Endothelial Dysfunction After Repeated *Chlamydia pneumoniae* Infection in Apolipoprotein E–Knockout Mice

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**Background**—Arterial relaxation is largely regulated by endothelial nitric oxide (NO). Its diminished activity has been associated with incipient atherosclerosis. We investigated the endothelium-dependent relaxation of aorta in apolipoprotein E–knockout (apoE-KO) mice exposed to single or repeated *Chlamydia pneumoniae* inoculation.

**Methods and Results**—Forty-eight apoE-KO mice, 8 weeks old, were inoculated intranasally with *C pneumoniae (n = 24)* or saline (*n = 24*) every 2 weeks over a 6-week period. Twenty mice (10 infected and 10 controls) were killed at 2 weeks and 6 weeks, respectively, after the first inoculation. The smooth muscle tone of aortic rings was measured in vitro at both time points. The norepinephrine-precontracted thoracic aortic rings were successively exposed to methacholine in the absence and presence of *N*-nitro-L-arginine methyl ester (L-NAME) and diclofenac. The methacholine-induced relaxation was attenuated in the infected mice at 6 weeks in both the absence and presence of L-NAME (*P*<0.05 and *P*<0.01, respectively). When administered together with L-NAME, diclofenac enhanced the relaxation of the L-NAME–pretreated aortas in infected mice at 2 weeks (*P*<0.05) but not in noninfected mice. The relaxation response from infected mice tended to differ in the same manner at 6 weeks (*P*<0.1). No intimal thickening was detected at either time point.

**Conclusions**—*C pneumoniae* impairs arterial endothelial function, and the NO pathway is principally involved. Cyclooxygenase-dependent vasoconstricting products may also account for the infection-induced impaired relaxation. These findings further support the role of *C pneumoniae* infection in atherosclerosis development. (*Circulation. 2000;102:1039-1044.*)

**Key Words:** endothelium ■ *Chlamydia pneumoniae* ■ nitric oxide ■ vasoconstriction

In increasing evidence suggests the participation of chronic *Chlamydia pneumoniae* infection in the pathogenesis of atherosclerosis.1–4 The atherogenicity of *C pneumoniae* may be related to its ability to cause local infection and inflammation of the arterial wall, consisting mainly of monocytes and T lymphocytes,5,6 which precede intimal migration of smooth muscle cells and foam cell formation, the hallmark for the development of atherosclerosis. These events are associated in atherogenesis with an abnormal function of or even damage to the arterial endothelium, consisting of decreased availability of vasodilating mediators such as endothelium-derived nitric oxide (NO) or increased production of vasoconstricting factors.7–10

The endothelium has a central role in the regulation of arterial tone,11–13 the control of vascular cell proliferation and platelet function, and the expression of adhesion molecules on the endothelial cell surface, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).14–16 These properties are mediated at least in part by endothelial NO.17 Muscarinic receptor stimulation of the normal coronary arteries produces vasodilatation via endothelial NO release.18 Atherosclerotic or preatherosclerotic coronary arteries display impaired vasodilatation or even vasoconstriction in response to muscarinic agonists.19 These abnormal responses are attributed mainly to decreased availability of endothelium-derived NO, but other endothelial products, such as cyclooxygenase (COX)-dependent vasoconstricting factors, may also be implicated.19,20 Interestingly, the COX inhibitor aspirin improved the endothelium-dependent relaxation of coronary arteries in humans at risk for atherosclerosis.20

Because of its similarity in distribution and appearance to human atherosclerosis, as well as high susceptibility to *C pneumoniae* infection, the apolipoprotein E–knockout (apoE-KO) mouse is considered an ideal model for studying the relationship between *C pneumoniae* infection and atheroscle-
In vitro studies on arterial rings from apoE-KO mice showed that endothelium-dependent relaxation in response to acetylcholine is mediated largely by NO. To the best of our knowledge, the direct relationship between C pneumoniae infection and endothelium-dependent relaxation has not been studied until the present study. To this end, the relaxation responses of thoracic aortic rings to methacholine, a muscarinic receptor agonist, were investigated in apoE-KO mice infected with C pneumoniae and fed a standard chow diet. Moreover, additional morphological and immunohistochemical studies were performed to give more insight into the ability of C pneumoniae infection to influence the endothelium-dependent arterial relaxation in these mice.

