Myocardial microvascular function during acute coronary artery stenosis: effect of hypertension and hypercholesterolaemia

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Received 2 December 2008; revised 24 April 2009; accepted 29 April 2009; online publish-ahead-of-print 7 May 2009

Time for primary review: 8 days

Aims Coronary collateral arteries (CCA) reduce cardiovascular events. We tested the hypothesis that new microvessels that proliferate in early atherosclerosis may be associated with myocardial protection during acute subtotal coronary artery obstruction (CAO).

Methods and results Acute left anterior descending CAO was induced by a balloon catheter in pigs after 12 weeks of high-cholesterol (HC) diet, renovascular hypertension (HTN), or normal control. Cardiac structure, myocardial perfusion, and functional response to iv adenosine and CAO were studied in vivo using electron beam computed tomography (CT). The intra-myocardial microvessels were subsequently evaluated ex vivo using micro-CT, and myocardial expression of growth factors using immuno-blotting. Basal myocardial perfusion and microvascular permeability were similar among the groups, whereas their responses to adenosine were attenuated in HC and HTN. A significant decline in myocardial perfusion in normal pigs during acute CAO was attenuated in HC and abolished in HTN. CAO also elicited an increase in normal anterior wall microvascular permeability (+202±59%, P<0.05), which was attenuated in HC and HTN (+55±9 and +31±8%, respectively, P<0.05 vs. normal). Microvascular (<200 μm) spatial density was significantly elevated in HC and HTN, accompanied by increased myocardial growth factor expression.

Conclusion This study demonstrates that early exposure to the cardiovascular risk factors HC and HTN protects the heart from decreases in myocardial perfusion during acute subtotal CAO. This protective effect is associated with and potentially mediated by pre-emptive development of intra-myocardial microvessels that might serve as recruitable CCA.

1. Introduction

An adequate coronary collateral circulation that can supply blood to the myocardium threatened by ischaemia is observed in about one-third of the patients with coronary artery disease (CAD). A well-developed net of coronary collateral arteries (CCA) has been observed to decrease infarct size and improve ventricular function in patients with ischaemic CAD, and to reduce non-fatal cardiovascular events. Interestingly, while the main pathogenic variable related to collateral flow is the severity of the proximal obstructive lesion in the coronary artery supplied by the CCA, ~20–25% of humans with angiographically normal coronary arteries have functional CCA that decreases myocardial ischaemia during brief vascular occlusions. However, the factors that account for the development of these vessels in the absence of obstructive CAD remain unclear.

A number of mechanisms have been suggested to contribute to development of the CCA. The relationship between the CCA observed during coronary angiography and the duration of preceding myocardial ischaemic symptoms implicates preconditioning in the development of CCA. Persistent or repetitive ischaemia and exposure to inflammatory and oxidative stress may impose cellular stress, leading to increased expression of mediators like hypoxia-inducible factor (HIF)-1α, a major upstream regulator of vascular endothelial growth factor (VEGF), which promote new vessel formation, as well as haem oxygenase (HO)-1. Interestingly, increased expressions of HIF-1 and...
VEGF have been demonstrated during exposure to major cardiovascular risks factors like hypercholesterolaemia (HC) and hypertension (HTN).\(^9\) We have previously shown that experimental HC\(^10\) and HTN\(^11\) are both characterized by increased generation of vasoconstrictors, endothelial dysfunction, and impaired myocardial perfusion. These in turn may lead to episodes of ischaemia, release of inflammatory mediators and reactive oxygen species,\(^12\) and consequently upregulation of VEGF expression,\(^13\) which in turn can trigger angiogenesis of myocardial microvessels. Indeed, microvascular proliferation was detected after 12 weeks of either diet-induced HC\(^13\) or experimental renovascular HTN.\(^9,14\) Alas, given the structural abnormalities of newly generated compared with native microvessels, including hyper-permeability, disorganized architecture, and blind endings,\(^15\) and their impaired response to cardiac challenge,\(^9\) their role has not been fully defined. The localization of these microvessels in the subendocardial layer, which is particularly susceptible to ischaemia, may be consistent with a compensatory response to preserve myocardial perfusion in the face of ischaemic insults. Indeed, these microvessels might possibly serve as recruitable CCA during development of obstructive lesions in the upstream coronary artery.

