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The Protective Effect of Lisinopril on Membrane-Bound Enzymes in Myocardial Preservation

C. SELIM ISBIR[1]*, RIZA DOĞAN[2], BORA FARSAK[2], MAKBULE AYDIN[3] AND KAMER KILINÇ[4]

[1]*Department of Cardiovascular Surgery, Marmara University Hospital, Istanbul, Turkey*
[2]*Department of Thoracic and Cardiovascular Surgery, Hacettepe University Faculty of Medicine, Ankara, Turkey*
[3]*Department of Molecular Medicine, Istanbul University Institute for Experimental Medicine and Research, Istanbul, Turkey*
[4]*Department of Biochemistry, Hacettepe University Faculty of Medicine, Ankara, Turkey*

A number of studies have reported that oxidant stress reduces the activity of isolated $Na^+ - K^+$ ATPase and Ca^{2+} ATPase which are known to affect the cell membrane integrity. The aim of the study is to determine whether the administration of lisinopril is able to protect the membrane-bound enzyme levels in isolated guinea pig hearts and also ascertain whether or not a relationship exists between oxygen free radicals and membrane bound $Na^+ - K^+$ ATPase and Ca^{2+} ATPase. Forty guinea pig hearts were studied in an isolated Krebs–Henseleit solution-perfused Langendorff cardiac model. In all groups cardioplegic arrest was achieved by administering St. Thomas' Hospital cardioplegic solution (STHCS). Group 1 (control, $n = 10$) received only STHCS. Group 2 ($n = 10$) were arrested with lisinopril (1 μmol l^{-1}) added STHCS. Group 3 ($n = 10$) were pretreated with oral lisinopril (0·2 mg kg^{-1} twice a day) for 10 days and then arrested with STHCS. Group 4 were also pretreated with oral lisinopril (0·2 mg kg^{-1} twice a day for 10 days), arrested with STHCS and reperfused with lisinopril added to Krebs–Henseleit solution (1 μmol l^{-1}). Hearts were subjected to normothermic global ischaemia for 90 min and then reperfused at 37°C. Pretreatment and addition of lisinopril in the reperfusion buffer improved the levels of membrane-bound enzymes. When the treated groups were compared with control hearts, the best results were achieved in group 4. The $Na^+ - K^+$ and Ca^{2+} ATPase levels increased from $466{\cdot}38 \pm 5{\cdot}99$ to $560{\cdot}12 \pm 18{\cdot}02$ and $884{\cdot}69 \pm 9{\cdot}13$ to $1287{\cdot}71 \pm 13{\cdot}01$ nmolPi mg^{-1} protein h^{-1} respectively ($p < 0{\cdot}05$). These results suggest that lisinopril protects the cell membrane integrity and lessens free radical-induced oxidant stress. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS — cardioplegia; Ca^{2+} ATPase; $Na^+ - K^+$ ATPase; Lisinopril

INTRODUCTION

Although many causal factors have been proposed for ischaemia–reperfusion injury, the exact mechanism for interdependent derangements of mechanical, electrical and metabolic events remains unclear. Intracellular calcium loading is considered to represent the common denominator of ischaemia–reperfusion-induced cell dysfunction and death.[1] Cytoplasmic calcium overload can occur either because of increased Ca^{2+} influx from the extracellular space to the cytosol or because of insufficient Ca^{2+} extrusion from the cytosol. These processes include Ca^{2+} channels, adrenergic receptors, Na^+–Ca^{2+} exchange and sodium–potassium-activated adenosine 5′ triphosphatase ($Na^+ - K^+$ ATPase). Another mechanism that also affects the cytosolic Ca^{2+} concentration is sarcoplasmic Ca^{2+} stores. This involves the calcium-activated adenosine 5′ triphosphatese (Ca^{2+} ATPase) pump of the sarcoplasmic membrane. Therefore the deleterious effect of ischaemia–reperfusion injury on membrane-bound enzymes such as $Na^+ - K^+$ ATPase and Ca^{2+} ATPase has been investigated. According to Korge and Campbell,[2] inhibited Ca^{2+} transport

* Correspondence to: Selim Isbir MD, Av. Sureyya Agaoglu sok. No: 4B d:17, Nisantasi Istanbul, Turkey 80200. Fax: +90 212 635 1959; +90 216 325 2426. E-mail: isbir@yahoo.com

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can be improved pharmacologically by interfering with Ca^{2+} ATPase.[2] Agents that limit Na^{+}–Ca^{2+} exchange during early reperfusion might also improve functional recovery.

