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C1q/TNF-related protein-3 (CTRP-3) attenuates lipopolysaccharide (LPS)-induced systemic inflammation and adipose tissue Erk-1/-2 phosphorylation in mice in vivo

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ABSTRACT

Background: The C1q/TNF-related proteins comprise a growing family of adiponectin paralogous proteins. CTRP-3 represents a novel adipokine with strong expression in adipose tissue and was shown to inhibit chemokine and cytokine release in adipocytes and monocytes in vitro. The aim of the study was to gain the proof of principle that CTRP-3 is a potent anti-inflammatory adipokine in vivo.
Methods: C57BL/6N mice were treated intraperitoneally (i.p.) with bacterial lipopolysaccharide (LPS) for 2 h. The effects of a 30 min pre-treatment with CTRP-3 i.p. or intravenously (i.v.) on systemic and on epididymal, perirenal and subcutaneous adipose tissue inflammation was analyzed via real-time RT-PCR, ELISA and Western blot analysis.
Results: LPS (1 μg i.p.) significantly increased serum IL-6 and MIP-2 levels as well as epididymal adipose tissue expression of IL-6 and MIP-2 in mice, whereas CTRP-3 (10 μg i.p.) alone or PBS (i.p.) had no effect. Pre-treatment of mice by CTRP-3 i.p. prior to LPS application significantly attenuated LPS-induced cytokine levels but had no effect on adipose tissue cytokine mRNA expression. In contrast to i.p. application of CTRP-3, systemic i.v. application was not sufficient to inhibit LPS-induced cytokine levels or mRNA tissue expression. CTRP-3 given i.p. significantly attenuated LPS-induced phosphorylation of Erk-1/-2 in inguinal adipose tissue.
Conclusion: The present study shows the proof of principle that the novel adipokine CTRP-3 is a potent inhibitor of LPS-induced systemic inflammation and LPS-induced signaling in adipose tissue in vivo.

1. Introduction

Besides its important and pleiotropic role in energy metabolism and hormonal homeostasis, the adipose tissue is increasingly being recognized as an immunological organ [1,2]. The C1q/TNF-related protein (CTRP) family represents a growing family of currently 15 adiponectin paralogous proteins [3] sharing common structural characteristics with C1q complement components and TNF receptor ligands [4]. CTRPs have pleiotropic functions in immunity and metabolism. The adipokine CTRP-3, formerly named CORS-26 [5,6], cartducin [7] or cartonectin [8], shows a predominant expression in subcutaneous and visceral adipose tissue [9] and exerts a dual role in energy metabolism and immune regulation [3]. Furthermore, a role as promoter of chondrocyte proliferation has been described [7]. CTRP-3 acts both on adipocytes and monocytes by targeting unknown receptors.

LPS inhibits terminal adipocyte differentiation and CTRP-3 expression in adipocytes [9]. LPS is a well known stimulator of adipocytic cytokine release such as IL-6 [10]. In adipocytes, CTRP-3 stimulates the release of the anti-inflammatory adiponectin and the proinflammatory resistin [8]. In vitro, it was shown that CTRP-3 is able to antagonize fatty acid- and LPS-induced proinflammatory activation in adipocytes [11]. Cellular knockdown of CTRP-3 in adipocytes by siRNA upregulates monocyte chemoattractant protein-1 (MCP-1) release and causes some degree of dedifferentiation and proinflammatory transformation [11,12]. Transgenic mice overexpressing CTRP-3 have reduced serum TNF-α levels and improved insulin sensitivity [13]. In mice, CTRP-3 levels are increased during fasting and decreased in obesity [14]. In contrast, CTRP-3 levels in humans were reported to be significantly higher in obese and type 2 diabetic patients as compared to control subjects [15]. CTRP-3 can be regarded as a metabolically

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active adipokine lowering glucose levels in mice by activation of the Akt signaling pathway [14].

