OPTIMAL REACTIVE OXYGEN SPECIES CONCENTRATION AND p38 MAP KINASE ARE REQUIRED FOR CORONARY COLLATERAL GROWTH

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Running Title: ROS, p38 and collateral growth

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Abstract

Reactive oxygen species (ROS) are implicated in coronary angiogenesis and collateral growth. In this study, we evaluated the requirement for ROS in endothelial cell (HCAEC) tube formation, coronary collateral development in vivo, and one component of the signaling pathway (redox sensitive p38 MAP kinase) by which ROS may stimulate vascular growth. Administration of the flavin-containing oxidase inhibitor, DPI (10 µM) or the superoxide dismutase (SOD) inhibitor, DETC (10 mM) blocked VEGF-induced (50 ng/ml) endothelial cell (HCAEC) tube formation in Matrigel (p<0.05, n=10). Next, we assessed the effect of DPI and DETC on coronary collateral development in a rat model of episodic ischemia: repetitive occlusion/ischemia (RI) of LAD (40 sec occlusion, every 20 min for 2h20min, 3 times a day, for 10 days). Each day of the protocol, DPI (0.2 mg/kg/day), or DETC (1 g/kg/day) were given by IP injection, or the NAD(P)H oxidase inhibitor, apocynin (0.25 mg/ml) was given in drinking water. Collateral-dependent flow (radioactive microspheres) was expressed as a ratio of flows between the normal and the ischemic zone. In sham animals, collateral flow in the ischemic zone was 18±6% of the normal zone flow; whereas in the RI group collateral flow in the ischemic zone was 83±5% of that in the normal zone. Blockade of ROS (superoxide and H₂O₂) production by DPI prevented the increase in collateral flow after RI (25±4% of that in the normal zone). Nearly identical results were obtained with apocynin following RI (32±7% of that in the normal zone). Increasing superoxide levels and decreasing H₂O₂ levels by SOD inhibition (DETC) achieved similar results (collateral flow after RI was 21±2% of that in the normal zone). Both DPI and DETC blocked RI-induced p38 MAP kinase activation in response to VEGF in vitro and to RI in vivo. These results demonstrate a requirement for optimal ROS concentration in HCAEC tube formation, coronary collateral development and p38 MAP kinase activation. In addition, p38 MAP kinase inhibition
prevented HCAEC tube formation and partially blocked RI-induced coronary collateral development (42±7% of normal zone flow, n=8) indicating that p38 MAP kinase is a critical signaling mediator of coronary collateral growth.
Introduction

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 upon re-establishment of blood flow and 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 re-occurrence 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 ROS are critical mediators of coronary collateral development in a canine model of repetitive ischemia. Treatment with N-acetyl cysteine (NAC) attenuated the repetitive ischemia (RI)-induced increase in coronary collateral flow. Likewise, myocardial interstitial fluid from dogs undergoing the RI protocol, but not from NAC 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, while 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. Previously, we have reported a requisite role for vascular endothelial growth factor (VEGF) in coronary collateral growth. (22) VEGF has been shown to activate several signaling pathways including ERK1/2, (21) JNK (17) and p38 (6) mitogen activated protein kinases (MAP kinases), the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway (5), the non-receptor tyrosine kinases c-Src (7), focal adhesion kinase (FAK) (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, 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 \( \text{H}_2\text{O}_2 \) allows for HCAEC tube formation in response to VEGF and for coronary collateral growth in response to repetitive ischemia/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/REPETITIVE ISCHEMIA. 3-4 month old, 300-350 g, male WKY rats 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: 8, 40 sec occlusions, ones every 20 minutes over a duration of 2 hours and 20 minutes followed by a period of “rest” for 5 hours and 40 minutes. This 8 hour cycle was repeated 3 times per day over a period of 10 days.

