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Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism

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Abstract

We used two experimental models to prove that resveratrol (*trans*-3,4′,5-trihydroxystilbene) reduces cardiac ischemic-reperfusion injury by means of a nitric oxide- and adenosine-dependent mechanism. (1) *Acute ex vivo*: resveratrol (10 μM, 10 min) infusion in Langendorff-perfused normoxic rat hearts significantly increased adenosine release and coronary flow compared with baseline. After 30-min low-flow ischemia, vasodilation, still present at reperfusion, was completely abolished by resveratrol plus adenosine antagonist 8-(*p*-sulfophenyl)theophylline (SPT, 50 μM) administration. (2) *Chronic in vivo*: rats received tap water containing 25 mg/l resveratrol for 15 days or normal water. Twenty-four hours after, their hearts were Langendorff-perfused and submitted to 60-min low-flow ischemia and reperfusion. The resveratrol-treated hearts showed better functional recovery at reperfusion and significant vasodilation, but no variation in high-energy phosphates (^{31}P Nuclear Magnetic Resonance). N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM), a nonselective nitric oxide synthase inhibitor, or SPT (50 μM) administered for 10 min prior to the low-flow ischemia cancelled the effects. This suggests that long-term moderate resveratrol consumption could play an important role in late cardioprotective effects.

Keywords: Adenosine; Preconditioning; ^{31}P-NMR (nuclear magnetic resonance); (Rat); Ischemia, low-flow

1. Introduction

The so-called "French paradox" (a very low mortality rate due to coronary heart disease despite a high-fat diet and smoking habits) has been partially ascribed to the consumption of wine, particularly red wines (Renaud and de Lorgeril, 1992; Renaud et al., 1998).

Recent studies have shown that, like with many other polyphenols, resveratrol (*trans*-3,4′,5-trihydroxystilbene, a polyphenol produced in grapes and present in wines) is responsible for the cardiovascular benefits associated with moderate wine consumption (Constant, 1997). Over the last decade, it has been shown that resveratrol modulates lipid and lipoprotein metabolism (Arichi et al., 1982; Kimura et al., 1985; Ragazzi et al., 1988), inhibits platelet activation/aggregation (Bertelli et al., 1995) and the activity of some protein kinases (Jayatilake et al., 1993), and has strong antioxidant activity (Frankel et al., 1993; Rotondo et al., 1998). Resveratrol also has anticancer, estrogenic and vasorelaxing activity (Fremont, 2000). It has recently been found that resveratrol exerts a cardioprotective action as a result of its antioxidant activity (Ray et al., 1999) and the upregulation of nitric oxide (NO) (Hung et al., 2000).

In a preliminary note, we (Bradamante et al., 2000) provided evidence that 10 min of resveratrol infusion (10 μM) in Langendorff-perfused rat hearts caused a 40% decrease in baseline phosphorylation potential ($P < 0.05$ vs. pre infusion value) without affecting contractility. The level of effluent adenosine was increased by 68% and paralleled a 50% increase in coronary flow. We suggested that the metabolic pattern following resveratrol infusion is similar to that produced by ischemic preconditioning, thus indicating that an increase in adenosine availability is

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involved in resveratrol cardioprotection. We have now tested this hypothesis using a low-flow ischemia protocol that we believe is more clinically relevant. The results show that resveratrol stimulates adenosine release, and significantly increases coronary flow. The effect is almost completely abolished using the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (SPT).

It has been shown that the activation of adenosine receptors is a pharmacological preconditioning (Guo et al., 1999; Zhao et al., 2000; Takano et al., 2001) that can elicit late-phase protection (12–24 h after the stimulus). In addition, Bolli (2001) pointed to NO as a trigger and mediator of late preconditioning.

We tested the effects of prolonged administration of resveratrol (15 days, 25 mg/l) in rats by observing the behaviour of their Langendorff-perfused hearts 24 h after the drug treatment. The hearts subjected to a low-flow ischemia protocol showed vasodilation and improved functional recovery at reperfusion. The use of the NO antagonist N^G-nitro-L-arginine methyl ester (L-NAME) as well as the adenosine antagonist SPT cancelled the effects.

2. Materials and methods

2.1. Heart perfusion

All of the animals were treated in accordance with the guiding principles in the care and use of animals approved by the American Physiology Society. The protocols were approved by the Animal Welfare Committee, Erasmus University, Rotterdam.

After anesthesia induced by an intraperitoneal injection of 0.5 ml sodium pentobarbital (Nembutal®, 60 mg/ml) supplemented with 0.1 ml heparin (Thromboliquine®, 5000 IU/ml), the hearts of fed male Sprague–Dawley rats (250–300 g) were rapidly isolated and arrested in ice-cold perfusion fluid. The aorta was cannulated and the heart was Langendorff-perfused at a constant pressure of 70 mm Hg. The perfusion medium was a modified Krebs–Henseleit (KH) solution (composition in mM): NaCl 118, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 20, α-D-glucose 10, saturated at 37 °C with 95% O_2–5% CO_2, pH 7.4. Temperature changes during ischemia were minimised by immersing the hearts in 37 °C perfusate controlled by a Bruker temperature controller accessory in the case of the Nuclear Magnetic Resonance (NMR) experiments; alternatively, they were immersed in a water-jacketed heart chamber and the temperature of their outer ventricular wall was monitored by means of a thermocouple (A-F6, Ellab A/S, Roedovre, Denmark). To monitor contractility, a balloon connected to a Statham pressure transducer was inserted into the left ventricle and filled to give an end-diastolic pressure of 4–8 mm Hg. Low-flow ischemia was achieved using a perfusion pump operating at a flow rate of 0.6 ml/min, with a reduction of the flow rate to approximately 5% of baseline. No fibrillation phenomena were observed during the experiments. Resveratrol was dissolved in ethanol (2.3 mg resveratrol/ml ethanol), and the obtained solution was added to the KH buffer; because resveratrol is sensitive to light, the complete perfusion system was kept in the dark.