In previous studies, angiotensin converting enzyme (ACE) inhibitors have been shown to reduce myocardial injury, enhance the contractile function of stunned myocardium and improve malignant reperfusion arrhythmias in experimental models of transient ischaemia.[3–8] Most of these studies were done with captopril, a sulfhydryl-containing ACE inhibitor. The beneficial effects of captopril were attributed to the superoxide radical scavenging activity of the sulfhydryl moiety in some of these studies. Our group recently showed that captopril also improves the membrane function in ischaemia–reperfusion injury (unpublished data). Recent studies suggest that the effect of captopril on free radicals cannot be explained by the sulfhydryl group because additional protective effects for non-sulfhydryl containing ACE inhibitors such as enalapril have also been suggested, but the mechanisms of action are still obscure.[9–10] The purpose of this study is (i) to evaluate the protective effect of lisinopril 'a non-sulfhydryl containing ACE inhibitor', on membrane-bound enzymes and in myocardial function after 90 min. of normothermic global ischaemia in Langendorff-perfused guinea pig hearts and (ii) to compare the effects of lisinopril treatment via different routes of administration (i.e. cardioplegic solution, pretreatment with oral lisinopril, and lisinopril-enriched reperfusion solution respectively).

MATERIALS AND METHODS

Hearts were obtained from male Duncan-Hartley guinea pigs weighing 270–330 g. All animals received humane care in compliance with the *Principles of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1986).

The animals were randomly divided into four groups ($n = 10$ animals in each group). In the first group, isolated hearts received only St. Thomas' Hospital Cardioplegic Solution (STHCS) (Table 1) and were used as controls. In the second group, hearts received lisinopril (l $\mu mol\, l^{-1}$) in addition to STHCS. In the third group, animals were pretreated with oral lisinopril (0·2 mg kg^{-1} twice a day by gastric gavage for 10 days) and then arrested with STHCS. In the fourth group, animals were also pretreated with oral lisinopril, arrested with STHCS, but reperfused with lisinopril-enriched (1 $\mu mol\, l^{-1}$) Krebs–Henseleit solution throughout the reperfusion period.

Table 1. St Thomas' Hospital cardioplegic solution (STHCS).

Compound	Concentration (mmol/L)
Sodium chloride	110·0
Potassium chloride	16·0
Magnesium chloride	16·0
Calcium chloride	1·2
Sodium bicarbonate	10·0

PH adjusted to 7·8.
Osmolarity = 324 mosm/kg H_2O.

The animals were anaesthetized with ether and after intraperitoneal administration of heparin (200 U), whole hearts were rapidly removed and quickly mounted on a non-recirculating Langendorff perfusion column. Retrograde aortic perfusion was initiated at a perfusion pressure of 100 cm H_2O with an oxygenated modified Krebs–Henseleit buffer solution with the following composition (in $mmol\, l^{-1}$): NaCl 118, KCl 4·7, $NaHCO_3$ 25, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 1·2 and glucose 11·1. The perfusate was bubbled continuously with 95 per cent oxygen and 5 per cent CO_2 to achieve a pH of 7·4 at 37°C.

Apical force displacement was used in order to measure the cardiac contractile force. A 7/0 silk ligature was placed to the left ventricular apex and connected to a Grass® FT 03 force displacement transducer (Grass Instrument Co, Quincy, MA, USA) and a resting tension of 5 g was applied. The transducer output (ventricular contractile force ($mm\, g^{-1}$) × heart rate ($beats\, min^{-1}$) was displayed continuously on a Grass® model 7 polygraph (Serial D531 V3, Grass Instrument Co, Quincy, MA, USA).

