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Apelin-13 limits infarct size and improves cardiac postischemic mechanical recovery only if given after ischemia

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1Department of Clinical and Biological Sciences, S. Luigi Gonzaga Hospital, University of Turin, Orbassano; 2Department of Neuroscience, Physiology Division, University of Turin, Turin; 3Italian Institute for Cardiovascular Research (INRC), Bologna; 4Department of Medicine, Surgery and Dentistry, University of Milan, Milano, Italy

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Rastaldo R, Cappello S, Folino A, Berta GN, Sprio AE, Losano G, Samaja M, Pagliaro P. Apelin-13 limits infarct size and improves cardiac postischemic mechanical recovery only if given after ischemia. Am J Physiol Heart Circ Physiol 300: H2308–H2315, 2011. First published March 4, 2011; doi:10.1152/ajpheart.01177.2010.—We studied whether apelin-13 is cardioprotective against ischemia/reperfusion injury if given as either a pre- or postconditioning mimetic and whether the improved postischemic mechanical recovery induced by apelin-13 depends only on the reduced infarct size or also on a recovery of function of the viable myocardium. We also studied whether nitric oxide (NO) is involved in apelin-induced protection and whether the reported ischemia-induced overexpression of the apelin receptor (APJ) plays a role in cardioprotection. Langendorff-perfused rat hearts underwent 30 min of global ischemia and 120 min of reperfusion. Left ventricular pressure was recorded. Infarct size and lactate dehydrogenase release were determined to evaluate the severity of myocardial injury. Apelin-13 was infused at 0.5 μM concentration for 20 min either before ischemia or in early reperfusion, without and with NO synthase inhibition by Nω-nitro-l-arginine (l-NNA). In additional experiments, before ischemia also 1 μM apelin-13 was tested. APJ protein level was measured before and after ischemia. Whereas before ischemia apelin-13 (0.5 and 1.0 μM) was ineffective, after ischemia it reduced infarct size from 54 ± 2% to 26 ± 4% of risk area (P < 0.001) and limited the postischemic myocardial contracture (P < 0.001). l-NNA alone increased postischemic myocardial contracture. This increase was attenuated by apelin-13, which, however, was unable to reduce infarct size. Ischemia increased APJ protein level after 15-min perfusion, i.e., after most of reperfusion injury has occurred. Apelin-13 protects the heart only if given after ischemia. In this protection NO plays an important role. Apelin-13 efficiency as postconditioning mimetic cannot be explained by the increased APJ level.

APJ receptor; ischemia/reperfusion; myocardial cardiac protection; preconditioning

Apelin mRNA is expressed in several organs and tissues. In the cardiovascular system, apelin and APJ receptors occur in vascular smooth muscle, endothelial cells, and cardiomyocytes (21, 22). Apelin exerts a positive inotropic effect (2, 42) and a nitric oxide (NO)-driven vasodilator activity (14, 15). It protects the heart against ischemia/reperfusion (I/R) injury both in vitro and in vivo, but the underlying mechanism is still controversial (20, 40). In particular, a role of NO in mediating apelin-induced cardioprotection has not yet been defined (40, 50).

Zeng et al. (50) observed antiapoptotic and antistunning activities by apelin administered in the same heart before and after global ischemia, a protocol that cannot clearly indicate the exact timing required to produce the protective effect.

A remarkable protection against I/R injury can be induced with either ischemic preconditioning (preC) or postconditioning (postC). Whereas preC is obtained with one or more brief (a few min) coronary occlusions performed 5–10 min before the onset of ischemia, postC consists of very brief (a few seconds) occlusions carried out after the end of ischemia (36). Some substances, e.g., adenosine, bradykinin, opioids, sildenafil, and sphingosine, are reported to behave like effective preC or postC mimetic (4, 9, 12, 24, 27, 38, 39, 45, 49).

Nevertheless, no investigation has hitherto been performed with apelin given as a preC mimetic, i.e., administered before but not during ischemia.

