Novel Antiinflammatory Vascular Benefits of Systemic and Stent-Based Delivery of Ethylisopropylamiloride

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Background—Recently, we demonstrated that the amiloride derivative ethylisopropylamiloride (EIPA) limits vascular smooth muscle cell growth and migration. The purpose of the present experiments was to determine whether EIPA can also reduce the inflammatory component of atherogenesis and stent neointima formation.

Methods and Results—To determine the effect of EIPA on the early inflammatory stages of atherogenesis, apolipoprotein E null mice (apoE−/−) fed an atherogenic diet received a subcutaneous pump infusion of either EIPA (3 mg · kg−1 · d−1) or the control vehicle for 4 weeks. The en face aortic area of atherosclerotic lesions and the subendothelial accumulation of macrophages were reduced by 46% and 38%, respectively, in EIPA-treated mice. Moreover, the number of vascular cell adhesion molecule-1 (VCAM-1) immunopositive luminal endothelial cells was 59% less in the EIPA treatment group. In vitro, there was a concentration-dependent inhibition of lipopolysaccharide (LPS)-induced VCAM-1 expression with a corresponding 37% reduction in U-937 cell adhesion to endothelial cells. EIPA also reduced LPS-stimulated nuclear factor-κB (NF-κB) activation as reflected by a 66% reduction in NF-κB nuclear translocation. Finally, to test the effect of EIPA on the early inflammatory reaction to stent implantation, stents coated with jelly alone or jelly plus EIPA were implanted into rabbit iliac arteries. Four weeks later, the stent neointimal area, abundance of peristrut macrophages, and density of intimal smooth muscle cells were reduced by 38%, 47%, and 37%, respectively, for EIPA stents.

Conclusions—EIPA downregulates endothelial cell activation of NF-κB and VCAM-1 expression and attenuates the early inflammatory stages of atherogenesis and stent intimal formation. (Circulation. 2004;110:3721-3726.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ inflammation ■ restenosis ■ stents

Circulating blood leukocytes play a key role in the early stages of atherogenesis as they adhere to activated endothelial cells and transmigrate into the subendothelial space, where they generate various growth factors and cytokines.1,2 Leukocyte-endothelium adhesion is mediated by endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), as well as the leukocyte integrin very late-acting antigen-4 (VLA-4).3–6 Enhanced expression of VCAM-1 in endothelial cells plays an important role in atherogenesis.7,8 In particular, several in vivo studies involving hypercholesterolemic mice or rabbits demonstrate that VCAM-1 expression is upregulated in arterial endothelial cells at lesion-prone areas.9,10 Antibody-mediated antagonism of VCAM-1 or VLA-4 and targeted disruption of VCAM-1 that involves deletion of the α4 binding site result in a reduction of monocyte rolling and adhesion, vessel wall inflammation, and atherogenesis.4,5,11–13 Moreover, inflammation is now recognized as a key component of neointimal formation after stent implantation, sharing many common features with atherogenesis.14–16 For example, we recently demonstrated that transient blockade of VLA-4 with a single infusion of an antibody directed against the α4 integrin subunit at the time of stent implantation results in attenuation of stent neointimal formation on days 3 and 7.17 Hence, addressing the early inflammatory response to stenting may not only help reduce neointimal formation but may potentially diminish the frequency of in-stent restenosis.

Recently, we demonstrated that the potent Na+/H+ exchanger (NHE) inhibitor ethylisopropylamiloride (EIPA) not only attenuates vascular smooth muscle cell (SMC) proliferation and migration but augments SMC apoptosis and antagonizes SMC urokinase plasminogen activator activity.18 Although the NHE is known to play an important role in the regulation of macrophage function, little is known about its potential antiinflammatory properties in the vessel wall.19–21 Therefore, the experiments described herein were designed to determine whether EIPA can also reduce the inflammatory component of lesion formation in atherosclerosis-susceptible
Methods

Three approaches were used to explore the role of EIPA in vascular inflammation. First, in vivo studies involving atherosclerosis-prone mice randomized to systemic treatment with EIPA or placebo were performed to determine the effect of this drug on the early stages of atherogenesis. Second, because the above-described murine studies showed an important reduction in VCAM-1 expression with EIPA therapy, we used in vitro studies to elaborate on the involved mechanisms. Finally, because inflammation is the initial event in stent neointimal formation, an in vivo stent model was used to explore whether stents coated with EIPA would reduce neointimal formation relative to control stents. All murine and rabbit experimental procedures were performed with the approval of the University of Ottawa Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. For a detailed account of the methodologies used in this study, please refer to the online-only Data Supplement.

