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# Regular dipyridamole therapy produces sustained protection against cardiac ischemia–reperfusion injury: Is it time to revisit PARIS?

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ABSTRACT

*Background:* Increased activated Akt and eNOS expression coincide with this persistent cardioprotection. Emergent coronary reperfusion therapies are rarely carried out before considerable myocardial injury has occurred. Moreover, reperfusion after prolonged ischemia produces paradoxical ischemia–reperfusion injury, attenuating the efficacy of reperfusion therapies. This has provided impetus for identifying chronic therapies to protect against ischemia–reperfusion injury in those at risk. We previously found that regular dipyridamole therapy produces a chronic preconditioning-like effect mediated through adenosine A1 receptors.
*Methods:* To determine how long this chronic preconditioning effect of dipyridamole remains present after discontinuing therapy, guinea pigs received 4 mg/kg/day in their water for 6 weeks. Ischemia–reperfusion was performed at 0, 2, 3, and 4 days after dipyridamole discontinuation (0 day, 2 days, 3 days and 4 days; n=8 per group). Left ventricular developed pressure (LVDP), end-diastolic pressure (LVEDP), coronary flow (CF), infarct size, and western blot analyses for Akt and endothelial nitric oxide synthase (eNOS) were studied.
*Results:* After ischemia–reperfusion, 0 day, 2 days and 3 days, but not 4 days, had significantly higher LVDP and lower LVEDP compared to control. Myocardial infarct size was significantly reduced at 0 day, 2 days and 3 days, but not 4 days, compared to control. Western blot analyses demonstrated upregulation of phospho-Akt and phospho-eNOS expression at 0 day, 2 days, and 3 days, but not 4 days.
*Conclusions:* A chronic preconditioning-like cardioprotection by regular dipyridamole treatment persists for 3 days after discontinuing therapy. Increased activated Akt and eNOS expression may play a role in this persistent cardioprotection.



## 1. Introduction

The Persantine–Aspirin Reinfarction Study (PARIS) Research Group examined the efficacy of Persantine (dipyridamole 75 mg) in combination with aspirin (324 mg) versus aspirin alone or placebo for preventing reinfarction in 2026 patients with a prior history of myocardial infarction in the preceding 8 weeks to 5 years [1]. No significant differences were observed between dipyridamole plus aspirin and aspirin alone in total mortality, coronary mortality, or nonfatal myocardial infarction (MI). However, patients were followed from 1975 to 1979, before revascularization procedures were widely available. Follow-up studies and meta-analyses also supported the lack of benefit of adding dipyridamole to aspirin to prevent cardiovascular events, with the possible exception of strokes in at risk patients.

Acute MIs are now routinely treated with emergent reperfusion therapies. However, these reperfusion therapies are rarely carried out before considerable myocardial injury has occurred. Moreover, reperfusion after prolonged ischemia produces a paradoxical ischemia–reperfusion injury [2,3], potentially attenuating the efficacy of reperfusion therapies. This has provided impetus for identifying therapies which protect against ischemia–reperfusion injury. Acute interventions, including ischemic preconditioning and infusion of adenosinergic agents (including dipyridamole) [2–4], reduce ischemia–reperfusion injury in animal models and human myocardium. To date, continual protection against ischemia–reperfusion injury is not available for patients at high risk for MI who may require emergent reperfusion therapy.

Several lines of evidence suggest that nucleoside transport inhibitors, which increase extracellular adenosine levels by inhibiting uptake into myocytes and endothelial cells, might offer sustained or chronic protection against ischemia–reperfusion injury. We previously demonstrated that chronic exposure to ethanol, an adenosine uptake inhibitor,

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reduces ischemia–reperfusion injury in guinea pig hearts, requiring adenosine A1 receptor signaling and εPKC translocation within the myocyte to effect cardioprotection [5,6]. While mechanistically interesting, the search for a safer therapy against ischemia–reperfusion injury was necessary. We subsequently found that regular dipyridamole therapy, a clinically usable nucleoside transport inhibitor, induces similar continual cardioprotection to that seen with chronic ethanol exposure, also mediated through adenosine A1 receptor activation [7].

As dipyridamole is clinically available, we asked whether chronic therapy with this adenosine uptake inhibitor produces continued protection against ischemia–reperfusion injury that lasted beyond 24 h after the last dosing. We further sought to identify potential mechanisms underlying this sustained cardioprotective effect. We found that regular dipyridamole therapy protects against ischemia–reperfusion injury, a protective effect persisting for at least 3 days following the last dosing. This sustained protection was associated with continued increased expression of activated Akt and endothelial nitric oxide synthase (eNOS), known mediators of myocyte preconditioning signaling cascades.

## 2. Materials and methods

This study was conducted in accordance with the Guidelines for Animal Research at Osaka Dental University, and with the approval of the Animal Experiment Committee of Osaka Dental University, Osaka, Japan. These guidelines conform to the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences, Washington D.C., USA. Male Hartley guinea pigs were fed Lab Diet (RC4, Oriental Yeast, Tokyo, Japan) and given water ad libitum. Dipyridamole was added to the drinking water of the dipyridamole-treated guinea pigs (11.4 mg/l) for 6 weeks. Guinea pigs drink approximately 3.5 ml water per 10 g of body weight per day, resulting in a dipyridamole intake of 4 mg/kg/day. This dose approximates the daily human dose of 75–100 mg four times daily. A diagram of the experimental protocol is shown in Fig. 1.