After a stabilization period of 15 min, the preischaemic heart rate and ventricular contractile force were recorded. Ischaemia was induced by clamping the aortic cannula and cardiac arrest was achieved by administration of retrograde STHCS for 3 min. via a reservoir located 60 cm above the heart, attached to a side arm of the aortic cannula. Throughout the period of ischaemic arrest the hearts were kept at 37°C in the isotonic saline-jacketed heart chamber. At the end of the 90 min. of global ischaemia, hearts were reperfused with normal Krebs–Henseleit solution (groups 1,2,3)

and with lisinopril (1 μmol l^{-1}) added to Krebs–Henseleit solution (group 4) for 30 min. The heart rate and ventricular contractile force were recorded every 5 min. of the reperfusion period.

At the end of the experiment the hearts were excised and weighed. In all instances, the left ventricular free wall was immediately resected and kept in −70°C liquid nitrogen until the assessments of membrane-bound enzyme activity ($Na^+ - K^+$ ATPase and Ca^{2+} ATPase), tissue reduced glutathione (GSH) and tissue thiobarbituric acid reactive substance (TBARS) levels were carried out.

Biochemical Determination

Frozen tissues were immediately weighed and homogenized in 10 volumes of ice-cold phosphate buffer (50 mM, pH 7·4), using a glass–glass homogenizer. The biochemical determinations were done on this homogenate. Tissue lipid peroxide levels (TBARS) were determined by the method of Uchiyama and Mihara.[11] The thiobarbituric acid reactive substances (TBARS) were calculated as $nmol^{-1}$ wet tissue, and tetramethoxy-propane was used as standard. TBARS formed from the breakdown of polyunsaturated fatty acids may serve as a convenient parameter for assessment of the extent of peroxidation. The spectrophotometric assay of Ellman[12] was utilized to determine the tissue concentrations of acid-soluble sulfhydryls.

Tissue levels of reduced glutathione were determined according to the method described by Fairbanks and Klee.[13] Levels were expressed as milimolar (mM).

Determination of $Na^+ - K^+$ ATPase and Ca^{2+} ATPase activities were done according to the method of Reading and Isbir.[14, 15] Ten per cent tissue homogenate was prepared in 0·3 M sucrose with 1 M magnesium by homogenizing for 90 s, using a glass pestle with a clearance of 0·25 to 0·38 mm at 1000 r.p.m. cooled in melting ice. The homogenates were then centrifuged at 1000 g × 15 min to remove nuclei and debris and ATPase activities were determined in the resulting supernatants using buffers with compositions as follows: (a) For $Na^+ - K^+$ ATPase (mM): $MgCl_2$ 6, KCL 5, NaCl 100, EDTA 0·1 and Tris buffer pH 7·4. 135. (b) for Ca^{2+} ATPase (mM): $MgCl_2$ 6, $CaCl_2$ 2·5, EDTA 0·1, Tris buffer pH 7·4. 135.

After preincubation for 5 min. at 37°C, disodium ATP (Boehringer, Mannheim) was added to a final concentration of 3 $mmol^{-1}$. The sample blank containing no assay standards and experimental samples were incubated at 37°C for 30 min. The reaction was stopped by putting the samples on ice. Inorganic phosphate (Pi) liberated was determined in 1 ml aliquots of the incubated mixtures by the addition of 2 ml of lubrol-molybdabte solution prepared according to the method of Atkinson *et al.*,[16] followed by vortexing and standing at ambient temperature for 10 min. Extinction at 390 nm was measured in a Shimadzu spectrophotometer. Samples were compared for phosphate content with a standard of KH_2PO_4. Specific activities were expressed as nmol Pi^{-1} mg^{-1} protein. Protein content was determined according to Lowry *et al.*[17] Bovine serum albumin was used as standard. All chemicals were of Analar grade, unless stated otherwise. Solutions were made up in glass-distilled deionized water.

Statistical Methods

For statistical analysis a one-way analysis of variance, followed by a Newman–Keul Test was applied to the results to determine which groups were different from each other. A p value $<0·05$ was considered to be statistically significant. All values are expressed as the mean ± standard error of the mean (SEM).

RESULTS

Baseline Measurement

There were no significant differences among the groups with regard to baseline hemodynamic data (Table 2).

Table 2. Initial (a) and reperfusion period (b) contractile force (gr. contractility/gr. heart weight) values in control and study groups.