Therefore, this study was designed to test the hypothesis that myocardial microvessels, which proliferate in early atherosclerosis in the absence of obstructive lesions, may be associated with myocardial protection during acute subtotal coronary artery obstruction (CAO). For this purpose, myocardial perfusion, structure, and function were studied in vivo in HC and HTN pigs using electron beam computed tomography (EBCT). The density and distribution of intra-myocardial CCA were subsequently evaluated ex vivo using micro-CT.

2. Methods

The Mayo Clinic Institutional Animal Care and Use Committee approved this study (approval reference number A9107), which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Seventeen female domestic cross-bred pigs matched for age and body weight (40–50 kg) were studied using EBCT after 10–12 weeks of observation. At baseline, animals were randomized for no intervention (normal, \(n = 6\)), HC (\(n = 5\)), which were then fed for 10–12 weeks with a 2% HC diet\(^10\) including 15% Lard (Harlan Teklad, Madison, WI, USA), or HTN (\(n = 6\)), achieved by implanting a local-irritant coil in the left renal artery at baseline.\(^9,14\) Mean arterial pressure (MAP) was obtained using a Physio-Tel\(^6\) telemetry system (Data Sciences) implanted at baseline in the left femoral artery in all animals. Blood samples were taken at the day of in vivo study for lipids and creatinine measurement.

2.1 EBCT studies

At the end of the 12-week observation, animals were sedated by intramuscular telazol (5 mg/kg) and xylazine (2 mg/kg), subsequently maintained with intravenous ketamine (0.2 mg/kg per min) and xylazine (0.01 mg/kg/min) in normal salines, intubated, and mechanically ventilated for in vivo studies. A 7 F guide catheter was advanced through a left carotid arterial sheath to the left main coronary artery. The presence of obstructive CAD or spontaneously visible CCA >0.5 mm in diameter\(^9\) was excluded using selective coronary angiography. A low-profile 3 mm balloon (13 mm in length) catheter was then positioned under fluoroscopic guidance in the left anterior descending (LAD) coronary artery for selective intra-coronary injection of contrast media and for measurement and modulation of intra-coronary perfusion pressure. The guide catheter was then withdrawn to the ascending aorta for online measurement of arterial pressure. The balloon catheter was inflated during coronary angiography to calibrate the degree of inflation (usually under 2 atm) needed to decrease coronary artery diameter by ~80% (a haemodynamically significant obstruction\(^15\)), and then was completely deflated. The degree of obstruction was subsequently confirmed offline using a quantitative coronary angiography (QCA) system.\(^7\) An additional 5 F pigtail catheter, advanced through a left jugular venous sheath, was positioned in the right atrium for subsequent contrast media injections. The venous access was also used for drug infusions. Following catheter placement, animals were positioned supine in the scanning gantry of the EBCT scanner (Imatron C-150, Imatron Inc., South San Francisco, CA, USA). A 30 min recovery period was allowed, during which infusion of saline (5 mL/min) was initiated.

Using localization scans, the levels displaying the left ventricle (LV) were selected for measurement of LV muscle mass (LVM). Eight tomographic scans were then acquired almost simultaneously, covering 8 cm of myocardial tissue from the apex to the LV outflow tract, as we showed before.\(^14\) Scanning was triggered at end-diastole and performed during infusion of the non-ionic, low-osmolar contrast agent iopamidol (Isovue\(^{-370}\), Squibb Diagnostics, Princeton, NJ, USA; 4 mL/s over 7 s) using four target rings in the multi-slice mode.

