The CX3C Chemokine Fractalkine Induces Vascular Dysfunction by Generation of Superoxide Anions

Andreas Schäfer, Christian Schulz, Daniela Fraccarollo, Piet Tas, Meike Leutke, Martin Eigenthaler, Stefan Seidl, Peter Heider, Georg Ertl, Steffen Massberg, Johann Bauersachs

Objective—The chemokine fractalkine activates platelets and induces leukocyte adhesion to the endothelium. Expression of fractalkine and its receptor, CX3CR1, is elevated in coronary artery disease. We assessed the effects of fractalkine on vascular function in isolated rat aorta.

Methods and Results—CX3CR1 expression was demonstrated in rat aortic endothelial and smooth muscle cells by immunohistochemistry, Western blot, and polymerase chain reaction (PCR). Fractalkine (up to 1 μg/mL) did not directly induce contractile or relaxant responses when applied to rat aortic rings in organ baths. Short-term incubation with fractalkine (1 μg/mL) for 5 minutes did not affect vascular reactivity. Pretreatment of isolated rat aortic rings with fractalkine for 2 hours impaired acetylcholine-induced nitric oxide (NO)-mediated relaxation after preconstriction with phenylephrine in a concentration-dependent manner. The concentration response to the NO donor DEA-NONOate was significantly shifted to the right. The radical scavenger tiron normalized the attenuated acetylcholine-induced relaxation after fractalkine incubation. Aortic superoxide formation was enhanced by fractalkine, which was inhibited by diphenyleneiodonium but not by inhibitors of xanthine oxidase or NO synthase.

Conclusion—In addition to its role as a chemokine and adhesion molecule, fractalkine induces vascular dysfunction by stimulating vascular reactive oxygen species resulting in reduced NO bioavailability. (Arterioscler Thromb Vasc Biol. 2007;27:55-62.)

Key Words: chemokines • endothelial dysfunction • nitric oxide • reactive oxygen species

Chemokines (chemotactic cytokines) are a large family of small proteins that induce the chemotaxis of cells and direct the trafficking of white blood cells in immune surveillance. They are distinguished by a cysteine signature motif based principally on the relative position of the first 2 highly conserved cysteine residues: CXC, CXC, CC, and C, where C is a cysteine and X any amino-acid residue.1 The unique difference between fractalkine and other chemokines except CXCL16 is a polypeptide chain that carries the chemokine domain on top of an extended mucine-like stalk, which allows the molecule to exist either as a membrane-anchored or as a soluble glycoprotein.1 Fractalkine is constitutively expressed in a variety of nonhematopoietic tissues such as brain, heart, kidney, and lung1-3 and its expression in endothelial cells is increased by pro-inflammatory agents.2 The fractalkine receptor (CX3CR1) is expressed in several cells including predominantly leukocytes.4,5 We recently demonstrated that platelets also express functional CX3CR1. Correspondingly, incubation of platelets with fractalkine resulted in CX3CR1-dependent platelet activation and adhesion.6 Importantly, platelet activation via the CX,C/C,CX3CR1-axis triggers leukocyte adhesion to activated endothelium in flowing whole blood.7 Apart from leukocytes and platelets, smooth muscle cells (SMCs) express CX3CR1 and migrate in response to fractalkine stimulation.8 Fractalkine and CX3CR1 expression is enhanced in vascular injury, atherosclerosis, and coronary artery disease.9-11

The fractalkine/CX3CR1 axis has recently been implicated in the pathogenesis of vascular dysfunction and vascular disease. Fractalkine and CX3CR1 expression is enhanced in atherosclerosis and after vascular injury.9,12,13 In fact, fractalkine is thought to contribute considerably to the atherosclerotic process: in mice lacking CX3CR1, progression of atherosclerotic lesion formation is significantly reduced.14,15 In human subjects, 2 common nonsynonymous single-nucleotide CX3CR1 polymorphisms causing amino acid changes from valine to isoleucine at position 249 (V249I) and from threonine to methionine at position 280 (T280M) have
been described and associated with modulation in cardiovascular risk: M280 is associated with reduced frequency of cardiovascular disease,16 significant coronary artery disease,17 and acute coronary syndrome,18 whereas the role of I249 in the absence of M280 is associated with elevated risk for acute coronary syndromes19 as well as restenosis after coronary stenting,20 whereas there was no harmful risk modification in patients bearing both I249 and M280.19,20 However, initial investigations showed that subjects with the I249 allele have greater endothelium-dependent coronary vasodilation indicating a direct functional role of the fractalkine system in the vasculature possibly by modifying endothelial function.17 In addition, fractalkine has recently been involved in vascular dysfunction after ischemia-reperfusion. Correspondingly, fractalkine expression is enhanced after transient cerebral ischemia in the rat13 and cerebral infarct size was significantly reduced in fractalkine-deficient mice.21 Although these findings clearly indicate a role for fractalkine in the modulation of vascular homeostasis, the mechanisms underlying the effects of fractalkine on vascular function remain as yet unexplored. In particular, it remains unclear whether fractalkine affects the vessel wall directly or rather acts indirectly by modulating the function of circulating cells, including leukocytes and platelets. In the present study, we investigated the direct effect of fractalkine on vascular function and superoxide formation in isolated rat aorta as well as in cultured human aortic endothelial cells and SMCs.

