Dipyridamole enhances ischaemia-induced arteriogenesis through an endocrine nitrite/nitric oxide-dependent pathway

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Aims

Anti-platelet agents, such as dipyridamole, have several clinical benefits for peripheral artery disease with the speculation of angiogenic potential that could preserve ischaemic tissue viability, yet the effect of dipyridamole on ischaemic arteriogenesis or angiogenesis is unknown. Here we test the hypothesis that dipyridamole therapy augments arteriolar vessel development and function during chronic ischaemia.

Methods and results

Mice were treated with 200 mg/kg dipyridamole twice daily to achieve therapeutic plasma levels (0.8–1.2 µg/mL). Chronic hindlimb ischaemia was induced by permanent femoral artery ligation followed by measurement of tissue perfusion using laser Doppler blood flow along with quantification of vascular density, cell proliferation, and activation of nitric oxide (NO) metabolism. Dipyridamole treatment quickly restored ischaemic hindlimb blood flow, increased vascular density and cell proliferation, and enhanced collateral artery perfusion compared with control treatments. The beneficial effects of dipyridamole on blood flow and vascular density were dependent on NO production as dipyridamole did not augment ischaemic tissue reperfusion, vascular density, or endothelial cell proliferation in endothelial NO synthase (eNOS)-deficient mice. Blood and tissue nitrite levels were significantly higher in dipyridamole-treated mice compared with controls and eNOS−/− mice, verifying increased NO production that was regulated in a PKA-dependent manner.

Conclusion

Dipyridamole augments nitrite/NO production, leading to enhanced arteriogenesis activity and blood perfusion in ischaemic limbs. Together, these data suggest that dipyridamole can augment ischaemic vessel function and restore blood flow, which may be beneficial in peripheral artery disease.

Keywords

Ischaemia • eNOS • Nitrite • Nitric oxide • Blood flow • Angiogenesis

1. Introduction

Peripheral arterial disease (PAD), a marker of systemic atherosclerosis, affecting millions of people worldwide, is associated with significant morbidity and mortality.1–3 Critical limb ischaemia (CLI) represents the end-stage of PAD with severe obstruction of blood flow resulting in ischaemic rest pain, ulcers, significant risk of limb loss, and significant socioeconomic costs. Aside from endovascular or surgical revascularization, therapeutic angiogenesis could have significant impact in the care of PAD and CLI patients by preventing further tissue dysfunction. Although experimental treatments such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), endothelial nitric oxide synthase (eNOS), nitric oxide (NO), and other therapies have been explored, successful clinical results are limited, emphasizing the need for continued investigation of alternative approaches to restore vascular function during ischaemia.4,5 Various anti-platelet agents (e.g. dipyridamole and clopidogrel) have shown some clinical efficacy in managing peripheral...
artery disease with the speculation that they may alter arteriogenesis/angiogenesis or ischaemic vascular function. \textsuperscript{6,7}

Nitric oxide and its metabolites have been implicated as key regulators of angiogenesis with several cellular mechanisms (increased endothelial cell motility, proliferation and survival, and activity of signaling pathways) implicated in angiogenesis. \textsuperscript{8–10} Recently, the use of NO donors, biotransformable metabolites (nitrite anion), and eNOS activators (e.g. statins or VEGF-A) have been reported to promote angiogenesis and/or arteriogenesis in models of chronic hindlimb ischaemia. \textsuperscript{5,8,11} Pharmacologic potentiation of NO metabolic pathways has significant potential for augmenting therapeutic angiogenesis during chronic ischaemic tissue disorders.

Dipyridamole, a conventionally used anti-platelet agent for the secondary prevention of cerebrovascular disease, has beneficial effects beyond platelet inhibition, including antithrombotic, anti-inflammatory, anti-proliferative, thrombolytic, and antioxidative properties. Dipyridamole has been shown to inhibit phosphodiesterases and potentiate the NO system. Dipyridamole also increases local extracellular concentrations of adenosine by blocking its uptake which is implicated in preconditioning and in stimulating VEGF production. \textsuperscript{12–14} However, its utility for therapeutic angiogenesis has not been evaluated under chronic ischaemic conditions. Here we examined the efficacy of dipyridamole in ischaemia-induced angiogenesis using the mouse hindlimb ischaemia model. We report that dipyridamole rapidly restores ischaemic tissue blood flow and stimulates angiogenesis through a protein kinase A (PKA)-dependent eNOS pathway. Surprisingly, the beneficial effects of dipyridamole therapy are dependent on an endocrine nitrite/NO pathway highlighting a novel pharmacological approach for altering NO metabolism.