The adjacent mid-LV tomographic levels were then selected for EBCT functional studies (myocardial perfusion and microvascular permeability). The first study was performed during an intra-coronary bolus injection (4 mL over 2 s) of iopamidol, to highlight the LAD perfusion territory distal to the catheter. Normal saline (1 mL/min) was then infused into the intra-coronary catheter for 10 min. This was followed by a baseline functional study performed in the standard resolution (50 ms/image), multi-slice flow mode using target-ring C, with a 360 × 360 matrix, 8 mm slice thickness, and 21 cm field-of-view (pixel size 0.58 × 0.58 mm). Forty consecutive scans, ECG-triggered at end-diastole, were obtained over the pre-selected levels at one to three heartbeat intervals (depending on heart rate). Scanning initiated 3 s before a 2 s bolus injection of iopamidol (0.33 cc/kg) into the right atrial catheter using a power injector.

The functional studies were then repeated, after a 20–30 min washout period, during iv infusion of adenosine (400 \(\mu\)g/kg/min). After a 20 min recovery, the LAD balloon was inflated to reduce intra-coronary diameter by ~80% for 15 min, as calibrated during coronary angiography, and the flow study was then repeated. MAP and heart rate were also recorded during adenosine and coronary balloon inflation.

To verify the haemodynamic significance of the coronary obstruction and collateral flow distal to the balloon, in additional matching five normal and six HTN pigs, a 0.014” pressure monitoring wire (RADI Medicinal System, Uppsala, Sweden) was advanced to the LAD distal to the balloon. During balloon inflation, distal pressure was recorded as coronary wedge pressure, and collateral flow index was determined by the ratio of coronary wedge pressure to mean proximal arterial pressure.\(^14\) The degree of obstruction was subsequently determined using QCA.

After completion of in vivo studies, the pigs were allowed to recover for 2–3 days and were then euthanized using intravenous pentobarbital sodium (100 mg/kg, Sleepaway\(^6\), Fort Dodge Laboratory, IA, USA).

2.2 CT data analysis

All EBCT images were reconstructed using a filtered back-projection algorithm, and then transferred for analysis on a Sun\(^8\) workstation. End-diastolic images were analysed by manually tracing regions of interest in the LV chamber and in the territories of the LAD and...
2.3 Micro-CT procedure

After euthanasia, the LV myocardium was harvested for in vitro studies. Before micro-CT scanning, the intravascular contrast agent microfil (MV-122, Flow Tech, Inc.) was perfused through the cannulated LAD coronary artery at physiological pressure and a flow rate of 0.9 mL/min. LV myocardium was sectioned and scanned at 0.49º angular increments. Images were digitized for reconstruction of 3D volume images with cubic voxels of (20)³ μm³. Using Analyze™, the myocardium was then tomographically divided into subepicardium and subendocardium, as described previously. In each region, the spatial density, average diameter, and vascular volume fraction (sum of microvascular cross-sectional area/area of the region of interest) of myocardial microvessels (diameters <500 μm) were calculated. To assess angiogenic characteristics, microvascular tortuosity of the main branches was determined (3D path distance divided by the linear distance).

2.4 Protein expression

Western blotting protocols were followed to evaluate growth factor expression in the anterior wall using antibodies against HIF-1α, VEGF, and HO-1 (Santa Cruz, Inc., CA, USA; 1:200), whereas β-actins (Sigma) were used as loading controls. The intensities of the protein bands were quantified using densitometry, normalized for loading controls, and averaged in each group. Myocardial protein level of fibroblast growth factor (FGF, R&D System, MN, USA) was measured by ELISA following manufacturer’s instruction. Furthermore, the correlations between protein expression of VEGF and HO-1 and subendocardial microvascular density and myocardial perfusion responses to adenosine and CAO were also calculated.

2.5 Statistical analysis

Continuous data are expressed as mean ± SEM. Multiple group comparisons utilized analysis-of-variance, followed by unpaired t-test, when applicable. Regressions were calculated by the least-squares method. Statistical significance was accepted if \( P \leq 0.05 \).

