Optimal reactive oxygen species concentration and p38 MAP kinase are required for coronary collateral growth

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Rocic P, Kolz C, Reed R, Potter B, Chilian WM. Optimal reactive oxygen species concentration and p38 MAP kinase are required for coronary collateral growth. Am J Physiol Heart Circ Physiol 292: H2729–H2736, 2007. First published February 16, 2007; doi:10.1152/ajpheart.01330.2006.—Reactive oxygen species (ROS) are critical mediators of coronary collateral growth (CCG). We evaluated the requirement for ROS in human coronary artery endothelial cell (HCAEC) tube formation, CCG in vivo, and signaling (p38 MAP kinase) by which ROS may stimulate vascular growth. The flavin-containing oxidase inhibitor diphenyleneiodonium (DPI) or the superoxide dismutase inhibitor diethyldithiocarbamate (DETC) blocked vascular endothelial growth factor-induced HCAEC tube formation in Matrigel. We assessed the effect of DPI and DETC on CCG in a rat model of repetitive ischemia (RI) (40 s left anterior descending coronary artery occlusion every 20 min for 2 h 20 min, 3 times/day, 10 days). DPI or DETC was given intraperitoneally, or the NAD(P)H oxidase inhibitor apocynin was given in drinking water. Collateral-dependent flow (measured by using microspheres) was expressed as a ratio of normal and ischemic zone flows. In sham-operated rats, collateral flow in the ischemic zone was 18 ± 6% of normal zone; in the RI group, collateral flow in the ischemic zone was 83 ± 5% of normal zone. DPI prevented the increase in collateral flow after RI (25 ± 4% of normal zone). Similar results were obtained with apocynin following RI (32 ± 7% of that in the normal zone). DETC achieved similar results (collateral flow after RI was 21 ± 2% of normal zone). DPI and DETC blocked RI-induced p38 MAP kinase activation in response to vascular endothelial growth factor and RI. These results demonstrate a requirement for optimal ROS concentrations in HCAEC tube formation, CCG, and p38 MAP kinase activation. p38 MAP kinase inhibition prevented HCAEC tube formation and partially blocked RI-induced CCG (42 ± 7% of normal zone flow), indicating that p38 MAP kinase is a critical signaling mediator of CCG.

ISCHEMIA-REPERFUSION INJURY is a biphasic process in which exposure of the myocardium to prolonged hypoxia/ischemia initiates massive cell death in the affected region of the heart. This is followed by further reperfusion injury commencing on reestablishment of blood flow and is defined as further destruction, including stunning and death, of tissue still alive at the onset of reperfusion (17). However, under certain conditions, ischemia-reperfusion can lead to adaptive mechanisms that diminish and/or prevent the reoccurrence of injury. Coronary collateral development is an example of such an adaptive response to myocardial ischemia caused by chronic occlusion of major coronary arteries. It has been shown that ischemic preconditioning renders the myocardium tolerant to prolonged ischemia-reperfusion injury (10), in part through promoting collateral development (4, 16, 20).

Our laboratory has previously shown that reactive oxygen species (ROS) are critical mediators of coronary collateral development in a canine model of repetitive ischemia (15). Treatment with N-acetyl cysteine attenuated the repetitive ischemia (RI)-induced increase in coronary collateral flow. Likewise, myocardial interstitial fluid from dogs undergoing the RI protocol, but not fluid from N-acetyl cysteine-treated dogs, induced endothelial cell tube formation and vascular smooth muscle cell proliferation in cultured cells (27). Interestingly, NAD(P)H oxidase-derived ROS have been shown to play a critical role in promoting endothelial cell tube formation in vitro (26). Furthermore, in cell culture studies, low concentrations of superoxide and H2O2 induced endothelial cell migration, adhesion, and tube formation, whereas high concentrations of these ROS inhibited these processes (8).