Methods

CX3CR1 expression was determined by polymerase chain reaction, Western blot, and flow cytometry. Functional studies on vascular reactivity were performed on isolated rat aortic rings in organ bath chambers. Superoxide generation was detected by lucigenin-enhanced chemiluminescence and hydroethidine staining. For more details, please refer to the online supplement at http://atvb.ahajournals.org.

Substances

Unless stated otherwise, all chemicals were obtained from Sigma (Deisenhofen, Germany) in the highest purity available. The used recombinant fractalkine was recombinant human fractalkine (365-384, R&D Systems, Minneapolis, Minn), which exerted similar reactive oxygen species (ROS) generation and impairment of vasorelaxation as recombinant rat fractalkine, and its activity was completely inhibited by pre-incubation of rat aortic rings with the anti-rat CX3CR1 antibody (data not shown).

Statistics

Data are presented as means ± SEM and analyzed using Student t test or 1-way ANOVA with a Tukey post-hoc test when appropriate. A repeated measures ANOVA was applied for dose response curves. A P<0.05 was considered statistically significant.

Results

Expression of CX3CR1 on Endothelial Cells and in Rat Aorta

Surface expression of CX3CR1 was determined in unstimulated cultured human endothelial cells by flow-cytometry (Figure 1A and 1B). Surface-expression of CX3CR1 was detected on endothelial cells, and was neither modulated by stimulation with fractalkine for 2 or 6 hours nor by activation of endothelial cells with either tumor necrosis factor (TNF)-α alone or in combination with interferon-γ, which are known to increase fractalkine expression on endothelial cells.1,7 Likewise, the rat aorta showed considerable CX3CR1 expression as assessed by polymerase chain reaction (Figure 1C) and Western blot (Figure 1D and supplemental Figure I, available online at http://atvb.ahajournals.org) to verify CX3CR1 expression in native vascular tissue. Incubation with fractalkine did not alter aortic CX3CR1 protein expression (Figure 1D). Whereas TNFα (50 ng/mL) induced the expression of adhesion molecules suggestive for endothelial activation such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, or E-selectin in human endothelial cells, fractalkine did not stimulate adhesion molecule expression (Figure 1E).

Immunohistochemistry demonstrated strong expression of CX3CR1 in endothelial cells (ECs) and smooth muscle cells (SMCs) within rat aortae (Figure 2A and 2D), which was not altered by stimulation with recombinant fractalkine (Figure 2B and 2E). ECs and SMCs were verified by CD31 and smooth muscle actin staining (supplemental Figure II). In parallel, we excluded infiltration by dendritic (CD21+) cells or macrophages (CD68+) as possible nonvascular sources for CX3CR1 (supplemental Figure III).

Acute Vascular Effects of Fractalkine

Direct application of fractalkine (1 μg/mL) on aortic rings preconstricted to ~20% of their maximal constriction did neither exert acute vasoconstriction nor alter L-NNA–induced vasoconstriction (supplemental Figure IVA), which is used to indirectly assess stretch-induced, calcium-independent nitric oxide (NO) release.22 Direct stimulation of phenylephrine-preconstricted aortic rings with recombinant fractalkine did not induce acute vasodilation, but slightly, however, nonsignificantly shifted the acetylcholine-induced vasorelaxation to the right (EC50 [nmol/L]: control 66.7±12.0, fractalkine [100 ng/mL] 140.0±23.9, fractalkine [1 μg/mL] 141.1±23.7). Similarly, maximum relaxation was not significantly reduced (supplemental Figure IVB).

