Effects of Bradykinin on Aortic Endothelial Function in ApoE-Knockout Mice With Chronic Chlamydia Pneumoniae Infection

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Background Impaired muscarinic receptor-mediated vasodilation is an important feature of early atherosclerosis. Earlier studies on apolipoprotein E-knockout mice (apoE-KO) mice suggested adverse effects of Chlamydia pneumoniae infection on the endothelial vasomotor responses of aortas to the muscarinic agonist methacholine. Using additional aorta samples the present study investigated the responses to bradykinin.

Methods and Results ApoE-KO mice were repeatedly inoculated with either Chlamydia pneumoniae (*C. pneumoniae*) or saline. At 2, 6, and 10 weeks after the first inoculation, precontracted aorta rings from both groups were exposed to bradykinin in the absence and presence of L-NAME and diclofenac. In noninfected animals, the vasomotor responses to bradykinin were similar at all timepoints ($p>0.5$). Compared with noninfected animals, the responses in infected animals tended to increase through the study period ($p<0.05$ at 10 weeks). Although diclofenac and L-NAME had no effect in noninfected mice, they inhibited the responses to bradykinin in infected mice at 6 and, more markedly, 10 weeks ($p<0.05$ for both).

Conclusion Bradykinin stimulation of aorta endothelium from *C. pneumoniae*-infected apoE-KO animals appears to activate compensatory kinin receptor-related mechanisms that could involve nitric oxide and vasorelaxing prostanoids. Although the precise molecular mechanisms require further investigation, one could speculate that strategies increasing bradykinin availability might reverse the arterial dysfunction during chronic infectious disease. (*Circ J* 2007; 71: 1480–1484)

Key Words: Bradykinin; Endothelium; Infection; Relaxation

There is evidence suggesting that infection could contribute to the development of atherosclerosis.^{1–3} Of infectious pathogens, Chlamydia pneumoniae (*C. pneumoniae*) has been most often associated with atherosclerosis and its clinical complications.^{4–6} Damage to the vascular endothelium has been hypothesized to underlie in part the putative link between infection and atherosclerosis.^{7–9} We have earlier demonstrated that infection of apolipoprotein E-knockout mice (apoE-KO) with *C. pneumoniae* leads to arterial endothelial dysfunction, as illustrated by impaired endothelium-dependent relaxation of aortas in response to the muscarinic agonist methacholine.¹⁰ The impaired vasomotor response to muscarinic agonists is generally regarded as a hallmark of early atherosclerosis.

A particular phenotype of vascular endothelial dysfunction has been detected in humans with cardiovascular risk factors and without signs of advanced atherosclerosis, being characterized by an impaired muscarinic-mediated vasodilator response associated with normal^{11–13} or even enhanced¹⁴ responses to bradykinin, another endothelium-dependent vasodilator. Although the mechanisms of this discrepancy

in the vasomotor responses to muscarinic and kinin receptor agonists remain elusive, this observation is in keeping with the hypothesis that the kinin pathway could be used as a pharmacological target in order to improve the endothelial function, and, thereby, to retard the development of atherosclerosis.^{15,16}

To date, there are no data connecting the endothelial kinin pathway with chronic *C. pneumoniae* infection, so we investigated the bradykinin-induced vasomotor responses in relation to *C. pneumoniae* infection in apoE-KO mice. Additional rings from thoracic aorta segments that had been tested with methacholine¹⁰ were exposed to bradykinin in the present study. The apoE-KO mouse develops atherosclerosis in a similar fashion to humans,¹⁷ and has, therefore, been increasingly used in the past decade for studying the pathogenesis of atherosclerosis in relation to several pathological conditions, including infection.^{18–20}

Methods

Material and Study Design

Forty-eight apoE-KO mice were purchased from Jackson Laboratories, and fed a regular mouse chow containing 4.5% crude fat. At the age of 8 weeks, half of the mice were inoculated intranasally with either *C. pneumoniae* (strain IOL-207; 400,000 infection forming units/mouse/inoculation) or saline. Additional 3 inoculations were carried out at 10, 12, and 14 weeks of age. Prior to inoculation, the mice were sedated with Avertin (0.1 ml/10 g).

Four to eight mice from both infected and noninfected

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Fig 1. Relaxation curves (A) and maximal relaxations (B) of aortas from noninfected and *Chlamydia pneumoniae*-infected apolipoprotein E-knockout mice (apoE-KO) mice in response to bradykinin at 2 weeks, 6 weeks, and 10 weeks, respectively. Data are expressed as mean $\pm$ SEM ($n=4-8$); * $p<0.05$ vs noninfected.

groups were killed at 2, 6 and 10 weeks after the first inoculation. Prior to this, mice were anaesthetized with sodium pentobarbital (0.2mg/g body weight, ip). This study was conducted in accordance to the local ethics committee guidelines for animal research.

