C1q/TNF-Related Protein-3 Represents a Novel and Endogenous Lipopolysaccharide Antagonist of the Adipose Tissue

Andrea Kopp, Margarita Bala, Christa Buechler, Werner Falk, Philipp Gross, Markus Neumeier, Jürgen Schölmerich, and Andreas Schäffler

Department of Internal Medicine I, Regensburg University Hospital, D-93042 Regensburg, Germany

Proteins secreted by adipocytes (adipokines) play an important role in the pathophysiology of type 2 diabetes mellitus and the associated chronic and low-grade state of inflammation. It was the aim to characterize the antiinflammatory potential of the new adipocytokine, C1q/TNF-related protein-3 (CTRP-3), which shows structural homologies to the pleiotropic adipocytokine adiponectin. mRNA and protein expression of CTRP-3 was analyzed by RT-PCR and Western blot. Recombinant CTRP-3 and small interfering RNA-based strategies were used to investigate the effect of CTRP-3 on toll-like receptor (TLR) ligand, lipopolysaccharide (LPS)-, and lauric acid-induced chemokine release of monocytes and adipocytes. Together with complex ELISA-based techniques, a designed TLR4/myeloid differentiation protein-2 fusion molecule shown to bind LPS was used to prove the ability of CTRP-3 to act as endogenous LPS antagonist. CTRP-3 is synthesized in monocytes and adipocytes. The recombinant protein dose-dependently inhibits the release of chemokines in monocytes and adipocytes that were induced by lauric acid, LPS, and other TLR ligands in vitro and ex vivo. CTRP-3 inhibits monocyte chemoattractant protein-1 release in adipocytes, whereas small interfering RNA-mediated knockdown of CTRP-3 up-regulates monocyte chemoattractant protein-1 release, reduces lipid droplet size, and decreases intracellular triglyceride concentration in adipocytes, causing a dedifferentiation into a more proinflammatory and immature phenotype. By using a designed TLR4/MD-2 fusion molecule, it is shown by different techniques that CTRP-3 specifically and effectively inhibits the binding of LPS to its receptor, TLR4/MD-2. CTRP-3 inhibits three basic and common proinflammatory pathways involved in obesity and type 2 diabetes mellitus (adipo-inflammation) by acting as an endogenous LPS antagonist of the adipose tissue. (Endocrinology 151: 5267–5278, 2010)

Visceral obesity, insulin resistance, and type 2 diabetes mellitus are characterized by a chronic and low-grade state of inflammation (1–3). Cytokines, chemokines, and adipokines seem to be involved in this local and systemic proinflammatory activation. Immunomodulating proteins of the adipose tissue are of major interest regarding underlying pathophysiology and identification of future drug targets. Since the discovery of TNF synthesis by adipocytes in 1993 (4) and toll-like receptor (TLR) expression and lipopolysaccharide (LPS) responsiveness in adipocytes in 2000 (5), it has become evident that the adipose tissue exerts multiple and important immune functions (6, 7).

It was demonstrated by structural analysis of the anti-inflammatory and antidiabetic adipocytokine adiponectin in 1998 (8) that the TNF-ligand family proteins and the C1q complement family proteins arose by divergence from an ancient precursor recognition molecule of the innate immune system. Based on these structural considerations, the name C1q/TNF-related protein (CTRP) family has been introduced (7).

Concerning the family member CTRP-3 (synonyms are CORS-26, cartonectin, and cartducin), only sparse data exist. The genomic structure of human (9) and murine (10–12) CTRP-3 clearly shows striking homologies to the...
CTRP family member adiponectin. CTRP-3 has been shown to regulate adipocyte adipokine secretion by inducing the release of adiponectin and resistin (13). CTRP-3 mRNA levels are strongly induced during adipocyte differentiation, and the promoter region (14) of CTRP-3 carries a functional peroxisome proliferator-activated receptor-y response element. Besides its role in adipocyte differentiation and metabolic regulation, CTRP-3 is also expressed in cartilage/chondrocytes (15) and stimulates the proliferation of endothelial cells (16), chondroprogenitor cells (17), and osteosarcoma cells (17). Moreover, CTRP-3 expression was also reported in monocytes cells (18). CTRP-3 forms homotrimeric protein structures after recombiant expression (18), and it circulates in human plasma and in peritoneal fluid surrounding visceral adipose tissue (19) as a high molecular weight form (20).

