Cellular Cholesterol Depletion Triggers Shedding of the Human Interleukin-6 Receptor by ADAM10 and ADAM17 (TACE)\textsuperscript{*}$\S$

Received for publication, October 16, 2002, and in revised form, June 11, 2003
Published, JBC Papers in Press, June 27, 2003, DOI 10.1074/jbc.M210584200

Vance Matthews, Björn Schuster, Stefan Schütz\textsuperscript{†}, Ingo Bussmeyer, Andreas Ludwig, Christian Hundhausen, Thorsten Sadowski, Paul Saftig, Dieter Hartmann\textsuperscript{§}, Karl-Josef Kallen\textsuperscript{¶}, and Stefan Rose-John\textsuperscript{¶}

From the Biochemisches Institut, Christian Albrechts Universität zu Kiel, Olshausenstrasse 40, D-24098 Kiel, \textit{S}Institut für Immunologie, Christian Albrechts Universität zu Kiel, Michelastrasse 5, D-24105 Kiel, Germany, and \textit{¶}Center for Human Genetics, Leuven and Flanders Interuniversity, B-3000 Leuven, Belgium

Interleukin-6 (IL-6)\textsuperscript{1} activates cells by binding to the membrane-bound IL-6 receptor (IL-6R) and subsequent formation of a glycoprotein 130 homodimer. Cells that express glycoprotein 130, but not the IL-6R, can be activated by IL-6 and the soluble IL-6R which is generated by shedding from the cell surface or by alternative splicing. Here we show that cholesterol depletion of cells with methyl-β-cyclodextrin increases IL-6R shedding independent of protein kinase C activation and thus differs from phorbol ester-induced shedding. Contrary to cholesterol depletion, cholesterol enrichment did not increase IL-6R shedding. Shedding of the IL-6R because of cholesterol depletion is highly dependent on the metalloproteinase ADAM17 (tumor necrosis factor-\textalpha-converting enzyme), and the related ADAM10, which is identified here for the first time as an enzyme involved in constitutive and induced shedding of the human IL-6R. When combined with protein kinase C inhibition by staurosporine or rotterlin, breakdown of plasma membrane sphinogmyelin or enrichment of the plasma membrane with ceramide also increased IL-6R shedding. The effect of cholesterol depletion was confirmed in human THP-1 and Hep3B cells and in primary human peripheral blood monocytes, which naturally express the IL-6R. For decades, high cholesterol levels have been considered harmful. This study indicates that low cholesterol levels may play a role in shedding of the membrane-bound IL-6R and thereby in the immunopathogenesis of human diseases.

\textsuperscript{1} Interleukin-6 (IL-6)\textsuperscript{1} is a pleiotropic cytokine that plays a major role in a variety of human diseases (1). Cellular responses to IL-6 are elicited by binding of IL-6 to the transmembrane IL-6 receptor (IL-6R) which is followed by recruitment of two gp130 molecules into an active, multisubunit receptor complex (2). Homodimerization of gp130 triggers activation of several intracellular signaling pathways, which include the Janus kinase/STAT-, Ras/mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways (3). Neither IL-6 nor the IL-6R alone bind or activate gp130, which indicates that the heterodimeric complex IL-6L-IL-6R constitutes the “active cytokine,” similar to the p35 and p40 subunits of interleukin-12 and interleukin-23, respectively (4, 5). Importantly, the IL-6R is not involved in signal transduction. A soluble form of the IL-6R (sIL-6R) is able to bind IL-6, and the complex of IL-6 and the sIL-6R activates target cells expressing gp130 in a process termed “trans-signaling” (6). The IL-6/sIL-6R complex may therefore either potentiate the IL-6 activity on cells expressing the transmembrane IL-6R or substantially widen the array of potential IL-6 targets to cells devoid of the transmembrane IL-6R, because all body cells express gp130 (7). Recent evidence indicates that the pathophysiological effects of IL-6 may depend strongly on a soluble form of the receptor (8–10).

The soluble form of the IL-6 receptor is generated either by alternative splicing (11) or by proteolytic release of the ectodomain of the membrane-bound IL-6R (12). Shedding is also observed for a variety of other transmembrane proteins, comprising growth factors and their receptors, membrane-bound chemokines, and cell adhesion molecules (13). Phorbol esters trigger shedding of the IL-6R and other proteins, implicating protein kinase C (PKC) in the process (14). In human multiple myeloma, the isoenzymes PKC\textalpha and PKC\textgamma regulate shedding of the IL-6R (15).

Inhibition of cleavage of the human IL-6R and the p60 TNF\textalpha receptor from the cell surface by hydroxymethyl acid-based inhibitors indicated that one or more metalloproteinases are involved in ectodomain shedding (16). TNF\textalpha-Converting enzyme (TACE or ADAM17) belongs to the adamalysins (ADAM, disintegrin and metalloproteinase domain) family of zinc-dependent metalloproteases, which also includes ADAM10. Elimination of the TACE gene was perinatally lethal for most homozygous knock-out mice, although some mice survived for several weeks (17, 18). In TACE\textsuperscript{−/−} cells, ectodomain shedding

---

\textsuperscript{*} This work was supported by Grants SFB415, Project B5, and KA15501-1 from the Deutsche Forschungsgemeinschaft (to S. R.-J. and K.-J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{§} The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

\textsuperscript{†} Both authors contributed equally to this work.