	Group 1	Group 2	Group 3	Group 4
a-Initial	22·7/4·8	24·1/5·2	23·5/3·9	21·9/4·1
b-Reperfusion period (min)				
5	10·9/3·1	12·8/2·1	16·1/3·0	16·2/4·2
10	9·1/2·9	11·6/3·3	15·2/2·1	15·8/3·9
15	8·8/3·0	11·0/2·9	14·9/2·0	14·7/3·9
20	7·8/2·5	11·5/3·0	14·1/3·9	14·6/4·5
25	7·5/3·1	10·6/4·1	13·4/2·4	13·6/3·7
30	6·7/5·0	10·1/2·0	13·0/2·8	12·8/4·3

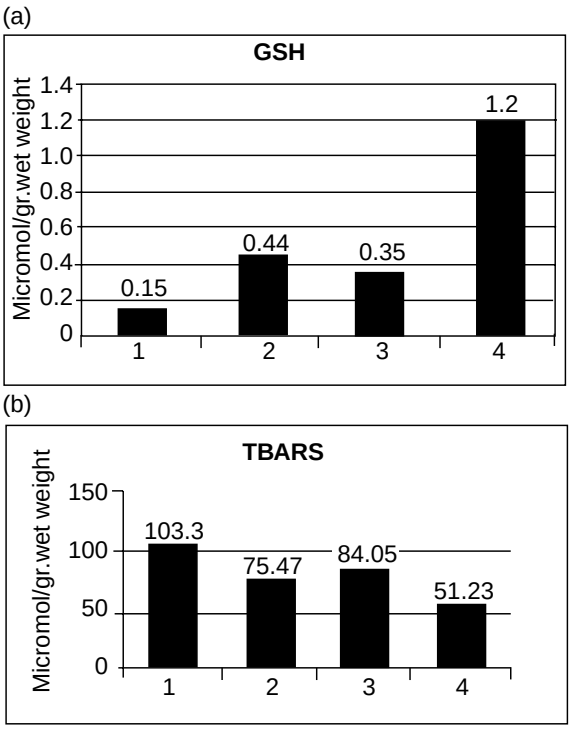

Figure 1. Tissue GSH (a) and TBARS (b) levels in groups 1, 2, 3, 4.

Left Ventricular Function

There was no recovery of contractile force. At 30 min it was approximately 50 per cent of the initial value for all groups ($p < 0{\cdot}05$) (Table 2).

TBARS

Hearts treated with lisinopril achieved significantly lower levels at the end of the reperfusion period ($103{\cdot}30 \pm 5{\cdot}79$ μmol g^{-1} wet weight in the controls versus $75{\cdot}47 \pm 1{\cdot}29$ μmol g^{-1} wet weight in group 2, $84{\cdot}05 \pm 5{\cdot}83$ μmol g^{-1} wet weight in group 3 and $51{\cdot}23 \pm 2{\cdot}1$ μmol g^{-1} wet weight in group 4, $p < 0{\cdot}0001$). Among the groups; the best results were achieved with group 4. Again the differences were statistically significant between groups 2–4 and 2–3 ($p < 0{\cdot}001$). However levels were similar in groups 3 and 4 ($p > 0{\cdot}05$) (Figure 1).

Reduced Glutathione Levels

In hearts pretreated with Lisinopril and reperfused with lisinopril-enriched Krebs–Henseleit solution, GSH levels were significantly higher at the end of the reperfusion period ($0{\cdot}15 \pm 0{\cdot}001$ in the control versus $1{\cdot}20 \pm 0{\cdot}22$ in group 4, $p < 0{\cdot}001$). However hearts receiving lisinopril in STHCS only (group 2) and pretreated with lisinopril only (group 3) did not show any significant difference from the control group. Among the groups 2–3 and 3–4; again the best levels were achieved with group 4. There was a statistically significant difference between groups 2 and 4 and groups 3 and 4 ($p < 0{\cdot}001$). However levels were similar in groups 2 and 3 ($p > 0{\cdot}005$) (Figure 1).