In Vitro Study of Vascular Relaxation Response to Bradykinin

The thoracic aorta was carefully removed, and placed immediately in fresh Krebs buffer. The vessel was then carefully cleaned of adjacent fatty tissue and cut into 3-mm rings. The aortic rings were suspended by 2 stainless steel hooks in organ chambers containing Krebs solution at 37°C, bubbled with a mixture of 95%O₂ and 5%CO₂. The arterial smooth muscle tone was measured with a force-displacement transducer connected to a Grass polygraph (Model 7D, Grass Instrument Co, Quincy, MA, USA). The aortic rings were exposed to noradrenaline (0.1 mol/L) in order to obtain a 70% submaximal contraction, and, after reaching a plateau level, relaxed by cumulative addition of sodium nitroprusside (SNP) and bradykinin (10⁻⁹ to 10⁻⁵ mol/L) in the absence and presence of 100 μ mol/L N^G-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthesis) or 10 μ mol/L diclofenac (an inhibitor of prostaglandin synthesis). These drugs were dropped into the organ bath 30 min before the administration of noradrenaline.

Drugs

All drugs were obtained from Sigma Chemical Co (St Louis, MO, USA). All concentrations refer to final concentrations in the organ bath.

Statistical Analyses

The relaxation responses to bradykinin are expressed as percent changes from the pre-contraction levels. Differences between the infected and noninfected mice were calculated by 2-way ANOVA for repeated measures followed by Newman-Keuls test. Statistical significance was accepted at $p<0.05$ level. All data are expressed as mean $\pm$ SEM; n indicates the number of animals.

Results

Aortic Relaxation Responses to Bradykinin and SNP

In both infected and noninfected animals, bradykinin caused relaxation of the aorta rings in a concentration-dependent fashion (Fig 1A). For the sake of clarity, the standard errors for maximal relaxation responses are illustrated in Fig 1B. As expected, and conforming to the methacholine-induced relaxation responses observed in our previous studies,¹⁰ there was no difference in the responses of aortas from noninfected mice at 2, 6, and 10 weeks (50 $\pm$ 11%, 56 $\pm$ 9%, and 51 $\pm$ 16%, respectively). As compared with the responses from noninfected animals, the maximal relaxation responses of aortas from infected animals showed a paradoxical trend toward an increase from 2 to 10 weeks, with a significant difference at 10 weeks (82 $\pm$ 12%, $p<0.05$, Fig 1B).

Similarly, the threshold-relaxing concentration (10⁻⁸ mol/L) of bradykinin, which had only minimal or no effect in noninfected animals (Fig 1A, Left panel), slightly relaxed the aortas from infected mice at 2 and 6 weeks (22 $\pm$ 6% and 26 $\pm$ 8%, respectively), and caused a significant increase in the relaxation response in the infected animals at 10 weeks (65 $\pm$ 13%, $p<0.05$, Figs 1A (Right panel), 2B).

Fig2. Effects of inhibition of prostaglandin and nitric oxide (NO) synthesis on the relaxation response to bradykinin in *Chlamydia pneumoniae*-infected apolipoprotein E-knockout mice (apoE-KO) mice at 2 weeks, 6 weeks, and 10: (A) cumulative addition and (B) 10^{-8} mol/L. Data obtained without pretreatment (solvent, S), and in the presence of L-NAME (L) or diclofenac (D). Data are expressed as mean $\pm$ SEM ($n=5-8$); * $p<0.05$, and ** $p<0.01$ vs solvent at 10 weeks.

Fig3. Maximal relaxation responses to methacholine at 2, 6 and 10 weeks in noninfected and *Chlamydia pneumoniae*-infected apolipoprotein E-knockout mice. Data are expressed as mean $\pm$ SEM ($n=5-8$); * $p<0.05$.

The relaxation responses to SNP were similar in both groups at each time point (2 weeks: $p=0.5$; 6 weeks: $p=0.4$; 10 weeks: $p=0.5$).

Effects of L-NAME and Diclofenac on Bradykinin-Induced Relaxation

Inhibition of either NO synthesis by L-NAME or prostaglandin synthesis by diclofenac, or the combination of these 2 had no significant effect on the bradykinin-induced relaxation of aortas from noninfected animals (data not shown). The relaxation responses to bradykinin were not significantly modified by these 2 inhibitors in infected animals at 2 and 6 weeks (Figs 2A,B). Similarly, no significant effect was obtained with combined pre-treatment (data not shown). However, in infected mice at 10 weeks, L-NAME and diclofenac decreased the relaxation to the threshold-relaxing concentration (10^{-8} mol/L) by approximately 30% ($p<0.05$) and 50% ($p<0.01$), respectively (Fig 2B). The maximal responses were also inhibited by these drugs, with a significant difference for diclofenac ($56\pm10\%$, $p<0.05$). At this time-point, when used together, L-NAME and diclofenac had a slightly greater, yet statistically nonsignificant inhibitory effect compared with that caused by diclofenac alone ($p=0.2$).

Effects of B2-Receptor Antagonist Hoe-140 on Bradykinin-Induced Relaxation

Pretreatment of aortas with Hoe-140 abolished the responses to bradykinin in all animals at each time-point (mean value of maximal relaxation: 0–10%) with no significant difference between the groups ($p>0.5$ at each time-point).