\textsuperscript{¶} To whom correspondence should be addressed: Biochemisches Institut, Christian Albrechts Universitätszu Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany. E-mail: roosejohn@biochem.uni-kiel.de.

\textsuperscript{1} The abbreviations used are: IL-6, interleukin-6; ADAM, a disintegrin and metalloproteinase domain; APP, amyloid precursor protein; IL-6R, interleukin-6 receptor; mCD, methyl-β-cyclodextrin; 2-OH-P\textalpha-CD, 2-hydroxyl-propyl-β-cyclodextrin; PBS, phosphate-buffered saline; PB\textalpha, phorbol dibutyrate; PKC, protein kinase C; PMA, 4-phorbol 12-myristate 13-acetate; sIL-6R, soluble IL-6R; SM, sphingomyelin; sMase, sphingomyelinase; STAT3, signal transducer and activator of transcription; TNF\alpha, tumor necrosis factor-\alpha; TACE, TNF\alpha-converting enzyme, ADAM17; VSMC, vascular smooth muscle cells; HMG, hydroxymethylglutaryl; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; sIL-6R, soluble IL-6R; RT, reverse transcriptase; gp, glycoprotein; LIFR, leukemia inhibitory factor receptor; TAPI, tumor necrosis factor-\alpha protease inhibitor.

This paper is available on line at http://www.jbc.org

38829
Cholesterol Depletion Triggers Shedding of the IL-6 Receptor

EXPERIMENTAL PROCEDURES

Reagents—The metalloprotease inhibitor TAPI was kindly donated by Dr. R. Black (Amgen, Seattle, WA). Phospho-antibodies against STAT3 and -site amylid precursor protein were from Cell Signaling Technology (Beverly, CA); horseradish peroxidase-coupled goat anti-rabbit and anti-mouse antibodies were obtained from Pierce.

Cell Culture—COS-7 cells were obtained from ATCC (Manassas, VA). TACE−/− and TACE−/− mouse ear fibroblasts have been described previously (17, 26). SV40 immortalized ADAM10−/− and ADAM10+/+ fibroblasts were generated as described (47), and both fibroblast lines were obtained from DSMZ (Braunschweig, Germany). All cells were grown in DMEM or RPMI 1640 (PAA Laboratories, Linz, Austria), supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% fetal calf serum. Peripheral blood samples were freshly collected into EDTA (5 mM) containing tubes for isolation of human blood monocytes. The monocytes were adjusted to 1.5 × 109 cells/ml, seeded into culture dishes, and grown in RPMI 1640 supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), 5 × 10−5 M 2-mercaptoethanol, and 5% human AB serum (Sigma). In all lipid depletion experiments, fatty acid-free BSA (10 μg/ml) was substituted for fetal calf or human AB serum.

Alterations of the Cellular Cholesterol and Sphingomyelin Content—Cells undergoing cholesterol depletion were washed with PBS and then treated with mβCD (20 mM), nystatin (240 units/ml), or 2-hydroxypro- pyl-β-cyclodextrin (2-0H-P-β-CD; 20 mM) for 90 min at 37 °C, 5% CO2. All depletion agents were diluted in DMEM or RPMI 1640 containing fatty acid-free BSA (10 mg/ml). Cholesterol enrichment was conducted as described previously (37). Plasma membrane sphingomyelin was digested by exogenous sphingomyelinase (SMase) from S. aureus (Sigma) (48).

Filipin Staining—Cells were grown on polylysine-coated coverslips, rinsed with PBS, and fixed in 1% glutaraldehyde (Fluka, Taufkirchen, Germany) on ice for 15 min. Cells were then rinsed three times with PBS and treated with 50 μg/ml filipin for 30 min at room temperature. Cells were again rinsed thoroughly and mounted in slow Fade Anti-Fade (Molecular Probes, MoBiTec, Goettingen, Germany) and examined using a UV filter.

Lipid Analysis—Cellular lipid composition was determined using methods described previously (48). Lipids were observed using charring densitometry. Briefly, TLC plates (Merck) were dried for 10 min at 180 °C, allowed to cool, and then exposed to a solution of 10% copper sulphate in 5% aqueous phosphoric acid. The plates were then dried for 2 min at 110 °C and charred at 175 °C for 10 min or until lipid bands became visible. After scanning the TLC plates, lipids were quantified using the ImageQuant program.

