Development of Endothelium-Dependent Relaxation in Canine Coronary Collateral Arteries

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Background—Little information exists regarding development of vasomotor control mechanisms during coronary collateral artery maturation. Therefore, we studied endothelium-dependent relaxation of canine collateral arteries isolated 2, 4, and 9 months after placement of an ameroid occluder around the proximal left circumflex coronary artery.

Results—Collateral arteries isolated after 2 months exhibited markedly reduced endothelium-dependent relaxation in response to acetylcholine (ACh; 10⁻¹⁰ to 10⁻⁴ mol/L) and bradykinin (BK; 10⁻¹¹ to 10⁻⁶ mol/L) compared with relaxation of noncollateral coronary arteries (P<0.01). In contrast, endothelium-independent relaxation of collateral arteries to nitroprusside was only slightly reduced compared with relaxation of noncollateral arteries (P<0.05). Endothelium-dependent relaxation of collateral arteries isolated after 4 and 9 months was increased significantly, to the extent that relaxation to ACh and BK was not significantly different between collateral and noncollateral arteries at these periods. Inhibition of nitric oxide synthesis with N²-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) markedly inhibited ACh-induced relaxation in all noncollateral arteries and in collateral arteries isolated after 9 months. However, neither L-NAME nor indomethacin (5 μmol/L) alone inhibited ACh-mediated relaxation of collateral arteries isolated after 4 months. ACh-induced relaxation of these collateral arteries was only inhibited when arteries were preconstricted with 30 mmol/L K⁺ and pretreated with L-NAME and indomethacin (ie, when synthesis/effects of nitric oxide, prostaglandins, and endothelium-derived hyperpolarizing factor were inhibited).

Conclusions—Development of endothelium-dependent relaxation in canine coronary collateral arteries is not complete after 2 months. After 4 months, endothelium-dependent relaxation of collateral arteries is similar to relaxation of noncollateral arteries, but the relaxation exhibits decreased dependence on synthesis of nitric oxide and increased involvement of prostaglandins and endothelium-derived hyperpolarizing factor(s). After 9 months of development, collateral arteries exhibit normal nitric oxide–dependent relaxation, similar to noncollateral arteries. (Circulation. 1998;98:1675-1683.)

Key Words: acetylcholine ■ bradykinin ■ nitric oxide ■ collateral circulation ■ coronary occlusion
Methods

Induction of Collateral Artery Growth

Adult mongrel male dogs (23 to 37 kg) were anesthetized with acepromazine maleate (0.8 mg/kg SC) and sodium pentobarbital (25 mg/kg IV) and ventilated mechanically. Using sterile techniques, we placed an ameroid constrictor (2.75 to 4.0 mm inner diameter, Research Animal Use, Mountain Orthodontics) around the proximal left circumflex coronary artery (LCx). During surgery and recovery, dogs received buprenorphine hydrochloride (0.3 mg IV or IM) as needed for pain relief. Antibiotics were given immediately before surgery (900,000 U penicillin IM) and for 5 days after surgery (800 mg sulfamethoxazole and 160 mg trimethoprim). All experimental procedures were in accordance with the “Position of the American Heart Association on Research Animal Use,” adopted November 11, 1984, and approved by the Animal Care and Use Committee of the University of Missouri.

Preparation of Coronary Artery Rings

We studied the function of coronary arteries isolated from dogs 2, 4, and 9 months after implantation of the ameroid. Dogs were anesthetized with sodium pentobarbital (40 mg/kg), and hearts were rapidly removed 9 months after implantation of the ameroid. Dogs were anesthetized with sodium pentobarbital (40 mg/kg), and hearts were rapidly removed. Collateral arteries were easily identified as tortuous, epicardial vessels extending from and placed in aerated cold Krebs bicarbonate buffer. Collateral arteries with sodium pentobarbital (40 mg/kg), and hearts were rapidly removed 9 months after implantation of the ameroid. Dogs were anesthetized