Most studies with apelin have addressed either the postischemic mechanical recovery or the reduction of the infarct size (IS). Thus it remains unclear whether the mechanical recovery is attributable to the reduction of IS only and/or to a direct effect of apelin on postischemic mechanical function of myocardium. Finally, although it has been reported that ischemia induces APJ receptor overexpression (50), to our knowledge the relationship between APJ overexpression and the cardioprotective effect of apelin has not yet been investigated. This issue is pivotal because APJ overexpression induced by ischemia may be functional to assess the role of apelin as a postC mimetic. Therefore, the aims of the present investigation are 1) to assess the cardioprotective effects of apelin as a preC or postC mimetic, 2) to determine whether the improved postischemic mechanical recovery induced by apelin is mainly attributable to the reduced IS and stunning or to the apelin inotropic effects, 3) to determine whether NO is involved in the protective mechanism, and 4) to evaluate APJ protein amount after ischemia.

To these aims, we designed experiments whereby apelin-13 was given either before ischemia (Ap-preC) or during early reperfusion (Ap-postC).
MATERIALS AND METHODS

Animals

Six-mo-old male Wistar rats (Harlan-Italy, S. Pietro al Natisone, Italy) weighing 300–400 g were housed three per cage in a ventilated cage rack system under standard conditions. The present investigation conforms with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health [DHEW Publication No. (NIH) 85-23, revised 1996, Office of Science and health Reports, DRR/NIH, Bethesda, MD20205] and are in accordance with the Italian ethical guidelines (DL. 116, 27 Jan, 1992). The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy and by the Ethical Committee of the University of Turin.

Isolated Heart Preparation

Ten minutes after heparin injection, animals were anesthetized with urethane (1 g/kg) intraperitoneally. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer (127 mM NaCl, 17.7 mM NaHCO₃, 1.26 mM MgCl₂, 5.1 mM KCl, 1.5 mM CaCl₂, 11 mM d-glucose, and 10 μg/ml xylocaine). Then the hearts were suspended on a Langendorff apparatus and retrogradely perfused via aorta at constant flow with the above Krebs-Henseleit buffer in a nonrecirculating way. The perfusate buffer was saturated with a 95% O₂-5% CO₂ gas mixture and infused at 37°C as previously described (3, 35).

The constant coronary flow was adjusted with a constant-flow perfusion pump (Watson-Marlow 505DU; Falmouth, Cornwall, UK) to obtain a level of 9 ml per min per g corresponding to a perfusion pressure of about 80 mmHg during the stabilization period. Thereafter, the pump maintained the same flow level throughout the experiments. A small hole in the left ventricular wall allowed the drainage of the thebesian flow. Coronary perfusion pressure (CPP) was measured throughout the experiments with an electronic pressure transducer (Monitoring kit mk5-02 DTNVF; Abbott, Milan, Italy) connected to the perfusion line (3, 35). Left ventricular pressure (LVP) was measured with a similar pressure transducer connected via a catheter to a polyvinyl chloride balloon placed in the left ventricle through the left atrium. The balloon was filled with saline to achieve a left ventricular end diastolic pressure (LVEDP) of about 5 mmHg. No further changes of ventricular volume were made during the experiment (3, 35). CPP and LVP were continuously recorded by a data acquisition system (Lab-View software; National Instrument, Austin, TX).

To keep the heart rate constant throughout the experiments, the hearts were electrically paced at 280 beat/min by a Grass S11 stimulator (Grass Instruments, Quincy, MA). Pacing was stopped at the beginning of the ischemia and restarted after the third minute of reperfusion (3, 35).

Chemicals for buffer solution were purchased from Sigma-Aldrich (St. Louis, MO). All the reagents necessary to assess myocardial infarction were purchased from Merck (Garcia, NJ).

Experimental Protocols

Studies on myocardial protection by apelin. The apelin-13 fragment (American Peptide, Sunnyvale, CA) was chosen because it has previously been reported to exhibit the most potent effect on myocardial protection (40). After 20 min of stabilization, each heart underwent I/R consisting of 30 min of global ischemia followed by 2 h of reperfusion. Global ischemia was obtained by arresting the perfusion pump. During ischemia the hearts were maintained at 37°C by the surrounding medium.