MICROSPHERE MEASUREMENTS OF MYOCARDIAL AND COLLATERAL-DEPENDENT BLOOD FLOW. Microspheres (5 x 10^5) labeled with gamma emitters ^57Co (at initial surgery) or ^103Ru (at end of RI protocol) were injected into the left ventricle over 20 sec. Collateral (LAD)-dependent zone was identified 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 Cm = activity (cpm/g) of the tissue samples from the LAD-dependent and normal zone. All experiments were n=8, and data were analyzed by two-way ANOVA followed by t-test. Collateral flow was measured in the following groups: a sham group (sham) that was instrumented but not subjected to RI (n=8), a control group/RI (n=8), RI + diphenyleneiodonium (DPI, 0.2 mg/kg/day, n=8), RI + apocynin (0.25 mg/ml in drinking water, n=3), and RI + diethyldithiocarbamate (DETC, 1 g/kg/day, n=8).
MEASUREMENT OF O$_2$$^-$ and H$_2$O$_2$. O$_2$$^-$ production was evaluated using dihydroethidium (DHE) in vitro and in vivo and electroparamagnetic resonance (EPR) in vivo. H$_2$O$_2$ production was evaluated by dihydrodichlorofluorescein (DCF) in vivo. For HCAEC cell culture, DHE was administered during the last 20 minutes of treatment. Cells were then observed immediately under a fluorescent microscope. For in vivo studies, DHE or DCF were injected into the left ventricle (LV) (60 µg/kg) for 20 min prior to two consecutive periods (40 sec occlusion, followed by 20 min reperfusion and another 40 sec occlusion and 20 min reperfusion) of ischemia/reperfusion. Animals were then sacrificed. The heart was removed, frozen in OCT on dry ice and stored at –70°C until sectioning. 5 µm sections were made in a cryo-microtome, and 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 obtained by Metamorph Software on an n=3 hearts (5 consecutive sections per heart). A Bruker EMX spectrometer was used for X-Band EPR measurements of O$_2$$^-$ using 1-hydroxy-3-carboxy-pyrrolidine (CP-H) as a spin-trap. Animals underwent two consecutive periods (40 sec occlusion, followed by 20 min reperfusion and another 40 sec occlusion and 20 min reperfusion) of ischemia/reperfusion. Animals were then sacrificed, the heart removed, LAD-dependent and normal zone separated and CP-H (238µg/100mg tissue) added to the tissue samples immediately. Tissue was then homogenized by sonication on ice, and frozen in liquid nitrogen until EPR measurements. O$_2$$^-$ concentration was calculated from arbitrary units (AU) (3.4x10$^6$ AU/nM).
HCAEC CELL CULTURE. Cells were purchased from Clonetics, and cultured at low passages (passage 3-8) in EGM-2 BulletKit medium (Clonetics) that contains 25% fetal bovine serum (FBS), 0.2% hydrocortisone, 2% hFGF-B, 0.5% IGF-1, 0.5% ascorbic acid, 0.5% hEGF and 0.5% GA-1000.

ENDOTHELIAL TUBE FORMATION. HCAECs (passage 3-10) were seeded on Matrigel (BD Bioscience) coated 24 well plates according to company instructions in EGM-2 BulletKit medium at the density of 30,000 cells/well. Cells were allowed to attach for 24 hours before the addition of 50ng/ml VEGF, DPI (10 µM), DETC (10 mM), or the p38 MAP kinase inhibitor (SB203580 (20 µM)). All treatment groups were n=10 wells. The extent of tube formation was quantified after 2 days using Scion Image software. An electronic grid was superimposed on microscopic images, and the number of squares containing tubes counted and averaged from 5 randomly selected fields for each well to obtain % of total field containing tubes.

WESTERN BLOT ANALYSIS. Proteins, from cells and myocardial tissue, were extracted in a lysis buffer containing 0.1% SDS and 1% Triton as previously described.(18) For extraction from tissue, hearts were excised, LV dissected, the LAD-dependent zone 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 transferred to Hybound-ECL nitrocellulose membranes. Phospho-specific anti-p38 and anti-total p38 antibodies (Cell Signaling) were used for Western blotting. Bands were visualized by enhanced chemiluminescence (ECL, Amersham) and quantified using NIH Image software. Since none of the treatments altered p38
MAP kinase expression, its total levels were used to check for equal lane loading and to normalize phosphorylation levels. All experiments were performed in triplicate (n=3 animals per group).

DATA ANALYSIS. ANOVA followed by t-tests with the Bonferroni Inequality was used to determine 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 the density of 30,000 cell/well, allowed to attach for 24 hours then treated with VEGF (50 ng/ml). Tube formation was observed after 2 days and was significantly increased in response to VEGF treatment as compared to control, non-treated cells (19±0.02 vs. 8±1% area covered, n=10, p<0.05)(Figure 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) (Figure1). Similarly, treatment with the SOD-inhibitor DETC (10 mM) also prevented VEGF-induced HCAEC tube formation (5±2% vs. 19±2%, n=10, p<0.05) (Figure1). Treatment with DPI or DETC alone (without VEGF) was not significantly different from control (5±1% and 5±2%, respectively).