In the context of obesity and a high-fat diet, the visceral adipose tissue becomes infiltrated by high quantities of macrophages (1, 2, 21), a mechanism that is mediated by local monocyte chemoattractant protein (MCP)-1 release of adipocytes. By using TLR4-deficient mice, Davis et al. (22) demonstrated that mice lacking TLR4 are protected from obesity induced by a diet rich in palmitic acid. Moreover, these TLR4-deficient animals have a lower grade of macrophage infiltration and MCP-1 expression in their visceral adipose tissue. These data strongly argue for an important role of TLR4 and MCP-1 in regulating the obesity- and fatty acid-related inflammatory response in visceral adipose tissue. Three recent publications (23–25) highlight the role of visceral adipose tissue inflammation and infiltration by distinct immune cells. Monocytic infiltration and subsequent generation of a proinflammatory micro-milieu in the adipose tissue causes local and systemic insulin resistance and might lead to type 2 diabetes mellitus and vascular disease (26). The local cross talk (7) between adipose tissue macrophages and adipocytes is poorly characterized, but TNF, LPS, and fatty acids (27–31) seem to play a role as mediators between these two cell types.

It was the aim of the present study to characterize the molecular mechanisms of the antiinflammatory effects of CTRP-3 on three basic and common proinflammatory pathways in the context of obesity, namely LPS-, other TLR ligands-, and fatty acid-induced chemokine release. By using small interfering RNA (siRNA)-mediated knockdown of CTRP-3 and a designed TLR4/MD-2 fusion molecule, we further aimed to investigate the potential role of CTRP-3 as a novel LPS antagonist of the adipose tissue.

**Materials and Methods**

**Recombiant CTRP-3 expression**

Recombiant CTRP-3 protein expression was performed in High 5 insect cells (Invitrogen, Karlsruhe, Germany) using the BacPAK baculovirus expression system (BD Biosciences, Palo Alto, CA) as published earlier by our group (18). Supernatants were collected 3 d after infection for purification of CTRP-3 using the BD Talon purification kit (BD Biosciences). Integrity and purity of the protein were analyzed by immunoblot and silver staining of SDS-PAGE. In contrast to Escherichia coli-based expression systems, the recombiant expression in insect cells usually maintains glycosylation and phosphorylation. Based on this, we could demonstrate earlier that our expression system generates trimeric CTRP-3 that was used for stimulation experiments (18, 20).

**SDS-PAGE and immunoblotting**

Proteins were transferred to an Immob-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules CA). Incubations with antibodies were performed in 5% nonfat dried milk in PBS and 0.1% Tween 20. The expression of proteins was investigated by using antibodies against CTRP-3 (rabbit; Abcam, Cambridge, UK; 1:1000); β-actin, p38 MAPK, and phospho (p)-p38 MAPK (Thr180/Tyr182); p-ERK-1/-2 (p-p44/42; Thr202/Tyr204) (rabbit; Cell Signaling Technology, Beverly, MA; 1:1000); and ERK1/2 (Upstate Biotechnology, Lake Placid, NY). The secondary horseradish peroxidase (HRP)-coupled antirabbit IgG antibody (Jackson ImmunoResearch, Newmarket, UK) was used in a 1:5000 dilution in a 5% nonfat dried milk in PBS suspension. Detection of immune complexes was carried out with the enhanced chemiluminescence Western blot detection system (Amersham ECL Western blotting system; GE Healthcare, Freiburg, Germany). All Western blot experiments were performed at least in triplicate.

**3T3-L1 adipocyte cell culture**

3T3-L1 preadipocytes (32) were cultured at 37 C and 5% CO2 in DMEM (Biochrom AG, Berlin, Germany) supplemented with 10% newborn calf serum (Sigma Biosciences, Deisenhofen, Germany) and 1% penicillin/streptomycin (PAN, Aidenbach, Germany). At confluence, cells were differentiated into adipocytes by DMEM/F12/glutamate medium (Lonza, Basel, Switzerland) supplemented with 0.5 mm 3-isobutyl-methyl-xanthine (Serva, Heidelberg, Germany), 10−7 M corticosterone, 10−7 M insulin, 200 mm ascorbate, 2 μg/ml transferrin, 5% newborn calf serum, 1 mm biotin, 17 mm pantothenate, 1% penicillin/streptomycin, and 300 mg/liter Pedersen-fetuin (MP Biomedicals, Illkirch, France) (33, 34) for 9 d using a slightly modified protocol as described in the literature (32, 35–38). Phenotype was controlled by light microscopy (typical appearance of extensive accumulation of lipid droplets) and by measuring the secretion of differentiation markers such as adiponectin and resistin. Only mature adipocytes at d 8 of differentiation were used for stimulation experiments. Preceding the stimulation experiments, cells were washed with PBS and incubated under serum-free culture conditions. 3T3-L1 adipocytes were stimulated for 18 h with LPS and other specific TLR ligands. LPS (TLR4), Pam3Cys (TLR1/2), polyinosinic-polycytidylic acid [poly(I:C)] (TLR3), flagellin (TLR5), macrophage activating lipopeptide-2 (TLR2/6), polyuridine (TLR7/8), and CpG (ODN 2395) (TLR9) were
used for stimulation experiments in the presence and absence of increasing doses of CTRP-3 (0.2, 1.0, and 10 μg/ml) and purchased from Axxora, Lörrach, Germany.