Effect of Fractalkine Incubation on Vascular Reactivity

After prolonged (2-hour) stimulation with recombinant fractalkine (1 μg/mL), vasoconstriction in response to cumulative concentrations of phenylephrine was slightly but significantly increased compared with unstimulated rings (Figure 3A). L-NNA–induced vasoconstriction after slight preconstriction with phenylephrine was significantly reduced indicating reduced NO bioactivity (Figure 3B).

Endothelium-dependent, acetylcholine-induced vasorelaxation was significantly attenuated after 2-hour stimulation with recombinant fractalkine (1 μg/mL; Figure 3C). Antagonism of the chemokine domain with a neutralizing anti-fractalkine antibody or pre-incubation of aortic rings with a neutralizing antibody against the fractalkine receptor (anti-CX3CR1) completely prevented fractalkine-induced endothelial dysfunction (Figure 3C). These data indicate that the observed effects are mediated by the chemokine domain of
Fractalkine and require functional interaction with its specific receptor CX3CR1. The concentration response curve to the NO donor DEA-NONOate was significantly shifted to the right (EC50 [nmol/L]: control 173.3/36.3, fractalkine [1 g/mL] 611.1/209.8, P<0.05, Figure 3D). Incubation of aortae with fractalkine for 4 and 6 hours did not result in further depression of relaxant responses (data not shown). Based on these data, further analysis was performed in aortic rings incubated with fractalkine for 2 hours.

Similar experiments were performed with different concentrations of fractalkine (30 ng/mL, 100 ng/mL, 300 ng/mL, and 1 g/mL). Higher concentrations of fractalkine increased phenylephrine-induced vasoconstriction (Figure 4A). Endothelium-dependent vasorelaxation was concentration-dependently impaired (Figure 4B and 4C). Endothelium-independent vasorelaxation was impaired by all concentrations of fractalkine (data not shown). Fractalkine-induced attenuation of vasorelaxation was also observed in endothelium-denuded aortic rings (Figure 4D).

We investigated whether de novo protein-synthesis is required for the observed effects of fractalkine. The experiments were performed in the presence of cycloheximide, which at least partially prevented the impairment of acetylcholine-induced and NONOate-induced vasorelaxation (EC50 [nmol/L]: fractalkine 468.1±95.9, fractalkine + cycloheximide 78.0±12.7, P<0.05; supplemental Figure V).

Fractalkine (1 g/mL, 2 hours) did not alter endothelial nitric oxide synthase (eNOS) expression or changes in eNOS phosphorylation as demonstrated by the respective Western blot analyses (supplemental Figure VI).

Fractalkine Incubation Increases Vascular Superoxide (O2−) Formation

Because the bioactivity of NO can be affected substantially by increases in oxidative stress, we addressed whether the generation and release of ROS might contribute to the vascular effects of fractalkine. When the unspecific radical scavenger tiron (1 mmol/L) was added to fractalkine-stimulated aortic rings in the organ bath prior to acetylcholine-induced vasorelaxation, endothelium-dependent relaxation was significantly improved indicating the potential relevance of ROS for endothelial dysfunction following stimulation with fractalkine (Figure 5A).

In separate experiments aortic rings were stimulated with fractalkine for 2 hours and shock-frozen after stimulation. The detection of O2− formation was then visualized using hydroxyethidium and was significantly increased in fractalkine-stimulated aortic rings (Figure 5B). Representa-
Confocal images showed stronger signal throughout the vascular wall in the fractalkine-treated group (Figure 5C bottom) versus the unstimulated controls (Figure 5C top). O$_2^-$ formation as detected by lucigenin (5 μmol/L)-enhanced chemiluminescence was significantly increased in fractalkine-stimulated aortic rings. Antagonizing the chemokine domain of fractalkine by pre-incubation with a neutralizing anti-fractalkine antibody inhibited fractalkine-induced O$_2^-$ production (Figure 5D). Selective inhibition of xanthine oxidase by allopurinol, NOS inhibition by L-NNA, or endothelial denudation did not affect O$_2^-$ formation by fractalkine. However, the flavoprotein inhibitor diphenyleneiodonium (DPI) inhibited fractalkine-induced O$_2^-$ formation (Figure 5D).

We performed additional experiments selectively addressing the question of cell-type specific fractalkine-induced ROS formation in isolated, cultured human aortic endothelial and SMCs. Fractalkine induced ROS formation in both cell types; however, only in SMC was there a nonsignificant trend toward additive ROS formation after co-stimulation with TNFα (Figure 5E).