Results

Murine Model of Atherogenesis

All of the apoE<sup>−/−</sup> mice survived the 4-week experimental protocol, with no difference in body weight noted between the control and drug-treatment groups (28.4±1.7 versus 29.4±1.8 g; respectively; P=0.25). Similarly, the total cholesterol levels of the control and EIPA-treated mice were similar (1756±196 versus 1683±224 mg/dL, respectively; P=0.55). Analysis of 10 high-power fields (HPFs) on peripheral blood smears revealed equal numbers of leukocytes in the control and EIPA groups (18.7±3.3 versus 19.5±0.4, respectively; P=0.2).

The ascending aorta, aortic arch, and descending aorta of the control mice showed widespread early atherosclerotic changes with fatty streaks. En face preparations revealed that these changes were most abundant in the aortic arch and descending aorta of control mice. In contrast, EIPA-treated mice showed 46% less atherosclerotic involvement of the aortic arch and descending aorta (eg, percent en face atherosclerotic lesion area of control 29.8±5.9% versus EIPA 16.1±4.8%; P<0.001; Figure 1). Cross-sectional analyses of the ascending aorta above the level of the coronary arteries demonstrated that macrophages were the predominant cells in these early atherosclerotic lesions (Figure 1). Quantification of cross sections immunolabeled with an anti-mouse macrophage antibody revealed that the percentage macrophage area was 41% reduced in EIPA-treated mice compared with control mice (control 45.8±5.0% versus EIPA 27.2±3.2%; P<0.001). Manual counting of the number of macrophages per square millimeter of ascending aorta vessel wall area revealed a 38% reduction with EIPA treatment versus controls (2944±509 versus 4765±789; P<0.001). With an anti-CD3 antibody, it was determined that T lymphocytes were virtually absent in arterial tissue from both the control and treatment groups (Data Supplement, Figure 1). To determine the frequency of cell proliferation at time of tissue harvesting, immunolabeling with an antibody to the proliferating cell nuclear antigen was used (Data Supplement, Figure 2). No difference was found in the prevalence of intimal proliferating cells in the control and EIPA-treated mice when expressed as either a function of the vessel wall area (mm<sup>2</sup>) or per intimal HPF (28.5±5.1 versus 27.2±4.8, P=0.53 or 1330±452 versus 1147±475, P=0.47, respectively). Moreover, scanning electron microscopy showed that adherent monocytes were abundant on the endoluminal surface of the aortas of control mice but not in the EIPA-treated mice (Figure 2). In control mice, VCAM-1 immunolabeling was commonly observed in the endothelium lining an atherosclerotic lesion, whereas more normal segments of the aorta were immunonegative for VCAM-1. However, the expression of VCAM-1 was markedly attenuated in all aortic areas for the EIPA-treated mice. Indeed, there was a 59% reduction in the number of VCAM-1–immunopositive lumenal endothelial cells in the EIPA-treatment group (P<0.001; Figure 2).

In Vitro Effects of EIPA on Mononuclear Cell Adhesion and VCAM-1 Expression

Treatment of human aortic endothelial cells (HAECs) with lipopolysaccharide (LPS) resulted in an increase in VCAM-1 expression (5 μg/mL of LPS produced a 390% increase in VCAM-1; Figure 3). This effect was independent of cell
number, because the ELISA optical density reading was normalized to the number of cells per well. However, there was a progressive reduction in VCAM-1 expression with increasing concentrations of EIPA, such that 10 and 20 μmol/L of EIPA resulted in 29% and 52% decreases in LPS-induced VCAM-1 expression, respectively. Moreover, Western blots of HAECs stimulated with LPS revealed a 21% reduction in VCAM-1 expression with EIPA treatment versus control \( (P=0.009; \text{Figure 3}) \).