2. Methods

2.1 Reagents

Pharmaceutical grade dipyridamole was provided by Boehringer-Ingelheim, Germany. Total and phospho-eNOS (Serine 1176) antibodies were purchased from Cell Signaling Technology. The PKA inhibitor KT5720 was purchased from Calbiochem. Anti-Ki67 antibody was obtained from Abcam Inc. Anti-CD31 antibody was obtained from BD Biosciences. Vectashield plus DAPI was obtained from Vector Laboratories. All secondary fluorophore labelled antibodies were obtained from Jackson Immunoresearch Inc. All other chemical reagents were obtained from Sigma Chemicals.

2.2 Animals and experimental procedures

2.2.1 Animals

Wild-type C57BL/6J mice weighing 20–25 g were used for experiments in this study. In some experiments, Harvard eNOS\textsuperscript{−/−} C57BL/6J mice were used to compare the importance of eNOS gene expression against wild-type mice. Mice were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, International accredited Louisiana State University Health Science Center-Shreveport animal resource facility, and maintained in accordance with the National Research Council’s Guide for Care and Use of Laboratory Animals. All animal studies were approved by the institutional animal care and use committee (protocol P-08-041) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2.2 Surgical models

Chronic hindlimb ischaemia was induced in C57BL/6J mice by ligating and transecting the left common femoral artery proximal to the origin of the profunda artery and its collateral branches. \textsuperscript{11,15} Mice were anaesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). After measuring hindlimb blood flows using laser tissue Doppler (see what follows), aseptic surgery was performed by a linear incision at the left groin and exposing the left common femoral artery identified by its pale pink and pulsatile nature. Immediate pallor was confirmed in the distal hindlimb post ligation.

Ischaemic angiogenesis was evaluated as previously reported. \textsuperscript{11,15} Briefly, acid water or dipyridamole-treated mice were anaesthetized and the femoral artery distal to the femoral profundus was ligated with 6-0 silk. On days 3 and 5 post ligation, the animals were euthanized and the vena cava and abdominal aorta distal to the kidneys immediately ligated with 6-0 silk suture. The aorta was canulated distal to this ligature to isolate the hindlimb vasculature for vascular cast perfusion. PBS was perfused through the catheter and the vena cava distal to the initial suture was severed allowing exit of the perfusate. A papaverine/adenosine (10.6 μM/3.7 μM) mixture was injected and 10% phosphate buffered formalin was administered to fix the vasculature. Blue pigmented Microfil was perfused at a 7.2 ratio (Microfil/diluent) and the hindlimbs cleared using graded glycerol solutions of 50, 75, 85, and 100% for 24 h each.

2.2.3 Measurement of plasma dipyridamole levels

Blood samples were collected by retroorbital venupuncture into 7.5% EDTA anticoagulant solution. Specimens were centrifuged at 3500 rpm for 15 min to obtain the plasma layer. Plasma levels of dipyridamole and its glucuronide were measured by HPLC with fluorescence detection (360 ex; 460 em). \textsuperscript{16}

2.2.4 Inhibition of PKA with KT5720

In some experiments, KT5720, a selective inhibitor of PKA, was dissolved in DMSO and administered intraperitoneally at 200 μg/kg daily to a cohort of mice receiving dipyridamole. KT5720 treatments were begun 2 h post-ligation of the femoral artery.

2.2.5 Laser Doppler measurement of tissue blood flow

The Vasamedics Laserfio BPM2 deep tissue laser Doppler device was used to measure hindlimb blood flows. The laser probe was placed over the medial gastrocnemius muscle region of both the hindlimbs of the mice. Areas of blood vessels visible through the skin were avoided to ensure readings indicative of tissue blood flow in the muscle. Readings were recorded in millilitre of blood flow per 100 g tissue per minute. Percent blood flows were calculated as: (ischaemic limb average blood flow /non-ischaemic limb average blood flow) × 100.

