Pretreatment with the nitric oxide donor SNAP or nerve transection blocks humoral preconditioning by remote limb ischemia or intra-arterial adenosine

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Am J Physiol Heart Circ Physiol 299: H1598–H1603, 2010. doi:10.1152/ajpheart.00396.2010.—We have previously shown that remote ischemic preconditioning (rIPC) by transient limb ischemia leads to the release of a circulating factor(s) that induces potent myocardial protection. Intra-arterial injection of adenosine into a limb also leads to cardioprotection, but the mechanism of its signal transduction is poorly understood. Eleven groups of rabbits received saline control or rIPC or adenosine administration with additional pretreatment with the nitric oxide (NO) synthase blocker Nω-nitro-arginine methyl ester, the NO donor S-nitroso-N-acetylpenicillamine, its non-NO-donating derivative N-acetylpenicillamine, or femoral nerve section. Blood was then drawn from each animal, and the dialysate of the plasma was used to perfuse a naïve heart from an untreated donor. Infarct size was measured after 30 min of global ischemia and 120 min reperfusion. When compared with that of the control, mean infarct size was significantly smaller in groups treated with rIPC alone (P < 0.01) and intra-arterial adenosine (P < 0.01). Pretreatment with Nω-nitro-arginine methyl ester or N-acetylpenicillamine did not affect the level of protection induced by rIPC (P = not significant, compared with rIPC alone) or intra-arterial adenosine (P = not significant, compared with intra-arterial adenosine alone), but prior femoral nerve transection or pretreatment with S-nitroso-N-acetylpenicillamine abolished the cardioprotective effect of intra-arterial adenosine and rIPC. Intra-arterial adenosine, like rIPC, releases a blood-borne cardioprotective factor(s) that is dependent on an intact femoral nerve and is inhibited by pretreatment with a NO donor. These results may be important when designing or assessing the results of clinical trials for the downstream signaling of remote preconditioning, but previous studies, e.g., using ganglion blockade in a mesenteric transient ischemia model (8), have concluded otherwise. This may be because these other studies have failed to separate the influence of neural pathways in the organ (e.g., limb) generating the stimulus from the effects in the target organ.

It does appear, however, that intact local neural pathways in the stimulus organ are required for effective signal transduction of the rIPC stimulus. Dong and coworkers (5) showed that femoral nerve transection (FNS) before transient limb ischemia abolished in vivo cardioprotection in a rat model. They also showed that intrafemoral arterial, but not intravenous, adenosine recapitulated the effects of rIPC and that this effect of intra-arterial adenosine was only partially abrogated by prior treatment with an adenosine A1 receptor blocker. Similarly, in another study an intramesenteric arterial, but not venous, infusion of adenosine induced in vivo cardioprotection identical to that induced by transient mesenteric ischemia and was blocked by pretreatment with hexamethonium and the nonspecific adenosine blocker d-(p-sulfophenyl)theophylline in a rat model (13). The authors in the latter study concluded that “in intraorgan preconditioning by small intestinal ischemia, locally released adenosine triggersafferent nerves that in turn lead to the stimulation of myocardial adenosine receptors.” However, the in vivo nature of their model (infarct size was measured in the heart of the same animal subjected to mesenteric rIPC, and so the heart itself might be affected by circulating adenosine receptor blockade) makes it difficult to dissect the components of this stimulus. Thus, although the biological pathways by which rIPC and intra-arterial adenosine achieve cardioprotection involve an intact neural pathway, the exact mechanisms remain uncertain.

Our understanding of the role of nitric oxide (NO) signaling in rIPC is similarly challenged by a lack of separation of its role in the transduction within the organ (e.g., limb, kidney, and mesentery) generating the stimulus, from its protective effects in the target organ. In terms of cardioprotection, it is well known that NO donors infused into the heart (10), agents that increase myocardial bioavailability of NO (7), and increased intracellular NO signaling within cardiomyocytes may all be crucially important to the preconditioning effect (3). Conversely, an interruption of intramyocardial NO signaling by systemic pretreatment with NO synthase blockers may abrogate local IPC (24), rIPC by limb ischemia (2), and after a high-dose systemic administration of adenosine (16). However, such experiments also fail to examine the potential divergent role of NO in the organ generating the stimulus and its...
downstream effects within the target organ. For example, local NO release and exogenous NO donors may be neuroinhibitory to renal sensory nerve stimulation (14), which in turn may be integral to the preconditioning stimulus resulting from renal ischemia (15). We therefore hypothesized that this may be related to interruption of local release of humoral effectors of preconditioning. This may be important as NO donors are commonly used as adjunctive treatments in clinical ischemia-reperfusion syndromes (e.g., coronary angioplasty, cardiac surgery) and may have an impact on the degree of cardioprotection afforded by rIPC.