Transient Transfections, Metabolic Labeling, and Immunoprecipitation of Cell Surface Proteins—COS-7 cells were transiently transfected with the expression vector pCDM5-IL-6R (12), pIREsG-ADAM10 hemagglutinin-tagged construct (50), or empty pIREsG using the DEAE/dextran method. One day after transfection, cells were trypsinized, pooled, and reseeded onto 10-cm cell culture dishes to ensure equal transfection efficiency for all plates. ADAM10+/− and ADAM17−/− cells were transiently transfected with the pCDM8-FuGENE transfection reagent (Roche Diagnostics). Transfection efficiencies on average were 80–90% for COS-7 cells and 20% for ADAM10+/− and ADAM17−/− cells. For pulse-chase experiments, transfected cells (48 h post-transfection) were incubated in methionine/cysteine-free DMEM at 37 °C for 30 min. The cellular proteins were then radioactively labeled with 50 μCl/ml Tras-(35S)label in methionine/cysteine-free medium for 2 h. Subsequently, cells were chased with DMEM containing methionine/cysteine. Cells were then incubated with or without compounds as indicated in the figure legends. Immunoprecipitation of the IL-6R in supernatants and cell lysates followed by autoradiography was performed as described before (51).

Semi-quantitative RT-PCR for Differentially Spliced IL-6R mRNA—Total RNA was extracted from cells by using Triozl reagent (Invitrogen). 1 μg of RNA was reverse-transcribed to cDNA using the Omniscript RT kit (Qiagen, Hilden, Germany) with oligo(dT) primers (0.1 μg/ml, Stratagene, Amsterdam, Netherlands) and then treated with RNase B (10 units, Strat- age) at 37 °C for 1 h. cDNA was amplified in a Robocycler Gradient 96 (Stratagene) using 1 unit of Taq polymerase (MBI Fermentas, St. Leon, Germany) in a 50-μl reaction containing 2 mM MgCl2, 5 μl of 10× buffer with
(NH₄)₂SO₄ and 10 mM dNTPs. PCR conditions and primers were as stated in Horiuchi et al. (11).

Soluble IL-6R ELISA—The levels of soluble IL-6R in culture media were determined as described before (9). Briefly, 960-well ELISA plates (Microlon, Greiner, Frickenhausen, Germany) were coated with the monoclonal anti-sIL-6R 227 antibody (R & D Systems, Wiesbaden, Germany). After blocking, samples and standards (rhIL-6sR; R&D Systems) were added as a dilution in PBS and 1% BSA. The plates were washed, and the biotinylated polyclonal anti-sIL-6R BAF-227 antibody was added, followed by streptavidin/horseradish peroxidase (1:1000 dilution) for 1 h. BM Blue peroxidase (Roche Diagnostics) was added as substrate and incubated in the dark for 10 min. The reaction was stopped by adding 1.8M sulfuric acid (50 μl/well). Absorbance values were measured at 450 nm.

Cholesterol Depletion and Cytokine Stimulation of Hep3B Cells—Hep3B cells were grown to 70–80% confluency in 10-cm dishes after which cells were trypsinized and seeded into 24-well culture plates (TPP, Biochrom, Berlin, Germany). After adherence, cells were washed twice with PBS and starved in serum-free medium. On the following day, serum-free medium was added. After 4 h, cells underwent the following treatments: (i) DMEM containing fatty acid-free BSA with or without 12.5 mM mβCD for 15 min or (ii) DMEM containing fatty acid-free BSA with or without 20 mM mβCD for 90 min. After treatment, supernatants were collected and subjected to sIL-6R ELISA measurements. Medium with or without cytokines (IL-6, 50 ng/ml, or oncostatin M, 50 ng/ml) was added for 10 min. Medium was removed; cells were washed once with PBS, and cells were lysed directly in 2× Laemmli buffer. Samples were heated for 5 min at 95 °C, subjected to SDS-PAGE, and blotted to a polyvinylidene difluoride membrane (Amersham Biosciences). The membranes were incubated with the anti-phospho-STAT3 antibody (Cell Signaling, Schwabach, Germany) before being labeled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed using the Amersham Biosciences ECL PLUS chemiluminescence kit. Loading was controlled by a Western blot against β-actin. In other Western blotting experiments, anti-ADAM10 (B 42.1; 1 in 1000), anti-ADAM9 (5 μg/ml; Chemicon, Hofheim, Germany), anti-ADAM17 (M220; 0.1 μg/ml), anti-hemagglutinin (0.4 μg/ml; Santa Cruz Biotechnology, Heidelberg, Germany), or anti-PC-tag (HPC4, 0.8 μg/ml; Roche Diagnostics) were used as primary antibodies. The M220 antibody was kindly provided by Dr. R. Black (52), whereas the B 42.1 antibody recognizing murine ADAM10 was generated in the laboratory of Dr. D. Hartmann (Leuven, Belgium) (47).