Measurement of chemokine and cytokine concentrations in the supernatants of primary human monocytes and 3T3-L1 adipocytes

The concentrations of macrophage migration inhibitory factor (MIF), MCP-1, CC-chemokine ligand (CCL)3/macrophage inflammatory protein (MIP)-1α, CCL4, CCL5, IL-6, adiponectin, and TNF were measured by ELISA. All ELISA-based detection systems were purchased from R&D Systems, Wiesbaden, Germany (DuoSet ELISA development systems). For data normalization, total protein concentration was measured. For the detection of potential cytotoxic effects, lactate dehydrogenase concentration was measured in the supernatants (cytotoxicity detection kit; Roche, Mannheim, Germany). Each sample was measured in duplicate by ELISA. Values were normalized to total protein content of each well and are expressed as means ± SEM.

Transfection of adipocytes with siRNA

For siRNA transfections, the X-tremeGENE siRNA transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) was used. CTRP-3 siRNA and Silencer Negative Control 1 siRNA were purchased from Applied Biosystems/Ambion (Darmstadt, Germany). A pool of three CTRP-3 siRNAs was used for all experiments (no. 1, sense, UGGAUUUUGGUUGUACCAtt, and antisense, UUGGUAACCCAGAAAUCt; no. 2, sense, GAUGAAGCAUGAGGAGCUAtt, and antisense, UACGUCCUCAUGCUUCAUc; and no. 3, sense, UUGUACGUAUGAAAACAAAtt, and antisense, UUUUGUCAUAUGCUAGATg). siRNA (100 nm) was incubated with 5 μl transfection reagent (X-tremeGENE) in 100 μl culture medium for 20 min at room temperature before transfection. Cells transfected with control or CTRP-3 siRNA were used for Oil Red O staining and measurement on intracellular triglycerides. Supernatants were used for measurement of MCP-1 by ELISA.

Oil Red O staining of 3T3-L1 adipocytes and measurement of lipid droplet size

After removing the supernatants, cells were washed with PBS and fixed for 5 min with 10% formaldehyde in PBS. Cells were washed with 60% isopropanol and dried. Oil Red O solution (purchased from Sigma-Aldrich, Deisenhofen, Germany) was added for 20 min, and after washing the cells with sterile water, cells were used for microscopy. The diameters of 23 lipid droplets were measured within a representative microscopic field (×200 magnification) per experimental group.

Measurement of intracellular triglyceride concentration

Intracellular triglyceride concentrations were measured using the triglyceride GPO-PAP micro-test (purchased from Roche, Mannheim, Germany). Triglyceride concentration was divided by intracellular total protein concentration, and these ratios are given as arbitrary units.

Isolation and purification of primary human monocytes

Ten female and 10 male healthy probands (students at the University Hospital of Regensburg, Regensburg, Germany) served as controls (n = 20). Thirty patients (11 females and 19 males) suffering from type 2 diabetes mellitus were included in the study. All individuals participating in the study gave informed consent, and the study was approved by the local ethical committee. The characteristics of the entire study population are summarized in Table 1. Patients suffering from acute/chronic infections and chronic inflammatory diseases were excluded. Whole blood was drawn from controls and patients after an overnight fast using the Vacutainer CPT system (Becton Dickinson, Franklin Lakes, NJ). Primary human monocytes were isolated and cultured as described previously (39) using magnetic separation with CD14 beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Before the stimulation experiments, the medium was replaced. Monocytes (3 × 10⁶) were stimulated for 18 h with LPS (1 μg/ml) alone or a combination of LPS (1 μg/ml) and CTRP-3 (1 μg/ml). When a combination of LPS and CTRP-3 was used, CTRP-3 (1 μg/ml) was added to the cells 30 min before LPS to allow CTRP-3 to exert potential antiinflammatory effects. LPS (Escherichia coli, serotype 055:B5) was purchased from Sigma-Aldrich. In addition, monocytes were also stimulated by lauric acid (200