**Expression and Regulation of NADPH-Oxidase Subunits**

To determine the origin of fractalkine-induced O$_2^-$ generation, we analyzed the expression of NADPH-oxidase subunits. Aortic expression of gp91phox (supplemental Figure VIIA), p67phox (supplemental Figure VIIB), and p22phox (supplemental Figure VIIC) remained unaltered after 2 hours of incubation with fractalkine. Expression of p47phox showed a nonsignificant trend toward an increase after stimulation (P=0.18; supplemental Figure VIID). However, vascular expression of thioredoxin, which functionally modulates NADPH oxidase, was significantly reduced by fractalkine (supplemental Figure VIIE).

**Discussion**

In the present study we demonstrate that fractalkine directly modifies vascular reactivity via its specific receptor CX$_3$CR1 expressed on endothelial and SMCs. Fractalkine induces vascular O$_2^-$ production resulting in decreased NO bioavailability, a well-distinguished feature of endothelial dysfunction.

Inflamed or activated ECs and SMCs express fractalkine and its receptor CX$_3$CR1. In the present study, we demonstrate that cultured endothelial cells as well as native endothelium and SMCs in healthy rat aorta express CX$_3$CR1. Stimulation with its ligand fractalkine did not affect CX$_3$CR1 expression. These results imply that endothelial cells are not only a possible source of fractalkine, but can also act as target cells for this chemokine. Therefore, enhanced expression/shedding of fractalkine from activated endothelium will not only triggers leukocyte chemotaxis and platelet activation, through CX$_3$CR1 on leukocytes and platelets, but they also exert paracrine effects in the vicinity of the inflamed endothelium and directly contribute to endothelial dysfunction and vascular damage via endothelial CX$_3$CR1.
First, we addressed whether brief (5 minutes) application of fractalkine would either induce direct vasomotor effects or modulate vascular reactivity. Although there was no direct relaxant or contractile effect of fractalkine itself, we found a slight but non-significant reduction of acetylcholine-induced NO-mediated relaxation after 5 minutes of incubation with fractalkine. These data suggest that brief or coincidental interactions of fractalkine with the endothelium are not sufficient to initiate detrimental changes like endothelial dysfunction or endothelial activation. However, prolonged incubation of intact aortic rings with fractalkine for 2 to 6 hours resulted in highly significant attenuation of endothelial NO bioavailability, which was concentration-dependent and already maximal at 2 hours. To the best of our knowledge, these data provide the first evidence for a direct, leukocyte- and platelet-independent effect of fractalkine in the regulation of endothelial function.17 Our data demonstrate that fractalkine indeed severely affects vascular function in addition to its effects on platelets and leukocytes. The complete prevention of fractalkine effects by inhibiting either the chemokine domain or antagonizing CX3CR1 in the vascular segments prior to incubation with the chemokine further underlines that the observed effect is in fact dependent on a functional ligand-receptor interaction. On the other side, it rules out that any unspecific interaction of parts of the full-length chemokine used in the experiments is responsible for the observed modification of vascular function.

Both pathways of NOS activation, Ca\(^{2+}\)-dependent (agonist-induced) as well as Ca\(^{2+}\)-independent (stretch-induced), were impaired after stimulation with fractalkine, suggesting that the reduction of NO bioavailability might be downstream of NO generation itself. This is also supported by the findings that incubation with fractalkine did not alter eNOS expression or its phosphorylation. When applying an exogenous NO donor on the aortae, we found a significant reduction of endothelium-independent relaxation after incubation with fractalkine. Therefore, the observed effect on endothelium-dependent, NO-mediated relaxation can be partially ascribed to reduced sensitivity of the SMC layer as indicated by decreased sensitivity in response to the NO donor DEA-NONOate.

A well-characterized mechanism for reduced NO bioavailability associated with endothelial dysfunction is an increased formation of ROS, mainly superoxide anions. Reduced NO bioavailability and abundant formation of ROS within the vascular wall are the key determinants in endothelial dysfunction resulting in an imbalance between NO and ROS. Indeed, impaired endothelial function in several cardiovascular dis-
eases has been linked to increased oxidative stress. In the present study, the role of ROS for the vascular effects of fractalkine was indicated by the radical scavenger tiron, which reversed fractalkine-induced attenuation of acetylcholine-induced vasorelaxation. Inhibition of superoxide formation by blocking the chemokine domain demonstrated that fractalkine increases vascular superoxide formation via ligand-dependent activation of CX3CR1.