RESULTS

Treatment with mβCD was used to alter the cellular cholesterol levels. Incubation with 20 mM mβCD for 90 min reduced cellular cholesterol levels by around 50% (Fig. 1A). Lower mβCD concentrations and shorter treatments (data not shown) resulted in less efficient cholesterol reduction, and treatment with 20 mM mβCD was therefore used throughout this study. Cholesterol enrichment of around 30% was achieved by overnight incubation of cells with mβCD preloaded with cholesterol (Fig. 1B). Treatment of COS-7 cells with S. aureus SMase...
reduced the cellular SM levels by 50–70% (Fig. 1C), consistent with the results achieved in other cell lines (53, 54). Successful cholesterol depletion of the plasma membrane by mβCD treatment was confirmed by reduced binding of filipin to cholesterol-depleted cells (Fig. 1, D and E). All cells used in this study remained attached to culture surfaces, and viability was ≥98%. Apoptosis was not induced by any of the lipid-altering treatments used in this study as demonstrated by the absence of characteristic DNA fragmentation (data not shown).

To study the effect of cholesterol depletion on IL-6R shedding, COS-7 cells were transiently transfected with a human IL-6R cDNA. On the 2nd day after transfection, the cells were pulse-labeled with radioactive [35S]cysteine and [35S]methionine for 2 h followed by a 2-h chase. Sheding of the IL-6R was ascertained by immunoprecipitation of the radioactively labeled IL-6R in the supernatant and cell lysates. This assay detects only sIL-6R generated by shedding (12, 28, 51). IL-6R shedding was consistently increased after 1.5 h of cholesterol depletion with 20 mM mβCD compared with basal IL-6R shedding (control; Fig. 2A). The time course of IL-6R shedding induced by cholesterol depletion differed from that observed after activation of PKC by phorbol myristate acetate (PMA) (12). Shedding of the IL-6R was hardly detectable after 30 min and first became apparent after around 1 h. IL-6R shedding caused by treatment with 20 mM mβCD was stronger than that after 10 mM mβCD and comparable with PMA-induced IL-6R shedding (Fig. 2A). In contrast to cholesterol depletion, cholesterol enrichment did not result in increased IL-6R shedding (Fig. 2B). The absence of cleaved sIL-6R in the supernatant of cholesterol-enriched cells was not due to high level shedding of the IL-6R prior to or during metabolic labeling, because there were equal levels of labeled IL-6R in the cell lysates of control and cholesterol-enriched cells (Fig. 2C). Furthermore, other cholesterol-depleting agents like nystatin or 2-OH-PβCD also resulted in increased shedding of the IL-6R (Fig. 2D).

We next asked whether PKC was involved in IL-6R shedding induced by cholesterol lowering. Whereas the broad spectrum
PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase

PKC inhibitors staurosporine and bisindolylmaleimide effectively inhibited PMA-induced shedding, they did not influence IL-6R shedding due to cholesterol depletion, suggesting that the sheddase was activated in a PKC-independent manner (Fig. 3, A and B). This was confirmed by analyzing shedding in COS-7 cells that had been depleted of PKC by prolonged pretreatment of cells with the phorbol ester analogue PBt2. Cellular levels of PKC remain low for several hours after washing out the PBt2 (55). Although PMA-induced shedding was largely reduced in these cells, mβCD induced shedding was unaffected by PKC depletion (Fig. 3C). In contrast to cholesterol depletion, SMase treatment of COS-7 cells transfected with the huIL-6R had only a weak influence on shedding (Fig. 3A, 6th lane; Fig. 4A). Unexpectedly, however, shedding of the IL-6R after SMase treatment was largely enhanced by co-treatment with the PKC inhibitor staurosporine (Fig. 4A). Co-treatment with SMase
FIG. 6. IL-6 shedding in ADAM<sup>−/−</sup> and TACE<sup>−/−</sup> cell lines. A, metalloproteinase expression in knock-out cell lines. Tace<sup>−/−</sup>, Tace<sup>−/+</sup>, ADAM10<sup>−/−</sup>, and ADAM10<sup>−/+</sup> cells were examined for expression of the three major metalloproteinases involved in ectodomain shedding. The total protein concentrations assayed in each Western blot were kept constant for each comparable cell line. B, influence of TACE on the shedding of the IL-6R. Tace<sup>−/−</sup> and Tace<sup>−/+</sup> fibroblasts were transiently transfected with a human IL-6R cDNA using the FuGENE transfection reagent. One day after transfection, cells were trypsinized, pooled, and reseeded onto 35-mm diameter plates to ensure transfection efficiency was equal for all plates. 48 h after transfection, cells were treated with TAPI (100 μM), PMA (10 μM), mβCD (20 mM), sphingomyelinase (0.1 units/ml; SMase), staurosporine (1 μg/ml), or mβCD-cholesterol complex (1 mM; cholesterol enrichment) for 90 min. sIL-6R protein in the culture media was quantified using an ELISA specific for human IL-6R. Data are shown as mean ± S.E. of triplicates. C, influence of ADAM10 on the shedding of the IL-6R. ADAM10<sup>−/−</sup> and ADAM10<sup>−/+</sup> mouse fibroblasts were transfected and treated as described in B. sIL-6R protein in the culture media and the cell lysates was quantified using the shIL-6R-specific ELISA. Data are shown as mean ± S.E. of triplicates. D, influence of ADAM10 on the shedding of the IL-6R. ADAM10<sup>−/−</sup> fibroblasts were transiently transfected with hIL-6R cDNA together with empty pIREScg vector or pIREScg(ADAM10) plasmid. Cells were treated with media, PMA (10 μM), or mβCD (20 mM) for 30 min. The sIL-6R protein in the culture media and lysate was quantified using an ELISA specific for human IL-6R. Data are shown as mean ± S.E. of quadruplicates. E, ADAM10 overexpression enables earlier constitutive and inducible shedding of the IL-6R. COS-7 cells were transiently transfected with a human IL-6R cDNA in
and the PKC inhibitor rottlerin, originally considered to be PKCδ-specific, had the same effect as SMase/staurosporine (Fig. 4B). Recent evidence demonstrates that rottlerin also acts by uncoupling mitochondrial ATP production and is not a specific PKCδ inhibitor (56).