Thus we specifically examined the role of local neuronal pathways and NO signaling in the cardioprotective stimulus induced by limb ischemia and adenosine using our previously described dialysate-Langendorff model to better understand the local influence of these interventions on the signal transduction of the rIPC stimulus itself assessed as release of the circulating factor.

**MATERIALS AND METHODS**

All animal protocols were approved by the Animal Care and Use Committee of the Hospital for Sick Children in Toronto and conformed with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

*Donor rabbits.* New Zealand white rabbits (male: weight, 3–3.5 kg) were anesthetized with Akmezine (0.25 mg/kg) and pentobarbital sodium (30 mg/kg), intubated and ventilated, and anticoagulated with heparin (100 IU/kg) via a marginal ear vein. The left carotid artery of sham-operated and treated rabbits was exposed and cannulated with a 5-Fr catheter to collect blood for dialysate preparation. Two separate groups (Fig. 1A) were prepared in the same way as in *study 1* and divided into five groups (Fig. 1B): *group 1* (FA ADO), animals were treated with 10 nmol/kg adenosine (Sigma) injected in the femoral artery over ~30 s; *group 2* (FV ADO), animals were treated with 10 nmol/kg adenosine injected in the femoral vein over ~30 s; *group 3* (FNS + FV ADO), animals underwent FNS (via the inguinal incision), followed 10 min later by rIPC; *group 4* (L-NAME + rIPC), animals were pretreated with an intravenous (femoral vein) infusion of 100 μmol/kg Nω-nitro-l-arginine methyl ester (L-NAME; NO inhibitor, Sigma), followed 20 min later by rIPC; *group 5* (SNAP + rIPC), animals were pretreated with a 10 min intra-arterial (femoral artery) infusion of 65 μg·kg⁻¹·min⁻¹ S-nitroso-N-acetylpenicillamine (SNAP; NO donor, Sigma) via a 27-gauge needle, immediately followed by rIPC; and *group 6* (NAP + rIPC), animals were treated with a 10 min intra-arterial infusion of 65 μg·kg⁻¹·min⁻¹ N-acetylpenicillamine (NAP, as SNAP negative control, non-NO-donating chemical, Sigma), immediately followed by rIPC. Blood was taken for dialysate preparation ~10 min after the end of each protocol (see Dialysate preparation).

Experimental design—*study 2*: local neural influences and effects of NO on the rIPC stimulus by intra-arterial adenosine. The rabbits were anesthetized with 100 IU/kg heparin (100 IU/kg) via a marginal ear vein. The left carotid artery of sham-operated and treated rabbits was exposed and cannulated with a 5-Fr catheter to collect blood for dialysate preparation. Two separate experiments were performed.

Experimental design—*study 1*: local neural influences and effects of NO on the rIPC stimulus by limb ischemia. An inguinal cut-down was performed to expose the right femoral artery, femoral vein, and nerve. The rabbits were divided into six groups (Fig. 1A): *group 1* (sham), animals were anesthetized for 40 min, and each animal was treated with an injection of 2 ml of normal saline into the femoral artery over ~30 s; *group 2* (rIPC), rIPC was induced by four cycles of 5 min of limb ischemia (by tourniquet), followed by 5 min reperfusion as previously described (22); *group 3* (FNS + rIPC), animals underwent FNS (via the inguinal incision), followed 10 min later by rIPC, using a tourniquet placed at the midthigh level; *group 4* (FV ADO), animals were treated with 10 nmol/kg adenosine injected into the femoral artery; *group 5* (SNAP + rIPC), animals were pretreated with a 10 min intra-arterial (femoral artery) infusion of 65 μg·kg⁻¹·min⁻¹ S-nitroso-N-acetylpenicillamine (SNAP; NO donor, Sigma) via a 27-gauge needle, immediately followed by rIPC; and *group 6* (NAP + rIPC), animals were treated with a 10 min intra-arterial infusion of 65 μg·kg⁻¹·min⁻¹ N-acetylpenicillamine (NAP, as SNAP negative control, non-NO-donating chemical, Sigma), immediately followed by rIPC. Blood was taken for dialysate preparation ~10 min after the end of each protocol (see Dialysate preparation).

**Dialysate preparation.** Both control and treated rabbits had a large bore cannula placed in the left carotid artery to rapidly draw blood at *30 s*. Blood was taken for dialysate preparation *30 s;* blood was taken for dialysate preparation *10 min after the end of each protocol (see Dialysate preparation).