Both in vitro and in vivo studies suggest that coronary collateral growth is mediated by a highly coordinated signaling cascade. We have previously reported (22) a requisite role for vascular endothelial growth factor (VEGF) in coronary collateral growth. VEGF has been shown to activate several signaling pathways, including ERK1/2 (21), JNK (17), and p38 (6) MAP kinases; the phosphatidylinositol 3-kinase/Akt pathway (5); and the nonreceptor tyrosine kinases c-Src (7), focal adhesion kinase (30), and Pyk2 (13). Of these, p38 MAP kinase (11, 23), Akt (11, 24), and c-Src (25) have been shown to be ROS sensitive in other cell types.

The potential role of p38 MAP kinase in coronary collateral growth is particularly interesting for a number of reasons. First, p38 MAP kinase has been shown to be specifically activated in response to hypoxia in human cancer cells (28). Second, human umbilical vein endothelial cell (HUVEC) tube formation in Matrigel was associated with rapid and transient p38 activation (12), and VEGF-induced endothelial cell migration was inhibited by specific p38 MAP kinase inhibition (29), indicating a required and beneficial effect of p38 MAP kinase on endothelial cell tube formation in vitro. The role of p38 MAP kinase has not been investigated in coronary collateral development in vivo. Third, in our preliminary studies, its activation in response to RI was ROS sensitive, but unlike the activation of other signaling molecules, p38 MAP kinase was only transiently activated.

In this study, we show that an optimal concentration of superoxide and H2O2 allows for human coronary artery endothelial cell (HCAEC) tube formation in response to VEGF and for coronary collateral growth in response to RI-reperfusion.
Furthermore, we demonstrate, for the first time, that the ROS-sensitive and transient activation of p38 MAP kinase is essential for the underlying positive effect of ROS on stimulating coronary collateral growth.

MATERIALS AND METHODS

Rat model of collateral growth/RI. Male Wistar-Kyoto rats (3–4 mo old, 300–350 g) were used for chronic (10 days) implantation of a pneumatic occluder over the left anterior descending coronary artery (LAD), as described by Toyota et al. (22). The RI protocol for rat consisted of eight 40-s occlusions, one every 20 min over a duration of 2 h and 20 min followed by a period of “rest” for 5 h and 40 min. This 8-h cycle was repeated three times per day over a period of 10 days.

Microsphere measurements of myocardial and collateral-dependent blood flow. Microspheres (5 × 10⁵) labeled with gamma emitters ⁵²⁷Co (at initial surgery) or ⁴⁰⁰Ru (at end of RI protocol) were injected into the left ventricle (LV) over 20 s. Collateral (LAD)-dependent zone was identified by using fluorescence microscopy in which the area without the fluorescent microspheres (injected into the LV at the time of initial surgery while LAD is occluded) is the LAD-dependent zone. Tissue flow was calculated as a ratio between activity (cpm/g) of the tissue samples from the LAD-dependent and normal zones. For all experiments, data were analyzed by two-way ANOVA followed by t-test. Collateral flow was measured in the following groups: a sham-operated group (n = 8) that was instrumented but not subjected to RI, a control group/RI (n = 8), RI + diphenyleneiodonium (DPI, 0.2 mg·kg⁻¹·day⁻¹; n = 8), RI + apocynin (0.25 mg/ml in drinking water; n = 3), and RI + diethylidithiocarbamate (DETC, 1 g·kg⁻¹·day⁻¹; n = 8).

Measurement of superoxide and H₂O₂. Superoxide production was evaluated by using dihydroethidium (DHE) in vitro and in vivo. DHE production was evaluated by dihydrochlorofluorescein (DCF) in vivo. For HCAEC cell culture, DHE was administered during the last 20 min of treatment. Cells were then observed immediately under a fluorescent microscope. For in vivo studies, DHE or DCF were injected into the LV (60 μg/kg) for 20 min before two consecutive periods of ischemia-reperfusion (40 s occlusion followed by 20 min reperfusion and another 40 s occlusion and 20 min reperfusion). Animals were then killed. The heart was removed, frozen in optimum cutting temperature compound on dry ice, and stored at −70°C until sectioning. Sections (5 μm) were made in a cryomicrotome and were mounted on glass slides. DHE or DCF fluorescence was detected with excitation/emission at 518/605 nm (for DHE) or 480/515 nm (for DCF). All images were analyzed at the same microscope settings, and relative fluorescence readings were obtained by Metamorph Software on three hearts (5 consecutive sections per heart). A Bruker EMX spectrometer was used for X-band EPR measurements of superoxide by using 1-hydroxy-3-carboxy-pyrorolidine (CP-H) as a spin-trap. Animals underwent two consecutive periods of ischemia-reperfusion (as above). Animals were then killed, hearts were removed, LAD-dependent and normal zones were separated, and CP-H (238 μg/100 mg tissue) was added to the tissue samples immediately. Tissue was then homogenized by sonication on ice and was frozen in liquid nitrogen until EPR measurements. Superoxide concentration was calculated from arbitrary units (AU; 3.4 × 10⁶ AU/mM).