Methods

Material and Study Design

Forty-eight homozygous apoE-KO mice (purchased from Jackson Laboratories, Bar Harbor, Me) and 3 wild-type (C57BL/6) mice, 8 weeks old, were taken into the study and fed a regular mouse chow (4.5% crude fat) ad libitum. Twenty-four apoE-KO mice were inoculated intranasally with C pneumoniae strain IOL-207 every 2 weeks (400 000 infectious units per mouse per inoculation) over a 6-week period. The rest of the mice were sham-inoculated with PBS. Before inoculation, the mice were sedated with Avertin (0.1 mL/10 g). This study strictly followed the local ethics committee guidelines.

Twenty apoE-KO mice (10 infected and 10 noninfected) were euthanized at 2 weeks and 6 weeks, respectively, after the first inoculation. Eight apoE-KO mice (4 infected and 4 noninfected) and 3 noninfected C57BL/6 were euthanized at 10 weeks after the first inoculation. On each occasion, the mice were anesthetized with sodium pentobarbital (0.2 mg/g body wt IP), and blood was collected in Tris buffer containing 5% BSA (TBS/BSA). Slides were incubated with relevant monoclonal or polyclonal antibodies at room temperature for 1 hour, washed in TBS, and incubated with second antibody absorbed with normal mouse serum (1:250 goat anti-rabbit IgG-biotin [Dako] or rabbit anti-goat IgG-biotin [Biogenex]) at room temperature for 30 minutes. Binding was revealed with alkaline-phosphatase–conjugated streptavidin (Biogenex) diluted 1:2 for 30 minutes at room temperature. Rat monoclonal antibodies were revealed with 1:20 anti–rat alkaline phosphatase (Binding-site). The sections were then reacted for 10 minutes in Fast Red substrate mixture (Dako) to reveal alkaline phosphatase activity. The sections were washed in running water, counterstained with hemalun for 3 minutes, rinsed, and mounted with Glycergel (Dako). Negative control consisted of omission of the primary antibody. The slides were then examined under light microscopy.

Serology and Polymerase Chain Reaction for C pneumoniae

C pneumoniae antibodies were determined by microimmunofluorescence at 2 and 6 weeks after the first inoculation. Serum anti–C pneumoniae IgG antibodies were measured.

Lung and ascending aortic specimens of 3 to 4 apoE-KO mice from each group were also investigated for C pneumoniae DNA by polymerase chain reaction (PCR) at 2 and 6 weeks, respectively.

In Vitro Study of Aortic Endothelial Function

Mice killed at 2 and 6 weeks (n=6 to 10 on each occasion) were included in this investigation. The thoracic aorta was removed and immediately placed in oxygenated Krebs buffer. The connective tissue adjacent to the adventitia of the thoracic aorta was carefully removed, and the specimens were cut into 2 rings of 3 mm each. The aortic rings were suspended by means of 2 stainless steel hooks in an organ bath 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). The aortic rings were exposed to norepinephrine (0.1 mol/L) to obtain a 70% submaximal contraction and then relaxed with increasing concentrations of methacholine in the absence and in the presence of N⁶-nitro-L-arginine methyl ester (L-NAME). The effect of diclofenac pretreatment was studied alone and in combination with L-NAME. These drugs were given 30 minutes before the administration of norepinephrine.

Drugs

All drugs were obtained from Sigma Chemical Co. L-NAME was used as a nitric oxide synthase (NOS) inhibitor and diclofenac as a COX inhibitor. All concentrations represent final concentrations in the organ bath.

Statistical Analysis

Differences between the infected and noninfected mice were studied with 2-way ANOVA for repeated measures. Statistical significance was accepted at the P<.05 level. All data are expressed as a percentage of maximal relaxation to methacholine (mean±SEM).

Immunohistochemistry

A total of 11 thoracic aorta samples were obtained from 8 apoE-KO mice (4 noninfected and 4 infected) and 3 C57BL/6 mice killed at 10 weeks. Samples were snap-frozen in liquid nitrogen and stored at −70°C. Serial 4-μm sections were mounted on Silane-coated slides, fixed, and washed in Tris buffer containing 5% BSA (TBS/BSA). Slides were incubated with relevant monoclonal or polyclonal antibodies at room temperature for 1 hour, washed in TBS, and incubated with second antibody absorbed with normal mouse serum (1:250 goat anti-rabbit IgG-biotin [Dako] or rabbit anti-goat IgG-biotin [Biogenex]) at room temperature for 30 minutes. Binding was revealed with alkaline-phosphatase–conjugated streptavidin (Biogenex) diluted 1:2 for 30 minutes at room temperature. Rat monoclonal antibodies were revealed with 1:20 anti–rat alkaline phosphatase (Binding-site). The sections were then reacted for 10 minutes in Fast Red substrate mixture (Dako) to reveal alkaline phosphatase activity. The sections were washed in running water, counterstained with hemalun for 3 minutes, rinsed, and mounted with Glycergel (Dako). Negative control consisted of omission of the primary antibody. The slides were then examined under light microscopy.