In human primary monocytes, CTRP-3 effectively inhibits LPS-induced release of IL-6 and TNF [16]. Another study [11] reported that CTRP-3 dose-dependently inhibits LPS-, lauric acid- and Toll-like receptor ligand-induced chemokine release such as MCP-1 by monocytes and adipocytes. Although a specific receptor for CTRP-3 waits to be identified, a previous study [11] using a designed TLR4/MD-2 fusion molecule demonstrated that CTRP-3 exerts anti-inflammatory actions by inhibiting the binding of LPS to its receptor TLR4/MD-2. Based on these data, CTRP-3 effects have been cited as "blocking a toll booth to obesity-related inflammation" [17]. CTRP-3 also exerts anti-inflammatory and anti-fibrotic effects on human primary colonic fibroblasts by antagonizing the LPS-pathway and by targeting the TGF-β-CTGF-collagen I pathway [18].

Since CTRP-3 acts anti-inflammatory both in adipocytes and in monocytes and antagonizes LPS-signaling in vitro, the aim of the present study was,

- to investigate the potential anti-inflammatory effects of CTRP-3 in vivo in mice,
- to analyze whether CTRP-3 is able to antagonize the systemic LPS-mediated inflammatory response in vivo in mice,
- to test whether LPS and CTRP-3 alone or in combination alter cytokine and chemokine expression in epididymal, perirenal and subcutaneous adipose tissue,
- to investigate whether the route (i.p. vs. i.v.) of CTRP-3 application plays a role.

2. Material and methods

2.1. Recombinant expression of CTRP-3

Recombinant CTRP-3 protein expression was performed in H5 insect cells (Invitrogen, Karlsruhe, Germany) using the BacPak™ Baculovirus Expression System (BD Biosciences, Palo Alto, CA, USA) as published earlier [6]. Unlike *Escherichia coli*-based expression systems, recombinant expression in insect cells usually maintains glycosylation and phosphorylation. Our expression system was proven to generate trimeric CTRP-3 [6]. High purity of the recombinant CTRP-3 protein in the preparation was verified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS–PAGE, see below).

2.2. Experimental animals

After an overnight fast, C57BL/6 mice (male, 8–12 weeks old, 24–28 g, Charles River, Sulzfeld, Germany) were treated with an intraperitoneal (i.p.) injection of either sterile phosphate-buffered saline (200 µl PBS) as a vehicle control or CTRP-3 (10 µg dissolved in 200 µl PBS). After 30 min, either lipopolysaccharide (LPS; 1 µg dissolved in 50 µl sterile H_2O) or sterile H_2O (50 µl) were injected i.p. When a combination of LPS/CTRP-3 was given, CTRP-3 was injected 30 min prior to LPS in order to avoid rapid LPS-induced overflowing effects on possible anti-inflammatory mechanisms of CTRP-3. The dose of 10 µg CTRP-3 per animal was chosen on the basis of recent studies showing a dose-dependent anti-inflammatory effect with a maximum at 10 µg [11]. A relatively low but effective quantity of LPS was chosen for the in vivo experiments [19]. We intended to generate a modest rather than an overwhelming inflammatory response syndrome since otherwise potential anti-inflammatory effects of CTRP-3 could be overridden by LPS. Mice were killed 2 h after LPS-injection and whole venous blood was collected. Epididymal (intra-abdominal), perirenal and subcutaneous adipose tissue specimen were resected and shock-frosted in fluid nitrogen for further analysis of mRNA expression. By using a different experimental setting but identical vehicle controls, pretreatment of mice with CTRP-3 was performed by intravenous application (i.v.) followed by i.p. LPS injection as described above. Animal experiments were performed at the University of Regensburg, Germany and all animal studies were approved by the local government agency (approval No. 54-2532, 1.14/10).

2.3. Monitoring of adipose tissue gene expression by real-time RT-PCR

The mRNA expression in murine epididymal, perirenal and subcutaneous adipose tissue was investigated by quantitative real-time (RT)-PCR. Total mRNA was isolated from adipose tissue (~50 mg) using TRIzol®-Reagent (Life Technologies GmbH, Darmstadt, Germany) in combination with gentleMACS dissociator and M-tubes (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for dissociation. After tissue preparation, mRNA was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) including DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany). 1 µg of total mRNA was reversely transcribed by QuantiTect® Reverse Transcription Kit (Qiagen) and was used for quantitative RT-PCR analysis (LightCycler®, Roche Applied Science, Mannheim, Germany), using intron-spanning primers (Metabion, Planegg-Martinsried, Germany) specific for mouse 18S rRNA (forward: 5′-gattgatagctctttctcgattcc-3′; reverse: 5′-catctaagggcatcacagacc-3′), mouse β-Actin (forward: 5′-tggaatcctgtggcatccatg-3′; reverse: 5′-taaaacgcagctcagtaacag-3′), mouse MIP-2 (C-X-C motif ligand-2) (forward: 5′-tccagagcttgagtgtgacg-3′; reverse: 5′-aggcacatcaggtac-gatcc-3′), and mouse IL-6 (forward: 5′-ttccatccagttgccttctt-3′; reverse: 5′-ttctgcaagtgcatcatcgt-3′) were used. Results of RT-PCR were quantified using the standard curve method. Expression of IL-6 and MIP-2 were normalized to relative mRNA expression levels of housekeeping genes 18S rRNA and β-actin.