3. Results

After 12 weeks of HC diet, serum total and LDL cholesterol levels in HC pigs were significantly higher than in normal and HTN, whereas HTN pigs had increased MAP compared with normal and HC (Table 1). Serum creatinine levels were similar among the three groups. Basal LV ejection fraction and cardiac output were similar in all the experimental groups, but systemic vascular resistance and LVMM were significantly increased in HTN compared with normal and HC (Table 1).

3.1 Myocardial microvascular function in response to adenosine

Adenosine infusion induced a slight decrease in MAP in all groups, which became significant in HTN animals (Table 1), whereas heart rate was unaffected and not significantly different among the groups (Table 1). Baseline anterior wall perfusion in the LAD territory (Figure 1) was not significantly different among the groups, but the response to adenosine in HC and HTN was significantly attenuated compared with normal (\( P < 0.05 \) vs. baseline, Figure 2). In the lateral wall (LCX territory), baseline perfusion also showed no statistical difference among the groups and the response to adenosine followed a pattern similar to the anterior wall (Figure 2).

Basal anterior wall MVP was similar among the experimental groups (Figure 3). In normal animals, adenosine infusion was not associated with any change in MVP (Figure 3). In contrast, adenosine led to a significant increase in MVP in the HC and HTN groups (\( P < 0.05 \) vs. baseline, Figure 3). At baseline, anterior wall MVR was similar among the groups (Figure 3). During infusion of adenosine, MVR significantly declined in normal animals (\( P < 0.05 \)), but not in HC and HTN, and the degree of response was significantly attenuated compared with normal (\( P < 0.05 \)). Similarly, there were no significant differences in baseline myocardial blood flow and blood volume among the three groups (Table 1). However, myocardial blood flow response to adenosine was significantly attenuated in HC (+16.8 ± 9.3%)
Table 1  Basal cardiac and systemic haemodynamics in normal, HC, and HTN pigs

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HC</th>
<th>HTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>44.6 ± 6.4</td>
<td>46.7 ± 8.9</td>
<td>47.2 ± 7.5</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.8 ± 0.2</td>
<td>9.8 ± 2.2*</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.7 ± 0.1</td>
<td>6.9 ± 0.9*</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.5 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>77.6 ± 4.2</td>
<td>78.0 ± 4.1</td>
<td>72.7 ± 5.2</td>
</tr>
<tr>
<td>Adenosine</td>
<td>80.9 ± 5.1</td>
<td>72.6 ± 2.5</td>
<td>71.3 ± 4.9</td>
</tr>
<tr>
<td>CAO</td>
<td>77.0 ± 4.1</td>
<td>64.4 ± 1.5</td>
<td>69.0 ± 3.8</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>95.6 ± 3.3</td>
<td>94.6 ± 4.66</td>
<td>120.2 ± 3.9*</td>
</tr>
<tr>
<td>Adenosine</td>
<td>81.9 ± 4.8</td>
<td>68.8 ± 12.77</td>
<td>80.8 ± 10.0*</td>
</tr>
<tr>
<td>CAO</td>
<td>90.1 ± 6.0</td>
<td>76.3 ± 15.5</td>
<td>100.6 ± 7.7</td>
</tr>
<tr>
<td>LVMM (g/kg/body weight)</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.6 ± 0.4*</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>50.8 ± 6.6</td>
<td>60.4 ± 3.5</td>
<td>47.8 ± 5.9</td>
</tr>
<tr>
<td>Cardiac output (L/min)</td>
<td>3.2 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Myocardial blood flow (mL/min)</td>
<td>116.6 ± 17.7</td>
<td>98.0 ± 7.9</td>
<td>131.2 ± 17.7</td>
</tr>
<tr>
<td>Myocardial blood volume (mL blood/mL)</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Systemic vascular resistance (mmHg/mL/min)</td>
<td>2532.2 ± 237.1</td>
<td>2414.7 ± 297.8</td>
<td>4443.3 ± 484.8*</td>
</tr>
</tbody>
</table>

LVMM, left ventricular muscle mass; CAO, coronary artery obstruction.
*P < 0.05 vs. normal.
†P < 0.05 vs. baseline.