Antibodies

Rat anti-mouse ICAM-1 and VCAM-1 (monoclonal, 1:100) were obtained from R&D Systems. Rabbit anti-iNOS (1:500) and rabbit anti-pNOS (1:200) were obtained from Calbiochem and Translab, respectively. Goat anti–COX-1 (1:100) and –COX-2 (1:200) were obtained from Santa Cruz.

Morphometry and Histology

Morphometry/histology was performed at 2 and 6 weeks from all animals tested for endothelial function. Early atherosclerotic lesions are located along the inner curvature of the aortic arch and ascending aorta, and therefore, samples were taken from these segments. After the removal of the descending thoracic aorta, the heart and the proximal aorta (ascending aorta and aortic arch) were perfused with 4% paraformaldehyde for 3 minutes via the left ventricle in situ and placed in formalin. Cross sections 5 μm thick were cut from each specimen, 2 from the ascending aorta (1 mm distal to the aortic valves and 1 mm proximal to the first aortic bifurcation) and 2 from the aortic arch (at one and two thirds of the distance between the origins of innominate artery and left carotid artery). The cross sections were stained with Verhoeff–van Gieson’s stain. Morphometric evaluation of the intima and media size was performed with an image analyzer, as described elsewhere.

Results

Clinical Status of the C pneumoniae–Infected Mice

The C pneumoniae–inoculated mice developed mild symptoms consisting of naso-ocular discharge, irritability or somnolence, and tachypnea. These signs were observed on the second day after the inoculation and disappeared within a week. The second and third C pneumoniae inoculations resulted in similar symptoms.

Serology and PCR for C pneumoniae

All infected animals showed IgG antibodies to C pneumoniae as early as at 2 weeks after the first inoculation (IgG >1/64). After repeated inoculations, the IgG antibody titers increased at 6 weeks. No C pneumoniae antibodies were detected in noninfected animals.
C pneumoniae DNA was detected by PCR in all lung specimens at 2 weeks and at 6 weeks. The ascending aortic specimens were only intermittently positive at both time points.

Endothelial Function of the Thoracic Aorta

The values of maximal relaxations and EC₅₀ are presented for all experiments in Table 1.

Effect of the Muscarinic Receptor Agonist Methacholine

In the absence of inhibitors, methacholine induced a concentration-dependent relaxation of the aortic rings in both infected and noninfected mice (Figures 1A and 1B and 2A through 2D). At 2 weeks, the methacholine-induced maximal relaxation response showed a trend toward being less in infected than in noninfected mice (Figure 1A). At 6 weeks, the aortas from the infected mice relaxed significantly less than those from noninfected mice (P<0.05; Figure 1B).

Effect of the COX Inhibitor Diclofenac

In the absence of NOS inhibition, the incubation of aortic rings with diclofenac did not change the relaxation response to methacholine at 2 or 6 weeks, independent of the presence or absence of infection (Figure 2A through 2D).

Effect of the NOS Inhibitor L-NAME

The methacholine-induced relaxation was significantly attenuated by L-NAME pretreatment at 2 and 6 weeks in both infected (Figure 2A and 2B) and noninfected (Figure 2C and 2D) mice. At 6 weeks, the aortic rings from the infected mice relaxed significantly less than those from the noninfected mice (P<0.01; Figure 1B), whereas the difference was not significant at 2 weeks (P<0.1; Figure 1A).

Combined Effect of L-NAME and Diclofenac

In noninfected mice, addition of diclofenac to L-NAME did not alter the relaxation responses compared with L-NAME alone (Figure 2C and 2D). In contrast, similar pretreatment of aortas from infected mice at 2 weeks enhanced the relaxation response compared with L-NAME alone (P<0.05; Figure 2A). At 6 weeks, relaxation was also enhanced in infected mice (maximum relaxation increased from 5% to 22%, see Table 1), but the difference was not significant (P<0.1; Figure 2B).