2.4. Measurement of cytokine and chemokine serum levels

Serum cytokine and chemokine levels were analyzed by enzyme-linked immunosorbent assay (ELISA) using IL-6 and MIP-2 sandwich ELISA detection systems (DuoSet® ELISA development systems; R&D Systems, Wiesbaden, Germany). Samples were measured in duplicate and mean values ± standard error of the mean (SEM) were used for statistical calculations of significance. For graphical illustrations, the median with upper and lower quartiles is depicted.

2.5. SDS–PAGE and immunoblotting

Adipose tissue was homogenized in radio-immunoprecipitation assay (RIPA) buffer using gentleMACS dissociator and M-tubes (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). SDS–PAGE was performed following standard procedures. Proteins were transferred to an Immun-Blot™ PVDF Membrane (Biorad, Hercules CA, USA). Incubations with antibodies were performed in 5% non-fat dried milk in PBS or Tris-buffered saline (TBS) with 0.1% Tween 20. Expression of Erk-1/-2 and phospho-Erk-1/-2 protein was investigated by using anti-Erk-1/-2 and anti-phospho-Erk-1/-2 (Thr202/Tyr204) polyclonal antibodies (rabbit; 1:1000; Cell Signaling, Boston, USA). An anti-PI3K (phosphatidylinositol-3 kinase) antibody was used as a control (rabbit; 1:1000; upstate/Millipore, Billerica, USA). Secondary horse radish peroxidase (HRP)-coupled anti-rabbit IgG antibody (Jackson Immuno Research, Newmarket, UK) was used in 1:5000 dilution in 5% non-fat dried milk in PBS or TBS. Detection of immune complexes was carried out with the enhanced chemiluminescence Western blot detection system (Amersham ECL™ Western blotting system, GE healthcare, Freiburg, Germany). Western blot experiments were performed at

least in triplicate. For densitometric quantification of bands, ImageJ software 1.47 (National Institutes of Health, Bethesda, MD) was used.

2.6. Statistics

For calculating mean values and standard error of the mean (±SEM), a statistical software package (SPSS 18.0; SPSS Inc., Chicago, IL, USA) was used. Means were compared by the non-parametric Mann–Whitney *U*-test. A p value < 0.05 (two tailed) was considered as statistically significant. For graphical illustration, box plots are depicted within the figures showing the median with lower and upper quartiles (box) and whiskers indicating the minimum and maximum of the samples. Extreme values lying outside the whiskers' range are depicted as small circles. Data obtained by densitometry were statistically analyzed by Mann–Whitney-*U*-Test and are depicted as box plots.

3. Results

3.1. Intraperitoneal application of CTRP-3 significantly attenuates LPS-induced systemic inflammation (Fig. 1A and B)

Serum IL-6 (Fig. 1A) and serum MIP-2 (Fig. 1B) levels were low under basal conditions in vehicle control animals. Intraperitoneal (i.p.) injection of CTRP-3 (10 μg) did not show any effects on basal IL-6 and MIP-2 serum levels. As expected, intraperitoneal injection of LPS (1 μg) strongly increased systemic IL-6 levels up to 22498.5 ± 1323.8 pg/ml (p = 0.005 vs. PBS) and MIP-2 levels up to 25314.9 ± 3867.0 pg/ml (p = 0.005 vs. PBS). Pre-treatment of the animals with CTRP-3 (10 μg) 30 min prior to LPS (1 μg) significantly reduced IL-6 levels down to 16974.0 ± 1132.9 pg/ml (p = 0.011 vs. LPS) and MIP-2 levels down to 16772.1 ± 1660.8 pg/ml (p = 0.041 vs. LPS).

