Cholesterol Enhances Thrombin-Induced Release of Fibroblast Growth Factor-2 in Human Vascular Smooth Muscle Cells

Bernhard H. Rauch, Godehard A. Scholz, Dana Baumgärtel-Allekotte, Petra Censarek, Jens W. Fischer, Artur-Aron Weber, Karsten Schrör

Objective—The mitogenic response to the G protein–coupled receptor agonist thrombin in human vascular smooth muscle cells (SMCs) depends on release of fibroblast growth factor-2 (FGF-2). Yet, intracellular mechanisms triggering FGF-2 release are unknown. The present study investigates possible effects of cholesterol enrichment and depletion, which have been shown to influence FGF-2–dependent signaling and SMC mitogenesis, on thrombin-induced FGF-2 release.

Methods and Results—Cultured human aortic and saphenous vein SMCs were enriched with cholesterol by using a cyclodextrin-cholesterol complex. Cholesterol accumulation was determined by a fluorometric assay. ELISA, Western blotting, and RT-PCR were used for quantification of FGF-2 levels. DNA synthesis was determined by [3H]-thymidine incorporation, proliferation by cell counting. Stimulation of SMCs with thrombin (30 nmol/L) resulted in release of FGF-2 into the pericellular space within 10 minutes. Preincubation with cyclodextrin-cholesterol caused accumulation of cellular cholesterol, increased thrombin-induced FGF-2 release, and stimulated FGF-2 de novo synthesis. Thrombin-induced DNA synthesis and proliferation were enhanced in cholesterol-rich SMCs. This effect was inhibited by FGF-2-neutralizing antibodies.

Conclusions—Enhanced cellular cholesterol stimulates thrombin-induced release of FGF-2 and increases the mitogenic response toward thrombin in human SMCs. This mechanism might also be relevant for thrombin induced mitogenesis in hypercholesterolemia in vivo. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: thrombin • FGF-2 • cholesterol • smooth muscle cells • mitogenesis

Proliferation of vascular smooth muscle cells (SMCs) is a key event in atherosclerosis and restenosis after vascular injury.1,2 In addition to its function as coagulation factor, thrombin acts as a mitogen for SMCs by activating the G protein–coupled protease-activated receptors (PARs).3 In previous studies, we and others have shown that the mitogenic signaling of G protein–coupled receptors (GPCRs) involves transactivation of receptor tyrosine kinases (RTKs).4–6 In human SMCs, we found that thrombin-induced mitogenesis depends on a rapid release of fibroblast growth factor-2 (FGF-2) into the pericellular matrix and consecutive activation of the FGF receptor-1 (FGFR-1).6 The role of FGF-2 as a mitogen and chemoattractant for SMCs in vitro and in vivo is well characterized.7,8,9,10,11 Interestingly, it is still unknown how FGF-2 is released from intact cells. Because FGF-2 lacks a typical amino acid sequence for externalization,12 earlier studies speculated that FGF-2 may be released on cell damage or by an exocytotic mechanism independent of the endoplasmic reticulum–Golgi pathway.13 Elevated non–high density lipoprotein (non-HDL) cholesterol levels are not only an established risk factor for the development and progression of atherosclerosis, but have also been correlated with complications of the disease, for example restenosis after percutaneous transluminal coronary angioplasty (PTCA) and bypass surgery.14,15,16 In experimental studies performed in rat and rabbit SMCs, enrichment of SMCs with cholesterol has been shown to increase the mitogenic response to growth factors (PDGF-BB)17 or to induce synthesis of the RTK ligand FGF-2.18 However, whether elevated cholesterol levels also affect the mitogenic response toward the GPCR ligand thrombin in vascular SMCs has not yet been studied. In a recent study, a complex of methyl-β-cyclodextrin (MbCD) and cholesterol has been used to enrich cellular cholesterol levels.19 After treatment of SMCs with MbCD-cholesterol, a foam cell-like transformation of SMCs has been described, indicating that this method may be useful to increase cholesterol-uptake in SMCs.19 In addition, treatment of cells with MbCD has been described to deplete cell membranes from cholesterol. This results in increased cell membrane stiffness20 and disturbed FGF-2 signaling.21 These effects of MbCD were antagonized and
intracellular cholesterol was enriched by exposing the cells to MbCD saturated with cholesterol.\textsuperscript{20,21} The present study investigates the impact of cholesterol enrichment and depletion on thrombin-induced release of FGF-2 and cell function in human vascular SMCs. In addition to cultured aortic SMCs, saphenous vein SMCs were also used because these cells are widely used as bypass graft material. Our data demonstrate for the first time that accumulated cellular cholesterol enhances thrombin-dependent mitogenesis in human SMCs and does so via increased release of FGF-2.