To find out the minimum dose of apelin-13 capable to reduce reperfusion injury, in pilot experiments (n = 2 for each concentration), the peptide was infused during the first 20 min of reperfusion at 0.1, 0.2, 0.5, and 1.0 μM concentrations. Because 0.1 and 0.2 μM were ineffective (see RESULTS), the bulk of this study was performed at apelin concentrations of 0.5 and 1.0 μM. Hearts were randomly assigned to one of the following experimental protocols (Fig. 1): Group 1 (control; n = 8): these hearts underwent I/R without any addition. Group 2 (Ap-pre-0.5; n = 7): these hearts were perfused before ischemia with 0.5 μM apelin-13 for 20 min followed by 10 min of washout to mimic ischemic preconditioning. At the end of washout, I/R was performed. Group 3 (Ap-post-0.5; n = 6): after ischemia, these hearts were perfused with 0.5 μM apelin-13 during the initial 20 min of reperfusion to mimic ischemic postconditioning. Group 4 [Ap-post-0.5 + N⁶-nitro-L-arginine (L-NNA); n = 6]: these hearts, perfused with apelin-13 after ischemia as in group 3, were treated with 100 μM of the NO synthase (NOS) inhibitor L-NNA 5 min before ischemia and during the first 25 min of reperfusion bracketing the 20-min infusion of apelin. Group 5 (L-NNA; n = 4): in these hearts L-NNA was administered as in group 4 but without apelin-13. Group 6 (Ap-pre-1; n = 4): because 0.5 μM apelin-13 was ineffective before...
ischemia (see RESULTS), we wanted to see whether the highest concentration (1 μM) reported to be effective in reperfusion (40) protects myocardium if used as a preconditioning agent. Thus in these hearts apelin-13 was given before ischemia as in group 2 but at 1 μM concentration.

Assessment of myocardial injury. IS was measured with the nitroblue-tetrazolium technique. In brief, at the end of the experiments, each heart was rapidly removed from the perfusion apparatus, and the left ventricle was cut in 1–2-mm-thick circumferential slices. Following 20 min of incubation at 37°C in 0.1% solution of nitro-blue-tetrazolium in phosphate buffer, stained viable tissue was carefully separated from unstained necrotic tissue and then weighed by an independent observer. Because ischemia was global, the total left ventricle mass corresponded to the risk area. The necrotic mass was expressed as a percentage of the total left ventricular mass (3, 35).

Because, in isolated rat hearts, IP and PostC are known to reduce the release of lactate dehydrogenase (LDH) during reperfusion (47), this enzyme was measured in the effluent during the 2 h of reperfusion as previously described (3). Data are expressed as cumulative values throughout the reperfusion period.

Cardiac function assessment. LVP was measured. Developed (LVDevP) pressure was calculated as the difference between left ventricular systolic pressure (LVSP) and LVEDP. Also the maximum rates of positive and negative changes in LVP (±dP/dt) were calculated. The changes in LVDevP and dP/dt during reperfusion were expressed as percents of the baseline value. Both of them were taken as indices of contractility, whereas LVEDP increase was taken as an index of myocardial contracture. Comparisons of these contractile variables were made throughout the entire time course of reperfusion.

Studies of ischemia-induced APJ protein expression. To test the effect of ischemia on APJ receptor protein level, 15 hearts, which did not receive apelin-13, underwent 30 min of global ischemia followed by 0, 7, 15, 30, 120 min of reperfusion (n = 3 for each conditions). To detect APJ protein basal levels, in three hearts ischemia was not induced and the perfusion lasted only 20 min. All the hearts were snap-frozen, stored at −80°C, and used for APJ protein analyses as follows: frozen samples (100 mg) were homogenized to a powder and resuspended in RIPA buffer. A sample of total proteins (100 μg) was then separated on a 10% SDS-PAGE and electrotransferred to a PVDF membrane (Macherey-Nagel, Düren, Germany). Western blot analysis was performed with the antibody APLNR (H300) (Santa Cruz Biotechnology, Heidelberg, Germany) at 1:2,000 dilution. Anti-rabbit horseradish peroxidase-conjugate secondary antibody (1:2,000) (Sigma-Aldrich) was used. Labeled bands were detected with a chemiluminescence kit (BioVision, Mountain View, CA).

Image acquisition and quantification was made with a Kodak Image Station 440CF (Kodak, Rochester, NY). Each experiment was done in triplicate.