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of the study population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 20)</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus (n = 30)</td>
</tr>
<tr>
<td>Females (n)</td>
</tr>
<tr>
<td>10 (50%)</td>
</tr>
<tr>
<td>11 (36.7%), NS</td>
</tr>
<tr>
<td>Males (n)</td>
</tr>
<tr>
<td>10 (50%)</td>
</tr>
<tr>
<td>19 (63.3%), NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>21.9 ± 0.3</td>
</tr>
<tr>
<td>31.0 ± 2.3²</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>25.1 ± 1.5</td>
</tr>
<tr>
<td>62 ± 2.0²</td>
</tr>
<tr>
<td>Lipoprotein metabolism</td>
</tr>
<tr>
<td>Cholesterol [mg/dl (range)]</td>
</tr>
<tr>
<td>156 ± 5 (40–244)</td>
</tr>
<tr>
<td>Triglycerides [mg/dl (range)]</td>
</tr>
<tr>
<td>129 ± 6 (56–279)</td>
</tr>
<tr>
<td>HDL [mg/dl (range)]</td>
</tr>
<tr>
<td>40 ± 1 (17–88)</td>
</tr>
<tr>
<td>LDL [mg/dl (range)]</td>
</tr>
<tr>
<td>88 ± 3 (9–146)</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>HbA₁c [% (range)]</td>
</tr>
<tr>
<td>7.5 ± 0.3 (4.6–18.0)</td>
</tr>
<tr>
<td>Fasting glucose [mg/dl (range)]</td>
</tr>
<tr>
<td>157 ± 9 (81–500)</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>CRP (mg/liter)</td>
</tr>
<tr>
<td>29 ± 4</td>
</tr>
<tr>
<td>WBC (nl)</td>
</tr>
<tr>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>Antidiabetic treatment</td>
</tr>
<tr>
<td>Diet/exercise (n)</td>
</tr>
<tr>
<td>3 (10%)</td>
</tr>
<tr>
<td>Oral treatment (n)</td>
</tr>
<tr>
<td>9 (30%)</td>
</tr>
<tr>
<td>Insulin (n)</td>
</tr>
<tr>
<td>14 (46.7%)</td>
</tr>
<tr>
<td>Insulin plus oral treatment (n)</td>
</tr>
<tr>
<td>4 (13.3%)</td>
</tr>
</tbody>
</table>

Mean values ± SEM are shown unless indicated otherwise. Human primary monocytes were isolated successfully in all 50 individuals. CRP, C-reactive protein; HbA₁c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; WBC, white blood cells.

² P < 0.01, body mass index (BMI) in controls vs. BMI in patients with type 2 diabetes mellitus.

² P < 0.01, age in controls vs. age in patients with type 2 diabetes mellitus.
Effects of CTRP-3 on the binding of biotinylated LPS to a designed TLR4/MD-2 fusion protein (LPS trap) by using a novel ELISA technique and immunoprecipitation

The binding of biotinylated LPS (bio-LPS) to a designed (40) (41) Flag-tagged TLR4/MD-2 fusion protein (LPS-trap) was investigated by an ELISA-based assay (biotin-streptavidin-peroxidase-tetramethylbenzidine detection system) at a wavelength of 450 nm. The TLR4/MD-2 designer molecule was prepared by fusing MD-2 with a C-terminal His tag to the C terminus of soluble TLR4 (comprising the whole extracellular domain) via a flexible linker. This so-called LPS trap was successfully proven to bind LPS and to inhibit TNF production by LPS-stimulated RAW 264.7 cells (41). The fusion protein was coated to plastic wells via an anti-Flag antibody (anti-FLAG HS, M2 coated 96-well plate; Sigma-Aldrich, Taufkirchen, Germany).

Bio-LPS strongly binds to the TLR4/MD-2 fusion protein and a 100-fold excess of nonbiotinylated LPS was used to confirm specific and competitive inhibition of bio-LPS binding to the LPS trap. Four doses of CTRP-3 (10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml) were tested for the ability of CTRP-3 to inhibit the specific binding of bio-LPS to the LPS trap. CTRP-3 alone was used as a negative control. A reduction of bio-LPS binding to the TLR4/MD-2 fusion protein indicates a specific inhibition of LPS binding to the TLR4/MD-2 fusion protein by CTRP-3. As a second approach, immunoprecipitation of the LPS trap was used. The LPS trap was incubated with bio-LPS, bio-LPS plus 100-fold unlabeled LPS (specific and competitive inhibition), and CTRP-3 alone (negative control). Increasing doses of CTRP-3 (10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml) were used that should compete effectively with LPS for the binding to the LPS trap. The LPS/LPS trap complex was pulled down with streptavidin-agarose and Western blotted, and the blot was probed with an anti-Flag antibody (M2).