HCAEC cell culture. Cells were purchased from Clonetics and were cultured at low passages (passages 3–8) in Clonetics EGM-2 BulletKit medium (Lonza) that contains 25% FBS, 0.2% hydrocortisone, 2% human FGF-B, 0.5% IGF-I, 0.5% ascorbic acid, 0.5% human EGF, and 0.5% GA-1000.

Endothelial tube formation. HCAECs (passages 3–10) were seeded on Matrigel (BD Biosciences)-coated 24-well plates according to manufacturer instructions in EGM-2 BulletKit medium at a density of 30,000 cells/well. Cells were allowed to attach for 24 h before the addition of 50 ng/ml VEGF, DPI (10 μM), DETC (10 μM), or the p38 MAP kinase inhibitor (SB-203580; 20 μM). All treatment groups consisted of 10 wells. The extent of tube formation was quantified after two days by using Scion Image software. An electronic grid was superimposed on microscopic images, and the number of squares containing tubes were counted and averaged from five randomly selected fields for each well to obtain the percentage of total field that contained tubes.

Western blot analysis. Proteins from cells and myocardial tissue were extracted in a lysis buffer containing 0.1% SDS and 1% Triton X-100 as previously described (18). For extraction from tissue, hearts were excised, LVs were dissected, and the LAD-dependent zone was separated from the normal zone and snap-frozen in liquid nitrogen before homogenization in lysis buffer. Equal amounts of protein (60 μg) were separated by SDS-PAGE and were transferred to Hybond-ECL nitrocellulose membranes. Phospho-specific anti-p38 and anti-total p38 antibodies (Cell Signaling) were used for Western blot analysis. Bands were visualized by enhanced chemiluminescence (Amersham) and were quantified by using National Institutes of Health Image software. Because none of the treatments altered p38 MAP kinase expression, its total levels were used to check for equal loading and to normalize phosphorylation levels. All experiments were performed in triplicate (n = 3 animals/group).

Data analysis. ANOVA followed by t-tests with Bonferroni inequalities was used for statistical analysis. A probability value of P < 0.05 was used to determine statistical significance.

RESULTS

VEGF-induced HCAEC tube formation requires optimal ROS concentration. To assess VEGF's ability to induce HCAEC tube formation, cells were seeded on Matrigel at a density of 30,000 cells/well, allowed to attach for 24 h and then treated with VEGF (50 ng/ml). Tube formation was observed after two days and was significantly increased in response to VEGF treatment compared with control nontreated cells (19.0 ± 0.02 vs. 8 ± 1% area covered, n = 10, P < 0.05; Fig. 1). Next, we investigated the effect of agents that increase or decrease ROS on VEGF-induced tube formation. To decrease ROS generation, cells were treated with the flavin-containing oxidase inhibitor DPI (10 μM), which blocked VEGF-induced HCAEC tube formation (6 ± 1 vs. 19 ± 2%, n = 10, P < 0.05; Fig. 1). Similarly, treatment with the superoxide dismutase (SOD) inhibitor DETC (10 mM) also prevented VEGF-induced HCAEC tube formation (5 ± 2 vs. 19 ± 2%, n = 10, P < 0.05; Fig. 1). Treatment with DPI or DETC alone (without VEGF) was not significantly different from control (5 ± 1 and 5 ± 2%, respectively).