To clarify whether the effect was due to a general disruption of the lipid phase as a consequence of SM breakdown or due to increased amounts of plasma membrane ceramide generated by the SMase treatment (54), we enriched the plasma membrane with C16- and C18-ceramide analogues (57). The ceramide analogues alone did not affect IL-6R shedding, but in conjunction with staurosporine, IL-6R shedding was increased, which did not reach the levels observed after PMA induction of shedding (Fig. 4, C and D). Both the enhanced IL-6R shedding after cholesterol depletion and after SMase/staurosporine treatment were subject to inhibition by TAPI (Fig. 5). To exclude the possibility that cholesterol depletion caused pore formation in the plasma membrane, thereby allowing extracellular calcium to trigger IL-6R shedding (30), cholesterol-depleted cells were also incubated in the presence of EGTA. However, this did not alter release of the IL-6R from the cell surface (Fig. 5). Pretreatment with cycloheximide or actinomycin did not affect IL-6R shedding after SMase/staurosporine treatment (data not shown) which suggests that de novo protein biosynthesis and mRNA synthesis are not required for the increased level of IL-6R shedding.

To ascertain whether TACE was involved in shedding of the IL-6R after cholesterol depletion, we investigated the effect of cholesterol depletion and plasma membrane SM breakdown in TACE-/- and in TACE-/- fibroblasts, which are retransfected with TACE (17, 26). The genotype of these cells was confirmed by Western blot (Fig. 6A) and Southern blot, utilizing a probe corresponding to exon 11 (the Zn2+-binding motif) of the murine TACE gene, thus demonstrating the absence of exon 11 in TACE-/- cells and the presence of the exon 11 sequence in TACE-/- fibroblasts (data not shown). There was clear constitutive shedding in TACE-/- fibroblasts, which was enhanced in the TACE-/-/+ fibroblasts (Fig. 6B). Cholesterol depletion caused a significant increase of IL-6R shedding in both TACE-/- and in TACE-/-/+ fibroblasts, and this could be inhibited by TAPI (Fig. 6B). Cholesterol enrichment did not result in significantly increased shedding of the IL-6R. As in COS-7 cells, PMA or the combination of SMase and staurosporine significantly increased IL-6R shedding in TACE-/-/+ cells. Because the ELISA used in the experiments is specific for the human IL-6R, increased expression of the murine sIL-6R by alternative splicing could not confound our data. Thus, these results demonstrate an important role of TACE in the enhanced shedding of the IL-6R after cholesterol depletion but also indicated involvement of another metalloproteinase in IL-6R shedding (see below).

Therefore, IL-6R shedding was also compared between fibroblasts from wild type and ADAM10-/- mice (Fig. 6C) (47), in which the genotype was also confirmed by Western blot (Fig. 6A). Constitutive as well as PMA-induced IL-6R shedding was increased in wild type fibroblasts compared with ADAM10-/- fibroblasts indicating for the first time that the IL-6R might be a substrate also of ADAM10. A similar effect was also seen in cholesterol-depleted cells, suggesting that ADAM10 contributes to shedding of the IL-6R after cholesterol depletion. The apparent stimulatory effect after cholesterol enrichment and SMase treatment is most likely due to different transfection efficiencies of ADAM-/- cells and normal fibroblasts because this difference is not visible when ratios of sIL-6R versus membrane bound IL-6R are compared (data not shown).

To ask whether ADAM10 increased IL-6R shedding, we performed additional studies in ADAM10-/- fibroblasts and COS-7 cells. ADAM10-/- fibroblasts were transiently transfected with a human IL-6R cDNA in combination with empty pIREScg vector or pIREScg(ADAM10). As can be seen in Fig. 6D, constitutive shedding, PMA-induced shedding, and in particular mβCD-induced shedding was strongly increased after co-transfection of the ADAM10-/- cells with an ADAM10 cDNA indicating that ADAM10 might be involved in IL-6R shedding in these cells.