<table>
<thead>
<tr>
<th>Time</th>
<th>Artery</th>
<th>Outer Diameter</th>
<th>Inner Diameter</th>
<th>Wall Thickness</th>
</tr>
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<tr>
<td>2 Months</td>
<td>LAD (10)</td>
<td>923±82</td>
<td>584±52</td>
<td>139±9</td>
</tr>
<tr>
<td></td>
<td>LCx (10)</td>
<td>853±65</td>
<td>528±43</td>
<td>139±6</td>
</tr>
<tr>
<td></td>
<td>COL (10)</td>
<td>789±48</td>
<td>312±26*</td>
<td>245±22*</td>
</tr>
<tr>
<td>4 Months</td>
<td>LAD (64)</td>
<td>908±31</td>
<td>558±22</td>
<td>146±5</td>
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<tr>
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<td>LCx (64)</td>
<td>873±23</td>
<td>551±16</td>
<td>143±4</td>
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<tr>
<td></td>
<td>COL (52)</td>
<td>893±28</td>
<td>400±23*</td>
<td>255±11*</td>
</tr>
<tr>
<td>9 Months</td>
<td>LAD (12)</td>
<td>953±45</td>
<td>604±42</td>
<td>137±9</td>
</tr>
<tr>
<td></td>
<td>LCx (11)</td>
<td>901±36</td>
<td>599±32</td>
<td>127±8</td>
</tr>
<tr>
<td></td>
<td>COL (12)</td>
<td>903±68</td>
<td>498±61*</td>
<td>180±12*</td>
</tr>
</tbody>
</table>

LAD indicates left anterior descending coronary artery; LCx, left circumflex coronary artery; COL, collateral artery.

*P<0.05 COL vs LAD and LCx. Values are expressed as mean±SEM (μm). Numbers in parentheses indicate number of vessels studied.

In Vitro Evaluation of Relaxation Responses

Relaxation responses were studied with arteries preconstricted with 30 to 40 mmol/L K+ or 30 mmol/L endothelin. Some rings were incubated continuously with enzyme inhibitors beginning at least 30 minutes before evaluation of relaxation responses. Concentration-response relationships to various agonists were determined by cumulative additions of small aliquots (20 to 150 μL; total volume <5% of bath volume) of concentrated stock solutions directly to the tissue bath. Unless otherwise indicated, drugs were purchased from Sigma Chemical Co. We purchased endothelin-1 from Peninsula Laboratories, Inc and BK from Bachem.

Data Analyses

Relaxation responses were expressed as percent decrease of the precontraction. The concentration of vasodilator causing 50% of the maximal relaxation response was designated as the EC50 and was determined by nonlinear regression analysis of the concentration-response data for each vessel. Concentration-response curves were compared by two-way ANOVA for repeated measures followed by Student-Newman-Keuls test. A P value <0.05 was considered significant.

![Figure 1. Endothelium-dependent relaxation in response to ACh in canine collateral artery (COL), LAD, and LCx isolated 2, 4, or 9 months after placement of an ameroid occluder. Arteries were preconstricted with 30 to 40 mmol/L K+; Values are expressed as the mean±SEM. The number of animals used for each study is indicated in parentheses. Relaxation of COL isolated after 2 months was attenuated significantly compared with relaxation of LAD and LCx (P<0.01). In contrast, 4 and 9 months after ameroid placement, ACh relaxation responses were not significantly different between collateral and noncollateral arteries. Relaxation of collateral-dependent LCx and normal LAD isolated after 2 and 4 months was not different. However, relaxation of LCx isolated after 9 months was reduced significantly compared with relaxation of LAD (P<0.05).]
significant. Data are presented as mean±SEM, and n values reflect the number of animals.

Results

Vessel Dimensions

Dimensions of the collateral, LAD, and LCx studied 2, 4, and 9 months after ameroid placement are presented in Table 1. All dimensions of LAD and LCx arterial rings were not significantly different. Outer diameters of collateral arteries isolated after all three periods were not significantly different from outer diameters of LAD and LCx. However, collateral arteries had thicker walls than noncollateral arteries, resulting from greater thickness of the intimal layer of collateral vessels. Inner diameters of all collateral arteries were smaller than inner diameters of LAD and LCx isolated at the same periods.

Development of Endothelium-Dependent Relaxation

We evaluated endothelium-dependent relaxation of collateral and noncollateral coronary arteries 2, 4, and 9 months (60±2, 120±2, and 279±2 days) after placement of an ameroid occluder around the proximal LCx. K⁺-induced preconstrictions of collateral arteries isolated after all periods were significantly smaller than contractions of noncollateral vessels, averaging 34%, 41%, and 47%, respectively, of contractions of the LAD and LCx. Relaxation responses of all arteries were expressed as a percentage of the stable K⁺ preconstriction.