Intraperitoneal application of CTRP-3 does not significantly affect LPS-induced adipose tissue expression of IL-6 and MIP-2 (Fig. 1C and D).

Neither intraperitoneal vehicle (PBS) nor CTRP-3 (10 μg) injection increased basal IL-6 (Fig. 1C) or MIP-2 (Fig. 1D) mRNA expression in epididymal adipose tissue, whereas LPS (1 μg) strongly induced IL-6 expression (mean value 40.0 ± 5.2; p = 0.001) and MIP-2 expression (mean value 13.4 ± 2.4; p = 0.001). Animals which were pre-treated with recombinant CTRP-3 (10 μg) intraperitoneally showed a slightly but not significant reduction of mRNA expression for IL-6 from 40.0 ± 5.2 to 31.9 ± 3.8) and for MIP-2 from 13.4 ± 2.4 to 10.8 ± 1.0).

As observed in epididymal adipose tissue, LPS significantly induced IL-6 and MIP-2 gene expression in perirenal and subcutaneous adipose tissue. However, intraperitoneal CTRP-3 pre-treatment had no anti-inflammatory effect in these two adipose tissue localization (data not shown).

Intravenous application of CTRP-3 does not antagonize the pro-inflammatory response upon intraperitoneal LPS application (Fig. 2).

In this experimental setup, the animals were given LPS intraperitoneally as described above, but CTRP-3 pre-treatment in this setting was performed by intravenous application (i.v.). This experimental approach was chosen in order to discriminate local intraperitoneal effects of CTRP-3 from potential systemic effects. LPS-induced increase of systemic IL-6 and MIP-2 levels could not be antagonized by intravenous application of CTRP-3 (10 μg). In contrast, there was further increase of MIP-2 in the LPS/CTRP-3 subgroup with marginal statistical significance (p = 0.028). Similarly, LPS-induced induction of IL-6 and MIP-2 mRNA expression could not be antagonized by intravenous pre-treatment with CTRP-3. There was a marginal (p = 0.047) reduction of LPS-induced MIP-2 gene expression by CTRP-3 pre-treatment.

LPS-induced phosphorylation of Erk-1/-2 in adipose tissue is significantly inhibited by intraperitoneal injection of CTRP-3 (Fig. 3).

In order to analyze the potential pathways involved in the anti-inflammatory effects of CTRP-3, Erk-1/-2 activation in epididymal adipose tissue samples of mice was investigated by semi-quantitative Western blot analysis using densitometry. As shown in Fig. 3 A and B, Erk-1/-2 is not phosphorylated under basal conditions using PBS vehicle control. Moreover, CTRP-3 alone does not activate phosphorylation. As expected, LPS given intraperitoneally strongly increases phosphorylation of Erk-1/-2 (p = 0.014). Pre-treatment of mice by intraperitoneal injection of CTRP-3 (10 μg) almost completely (p = 0.043) abolished phosphorylation of Erk-1/-2 (mean value 23.5 ± 5.7 after LPS exposure vs. 3.8 ± 1.8 after pre-treatment with CTRP-3).

4. Discussion

The present data demonstrate in vivo in mice that LPS increases systemic levels of inflammatory mediators such as IL-6 and MIP-2 and upregulates gene transcription of IL-6 and MIP-2 in epididymal (but not perirenal or subcutaneous) adipose tissue. Since LPS was injected intraperitoneally, the systemic effects could be caused (i) by peritoneal resorption of LPS and its action on peripheral blood monocytes, (ii) by peritoneal inflammation with concomitant spill over of cytokines into the systemic circulation, (iii) by induction of a local, intra-abdominal adipose tissue inflammation with spill over of adipocytic cytokines into the systemic circulation, or (iv) by a combination of all.

