Increased prostanoid dependency of arterial relaxation in *Chlamydia pneumoniae*-infected mice

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Endothelial dysfunction plays an important role in the development of atherosclerosis. Previous studies have shown that inoculation with *Chlamydia pneumoniae* contributes to atherosclerotic development in rabbits and hypercholesterolaemic mice and causes endothelial dysfunction in apolipoprotein E-deficient mice. The effect of acute *C. pneumoniae* infection on endothelial function in normocholesterolaemic C57BL/6J mice was studied by measuring the force of contraction of the descending aorta after noradrenaline stimulation and in response to methacholine-induced relaxation. In addition, the effects of the nitric oxide synthase inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME) and the cyclooxygenase inhibitor diclofenac on relaxation were assessed. Pre-treatment of the aortas with L-NAME decreased the relaxation response in both the infected and uninfected groups and no significant difference was detected between these groups, whereas diclofenac significantly attenuated the relaxation response only in the infected animals. In conclusion, infection shifted the balance of endothelium-derived relaxing factors from nitric oxide towards vasorelaxing prostanoids in C57BL/6J mice.

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INTRODUCTION

The intracellular, Gram-negative pathogen *Chlamydia pneumoniae*, which generally causes mild upper respiratory tract infections and occasionally bronchitis and pneumonia in humans worldwide, has been suggested to participate in the development of cardiovascular disease (Leinonen & Saikku, 2002). In murine models, repeated intranasal inoculations of *C. pneumoniae* can accelerate atherosclerotic development, especially in hyperlipidaemic mice (Hu *et al.*, 1999; Moazed *et al.*, 1999) and, recently, *C. pneumoniae* inoculation was also shown to cause aortic endothelial dysfunction in apolipoprotein E-knockout (apoE-ko) mice after repeated *C. pneumoniae* infections (Liuba *et al.*, 2000) or after a combined infection with *Helicobacter pylori* (Liuba *et al.*, 2003). This was suggested to be due to a decrease in nitric oxide (NO) availability, caused by the infections, as aortic relaxation in the system was significantly impaired by an inhibitor of nitric oxide synthase (NOS), *N*^ω-nitro-L-arginine methyl ester (L-NAME). A possible effect of cyclooxygenases (COXs) and prostanoids was also proposed, but these results were not quite as conclusive (Liuba *et al.*, 2000).

Endothelial relaxation is commonly known to be mediated via NO and via prostaglandin I₂ (PGI₂) produced by vascular COX but, a third mechanism, an endothelium-derived hyperpolarizing factor (EDHF) that does not respond to NOS or COX inhibitors, could also mediate vascular relaxation, possibly by hyperpolarizing smooth muscle cells through activation of K⁺ channels in both endothelial and smooth muscle cells (Feletou & Vanhoutte, 1999). However, the chemical identity of EDHF, if any, is not clear.

Endothelial dysfunction plays an important role in the development of atherosclerosis (Ross, 1999). In this study, dysfunction detected as impaired relaxation responses was assessed. Wild-type mice fed a standard chow diet are normocholesterolaemic and do not develop atherosclerotic lesions spontaneously; however, C57BL/6J mice have been shown to be susceptible to atherosclerosis when fed an atherogenic diet (Paigen *et al.*, 1985) and are also vulnerable to changes similar to the human metabolic syndrome (Collins *et al.*, 2004). We have shown previously that repeated *C. pneumoniae* inoculations can lead to an increase in sub-intimal lipid accumulation in aortic sinuses of normocholesterolaemic C57BL/6J mice fed a diet with a slight cholesterol supplement (Törmäkangas *et al.*, 2005a). Thus, the aim of the present study was to determine whether acute *C. pneumoniae* infection is able to induce endothelial dysfunction measured as altered relaxation responses in these wild-type C57BL/6J mice.

Abbreviations: COX, cyclooxygenase; EDHF, endothelium-derived hyperpolarizing factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 6-keto PGF_{1α}, 6-keto prostaglandin F_{1α}; MC, methacholine; L-NAME, *N*^ω-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; PGI₂, prostaglandin I₂.