Statistical Analysis

Data are expressed as means ± SE. One-way ANOVA and Newman-Keuls multiple-comparison test (for post-ANOVA comparisons) were used to evaluate the statistical significance of the differences of the variables among groups and for the molecular analysis data. All analyses were carried out with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA), with P < 0.05 as the significant cutoff.

RESULTS

Infarct Size

In control hearts, IS was 54 ± 2% of the left ventricular mass (Fig. 2A). Pilot experiments showed that 0.1 and 0.2 μM apelin-13 during reperfusion did not affect IS (49–54% and 51–57% of the left ventricle, respectively). By contrast, IS was reduced to 18–30% and 25–33% when apelin-13 concentration was 0.5 and 1 μM, respectively. Therefore, the minimum apelin-13 concentration required to reduce IS in reperfusion is 0.5 μM.

IS was not significantly changed with respect to the control when apelin-13 was given before ischemia (Ap-pre), either at
0.5 μM (45 ± 4%) or at 1 μM (44 ± 6%). However, IS was significantly ($P < 0.001$) reduced to 26 ± 4% when apelin-13 was infused after ischemia (Ap-post 0.5). Infarct size in Ap-post-0.5 was also significantly lower than in both Ap-pre-0.5 and Ap-pre-1 ($P < 0.05$).

In presence of l-NNA alone, IS was similar to the control (66 ± 5%; $P = \text{ns vs. control}$). However, the protective effect of Ap-post-0.5 was abolished by this NOS inhibitor (64 ± 5%; $P = \text{ns vs. control}$).

**LDH Release**

In the control hearts the cumulative LDH release collected during 2 h of reperfusion was 672 ± 139 U·l⁻¹·g⁻¹ (Fig. 2B). LDH release was not significantly different when apelin-13 was given before ischemia either at 0.5 (443 ± 44 U·l⁻¹·g⁻¹) or at 1 μM concentration (416 ± 74 U·l⁻¹·g⁻¹), but it decreased significantly ($P < 0.05$) to 186 ± 68 U·l⁻¹·g⁻¹ when apelin-13 was given during reperfusion (Ap-post-0.5). In Ap-post-0.5 + l-NNA and l-NNA hearts, LDH release were not significantly different (863 ± 107 U·l⁻¹·g⁻¹ and 833 ± 134 U·l⁻¹·g⁻¹, respectively) from the control group but were significantly higher ($P < 0.01$ and $P < 0.05$, respectively) with respect to Ap-post-0.5.

**Cardiac Function**

When given before ischemia at both 0.5 and 1 μM concentrations, apelin-13 showed the well-known transient inotropic effect (6), which lasted a few minutes only and was over before the end of the infusion.

In the control group, global ischemia and the subsequent reperfusion caused an increase in LVEDP that began before the end of ischemia (Fig. 3A), continued during the early reperfusion, and then slightly decreased throughout the experiment. At constant volume of the intraventricular balloon, LVEDP increase can be taken as an index of myocardial contracture. Whereas at both concentrations Ap-pre did not prevent LVEDP from increasing, Ap-post strongly attenuated such increase.

The effect of Ap-post-0.5 on LVEDP was blunted in Ap-post-0.5 + l-NNA, so that LVEDP was not different from the data observed in the control (Fig. 3A). Interestingly, despite the marked increase ($P < 0.001$) of contracture brought about by l-NNA alone, Ap-post-0.5 in the presence of l-NNA was still able to reduce LVEDP.

LVSP during reperfusion was not different from the baseline values in all groups with exception of l-NNA group (Fig. 3B). In fact, with respect to the other groups, in l-NNA group LVSP increased to a significantly ($P < 0.001$) greater extent.
In reperfusion, LVDevP recovered to about 80% of the basal value in Ap-post-0.5 (Fig. 3). The recovery was significantly (P < 0.001) greater not only with respect to the control group but also with respect to both Ap-pre-0.5 and Ap-pre-1, Ap-post-0.5 + 1-NNa and 1-NNa groups, which showed LVDevP values nonsignificantly different from the control group (Fig. 3).