2.2.6 Vascular density measurement

Vascular density measurements were performed as we have previously reported. \textsuperscript{11,15} The gastrocnemius muscles from ischaemic and non-ischaemic hindlimbs were dissected and embedded in OCT freezing medium, frozen, and cut into 5 μm sections. The slides were fixed at −20°C in 95% ethanol/5% glacial acetic acid for 1 h. Slides were washed three times in cold PBS with 1% horse serum (5 min per wash) and blocked overnight with 5% horse serum in PBS at 4°C. Primary antibody against CD31 (platelet/endothelial cell adhesion molecule-1 (PECAM-1)) was added at 1:200 dilution (in PBS with 0.05% horse serum) and incubated at 37°C for 1 h. Slides were then washed and Cy3 conjugated anti-rat secondary antibody was added at 1:250 dilution (in PBS with 0.05% horse serum) and incubated at room temperature for 1 h. Slides were washed again and mounted using Vectashield DAPI (4’,6-Diamidine-2’-phenylindole dihydrochloride) mounting medium. At least four slides per hindlimb with three sections per slide were made for vascular staining and analysis. At least two fields were acquired per section of muscle. Pictures were taken with a Hamamatsu digital camera using a Nikon TE-2000 epifluorescence microscope (Nikon Corporation, Japan) at ×200 magnification for CD31 and DAPI staining.
respectively. Simple PCI software version 6.0 (Compix Inc., Sewickly, PA, USA) was used to quantitate the area of CD31 and DAPI staining of the vascular endothelium and nuclei of all cells, respectively. Tissue vascular density was determined as the ratio between the quantitative measurement of CD31 and DAPI staining.

2.2.7 Cell proliferation
Immunofluorescent staining of frozen tissue sections was performed by dual staining with anti-Ki67 (1:350 dilution) cell proliferation marker and anti-CD31 endothelial cell marker. Sections were washed and stained with DTAF anti-rabbit secondary (1:150 dilution) and Cy3 anti-rat secondary (1:250 dilution) antibodies. Sections were washed again and mounted using Vectashield DAPI mounting medium with co-localization images acquired as above.

2.2.8 Measurement of tissue nitrite levels and eNOS western blotting
Blood and tissue nitrite levels were measured using chemiluminescence techniques as we have previously reported. Tissues were homogenized with RIPA buffer containing protease and phosphatase inhibitors. Total protein concentrations were determined by Bradford assay and 30 μg were run on SDS–polyacrylamide gels. Phospho Ser1176 and total eNOS western blots were performed as we have previously reported.

2.2.9 Scavenging of NO using cPTIO
cPTIO, a NO scavenger, was dissolved in PBS and administered intraperitoneally at 1 mg/kg daily to a separate cohort of mice receiving dipyridamole to study the role of NO for dipyridamole augmentation of ischemic limb perfusion and vascular density, as we have previously reported.

2.2.10 Statistical analysis
One way analysis of variance with Bonferroni’s post test was used to statistically compare changes in ischemic hindlimb blood flow over time as well as between treatment groups at each time point. Student’s t-test (unpaired) was used to analyse differences between the dipyridamole and control groups for tissue analysis of vascular density, proliferation, nitrite production, and eNOS western blotting as well as differences in blood flow at specific time points between two groups. A P-value of <0.05 was required for statistical significance. Statistics were performed with GraphPad Prism 4.0 software. The number of mice used per experiment is reported in the figure legends.

3. Results
3.1 Therapeutic dosing regimen of dipyridamole in mice
Since dipyridamole has not been studied extensively in mice, it was necessary to establish a dosing regimen to achieve established therapeutic plasma levels between 0.8 and 1.2 μg/mL in C57BL/6J mice. Dipyridamole was administered by gavage in a mixture of acid water (pH 2.0) plus 10% glycofurol (every 12 h). Retro-orbital sinus bleeds were performed 3 h after the morning dose on the assigned day to collect 500 μL of whole blood to obtain plasma. Figure 1A