Several mechanisms have to be considered as potential contributors for increased superoxide anion formation: increased activity of NADPH oxidases or xanthine oxidase as well as uncoupled eNOS. Xanthine oxidase does not seem to play a major role for fractalkine-induced superoxide generation, because its inhibition by allopurinol did not reduce superoxide formation. eNOS uncoupling was functionally ruled out, because neither NOS inhibition nor endothelial denudation reduced the superoxide signal. Furthermore, enhanced formation of superoxide was present in all layers of the aortic wall favoring a source of ROS outside the endothelium. Our experiments in isolated cultured aortic endothelial and SMCs provided evidence that fractalkine induces ROS formation in both cell types. One such source, which is present throughout the vascular wall and sensitive to DPI, is NADPH oxidase. However, the expression of NADPH oxidase subunits was not altered after stimulation with fractalkine in this study. NADPH oxidase activity is physiologically modulated by thioredoxin, which exerts important protective roles against ROS. The thioredoxin system may effectively regenerate proteins that were inactivated by oxidative stress. Thioredoxin functions as a crucial mediator of redox-mediated transcriptional activation. Although we did not elucidate the underlying mechanisms, our data show that incubation with fractalkine was associated with reduced expression of thioredoxin. Thereby, increased NADPH oxidase activity results in increased formation of superoxide anions despite unaltered expression of NADPH oxidase subunits.

In addition to its acute effects, repetitive or chronic stimulation of the vessel wall by fractalkine in vivo may exert even more unfavorable effects: chronic endothelial dysfunction results in a more contractile, pro-atherogenic, and pro-inflammatory environment in the vascular system. Besides the impairment of vasodilation, the reduction of NO bioavailability by fractalkine observed in this study further facilitates fractalkine-induced leukocyte and platelet activation and recruitment. Therefore, the protection from atherosclerosis observed in ApoE−/−-mice deficient for CX3CR1 may not only be attributed to less fractalkine-mediated leukocyte activation and secondary endothelial damage. Indeed, the results of our study demonstrate direct, leukocyte-independent fractalkine-induced damage on the endothelium, which may contribute to endothelial dysfunction and lesion formation in ApoE−/− mice.

In conclusion, the present study demonstrates that fractalkine induces endothelial dysfunction in isolated vascular segments under leukocyte-free conditions. Fractalkine increased NADPH oxidase dependent superoxide formation resulting in decreased NO bioavailability, an additional para-crime action of this chemokine in vascular disease.

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Figure 5. Addition of the radical scavenger tiroli reversed endothelial dysfunction induced by fractalkine (FKN) (1 μg/mL, 2 hours) in isolated rat aortic rings (A, n=10). Confocal microscopy of 10-μm-thick aortic sections incubated with the fluorescent dye hydroxyethidium to visualize O2− formation throughout the vascular wall (B, n=6). Representative confocal images are shown (C). Aortic O2− formation detected by lucigenin-enhanced chemiluminescence was increased after 2 hours of pretreatment with FKN. Antagonism of the chemokine domain with an antagonizing antibody or inhibition of oxidases by diphenyleneiodonium chloride (DPI) significantly reduced O2− formation in aortic rings (D, Allo=xanthine oxidase inhibition by allopyrulon, L-NNA=NOS inhibition by L-arginine analogue, E=vascular rings lacking intact endothelium following denudation, n=7 to 12). O2− formation in isolated, cultured human aortic endothelial cells (HAEC) as well as human aortic smooth muscle cells (HASMCs) induced by either fractalkine (1 μg/mL, 90 minutes) and/or TNFα (10 ng/mL, 90 minutes) (E, n=5). **P<0.01 vs FKN. §§P<0.01 vs Co; #P<0.05, ##P<0.01 vs FKN.

Disclosures

None.

References


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The CX3C Chemokine Fractalkine Induces Vascular Dysfunction
by Generation of Superoxide Anions