*Repetitive ischemia causes coronary collateral development in the rat model of repetitive ischemia in vivo.* Results in Figure 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 (Figure 2), demonstrating significant collateral development in this region of the myocardium.

Blockade of superoxide production by DPI (0.2 mg/kg/day) prevented the increase in collateral flow induced by RI (25±4% of that in the normal zone, p<0.05, n=8) (Figure 2). Similar results were obtained with apocynin (0.25 mg/ml in drinking water = 43 mg/kg/day), a more specific inhibitor of NAD(P)H oxidases (collateral flow was 32±7% of that in the normal zone after RI, p<0.05, n=3) (Figure 2). Likewise, increasing superoxide by SOD inhibition (DETC, 1 g/kg/day) achieved similar results (collateral flow was 21±2% of that in the normal zone following RI, p<0.05, n=8) (Figure 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 µg/kg) for 20 minutes prior to two consecutive periods of ischemia/reperfusion. Ischemia/reperfusion caused an increase in superoxide generation in the LAD-dependent zone as compared to sham (RI=450±13 vs. sham=124±6 AU, n=3, p<0.05) (Figure 3A). When DPI was administered for 2 days prior to 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 2 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) (Figure 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) (Figure 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 (Figure 3B). Thus, RI increased superoxide concentrations in both the LAD-dependent and the normal zone, 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, while the effect of DPI was pronounced in the LAD-dependent but negligible in the normal zone.

Since SOD inhibition might also lower myocardial H$_2$O$_2$ concentration, DCF (60 µg/kg) was injected LV (in a manner identical to DHE administration) in a separate group of animals, and DCF fluorescence evaluated in arbitrary units (AU) to evaluate this possibility. Figure 3C shows that H$_2$O$_2$ levels were decreased approximately 4 fold by both DPI and DETC (122 AU for RI+DPI; 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 minutes, 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 (4.5 ± 0.23 vs. control, 1 ± 0.19,
p<0.05, n=3) (Figure 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, p<0.05, n=3) 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, SB203580 (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) (Figure 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 sacrificed, hearts excised, LAD-dependent and normal zones separated and prepared for Western blotting. Western blots with phospho-specific anti-p38 antibodies revealed a
significant increase in p38 MAP kinase activation in response to ischemia/reperfusion (2.7 ± 0.23 fold vs. sham 1.0 ± 0.17, p<0.05, n=3) in the LAD-dependent zone, which was completely blocked by both DPI and DETC (1.0 ± 0.18 and 1.1 ± 0.3 fold, respectively, p<0.05, n=3) (Figure 5A). Expression (total p38 demonstrated by Western blot 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) (Figure 5A).

To ascertain whether p38 MAP kinase is required for coronary collateral development in response to ischemia/reperfusion in vivo, rats were treated with SB203580 (3.2 mg/kg/day) for the duration of the 10 day RI protocol via IP injection once/day. p38 MAP kinase inhibition resulted in a significant (~50%) decrease in coronary collateral development (42±7% of that in the normal zone, p<0.05, n=8) (Figure 5B).

Taken 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 IP administration of SB203580 (3.2 mg/kg/day) 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 zone, while activation of several other kinases, including Akt and ERK1/2, was not effected (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 regards 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 repetitive ischemia is critically dependent upon 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 (Figure 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, while treatment with DPI decreased concentrations of both of these ROS. Thus, the results in this study do not answer the question whether this process is inhibited by too high superoxide concentrations alone, by too low H₂O₂ concentrations, or a combination of both.

Our results implicate superoxide derived from flavin-containing oxidases to be critical for coronary collateral growth, since DPI blocks this process in response to
ischemia/reperfusion in vivo. Furthermore, inhibition of NAD(P)H oxidases by apocynin yielded nearly identical results (Figure 2). Ushio-Fukai et al. established a role for NAD(P)H oxidase in endothelial cell tube formation,(26) and Angermyer et al demonstrated that NAD(P)H oxidase modulates angiogenesis and portal collateral development in rat.(2) 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 NOS 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 (Figure 3A 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 by maximally dilating coronary arteries by dipyridamole during the initial occlusion. Dipyridamole did not increase coronary flow beyond that during occlusion.(22) In addition, in previous work published by our laboratory, micro-CT images clearly show an increase in coronary collateral development following the RI protocol(22). 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 (Figure 4). In contrast, Lin et al. found that the antioxidant resveratrol did not affect p38 phosphorylation in HUVECs. (1) 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 were likewise remain unknown. In fact, both pro (29)- and anti (3)-angiogenic 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 (Figure 4B).