**Materials and Methods**

**Materials**

Methyl-\(\beta\)-cyclodextrin (MbCD)-cholesterol-complex, cholesterol-free MbCD, heparin, and \(\beta\)-actin antibodies (abs) were from Sigma-Aldrich (München, Germany). Abs against extracellular-regulated kinase (ERK1/2) were from New England Biolabs (Frankfurt, Germany). Neutralizing abs against human basic fibroblast growth factor (FGF-2) and nonspecific control IgG were a generous gift (Friedrich-Schiller-Universität, Jena, Germany). Specific PAR-activating peptides (PAR-AP) were from Biosyntan (Berlin, Germany). GF109203X, PD98059, genistein, BAPTA/AM, colchicine, and adenylyl-imidodiphosphate were from Calbiochem (Darmstadt, Germany).

**Cell Culture**

Human SMCs were prepared from aorta or saphenous veins by the explant technique and cultured as described previously.\textsuperscript{5,22} Cells were synchronized by serum deprivation for 48 hours before the experiments.

**Cholesterol-Enrichment of Human SMC**

Cellular cholesterol was stained by oil-red \(\mathrm{O}\) as described by others.\textsuperscript{19} Nuclei were stained with hemalum. Images were taken with an Olympus BX 50 microscope connected to a color view II camera (Soft Imaging System) as described.\textsuperscript{23} Intracellular concentrations of cholesterol were determined after incubation of SMCs with MbCD-cholesterol (10 \(\mu\)g/mL cholesterol) or MbCD for 24 hours with the Amplex red cholesterol assay kit (Invitrogen) according to the manufacturer’s instructions. Fluorescence was determined at 590 nm using a Fluoroskan Ascent microplate reader (Thermo Labsystems).

**Enzyme-Linked Immunosorbent Assay**

Basic FGF was determined in the media, the pericellular matrix, and intracellularly by using an ELISA (R&D Systems) according to the manufacturer’s instructions. Data were analyzed with the Sequence Detection Software v1.2.3.

**Results and Discussion**

**DNA Synthesis and Cell Count**

DNA synthesis was determined by \([\mathrm{H}]\)-thymidine incorporation, proliferation by cell count as described.\textsuperscript{6,24}

**Statistics**

Data are mean±SEM of \(n\) independent experiments as indicated. Statistical analysis was performed using one-way ANOVA followed by Bonferroni multiple comparison test. Probability values of <0.05 were considered significant.

Human saphenous vein SMCs were incubated with thrombin (30 \(\mathrm{mL}\)) for 5 to 60 minutes (Figure 1A). A maximum release of FGF-2 to the pericellular matrix was seen after 10 to 15 minutes of stimulation, and control levels were observed after 1 to 24 hours (not shown). In contrast, FGF-2 concentrations in the culture media were unchanged over the time of incubation with thrombin (not shown). This is in agreement with previous data in human aortic SMCs.\textsuperscript{6} In addition to our previous study, specific PAR-activating peptides (PAR-AP) were used to evaluate which PARs may be involved in thrombin-induced FGF-2 release. Although RNA for all PARs is expressed in these cells (not shown), only PAR1- and PAR3-AP increased the release of FGF-2 into the pericellular matrix (Figure 1B). PAR4-AP had a similar effect, whereas PAR2-AP was ineffective (Figure 1B). This is consistent with the observation that thrombin activates only PAR1, 3, and 4 but not PAR2.\textsuperscript{20} Of note is that PAR3-AP may not be specific for PAR3. A recent report suggests that it can also activate PAR1.\textsuperscript{27} Human vascular SMCs were enriched with cholesterol by incubating them with MbCD-cholesterol (Figure 2). This method has been reported to induce cholesterol accumulation and to cause transformation of mouse SMCs into a foam cell-like phenotype after incubation for several days.\textsuperscript{19} In human SMCs, the morphology of the cells after incubation with MbCD-cholesterol for 24 hours appeared rather unchanged, except for a vesicular staining with oil-red-O indicating the excessive cholesterol (Figure 2). Intracellular cholesterol concentrations were increased significantly from 2.21±0.17 \(\mu\)g/mL in control cells to 3.88±0.27 \(\mu\)g/mL \((n=6)\) after incubation with MbCD-cholesterol. In contrast, treatment with MbCD has been reported to extract cholesterol from cells and to increase cell membrane stiffness.\textsuperscript{20} In agreement with this report, MbCD significantly reduced cellular cholesterol levels (to 1.66±0.10 \(\mu\)g/mL). The intracellular cholesterol contained about 20% cholesterol-ester, whereas 80% were free cholesterol in all groups (data not shown).