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Methods

Vascular reactivity studies

After induction of terminal anaesthesia with isoflurane in male Wistar rats (~250g, Harlan Winkelmann, Borken, Germany), the descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue. Aortic segments were either frozen for Western blot analysis, used for measurement of superoxide anion (O$_2^-$) production or cut into rings which were mounted in an organ bath (FMI, Seeheim, Germany) for isometric force measurements. The rings were equilibrated for 30 min under a resting tension of 2g in oxygenated (95%O$_2$; 5%CO$_2$) Krebs-Henseleit solution (NaCl 118mmol/L, KCl 4.7mmol/L, MgSO$_4$ 1.2mmol/L, CaCl$_2$ 1.6mmol/L, KH$_2$PO$_4$ 1.2mmol/L, NaHCO$_3$ 25mmol/L, glucose 12mmol/L; pH 7.4, 37°C) containing diclofenac (1µmol/L) and in the case of chronic stimulation with fractalkine were incubated with different concentrations (30ng/mL, 100ng/mL, 300ng/mL, and 1µg/mL) of the recombinant chemokine for 2 hours. Rings were repeatedly contracted by KCl (with a maximum of 100mmol/L) until reproducible responses were obtained, before a dose response curve for phenylephrine was performed. Thereafter, the rings were preconstricted with phenylephrine to comparable constriction levels and the relaxant responses to cumulative doses of acetylcholine and 2-(N,N-Diethylamino)diazenolate-2-oxide (DEA-NONOate, Alexis Biochemicals, Lausen, Switzerland) were assessed. In separate experiments aortic rings were slightly preconstricted to about 15-20% of their maximal constriction with low, incrementing doses of phenylephrine and the additional contraction to N$^\text{G}$-nitro-L-arginine (L-NNA, 100µmol/L) was measured as a marker of physiological stretch-induced, calcium-independent NO formation as previously described$^1$. In a subset of the experiments the radical scavenger tiron or an antagonizing antibody against CX$_3$CR1 (TP501, Torrey Pines) was added. Some aortic rings had been pre-incubated with cycloheximide (100µmol/L) from the beginning of the equilibration period to assess the
necessity of de novo protein synthesis for fractalkine-induced effects and others were endothelium-denuded to observe endothelium-independent vascular effects.

**Measurement of superoxide anion formation**

Vascular $O_2^-$ formation was measured using lucigenin-enhanced chemiluminescence\(^2\). The light reaction between $O_2^-$ and lucigenin (5µmol/L\(^3\)) was detected in a luminometer (Wallac, Freiburg, Germany) during incubation of rings in a HEPES-modified Krebs buffer (pH 7.40). The oxidative fluorescent dye hydroethidine was used to evaluate in situ production of superoxide. Hydroethidine is freely permeable to cells and in the presence of $O_2^-$ is oxidized to hydroxyethidium, where it is trapped by intercalating with the DNA\(^4\). Hydroxyethidium is excited at 488nm and the emission measured at 610nm. In cell-free assays, addition of hydrogen peroxide to hydroethidine does not significantly increase hydroxyethidium fluorescence.

Unfixed frozen ring segments were cut into 10-µm-thick sections and placed on a glass slide. Hydroethidine (2µmol/L) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Aortic rings stimulated with fractalkine and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from both groups. Fluorescence was detected with a 585nm long-pass filter. Quantitative analysis of hydroxyethidium fluorescence was performed using NIH ImageJ.

ROS production in human aortic endothelial (HAEC) and human aortic smooth muscle cells (HASMC) purchased from PromoCell (Heidelberg, Germany) was measured as previously described \(^5\). Briefly, cells were grown to confluency at passages 7 to 12 in appropriate cell growth medium in a 6-well plate. Confluent cells were washed twice with PBS. All
stimulations were performed in PBS supplemented with 5.5mmol/L D-Glucose, 1mmol/L CaCl$_2$ and MgCl$_2$ (PBS/Glc/Ca$^{2+}$/Mg$^{2+}$) at 37°C in humidified atmosphere of 95% air – 5% CO$_2$. Cells were stimulated with 10ng/mL TNF-α for 90 min and/or 1µg/mL fractalkine for 90 min. During the last 10 min of incubation, cells were loaded with 10µmol/L (final concentration) H$_2$O$_2$-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate. After that, cells were washed with PBS twice, scraped and collected in 0.5mL of PBS into flow cytometry tubes. Fluorescence intensity of cells was measured by flow cytometry.

**PCR analysis**

1µg of total RNA from aorta samples was subjected to cDNA synthesis using SuperScript II RNase H’ Reverse Transcriptase (Invitrogen) and oligo-dT primer, according to the manufactures protocol. The cDNA was amplified using Taq DNA Polymerase (Amersham Biosciences). The primers were: fractalkine, (sense) 5’-GAATTCCCTGGCGGGTCAGCACCTCGGCATA-3’, (anti-sense) 5’-AAGCTTTTACAGGGCAGCGGTCTGGTGGT-3’ and CX3CR1, (sense) 5’-AGCTGCTCAGGACCTCACCAT-3’, (anti-sense) 5’-GTTGTGGAGGCCCTCATGGCTGAT-3’. The PCR conditions were as follows: After 3 min at 95°C, amplification was repeated for 35 cycles of 95°C for 1 min, 60°C for 30 sec, and 72°C for 1 min, followed by a final step at 72°C for 3 min. The PCR products were visualized by 2.0% agarose gel electrophoresis and ethidium bromide staining.