2.2. ^{31}P-NMR spectroscopy

The hearts were inserted into a broadband 20-mm probe of a Bruker AMX 500 wide-bore NMR spectrometer operating at 11.74 T. Field homogeneity was optimised by shimming the water proton signal using the decoupling coil. The ^{31}P-NMR spectra were obtained at 202.4 MHz with a 60° pulse of 20 μs, a 2-s delay, and blocks of 150 transients corresponding to 5 min of accumulation. We have previously described the measurement of myocardial phosphorus metabolites, pH and phosphorylation potential in detail (Bradamante et al., 1993, 1995). Briefly, peak areas of phosphocreatine, β-ATP and inorganic phosphate (Pi), corrected for saturation by comparison with spectra obtained under fully relaxed conditions, were converted to intracellular concentrations by calibration against a capillary inserted close to the heart in the NMR tube and containing Na-methylenediphosphonate (100 mM). Intracellular pH was derived from the chemical shift of Pi in relation to phosphocreatine (δ) using the equation $pH = 6.77 - \log(\delta - 5.78)/(3.27 - \delta)$. Zero ppm was assigned to phosphocreatine. The validity of this equation has been checked for our experimental conditions (Flaherty et al., 1982). The phosphorylation potential ([ATP]/([ADP]·[Pi])) was estimated from the creatine-kinase equation:

$$[ATP]/([ADP] \cdot [Pi]) = \{[PCr]/([Cr] \cdot [Pi])\} \cdot ([H^+]/K_{ck}) \quad (1)$$

where $[H^+]$ is the cytosolic H^+ concentration and K_{ck} is the pH- and $[Mg^{2+}]$-dependent creatine-kinase equilibrium constant. The following relationship was used to calculate $[H^+]/K_{ck}$, according to Bunger et al. (1991):

$$\log([H^+]/K_{ck}) = 7.52 - 0.97pH + 3.12[Mg^{2+}]^{0.11} \quad (2)$$

The creatine concentration [Cr] was determined according to Bunger et al. (1991) and the creatine pool (phosphocreatine + Cr) was checked to remain constant during each experiment.

2.3. Experimental protocols

2.3.1. Acute experiments

The three experimental groups consisted of randomly assigned rats, killed and Langendorff-perfused as described in Section 2.1. In each experiment, after 20 min of stabilisation, the hearts received different treatments for 10 min before low-flow ischemia (0.6 ml/min) lasting 30 min followed by 30 min of reperfusion (Fig. 1A). The *Res* group

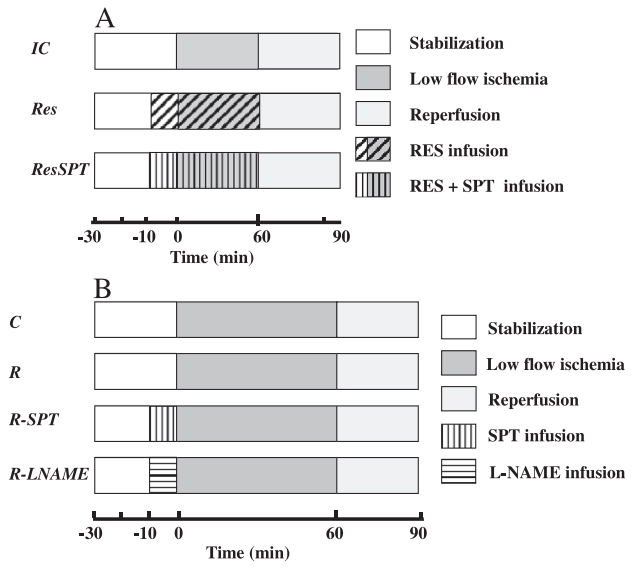

Fig. 1. (A) Acute experimental protocol. After 20 min of stabilisation, the *Res* group received 10 μM resveratrol, the *ResSPT* group received 10 μM resveratrol and 50 μM 8-(*p*-sulfophenyl)theophylline (SPT). For the same period, 10 min, the control hearts (*IC*) received only KH buffer. With drug treatment continued, the hearts ($n=17$) were exposed to 30 min of low-flow ischemia (0.6 ml/min) followed by 30 min of reperfusion. Neither resveratrol nor SPT were present in the buffer during reperfusion. (B) Chronic experimental protocol. After 20 min of stabilisation, the *R-SPT* group received 50 μM SPT, the *R-LNAME* group received 30 μM L-NAME. For the same period, 10 min, the control hearts (*C*) and the resveratrol treated hearts (*R*) received only KH buffer. All of the hearts ($n=23$) were exposed to 60 min of low-flow ischemia (0.6 ml/min) followed by 30 min of reperfusion. Neither SPT nor L-NAME were present in the buffer during ischemia–reperfusion.

($n=6$) received 10 μM resveratrol in the perfusate 10 min before and during low-flow ischemia, but not during reperfusion. The *ResSPT* group ($n=4$) received 10 μM resveratrol + 50 μM SPT 10 min before and during low-flow ischemia, but not during reperfusion. The control group *IC* ($n=7$) was only monitored before being subjected to ischemia–reperfusion.

2.3.2. Chronic experiments

Rats were divided into four groups: *R* ($n=5$), *R-SPT* ($n=5$) and *R-LNAME* ($n=5$) received tap water containing 25 mg/l resveratrol for a period of 15 days, and group *C* ($n=8$) was given normal drinking water for the same period. Twenty-four hours after this treatment, their hearts were Langendorff-perfused at constant pressure and subjected to a protocol of low-flow ischemia (Fig. 1B). The ischemia was obtained by reducing the natural flow to 0.6 ml/min and lasted for 60 min, after which the hearts underwent 30 min of reperfusion. During the last 10 min of the stabilisation period, the *R-SPT* group received 50 μM SPT and the *R-LNAME* group 30 μM L-NAME. The perfusion medium was free of both SPT and L-NAME during low-flow ischemia and reperfusion. The hearts were weighed after the procedure.