METHODS

Animal model and samples. Inbred C57BL/6J female mice purchased from Harlan Netherlands were divided into two groups of nine animals at the age of 8 weeks. *C. pneumoniae* isolate Kajaani 7, free from mycoplasma, was used to inoculate the mice intranasally. The first group was inoculated with 7×10^5 inclusion-forming units of *C. pneumoniae* per mouse in sucrose/phosphate/glutamate (SPG) buffer under inhaled methoxyflurane (Medical Developments Australia) anaesthesia. The second group was inoculated with SPG instead of chlamydia. The mice were sacrificed using CO₂ and samples were collected 4 days post-inoculation (p.i.). *Chlamydia* culture, quantitative COX analyses and measurement of PGI₂ levels were done for each of the nine animals in both groups. The aortas from only six randomly selected mice from each group were taken for vascular reactivity studies because of limited instrumentation. The Animal Care and Use Committee of the National Public Health Institute, Helsinki, Finland approved all procedures involving animals.

Chlamydia culture. Culture from mechanically homogenized lung tissue was performed as described in detail previously (Erkkilä *et al.*, 2002). Pathfinder (Sanofi Diagnostics Pasteur) *Chlamydia* genus-specific monoclonal antibody conjugated to fluorescein isothiocyanate was used to detect the chlamydia inclusions in HL (a human epithelial cell line) cells.

Detection of chlamydial DNA in aortic tissue. Sections of the aortas collected from the six infected and uninfected mice for vascular reactivity measurements were analysed for the presence of chlamydial DNA in the tissue. Descending aortic tissue was homogenized for Western blot analysis and the supernatant collected after centrifugation of the homogenate was studied by using PCR. Purification of DNA using a commercially available QIAamp tissue kit and a nested-PCR assay combining conventional and LightCycler real-time methods for detecting the *C. pneumoniae* *pst* fragment were done as described previously (Törmäkangas *et al.*, 2005a). *PstI* is a *C. pneumoniae*-specific genome fraction with no known function at present.

Quantitative mouse COX-1 and COX-2 mRNA analyses. Pieces of about 10–25 mg of the right lung from each of the nine mice per study group were stored in RNeasy RNA Stabilization reagent (Qiagen). Total RNA was extracted and reverse transcription was performed as described elsewhere (Törmäkangas *et al.*, 2005a). A quantitative LightCycler analysis for cDNA was performed with specific primers based on COX-1 and COX-2 mRNA sequences (Tanigawa *et al.*, 2004). The PCR reaction mixtures (final volume 20 µl) consisted of 2.5 mmol MgCl₂ l⁻¹ for COX-2 and 3 mmol MgCl₂ l⁻¹ for COX-1, 0.5 mmol l⁻¹ of each primer, 2 µl FastStart DNA Master SYBR Green I mix and 2 µl cDNA template. After pre-incubation at 95 °C, the amplification cycles were: COX-1, 95 °C/10 s, 60 °C/10 s and 72 °C/5 s; COX-2, 95 °C/15 s, 61 °C/10 s and 72 °C/9 s. Specific standards for all genes were produced by purification of the correct PCR products from agarose gels, amplification of these products with LightCycler and concentration of the amplified products by ethanol precipitation. The amount of the specific sequence in each stock was determined and the stocks were diluted to obtain standards of 1–10⁴ genomes µl⁻¹. Quantification was done by using a standard curve and calculating the results using the second-derivative maximum method of the LightCycler Data Analysis software (version 3.5.28). A melting curve analysis for each LightCycler run was performed and the expression of a housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured from the cDNA samples, according to the method described by Simpson *et al.* (2000). The crossing point (C_p) value for the GAPDH and COX analyses represents the PCR cycle at which the increase in the SYBR Green fluorescence signal above baseline was detected and indicates the level of template concentration in the

sample. When the C_p values for GAPDH cDNA were studied, it was shown that the mRNA levels were affected by infection: the mean C_p (SD) for the uninfected group was 25.4 (0.99) and 24.6 (0.99) for the infected group. Infection therefore increased the expression of GAPDH, and GAPDH results were not used for the relative quantification analysis. COX cDNA quantification calculated using the LightCycler software with a standard curve was adjusted for the weight of the tissue taken for RNA extraction. The results are presented per 25 mg lung tissue.