**Figure 1** Dipyridamole dosing regimens in C57BL/6J mice. (A) Plasma levels of dipyridamole from an escalating dosing regimen of dipyridamole b.i.d. over 4 days. (B) Plasma levels of dipyridamole from a single dosing regimen of 100 mg/kg dipyridamole b.i.d. over 4 days. (C) Continuous dosing regimen of dipyridamole 200 mg/kg b.i.d. achieves therapeutic plasma levels (0.8–1.2 μg/mL) by day 3. n = 10 mice per time point. **p < 0.01 vs. day 0.
shows the results of a dose escalation study where dipyridamole was given b.i.d. at 25 mg/kg for 2 days, then 50 mg/kg on day 3, and 100 mg/kg on day 4. While plasma levels of dipyridamole increased, the levels were subtherapeutic peaking at 0.115 ± 0.004 µg/mL at day 4. Figure 1B shows results from administration of dipyridamole at 100 mg/kg b.i.d. Plasma analysis revealed the 100 mg/kg b.i.d. dosing regimen achieved a steady-state plasma dipyridamole concentration of 0.105 ± 0.006 µg/mL that is well below established therapeutic levels. Therefore, a third dosing regimen was performed using a dose of 200 mg/kg dipyridamole b.i.d. over a 4-day period. Figure 1C illustrates that a 200 mg/kg b.i.d. dosing regimen significantly increased plasma dipyridamole levels to therapeutic levels of 0.925 ± 0.14 µg/mL by day 3 reaching a plateau at day 4. Thus, all subsequent experiments reporting the use of dipyridamole in the chronic hindlimb ischaemia model are dosed with 200 mg/kg dipyridamole b.i.d. 3 days prior to induction of hindlimb ischaemia and maintained on this dosing regimen throughout the remainder of the study.

3.2 Dipyridamole significantly enhanced ischaemic hindlimb blood flow and vascular density

Temporal serial measurements of tissue blood flows by laser Doppler are shown in Figure 2A. Dipyridamole-treated mice restored ischaemic hindlimb blood flow to baseline levels by day 5 post ligation which persisted through day 21 unlike acid water control mice. Vascular density was evaluated by measuring the ratio of endothelial CD31 surface expression to DAPI nuclear counterstaining as we have previously reported. Figure 2B and C shows representative immunofluorescent staining for CD31 (red) and DAPI (blue) nuclear counterstain in ischaemic tissues at day 7 from acid water control

Figure 2 Dipyridamole therapy restores ischaemic hindlimb blood flow and stimulates angiogenesis. (A) Effect of 200 mg/kg dipyridamole therapy on ischaemic hindlimb blood flow compared with acid-water control. *P < 0.01 vs. post-ligation blood flow. (B and C) Day 7 CD31 and DAPI staining of ischaemic tissue from acid water and dipyridamole-treated mice, respectively. (D) and (E) show day 7 CD31, Ki67, and DAPI staining of ischaemic tissue from acid water and dipyridamole-treated mice, respectively. Bar equals 100 µm. (F) Vascular density in non-ischaemic and ischaemic tissue from acid water or dipyridamole-treated mice at days 7 and 21 per 500 µm². (G) Proliferation index of non-ischaemic and ischaemic tissue from acid water or dipyridamole-treated mice at day 7 per 500 µm². *P < 0.01 dipyridamole ischaemic vs. control ischaemic. n = 8 mice per cohort.
and dipyridamole-treated mice, respectively. Ischaemic tissue from dipyridamole-treated mice revealed a greater vascular density (Figure 2C) compared with ischaemic tissue from acid water control mice (Figure 2B) as well as an increase over dipyridamole-treated non-ischaemic tissue (data not shown). Figure 2D and E shows the amount of staining for Ki67 cell proliferation marker (green) in day 7 ischaemic tissue of mice treated with either acid water or dipyridamole, respectively. Dipyridamole treatment resulted in greater Ki67 staining of ischaemic tissue with colocalization and adjacent staining of CD31. Figure 2F reports CD31 vascular density measurement at day 7 and day 21 that shows dipyridamole therapy selectively and significantly increased ischaemic muscle vessel density. Likewise, Figure 2G demonstrates that at day 7 dipyridamole therapy significantly increased ischaemic tissue proliferation index compared with ischaemic acid water control and non-ischaemic tissues.

3.3 Dipyridamole influence on arteriogenesis of ischaemic tissue
Given the rapid restoration of ischaemic tissue perfusion, we examined whether dipyridamole therapy altered arterial function or number in ischaemic tissues at early time points of ischaemia. Figure 3A–D illustrates Microfil vascular casting at day 3 of acid water control non-ischaemic and ischaemic hindlimbs (Figure 3A and B), and dipyridamole non-ischaemic and ischaemic hindlimbs (Figure 3C and D), respectively. Dipyridamole treatment augmented collateral artery perfusion through the adductor muscle as indicated by Microfil contrast (arrows). Figure 4 reports measurement of arterial branching and the distance between branches in both ischaemic and non-ischaemic limbs upon dipyridamole or vehicle treatment. Dipyridamole therapy showed a moderate but insignificant increase in the number of arterial branches off first-order arteries at day 3 (Figure 4A). Conversely, dipyridamole therapy significantly enhanced branching at day 5 (Figure 4B) compared with acid water vehicle control. Dipyridamole treatment did not alter the distance between arterial branches at day 3 (Figure 4C), but significantly decreased the distance between branch points at day 5 (Figure 4D). These data suggest dipyridamole therapy augments collateral perfusion in both non-ischaemic and ischaemic tissue and augments arterial branch number and decreases distances between branches.