RI causes coronary collateral development in the rat model of RI in vivo. Results in Fig. 2 demonstrate impaired coronary flow in the LAD-dependent (collateral-dependent) zone in animals that underwent a sham surgical procedure and thus were not subjected to RI (collateral flow was 18 ± 6% of the normal zone flow and represents native collateral flow; n = 8). In contrast, collateral flow in animals that underwent the 10-day RI protocol was 83 ± 5% of that in the normal zone (Fig. 2), demonstrating significant collateral development in this region of the myocardium.

Blockade of superoxide production by DPI (0.2 mg·kg⁻¹·day⁻¹) prevented the increase in collateral flow induced by RI (25 ± 4% of that in the normal zone, n = 8, P < 0.05; Fig. 2). Similar results were obtained with apocynin (0.25 mg/ml in drinking water = 43 mg·kg⁻¹·day⁻¹), a more specific inhibitor of NAD(P)H oxidases (collateral flow was 32 ± 7% of that in
the normal zone after RI, \( n = 3 \), \( P < 0.05 \); Fig. 2). Likewise, increasing superoxide by SOD inhibition (DETC, 1 \( \mu \)g·kg\(^{-1} \)·day\(^{-1} \)) achieved similar results (collateral flow was 21 ± 2% of that in the normal zone following RI, \( n = 8 \), \( P < 0.05 \); Fig. 2).

To confirm the effects of ischemia-reperfusion and efficacy of DPI and DETC treatments on superoxide generation in vivo, in some animals DHE was given by LV injection (60 \( \mu \)g/kg) for 20 min before two consecutive periods of ischemia-reperfusion. Ischemia-reperfusion caused an increase in superoxide generation in the LAD-dependent zone compared with sham (RI = 450 ± 13 vs. sham = 124 ± 6 AU, \( n = 3 \), \( P < 0.05 \); Fig. 3A). When DPI was administered for two days before DHE injection, superoxide production was markedly lower (RI + DPI = 118 ± 9 vs. RI = 450 ± 13 AU, \( n = 3 \), \( P < 0.05 \)). In contrast, DETC treatment for two days resulted in an increase in superoxide concentration above that produced by ischemia-reperfusion (RI + DETC = 861 ± 16 vs. RI = 450 ± 13 AU, \( n = 3 \), \( P < 0.05 \); Fig. 3A). Identical results were observed following both one and three consecutive periods of ischemia-reperfusion (data not shown). Furthermore, in some animals CP-H was added and tissue samples were subjected to X-band EPR to obtain a quantitative measurement of superoxide. In all treatment groups the observed superoxide concentrations in the LAD-dependent zone are in precise agreement with the DHE fluorescence intensity (0.12 nM for sham, 0.53 nM for RI, 0.10 nM for RI + DPI, and 1.18 nM for RI + DETC; Fig. 3B). Superoxide concentrations in the normal zone were 0.10 nM for sham, 0.36 nM for RI, 0.29 nM for RI + DPI, and 0.64 nM for RI + DETC (Fig. 3B). Thus RI increased

![Fig. 1. Human coronary artery endothelial cell (HCAEC) tube formation in Matrigel. Left: representative images of HCAEC 2 days after addition of 50 ng/ml vascular endothelial growth factor (VEGF) with or without inhibitors [10 \( \mu \)M diphenyleneiodonium (DPI) or 10 mM diethyldithiocarbamate (DETC)] as indicated. Right: percent of total field with tubes (\( n = 10 \) per group, means ± SE of 5 random fields per well; * and #, \( P < 0.05 \)).](image)

![Fig. 2. Collateral-dependent flow expressed as percentage of flow in the normal zone. DPI, DETC, and apocynin blocked the repetitive occlusion/ischemia (RI)-induced increase in collateral-dependent flow (\( n = 8 \) for each treatment group).](image)
superoxide concentrations in both the LAD-dependent and the normal zones, but this increase was greater in the LAD-dependent zone. Likewise, the effect of DETC was more pronounced in the LAD-dependent than in the normal zone, whereas the effect of DPI was pronounced in the LAD-dependent but negligible in the normal zone.