Immunohistochemistry

The immunohistochemical stainings of ICAM-1, VCAM-1, endothelial constitutive NOS (NOS III)/iNOS, and COX enzymes in the thoracic aortic wall are semiquantitatively expressed by grading of positivity by animal (Table 2). ICAM-1 was intensely expressed by endothelial cells in all the samples, which attests to the integrity of these cells in both noninfected and infected apoE-KO mice. VCAM-1 was detected on endothelial cells and also within the arterial wall in similar proportions in infected and noninfected mice. Of note, samples from C57BL/6 mice did not show any staining for VCAM-1. The VCAM-1 findings confirm the susceptibility of apoE-KO mice to develop atherosclerosis.26 Induc-

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### Table 1. Mean Maximal Relaxation Percentages at 2 and 6 Weeks After First Inoculation and EC₅₀ Values

<table>
<thead>
<tr>
<th></th>
<th>Maximal Relaxation, %</th>
<th>EC₅₀, log mmol/L</th>
<th>Maximal Relaxation, %</th>
<th>EC₅₀, log mmol/L</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Noninfected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>76±5</td>
<td>−5.92±0.27</td>
<td>69±8</td>
<td>−6.12±0.39</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>65±9</td>
<td>−6.25±0.47</td>
<td>76±5</td>
<td>−6.34±0.36</td>
</tr>
<tr>
<td>L-NAME</td>
<td>50±10</td>
<td>−5.98±0.45</td>
<td>36±12</td>
<td>−5.35±0.47</td>
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<tr>
<td>Diclofenac + L-NAME</td>
<td>56±13</td>
<td>−6.59±0.19</td>
<td>60±10</td>
<td>−6.20±0.42</td>
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<tr>
<td>6 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>79±4</td>
<td>−6.07±0.29</td>
<td>60±7</td>
<td>−5.55±0.28</td>
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<tr>
<td>Diclofenac</td>
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<td>63±10</td>
<td>−6.15±0.24</td>
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<tr>
<td>L-NAME</td>
<td>33±4</td>
<td>NA</td>
<td>5±10</td>
<td>NA</td>
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<tr>
<td>Diclofenac + L-NAME</td>
<td>37±8</td>
<td>−4.89±0.10</td>
<td>22±14</td>
<td>−5.00</td>
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</table>

NA indicates not assessable. n=6 to 10 animals. Values are mean±SEM.

Figure 1. Relaxation of thoracic aorta to methacholine after pretreatment with solvent or L-NAME. Open symbols denote noninfected and solid symbols infected animals. A, Changes at 2 weeks and B, changes at 6 weeks after first inoculation. Probability values represent statistical difference between infected and noninfected groups. For clarity, standard errors are not shown but are presented in Table 1.
NOS was slightly and focally expressed in apoE-KO mice but not in wild-type mice. NOS III was similarly expressed in all the mouse samples. Finally, this semiquantitative rendering of the immunohistochemical results did not show a difference in the expression of COX-1 and COX-2 between infected and noninfected mice.

**TABLE 2. Immunohistochemistry of Thoracic Aorta**

<table>
<thead>
<tr>
<th>ApoE-KO</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>NOS III</th>
<th>COX-1</th>
<th>COX-2</th>
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<tbody>
<tr>
<td>Noninfected</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Infected</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Noninfected</td>
<td>+++</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Infected</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Noninfected</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Infected</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Noninfected</td>
<td>+++</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Infected</td>
<td>+++</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

C57BL/6

1. +++ + + +++ +
2. +++ + + +++ +
3. +++ + + +++ +

Table shows grading of positivity in 4 infected and 4 noninfected apoE-KO and 3 wild-type (C57BL/6) mice at 10 weeks. – indicates negative; (+), rare positive cells; +, ++, and ++++ grades of positivity.

**Morphometry and Histology of the Aorta**

No intimal thickening was detected along the thoracic aorta (ascending aorta and aortic arch) in either noninfected or infected mice (Figure 3).

**Discussion**

Repeated inoculation with *C pneumoniae* significantly impaired the aortic relaxation in response to methacholine in apoE-KO mice. Muscarinic receptor agonists, such as methacholine and acetylcholine, cause endothelium-dependent re-
laxation, which is mediated largely by endothelial NO.18,27 This also holds true for genetically altered hypercholesterolemic apoE-KO mice24 and was reconfirmed in the present study by the significantly diminished muscarinic relaxation in the presence of L-NAME, an inhibitor of NO synthesis, in all apoE-KO mice tested. On normal chow diet, apoE-KO mice display a normal muscarinic relaxation during the first 20 weeks of age, despite their hypercholesterolemia.24,28 On the basis of this previous finding, the noninfected apoE-KO mice used in the present study had no impairment of endothelial function at either 10 or 14 weeks of age. We therefore conclude that the impaired endothelium-dependent relaxation observed in the present study could be attributed to C pneumoniae infection.