Noncollateral LAD and LCx isolated 2 months after ameroid placement relaxed 70% to 80% in response to ACh and BK (Figures 1 and 2). In contrast, maximal relaxation in response to ACh and BK averaged only 35% and 25%, respectively, in collateral arteries isolated after 2 months (P<0.01 versus noncollateral arteries). Relaxation of collateral arteries isolated after 4 and 9 months was increased significantly compared with relaxation of collateral arteries isolated after 2 months. Relaxation in response to ACh and BK was not significantly different between collateral and noncollateral arteries at these later periods.

ACh- and BK-induced relaxation was not significantly different between collateral-dependent LCx and normal LAD.
isolated 2 and 4 months after ameroid placement. However,
ACh-induced relaxation of collateral-dependent LCx isolated
after 9 months was reduced significantly compared with
relaxation of LAD isolated from the same hearts. In contrast,
relaxation in response to BK was not significantly different
between LCx and LAD isolated after 9 months.

Development of
Endothelium-Independent Relaxation
Maximal relaxation to the endothelium-independent vasodi-
lator averaged >80% in collateral and noncollateral arteries
isolated at all periods (Figure 3). However, sodium nitroprus-
side (NP)-induced relaxation of collateral arteries studied at
all periods was slightly but significantly attenuated compared
with relaxation of noncollateral arteries.

Role of Nitric Oxide

Acetylcholine Relaxation
We determined the effect of inhibition of nitric oxide synthe-
sis with L-NAME (100 μmol/L) on endothelium-dependent
relaxation of collateral arteries isolated after 4 and 9 months.
In studies performed 4 months after ameroid placement,
arteries were preconstricted either with 30 to 40 mmol/L K+
(Figure 4A) or 30 nmol/L endothelin (B). Relaxation of the LAD
and LCx was significantly inhibited by L-NAME
($P<0.01$). In contrast, relaxation of COL was
not significantly altered by L-NAME ($P>0.05$).
Similar results were obtained in arteries pre-
constricted with either K+ or endothelin.
collateral arteries in response to ACh was markedly inhibited by L-NAME (P<0.01, Figure 5).

**Bradykinin Relaxation**

The relative role of nitric oxide in mediating relaxation of noncollateral coronary arteries to BK appeared to depend on the type of precontraction (K+ versus endothelin). L-NAME markedly attenuated BK-mediated relaxation of noncollateral arteries preconstricted with K+ (P<0.01, Figure 6A). However, when noncollateral arteries were preconstricted with endothelin, L-NAME produced only a slight decrease (≤15%) in maximal BK-induced relaxation of LAD and LCx (P<0.05; Figure 6B) and a small increase in EC50 (P<0.05, Table 3).

The effects of L-NAME on relaxation to BK were similar in collateral arteries preconstricted with either K+ or endothelin. L-NAME did not significantly decrease maximal BK-induced relaxation of collateral arteries isolated after 4 months, which was in contrast to marked inhibition of relaxation of noncollateral arteries preconstricted with K+ (Figure 6A). However, L-NAME significantly increased the EC50 (decreased pEC50) for BK in collateral arteries (Tables 2 and 3). BK-induced relaxation of collateral arteries isolated after 9 months was markedly inhibited by L-NAME, similar to the relaxation of noncollateral arteries (P<0.05, Figure 7).

**Nitroprusside Relaxation**

L-NAME did not significantly alter the relaxation of LAD or LCx to NP (data not shown). L-NAME slightly enhanced the relaxation of collateral arteries to higher concentrations of NP (10^-7 mol/L; P<0.05), but did not significantly alter the EC50 value for NP (Table 2).

**Role of Prostaglandins**

**Acetylcholine Relaxation**

The cyclooxygenase inhibitor indomethacin (5 μmol/L) did not significantly alter ACh-induced relaxation of either collateral or noncollateral arteries isolated after 4 months (Figure 8A).