Figure 2  Basal myocardial perfusion (top) and relative changes in myocardial perfusion in response to adenosine (middle) and CAO (bottom) in the anterior (left) and lateral (right) cardiac walls in normal (N), HC, and HTN pigs. *P < 0.05 vs. normal; †P < 0.05 vs. baseline.
and HTN (+4.0 ± 3.6%) compared with normal (+59.1 ± 20.1%, P < 0.05).

3.2 Myocardial microvascular response to subtotal occlusion

CAO slightly but not significantly reduced MAP and heart rate in all animals (Table 1). QCA revealed an average degree of CAO of 78.2 ± 3.2%. Acute LAD obstruction caused a significant reduction in anterior wall myocardial perfusion in normal animals (P < 0.05), but not in HC or HTN. Furthermore, the degree of response in HTN was smaller than in normal (P < 0.05, Figure 2). Interestingly, myocardial blood volume significantly declined during CAO in normal (−67.5 ± 7.8% P = 0.0002 vs. baseline) and HC (−38.6 ± 8.0%, P = 0.006) animals, but has not significantly changed in HTN animals (+23.2 ± 17.3%, P = 0.79 vs. baseline), and anterior wall blood volume in HC and HTN during the LAD obstruction was significantly higher than normal (P = 0.001 and P = 0.038 vs. normal, respectively). In the LCX territory of normal pigs, this was associated with no change in lateral wall perfusion, whereas in HC and HTN, a significant reduction in lateral wall perfusion suggested potential ‘steal’ of blood flow from the lateral to the obstructed anterior wall territory (Figure 2). However, there was no significant difference in the degree of response among the three groups.

Acute LAD obstruction also caused a significant increase in anterior wall MVP in normal pigs (P < 0.05 vs. baseline), which was significantly attenuated in HC and HTN compared with normal (P < 0.05 vs. N, Figure 3). A slight decrease in MVR in normal was not significant. In HC and HTN animals, MVR significantly decreased compared with baseline (P < 0.05) (Figure 3).

The experiment employing the pressure wire confirmed that a pressure gradient across the balloon during inflation was observed in all animals. However, despite similar obstruction, the pressure gradient was smaller in HTN animals. Hence, collateral flow index was significantly higher in HTN than in normal pigs (Figure 4).

3.3 Myocardial microvascular architecture in HC and HTN

The spatial density of small microvessels (<200 μm) was significantly higher in HC and HTN than normal (Table 2), especially in the subendocardium (ANOVA, P = 0.007) (Figure 5). In addition, HC and HTN showed increased microvascular tortuosity, suggesting the presence of angiogenic microvessels, whereas tomographic microvascular diameter was not significantly changed (Table 2).

Furthermore, HC and HTN pigs showed increased myocardial expression of the growth factors HIF-1α and VEGF, with HIF-1α expression yet greater in HTN than HC. Myocardial
levels of basic FGF were elevated only in HTN, and myocardial expression of HO-1 was markedly increased in HTN compared with both normal and HC (Figure 5). Interestingly, VEGF and HO-1 expressions were linearly and directly correlated with subendocardial microvascular density and perfusion responses to CAO, and inversely with myocardial perfusion response to adenosine (Figure 6).

### 4. Discussion

The present study demonstrates that experimental exposure to the cardiovascular risk factors HC and HTN partly protects the heart from subsequent decreases in myocardial perfusion during an acute subtotal CAO. This protective effect may be mediated by preconditioning, reflected in neovascularization of intra-myocardial microvessels, which despite impaired function in response to adenosine, might potentially serve as recruitable CCA and attenuate the decrease in myocardial perfusion during superimposed acute vascular obstruction.