The attenuation in VCAM-1 expression paralleled the change in human monocytic U-937 cell attachment to HAECs. Relative to control conditions, LPS stimulation resulted in a 338% increase in the number of U-937 cells that attached to HAECs. However, compared with peak levels noted after LPS stimulation, EIPA treatment reduced the number of U-937 cells adherent to HAECs per HPF by 37% (control 8±2, LPS 27±4, and LPS plus 20 μmol/L EIPA 17±2 cells/HPF; \( P<0.001; \text{Figure 4} \)).

To determine whether the reduced VCAM-1 expression might be related to inhibition of nuclear translocation of nuclear factor-κB (NF-κB) by EIPA, we used the nuclear translocation of NF-κB as an index of NF-κB activation. HAECs were stimulated with LPS, and the number of cells that demonstrated nuclear translocation of NF-κB was assessed in the presence or absence of EIPA. Compared with LPS group, EIPA produced a 66% reduction in the number of cells that demonstrated nuclear translocation of NF-κB (Figure 5).

Rabbit Drug-Coated Stent Experiment

Mean injury scores on day 28 were similar for the arteries that received either the control or the EIPA-coated stents (1.22±0.40 and 1.32±0.40, respectively; \( P=0.71 \)). Although a modest stent neointima formed in all rabbit arteries, stent neointimal area was 38% less in those rabbits that received the EIPA jelly–coated stent than in those with the control stent (0.61±0.13 versus 0.98±0.15 mm²; \( P=0.01 \)). RAM-11–immunopositive macrophages were found exclusively within 1 HPF (magnification ×400) of vacant tissue areas originally occupied by stent struts (hereafter referred to as the peristrut area, or PSA), where they were intermixed with stellate-shaped SMCs that were immunolabeled with an anti-smooth muscle α-actin antibody (Figure 6). PSA macrophages were more abundant in the control than in the

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**Figure 2.** Scanning electron photomicrographs (A, B; magnification ×1272) of aorta demonstrating adherence of mononuclear cells on surface of control but not EIPA-treated mice. Aortic cross sections showing positive immunolabeling reaction (brown; hematoxylin nuclear counterstain) for VCAM-1 in luminal endothelial cells of control but not EIPA-treated mice (C and D, arrows indicate internal elastic lamina; magnification ×400). Manual counting of number of luminal endothelial cells expressing VCAM-1 revealed 59% reduction in EIPA group relative to controls \( (*P<0.001) \).

**Figure 3.** A, Treatment of HAECs with LPS resulted in concentration-dependent increase in VCAM-1 expression, as assessed by optical density (OD) standardized to number of cells per well. Addition of EIPA resulted in dose-related reduction in abundance of VCAM-1 (eg, 10 and 20 μmol/L of EIPA resulted in 29% and 52% reductions in VCAM-1 expression; \( *P<0.01 \) and \( **P<0.001 \), respectively). B, HAECs stimulated with LPS showed increase in VCAM-1 expression. However, in presence of EIPA, there was 21% reduction in VCAM-1 expression relative to LPS alone \( (*P=0.009) \). All VCAM-1 protein expression normalized to β-actin levels.
EIPA-treated vessels (6.2 ± 4.2 versus 3.3 ± 3.1 cells/HPF; \( P < 0.05 \)). The abundance of SMCs per HPF was also assessed in the non-PSA of the stent neointima. Because the non-PSA consisted solely of smooth muscle \( \alpha \)-actin immunopositive cells, the number of these cells per HPF was manually counted, divided by the area of an HPF, and expressed as SMC density. SMC density was 37% lower in the stent neointima from rabbits treated with EIPA than from control rabbits (3114 ± 747 versus 4966 ± 2727 cells per mm\(^2\); \( P = 0.02 \)). Finally, the frequency of proliferating cells on day 28 after stenting, as determined by anti-bromodeoxyuridine immunopositivity, did not differ significantly between control and EIPA groups (0.21 ± 0.0.08% versus 0.29 ± 0.19%; \( P = 0.48 \)).