To support these findings further, we transiently transfected COS-7 cells with a human IL-6R cDNA in combination with empty pIREScg vector or pIREScg(ADAM10). We stimulated the cells with PMA and mβCD. Shedding of the IL-6R after PMA stimulation was analyzed after 30 min, since we have shown that such shedding is complete after 40 min (12, 14). Similarly, shedding of the IL-6R after mβCD stimulation was analyzed after 30 and 60 min because shedding was complete combination with empty pIREScg vector or pIREScg(ADAM10) plasmid and incubated with PMA (10-7 M), mβCD (20 mM), or left untreated (control) for the times indicated in the figure. Radioactively labeled human sIL-6R (60 kDa) was subsequently immunoprecipitated from the supernatants. F, ADAM10 overexpression increases long term constitutive shedding of the human IL-6R. COS-7 cells were transiently transfected with a human IL-6R cDNA in combination with empty pIREScg vector or pIREScg(ADAM10) plasmid and were incubated for 8 and 23 h with media. Soluble IL-6R was subsequently immunoprecipitated from the supernatants.
after 90 min (Fig. 2A). As can be seen in Fig. 6E, uninduced and PMA-induced shedding was markedly accelerated after transfection of the cells with an ADAM10 expression plasmid. When ADAM10-transfected cells were stimulated with mβCD, shedding of the IL-6R was already stimulated after 30 min, while in cells transfected with empty vector shedding was only observed after 60 min. Again, these data indicate that ADAM10 might be involved in constitutive and induced shedding of the human IL-6R. Constitutive shedding in transfected COS-7 cells was further investigated for longer incubation times. We had shown previously that shedding of the IL-6R in COS-7 cells became detectable after 8 h and was complete after 24 h (12, 14) when using metabolic labeling. As can be seen in Fig. 6F, constitutive IL-6R shedding of COS-7 cells overexpressing ADAM10 was strongly induced after 8 and 23 h without further stimulation. Taken together these data show that not only complementation of ADAM10−/− cells with ADAM10, but also overexpression of ADAM10 in COS-7 cells leads to increased levels of constitutive as well as of PMA- and mβCD-induced shedding of the human IL-6R.

The above experiments demonstrated that in vitro, cholesterol depletion due to mβCD resulted in marked increases in IL-6R shedding in several independent non-human cell lines. To determine whether mβCD treatment also resulted in increased shedding of the IL-6R in human cells naturally expressing the IL-6R, we utilized THP-1 cells, a human acute monocytic leukemia cell line (Fig. 7A) and peripheral blood monocytes isolated from different donors (Fig. 8). The THP-1 cells showed a strong increase in IL-6R shedding after treatment with PMA and mβCD, whereas there was only a slight increase in shedding following cholesterol enrichment (Fig. 7A). In contrast to the results obtained in COS-7 cells, treatments affecting plasma membrane SM did not cause increased shedding (Fig. 7A). TAPI significantly decreased the shedding of the IL-6R for all treatments. To exclude the possibility that expression of the alternatively spliced variant of the sIL-6R was induced by cholesterol depletion, expression of the alternatively spliced form of the sIL-6R was examined by RT-PCR. If anything, there was a decrease in the expression of the spliced form of the sIL-6R (304 bp) (Fig. 7B), supporting that the increased sIL-6R level observed after cholesterol depletion is due to shedding.

We also found that cholesterol depletion increases shedding of the IL-6R from human monocytes. In all four donors, the IL-6R shedding induced by mβCD was greater than that induced by PMA (Fig. 8A). This trend was previously seen for TACE−/− cells (Fig. 6B) and THP-1 cells (Fig. 7). Only the mβCD-induced shedding proved to be significantly increased compared with constitutive shedding in monocytes from all donors.

A recent study suggested that after treatment of human Hep3B hepatoma cells with mβCD, IL-6 was no longer able to induce activation of STAT3 (58). Disruption of lipid rafts was given as an explanation. We therefore investigated whether shedding of the IL-6R might have contributed to the inhibition of STAT3 under the conditions used by these authors. Hep3B cells were treated for 15 min with 12.5 mM mβCD prior to stimulation with IL-6 or human oncostatin M (Fig. 9A), which was used as an additional negative control, because Hep3B cells do not express LIFR or oncostatin M receptor. Neither shedding of the IL-6R nor inhibition of STAT3 phosphorylation was observed. However, when we used the conditions established in this study for the treatment of Hep3B cells, we observed strongly enhanced shedding of the IL-6R and complete abolition of STAT3 phosphorylation (Fig. 9B). As in THP-1 cells, cholesterol depletion did not increase but rather decreased expression of the alternatively spliced sIL-6R variant (Fig. 9C). Thus, impaired signaling of IL-6 in cholesterol-depleted cells could be due to increased shedding of the IL-6R.

