Detection of adiponectin in cerebrospinal fluid in humans

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Neumeier M, Weigert J, Buettner R, Wanninger J, Schäffler A, Müller AM, Killian S, Sauerbruch S, Schlachetzki F, Steinbrecher A, Aslanidis C, Schölmerich J, Buechler C. Detection of adiponectin in cerebrospinal fluid in humans. Am J Physiol Endocrinol Metab 293: E965–E969, 2007. First published July 10, 2007; doi:10.1152/ajpendo.00119.2007.—Adiponectin circulates in the body in high concentrations, and 100-fold lower amounts were described in the cerebrospinal fluid (CSF) of mice, whereas in humans, contradictory results have been published. To clarify whether adiponectin is present in human CSF and is derived from the circulation, it was determined in human CSF and plasma of 52 nonselected patients. Adiponectin was detected by immunoblot in CSF and was quantified in CSF and serum by ELISA. CSF adiponectin was positively correlated to systemic levels, and the CSF/serum adiponectin ratio was correlated to the CSF/serum albumin ratio. Furthermore, disturbed function of the blood-brain barrier (BBB) was associated with an elevated CSF/serum adiponectin ratio. Adiponectin mRNA was not found in the brain, indicating that adiponectin crosses the BBB and/or the blood-cerebrospinal fluid barrier (BCB). Rat adiponectin with a COOH-terminal tag was injected into the tail vein of rats and was detected 3 h later in CSF. However, CSF adiponectin in humans and rats was ~0.1% of the serum concentration and therefore was below the 0.5% expected in the CSF because of the residual leakage of an undisturbed BBB/BCB. Taken together, data from the present study show that adiponectin in human CSF is far below the level expected by the baseline BBB/BCB permeability, indicating that adiponectin enters the brain much less efficiently than albumin, thus supporting recent data that exclude adiponectin transport to the CSF. Additional studies are needed to reveal whether these low levels of adiponectin in CSF have a physiological function.

blood-brain barrier; inflammation

ADIPONECTIN IS HIGHLY ABUNDANT in human serum, and low adiponectin is associated with the metabolic syndrome (13). Adiponectin circulates as trimers, hexamers, and high molecular mass forms, and these isoforms do not interconvert in vivo (8). Impaired multimerization of adiponectin in humans is associated with type 2 diabetes mellitus, showing that higher order structures are important for the biological function of adiponectin (14).

Studies in mice show that peripheral and intracerebroventricular (icv) administration of adiponectin decreases body weight by stimulating energy expenditure. Intravenous application (iv) of adiponectin led to a rise of cerebrospinal fluid (CSF) adiponectin 3 h after application, indicating that adiponectin crosses the blood-CSF barrier (BCB) and/or the blood-brain barrier (BBB) (9). Leptin-deficient obese mice were more sensitive to icv and iv injection of adiponectin, whereas Agouti mice were resistant to adiponectin, and therefore the effects of adiponectin may depend on the melanocortin pathway (9).

In contrast to rodents, a recent study could not detect adiponectin in the CSF of healthy human volunteers (12). Expression of adiponectin receptor-1 and -2 (AdipoR1/2) mRNA was demonstrated in brain endothelial cells, and treatment of these cells with recombinant adiponectin reduced IL-6 release. Therefore, it was suggested that adiponectin modifies cytokine release of brain endothelial cells and thereby may influence energy expenditure (12). A study by Pan et al. (7) compared BBB permeation of mouse obestatin, human ghrelin, and mouse adiponectin in mice, and adiponectin did not cross the BBB in their experiments (7). Two very recent reports (3, 4), however, identified adiponectin in human CSF at a 1,000-fold lower concentration compared with serum. AdipoR1 and AdipoR2 proteins were found expressed in the hypothalamus and the paraventricular nucleus, further indicating that adiponectin exerts a specific role in the brain (3). In contrast to systemic adiponectin, CSF adiponectin showed no sex difference or correlation with insulin resistance (3), and the trimERIC isoform was the predominant form identified in human CSF (4).

Taken together, these recent publications report contradictory findings concerning the abundance of adiponectin in human CSF. In the present study, the presence of adiponectin in human CSF was demonstrated by immunoblot and ELISA. In addition, using recombinant rat adiponectin with a COOH-terminal tag, we could show that systemic adiponectin enters the CSF in rats.

EXPERIMENTAL PROCEDURES

Patients. CSF and serum from 52 random patients (26 females) from the University Hospital, Department of Neurology, were collected. Standard lumbar puncture was performed under regional anesthesia (2). Body mass index (BMI) was measured for 41 patients, and the mean BMI was 26.1 ± 5.0 kg/m2. Mean age was 52 ± 15.5 yr. Patients with the following diagnoses were included: multiple sclerosis/optic neuritis (17), other central nervous system (CNS) inflammatory disorders (7), noninflammatory CNS disorders (i.e., normal pressure hydrocephalus) (18), diagnostic workup for peripheral neuritis/neuropathy, and white matter hyperintensities (10).

Normal BBB/BCB function was found in 29 patients, impaired barriers and normal IgG were diagnosed in 12 patients, and normal barrier function with elevated IgG was found in 8 patients. BBB/BCB disturbance was assessed using the CSF/serum ratio of albumin as well as CSF/serum ratio for albumin correlated to the CSF/serum ratio.
for immunoglobulins (10). Barrier function was unknown in three patients, as no serum controls were available. Experimental procedures were performed with the informed patient’s consent and were approved by the local ethics committee of the University of Regensburg.