**A**

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**C**

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Fig. 1. Schematic overview of experimental protocols applied to each of the groups in *study 1* (A) and *study 2* (B). In *study 1*, remote ischemic preconditioning (rIPC) [4 cycles of transient ischemia (black box, 5 min) and reperfusion (5 min)] was performed with or without prior femoral nerve transection (FNS), injection of Nω-nitro-l-arginine methyl ester (L-NAME, L), N-acetylpenicillamine (NAP, N), or S-nitroso-N-acetylpenicillamine (SNAP, S). In *study 2*, adenosine (ADO; shaded box) was injected into the femoral artery (FA, A) or vein (FV, V) with or without prior femoral nerve transection (FNS; denoted by boxed X), injection of L-NAME (L) or SNAP (S). Dialysate resulting from each of the groups in *study 1* and 2 was perfused in naïve rabbit donor hearts on a Langendorff apparatus according to the protocol outlined graphically (C). TTC, triphenyltetrazolium chloride. See MATERIALS AND METHODS for additional details.

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the end of the study protocol. The protocol for preparation of plasma dialysate was as previously described (23). Approximately 100 ml of blood was obtained from each animal at the end of the treatment. Bleeding was limited to <2 min to avoid secondary hemodynamic effects. Blood gases and electrolyte measurements were taken at the end of the blood draw to confirm that each rabbit remained well oxygenated and neither acidic nor hyperkalaemic. Plasma was obtained by centrifugation (3,000 rpm for 20 min) of the whole blood. To prepare the dialysate, 50 ml of plasma were placed in dialysis tubing with a 12–14-kDa cutoff membrane (Spectrapor) and dialyzed against a 20-fold volume of Krebs-Henseleit buffer (1,000 ml). Before perfusion of the donor hearts, D-glucose and NaHCO3 were added to the dialysate and then filtered through a 0.2-μm filter. Perfusate was equilibrated with 95% oxygen-5% CO2 and adjusted to a pH of 7.35 to 7.4.

**Langendorff preparation.** Once the dialysate was prepared, a heart from an untreated rabbit (male, 2.7–3.5 kg body wt) was quickly excised, mounted on a Langendorff perfusion apparatus, and perfused under normocirculating conditions at constant pressure (80 mmHg) at 37°C. Krebs-Henseleit buffer contained (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.8 CaCl2, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose, gassed with 95% O2-5% CO2. The solution was continuously oxygenated with 95% O2-5% CO2 to maintain a final pH of 7.4. The buffer was vacuum filtered with a 0.2-μm nitrocellulose filter to remove particulates. Once placed onto the perfusion apparatus, each heart was submerged in 37°C Krebs-Henseleit buffer during the experiment. A water-filled balloon, previously made with thin plastic Saran Wrap on PE-160 polyethylene tubing, was inserted into the left ventricle (LV) through the mitral valve and connected to a pressure transducer (ML844, ADInstruments, Colorado Springs, CO). The balloon was inflated with water to adjust LV end-diastolic pressure (LVEDP) to 6–8 mmHg at the beginning of the experiment, and the volume was kept constant for the duration of the study.

Hemodynamic measurements, including heart rate, peak LV pressure, maximum rate of contraction (+dP/dt), maximum rate of relaxation (−dP/dt), and LVEDP, were recorded with a PowerLab data acquisition system (ADInstruments). The LV developed pressure was calculated as the difference between the systolic and end-diastolic LV pressures. Coronary flow was measured continuously with an in-line transit-time Doppler probe (Transonic Instruments). Each heart was allowed to stabilize for 20 min and then perfused with dialysate for 14 to 22 min, dependent on the coronary flow and the amount of dialysate. Then standard buffer was added for the remaining 8 to 16 min of a total of 30 min of pretreatment after stabilization. The hearts were then subjected to 30 min of global ischemia (37°C) and 120 min of reperfusion (Fig. 1C). Hearts were excluded from subsequent analysis for the following predetermined reasons: 1) stabilized hearts that displayed poor contractile function (<100 mmHg developed pressure after stabilization), 2) bradycardic (heart rate < 140 beats/min) or unacceptably arrhythmic during stabilization, and 3) sustained ventricular fibrillation (VF) during reperfusion.

**Measurement of infarct size.** At the end of reperfusion period, the LV was excised and frozen at −80°C. The frozen heart was cut transversely into 2-mm-thick slices using a rabbit Heart Slicer Matrix (Zivic Instruments) and stained with 1.25% triphenyltetrazolium chloride (Sigma) in 200 mM Tris (pH 7.4) for 20 min in a water bath at 37°C. After fixation for 2 h in 10% neutral-buffered formaldehyde, each slice was photographed by electronic scanning (CanoScan 4400F). The viable myocardium stained brick red, and infarct tissues appeared pale. The infarct and LV areas were measured via automated planimetry using Adobe Photoshop CS2 software, with the infarct size expressed as a percentage of the total LV area.