**DISCUSSION**

The results of this study clearly demonstrate that cellular cholesterol levels affect the shedding of the IL-6R. The effect is in the same range as that seen after phorbol ester stimulation.
The enhanced cleavage of the IL-6R after cholesterol depletion is mediated by metalloproteinases as evidenced by the inhibitory effect of TAPI on this process and expression of the alternatively spliced form of the sIL-6R not being increased upon cholesterol depletion (Figs. 7B and 9C). Experiments with staurosporine, bisindolylmaleimide, and PKC-depleted cells showed that cholesterol depletion stimulates enhanced activity of these metalloproteinases independently of PKC. The difference in IL-6R shedding between TACE/H11002/H11002/H11002 cells and TACE/H11002/H11002/H11002/H11002 cells after cholesterol depletion clearly demonstrates that TACE-dependent shedding of the IL-6R is influenced by the plasma membrane cholesterol content. However, there was clearly enhanced IL-6R shedding also in the TACE−/− cells, which is also inhibited by TAPI (Fig. 6B). Thus, TACE is not the only metalloproteinase involved in shedding of the IL-6R. Similar to ADAM10, TACE also participates in α-secretase cleavage of APP (22, 23). Compared with ADAM10+/− fibroblasts, wild type fibroblasts and ADAM10-transfected ADAM10−/− cells showed stronger shedding of the IL-6R after PMA stimulation and cholesterol depletion. Moreover, overexpression of ADAM10 in COS-7 cells led to earlier shedding after PMA and mβCD stimulation as well as to stronger constitutive shedding.

We conclude from these data that IL-6R might be a novel substrate for ADAM10 (Fig. 6, C–F), although we cannot exclude at the present time that ADAM10 increases IL-6R shedding via an indirect mechanism.

It is not known how TACE and ADAM10 recognize their different targets. Recently, cleavable chimeras were constructed by exchanging the juxtamembrane sequence of gp130 for the corresponding region of leukemia inhibitory factor receptor, although neither LIFR nor gp130 is subject to regulated or spontaneous shedding (29). Similarly, the exchange of metalloproteinase cleavage sites between IL-6R and pro-TNFα resulted in constitutive or largely abolished shedding of the chimeric proteins. Rather than minimal consensus shedding sequences, structural and kinetic characteristics of proteins were suggested to regulate access of the protease to the cleavage site (29).

Low cholesterol was shown to stimulate APP cleavage by an effect on the α-secretase ADAM10 (37). It is known that cholesterol depletion causes disruption of lipid rafts and may thus interfere with cellular processes such as receptor transactivation or antigen presentation (46). Although APP binds to caveolin, a protein associated with at least some forms of lipid rafts
Cholesterol Depletion Triggers Shedding of the IL-6 Receptor

(59, 60), disassembly of lipid rafts was excluded as a cause of the increased APP cleavage by ADAM10 in cholesterol-depleted cells. Changes of 1,6-diphenyl-1,3,5-hexatriene steady-state fluorescence anisotropy in biological membranes enables estimation of lipid structural order, which is strongly influenced by the cholesterol content of membranes (61, 62). By using this method, Kojro et al. (37) concluded that cholesterol depletion causes an increase of the fluidity of the plasma membrane and suggested this as a mechanism contributing to increased APP cleavage. In line with this notion, a recent report (63) showed strongly increased lipid phase separation in cholesterol-depleted cells, resulting in large confluent areas of lipid ordered and disordered states. Individual rafts are small and of heterogeneous protein composition and may thus keep raft-associated protein processes in an “off” state, because the proteins participating may be localized in different rafts (46). Clustering of similar lipid phases would then be required for activation. Therefore, it appears conceivable that increased IL-6R shedding by TACE and ADAM10 in cholesterol-depleted cells may be due to increased accessibility of the protease to the receptor cleavage site. This could result from either a direct effect of lipid phase change on the dynamic structure of the proteins involved in shedding, i.e. substrate and enzyme. Alternatively it could be due to the fact that larger subdomains of similar lipid order status exist in cholesterol-depleted cells, thus facilitating association of substrate and enzyme. A similar process was recently stated to be responsible for attenuated STAT3 phosphorylation after IL-6 stimulation of cholesterol-depleted cells (65). However, the ceramide response requires the kinase activity of PKC, i.e. substrate and enzyme. A similar mechanism is thus not expected to explain attenuation of STAT3 phosphorylation in cholesterol-depleted cells.

Breakdown of SM in the plasma membrane causes increased efferocytosis of cholesterol, but most of the cholesterol freed from the direct interaction with SM remains in the plasma membrane (64). Although we observed a somewhat increased shedding of the IL-6R after SM breakdown in some experiments, clearly increased shedding was only observed after cotreatment with the PKC inhibitors staurosporine and rottlerin. Substitution of SMase treatment by enrichment of the plasma membrane with C16– or C18–ceramide also causes increased shedding of the IL-6R when cells were co-treated with staurosporine. The mechanism underlying this observation is unclear. Ceramide interferes with the reverse translocation of classical PKC isoenzymes by inhibition of NF-kB activation (65). However, the ceramide response requires the kinase activity of PKC and therefore is unlikely to be at the basis of our observation. Ceramide also inhibits Akt kinase through PKCζ (66). Because several bacteria, e.g. S. aureus or Bacillus cereus, express potent SMases, one may speculate whether increased IL-6R shedding by this mechanism is involved in the pathology caused by these pathogens.