6-keto Prostaglandin F_{1α} (6-keto PGF_{1α}) levels. The main metabolite of PGI₂, 6-keto PGF_{1α}, in the serum samples was measured by using a commercial enzyme immunoassay kit (no. 515211; Cayman Chemical). The samples were diluted 1:2 using the kit EIA buffer and the analysis was performed according to the manufacturer's instructions.

Vascular reactivity studies. Six aortas from both infected and uninfected control mice were analysed for endothelial dysfunction. The fat tissue surrounding the upper section of the descending thoracic aorta was removed and the aorta was cut into rings (approximately 3 mm wide). The endothelium-intact rings were placed between steel hooks, mounted in an organ bath chamber and equilibrated for 60 min under a resting tension of 0.7 g in oxygenated (96 % O₂/4 % CO₂) Krebs solution (pH 7.4, 37 °C) after a first pre-contraction with 1.2 g. The force of contraction was measured with an isometric force-displacement transducer using a computerized system (EMKA 2000). The presence of intact endothelium in the vascular preparation was confirmed by observing the relaxation response to 1 µmol methacholine (MC) l⁻¹ (acetyl-β-methacholine chloride) in rings pre-contracted with 0.1 µmol noradrenaline l⁻¹ (bitartrate salt). To measure the endothelial function, the aortic rings were relaxed with increasing concentrations of MC after noradrenaline contraction. In addition, the effects of the non-selective NOS enzyme inhibitor L-NAME (at a concentration of 10⁻⁴ mol l⁻¹) and the non-selective COX inhibitor diclofenac (diclofenac sodium salt; at a concentration of 10⁻⁶ mol l⁻¹) on arterial relaxation were measured by incubating the aortic rings in these solutions in organ baths prior to relaxation assays. Noradrenaline, MC, L-NAME and diclofenac were obtained from Sigma-Aldrich.

Statistical analysis. The endothelial relaxation responses of the groups were compared using 2-way ANOVA for repeated measures. Differences between the groups after quantitative COX-expression analyses were tested with the non-parametric Mann-Whitney U test. Statistics were done using SPSS version 11.5.1.

RESULTS AND DISCUSSION

In this study, we analysed the effect of acute *C. pneumoniae* infection on aortic endothelial function in a C57BL/6J mouse model. Eight of nine mice were found to be culture-positive, with high inclusion numbers from lung tissue, thus proving that the inoculation was successful. The one mouse that was negative was excluded from further analyses and was not among the six animals studied for vascular reactivity. None of the SPG-inoculated control mice were positive. Aortic tissue from the six mice from each group that were studied for aortic relaxation was analysed for the presence of chlamydial DNA. Four of the six aortas from the group infected with chlamydia were found to be positive with the nested-PCR method used, thus indicating the presence of chlamydia in the aortic tissue samples, whereas all six aortas from the uninfected mice analysed in the same

PCR were negative. Although the nested-PCR method used in these analyses is not quantitative, it indicated that the genome numbers in all four positive samples were very low, thus increasing the possibility of false-negative results. Because of this and the fact that only a very small section of aortic tissue was taken for DNA extraction, the negative PCR result from two infected mice does not prove that these aortas were not infected. As these animals showed successful infection detected by culture, they were not excluded from the vascular reactivity analyses. The PCR analysis of the aortic tissue was done merely to show that chlamydia are able to invade the tissue.