**Statistical analysis.** Data are reported as means ± SE. Because it had a nonlinear distribution, we elected to apply a natural logarithmic transformation on infarct size. Thereafter, the linear regression models, with maximum likelihood algorithm for parameter estimation, were created using sham or rIPC (as indicated) as the reference group. The infarct size for each experimental group was then compared against the reference (all other groups included in the same regression models). Statistical significance is based on standard error ratio to the parameter estimates representing the difference in log percent infarct size between each experimental group and the reference group. All statistical analyses were performed using SAS v. 9.2 (SAS Institute, Cary NC).

**RESULTS**

A total of 76 animals was used in this study. The data from 10 hearts were excluded at the time of Langendorff preparation because of intractable VF during stabilization in two hearts, because of contractile failure during stabilization in two hearts, and intractable VF during reperfusion in six hearts. However, there were no differences in the number of excluded hearts between the groups. The baseline function between the groups is shown in Table 1.

**Study 1: local pretreatment with SNAP or nerve transaction blocks humoral preconditioning following rIPC.** Charted data and representative images of triphenyltetrazolium chloride staining are shown in Fig. 2. Perfusion with the rIPC dialysate resulted in a significantly reduced infarct size compared with sham dialysate (28 ± 6% in rIPC vs. 46 ± 3% in sham, P < 0.01). Pretreatment with l-NAME and NAP did not block the release of the cardioprotective factor following rIPC [l-NAME + rIPC, 34 ± 5%; and NAP + rIPC, 31 ± 2%; both P = not significant (NS) compared with rIPC alone]. However, there was no difference in infarct size between sham and FNS + rIPC-treated animals (42 ± 2%, P = NS) or the SNAP + rIPC group (43 ± 7%, P = NS), suggesting an abrogation of release of the cardioprotective factor.

Perfusion with the rIPC dialysate improved postischemic cardiac performance in isolated perfused hearts at 120 min reperfusion (Table 1). The recovery of LV developed pressure was greater in rIPC dialysate-perfused hearts (44 ± 3% of preischemic value) than in sham-operated hearts (32 ± 4% of preischemic value, P < 0.01). +dP/dt and −dP/dt were also improved in rIPC compared with sham-operated hearts (+dP/ dt, 49 ± 5% in rIPC vs. 34 ± 5% in sham, P < 0.01; and −dP/dt, 47 ± 2% in rIPC vs. 35 ± 5% in sham, P < 0.01). Diastolic recovery was also improved by rIPC, and LVEDP was lower than that of sham-operated hearts at the end of the reperfusion period (25 ± 4 vs. 38 ± 7 mmHg, P = NS). However, perfusion with the dialysate after FNS or pretreatment with SNAP did not improve postischemic cardiac performance.

**Study 2: local pretreatment with SNAP or nerve transaction blocks humoral preconditioning following intra-arterial adenosine injection.** As shown on Fig. 2, right, when compared with that of sham (infarct size, 46 ± 3%), the infarct size was significantly smaller in the hearts perfused with dialysate from group 2 (FA ADO, 33 ± 12%, P < 0.01) or group 5 (l-NAME + FA ADO, 33 ± 10%, P < 0.01) animals. There was no difference between sham-operated and group 3 animals (FV ADO, 51 ± 13%, P = NS), group 4 animals (FA ADO, 46 ± 15%, P = NS), or group 6 (SNAP + FA ADO, 45 ± 9%, P = NS) animals.

After 120 min reperfusion, all hearts had an increase in end-diastolic pressure and also a reduction in coronary flow, LV pressure, and maximal and minimal change in pressure
over time (+dP/dt and −dP/dt, respectively) compared with the baseline data. However, there were no statistically significant group differences in hemodynamics either at baseline or at 120 min of reperfusion (Table 1).

**DISCUSSION**

This is the first study to show that an intrafemoral arterial injection of adenosine leads to the release of a dialyzable cardioprotective factor into the bloodstream, with similar characteristics to that previously demonstrated by us in the setting of rIPC induced by transient limb ischemia (22). Furthermore, we were able to show that the preconditioning effect of intra-arterial adenosine and of rIPC by transient limb ischemia is completely abrogated by prior FNS and pretreatment with the NO donor SNAP but is unaffected by pretreatment with the NO synthase NO synthase blocker l-NNAME or the negative control for SNAP, NAP.