Because SOD inhibition might also lower myocardial H$_2$O$_2$ concentration, DCF (60 μg/kg) was injected into the LV (in a manner identical to DHE administration) in a separate group of animals, and DCF fluorescence was evaluated in AU to evaluate this possibility. Figure 3C shows that H$_2$O$_2$ levels were decreased approximately fourfold by both DPI and DETC (122 AU for RI + DPI and 125 AU for RI + DETC vs. 450 AU for RI), indicating that H$_2$O$_2$ concentrations below a certain level (RI alone) may result in inhibition of coronary collateral growth.

VEGF induces p38 MAP kinase activation in vitro and p38 MAP kinase inhibition prevents VEGF-induced HCAEC tube formation. To elucidate some of the signaling mechanisms that underlay these processes, HCAEC cultures were treated with 50 ng/ml VEGF for 20 min, harvested, and prepared for Western blot analysis with phospho-specific antibodies. One of the molecules that showed an increase in activation in response to VEGF was p38 MAP kinase (p38 = 4.5 ± 0.23 vs. control = 1 ± 0.19, n = 3, P < 0.05; Fig. 4A). Additionally, a 45-min pretreatment with both DPI and DETC completely abolished VEGF-induced p38 MAP kinase activation (1.4 ± 0.43 and 1.3 ± 0.18, respectively, n = 3, P < 0.05) demonstrating the redox sensitivity of its activation in response to VEGF. Importantly, VEGF, DPI, or DETC treatment did not alter the expression of the kinase. To establish a causal role for p38 in HCAEC tube formation, we determined the effect of...
p38 MAP kinase inhibition by its specific inhibitor, SB-203580 (20 μM), on VEGF-induced tube formation. Figure 4B shows that p38 MAP kinase inhibition prevents VEGF-induced HCAEC tube formation (0 ± 0% vs. 19 ± 2%, n = 10, P < 0.05), without any apparent effect on HCAEC survival or proliferation.

*p38 MAP kinase activation by ischemia-reperfusion is partially responsible for ischemia-reperfusion-induced coronary collateral growth. Figure 5A shows that p38 is specifically activated (2.7 ± 0.23-fold vs. sham, n = 3, P < 0.05) in the LAD-dependent region of the myocardium only on day 3 of the RI protocol. This is in contrast to other kinases, including Akt, which remain activated to a high degree for the duration of the protocol (data not shown). This activation is smaller in magnitude than the VEGF-induced response in HCAECs, which likely accounts for a large proportion of cardiac myocytes and fibroblasts in the tissue sample and a less robust activation of p38 in these cell types vs. in coronary endothelium. In addition, p38 activation by RI was completely blocked by DPI and DETC at the time point of maximal p38 activation by RI (day 3; Fig. 5A). In a protocol identical to that used for confirmation of DPI and DETC effects on superoxide concentration in vivo, we investigated the effect of these compounds on p38 MAP kinase activation. After 3 days of the RI protocol, animals were killed, hearts were excised, and LAD-dependent and normal zones were separated and prepared for Western blot analysis. Western blots with phospho-specific anti-p38 antibodies revealed a significant increase in p38 MAP kinase activation in response to ischemia-reperfusion (p38 = 2.7 ± 0.23-fold vs. sham = 1.0 ± 0.17, n = 3, P < 0.05) in the

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**A**

10 mM DETC - - - +
10 μM DPI - - + -
50 ng/ml VEGF - + + +

**B**

Control
50 ng/ml VEGF

50 ng/ml VEGF + 20 μM SB203580

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Fig. 4. **A**, top: Western blot with phospho- and total p38 MAP kinase antibodies in untreated endothelial cells or cells treated with VEGF, VEGF + DPI, or VEGF + DETC. Bottom: area × density of the Western blots (arbitrary units). Activation (phosphorylation) of p38 in VEGF-treated cells was prevented by either DPI or DETC. **B**: effects of p38 inhibition (SB-203580) on VEGF-induced tube formation. Inhibition of p38 completely prevented VEGF stimulation of endothelial cell tube formation.
LAD-dependent zone, which was completely blocked by both DPI and DETC (1.0 ± 0.18- and 1.1 ± 0.3-fold, respectively, n = 3, P < 0.05; Fig. 5A). Expression (total p38 demonstrated by Western blot analysis with anti-p38 MAP kinase antibodies) was not affected by any of the interventions, and p38 was not significantly activated in the normal zone (1.2 ± 0.2-fold in RI vs. sham, n = 3; Fig. 5A).