Discussion

Although the amiloride derivative EIPA is known to lessen proliferation and migration of SMCs, its potential for reducing the inflammatory component of atherosclerosis and stent neointimal formation was previously unstudied. The data from the present experiments reveal that systemic treatment with EIPA reduces fatty streak formation in the aortas of apoE \(^{-/-}\) mice by mechanisms that involve reduction in the number of macrophages adherent to the endothelium or found in the subendothelial space. This effect was associated with attenuation of aortic endothelial cell VCAM-1 expression. To corroborate the in vivo findings, in vitro studies were performed, which confirmed that

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**Figure 4.** Adhesion of human U-937 mononuclear cells to HAECs was assessed under control conditions (A) and after LPS induction of endothelium either in absence (B) or presence (C) of EIPA (20 \( \mu \)mol/L). U-937 cells showed infrequent adherence to quiescent HAECs (D); however, LPS stimulation yielded 338% increase in U-937 cell adherence (E) that was reduced by 37% when EIPA was added (F). \( * P < 0.001 \). Magnification: A–C, \( \times 100 \); D, \( \times 3036 \); E, \( \times 1500 \); F, \( \times 1000 \).

**Figure 5.** EIPA inhibits nuclear translocation of NF-\( \kappa \)B. Left column shows localization of NF-\( \kappa \)B p65 subunit expression (red reaction product) in HAECs under control (top row), LPS stimulation (middle row), or LPS stimulation plus EIPA (bottom row) conditions. Center column shows nuclear uptake of blue Hoechst 33258 dye, whereas right column is merged product of other 2 columns. Under control conditions, NF-\( \kappa \)B is located in cytoplasm, but with LPS stimulation, it translocates to nucleus. Addition of EIPA largely inhibits LPS-induced translocation of NF-\( \kappa \)B. Magnification \( \times 400 \). Bottom graph shows 66% reduction in number of HAECs that demonstrate NF-\( \kappa \)B nuclear translocation with EIPA treatment vs LPS (\( * P < 0.001 \)).
EIPA reduces LPS-induced VCAM-1 expression and adherence of U-937 cells to HAECs. Moreover, in endothelial cells, EIPA produces a 66% reduction in the nuclear translocation of NF-κB, an event that signals the activation of this key inflammatory transcriptional factor.22 Hence, these data suggest that VCAM-1 expression and mononuclear cell attachment to the endothelium may be indirectly reduced because of EIPA-induced attenuation of NF-κB activation. Finally, using a rabbit stent model of vascular inflammation, we showed that the neointimal area of stents coated with EIPA is smaller than that of control stents. Both PSA macrophage abundance and neointimal SMC density were reduced with EIPA-coated stents compared with controls. Therefore, these results suggest a novel anti-inflammatory role for EIPA in the vessel wall that is not species specific and may act synergistically with its anti-SMC effects.

In the murine model, the 46% reduction in aortic lesion area, 41% decrease in subendothelial percentage of macrophage area, and 38% reduction in macrophage cell number are particularly noteworthy when one considers that the serum cholesterol levels in these mice were ~1700 mg/dL. Indeed, with this degree of hyperlipidemia, one might expect an extraordinary induction of VCAM-1 expression that would be difficult to suppress by any means. Moreover, independent of VCAM-1 expression, serum cholesterol levels in this range might by themselves promote generic inflammatory responses in the vessel wall that can accelerate atherosclerosis. In addition, we observed a reduction in atherosclerotic aortic surface area that was similar to that observed in experiments performed by Cybulsky et al4 with genetically engineered mice that express <10% of VCAM-1 protein. Hence, although we did not have the capability of measuring EIPA levels in these mice, one might speculate there was near-maximal efficacy of EIPA in this experiment. The in vitro data showing a strong reduction in the nuclear translocation of NF-κB with EIPA treatment suggest that reduced VCAM-1 expression may occur via an inhibition of NF-κB. Although NF-κB is one of the most important mediators of inflammation in atherogenesis, it normally exists in an inactive form in the cytoplasm. Once activated, NF-κB translocates to the nucleus, where it regulates the expression of several key molecules that are involved in vascular inflammation (eg, VCAM-1, monocyte chemotactic protein-1, and interleukin-6).22 Although NHE activation is known to be an integral component of inflammatory responses and we now show that NHE inhibition by EIPA suppresses production of the key inflammatory mediator NF-κB, exactly how NHE inhibition attenuates NF-κB activation remains to be determined (eg, whether this is related to changes in intracellular pH secondary to NHE inhibition or alterations in degradation of IkB, the inhibitor of NF-κB).19–21,23