To dissolve resveratrol in water, ethanol was used as a carrier (25 mg resveratrol/ml ethanol), an ethanol dose that has no pharmacological or synergistic effects (Chan et al., 2000). As resveratrol is sensitive to light, all of the drinking bottles were covered with aluminum foil. The rats received standard food ad libitum. There were no differences in the amounts of water drunk by the four groups, the average being 40 ml/day (≈ 1 mg resveratrol/day for the animals in the resveratrol-treated groups). There was also no difference in average weight between the four groups at the end of the 15-day pretreatment.

2.4. Analysis of coronary effluent

The coronary effluents of the *Res* and *IC* groups used for the acute resveratrol protocol were analysed for purine and lactate release. During stabilisation, ischemia and reperfusion, coronary perfusate samples were collected at 1, 2, 3, 5 or 15 min intervals depending on the changes expected, immediately cooled in ice water, and stored at − 55 °C until further analysis. The purines in the samples were determined by means of reversed-phase high-performance liquid chromatography (HPLC) according to Smolenski et al. (1990). Briefly, a C_{18} column (Hypersil ODS 3 μm, 150 × 4.6 mm, Alltech, Deerfield, IL, USA) was used in combination with a C_{18} guard column (Hypersil ODS 5 μm, 7.5 × 4.6 mm). The system configuration consisted of an AS3000 cooled autosampler, an SCM1000 vacuum membrane degasser, a P2000 gradient pump, a Spectra Focus forward optical scanning detector, and PC1000 software (Spectra-Physics, San Jose, CA, USA). Peaks were detected at 254 nm (hypoxanthine, xanthine, inosine, adenosine) and at 280 nm (uric acid). The purines were identified on the basis of their retention times, co-elution with standards, and their 254 nm/280 nm ratios. The amount of lactate in the samples was determined enzymatically using an Elan auto-analyser (Eppendorf, Merck, Amsterdam, Netherlands) according to Sigma procedure #735 (St. Louis, MO, USA).

2.5. Exclusion criteria

During stabilisation, hearts were excluded if they showed unstable contractile function, coronary flows outside the range of 10–20 ml/min or severe arrhythmia.

2.6. Chemicals

L-NAME and SPT were purchased from Sigma. Resveratrol was a gift from Pharmascience, Montreal, Canada.

2.7. Statistical analysis

The data are expressed as mean values ± S.E.M. The hemodynamic and metabolic data were analysed using repeated measures and/or Student's *t*-test for the comparison

of two groups, or a paired *t*-test for within-group comparisons at different time-points. *P* values <0.05 were considered significant.

3. Results

3.1. Effects of acute resveratrol administration

Our preliminary results indicated that several changes occur during resveratrol infusion in normoxic hearts as shown in Table 1, and that resveratrol affects the function and high-energy phosphate metabolism of rat hearts subjected to 20-min *stop-flow* ischemia (Bradamante et al., 2000). We have now extended our research into the effects of resveratrol using a milder ischemic protocol (*low-flow* ischemia, Fig. 1) in order to evaluate purine and lactate release in coronary effluent and coronary flow changes before and after ischemia.

With this protocol, resveratrol infusion did not affect contractile function (data not shown), but affected coronary flow during both stabilisation and reperfusion (Fig. 2). After 10 min of infusion, coronary flow significantly increased in comparison with the baseline levels in the same group ($46.3 \pm 19.5\%$; $P<0.05$) and the *IC* group ($40.9 \pm 10.3\%$ $P<0.05$). After 15 min of reperfusion, the *Res* group showed a significant increase in coronary flow vs. the *IC* group ($P<0.01$), which was still apparent after 30 min ($P<0.05$).

The effect of 10 μM resveratrol on purine release is shown in Fig. 3A and B. As expected, 10 min of resveratrol infusion led to a significant increase in adenosine release vs. the *IC* group ($95.5 \pm 31.0\%$, $P<0.05$) and $115.6 \pm 41.0\%$ in comparison with $t=-10$ min in the same group ($P<0.05$). During reperfusion, no significant differences were seen in adenosine release in comparison with the *IC* group. No significant effect on inosine, hypoxanthine, xanthine or urate release was observed (data not shown), although there was a tendency towards higher purine release in the *Res* group (Fig. 3B). Lactate release levels in the two groups were similar throughout the protocol (Fig. 3C), thus indicating similar anaerobic glycolysis.

Table 1
Effects on metabolic and functional parameters of 10 min of 10 μM resveratrol infusion in Langendoff-perfused rat hearts ($n=6$) under normoxic conditions[a,b]

Time (min)	0	10	*P*
PCr (μmol gww^{-1})	4.99 ± 0.33	4.35 ± 0.22	<0.05
Pi (μmol gww^{-1})	1.57 ± 0.12	2.18 ± 0.25	<0.05
PP (mM^{-1})	107.6 ± 16.0	60.0 ± 7.4	<0.02
Ado (nmol min^{-1} gww^{-1})[c]	0.18 ± 0.02	0.38 ± 0.06	<0.02
CF (ml min^{-1})	9.0 ± 0.8	12.9 ± 1.5	<0.05

[a] PCr = phosphocreatine; Pi = inorganic phosphates; PP = phosphorylation potential; Ado = adenosine; CF = coronary flow.
[b] Data from Bradamante et al. (2000).
[c] In control hearts adenosine release was 0.20 ± 0.02 at $t=0$ min and 0.22 ± 0.02 at $t=10$ min.