Whereas induction of systemic inflammation by intraperitoneal injection of LPS is well documented in the literature, the present study shows for the first time in vivo that CTRP-3 is able to attenuate these proinflammatory effects. Together with the published in vitro findings, CTRP-3 can be regarded as an anti-inflammatory LPS-antagonist in vivo in the murine system. Importantly, only intraperitoneal but not intravenous pre-treatment with CTRP-3 was sufficient to attenuate LPS-induced systemic inflammation. This could indicate the possibility that LPS-induced adipose tissue inflammation after intraperitoneal injection of LPS contributes to or even causes systemic inflammation. In order to prove this hypothesis, three types of adipose tissue localizations were investigated. Only epididymal adipose tissue has anatomical contact with intraperitoneally administered LPS. However, all of the three adipose tissues investigated showed an induction of IL-6 and MIP-2 gene transcription indicating at least in part systemic effects of i.p. LPS administration. Since only i.p. injection but not i.v. injection of CTRP-3 reduces systemic LPS-related inflammation, one could speculate on a significant intra-abdominal antagonism between CTRP-3 and LPS. As a potential mechanism, CTRP-3 has been shown [11] to inhibit the binding of LPS to its receptor TLR4/MD-2. In a clinical context, intraabdominal adipose tissue inflammation is being increasingly recognized as a hallmark of visceral obesity and obesity-related insulin resistance [20,21].

An additional explanation for the absence of anti-inflammatory effects of intravenously injected CTRP-3 might arise from the limited protein quantities used due to restrictions of our experimental approach. Future studies using higher concentrations of CTRP-3 intravenously will have to address this question.

LPS has been reported to stimulate production of IL-6, IL-8 and MCP-1 in adipocytes [10,22]. In mice, LPS is a well-described systemic stimulator of MIP-2 release [23]. However, neither LPS-mediated induction of MIP-2 in adipose tissue nor inhibition of IL-6 or MIP-2 by CTRP-3 has been reported in the literature so