Importantly, we show a role for p38 MAP kinase in coronary collateral growth. Near complete inhibition (>90%) of p38 MAP kinase by SB203580 in vivo lead to a partial (~50%) attenuation in coronary collateral development in response to LAD occlusion (Figure 5B). These results demonstrate that collateral growth is partially but critically dependent upon 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 in 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 then 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 while 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, the demonstration of a requirement for a specific concentration range of ROS for coronary collateral growth, and the partial but definitive dependence of coronary collateral growth on p38 MAP kinase, provide an important step towards understanding the molecular mechanisms that regulate coronary collateral growth.


Figure 1. HCAEC tube formation in Matrigel. Left: Representative images of HCAEC 2 days after addition of 50ng/ml VEGF +/- inhibitors (10 µM DPI or 10 mM DETC) as indicated. Right: Percent of total field with tubes (n=10 per group, mean=average of 5 random fields per well, p<0.05).

Figure 2. Collateral-dependent flow expressed as % of flow in the normal zone. DPI, DETC and apocynin blocked the RI-induced increase in collateral-dependent flow (n=8 for each treatment group).

Figure 3. A. DHE fluorescence in the LAD-dependent zone in a sham animal, following two periods of ischemia (40 sec)/reperfusion (20 min) (RI), and RI + DPI or DETC treatment. RI increased DHE fluorescence, and this increase was abolished by DPI, but further augmented by DETC. B. Superoxide levels in Sham, IR, and in IR + DPI or DETC groups measured by X-band EPR in the LAD-dependent zone (black bars) and the normal zone (white bars). Similar to A, IR increased superoxide levels in the heart, and this increase was attenuated by DPI and accentuated by DETC to a greater extent in the LAD-dependent than in the normal zone. C. DCF fluorescence in Sham, IR, and IR + DPI or DETC groups. Elevated DCF fluorescence was observed following IR, but, either DETC or DPI decreased the signal.

Figure 4. A. Top: Western blot with phospho (top) and total (bottom) p38 MAP kinase antibodies in untreated endothelial cells, or cell treated with VEGF, or VEGF + DPI or DETC. Bottom: Area x 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 (SB203580) on VEGF-induced tube formation. Inhibition of p38 completely prevented VEGF stimulation of endothelial cell tube formation.

**Figure 5. A.** Left: Western blot analysis with anti-phospho specific (top) and anti-total (bottom) p38 MAP kinase antibodies in cardiac tissue from sham rats (day 0) and in rats at varying days of the RI protocol. Phospho-p38 was elevated at day 3, but not at day 6 or 9. DETC or DPI prevented the activation at day 3. Right: Comparison of phospho-p38 in the normal zone of rats undergoing RI at day 3 to that in a sham rats. There were no difference in activation between the normal zone and shams.  

**B.** Collateral flow (expressed as % of normal zone flow) in sham, RI, and RI rats treated with the p38 inhibitor, SB203580. Inhibition of p38 attenuated the increase in collateral-dependent flow by about 50% at day 10 of the RI protocol.
Figure 1.
Figure 2.

Sham: 18±5%
10 day RI: 83±5%
10 day RI + DPI: 25±4%
10 day RI + DETC: 21±3%
p<0.05, n=8
10 day RI + Apocynin: 32±7%
p<0.05, n=3
Figure 3.
Figure 3.
Figure 3.
**Figure 4.**

A.

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Fold Increase/Control

* p<0.05
B.

Control

50ng/ml VEGF

50ng/ml VEGF + 20 µM SB203580

% area covered

Control VEGF

VEGF

*vs. Control

# vs. VEGF

Figure 4.
Figure 5.

A.

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Anti-phospho p38

Anti-total p38

Fold Increase/Control

* p<0.05
B.

Sham: 18±5%
10 day RI: 83±5%
10 day RI + SB203580: 42±7%
*p<0.05, †<0.05, n=8

Figure 5.