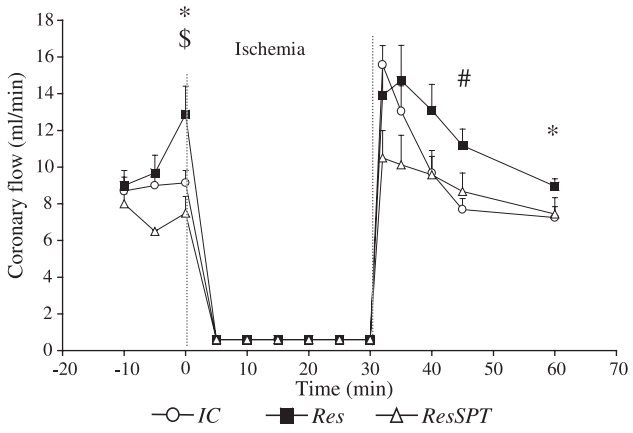

Fig. 2. Changes in coronary flow during the acute protocol. At $t=0$ min, the *Res* group ($n=6$) showed significantly increased coronary flow in comparison with $t=-10$ min ($ $P<0.05$), and $*P<0.05$ vs. *IC* ($n=7$) and *ResSPT* ($n=4$). During reperfusion, there was a significant increase in coronary flow in the *Res* group: $t=45$ min, $\#P<0.01$ vs. *IC* and *ResSPT*; $t=60$ min, $*P<0.05$ vs. *IC* and *ResSPT*. For abbreviations and protocol, see legend to Fig. 1.

We confirmed that the increase in coronary flow was adenosine-mediated by evaluating the effect of administering the adenosine receptor antagonist SPT (50 μM) in combination with resveratrol (10 μM) during the pre-ischemic period. The increase in coronary flow was completely cancelled by adenosine receptor antagonist administration (Fig. 2). There was no difference in contractile function between the *ResSPT* and *IC* groups at any time (data not shown).

3.2. Effects of chronic resveratrol administration

Chronic 15-day resveratrol administration (see Section 2.3.2) had no effect on the rats' growth rate and was stopped 24 h before the perfusion experiments. The treated and control groups (*R* and *C*, respectively) were submitted to 60 min of low-flow ischemia (reduction of the natural coronary flow to 0.6 ml/min) and 30 min of reperfusion.

Chronic resveratrol consumption affected cardiac function but not coronary flow (see Fig. 4). After 30 min of stabilisation, heart rate in the *R* group was significantly lower than in the *C* group (180.0 ± 8.9 vs. 235.7 ± 13.1 beats/min; $P=0.01$) as was the rate-pressure product (*R*: 10818 ± 1959 vs. *C*: 19254 ± 2517 mm Hg/min; $P<0.05$). The developed pressure in the *R* group was 25% lower, but this change was not significant (64.5 ± 14.5 vs. 85.6 ± 12.2 mm Hg; NS).

High-energy phosphate metabolism was monitored using NMR spectroscopy (Fig. 5). There was no significant difference in the behaviour of ATP, phosphocreatine and Pi levels or pH between the two groups throughout the ischemic protocol, but it is worth noting that very uniform behaviour of all of the resveratrol-treated hearts led to very small

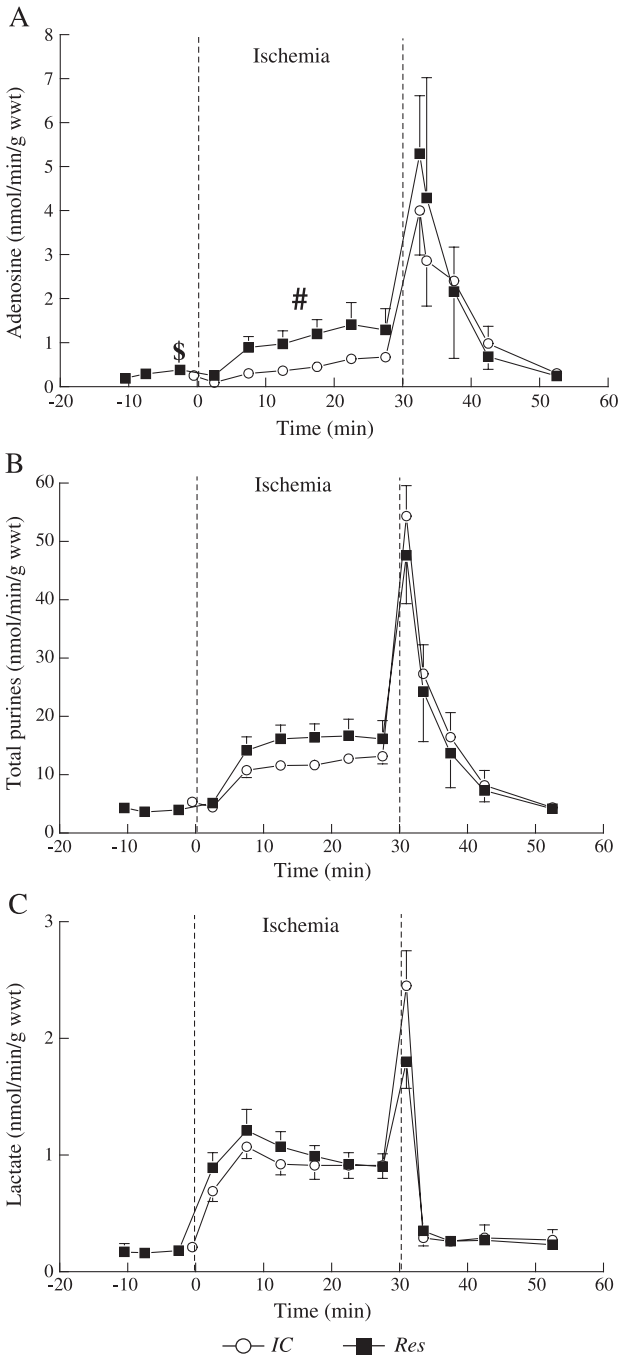

Fig. 3. Release of (A) adenosine, (B) total purines and (C) lactate during the acute protocol. Ten minutes of resveratrol infusion produced a significant increase in adenosine release in comparison with $t = -10$ min ($ $P < 0.05$). During low-flow ischemia, the *Res* group released significantly more adenosine than the *IC* group (# $P < 0.05$). No significant variations in total purine or lactate release were observed. For abbreviations and protocol, see legend to Fig. 1.