The inoculum dose and *C. pneumoniae* isolate used in this study have been shown in our previous studies to induce histologically severe, but self-restricted, pneumonitis in C57BL/6J mice (Törmäkangas *et al.*, 2005b), leading to the systemic spread of the pathogen into the vasculature and increased aortic sinus lipid accumulation (Erkkilä *et al.*, 2004; Törmäkangas *et al.*, 2005a). The infectious dose used does not cause serious clinical symptoms or deaths in this mouse model.

Quantification of COX mRNA expression in lung tissue

The infected group showed an increase in COX-2 expression levels compared with the uninfected group, which was close to significant ($P=0.074$; Fig. 1b). No significant differences in the expression levels of COX-1 were detected (Fig. 1a). Infection appeared to increase the expression of the inducible form of cyclooxygenase, COX-2, in lung tissue in our study; however, this finding could not be correlated with the aortic relaxation results. Further studies should also assess the mRNA and protein expression of COXs in aortic tissue. A previous *in vitro* study showed an increase in COX-2

Fig. 1. Expression levels of COX-1 (a) and COX-2 (b) mRNA in mouse lung tissue. Bars represent geometric means and 95 % upper confidence interval. The quantification results were adjusted for the weight of the tissue taken for mRNA extraction. LC, LightCycler.

expression that was sustained at a lower level after the rapid induction by *C. pneumoniae* infection in human peripheral blood mononuclear cells (Rupp *et al.*, 2004). The results here show the situation 4 days after infection and it is possible that the peak of the response also occurs soon after primary infection *in vivo*. PGI₂ is produced mainly by vascular endothelium and its metabolite 6-keto PGF_{1α} is commonly used to estimate PGI₂ synthesis. Production of PGI₂ is increased in response to an inflammatory stimulus via increased COX-2 expression, and the function of this prostanoid is to induce vasodilatation and inhibition of platelet aggregation. The slight increase in COX-2 levels detected did not predict the presence of increased 6-keto PGF_{1α} levels in the serum samples in this study; no difference was detected in the metabolite concentrations between infected and uninfected mice (results not shown). This finding does not support, but is not able to contradict, the results presented below showing that COX-mediated vasodilatation is increased in the aortas of the infected mice. COX-mediated vasodilatation can also be affected by other prostanoids, in particular prostaglandin E₂, which is the major prostanoid produced by COX-2 in response to inflammation.

Endothelial function

The effects of inoculation on aortic endothelial function are shown in Fig. 2. The aortas from both study groups relaxed similarly in response to increasing MC concentrations when no pre-treatments were used (Fig. 2a). Only a non-significant decrease of 5–10 %, depending on the MC concentration, was seen in the aortas from infected control mice compared with the uninfected group. In the uninfected group, the MC-induced relaxation was mainly NO-dependent, as shown by the inhibitory effect of L-NAME (Fig. 2b) and the lack of a similar effect of diclofenac (Fig. 2c). In the presence of infection, relaxation was dependent on NOS products (presumably NO), but also significantly on COX products (presumably prostanoids), as the relaxation effects of MC were antagonized by both L-NAME and diclofenac (Fig. 2b, c) and were completely abolished by their combination (Fig. 2d). The difference between the uninfected and infected groups was significant after diclofenac pre-treatment ($P=0.001$; relaxation with the highest MC concentration used was about 90 vs 60 %, respectively; Fig. 2c) and after combined pre-treatments with both inhibitors ($P=0.031$; Fig. 2d), but not after L-NAME pre-treatment ($P=0.17$; Fig. 2b).