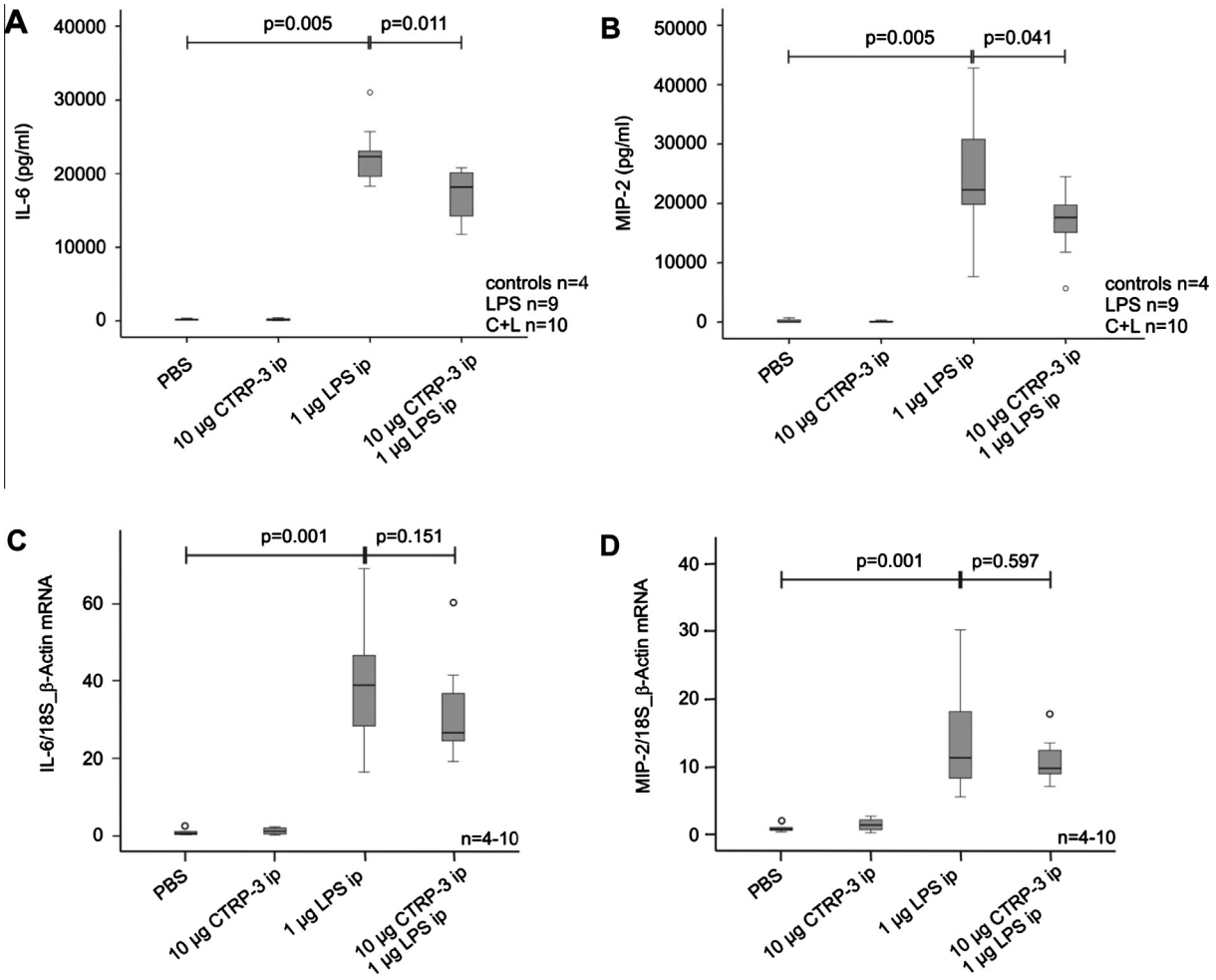

Fig. 1. Effects of intraperitoneal application of CTRP-3 on basal and LPS-induced systemic levels (A and B) and on epididymal adipose tissue expression (C and D) of IL-6 and MIP-2. Serum IL-6 and MIP-2 levels were measured by ELISA in duplicate. Data were statistically analyzed by Mann–Whitney-*U*-Test and values are depicted as box plots. In the control groups, 200 µl of PBS (n = 4 mice) and 10 µg CTRP-3 (n = 4 mice) were given intraperitoneally (i.p.). Animals were treated either with 1 µg LPS alone (n = 9 mice) or with a combination of LPS (1 µg) and CTRP-3 (10 µg), the latter given 30 min prior to LPS (n = 10 mice). mRNA expression of IL-6 and MIP-2 in epididymal adipose tissue was analyzed by real-time RT-PCR analyses. Data were statistically analyzed by Mann–Whitney-*U*-Test and values are depicted as box plots. (A) Serum IL-6 levels (pg/ml) in mice treated with PBS (i.p.), CTRP-3 (i.p.), LPS (i.p.), or a combination of CTRP-3/LPS (i.p.). (B) Serum MIP-2 levels (pg/ml) in mice treated with PBS (i.p.), CTRP-3 (i.p.), LPS (i.p.), or a combination of CTRP-3/LPS (i.p.). (C) Adipose tissue IL-6 mRNA expression in mice treated with PBS (i.p.), CTRP-3 (i.p.), LPS (i.p.), or a combination of CTRP-3/LPS (i.p.). (D) Adipose tissue MIP-2 mRNA expression in mice treated with PBS (i.p.), CTRP-3 (i.p.), LPS (i.p.), or a combination of CTRP-3/LPS (i.p.).

far. In this context it is important to remember that fatty acids [24], Toll-like receptor ligands [25,26] and LPS [11] have been shown to activate both adipocytes and monocytes. In vitro, it was shown that CTRP-3 is able to antagonize fatty acid- and LPS-induced pro-inflammatory activation both in adipocytes and in monocytic cells [11]. Although the exact mechanism is unknown, CTRP-3 seems to act anti-inflammatorily by inhibiting the binding of LPS to its receptor TLR4/MD-2 [11,17]. These in vitro data fit well with the presented in vivo data. Together they argue for an important physiological role of CTRP-3 as an anti-inflammatory and LPS-antagonistic adipokine. This hypothesis is supported by a recent study using a collagen-induced arthritis model in mice. Systemic knock out of CTRP-3 in these mice resulted in a more severe disease course of arthritis and an increased proinflammatory cytokine expression in several tissues and cells [27]. Moreover, there is increasing evidence that adipocyte-derived mediators interfere with LPS signaling. A recent study reported on a role of adipocyte-derived lipopolysaccharide-binding protein (LBP) in inflammation-associated adipose tissue dysfunction [28].

Notably, differences in IL-6 as well as in MIP-2 mRNA levels in whole epididymal adipose tissue preparations were not statistically significant (Fig. 1C and D). Thus, even though intracellular mRNA concentrations do neither necessarily reflect the exact levels of protein synthesis nor secretion, we cannot exclude that tissues other than adipose tissue are responsible for systemic serum levels of IL-6 and MIP-2 after intraperitoneal LPS injection. Future studies will have to focus on the role of for example dendritic cells and peritoneal macrophages for systemic inflammation induced by intraperitoneal LPS injection and its inhibition by CTRP-3.