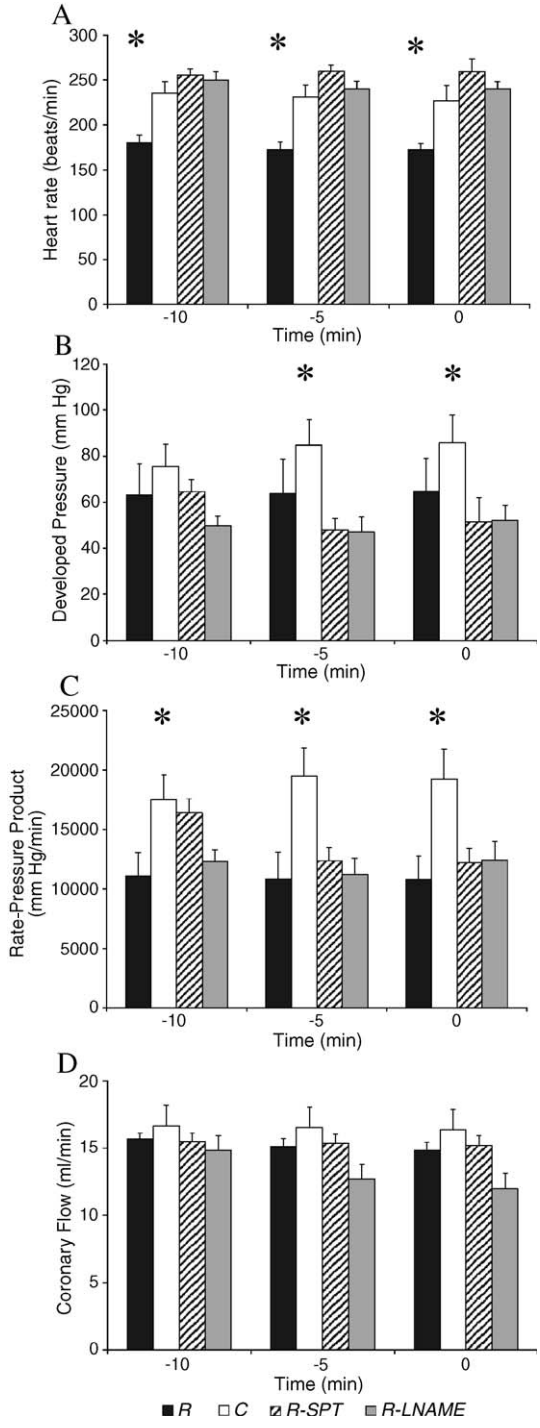

Fig. 4. Changes in (A) heart rate, (B) developed pressure, (C) rate-pressure product and (D) coronary flow measured during the last 10 min of stabilisation before the low-flow ischemic-reperfusion protocol B in Langendorff-perfused hearts of rats after chronic resveratrol consumption. Groups *R* ($n = 5$), *R-LNAME* ($n = 5$) and *R-SPT* ($n = 5$) received tap water containing resveratrol (25 mg/l) for 15 days. Group *C* ($n = 8$) received tap water for 15 days. During the observed 10 min, the groups *R-LNAME* and *R-SPT* received L-NAME (30 μM) and SPT (50 μM), respectively. (A) *$P < 0.05$ of *R* vs. *C*, *R-LNAME* and *R-SPT*. (B) *$P < 0.05$ of *C* vs. *R-LNAME* and *R-SPT*. (C) *$P < 0.05$ of *C* vs. R at $t = -10$ min; *$P < 0.05$ of *C* vs. *R*, *R-LNAME* and *R-SPT* at $t = -5$, 0 min.

standard errors in comparison with the expected variability shown by the controls.

The effect of chronic resveratrol consumption on coronary flow and contractile function is shown in Fig. 6. There was a significant difference in coronary flow recovery at the

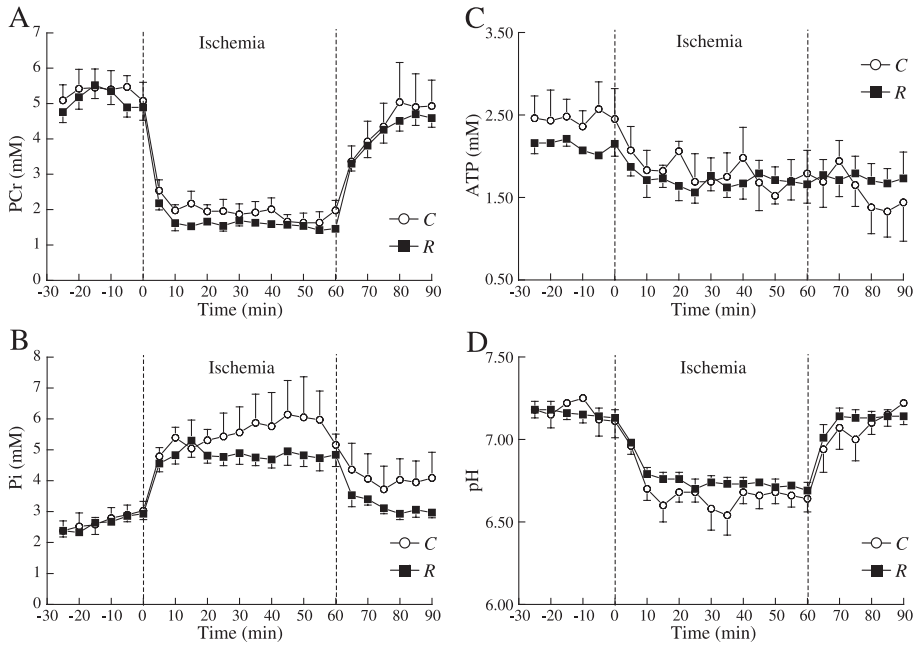

Fig. 5. Changes in (A) phosphocreatine (PCr), (B) inorganic phosphates (Pi), (C) ATP and (D) pH of Langendorff-perfused hearts of rats after chronic resveratrol consumption. The applied protocol consisted of 30 min of stabilisation, 60 min of low-flow ischemia (0.6 ml/min), 30 min of reperfusion. For abbreviations, see legend to Fig. 4.