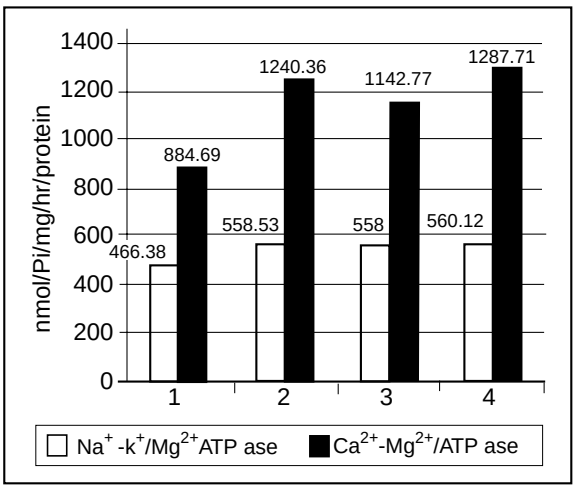

Figure 2. Tissue membrane bound enzyme levels in groups 1, 2, 3, 4.

$Na^+ - K^+$ ATPase Levels

Tissue $Na^+ - K^+$ ATPase activity at the end of the reperfusion period was significantly higher in groups 2, 3 and 4 compared to group 1 (Figure 2). However among the groups 2, 3 and 4 there were no significant differences.

Ca^{2+} ATPase Levels

In hearts treated with lisinopril Ca^{2+} ATPase levels at the end of the reperfusion period were significantly higher than the control group (Figure 2). Levels were also significantly different between groups 2 and 3 ($p < 0{\cdot}001$), 2 and 4 ($p < 0{\cdot}01$) and 3 and 4 ($p < 0{\cdot}001$).

DISCUSSION

The prevention of detrimental sequelae caused by successful reperfusion is an area of recent interest in

the field of cardiovascular surgery. Although many causal factors of ischaemia–reperfusion injury have been postulated, the integrated mechanisms linking mechanical dysfunction and arrhythmogenicity to metabolic derangements remain poorly understood. Two theories of how reperfusion injury might occur are (a) the calcium hypothesis and (b) the free radical hypothesis.[18] The former suggests that ischaemia induces a defect in the ability of the cell to regulate calcium such that upon reperfusion the cell accumulates toxic levels of calcium. The free radical hypothesis is based on the premise that partially reduced forms of molecular oxygen including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are produced at the time of reperfusion. Many studies have shown that free radical scavengers can reduce injury in the isolated heart model and argue in favour of a free radical mechanism. In fact free radicals might be inducing the membrane defect which promotes calcium entry, thus unifying both these theories. It has been previously reported that the primary target organelles attacked by the ischaemic process are sarcoplasmic reticulum and sarcolemma which may impair the sequestration of calcium into the sarcoplasmic reticular lumen. Sequestration of the released calcium is mediated by a Ca^{2+} ATPase enzyme. $Na^+ - K^+$ ATPase is another important sarcolemmal enzyme which regulates the membrane ionic potential. $Na^+ - K^+$ ATPase activity is an electrogenic process in which two Na^+ ions extrude out of the cell while one K^+ ion enters the cell, thereby maintaining an appropriate transmembrane Na^+ gradient. Normally $Na^+ - K^+$ ATPase is a major energy using process that accounts for as much as 40 per cent of the basal metabolism of the body. Further stimulation of the enzyme activity may be necessary when Na^+ influx is increased under various pathologic conditions such as post-ischaemic reperfusion. Na^+ accumulation and resultant Ca^{2+} overload could have serious consequences for the myocyte through activation of various enzymes such as lipases, phospholipases and ATPase. Various studies showed that $Na^+ - K^+$ and Ca^{2+} ATPase enzymes may play a key role in the prevention of ischaemia–reperfusion injury. Kim and Akera[19] reported that ischaemia–reperfusion of isolated guinea pig heart reduced the $Na^+ - K^+$ ATPase activity and this effect of ischaemia-reperfusion injury was prevented by oxygen free radical scavengers such as superoxide dismutase, catalase, dimethylsulfoxide, histidine or Vitamin E. Krause *et al.*[20] and Koneko *et al.*[21] showed that sarcoplasmic reticulum, isolated from stunned myocardium demonstrates a decrease in the ability to transport calcium, concomitantly with a reduction in the activity of the associated Ca^{2+} ATPase.