Although infection did not impair aortic relaxation, it clearly shifted the balance of endothelium-derived relaxing factors from NO towards vasorelaxing prostanoids. This was shown by pre-treatments with L-NAME and diclofenac. In non-infected aortas, MC-induced relaxation was predominantly NO-dependent (blocked greatly by the NOS inhibitor), whereas the blocking of prostanoid production by the COX inhibitor did not modify the relaxations. However, in vessels from infected mice, the relaxations were still strongly inhibited by L-NAME but the relaxation response was not

Fig. 2. Aortic relaxation responses to increasing MC concentrations. (a) Relaxation without pre-treatment, (b) relaxation after L-NAME pre-treatment, (c) relaxation after diclofenac pre-treatment and (d) relaxation after both L-NAME and diclofenac pre-treatments. ○, Uninfected control group; ▲, infected group. Values are means $\pm$ SD for the six mice. * $P < 0.05$; ** $P < 0.01$.

significantly different when compared with the uninfected group, whereas diclofenac strongly and significantly impaired relaxation in the infected group. The contribution of both NOS- and COX-mediated relaxation in the infected group was shown by the almost total absence of any vaso-relaxing activity of MC after combined blocking of both NOS and COX. In addition to these responses, the presence of another endothelial relaxing factor, possibly EDHF, in the normal uninfected aortas only could be suggested by the present results. A statistically significant relaxation in the presence of the combined blocking of both NOS and COX was observed in the uninfected group, but not in the chlamydia-infected group. However, the potential role of EDHF in this relaxation response should be verified in further studies using the NOS inhibitor and NO scavenger together with diclofenac, to exclude possible incomplete blocking of NOS.

The effects of *C. pneumoniae* infection on the aortic endothelial function of the apoE-ko mouse model have been studied previously (Liuba *et al.*, 2000). The effects of L-NAME in the present study were similar to those reported by Liuba *et al.* (2000) after primary inoculation, but the results from diclofenac pre-treatment are partly contradictory. In both studies, COX inhibition had no effect on uninfected mice, but Liuba *et al.* (2000) suggested that vasoconstricting prostanoids were present after both primary and repeated inoculations in the aortas of apoE-ko mice. In our study of C57BL/6J mice, prostanoid-mediated vasodilatation could be proposed in the infected animals. Use of a different mouse strain may explain the partial differences between our results and those reported by Liuba *et al.* (2000). In contrast to wild-type C57BL mice, apoE-ko mice are hypercholesterolaemic and spontaneously develop atherosclerotic changes comparable with human lesions in the aorta. Recent studies also suggest that the

inflammatory responses to infection are altered in these animals (Laskowitz *et al.*, 2000).

Blessing *et al.* (2000) reported that three repeated inoculations are able to cause signs of inflammation in the aortic sinuses of C57BL/6J mice, whereas acute infection alone had no such effect. Also, prolonged persistence of chlamydia in the aortic and other tissues has been reported only after repeated *C. pneumoniae* inoculations in hyperlipidaemic mice (Moazed *et al.*, 1997). However, the present study shows that transient aortic changes are induced by acute intranasal *C. pneumoniae* infection in C57BL/6J mice. Alterations in the aortic relaxation responses seen on day four after primary inoculation in this study were not detected on day 10 p.i. (results not shown). This is in accordance with results reported previously, in which acute *C. pneumoniae* inoculation did not lead to aortic endothelial dysfunction at 2 weeks p.i. in apoE-ko mice (Liuba *et al.*, 2000).

In conclusion, *C. pneumoniae* infection or multiple infections are able to affect and change the endothelium-dependent relaxation responses of aortic tissue in mice, as also shown in a previous report (Liuba *et al.*, 2000). In the present study, the mode of endothelium-derived relaxation was shifted from NO dependency towards prostanoid dependency and, in addition, the relaxation effect of a still unknown agent was abolished. Thus, although *C. pneumoniae* infection may not always impair endothelium-mediated relaxation, which was tested here with cholinergic stimulation, it may alter the balance of relaxing factors and even abolish some of their activity. This preliminary study warrants a thorough analysis of the effects of both acute and repeated inoculations on endothelial relaxation mediated by NOS, COX and smooth muscle cell-related pathways, measured at different time-points p.i. in this animal model. The results also call for further studies to answer two questions:

by which mechanisms is endothelial function altered in different disease states, and is the recently reported pro-thrombotic property of the selective COX-2 inhibitor, rofecoxib, also associated with adverse changes in vascular endothelial contractility.

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