To ascertain whether p38 MAP kinase is required for coronary collateral development in response to ischemia-reperfusion in vivo, rats were treated with SB-203580 (3.2 mg·kg⁻¹·day⁻¹) for the duration of the 10-day RI protocol via intraperitoneal injection once per day. p38 MAP kinase inhibition resulted in a significant (>50%) decrease in coronary collateral development (42 ± 7% of that in the normal zone, n = 8, P < 0.05; Fig. 5B).

Together, these results show that the redox-sensitive p38 MAP kinase is partially responsible for ischemia-reperfusion-induced coronary collateral development, although it remains unclear in which cell type its activation is most important. We confirmed the blockade of p38 MAP kinase by intraperitoneal administration of SB-203580 (3.2 mg·kg⁻¹·day⁻¹) in a modified RI protocol (3 days) and found an almost complete (>90%) inhibition of the kinase in both the LAD-dependent and the normal zones, whereas activation of several other kinases, including Akt and ERK1/2, was not affected (data not shown).

**DISCUSSION**

The major observation of our study is that the induction of endothelial cell tube formation in vitro and coronary collateral growth in vivo require a specific intracellular concentration of ROS. ROS levels below and above (with regard to superoxide) this optimal range prohibit coronary collateral development and endothelial cell tube formation. We also demonstrate that p38 MAP kinase activation plays a pivotal role in ROS signaling for both endothelial cell tube formation and, for the first time, coronary collateral growth in vivo. Our observations and conclusions are supported by some cogent work in the literature.

In support of the present study, it has been reported that short and repetitive exposure to hypoxia-reoxygenation produces ROS that are associated with angiogenesis (9, 16, 27). A role for ROS in VEGF-induced tube formation in cultured endothelial cells has been demonstrated (1, 26). Here we show, for the first time, that coronary collateral development in the rat
model of RI is critically dependent on an optimal concentration of ROS generated in the myocardium by our RI protocol. Specifically, we show that a specific concentration range of superoxide is required for coronary collateral growth and that elevated superoxide levels are detrimental to the process (Fig. 4). Our data are in agreement with in vivo studies demonstrating that SOD inhibition resulted in marked reduction of tumor growth and metastasis, which was dependent on vascular angiogenesis (14). Too-low concentrations of both superoxide and possibly H2O2 were not conducive to coronary collateral growth, because SOD inhibition results in both elevation in superoxide levels and a decrease in H2O2 levels in the myocardium, whereas treatment with DPI decreased concentrations of both of these ROS. Thus the results in this study do not answer the question of whether this process is inhibited by too-high superoxide concentrations alone, by too-low H2O2 concentrations, or by a combination of both.

Our results implicate superoxide derived from flavin-containing oxidases to be critical for coronary collateral growth, because DPI blocks this process in response to ischemia-reperfusion in vivo. Furthermore, inhibition of NAD(P)H oxidases by apocynin yielded nearly identical results (Fig. 2). Ushio-Fukai et al. (26) established a role for NAD(P)H oxidase in endothelial cell tube formation, and Angermayr et al. (2) demonstrated that NAD(P)H oxidase modulates angiogenesis and portal collateral development in rat. These results, together with our own, suggest that NAD(P)H oxidase-derived ROS play a critical role in regulation of coronary collateral growth.

Nitric oxide has been implicated in collateral development so that reduced NO bioavailability or inhibition of NO synthase resulted in decreased collateral growth (15). DETC, which increases superoxide concentrations, would potentially result in decreased NO bioavailability and leaves the question of NO involvement open for discussion. However, treatment with DPI markedly reduces superoxide concentrations in the heart (Fig. 3, A and B), thus potentially increasing or at least not altering NO concentrations. Therefore, we believe that an optimal concentration of superoxide and H2O2 but not NO is critical for coronary collateral growth.