Based on the present in vivo study, future experimental settings will have to clarify the detailed mechanism of how CTRP-3 inhibits LPS-induced systemic and local adipose tissue inflammation in normal-weight, obese and diabetic mice. Also, since our results were generated in a model of a modest inflammatory response, future studies will have to focus on potential protective effects of CTRP-3 in other models of local/systemic acute and chronic inflammation. Since visceral adipose tissue inflammation and macrophage infiltration play an important pathophysiologic role in metabolic syndrome, type 2 diabetes mellitus and associated

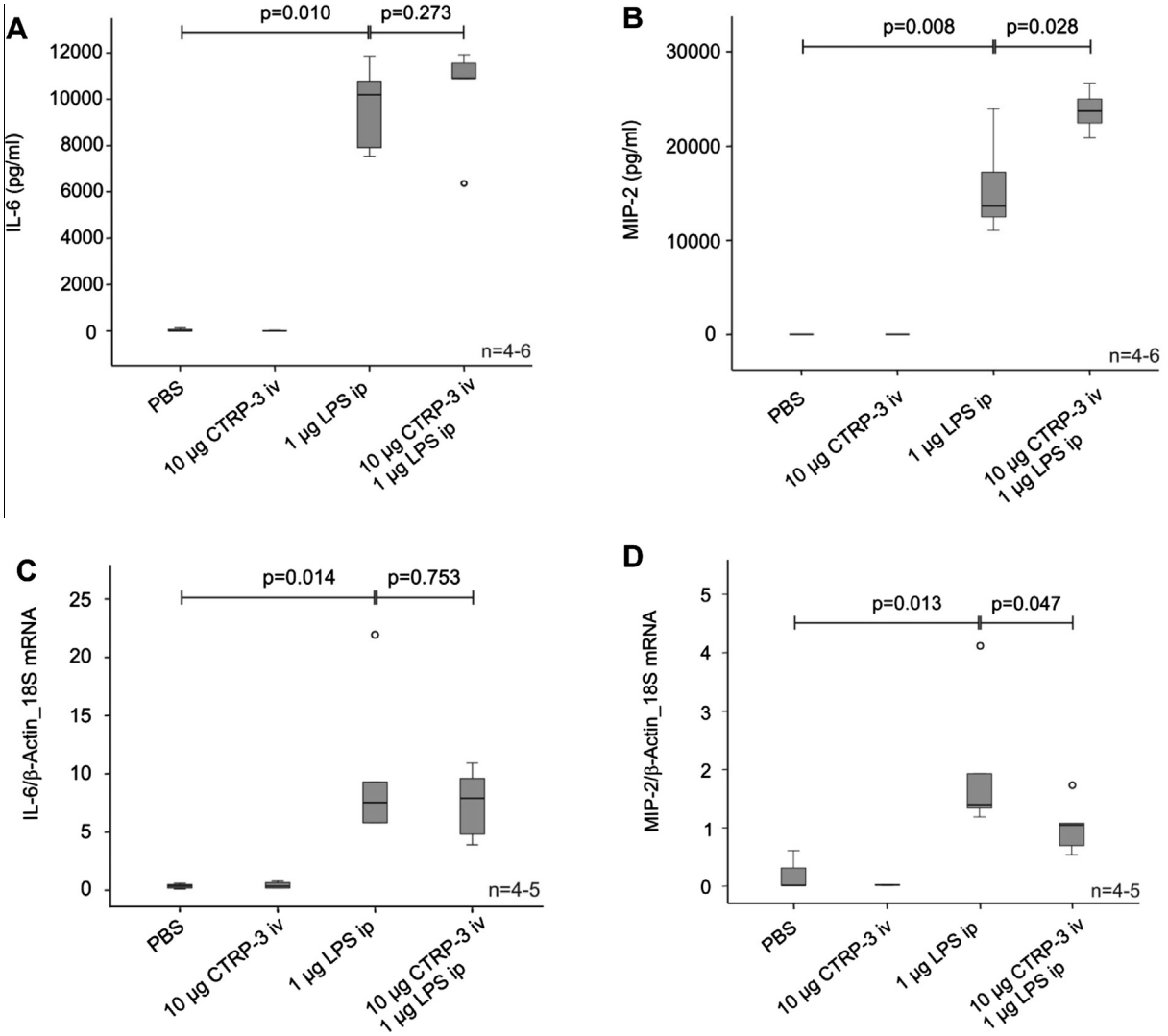

Fig. 2. Effects of intravenous application of CTRP-3 on basal and on LPS-induced systemic levels (A and B) and epididymal adipose tissue expression (C and D) of IL-6 and MIP-2. Serum IL-6 and MIP-2 levels were measured by ELISA in duplicate. Data were statistically analyzed by Mann–Whitney-*U*-Test and values are depicted as box plots. In the control groups, 200 µl of PBS (n = 4 mice) and 10 µg CTRP-3 (n = 4 mice) were given intravenously (i.v.). Further animals were treated with 1 µg LPS (i.p.) alone (n = 6 mice) or with a combination of LPS (1 µg i.p.) and CTRP-3 (10 µg i.v., n = 5 mice), the latter given 30 min prior to LPS. mRNA expression of IL-6 and MIP-2 in epididymal adipose tissue was analyzed by real-time RT-PCR analyses. Data were statistically analyzed by Mann–Whitney-*U*-Test and values are depicted as box plots. (A) Serum IL-6 levels (pg/ml) in mice treated with PBS (i.p.), CTRP-3 (i.v.), LPS (i.p.), or a combination of CTRP-3 (i.v.)