ELISA-based analysis of LPS binding to CTRP-3

To exclude that CTRP-3 effects on LPS binding to TLR4/MD-2 are caused by binding of CTRP-3 to LPS itself, an ELISA-based detection system for biotin-LPS was developed using plates coated with CTRP-3 (10 μg/ml CTRP-3) in carbonate/bicarbonate buffer (Sigma-Aldrich). As a control, uncoated plates (NUNC MaxiSorp, Langenbold, Germany) were used. After blocking with PBS/20% fetal calf serum, rising concentrations of bio-LPS (Invivogen, San Diego, CA) were added for 2 h. After washing the plates, detection was performed by streptavidin-HRP and tetramethylbenzidine (TMB). To demonstrate effective CTRP-3 binding to the plates, the presence of CTRP-3 on the wells was documented by an ELISA-based system using an anti-CTRP-3 antibody (rabbit; Abcam, Cambridge, UK; 1:10000) and detection by a secondary (1:5000) antirabbit IgG antibody (Jackson ImmunoResearch).

Statistics

For calculating mean values and SEM, a statistical software package (SPSS version 15.0) was used. Means were compared by the Mann-Whitney U test. A P value <0.05 (two tailed) was considered as statistically significant. Spearman’s analysis and regression analysis were used to test for potential correlations between numerical parameters. Highly significant P values < 0.001 are given as P < 0.001, whereas significant P values > 0.001 are given as actual values.

Results

Antiinflammatory effects of CTRP-3 on the proinflammatory activation of adipocytes

The effects of human recombinant CTRP-3 on LPS-, lauric acid-, and TLR ligand-induced MCP-1 release were investigated in mature 3T3-L1 adipocytes (Fig. 1).

The potent antiinflammatory and dose-dependent effect of CTRP-3 on LPS-induced release of MCP-1 in 3T3-L1 adipocytes is shown in Fig. 1A. LPS strongly elevates supernatant MCP-1 concentration (P < 0.001), and this proinflammatory effect of LPS is dose-dependently and significantly (P = 0.004 and P < 0.001, respectively) antagonized by adding increasing doses of CTRP-3 ranging from 0.1–1.0 and 10 μg/ml. At the highest dose used, the proinflammatory effect of LPS is nearly totally blocked (P < 0.001). CTRP-3 at 1 μg/ml is also effective (~25% reduction), whereas 0.1 μg/ml slightly but not significantly reduced MCP-1 levels. The antiinflammatory effect of CTRP-3 on lauric acid, the main lipid component of LPS, is depicted in Fig. 1B. Lauric acid strongly (P < 0.001) induces MCP-1 release, and this effect is significantly decreased by CTRP-3 (P < 0.001).

The potent and dose-dependent antiinflammatory effects of CTRP-3 on TLR1/2-induced MCP-1 release by Pam3Cys are summarized in Fig. 1C. Pam3Cys strongly induces MCP-1 release in 3T3-L1 adipocytes, and this effect is blocked stepwise and significantly by increasing doses of CTRP-3. The highest dose of CTRP-3 almost completely blocks Pam3Cys-induced MCP-1 (P = 0.008).

Figure 1D shows the dose-dependent antiinflammatory effects of CTRP-3 on TLR3-induced MCP-1 release by using poly(I:C) as specific agonist. CTRP-3 dose-dependently reduces the poly(I:C)-induced MCP-1 release by approximately 50% (P = 0.001).

The specific ligands for TLR5 (flagellin), TLR7/8 (polyuridine), and TLR9 (CpG) were additionally tested; these ligands did not stimulate MCP-1 release, and CTRP-3 had no effect (data not shown). The specific ligand for TLR2/6 (MALP-2) induced MCP-1 release; however, CTRP-3 was not able to inhibit this effect (data not shown).