Another proposed explanation for the increase in collateral flow is an increase in coronary dilation in response to ischemia-reperfusion. We doubt that this occurs because coronary circulation is maximally dilated by ischemia. We have confirmed this in a previous study (22) by maximally dilating coronary arteries with dipyridamole during the initial occlusion. Dipyridamole did not increase coronary flow beyond that during occlusion. In addition, in previous work published by our laboratory (22), microcomputed tomography images clearly show an increase in coronary collateral development following the RI protocol. Another point we are compelled to make is that an increase in the collateral-dependent region must reflect collateral growth, i.e., an increase in the caliber of the collateral vessels. The native collateral circulation is high in resistance (collateral flow is 20% of normal zone flow because of the high resistance of these vessels) because of the small caliber of the vessels. Therefore, the only way for flow to quadruple following the RI protocol is through growth of these vessels.

p38 MAP kinase has been shown to be redox dependent in cultured aortic smooth muscle (23), and VEGF has been shown to activate p38 MAP kinase in endothelial cultures (6), but these studies fall short of investigating the redox sensitivity of p38 MAP kinase in response to VEGF. Here, we show that p38 MAP kinase activation in response to VEGF is ROS sensitive (Fig. 4). In contrast, Amin et al. (1) found that the antioxidant resveratrol did not affect p38 phosphorylation in HUVECs. The reason for this discrepancy is unclear but may include differences in ROS dependency of p38 activation between HCAECs and HUVECs or whether the dose of resveratrol was sufficient to affect the redox state of cells. Functional consequences of p38 MAP kinase inhibition in endothelial cell tube formation likewise remain unknown. In fact, both proangiogenic (29) and antiangiogenic (3) effects of p38 MAP kinase activation have been reported in vitro. In this study, we demonstrate that specific inhibition of p38 MAP kinase activation prevents VEGF-induced HCAEC tube formation in Matrigel (Fig. 4B).

Importantly, we show a role for p38 MAP kinase in coronary collateral growth. Near-complete inhibition (>90%) of p38 MAP kinase by SB-203580 in vivo led to a partial (~50%) attenuation in coronary collateral development in response to LAD occlusion (Fig. 5B). These results demonstrate that collateral growth is partially but critically dependent on p38 MAP kinase and indicate that p38 MAP kinase activation in response to RI depends on an optimal level of ROS. Since 80% of cells, by volume, in the whole heart are cardiac myocytes, the observed p38 activation in these preparations is likely to be primarily related to its activation in myocytes. However, p38 activation may be critical in both endothelial cells, where its activation could directly regulate endothelial cell migration, and in cardiac myocytes, where it may affect the expression of growth factors and matrix-degrading enzymes to facilitate migration of vascular cells.

Another relevant question is whether regional ischemia is the driving force for coronary collateral growth. In our study, RI induced an increase in myocardial superoxide both in the LAD-dependent and the normal zone. The increase in superoxide in the normal zone may be related to the myriad observations of remote preconditioning (19). However, we are compelled to emphasize that superoxide levels generated by RI in the normal zone were lower then those in the LAD-dependent zone. In addition, the effect of DETC on further increasing superoxide concentrations was far greater in the LAD-dependent than in the normal zone, and the effect of DPI on decreasing superoxide concentrations in the normal zone was negligible. Importantly, p38 MAP kinase activation in the normal zone was not altered by RI. Thus we believe that although alterations in ROS levels in the remote region of the myocardium may contribute to regulation of coronary collateral growth, regulation of ROS concentrations and especially redox-dependent signaling in the ischemic zone is more significant.

The novel findings in this study, specifically 1) the demonstration of a requirement for a specific concentration range of ROS for coronary collateral growth and 2) the partial but definitive dependence of coronary collateral growth on p38 MAP kinase, provide an important step toward understanding the molecular mechanisms that regulate coronary collateral growth.

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REFERENCES