Similarly to LVDevP, the rates of contraction (+dP/dt) and relaxation (−dP/dt) during reperfusion were significantly increased (P < 0.001) in Ap-post-0.5 hearts with respect to all the other groups (Fig. 4). Notably, when given alone, 1-NNa significantly (P < 0.05) reduced −dP/dt with respect to the administration together with apelin-13 (Ap-post-0.5 + 1-NNa).

**APJ Receptor Level after I/R**

Western blot analysis was performed to evaluate the APJ protein level in cardiac tissue in response to ischemia/reperfusion. Whereas at 0 and 7 min of reperfusion we did not observe any alteration of APJ expression, a significant (P < 0.05) increase was observed only at 15 min of reperfusion (Fig. 5).

**DISCUSSION**

Here we show that apelin-13 has protective effects in postischemic hearts only when given as a postC mimetic immediately after ischemia, whereas it is not effective as a preC mimetic. As the protective effect of apelin becomes blunted when NO release is inhibited, its action is in part a NO-dependent mechanism. Furthermore, the Ap-post-induced improvement of postischemic mechanical recovery does not appear related to increased levels of APJ proteins.

In the isolated rat heart model, the effects of the various experimental maneuvers are assessed independently of extrinsic neural and humoral factors. Furthermore, this model allows performing global ischemia. Although regional I/R is the most prevalent clinical setting of acute myocardial infarction (11), global ischemia avoids the fact that little variations in IS may be relevant if they occur in the small risk area of a regional ischemia. In addition, global ischemia excludes any interference of the collateral circulation although this is predicted not to exceed 7% in the rat heart (13, 29).

It has been reported that, in regional ischemia experiments (20), the reduction of IS was obtained with the administration of a low concentration of apelin (10 nM) in the correspondence of early reperfusion. By contrast, in global ischemia experiments (40), a remarkable reduction of IS (43%) was obtained only when apelin was given after ischemia at 1 μM, whereas 10 and 100 nM were ineffective. Starting from these data, in preliminary experiments we tested different concentrations of apelin-13 in the range 0.1–1.0 μM and found that 0.5 μM apelin-13 provides a protection similar to that elicited by...
higher apelin-13 concentrations. Therefore, in mimicking postconditioning, apelin-13 was infused at 0.5 μM concentration during the first 20 min of reperfusion, thus including the period during which most of the injury takes place (35, 45, 52).

A point to consider is the selectivity of apelin-13 at the concentrations tested. Although apelin-13/APJ selectivity has been demonstrated for nanomolar concentrations (18) and we cannot exclude that at high concentration apelin-13 can bind to different receptors, experiments on APJ knockout mice showed that apelin effect was also abolished when the peptide was administered at micromolar concentrations, without suggesting any involvement of other receptors (10, 12).

**Is Apelin a PreC or a PostC Mimetic?**

As in previous studies apelin-13 infusion started before ischemia and either continued during a regional ischemia (20) or restarted after global ischemia (50); no data is available to assess the cardiac protection exerted by apelin as a preC mimetic (36). This study shows that 0.5 μM apelin-13 protects the rat heart only when infused during the first 20 min of reperfusion but not when given before ischemia. Thus, whereas several compounds mimic both preC and postC (12, 44, 48), apelin-13 mimics postC only.

The reason whereby apelin protects the heart if given after, but not before, ischemia is not clear. However, one can speculate that, because of the clear involvement of NO in the protective effect observed with apelin-postC (see also below), timing and kinetics of the apelin-induced release of NO plays an important role. This opinion is consistent with the our previous observation (35) that, despite the well-known protective effect of NO in both ischemic preC and postC as evidenced by the Reperfusion Injury Salvage Kinases (RISK) cascade, NO may sometimes fail in reducing IS if released by NO donors, independently of their concentration.