Because CTRP-3 expression is induced during the differentiation of preadipocytes into mature adipocytes (Fig. 1E), this protein can be regarded as an adipocyte-tokine and marker protein of mature adipocytes.
CTRP-3 expression is almost absent in undifferentiated preadipocytes and is strongly induced during adipocyte differentiation with a maximum in late adipocyte differentiation (d 9 in culture).

siRNA-mediated knockdown of CTRP-3 elevates supernatant MCP-1 concentrations and down-regulates adiponectin concentrations in adipocytes

To investigate whether cellular CTRP-3 deficiency is linked to an elevated release of proinflammatory MCP-1 and a reduced release of antiinflammatory adiponectin in adipocytes, siRNA-mediated knockdown of CTRP-3 was conducted in 3T3-L1 adipocytes (Fig. 2). If CTRP-3 acts as an antiinflammatory by inhibiting the release of MCP-1 as shown in Fig. 1, knockdown of this protein in adipocytes may result in an up-regulation of basal MCP-1 release. As depicted in Fig. 2A, the adipocytic supernatant MCP-1 concentrations were significantly \((P < 0.001)\) higher after siRNA-mediated knockdown of CTRP-3 when compared with control experiments.

In contrast, supernatant adiponectin concentrations were significantly \((P = 0.023)\) lower after siRNA-mediated knockdown of CTRP-3 when compared with control experiments (Fig. 2B).

Figure 2C demonstrates the successful siRNA-mediated knockdown of CTRP-3 expression by Western blot analysis in adipocytes.

siRNA-mediated knockdown of CTRP-3 reduces lipid droplet size and intracellular triglyceride concentration

As shown in Fig. 2D, siRNA-mediated knockdown of CTRP-3 results in extensive morphological alterations of adipocytes. When compared with control siRNA-transfected cells, adipocytes transected with CTRP-3 siRNA are characterized by reduced lipid droplet size and by lower quantities of intracellular triglycerides. For quanti-
fication of these morphological alterations, lipid droplet diameters were determined and intracellular triglyceride concentrations was measured. As depicted in Fig. 2E, siRNA-mediated knockdown of CTRP-3 significantly reduces lipid droplet size and intracellular triglyceride concentration. These changes reflect mechanisms leading to a dedifferentiation of mature adipocytes to a phenotype resembling immature adipocytes.

Antiinflammatory effects of CTRP-3 in primary human monocytes isolated from healthy controls and patients with type 2 diabetes

The effects of CTRP-3 on LPS-induced release of MIF, MCP-1, CCL3/MIP1α, CCL4, and CCL5 were investigated in primary human monocytes isolated ex vivo from 20 nondiabetic controls (Fig. 3, left) and 30 patients suffering from type 2 diabetes mellitus (Fig. 3, right).

LPS-induced supernatant concentrations of MIF (Fig. 3A), CCL4 (Fig. 3C), and CCL3 (Fig. 3D) were not significantly different between nondiabetic controls and type 2 diabetic patients. In contrast, LPS-induced supernatant concentration of MCP-1 (Fig. 3B) was significantly (P < 0.001) lower in controls than in type 2 diabetic patients.

Supernatant concentrations of MIF (Fig. 3A; P < 0.001), MCP-1 (Fig. 3B), CCL4 (Fig. 3C; P < 0.001), and CCL3 (Fig. 3D) were significantly lower in nondiabetic controls when compared with type 2 diabetic patients after costimulation with LPS and CTRP-3.

CTRP-3 potently reduces LPS-induced release of MIF (Fig. 3A; P = 0.003), MCP-1 (Fig. 3B; P = 0.03), and CCL4 (Fig. 3C; P < 0.001) by approximately 50% in nondiabetic controls, whereas CCL3/MIP1α (Fig. 3D) release was not affected. Regarding type 2 diabetic patients, CTRP-3 had no effect on LPS-induced release of MIF (Fig.
3A), CCL4 (Fig. 3C), and CCL3/MIP1α (Fig. 3D), whereas MCP-1 concentration was effectively reduced by CTRP-3 by approximately 50% (Fig. 3B; \( P < 0.001 \)).

Effects of LPS alone or in combination with CTRP-3 on CCL5 were also investigated (data not shown). CCL5 levels were not different between healthy controls and type 2 diabetic patients, and CTRP-3 was without any effect.

### CTRP-3 antagonizes lauric acid-induced proinflammatory activation of primary human monocytes

The effect of CTRP-3 on lauric acid-induced release of IL-6 and TNF in primary human monocytes isolated ex vivo was investigated by ELISA. Figure 4 depicts the results obtained in two (panels A and B) representative and healthy, nondiabetic individuals. As a potent proinflammatory fatty acid and major lipid component of LPS, lauric acid strongly induces IL-6 and TNF release. This stimulatory effect of lauric acid on IL-6 and TNF was nearly totally blocked after prestimulating the cells with CTRP-3.

As demonstrated in Fig. 4C, CTRP-3 is not only expressed in human adipocytes but also (to a lesser extent) in primary human monocytes and in the monocytic cell line THP-1.

