isoforms in cytosol. This study pointed out that the lack of protection against MI conferred by PC, if PKC is inhibited and with protective effect for MI when PKC is activated DOG is able to protect myocardium against MI but has no protective effect against the arrhythmias induced by preconditioning. These observations can be considered as ant infarct and anti-arrhythmic properties of preconditioning mediated by different signaling pathways. Our data accounts for PKC protective role against ischemia in rat myocardium. $K^{+}_{ATP}$ channels are involved in both phases of ischemic preconditioning, and their role in preconditioning was demonstrated using pharmacological agents. Opening the $K^{+}_{ATP}$ increases the efflux of $K^{+}$ from myocytes by shortening action potential duration, maintaining reserves of ATP and blocking the influx of $Ca^{2+}$ by blocking the voltage- $Ca^{2+}$ dependent channels, the final effect is the reduction of the $Ca^{2+}$ concentration in myocytes which ensures a high degree protection against ischemia.

## CONCLUSIONS

Ischemic preconditioning is an endogenous cardioprotective mechanism in which accessory nerve pathways are not involved, this fact being demonstrated by the effect of ischemic preconditioning on isolated rat heart. PKC isoforms are included in the cascade of the myocardial preconditioning mechanism as well as K+ATP channels, fact demonstrated by using selective agents like: PKC and K+ATP agonists and antagonists. Ischemic preconditioning brought about a recovery of contractyle as our results concerning LVDP values have shown. Infarct size as a golden standard has been reduced in ischemic preconditioning to similar degree when the activator DOG of PKC has been used.

This study offers the evidence that hypothesis concerning PKC activation is a pivotal stage in the pathway of intracellular signaling of preconditioning, playing a role in cell regulation. The protective mechanism of K+ channels opener seems to be the result of their capacity to activate KATP channels from ischemic myocardium, they seem to possess a unique anti-ischemic mechanism which cannot be explained by improving the oxygen supply or reduction of its consumption, and the anti-ischemic effect is produced by a direct cardioprotective action. The hypothesis is sustained by the observation that pre-treatments of dogs with GLY reduce the benefit of cromakalim upon $K^{+}$ channels. Ischemia/reperfusion modifies functional parameters in isolated heart, and the action of factors which act upon K+ channels (either blocking or deblocking them) is expressed by modification of these functional parameters. Despite the clinical observation mentioned before, treatments involving ischemic preconditioning or postconditioning are not advisable to be employed in patients with already compromised cardiovascular status. The usage of pharmacological agents in ischemic conditioning may provide a more benign approach for eliciting cardioprotection in the clinical setting. Even in the last two decades there were a lot of studies done in the effort to understand the mechanism of ischemic preconditioning, additional studies are still needed in order to elucidate the complex mechanism of its action.

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