In patients with hepatitis B infection, high sIL-6R and normal IL-6 concentrations indicated successful clearance of hepatitis B virus after interferon-α therapy (67). Hypercholesterolemia suppresses antiviral cytotoxic T cell responses and thereby exacerbates virus-induced hepatic diseases (68). In the light of our results, suppression or decreased cleavage of the IL-6R might be one of the causes for this. Increasing attention has been directed recently to the significance of inflammatory processes in the pathogenesis of cardiovascular disease and in particular of atherosclerosis (33). VSMC proliferation is one of the histological hallmarks of this disease and can be triggered by the complex of IL-6 and sIL-6R (32). Moreover, the uptake of enzymatically degraded LDL by blood monocytes causes IL-6 secretion and shedding of the IL-6R and induction of MCP-1 (69). On VSMC and endothelial cells, the IL-6-sIL-6R complex induces expression of MCP-1, MCP-3, IL-8, IL-6, gp130, LIFR, VCAM-1, ICAM-1, and protein S, thus providing the basis for leukocyte recruitment and adhesion (10). IL-8 can induce shedding of the IL-6R on neutrophils by stimulation of CXCR1, which can thus be a source of the sIL-6R for activation of endothelial cells (10). IL-6 and sIL-6R are thus at the basis of an autocrine-positive feedback loop, which can ultimately lead to inflammation and proliferation of cells constituting the vessel wall.

A plethora of studies using HMG-CoA reductase inhibitors to lower serum levels of cholesterol in the secondary prevention of cardiovascular disease has demonstrated the beneficial effects of such treatment (34). Low cholesterol levels are therefore considered a key end point to reduce lethality from cardiovascular diseases. However, recent evidence suggests that HMG-CoA reductase inhibitors have an anti-inflammatory effect, which is independent of their cholesterol-reducing action (35, 36). It has been pointed out that in men there is a trough-like relationship between cholesterol levels and mortality, with the latter rising at both low and high cholesterol levels, and that in women mortality is higher at lower cholesterol levels (70). A recent longitudinal study in a large cohort of patients found that the quartile with the lowest serum cholesterol levels showed the highest mortality independent of confounding variables, a finding for which there is no explanation so far (71).

Our observation that depletion, but not enrichment, of cellular cholesterol levels increases shedding of the IL-6R raises the possibility that constant low levels of cholesterol may favor inflammatory processes entertained also by the IL-6/sIL-6R complex (8–10, 32). Whether or not this is the cause for the increased mortality in patients with low cholesterol levels is an open question.

Acknowledgments—We thank Dr. R. Black (Amgen, Seattle) for the kind donation of TAPI, the gift of the M220 antibody, and the TACE−/− and TACE−/− fibroblasts. Dr. E. Kojro and Dr. F. Fahrenholz (Universität Mainz, Mainz, Germany) are warmly thanked for their gift of the control and TACE fibroblasts. Dr. E. Kojro and Dr. F. Fahrenholz (University of Turku, Finland) with the alterations of cellular cholesterol levels is warmly acknowledged.

REFERENCES

1. Kallen, K.-J., Galle, P. R., and Rose-John, S. (1999) Exp. Opin. Investig. Drugs 8, 1327–1349.
2. Grotzinger, J., Kernebeck, T., Kallen, K. J., and Rose-John, S. (1999) Biol. Chem. Hoppe-Seyler 380, 803–813.
3. Heinrich, P. C., Behrmann, I., Müller-Newton, G., Schaper, F., and Graeve, L. (1998) Biochem. J. 334, 297–314.
4. Gearing, D. P., and Cosman, D. (1991) Cell 66, 9–10.
5. Oppmann, B., Lexley, R., Elms, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaiberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., and Kastelein, R. A. (2000) Immunity 13, 715–727.
6. Rose-John, S., and Heinrich, P. C. (1994) Biochem. J. 300, 281–290.
7. Peters, M., Müller, A. M., and Rose-John, S. (1998) Blood 92, 3495–3504.
8. Arets, J., Mudder, J., Finnato, S., Mullberg, J., Justek, T., Wurtz, S., Schütz, M., Bartsh, B., Heitmann, M., Becker, C., Strand, D., Czaja, J., Schlaak, J. F., Lehr, H. A., Autschbach, F., Schurmann, G., Nishimoto, N., Yoshizaki, K., Inoue, H., Kishimoto, T., Galie, P. R., Rose-John, S., and Neurath, M. F. (2000) Nat. Med. 6, 583–588.
9. Hurst, S. M., Wilkinson, T. S., McLaughlin, R. M., Jones, S., Horisuchi, S., Yamamoto, N., Rose-John, S., Fuller, G. M., Topley, N., and Jones, S. A. (2001) Immunity 14, 705–714.
10. Kallen, K.-J. (2002) Biochim. Biophys. Acta 1592, 323–343.
11. Horisuchi, S., Koyanagi, Y., Zhou, Y., Miyamoto, H., Tanaka, Y., Waki, M.,