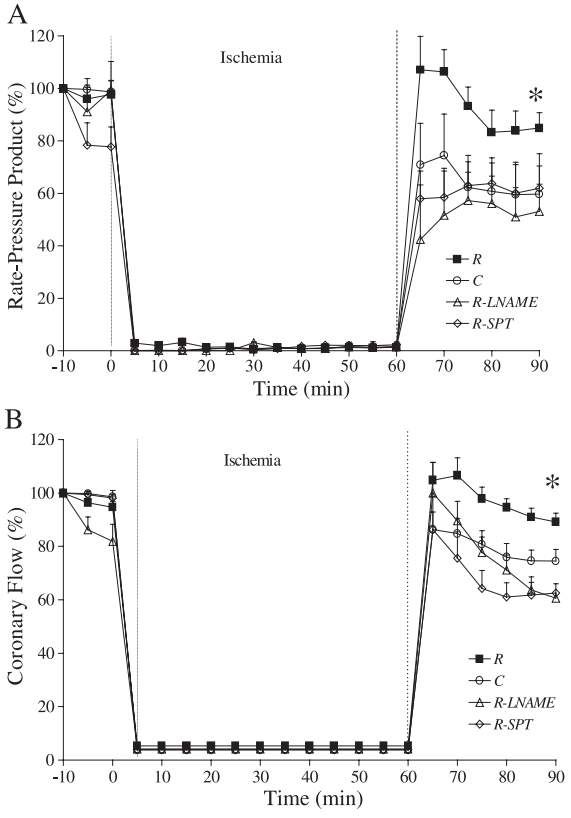

Fig. 6. Changes in (A) rate-pressure product and (B) coronary flow expressed as percentages of baseline values (for 100% values, see Fig. 4) in Langendorff-perfused hearts of rats after chronic resveratrol consumption. *$P<0.05$ of *R* vs. *C*, *R-LNAME* and *R-SPT*. For abbreviations and protocol, see legends to Figs. 4 and 5.

end of reperfusion (in comparison with the values at the end of stabilisation): 73.9 ± 4.0% for group *C* vs. 89.5 ± 3.1% for group *R* ($P<0.05$). The *R* group showed better functional recovery, with the restoration of almost initial values (rate-pressure product: 85.5 ± 5.0% vs. 59.6 ± 6.0%; $P<0.05$).

The recovery in function and coronary flow of the resveratrol-treated hearts was still present 24 h after resveratrol administration was stopped and this, together with the homogeneous metabolic behaviour, prompted us to consider that it may be a long-lasting effect. Taking into account the results of adenosine-mediated vasodilation (see acute protocol) and NO production (Hung et al., 2000), we examined the effects of the NO antagonist L-NAME and the adenosine receptor antagonist SPT on contractile function and coronary flow. The use of the antagonists prior to ischemia had no significant effect on either (Fig. 4), but the vasodilation effect and functional recovery observed *at reperfusion* were completely abolished by the SPT and L-NAME treatments (Fig. 6).

4. Discussion

Recent studies indicate that resveratrol-treated hearts are resistant to ischemic-reperfusion injury as shown by improved post-ischemic ventricular function and reduced infarct size on isolated perfused working rat hearts (Ray et al., 1999). Resveratrol has also been found to be effective in anaesthetised rat hearts subjected to transient regional ischemia (Hung et al., 2000). The observed cardioprotection

was mainly attributed to resveratrol's antioxidant activity, particularly its ability to act as a scavenger of peroxyl radicals. Our present results show that other mechanisms add to the antioxidant effect.

4.1. The acute experiment

We used the same resveratrol concentration of 10 μM that has been found to be effective in other ischemic-reperfusion experiments (Ray et al., 1999; Hung et al., 2000; Bradamante et al., 2000). Resveratrol administration immediately caused coronary vasodilation leading to an increase in coronary flow in the *Res* group. The simultaneous increase in adenosine levels observed during the stabilisation period suggested a possible cause–effect relationship. Furthermore, the significant increase in coronary flow during the reperfusion period may have been due to the significant increase in adenosine levels during ischemia, as shown in Fig. 3. We exclude a direct effect of resveratrol on vasodilation because its chemical structure does not fulfil the reported criteria for direct binding to any adenosine receptors (Jacobson et al., 1992).

We tested the hypothesis that the vasodilation was adenosine-mediated by using the nonselective adenosine receptor antagonist SPT during resveratrol administration (Fig. 2), which is known to impair vasodilation mediated in the heart by adenosine A_1 and A_2 receptors (Daly et al., 1985). The concentration used in our study (50 μM) has been shown to be sufficient to block adenosine receptors under similar experimental conditions (Headrick, 1996; De Jonge et al., 2001). The results show that the increase in coronary flow was completely abolished by SPT treatment, thus confirming that it was caused by an increase in adenosine.

This evidence extends our previous results (Table 1) concerning the reduced metabolism, as revealed by phosphorylation potential values, due to resveratrol administration under normoxic conditions; this reduction is probably mediated by the increase in adenosine, which acts on adenosine A_1 myocardial receptors.

It was thought that the vasodilating effect of adenosine is based on the direct stimulation of adenosine A_2 receptors on vascular smooth muscle cells, which mediate an increase in second messenger cAMP by stimulating adenylate cyclase. However, other investigations dispute the endothelium-independent character of adenosine-mediated vasodilation and indicate that a number of potential mechanisms may mediate the endothelial release of NO after adenosine formation (Smits et al., 1995). On the basis of published data, SPT treatment should also block the NO activity mediated by adenosine A_2 receptors (Vials and Burnstock, 1993), and so our conclusion does not contradict the evidence that resveratrol can stimulate aortic ring vasodilation by means of an NO-mediated process (Chen and Pace-Asciak, 1996) and increase NO production (Hung et al., 2000).