3.4 Dipyridamole-induced ischaemic limb reperfusion is eNOS dependent
Previous reports have suggested that dipyridamole can potentiate NO biological effects.20–22 The potent effect of dipyridamole therapy suggests its beneficial action likely involves key regulators of angiogenic activity (e.g. eNOS and NO). Therefore, we examined whether the protective effects of dipyridamole therapy involved eNOS enzyme activity. Figure 5A shows that dipyridamole therapy failed to restore ischaemic hindlimb blood flow in eNOS<sup>−/−</sup> mice compared with wild-type mice. Figure 5B reports that dipyridamole restoration of ischaemic tissue vascular density in eNOS<sup>−/−</sup> mice is significantly inhibited. Likewise, Figure 5C shows dipyridamole induction of the proliferation index is significantly blunted in eNOS deficient mice. Together, these data strongly suggest eNOS.
expression is required for dipyridamole mediated reperfusion and vascular density in the ischaemic hindlimb.

3.5 Dipyridamole stimulates eNOS activity and ischaemic limb reperfusion in a PKA-dependent manner

Since dipyridamole has been reported to increase intracellular cAMP levels which activate PKA, we examined whether the beneficial effects of dipyridamole require PKA activity. Figure 6A shows the PKA inhibitor KT5720 (200 μg/kg) significantly blocked dipyridamole enhancement of ischaemic tissue blood flow. Dipyridamole therapy appeared to preferentially enhance eNOS Ser1176 phosphorylation in non-ischaemic tissues which was PKA-dependent (see Supplementary material online, Figure S1). Figure 6B and C also demonstrates that dipyridamole-dependent increases in ischaemic tissue vascular density and cell proliferation indices were also inhibited by KT5720. These data suggest that dipyridamole exerts its protective effect through a PKA/eNOS pathway.

3.6 Dipyridamole increases ischaemic limb reperfusion through an endocrine nitrite/NO pathway

The observation of preferential dipyridamole-dependent eNOS phosphorylation in non-ischaemic tissues was surprising and prompted us to investigate whether dipyridamole may act as a preconditioning agent activating the recently described nitrite/NO endocrine system. Figure 7A shows increased nitrite levels at day 7 in both non-ischaemic and ischaemic tissues from dipyridamole-treated mice compared with acid water controls. Figure 7B reports that dipyridamole therapy increased whole blood nitrite levels which are clearly dependent on eNOS expression. The NO scavenger cPTIO (1 mg/kg) blunted dipyridamole induced restoration of ischaemic tissue blood flow (Figure 7C) demonstrating the importance of the nitrite/NO pathway for the beneficial effects of dipyridamole therapy. Lastly, Figure 7D and E demonstrates that cPTIO significantly attenuated dipyridamole increases in ischaemic tissue vascular density and cell proliferation indices.
4. Discussion

PAD is responsible for significant morbidity, mortality, disability, and socio-economic costs. Therapeutic angiogenesis may have significant impact in the care of these patients, by improving symptoms, restoring tissue perfusion, and preventing further tissue dysfunction. It may also serve as an important adjunct to revascularization therapy or be beneficial in patients who are not candidates for surgical revascularization. Although there have been many investigations on the role of various angiogenic growth factors they have proved largely ineffective in the clinical setting highlighting the continuing need for new effective therapeutic options.

Dipyridamole is an antithrombotic agent, primarily used for secondary prevention of cerebrovascular disease in combination with aspirin.24 Although dipyridamole was originally intended as an antianginal agent, there has been no conclusive evidence supporting its routine use in the care of coronary artery disease patients.25 Dipyridamole has also been studied in maintaining coronary artery graft patency,26 treatment prior to angioplasty27,28 and valve replacement;29 however, no convincing evidence supports its routine use in these scenarios. With regards to PAD, one report suggests dipyridamole when used in combination with aspirin may be beneficial in delaying disease progression.30 A few reports suggest dipyridamole could enhance coronary collateral growth31–33 and angiogenesis,34–36 yet the utility of dipyridamole as a therapeutic angiogenesis agent during chronic ischaemia remains unknown. Here we demonstrate that dipyridamole hastens ischaemic tissue reperfusion by augmenting ischaemic tissue vascular density and collateral arteriolar function through a PKA-dependent eNOS pathway. Moreover, dipyridamole enhanced utilization of the NO/nitrite endocrine system in non-ischaemic tissue revealing a novel method of preconditioning which affects distant organ function.