Given the attenuation of inflammatory cell attachment by EIPA in the murine and in vitro experiments, we sought to determine whether this effect would be beneficial in reducing the inflammatory response to stent implantation. Although we recently demonstrated that a single dose of an antibody to the α4 integrin subunit was effective in antagonizing VLA-4 and the transmigration of inflammatory cells into stent neointima on days 3 and 7, we discovered that these effects were lost by day 28 because of a “catch-up” phenomenon in neointimal formation that coincided with a progressive accumulation of inflammatory cells.17 Hence, to optimize delivery of an antiinflammatory agent into stented arteries, we proposed a drug-coated stent strategy. The magnitude of the lesion reduction with the EIPA-coated stents parallels that of the antiinflammatory effects observed in the rabbit stent model of vascular inflammation. Unfortunately, technical factors prevented us from determining whether the attenuation of stent neointimal area with EIPA-coated stents occurred in conjunction with a reduction in VCAM-1 expression, because immunolabeling for VCAM-1 in formalin-fixed rabbit stent neointima specimens was unsuccessful. However, in separate experiments involving cryopreserved rabbit iliac arterial tissue, we know that VCAM-1 is expressed in reendothelialized arteries 2 weeks after balloon injury (unpublished data, E.O.B. and X.M., 2004). Hence, we presume that the anti-inflammatory effects observed in the rabbit stent model share common mechanisms with the murine in vivo and human in vitro data and result from a reduction in mononuclear cell attachment to the stented segments as a result of diminished VCAM-1 expression.
Moreover, because we also noted a reduction in SMC density in the neointima of EIPA stents, we suspect that this was due to the previously documented antiproliferative, antiinflammatory and pro-apoptotic effects of EIPA that may have occurred before day 28.\textsuperscript{18}

The present results with EIPA-coated stents share similarities with preclinical studies performed with sirolimus- and paclitaxel-eluting stents. For example, using the same rabbit model, Klugherz and colleagues\textsuperscript{24} showed a 45% reduction in stent neointimal area 28 days after implantation of sirolimus-coated stents. Likewise, a preclinical rabbit paclitaxel-coated stent study demonstrated a 48% decrease in stent neointimal thickness.\textsuperscript{25} Interestingly, the dose of EIPA per stent used in the present study is approximately one sixth the dose of sirolimus and three fourths the dose of paclitaxel used in their respective preclinical studies. Subsequent clinical studies using stents coated with similar doses of sirolimus or paclitaxel demonstrate frequencies of in-stent restenosis that were previously thought to be unobtainable.\textsuperscript{26,27} One concern regarding the long-term success rate of drug-eluting stents is the potential for a late catch-up phenomenon that may, in effect, only postpone and not abolish the onset of in-stent restenosis. For example, a preclinical paclitaxel-eluting stent study demonstrated that although paclitaxel-eluting stents had thinner neointimas at 4 weeks, there was no difference from control stents at 90 days.\textsuperscript{25} One explanation for this was a hyperproliferative neointima in the paclitaxel-coated stents at 4 weeks. Although more long-term studies with EIPA-coated stents are required, it is important to note that on day 28, the neointima of EIPA stents has a very low frequency of proliferating cells that is indistinguishable from that of control stents.

In summary, the experiments in the present study describe provocative antiinflammatory properties of EIPA that may attenuate atherogenesis and the neointimal response to stent implantation. In vivo studies testing the role of EIPA in the secondary prevention (or even regression) of established lesions are indicated. Moreover, studies with more sophisticated polymeric formulations of EIPA loaded onto stents are needed, including those that involve combinations of EIPA with either sirolimus or paclitaxel to assess potential synergistic effects of multidrug-eluting stents. Given that we recently demonstrated that the pathobiology of human in-stent restenosis lesions is complex and includes the involvement of c-kit-immunopositive vascular progenitor cells, antiinflammatory strategies such as those involving EIPA may prove crucial in preventing later stages of lesion development.\textsuperscript{28}

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