The impaired relaxation of the infected mouse aorta in response to methacholine indicates an impaired availability of NO. The availability of the endothelial NO is determined by the balance between production and breakdown and is reflected by the level of arterial relaxation induced by muscarinic agonists.17,18,27,29 The more impaired aortic relaxation in the infected animals than in noninfected animals in the presence of L-NAME suggests that the impaired NO availability could be a net result from increased NO production counterbalanced by even more increased NO degradation. The concept of increased NO formation is supported by the observation that proinflammatory cytokines produced by exposure to Gram-negative bacterial lipopolysaccharides or C pneumoniae infection are associated with increased activity of NOS III and/or expression of iNOS.30,31 However, we did not see any difference in the immunostaining for iNOS of thoracic aorta between infected and noninfected animals. The positive staining for endothelial iNOS in apoE-KO mice but not in control (C57BL/6) mice is probably a consequence of their hyperlipidemia.32,33 Reduction in the endothelial availability of NO may be due to its increased degradation resulting from interaction with superoxide.34,35 This hypothesis, however, remains speculative, because the superoxide production was not assessed in the present study.

The relaxation responses at 6 weeks (3 inoculations) differed clearly from those at 2 weeks (1 inoculation). First, the differences between the infected and noninfected groups became greater, and second, increased efficacy of L-NAME in decreasing endothelium-dependent relaxation was observed in both infected and noninfected groups. Because the methacholine-induced relaxations in noninfected animals were similar at 2 and 6 weeks, the multiple C pneumoniae inoculation appears to be the most plausible explanation for the impaired relaxation in infected animals at 6 weeks. Our finding is in agreement with a previous study in which only C pneumoniae–reinfected rabbits showed inflammatory changes and intimal thickening of aortas.3 However, advancing age may be a contributing factor that increases the susceptibility to the C pneumoniae infection23 and subsequent C pneumoniae–induced endothelial dysfunction. The latter is suggested by the fact that in the noninfected groups, the efficacy of L-NAME increased with advancing age from 2 to 6 weeks. Another major finding was that inhibition of COX enzyme by diclofenac, in the presence of NOS inhibition, signifi-


cantly improved methacholine-induced relaxation of aorta from infected mice. The enhanced relaxation suggests the presence of COX-dependent vasoconstrictors in association with C pneumoniae infection. Diclofenac is a nonselective inhibitor of both constitutive (COX-1) and inducible (COX-2) isoforms of COX.37,38 The fact that diclofenac improved relaxation only in the infected animals may be due to upregulation of COX-2 by bacterial lipopolysaccharides or infection-related cytokines, resulting in production of proinflammatory prostanooids.39,40 COX-2 is capable of producing vasoconstricting prostanooids, such as prostaglandin F2α and thromboxane A₂.41 Accordingly, the diclofenac-induced improved relaxation could be explained in part by inhibition of production of vasoconstricting prostanooids.

Superoxide anion produced by hydperoxidase activity of COX has been proposed to act as an endothelium-derived contracting factor.42 However, further studies are warranted to establish the role of the COX-dependent superoxide production in this infection model. Moreover, possible induction of COX-2 and COX-dependent vasoconstricting products by NO43 may also contribute to the observed effects of diclofenac in the infected animals.

Our immunohistochemistry data failed to show a greater expression of COX-2 in infected animals. Moreover, the similar staining for COX-2 in endothelial cells of infected and noninfected apoE-KO mice probably reflects the basal endothelial COX-2,44 because there was no detectable difference from wild-type mice that do not develop atherosclerosis.

The present findings, together with the observation that endothelial COX-dependent constrictiong products may also be released in various pathological conditions, such as congestive heart failure and atherosclerosis,20,45,46 call for further studies to elucidate the role of COX in C pneumoniae–induced endothelial dysfunction.

In conclusion, C pneumoniae infection impairs the endothelial function in apoE-KO mice. The NO pathway of the endothelial cell signaling is principally involved. COX inhibitors seem to improve the impaired relaxation response, but further studies are needed to elucidate the role of COX in C pneumoniae–induced endothelial dysfunction. The infection-induced functional changes of the arterial endothelium precede the morphological changes (intimal thickening) in the aorta. Our findings further support the participation of C pneumoniae infection in the development of atherosclerosis.

Acknowledgments

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