### 2.1. Isolated heart perfusion and measurement of function

Male guinea pigs weighing 550–700 g (12–13 weeks old) were given heparin (1000 units intraperitoneally), then anesthetized with pentobarbital (60 mg/kg, intraperitoneally). Hearts were excised and immediately arrested in cold iso-osmotic saline containing 20 mM KCl. The aorta was cannulated and the isolated hearts were perfused at 70 mm Hg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit perfusate and paced at 240 beats/min as previously described [5]. Left ventricular developed pressure (LVDP; mm Hg) was measured using a 2.5 French, high-fidelity micromanometer (Nihon-Kohden, Tokyo, Japan) passed into a compliant latex balloon, inserted into the left ventricle, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other used to fill the left ventricular balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mm Hg. Coronary flow (CF) (ml/min) was measured by collecting effluent. Global ischemia was achieved by clamping the aortic inflow line. During ischemia, hearts were maintained at 37 °C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation. Heart temperature was continuously monitored by a digital thermometer (PTW-100A, Unique Medical, Tokyo, Japan).

### 2.2. Experimental protocol

Forty guinea pigs were divided into 5 groups of eight each (see Fig. 1). After a 20 min equilibration, baseline LVDP, LVEDP and CF were recorded. Eight hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion (control; CTL). Ischemia–reperfusion was performed at 0, 2, 3, and 4 days after discontinuation of dipyridamole in four additional groups of eight each (0 day, 2 days, 3 days and 4 days).

### 2.3. Determination of myocardial infarct size

At the end of experiments, hearts were quickly frozen at −80 °C for 15 min, and then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). After removing the right ventricle and defrosting, each slice was weighed and incubated at 37 °C with 1% triphenyltetrazolium chloride (Sigma Chemicals) in phosphate buffer (pH 7.4) for 10 min and then fixed in 10% formalin for at least 5 h to distinguish red stained viable tissue from pale unstained necrotic tissue [8]. Each slice was photographed and the necrotic area was determined using Adobe Photoshop® CS (Adobe, San Jose, CA, USA) and multiplied by the weight of the slice, then expressed as a fraction of the left ventricle.

### 2.4. Western blot analysis

Separate experiments were performed on eight additional hearts to examine expression of Akt and eNOS (n = 4 for each group). Myocardial tissue samples were collected after 120 min reperfusion, and homogenized in ice-cold homogenizing buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 25 mM NaF, 50 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM PMSF and protease inhibitor leupeptin (10 μg/ml). The homogenate was centrifuged at 1000 *g* for 5 min at 4 °C to clean up. The supernatant was re-centrifuged at 10,000 *g* for 15 min at 4 °C to obtain cytosolic fraction. The protein concentration was estimated with a Bradford assay. Equivalent amounts (20 μg) of protein samples were loaded and separated on a 10% SDS-PAGE gradient gel, then electrically transferred overnight to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 2 h at 4 °C in TBS-T containing 5% skim milk and overnight 1:1000 dilution of rabbit primary

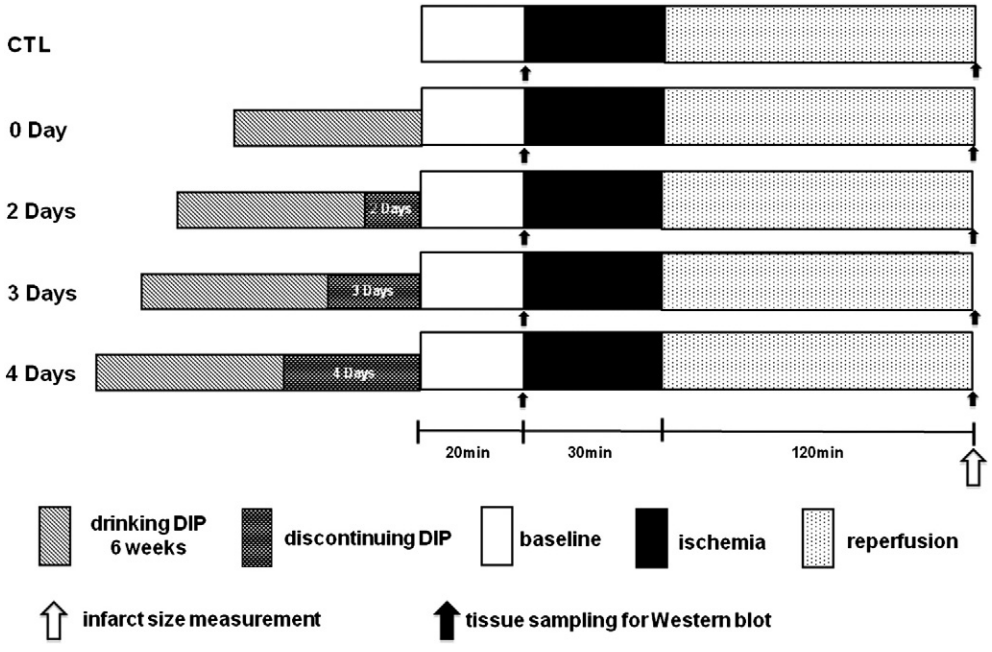


**Fig. 1.** Schematic illustration of experimental protocol. All hearts were subjected to 30 min of global ischemia followed by 120 min of reperfusion. Dipyridamole-treated guinea pigs received 4 mg/kg/day dipyridamole in their drinking water for 6 weeks. Ischemia–reperfusion was performed at 0, 2, 3, and 4 days after discontinuation of dipyridamole (0 day, 2 days, 3 days and 4 days; n = 8 for each group). Tissue samples were obtained at baseline and 120 min after reperfusion (n = 4 for each group).