**Western Blot analysis**

Aorta amples were homogenized in ice-cold RIPA buffer (150mmol/L NaCl, 50mmol/L Tris-HCl, 5mmol/L EDTA, 1%v/v Nonidet P-40, 0.5%w/v deoxycholate, 10mmol/L NaF, 10mmol/L sodium pyrophosphate, 100mmol/L phenylmethylsulfonyl fluoride, 2µg/mL aprotinin, and 2µg/mL leupeptin). Proteins were determined by Bradford assay. Aorta extracts
(10µg protein per lane) were mixed with sample loading buffer (B7703, BioLabs) and separated on 12% SDS-polyacrylamide gel. Proteins were electrotransferred onto PVDF membrane (Immun-Blot® 0.2µm, Bio-Rad). The bands were detected using chemiluminescence assay (ECL+Plus, Amershamb). Primary antibodies used recognize: endothelial nitric oxide synthase (eNOS; N-30020, Transduction Laboratories), phosphorylated eNOS at Ser1177 (9571, Cell Signaling Technology) thioredoxin (sc-20146, Santa Cruz Biotechnology), gp91phox (sc-5827, Santa Cruz Biotechnology), p67phox (sc-7663, Santa Cruz Biotechnology), p22phox (sc-11712, Santa Cruz Biotechnology), p47phox (sc-7660, Santa Cruz Biotechnology), CX3CR1 (ab8021, Abcam) and glyceraldehyd-3-phosphate-dehydrogenase (GAPDH; Ab8245, Abcam). Human spleen lysate (ab29699, Abcam) was used as a positive control for CX3CR1 and preincubation of the primary antibody with the blocking peptide (ab8125, Abcam) was used as a negative control as recommended by the manufacturer.

**Immunohistochemistry**

Formalin-fixed tissues were paraffin-embedded, sectioned, and mounted on slides as previously described. Sections were dewaxed and rehydrated, then immunohistochemical staining for CX3CR1 was performed using the Vectastain® ABC kit rabbit IgG (PK-6101, Vector laboratories, Burlingame, CA). Slides were incubated with the primary antibody recognizing CX3CR1 (rabbit anti-rat CX3CR1 TP-501P, Torrey Pines Biolabs, San Diego, CA) for 1 hour. Biotinylated goat anti-rabbit IgG (Vector) and StreptABComplex/POX (Vector) were incubated sequentially at room temperature. Antibodies were localized using the DAB Substrate Kit (Dako), followed by counterstaining with hamalaun. Rat spleen tissue was used as the positive test control tissue. Species and isotype-matched IgG (Dako) were used as negative controls on both control and test tissues.
Flow cytometry for CX3CR1 and adhesion molecules

CX3CR1 surface expression was analyzed in unstimulated HUVEC. HUVEC were incubated with a polyclonal rabbit anti-human CX3CR1 antibody (TP502, Torrey Pines Biolabs) and an FITC-labeled secondary antibody (10µg/mL) for 15 minutes. Thereafter, cells were washed, detached with trypsin/EDTA, and fixed with paraformaldehyde (0.5% final concentration). 20,000 events were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson Biosciences, Heidelberg, Germany). Cells were gated by their characteristic forward and side scatter distribution\(^7\) and the mean intensity of immunofluorescence was used as index of antigen surface expression.

To analyze the expression of adhesion molecules on stimulated endothelium, HUVEC were treated with TNF-\(\alpha\) (50 ng/mL) or fractalkine (1µg/mL) for 2 and 6 hours and monoclonal FITC-labeled mouse anti-human antibodies directed either against CD106 (Serotec, Düsseldorf, Germany), CD62E (Calbiochem, Schwalbach, Germany), CD54 (Beckman Coulter, Krefeld, Germany) or a mouse IgG1 isotype control were added. Flow cytometric analysis was performed as described above and the expression calculated in % of the unstimulated controls.

Substances

Unless stated otherwise, all chemicals were obtained from Sigma (Deisenhofen, Germany) in the highest purity available. The used recombinant fractalkine was recombinant human fractalkine (365-FR, R&D Systems, Minneapolis, MN) which exerted similar ROS generation and impairment of vasorelaxation as recombinant rat fractalkine, and its activity was completely inhibited by pre-incubation of rat aortic rings with the anti-rat CX3CR1 antibody (data not shown).