### CTRP-3 specifically antagonizes the binding of LPS to a designed Flag-tag TLR4/MD-2 fusion protein (LPS trap)

As explained in detail within the legends of Fig. 5, both an ELISA-based approach (Fig. 5A) and an immunoprecipitation-based (Fig. 5B) strategy were used to investigate whether CTRP-3 antagonizes the binding of LPS to the TLR4/MD-2 monomeric fusion protein. As demonstrated by the LPS trap-based ELISA technique, bio-LPS strongly binds to the TLR4/MD-2 fusion protein (positive control), and this binding can be abrogated by adding a 100-fold excess of unlabeled LPS. Whereas CTRP-3 in lower doses (10 and 100 ng/ml) did not reduce bio-LPS binding, the higher doses used (1 and 10 \( \mu \)g/ml) significantly inhibited bio-LPS binding (\( P = 0.005 \) and \( P < 0.001 \), respectively) by more than 50%. CTRP-3 (1 \( \mu \)g/ml) alone was used as a negative control.

These data were supported by immunoprecipitation experiments (Fig. 5A). Although this technique has a lower sensitivity for detecting CTRP-3 binding to the TLR4/MD-2 complex, CTRP-3 at higher doses of 10 \( \mu \)g/ml did almost completely abrogate the binding of LPS to its soluble receptor complex TLR4/MD-2.
CTRP-3-mediated inhibition of LPS binding to TLR4/MD-2 is not caused by unspecific binding of CTRP-3 to LPS

As a last step, we aimed to exclude the potential binding of CTRP-3 to LPS. It seems important to exclude that the CTRP-3-mediated inhibition of LPS binding to TLR4/MD-2 is solely caused by an unspecific binding of CTRP-3 to LPS. This might form higher molecular weight complexes consisting of CTRP-3-LPS dimers that could inhibit the interaction of CTRP-3 with TLR4/MD-2 by steric effects. As shown in Fig. 6A, different doses of biotin-labeled LPS were not able to bind to CTRP-3 coated onto plastic wells. Therefore, it can be concluded that CTRP-3-mediated effects on LPS binding to TLR4/MD-2 are not caused by a direct and unspecific interaction of LPS with CTRP-3. The effective binding of CTRP-3 onto the plastic wells as a prerequisite for this experimental approach is shown in Fig. 6B.

CTRP-3 induces the expression of p-p38 MAPK and p-ERK-2/-2 in adipocytes

To investigate the involvement of signal transduction proteins in CTRP-3 signaling, mature 3T3-L1 adipocytes were stimulated with recombinant CTRP-3 (1 μg/ml) for 5, 10, 30, 60, and 120 min. The expression of phosphorylated and unphosphorylated p38 MAPK and ERK-2/-2 was investigated by Western blot analysis (Fig. 7). Whereas the unphosphorylated proteins remained unchanged, p-p38 MAPK was transiently induced after 10 min, and p-ERK-1/-2 was transiently induced after 5 and 10 min after CTRP-3 stimulation.

Discussion

Because LPS and fatty acids are molecules inducing insulin resistance and inflammation, activation of TLR4 by these molecules (30, 42–44) might represent the molecular link
between metabolism/nutrition and immunity. LPS stimulation of adipocytes does not only induce proinflammatory activation but generates insulin-resistant adipocytes (43). These data strongly support the hypothesis that TLR4, either activated by LPS or saturated fatty acids, represents a metabolic gate linking inflammation or infection with whole-body metabolism and insulin signaling.

The present data demonstrate for the first time that CTRP-3 is an effective and negative regulator of TLR4-mediated proinflammatory response in adipocytes and in monocytes. CTRP-3 specifically and dose-dependently antagonizes the binding of LPS to its receptor TLR4. This antagonism seems to be due to an interference of lauric acid (the main component of the fatty acid moiety of lipid
A) with TLR4/MD-2, because CTRP-3 strongly inhibits the proinflammatory response not only of LPS but also of lauric acid. As proven by the LPS-trap experiments, CTRP-3 functions as an inhibitor of LPS binding to its receptor, TLR4/MD-2. Moreover, an unspecific binding of CTRP-3 to LPS could be excluded in the present experiments, suggesting that the inhibitory action of CTRP-3 is caused by a direct binding of CTRP-3 to TLR4/MD-2. Based on these data, CTRP-3 acts as a novel and endogenous LPS antagonist expressed by adipocytes and monocytes/macrophages, two cell types standing in direct cross talk within visceral adipose tissue adipocytes. Moreover, CTRP-3 does also effectively inhibit both the proinflammatory effects of TLR ligands other than LPS.