Increased number of angiographically apparent collaterals has been previously observed in HC\textsuperscript{21} and HTN\textsuperscript{22} patients, and linked to preserved cardiac function.\textsuperscript{21} Moreover, both spontaneously visible and recruitable CCA\textsuperscript{6} frequently proliferate in the presence of chronic occlusive CAD\textsuperscript{5} and may serve to supply blood to the jeopardized myocardial territory from neighbouring regions. Indeed, in patients with chronic stable CAD, the presence and the extent of well-functioning recruitable CCA, identified indirectly by sustained coronary arterial pressure distal to a brief coronary occlusion, is associated with reduced long-term cardiac mortality.\textsuperscript{23}

In our study, the majority of microvessels that proliferated in HC and HTN were \( \leq 200 \) \( \mu \)m, which are mostly those that progressively dilate shortly after coronary occlusion,\textsuperscript{24} and would be difficult to detect in humans. Extending...
our previous studies,13,14 this study interestingly shows that proliferation of such intra-myocardial microvessels in HC and HTN is associated with effective sustenance of adequate myocardial perfusion and intact microvascular permeability during a significant CAO. Formation of newly developed intra-myocardial microvessels in HC and HTN is suggested by their increased tortuosity, which characterizes angiogenic vessels. This might conceivably represent a compensatory response aimed at sustaining myocardial perfusion and is indeed particularly pronounced in the subendocardium, a region with greater oxygen consumption and thus vulnerability to ischaemia. Therefore, this study supports the notion that spawning of intra-myocardial microvessels (which might potentially serve as recruitable CCA) precedes development of occlusive lesions. Speculatively, impaired coronary blood flow responses, as often observed during exposure to cardiovascular risk factors,9,10 stimulate development of microvessels, which may play a role in myocardial preconditioning during progression of CAD.25,26 Although our HTN pigs had moderate HTN and increased LVMM, they had well-preserved systolic function, and thus preconditioning likely remained relatively in act.27,28 Our study is also supported by previous studies that showed that HC may render the myocardium more resistant to ischaemic stress.29

On the other hand, the present study reasserts the dysfunction of intra-myocardial microvessels in HT and HC,11,12 as demonstrated by the impaired myocardial perfusion and permeability responses to vasoactive challenge using adenosine. The altered architecture and impaired vasodilatory capacity of these microvessels in HC and HT are also reflected in the blunted response to adenosine of intra-myocardial blood volume, an index of resistance microvascular integrity and function,37 which is impaired during exposure to cardiovascular risk factors.38 Indeed, in contrast to spontaneously visible collateral vessels that accompany obstructive CAD, recruitable CCA may have impaired vasoreactivity,30 implying that the former is a more effective defence mechanism to mitigate episodes of myocardial ischaemia.

In response to CAO, myocardial perfusion may be initially preserved by an increase in distal intravascular blood volume, achieved by dilatation and recruitment of downstream myocardial microvessels.31 Hence, the efficacy of the CCA network in preserving myocardial perfusion in the face of an acute CAO might be facilitated by coronary steal (redistribution of blood) from the adjacent lateral wall.32 Indeed, in normal animals, the submaximal LAD obstruction elicited a significant decline in anterior wall myocardial perfusion and increased MVP, whereas lateral wall perfusion remained unchanged. Contrarily, the fall in anterior wall perfusion was relatively attenuated in HC and HTN, whereas lateral wall perfusion declined. The CCA of HC and HTN pigs might then afford a greater than normal decrease in distal MVR and blood flow steal from the adjacent territories. Nevertheless, since the differences among the groups in lateral wall perfusion responses were small, future studies will need to establish the activation of this mechanism.
The adaptive responses seem to be more effective in HTN pigs, possibly because of a greater increase in growth factor expression. In particular, the enzyme HO-1 exerts an array of protective effects that alleviate cellular stress, and plays a central role in myocardial preconditioning. HO-1 is regulated by stress-responsive transcription factors, mediates the proangiogenic effects of several growth factors like VEGF, and also regulates their expression. It is functionally significant in cardiovascular diseases such as myocardial infarction or atherosclerosis, but especially relevant in HTN, as biomechanical stress and angiotensin II signalling upregulate its gene expression in cardiomyocytes. Remarkably, protein expression of both VEGF and HO-1 directly correlated with subendocardial microvascular density and perfusion responses to CAO and inversely with myocardial perfusion response to adenosine. These findings support the contribution of VEGF and HO-1 to myocardial protection. Furthermore, FGF level was higher in HTN, which may enhance angiogenesis by induction of VEGF. Indeed, the density of subendocardial small microvessels seems slightly higher in HTN compared with HC pigs, but the difference has not reached statistical significance.