4.2. The chronic experiment

The amount of resveratrol used in the chronic experiment was chosen with the aim of reaching a plasma resveratrol concentration similar to that used in the acute experiment (10 μM). The dose was selected also bearing in mind some of the results obtained by means of the chronic administration of red wine (Bertelli et al., 1996) and pure resveratrol absorption in rats (Soleas et al., 2001a,b), despite the differences in administering the drug and the discrepancies in pharmacokinetic results possibly due to the use of pure resveratrol or a polyphenol mixture. However, all authors agree that, 24 h after resveratrol administration withdrawal, any protective effect must be due to resveratrol accumulation in the organs or long-lasting effects.

We cannot completely exclude the possibility that resveratrol accumulates in the heart and has a *direct* cardioprotective effect, although the quantities detected in rat hearts (Bertelli et al., 1996) are well below any possible pharmacological action. However, we investigated the possibility that resveratrol administration may provide a lasting *indirect* benefit. In order to avoid the superimposition of effects, the low-flow ischemia–reperfusion experiments were performed 24 h after the withdrawal of resveratrol treatment. The resveratrol-treated hearts subjected to 60-min low-flow ischemia and reperfusion showed better functional recovery than the controls. Under our experimental conditions, the significant vasodilation and recovery of contractile function seem to be indices of cardioprotection.

Our hypothesis is that continuous resveratrol administration stimulates adenosine production and thus activates adenosine A_1 and A_3 receptors, and that this treatment may be equivalent to pharmacological preconditioning. It has been shown that the activation of adenosine A_1 and A_3 receptors is a pharmacological preconditioning (Guo et al., 1999; Zhao et al., 2000; Takano et al., 2001) that can elicit late-phase protection (12–24 h after the stimulus). In addition, reviewing the role of NO in myocardial ischemia and preconditioning, Bolli (2001) pointed to NO as a trigger and mediator of late preconditioning. In particular, Bolli et al. identified the inducible isoform of NO synthase (iNOS) as a NO source (Guo et al., 1999) and Zhao et al. (2000) has recently suggested that iNOS has a direct cause–effect relationship with adenosine-induced late cardioprotection.

It is also known that NO modulates myocardial contractile function (negative inotropic effect) and directly inhibits the respiratory chain (Stumpe et al., 2001). These activities are in line with the reduced cardiac function observed in our *R* group before the ischemic protocol (Fig. 4A–C).

In order to test the hypothesis that NO mediates resveratrol delayed protection against ischemia, we used the NO synthase (NOS) inhibitor L-NAME. Our results show that L-NAME given for 10 min prior to low-flow ischemia blocks the long-lasting effects of resveratrol and allow us to conclude that the formation of NO is necessary for the development of the cardioprotection observed 24 h after

resveratrol withdrawal. At this stage, we cannot indicate iNOS as the NO source because we used a nonspecific NOS inhibitor. Although it has been shown that 10 μM resveratrol has no effect on endothelial NOS (eNOS) expression in intact cells (Hsieh et al., 1999), we cannot exclude a possible role of eNOS: one recent study of iNOS knockout mice has concluded that eNOS is upregulated during delayed adenosine A_1 receptor triggered pharmacological preconditioning (Bell et al., 2002).

The fact that the nonselective adenosine receptor antagonist SPT can also abolish the increase in coronary flow and function produced by prolonged resveratrol treatment suggests that the stimulation of adenosine receptors plays a primary role in the delayed effects of resveratrol. It is obvious that the mere occupation of adenosine receptors would not provide cardioprotection, but it may start a cascade of events including the upregulation of protective proteins.

4.3. Conclusion

Resveratrol infusion increases adenosine availability under normoxic conditions and is responsible for the vasodilation effects observed in acute low-flow experiments. Prolonged resveratrol treatment can protect the heart from ischemic-reperfusion injury as shown by greater post-ischemic functional recovery. This effect can be rationalised by considering that continuous resveratrol administration stimulates adenosine production and activates adenosine A_1 and A_3 receptors; this treatment may be equivalent to pharmacological preconditioning. The cardioprotective effects evaluated 24 h after resveratrol withdrawal, and cancelled by L-NAME or SPT administration, indicate that the protection is a long-lasting consequence of resveratrol infusion similar to late-phase preconditioning.

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References

Arichi, H., Kimura, Y., Okuda, H., Baba, K., Kozawa, M., Arichi, S., 1982. Effects of stilbene components of the roots of polygonum on lipid metabolism. Chem. Pharm. Bull. 30, 1766–1770.

Bell, R.M., Smith, C.C.T., Yellon, D.M., 2002. Nitric oxide as a mediator of delayed pharmacological (A_1 receptor triggered) preconditioning; is eNOS masquerading as iNOS? Cardiovasc. Res. 53, 405–413.

Bertelli, A.A., Giovannini, L., Giannessi, D., Migliori, M., Bernini, W., Fregoni, M., Bertelli, A., 1995. Antiplatelet activity of synthetic and natural resveratrol in red wine. Int. J. Tissue React. 17, 1–3.

Bertelli, A.A.E., Giovannini, L., Stradi, R., Bertelli, A., Tillement, J.-P., 1996. Plasma, urine and tissue levels of *trans*- and *cis*-resveratrol (3,4′,5-trihydroxystilbene) after short-term or prolonged administration of red wine to rats. Int. J. Tissue React. 18, 67–71.

Bolli, R., 2001. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. J. Mol. Cell. Cardiol. 33, 1897–1918.

Bradamante, S., De Jong, J.W., Piccinini, F., 1993. Intracellular magnesium homeostasis is involved in the functional recovery of preconditioned rat heart. Biochem. Biophys. Res. Commun. 196, 872–878.

Bradamante, S., Piccinini, F., Delu, C., Janssen, M., de Jong, J.W., 1995. NMR evaluation of changes in myocardial high energy metabolism produced by repeated short periods of ischemia. Biochim. Biophys. Acta 1243, 1–8.

Bradamante, S., Piccinini, F., Barenghi, L., Bertelli, A.A.E., De Jonge, R., Beemster, P., De Jong, J.W., 2000. Does resveratrol induce pharmacological preconditioning? Int. J. Tissue React. 22, 37–40.