Discussion

Previous studies from our laboratory¹⁰ have shown that chronic *C. pneumoniae* infection of apoE-KO mice may cause injury to the aortic endothelium, as indicated by the impaired aortic relaxation responses to the muscarinic agonist methacholine (Fig 3). In the same animal model, infection with *C. pneumoniae* caused lipid accumulation and inflammatory changes in the aorta wall.¹⁹

Using additional segments from the aortas previously tested with methacholine, the findings of the present study indicate that the responses to bradykinin are not altered in the early phase of chronic infection with *C. pneumoniae*. Moreover, the augmented relaxation responses to bradykinin in the later stages of infection suggest that active regulatory mechanisms in response to bradykinin stimulation could be responsible for the observed differences. Although pretreatment with the bradykinin B2-receptor antagonist Hoe140 abolished the relaxation response to bradykinin in infected animals, the contribution of vascular endothelial kinin B1 receptors, which may be upregulated by infections and inflammation,²¹ might be among the underlying mechanisms.

Earlier studies on vascular physiology in relation to various risk factors for atherosclerosis have suggested a discrepancy between the vasomotor responses to muscarinic agonists and those elicited by bradykinin. Indeed, in the majority of studies, impaired arterial vasomotor responses to muscarinic receptor stimulation were documented, whereas the responses to bradykinin were shown to be preserved^{11–13} or even enhanced.¹⁴ It has therefore been speculated that the endothelial injury in atherosclerosis is receptor-specific, being confined at least in the early stages to muscarinic receptors. The mechanisms proposed to explain these findings have primarily depicted 2 possible scenarios: either the kinin receptor pathway is spared in the early stages, or regulatory kinin-mediated, endothelium-dependent mechanisms are set in response to the injurious stimuli. Such mechanisms might involve kinin-mediated upregulation of endothelial pathways such as increased synthesis of cyclooxygenase (COX)-dependent relaxant prostaglandins.²²

Although the arterial relaxation responses to methacholine are largely mediated by NO, the relaxation to bradykinin might in part involve both NO and prostacyclin, a COX product with dilatory properties.¹⁶ However, in the present study, blocking of either NO synthesis or COX activity by L-NAME and diclofenac, respectively, did not have a consistent inhibitory effect on the bradykinin-induced relaxation in the noninfected apoE-KO mice. This suggests that at least in this animal model a non-NO, non-prostanoid factor, possibly endothelial-derived hyperpolarizing factor, is mainly responsible for the relaxant effects of bradykinin. In contrast, pretreatment of aortas from infected animals with L-NAME or diclofenac inhibited the relaxation responses to bradykinin, suggesting that increased production of both NO and relaxing prostaglandins, most likely prostacyclin, are responsible for the augmented

relaxation to bradykinin in the infected animals. Because the amounts of the COX and NOS enzyme proteins did not differ between infected and noninfected animals,¹⁰ it is possible that the activity of both the NOS and COX pathways could have been upregulated by bradykinin in infected animals, with a composite result of increased availability of NO and relaxing prostaglandins.

Bradykinin could improve the bioactivity of NO by serving as a substrate to NOS because of the L-arginine incorporated in its structure;²³ and by its interaction with tetrahydrobiopterin;²⁴ the coenzyme implicated in the NO synthesis. This results in a better coupling of L-arginine and NADPH oxidation, leading to improved bioactivity of NO. These bradykinin-induced mechanisms may be important in the setting of vascular pathologies, including atherosclerosis, in which the levels of both L-arginine and tetrahydropterin are suboptimal.^{25,26} Of note, L-arginine was shown to improve the coronary reactivity in swine with *C. pneumoniae* infection following repeated inoculations with this pathogen;²⁷ a finding that supports the hypothesis that decreased availability of endothelial L-arginine could be associated with this infection. The improvement in the NOS pathway by bradykinin could in turn influence the COX pathway, probably in part through a complex cross-talk between the NOS and COX enzymes;²⁸ with a subsequent increase in the release of relaxant prostaglandin.

Under normal circumstances, bradykinin contributes to regulation of both the resting tone and flow-mediated dilatation of arteries, and exerts important antiatherogenic effects through NO and prostacyclin.^{29,30} Of note, angiotensin-converting enzyme (ACE) inhibitors improve vascular endothelial function in various pathological conditions associated with atherosclerosis, in part through increased availability of bradykinin.^{31,32} Accumulating data from both experimental studies and clinical trials suggest that ACE inhibitors may retard the development of atherosclerosis and its manifestations.^{18,33,34} Based on the present findings, one might speculate that similar benefit could be attained by ACE inhibitors on *C. pneumoniae*-induced endothelial dysfunction and, perhaps, on the development of atherosclerosis with an infectious basis.

In conclusion, bradykinin stimulation of the endothelium of aortas from *C. pneumoniae*-infected apoE-KO animals appears to activate compensatory kinin receptor-related mechanisms that could involve NO and vasorelaxing prostanoids. Additional studies are needed to further investigate the molecular pathways, and, in particular, to verify whether similar effects may occur in other animal models.

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