To assess effects of MbCD-cholesterol and MbCD on thrombin-dependent FGF-2 release, both human saphenous vein and aortic SMCs were stimulated with thrombin for 10 minutes after incubation with MbCD-cholesterol or MbCD for 24 hours. Pretreatment with MbCD-cholesterol increased thrombin-released FGF-2 in both cell types, whereas MbCD

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DNA synthesis was determined by \([\mathrm{H}]\)-thymidine incorporation, proliferation by cell count as described.\textsuperscript{6,24}

**Statistics**

Data are mean±SEM of \(n\) independent experiments as indicated. Statistical analysis was performed using one-way ANOVA followed by Bonferroni multiple comparison test. Probability values of <0.05 were considered significant.
inhibited thrombin-induced release of FGF-2 (Figure 1C and 1D). Cell numbers were not affected by pretreatment with either MbCD or MbCD-cholesterol (not shown). These data suggest that accumulated intracellular cholesterol increases FGF-2 release, presumably via increased cell membrane stiffness.20 Another mechanism which could be involved in the observed increase in FGF-2 release by thrombin in cholesterol rich cells may be via increased expression of the PARs or by affecting intracellular GPCR signaling. However, expression levels of PAR mRNAs and of PAR1 protein on the cell surface as determined by flow cytometry were not affected by cholesterol (data not shown). Whether cholesterol affects GPCR-dependent intracellular signaling has not been investigated here.

Because cholesterol accumulation did not only neutralize the inhibitory effect of MbCD on thrombin-induced FGF-2 release but even caused an increased release of FGF-2 by thrombin, we investigated whether cholesterol may stimulate FGF-2 expression. An induction of FGF-2 by a different procedure of elevating cellular cholesterol has been described before in rabbit SMCs.18 In human SMCs, cholesterol loading caused a concentration-dependent induction of FGF-2 mRNA (Figure 3A). Figure 3B shows induction of FGF-2 mRNA determined by real-time PCR. This induction was maximal after 12 hours of cholesterol treatment and was maintained over 24 hours (not shown). Figure 3C and 3D demonstrates increased expression of FGF-2 protein by cholesterol, whereas MbCD had no effect on FGF-2 protein or mRNA levels. In comparison to upregulation of FGF-2, the known receptors involved in FGF-2 signaling—FGFR-1 and syndecan-424—were not affected by cholesterol or MbCD (data not shown).

Thus, increased expression of FGF-2 is likely to account for its enhanced release (Figure 1C and 1D) and the enhanced mitogenic response of cholesterol-treated SMCs after stimulation with thrombin as compared with control and MbCD-treated cells (Figure 4). Consistently, neutralizing FGF-2 by