Bunger, R., Mukohara, N., Kang, Y.H., Mallet, R.T., 1991. Combined glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase in catecholamine-stimulated guinea-pig cardiac muscle. Comparison with mass-action ratio of creatine kinase. Eur. J. Biochem. 202, 913–921.

Chan, M.M.-Y., Mattiacci, J.A., Hwang, H.S., Shah, A., Fong, D., 2000. Synergy between ethanol and grape polyphenols, quercitin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. Biochem. Pharmacol. 60, 1539–1548.

Chen, C.K., Pace-Asciak, C.R., 1996. Vasorelaxing activity of resveratrol and quercetin in isolated aorta. Gen. Pharmacol. 27, 363–366.

Constant, J., 1997. Alcohol, ischemic heart disease, and the French paradox. Coron. Artery Dis. 8, 645–649.

Daly, J.W., Padgett, W., Shamim, M.T., Butts-Lamb, P., Walters, J., 1985. 1,3-Dialkyl-8-(*p*-sulfophenyl)xanthines: potent water-soluble antagonist for A1- and A2-adenosine receptor. J. Med. Chem. 28, 482–487.

De Jonge, R., de Jong, J.W., Giacometti, D., Bradamante, S., 2001. Role of adenosine in ischemic preconditioning of rat hearts. Eur. J. Pharmacol. 414, 55–62.

Flaherty, J.T., Weisfeldt, M.L., Bulkley, B.H., Gardner, T.J., Gott, V.L., Jacobus, W.E., 1982. Mechanisms of ischemic myocardial cell damage assessed by phosphorus-31 nuclear magnetic resonance. Circulation 65, 561–570.

Frankel, E.N., Waterhouse, A.L., Kinsella, J.E., 1993. Inhibition of human LDL oxidation by resveratrol. Lancet 341, 1103–1104.

Fremont, L., 2000. Biological effects of resveratrol. Life Sci. 66, 663–673.

Guo, Y., Jones, W.K., Xuan, Y.T., Tang, X.L., Bao, W., Wu, W.J., Han, H., Laubach, V.E., Ping, P., Yang, Z., Qiu, Y., Bolli, R., 1999. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. Proc. Natl. Acad. Sci. U. S. A. 96, 11507–11512.

Headrick, J.P., 1996. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. J. Mol. Cell. Cardiol. 28, 1227–1240.

Hsieh, T.C., Juan, G., Darzynkiewicz, Z., Wu, J.M., 1999. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1), and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G2. Cancer Res. 59, 2596–2601.

Hung, L.-M., Chen, J.-K., Huang, S.-S., Lee, R.-S., Su, M.-J., 2000. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc. Res. 47, 549–555.

Jacobson, K.A., van Galen, P.J., Williams, M., 1992. Adenosine receptor: pharmacology, structure–activity relationships, and therapeutic potential. J. Med. Chem. 35, 407–422.

Jayatilake, G.S., Jayasuriya, H., Lee, E.S., Koonchanok, N.M., Geahlen, R.L., Ashendel, C.L., McLaughlin, J.L., Chang, C.J., 1993. Kinase inhibitors from *Polygonum cuspidatum*. J. Nat. Prod. 56, 1805–1810.

Kimura, Y., Okuda, H., Arichi, S., 1985. Effects of stilbene derivatives on arachidonate metabolism in leukocytes. Biochim. Biophys. Acta 837, 209–212.

Ragazzi, E., Froldi, G., Fassina, G., 1988. Resveratrol activity on guinea pig isolated trachea from normal and albumin-sensitized animals. Pharmacol. Res. Commun. 20, 79–82.

Ray, P.S., Maulik, G., Cordis, G.A., Bertelli, A.A.E., Bertelli, A., Das, D.K., 1999. The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. Free Radic. Biol. Med. 27, 160–169.

Renaud, S., de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526.

Renaud, S.C., Gueguen, R., Schenker, J., d'Houtaud, A., 1998. Alcohol and mortality in middle-aged men from eastern France. Epidemiology 9, 184–188.

Rotondo, S., Rajtar, G., Manarinis, S., Celardo, A., Rotillo, D., de Gaetano, G., Evangelista, V., Cerletti, C., 1998. Effect of *trans*-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function. Br. J. Pharmacol. 123, 1691–1699.

Smits, P., Williams, S.B., Lipson, D.E., Banitt, P., Rongen, G.A., Creager, M.A., 1995. Endothelial release of NO contributes to the vasodilator effect of adenosine in humans. Circulation 92, 2135–2141.

Smolenski, R.T., Lachno, D.R., Ledingsham, S.J., Yacoub, M.H., 1990. Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. J. Chromatogr. 527, 414–420.

Soleas, G.J., Yan, J., Goldberg, D.M., 2001a. Measurement of *trans*-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. Methods Enzymol. 335, 130–145.

Soleas, G.J., Angelini, M., Grass, L., Diamandis, E.P., Goldberg, D.M., 2001b. Absorption of *trans*-resveratrol in rats. Methods Enzymol. 335, 145–154.

Stumpe, T., Ulrich, K.M., Decking, M., Schrader, J., 2001. Nitric oxide reduces energy supply by direct action on the respiratory chain in isolated cardiomyocytes. Am. J. Physiol. 280, H2350–H2356.

Takano, H., Bolli, R., Black Jr., R.J., Kodani, E., Tang, X.-L., Yang, Z., Bhattacharya, S., Auchampach, J.A., 2001. A_1 and A_3 adenosine receptors induce late preconditioning against infarction in conscious rabbits by different mechanisms. Circ. Res. 88, 520–528.

Vials, A., Burnstock, G., 1993. A_2-purinoceptor-mediated relaxation in the guinea-pig coronary vasculature: a role for nitric oxide. Br. J. Pharmacol. 109, 424–429.

Zhao, T., Xi, L., Chelliah, J., Levasseur, J.E., Kukreja, R.C., 2000. Inducible nitric oxide synthase mediates delayed myocardial protection induced by activation of adenosine A(1) receptors: evidence from gene-knockout mice. Circulation 102, 902–907.