**Bradykinin Relaxation**

BK-mediated relaxation of collateral arteries isolated after 4 months was slightly inhibited by indomethacin (Figure 8B). The EC50 for BK in collateral arteries was increased in the presence of indomethacin from 32±6 to 138±11 nmol/L (P<0.05). Relaxation of noncollateral arteries in response to BK was not altered by indomethacin.

**Combined Effects of L-NAME and Indomethacin**

We evaluated the effects of combined inhibition of nitric oxide synthase and cyclooxygenase on relaxation of arteries preconstricted with either endothelin or high K+ to investigate the role of EDHF as a mediator of ACh relaxation of arteries.
collateral arteries isolated after 4 months. ACh-induced relaxation of LAD and LCx preconstricted with either endothelin or K$_1$ was markedly inhibited by combined pretreatment with L-NAME and indomethacin ($P<0.01$, Figures 9 and 10). In contrast, ACh-induced relaxation of collateral arteries preconstricted with endothelin was not altered by combined inhibition of nitric oxide synthase and cyclooxygenase with these drugs ($P>0.05$, Figure 9). However, when collateral arteries were preconstricted with K$_1$, ACh-mediated relaxation was markedly inhibited in the presence of L-NAME and indomethacin ($P<0.01$, Figure 10).

**Discussion**

The present study is the first systematic evaluation of the chronologic development of endothelium-dependent relaxation in maturing coronary collateral arteries. Our results indicate that development of agonist-mediated endothelium-dependent relaxation is not complete in collateral arteries 2 months after ameroid implantation. After 4 months of development, endothelium-dependent relaxation of collateral arteries in response to ACh and BK appears to be similar to endothelium-dependent relaxation of noncollateral coronary arteries; however, this relaxation exhibits decreased dependence on synthesis of nitric oxide compared with relaxation of noncollateral arteries. In contrast, 9 months after ameroid implantation, endothelium-dependent relaxation of collateral arteries appears to be mediated primarily by synthesis of nitric oxide, similar to noncollateral arteries. These observations may have important implications relative to the critical role of collateral arteries in providing blood flow to collateral-dependent myocardium.

**Time Course of Development of Endothelium-Dependent Relaxation**

This is the first study to evaluate endothelial function of collateral arteries isolated as early as 2 months (60 days) after ameroid placement. Previous studies have demonstrated intact endothelium-dependent responses to ACh and BK in well-developed coronary collateral arteries. However, the present study has provided a new finding, that collateral arteries in early stages of development exhibit reduced relaxation in response to
Figure 7. Effects of L-NAME on BK-mediated relaxation of COL, LAD, and LCx isolated 9 months after ameroid placement. Arteries were preconstricted with 30 to 40 mmol/L K$^+$. L-NAME significantly inhibited the relaxation of all three artery types ($P<0.05$).

Figure 8. Effects of indomethacin (INDO; 5 $\mu$mol/L) on ACh (A) and BK (B) relaxation of COL, LAD, and LCx isolated 4 months after ameroid placement. ACh-induced relaxation of COL, LAD, and LCx was not significantly altered by pretreatment with indomethacin. Relaxation responses of LAD and LCx in response to BK were also not significantly different in the presence of indomethacin. However, indomethacin produced a significant rightward shift in the concentration-response relationship for BK in COL ($P<0.05$). Maximal BK-mediated relaxation of COL was not altered by indomethacin.
endothelium-dependent vasodilator agonists. Potential mechanisms responsible for the marked attenuation of agonist-mediated endothelium-dependent relaxation in immature collateral arteries could include a paucity of muscarinic and kininergic receptors on endothelial cells, deficiencies of signal transduction elements coupling endothelial receptors to synthesis of mediators of relaxation, low intrinsic activity of nitric oxide synthase, and/or dysfunctional guanylyl cyclase–cGMP cascade in smooth muscle. Impairment of guanylyl cyclase–cGMP cascade in smooth muscle of collateral arteries appears to be unlikely as a primary mechanism of impairment because relaxation in response to the nitric oxide donor NP was only mildly decreased in collateral arteries isolated after 2 months compared with noncollateral arteries.

Findings from studies of endothelial function during re-growth after vascular injury are consistent with our results. For example, agonist-mediated endothelium-dependent relaxation has been shown to be impaired in canine coronary arteries with newly developed endothelial cells 5 weeks after balloon injury. 4 Thus, it is plausible that receptor/signal transduction coupling mechanisms in proliferating endothelial cells may require extended periods (>2 months) to develop in collateral arteries as well.