In the last 15 years there has been an impressive amount of work in the field of ischaemia–reperfusion injury. Most recently, studies have been targeted at finding the ideal antioxidant. Angiotensin converting enzyme inhibitors have been receiving some attention lately for their free radical scavenging properties. The mechanism of free radical scavenging is still obscure. Several types of ACE inhibitors were used as scavengers. In most of these studies captopril (a sulfhydryl-containing ACE inhibitor) was used. In some of these studies captopril was found to attenuate the ischaemia–reperfusion injury but in others it was not found to affect the O_2^- radicals and thus ischaemia–reperfusion injury. Recent studies with ACE inhibitors also showed that the presence of sulfhydryl groups in the structure of the drug did not affect superoxide radical formation.[22]

Thus there are still some questions remaining to be answered. Do all ACE inhibitors behave like antioxidants? Are there other different mechanisms for action as an antioxidant?

In our study, hearts receiving lisinopril either in the cardioplegia, by pretreatment or in the reperfusion solution had a significant improvement in myocardial membrane-bound enzyme levels compared with the control group. The best results were achieved when the drug was given for 10 days prior to ischaemia and reperfusion conducted with a lisinopril-enriched Krebs–Henseleit solution. We also found that this superiority is well correlated with the biochemical determinations such as higher levels of $Na^+ - K^+$ ATPase, Ca^{2+} ATPase, glutathione and lower levels of TBARS.

Our data showed that lisinopril added to STHCS or oral pretreatment increased the $Na^+ - K^+$ ATPase, Ca^{2+} ATPase activities and decreased TBARS. Good correlation was observed between inhibition of TBARS production and the degree of enzyme protection provided by lisinopril. GSH measurements showed a significant loss of glutathione from the tissues in group 1 when compared with group 4.

In a recent study by Golik *et al.*[23] it was shown that chronic treatment with either enalapril or captopril induces a significant rise in erythrocyte membrane $Na^+ - K^+$ ATPase and Ca^{2+} ATPase

activities. Another study by Falkner *et al.*[24] in hypertensive patients also showed that lisinopril treatment improved the Na^+ transport abnormalities. Our data showed that lisinopril was effective in restoring $Na^+ - K^+$ ATPase and Ca^{2+} ATPase levels in ischaemia–reperfusion injury. It is known that lisinopril does not contain sulfhydryl groups and since previous studies with elanapril and captopril showed that both drugs were effective in terms of restoring the membrane-bound enzymes we can conclude that the mechanism that restores the membrane ion transport system may not be related to sulfhydryl groups.[23, 24] This is also consistent with the results of Mehta *et al.*[22] They also concluded that the sulfhydryl moiety does not scavenge free radicals.

On the other hand, ACE inhibition results in a significant increase in K^+ levels which may also stimulate erythrocyte $Na^+ - K^+$ ATPase activity.

Koneko *et al.*[21] showed that inhibition of sarcolemmal Ca^{2+} pump activity by oxygen free radicals was prevented by sulfhydryl reducing agents such as DDT or cysteine. Based on our results and Koneko *et al.* the Ca^{2+} ATPase pump is not dependent on sulfhydryl groups but we can conclude that by maintaining the sulfhydryl groups (in reduced form) we can augment the activity of Ca^{2+} ATPase activity. Further studies need to be done to confirm this hypothesis.

There are several limitations to this study. First of all it was carried out in a clinical setting and it is therefore far from explaining the exact mechanism responsible for the scavenging effect of lisinopril. We could not measure the cellular calcium and sodium levels because of technical problems. $Na^+ - K^+$ ATPase, Ca^{2+} ATPase, TBARS and GSH levels were determined using homogenates instead of sarcolemmal preparations in order to circumvent possible changes in yield or purity of the sarcolemma following ischaemia–reperfusion injury. The results therefore do not specifically show the degree of sarcolemmal lipid peroxidation but provide an indication of the level of tissue peroxidation or its reversal by lisinopril.

In conclusion this study shows that protecting the membrane function by improving the membrane-bound enzyme levels is one of the critical issues in ischaemia–reperfusion injury. The results suggest that lisinopril as a non-sulfhydryl containing ACE inhibitor had a favourable effect on the recovery of enzyme activities and myocardial function after global ischaemia. Especially effective actions were produced with pretreatment, prior to cardioplegic arrest.

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