These observations confirm the results of other studies showing that a low dose of intra-arterial adenosine in a remote vascular bed leads to a preconditioning of the heart, whereas the same dose given intravenously fails to protect (13). Other studies have also shown that prior local [FNS (5)] or systemic [using the ganglion blocker hexamethonium (16)] peripheral neural blockade abolishes the remote cardioprotection induced by transient limb ischemia or intra-arterial adenosine. However, our data extend the understanding of the mechanisms of signal transduction of the intra-arterial adenosine and limb ischemia stimuli. Our data show that intra-arterial adenosine leads to the release of a blood-borne factor(s) that can be dialedyzed from plasma and protect a naïve heart in Langendorff preparation, much as we have previously shown with rIPC via transient limb ischemia. However, it may be inaccurate to assume that these stimuli are interchangeable given the lack of functional protection by intra-arterial adenosine despite a similar reduction in myocardial infarction. Nonetheless, the release of the circulating cardioprotective factors after intra-arterial adenosine or rIPC is dependent on intact local neural pathways, FNS before administration completely abolishing the protective effect of the dialysate.

While our experiments were not designed to establish whether rIPC by transient limb ischemia is also dependent on adenosine release as an additional autacoid factor, it is interesting to note that the effect of intra-arterial adenosine and rIPC by limb ischemia was also blocked by pretreatment with SNAP. This is initially somewhat counterintuitive given the wealth of data describing the crucial role of NO in the local initiation and the intracellular signaling of both local and remote preconditioning strategies (1). NO donors such as sodium nitroprusside (6), glyceryl trinitrate (9) and SNAP (17, 20), and drugs that increase the bioavailability of intracellular cyclic GMP, such as sildenafil (18), have all been shown to invoke myocardial preconditioning, whereas pretreatment with l-NNAME to block local NO signaling is a potent blocker of local (12) and drug-induced preconditioning (25).

Our data show quite contrary results and emphasize the importance of dissecting the signaling cascade of the stimulus (in this case assayed by the release of blood-borne cardioprotective factors by rIPC or intra-arterial adenosine)
from the direct effects on the target organ (myocardium) invoked by preconditioning agents and blockers. For example, in a recent study investigating rIPC induced by transient limb ischemia, a pretreatment with L-NAME appeared to abolish the preconditioning effect in an in vivo myocardial infarction model in rats (21). By using an in vitro model, we minimized the possibility of systemic effects from our “local” therapies. In our study L-NAME had no measurable effect on cardioprotection induced by dialysate obtained after either intra-arterial adenosine or rIPC, suggesting that the antagonistic effect of systemically administered L-NAME in previous studies must be working downstream, presumably within the myocardium itself. Consistent with our findings, Ma et al. (15) showed that inhibiting NO synthesis by L-NAME before intra-arterial adenosine injection into the renal artery increased afferent renal nerve activity, suggesting that NO in this setting may act as an inhibitory neurotransmitter. Furthermore, Petrishchev et al. (19) showed that systemic pretreatment with a relatively low dose of the NO synthase inhibitor L-NNA did not reduce the myocardial protection in rIPC induced by limb ischemia and reperfusion (19). While more studies are clearly required to fully understand the mechanisms, we would therefore speculate that excess NO released by SNAP inhibits neuronal signals induced by adenosine and limb ischemia, which is a necessary element to the signaling cascade leading to the release of the circulating cardioprotective factor.

Consequently, our data should be taken into account when designing clinical trials, or interpreting their results, when NO donors are used clinically, since clearly they may affect the effectiveness of the rIPC stimulus under those circumstances.

Limitations. We did not measure the concentration of adenosine, L-NAME, or SNAP in the dialysate, so we cannot exclude a spillover effect of these therapies on the naïve heart in the Langendorff preparation. However, the circulating half-life of adenosine is short, and the difference between the activity of dialysate from animals treated with identical doses of intravenous and intra-arterial adenosine makes this unlikely. Furthermore, given the counterintuitive effects of SNAP and L-NAME on ultimate infarct size, it is highly unlikely that there was a significant residual activity of these agents in the dialysate, which was also diluted 10-fold by comparison to plasma.

In conclusion, remote preconditioning induced by intra-arterial injection of adenosine or limb ischemia leads to the release of a blood-borne cardioprotective factor(s) that can be transferred to naïve hearts. These stimuli were unaffected by pretreatment with L-NAME but completely abrogated by FNS or pretreatment with the NO donor SNAP. This suggests that the release of the blood-borne preconditioning factor(s) by rIPC is dependent on intact local neural pathways that may be inhibited by NO.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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