/LPS (i.p.). (B) Serum MIP-2 levels (pg/ml) in mice treated with PBS (i.p.), CTRP-3 (i.v.), LPS (i.p.), or a combination of CTRP-3 (i.v.)/LPS (i.p.). (C) Adipose tissue IL-6 mRNA expression in mice treated with PBS (i.p.), CTRP-3 (i.v.), LPS (i.p.), or a combination of CTRP-3 (iv)/LPS (i.p.). (D) Adipose tissue MIP-2 mRNA expression in mice treated with PBS (i.p.), CTRP-3 (i.v.), LPS (i.p.), or a combination of CTRP-3 (iv)/LPS (i.p.).

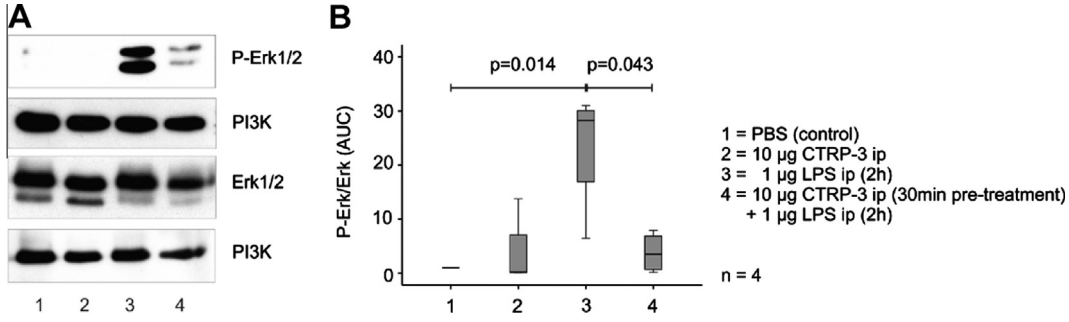

Fig. 3. Effects of CTRP-3 on LPS-induced phosphorylation of Erk-1/-2 in epididymal adipose tissue. Protein lysates of the epididymal adipose tissue were analyzed by Western blot analysis (n = 4 mice per group). PI3K expression was investigated as a loading control and as a housekeeping protein. Densitometric analysis was performed using ImageJ. Data were statistically analyzed by Mann–Whitney-*U*-Test and are depicted as box plots. PBS (200 µl), CTRP-3 (10 µg), and LPS (1 µg) were given intraperitoneally (i.p.). In the forth group, CTRP-3 (10 µg) was given i.p. 30 min prior to LPS (1 µg). Adipose tissue was obtained 2 h after LPS application.

insulin resistance [20,29,30], adipose tissue derived CTRP-3 could serve as future anti-inflammatory drug target [31].

Disclosure statement

The authors have nothing to declare and there is no conflict of interest.

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