![Figure 1. Thrombin-induced release of FGF-2 into the extracellular matrix is modulated by cholesterol in human SMCs. Basic FGF was removed from the cell layer by washing with heparin-containing PBS and determined by ELISA. Human saphenous vein SMCs were stimulated with thrombin (30 nmol/L) for the indicated times (A) or with specific PAR-activating peptides (AP1–4, 100 µmol/L each) for 10 minutes (B); mean ± SEM of 5 (A) and 4 (B) independent experiments, *P < 0.05 vs controls. Both human saphenous vein (C) and aortic SMCs (D) were treated with either MbCD-cholesterol (10 µg/mL cholesterol) or the equivalent amount of MbCD for 24 hours. After stimulation with thrombin (30 nmol/L) for 10 minutes, release of FGF-2 into the pericellular matrix was determined by ELISA (mean ± SEM of 4 independent experiments, *P < 0.05 vs control, #P < 0.05 vs cholesterol).](image1)

![Figure 2. Cholesterol enrichment in human SMCs. Cells were incubated with MbCD-cholesterol (10 µg/mL cholesterol) or with the equivalent amount of MbCD for 24 hours. Cholesterol was stained with oil-red O.](image2)
specific antibodies inhibited thrombin-induced DNA synthesis (Figure 4A) and cell proliferation (Figure 4B) in cholesterol-enriched cells to a similar level as pretreatment with MbCD. In agreement with previous results, neutralizing FGF-2 inhibited thrombin-induced DNA synthesis also in the untreated control group. However, when SMCs were pre-treated with MbCD the FGF-2-neutralizing antibody did not yield any additional inhibition on thrombin-mediated DNA synthesis (Figure 4A). Similar results were observed for cell counts (Figure 4B). Whereas MbCD-cholesterol enhanced SMC proliferation induced by thrombin, MbCD and FGF-2-neutralizing antibody inhibited thrombin-induced cell proliferation. In line with these effects of cholesterol loading on thrombin-induced DNA synthesis and proliferation, we observed a pronounced phosphorylation of extracellular signal regulated kinase (ERK) 1/2 in cholesterol-rich SMCs in the late phase of thrombin-induced ERK phosphorylation (Figure 4D). This is consistent with previous observations that the late phase of thrombin-induced ERK phosphorylation is FGF-2 dependent. To determine potential signaling pathways which may be involved in the release of FGF-2 by thrombin, we incubated the cells with various inhibitors of signaling pathways which are either known to be important in thrombin-dependent mitogenesis or are associated with the release of intracellular proteins. Whereas pretreatment with the Rho-kinase inhibitor Y27632 inhibited thrombin-induced release of FGF-2 in untreated and in cholesterol-rich SMCs (Figure 4C), the PKC inhibitor GF109203X, the MAPK inhibitor PD98059, the tyrosine kinase inhibitor genistein, the intracellular calcium chelator BAPTA/AM, EGTA, the cytoskeleton inhibitor colchicine, and the potassium channel inhibitor adenylyl-imidodiphosphate did not reduce thrombin-dependent FGF-2 release (not shown). Y27632 alone had no effect on basal FGF-2 release. In agreement with the literature, incubation with Y27632 (1 to 10 µmol/L) or with PD98059 (10 to 40 µmol/L) inhibited thrombin-induced DNA synthesis in both, control cells and cholesterol-rich cell (not shown). Taken together, these observations indicate that the Rho/Rho kinase pathway may be involved in thrombin-triggered FGF-2 release, whereas ERK activation—although required for thrombin- and cholesterol-mediated mitogenesis—is not essential for the release of FGF-2. However, further investigation will have to follow to clarify this pathway in more detail. In addition, MbCD has been shown to affect FGF-2 signaling probably by disruption of lipid rafts. Lipid rafts are cholesterol-rich membrane domains where FGF-2–binding HSPG like syndecan-4 cluster. Whether disruption of lipid rafts contributes to the MbCD effect on thrombin-induces mitogenesis or may be involved in the release of FGF-2, remains to be determined.

In summary, the present study demonstrates that thrombin-induced mitogenesis is enhanced at elevated intracellular...
cholesterol levels in human vascular SMCs. This might be attributable to enhanced release of FGF-2 by thrombin, presumably via cholesterol-induced transcriptional upregulation of FGF-2 gene expression. This suggests that cellular cholesterol levels may indirectly control thrombin-dependent mitogenesis in human SMCs by modulating FGF-2 expression.

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Disclosures
None.

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