Mechanisms of Endothelium-Dependent Relaxation in Developing Coronary Collateral Arteries

Our results indicate that endothelium-dependent relaxation of collateral arteries isolated after 4 months is not dependent on synthesis of nitric oxide. These findings are in distinct contrast to those from noncollateral coronary arteries isolated from the same hearts. Results of our studies suggest that all three known endothelium-derived mediators (nitric oxide, prostaglandins, and EDHF) are involved in ACh-induced relaxation of collateral arteries isolated after 4 months. Inhibition of the synthesis or action of any one or two of the known mediators of endothelium-dependent relaxation did...
not significantly attenuate ACh relaxation of collateral arteries. ACh relaxation of collateral arteries isolated after 4 months was attenuated significantly only when the synthesis and/or action of nitric oxide, prostaglandins, and EDHF were inhibited simultaneously. These results indicate that prostaglandins and EDHF have important roles as mediators of endothelium-dependent relaxation in collateral arteries isolated after 4 months and that endothelium-dependent relaxation of collateral arteries at this period involves a redundancy or reserve of mediators of relaxation. However, endothelium-dependent relaxation of collateral arteries isolated after 9 months was primarily dependent on synthesis of nitric oxide, which indicates that the roles of prostaglandins and EDHF as redundant mediators of endothelium-dependent relaxation diminish with time during collateral development.

In contrast to our findings, Flynn and colleagues reported that N^\text{\textdegree}^-\text{monomethyl-L-arginine} (L-NMMA) produced similar inhibition of ACh- and BK-mediated relaxation of collateral and noncollateral coronary arteries. The inconsistency in our results may be explained by the differences in the experimental design or in the time allowed for collateral development. Flynn et al studied collateral arteries isolated over a broad, unspecified range of time (“a minimum of 12 weeks”), whereas we studied collateral arteries isolated at specific time intervals after amioder implantation.

Dulas and coworkers studied endothelium-dependent relaxation of collateral arteries isolated 6 months after coronary occlusion. In contrast to the report of Flynn et al., Dulas and colleagues found that L-NMMA produced less inhibition of ACh relaxation of collateral arteries than of noncollateral coronary arteries, indicating decreased dependence on nitric oxide synthesis. These investigators concluded that the greater residual relaxation in collateral arteries induced by ACh after nitric oxide synthase and cyclooxygenase blockade could result from enhanced EDHF production in collateral vessels. The results of Dulas et al correlate well with our findings. Taken in concert, these results indicate that endothelium-dependent relaxation of maturing collateral arteries progressively develops increased dependence on nitric oxide synthesis between 4 and 9 months after coronary occlusion.

Implications and Conclusions

Our findings have potentially important implications relative to understanding collateral artery function and regulation of perfusion of collateral-dependent myocardium. Reduced endothelium-dependent vasodilation in collateral arteries early in development suggests the possibility of an increased propensity for vasospasm of collateral arteries and myocardial ischemia soon after occlusion of a major coronary artery. Endothelium-derived relaxing factors have been implicated as important mediators of flow-mediated vasodilation of coronary vessels and coronary metabolic vasodilation. Endothelium-derived relaxing factors (nitric oxide, prostacyclin) also exert protective antithrombotic effects. Thus, the decreased capability to produce endothelium-derived relaxing factors in developing collateral arteries may result in reduced vasodilation and increased thrombogenesis, potentially limiting blood flow to collateral-dependent myocardium.

In contrast to risks posed by delayed development of endothelium-dependent relaxation in collateral arteries early after coronary occlusion, the mechanisms underlying relaxation of collateral arteries studied after 4 months may enhance the ability of the collateral circulation to provide adequate perfusion of the collateral-dependent myocardium. In contrast to the marked dependence of noncollateral canine coronary arteries on nitric oxide synthesis, these collateral arteries exhibit an increased capacity for production of multiple endothelium-derived relaxing factors in response to receptor-dependent agonists. This redundancy or reserve of endothelium-derived mediators of relaxation may promote and maintain adequate perfusion of the collateral-dependent myocardium under diverse conditions and thereby prevent myocardial ischemia and infarction.

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References