Nevertheless, it is not unlikely that mechanisms other than a change in CCA architecture contributed to maintain an adequate myocardial perfusion in HTN. Microvascular dysfunction in the presence of obstructive CAD might play an important role in determining the ischaemic threshold. For example, a previous study in patients with unstable angina has demonstrated that microcirculatory vasoconstriction due to severe microvascular dysfunction distal to chronic epicardial CAO may also prevent trans-stenotic pressure drop during episodes of spontaneous myocardial ischaemia, but not during balloon angioplasty. However, in contrast to balloon angioplasty, our study was performed after a 15 min balloon inflation and might have therefore revealed native collaterals that may take up to 15 min to open up. Furthermore, we cannot exclude the possibility that some of the CCA that we observed subsequently regress during cardiac disease progression. Lastly, the vaso-dilatory properties of VEGF, and the potent antioxidant and anti-inflammatory properties of HO-1 reaction products,
may have also contributed to sustain vascular function during cellular stress evoked by acute CAO.

4.1 Limitations
Our studies were performed in young pigs after a relatively short (12-week) exposure to cardiovascular risk factors. Nonetheless, the HTN and HC interventions likely elicited systemic inflammation in the animals. In addition, the long-term cardioprotective efficacy of the newly generated myocardial microvessels in the presence of persistent myocardial ischaemia, chronic coronary artery stenosis, or long-term HC and/or HTN remains to be determined. On the other hand, the structure and function of the porcine cardiovascular system are similar to humans, and thus our results may have clinical relevance, although caution should be exercised when applying experimental results to clinical cardioprotection. Additional studies are also needed to demonstrate directly that the new microvessels function as recruitable collaterals, to determine whether they remain available or regress during chronic disease, and to investigate the possible involvement of these dysfunctional vessels in the process of myocardial injury. It is also possible that these microvessels are less available in HTN in vivo due to compressive forces within the myocardium or LV. In addition, we determined the response to acute submaximal obstruction, rather than complete LAD occlusion, and microvascular reactivity may also depend on the degree of obstruction. Nevertheless, this approach also mimics pathophysiological conditions, and our results clearly show preservation of myocardial perfusion in the groups exposed to HC and HTN. Notably, our functional data have been acquired using imaging techniques, which are non-invasive and widely used in clinical practice, but limited by the need for contrast media and exposure to x-ray. Furthermore, fast CT might underestimate very high myocardial flow rates (≥2.5 mL/min/g), but this is unlikely to interfere with our studies, since in a physiologic range of myocardial perfusion like those we study, CT measurements agree well with reference standards.

In summary, the present study demonstrates that new microvessels can develop as early as during exposure to cardiovascular risk factors and may potentially function as coronary collateral vessels upon the superimposition of occlusive CAD. Despite being dysfunctional, these vessels may play an important role in protecting the myocardium from ischaemic insults, at least in this early phase and in the presence of an acute subtotal occlusion. Indeed, this study suggests mechanisms consistent with clinical studies, implying that the presence of CCA is associated with better outcomes. Nevertheless, the protective effects of the CCA may be offset in the long run by coronary vascular dysfunction and CAD that develop in many patients with HC and HTN and contribute to myocardial ischaemia and cardiovascular events.

Conflict of interest: none declared.

Funding
This study was partly supported by NIH grant numbers HL77131, DK73608, DK77013, and P01HL85307, and by the Universita’ degli Studi di Pisa.

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