Restoration of ischaemic tissue perfusion not only requires increased vascular density through angiogenesis but also involves changes in conduit vessel number or function through enhanced arteriogenesis activity. While dipyridamole is well known to stimulate...
vasodilation, the effect of dipyridamole on ischaemic tissue arteriogenesis is less clear. We found dipyridamole therapy did enhance collateral arteriolar branching and decreased the distance between branches in ischaemic limbs from dipyridamole-treated mice. These data suggest that dipyridamole treatment augments ischaemic tissue blood flow in part by enhancing collateral artery-dependent perfusion of the microcirculation. Our data reveal that genetic deficiency of eNOS or scavenging NO significantly prevents the beneficial effects of dipyridamole therapy suggesting that NO is important for dipyridamole-dependent arteriogenesis. Several studies reveal a complex role for NOS/NO in altering ischaemic tissue collateral development and blood flow as eNOS expression and NO production are involved in cerebral ischaemia and exercise-induced arteriogenesis. Conversely, Mees et al. determined that eNOS expression was not obligatory for ischaemic hindlimb arteriogenesis. However, our findings indicate that the nitrite/NO endocrine system likely plays an important role in mediating the beneficial effects of dipyridamole, which we have previously shown to be beneficial for ischaemic arteriogenesis.

Dipyridamole is well appreciated to inhibit adenosine uptake and adenosine deaminase, thus increasing the extracellular concentration of adenosine. Adenosine classically modulates intracellular cAMP levels by activating adenylate cyclase activity and inhibiting phosphodiesterase activity that promotes preconditioning and vascular growth through growth factor induction. Dipyridamole has also been reported to inhibit phosphodiesterase-5 (PDE-5) activity, thereby increasing cGMP levels. We have recently reported that PDE-5 inhibition significantly augments ischaemic tissue reperfusion and angiogenesis involving a PKG-dependent mechanism that is downstream and independent of NO/NOS activity. It is unlikely that dipyridamole inhibition of PDE-5 plays a dominant role in this model due to the fact that the effects of dipyridamole therapy are NO-dependent and mediated by PKA. Together, our data strongly suggest that dipyridamole works through the signalling cascade of adenosine/receptor interactions, activation of PKA, activation of eNOS, and increased production of NO. Future studies will be needed to clarify the temporal and spatial nature of these signals during dipyridamole-mediated ischaemic arteriogenesis and angiogenesis.

We found dipyridamole therapy significantly increased circulating and tissue levels of nitrite compared with control mice. This observation is consistent with our recent report that augmenting tissue nitrite levels significantly enhances ischaemic arteriogenesis and angiogenesis. Our current observation is interesting as the level of eNOS phosphorylation was significantly higher in non-ischaemic
tissue yet both ischaemic and non-ischaemic tissues showed enhanced nitrite levels. This could be due to preferential uptake of nitrite in ischaemic tissues as we have previously suggested and is corroborated by our current data. A very striking and novel finding of our study is that dipyridamole is able to pharmacologically enhance the NO/nitrite endocrine system in non-target tissue that has significant effects on distant organs. These data suggest that dipyridamole may serve as a unique preconditioning agent to increase NO/nitrite endocrine responses. Additional studies will be needed to determine the precise roles adenosine and adenylate cyclase play in mediating the beneficial effects of dipyridamole for therapeutic angiogenesis.

In summary, we conclude that dipyridamole significantly enhances ischaemia-induced arteriogenesis thereby quickly restoring blood flow during chronic tissue ischaemia through a PKA-dependent NO pathway. Our results open new insights into pharmacological manipulation of the nitrite/NO endocrine system for pharmacological revascularization and have important implications in the care of CLI and peripheral artery disease.
Supplementary material

Supplementary Material is available at Cardiovascular Research online.

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Conflict of interest: C.G.K. has filed a provisional patent for dipyridamole induction of the endocrine nitrite/NO system for peripheral artery disease.

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