LPS, fatty acids, and alternative TLR ligands represent three basic and common metabolic and inflammatory signaling molecules that mediate the cross talk between adipocytes and adipose tissue macrophages. Because all of these three signaling pathways are antagonized by CTRP-3, this molecule represents a novel and promising drug target to treat adipo-inflammation and obesity-associated metabolic diseases.

MCP-1 is the predominant adipocyte-expressed chemokine that causes macrophage infiltration of visceral adipose tissue in the context of obesity (1, 2, 21, 22). Because we could demonstrate that CTRP-3 is a potent inhibitor of MCP-1 release in adipocytes, CTRP-3 represents an important regulatory factor in adipose tissue physiology and in controlling visceral adipose tissue inflammation.

As demonstrated by the siRNA experiments, knockdown of antiinflammatory CTRP-3 alters adipocyte morphology. CTRP-3 knockdown in adipocytes reduces intracellular triglyceride content and lipid droplet size. These alterations are typical for a dedifferentiation back into an immature adipocyte phenotype. This phenomenon is important in a metabolic context, because immature adipocytes are characterized by a more proinflammatory (e.g. higher IL-6 release) and a less insulin-sensitive phenotype. Moreover, the present experiments demonstrate that CTRP-3 knockdown reduces the release of the antiinflammatory, insulin-sensitizing, antidiabetic, and differentiation-induced adipokine adiponectin (45–48). These data argue not only for an antiinflammatory role of CTRP-3 but also for an insulin-sensitizing and differentiation-inducing role of CTRP-3 in adipocyte biology.

As shown earlier by Western blot analysis, we could demonstrate that CTRP-3 circulates in human plasma (18) and in peritoneal fluid surrounding visceral adipose tissue (19). In the plasma of 20 probands, CTRP-3 was found highly abundant as a high molecular weight complex of about 180–250 kDa, and quantification of the immuno-
blots revealed a 2-fold difference in CTPR-3 levels when all samples were compared (18). It would be of interest to know whether there is a correlation of circulating or local concentrations of CTRP-3 in humans suffering from obesity and type 2 diabetes mellitus. However, due to the current lack of commercial ELISA-based detection systems, this point awaits future elucidation. Using Western blot analysis (18), CTRP-3 was similarly abundant before and after an oral glucose uptake, but immunoblot is not suitable to detect changes of less than 20% in protein levels.

Data on CTRP-3-induced signal transduction proteins are rare and dependent on the cell type used. In endothelial cells (17), osteosarcoma cells (49), and chondrocyte progenitor cells (15), the involvement of MAPK kinase-1/-2, ERK-1/-2, and p38-MAPK was demonstrated. The present experiments support these data and demonstrate that up-regulation of p-p38-MAPK and p-ERK-1/-2 are involved in CTRP-3-induced signal transduction in adipocytes.

Conclusions

CTRP-3 represents a novel, endogenous, and potent LPS antagonist of the adipose tissue that inhibits the proinflammatory activation of adipocytes and monocytes by three basic pathways known to play an important role in immunity and metabolism. Because macrophage infiltration of adipose tissue is mainly caused by MCP-1, the inhibitory effect of CTRP-3 on adipocyte MCP-1 release is of major interest. CTRP-3 exerts antiinflammatory effects, and knockdown of CTRP-3 does not only reduce adiponectin release but also reverses adipocyte differentiation morphologically back to a more proinflammatory phenotype. Thus, CTRP-3 is a highly promising drug target in visceral adipose tissue inflammation, insulin resistance, and type 2 diabetes mellitus.

Acknowledgments

The technical assistance of Kerstin Winkler is highly appreciated.

Address all correspondence and requests for reprints to: Prof. Dr. Andreas Schäffler, M.D., Department of Internal Medicine I, University of Regensburg, D-93042 Regensburg, Germany. E-mail: andreas.schaeffler@klinik.uni-regensburg.de.

Disclosure Summary: The authors have nothing to declare, and there is no conflict of interest.

References

8. Shapiro L, Scherer PE 1998 The crystal structure of a complement 1q family protein suggests an evolutionary link to tumor necrosis factor. Curr Biol 8:335–338
17. Akiyama H, Furukawa S, Wakisaka S, Maeda T 2006 Cartducin stimulates mesenchymal chondroprogenitor cell proliferation through both extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways. FEBs J 273:2257–2263
22. Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME 2008 Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. Obesity (Silver Spring) 16:1248–1255