Statistics
Data are presented as means ± SEM and analyzed using Student’s t-test or one-way ANOVA with a Tukey post-hoc test where appropriate. A repeated measures ANOVA was applied for dose response curves. A p<0.05 was considered statistically significant.

Supplementary methods for immunohistochemistry

Formalin-fixed tissues were paraffin-embedded, sectioned, and mounted on slides as previously described⁶. Sections were dewaxed and rehydrated, then immunohistochemical staining for CX₃CR1 was performed using the Vectastain® ABC kit rabbit IgG (PK-6101, Vector laboratories, Burlingame, CA). Slides were incubated with the primary antibody recognizing CX₃CR1 (rabbit anti-rat CX₃CR1 TP-501P, Torrey Pines Biolabs, San Diego, CA), PECAM-1 (CD31, goat polyclonal antibody 200µg/ml 1:800, Santa Cruz M-20), macrophages (CD68, mouse anti-rat MCA341GA purified IgG 1.0mg/ml 1:100, Serotec) and follicular dendritic cells (CD21, monoclonal mouse anti-human CD21 Clone 1F8 mouse IgGconc. 350mg/l 1:50, MO784 Dako, cross-reacting with rat) for 1 hour. Species-specific biotinylated secondary antibodies (anti-rabbit, anti-mouse, and anti-goat; Vector) and StreptABComplex/POX (Vector) were incubated sequentially at room temperature. Antibodies were localized using the DAB Substrate Kit (Dako), followed by counterstaining with hamalaun. Rat spleen tissue was used as the positive test control tissue. Species and isotype-matched IgG (Dako) were used as negative controls on both control and test tissues. Smooth muscle actin (SMA, monoclonal mouse anti-human SMA clone 1A4 70mg/l 1:50, Dako MO851, cross-reacting with rat) staining was performed using the APAAP method (ChemMate Detection Kit K5000 APPAP mouse, Dako).


Legends

Figure I: Human spleen lysate was used as a positive control for CX$_3$CR1 (15 µL and 10 µL, respectively) in comparison to the positive staining for CX$_3$CR1 in rat aorta, which was abrogated by incubating the antibody with a specific blocking peptide.

Figure II: Immunohistochemistry specifically demonstrated endothelial cells by CD31-positivity (A, D) and smooth muscle cells by SMA-positivity (B, E). In parallel, expression of CX$_3$CR1 in rat aortae (C, F) was present in both cell types in the absence (A-C) and presence (D-F) of stimulation with fractalkine (FKN, 1 µg/mL, 2h). Images were taken with 20-fold magnifications.

Figure III: Immunohistochemistry showing no evidence of follicular dendritic cells (CD21$^+$) or macrophages (CD68$^+$) within the investigated vascular samples even after stimulation with fractalkine (FKN, 1 µg/mL, 2h). Positive controls were obtained from rat spleen. Images were taken with 20-fold magnifications.

Figure IV: Fractalkine (FKN, 1 µg/mL) did not exert direct vasoconstrictor effects nor did it acutely modulate NOS inhibitor (N$^G$-nitro-L-arginine, L-NNA, 100 µmol/L)-induced vasoconstriction (A, n=5). FKN (100 ng/mL or 1 µg/mL respectively, 5 min) did not significantly affect acetylcholine-induced, endothelium dependent-relaxation (B, n=9).

Figure V: Modification of fractalkine (FKN, 1 µg/mL, 2h)-induced endothelium-dependent (A) and endothelium-independent (B,C) vasorelaxation by inhibition of de novo protein synthesis with cycloheximide. **=p<0.01 vs. FKN, n=16.

Figure VI: Neither eNOS protein expression (A) nor its phosphorylation (B) was altered after stimulation with fractalkine (FKN, 1 µg/mL, 2h), n=6.

Figure VII: Protein expression of NADPH oxidase subunits gp91$^{\text{phox}}$ (A), p67$^{\text{phox}}$ (B), p22$^{\text{phox}}$ (C) and p47$^{\text{phox}}$ (D) was not significantly changed after stimulation with fractalkine (FKN, 1 µg/mL, 2h), whereas the expression of thioredoxin was reduced (E). **=p<0.01 vs. Co, n=6.
Figure I
Figure I

Figure II
Figure III

Rat spleen

CD21

CD68

Rat aorta
Figure IV
Figure V
Figure VI
Figure VII