**Is the Postischemic Mechanical Recovery Due to IS Limitation Only?**

The long-lasting postischemic mechanical recovery in Ap-post-0.5 experiments cannot be explained by the inotropic effect of the peptide. In fact, this effect is short-lasting (6) as also observed in both Ap-pre-0.5 and Ap-pre-1. Therefore the apelin-induced protection can be ascribed either to limitation of IS or to prevention of myocardial contracture. Here we show that apelin-13, in addition to the reduction of IS, also attenuates myocardial contracture and improves contractility during reperfusion (for putative mechanism see below). Indeed the long-lasting reduction of LVEDP corresponds to improved LVDevP. Thus the reduction in contracture becomes a reliable index of myocardial protection (8, 33) together with IS reduction. Because the contractile function recovery was faster and greater than that induced in various protocols of ischemic preC or postC (17, 32), this supports the importance of apelin-induced myocardial protection. We argue, therefore, that both IS and contracture reduction determine the apelin-13-induced increase in LVDevP, as also underlined by increased ±dP/dt (Figs. 3 and 4). When apelin-13 was used as a preC mimetic, it was ineffective at both 0.5 μM and 1.0 μM with regard not only to IS but also to mechanical performance.

**Is NO Involved in Ap-postC Protection?**

Although controversial (20), the RISK pathway was suggested to mediate IS limitation by apelin-13 and -36 (40). However, whereas Simpkin et al. (40) found that the total and the phosphorylated levels of endothelial NOS (eNOS) were unaltered by apelin, Zeng et al. (50) reported an apelin-induced increase of eNOS activity in neonatal cardiomyocytes after hypoxia and reperfusion. As preC and postC protection critically involves e-NOS activation (12, 37), we studied whether the blockade of NOS suppresses the protective effect of apelin against I/R injury. The effects of NOS inhibition in various models on IS (1, 3, 31) and postischemic mechanical recovery (23) have already been studied. Because L-NNA infusion impaired apelin-induced protection with regard to both IS and mechanical recovery, this finding points at NO as a mediator of the protective effects of apelin-13.

The LVEDP reduction and the increase in -dP/dt with apelin-13 as a postC mimetic is consistent with the apelin-induced increase of the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) activity (46), which reduces calcium overload via NO-dependent mechanisms. In fact, it has been reported that myocardial production of NO by eNOS and/or neuronal NOS may stimulate the activity of SERCA (7, 28, 51) by removing the inhibitory effect of phospholamban via nitrosylation of its cysteine residues (7). The role of NO on SERCA activity might explain the severe contracture attributable to L-NNA administration, when also the basal release of NO is impaired if not prevented. Because apelin-13 as a postC mimetic attenuates this contracture, the contribution of a NOS-independent mechanism cannot be excluded although our observations do not reveal any precise mechanism. Nevertheless, partial dichotomy is reported between the effects on IS and contractile function in the postischemic phase (5, 16, 26, 33).

**Is APJ Expression Involved in Ape-postC Protection?**

Because in rat hearts Zeng et al. (50) found a significant APJ protein overexpression 30 min after the end of ischemia, we investigated whether the protective activity driven by apelin as a postC mimetic is associated with earlier increase in APJ protein level. Our findings, however, show that APJ protein level is increased only after 45 min from the beginning of the ischemia, i.e., 15 min after the beginning of reperfusion. This result is in contrast with the notion that most of the ischemic injury takes place at the very beginning of reperfusion (34). Thus the link between apelin-13-induced protection and increase in APJ protein level is not a satisfactory mechanism to explain the protection obtained when apelin-13 is given as a postC mimetic. As a matter of fact, our finding is consistent with those by Smith et al. (41), who reported that apelin infusion in the initial 2.5–10 min of reperfusion triggers cardioprotection despite the lack of time correspondence with the increased amount of APJ receptor (Ref. 50 and present study). However, we cannot exclude that the APJ intracellular trafficking induced by ischemia involves delocalization of the APJ receptor from intracellular vesicles to the plasma membrane of surviving cells, a phenomenon that cannot be clearly detected by Western blot methods.

In conclusion, the cardioprotective effects of apelin mimic ischemic postC, but not preC interventions; the improved mechanical recovery induced by apelin is attributed, not only to reduced IS, but also to reduced postischemic diastolic...
contracture. Whereas the infarct limiting effect seems to be fully NO dependent, contracture limitation is only partially suppressed by apelin in the presence of NOS-blockade. The timing of the increase in APJ protein level after ischemia cannot explain the protective effect of apelin as postC mimic.

The endogenous origin of this effective compound as well as the peculiarity of being a postC mimic warrants further investigation into the mode of action of apelin-13 within the pool of treatments under study for postinfarction therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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