**Blood-to-CSF transport.** Animal procedures were performed under the guidelines set by the University Hospital Regensburg Institutional Animal Care and Use Committee. Rat adiponectin with a COOH-terminal FLAG tag (20 μg/animal) was injected into the tail vein of five rats. After 3 h, rats were killed with carbon dioxide, and CSF was obtained by suboccipital puncture. Erythrocytes were not detected in the samples before and after centrifugation.

**Culture media and reagents.** Oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). LightCycler FastStart DNA Master SYBR Green I was from Roche (Mannheim, Germany). Recombinant human adiponectin, polyclonal adiponectin antibody, and ELISA to detect human adiponectin were from R&D Systems (Wiesbaden-Nordenstadt, Germany). Recombinant human adiponectin expressed in insect cells was purified as recently described (5). Rat adiponectin ELISA was from BioCat (Heidelberg, Germany), and rat adiponectin from Axxora (Grünenberg, Germany). The ELISA to determine rat albumin was from Natucet (Frankfurt, Germany). Anti-Flag antibody was from Sigma (Deisenhofen, Germany). Human adult normal tissue total brain mRNA was ordered from BioCat (Heidelberg, Germany), and postmortem tissue was used.

**Cultivation of immortalized rat brain endothelial cells.** Rat brain endothelial cells (RBE4 cells), a kind gift of F. Roux (INSERM U26, Hospital Fernand Widal, 75010, Paris, France) (11), were cultivated in DMEM-F12 supplemented with 10% FCS, 1 ng/ml fibroblast growth factor (FGF) (Peprotech), and 225 μg/ml G418 on collagen-coated dishes. For transport studies, cells were cultivated on Transwells (12 wells) until confluency of the cells as assessed by light microscopy to confirm a tight layer of cells. Transendothelial electrical resistance was 21.0 ± 4.5 Ω/cm².

**Monitoring of gene expression by real-time RT-PCR.** Real-time RT-PCR was performed as recently described (6).

**SDS-PAGE and immunoblotting.** Plasma was diluted 1,000-fold in PBS, and CSF was used undiluted; 10 μl of each sample were separated by SDS-PAGE as recently described (1). **FLAG tag adiponectin ELISA.** Plasma and CSF were loaded overnight on the anti-FLAG HS M2 96-well plate from Sigma (Deisenhofen, Germany). After a washing with PBS-Tween (0.1%), 100 μl of the biotinylated secondary rabbit anti-rat adiponectin antibody from BioCat were dispensed to each well and incubated for 3 h at room temperature. Further procedures were performed as recommended for the rat adiponectin ELISA. The concentrations were calculated using the absorbance values of the serial-diluted FLAG-tagged adiponectin assayed at the same time.

**Statistical analysis.** Data are represented as mean values ± SD (SPSS 12.0). Statistical differences were analyzed by Student’s t-test, and a value of P < 0.05 was regarded as statistically significant. The Pearson correlation was calculated using the SPSS 12.0 software.

**RESULTS**

Adiponectin in CSF and serum of the patients. Mean adiponectin in CSF was 3.2 ± 1.9 ng/ml and 4.4 ± 2.8 μg/ml in the respective serum samples (Fig. 1A). Immunoblot was performed with undiluted CSF and serum diluted 1,000-fold in PBS. Samples were not boiled and were analyzed by nonreducing and reducing SDS-PAGE. Under nonreducing conditions, plasma and CSF adiponectin mainly formed complexes with a molecular mass of 150–180 kDa, whereas under reducing conditions, lower molecular mass forms became visible (Fig. 1B).

**A**

![Image](http://ajpendo.physiology.org/Downloaded/from/http://ajpendo.physiology.org/)

**B**

![Image](http://ajpendo.physiology.org/)

**C**

![Image](http://ajpendo.physiology.org/)

**D**

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**E**

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**F**

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Fig. 1. Adiponectin in human cerebrospinal fluid (CSF) and serum. A: adiponectin levels in CSF (ng/ml) and serum (μg/ml) as determined by ELISA in samples of 49 patients. B: CSF and serum (S) (1,000-fold dilution) were separated by SDS-PAGE without or with β-mercaptoethanol (ME), and the presence of adiponectin was analyzed by immunoblot. C: adiponectin in CSF of patients with normal blood-brain barrier (BBB)/blood-cerebrospinal fluid barrier (BCB) function (group 1), in CSF of patients with an impaired BBB/BCB and normal IgG (group 2), and in CSF of patients with a normal BBB/BCB and elevated IgG (group 3). D: CSF/serum adiponectin ratio in groups 1, 2, and 3. E: correlation of CSF and systemic adiponectin in samples from patients with an undisturbed BBB/BCB (group 1, open circles; group 3, solid circles). F: correlation of adiponectin and albumin CSF/serum ratio (group 1, open circles; group 2, solid gray circles; group 3, solid black circles). *P < 0.05.

Adiponectin in CSF of patients with a normal BBB/BCB function (group 1) was 3.0 ± 1.8 ng/ml; in CSF of patients with an impaired BBB/BCB and normal IgG, it was 4.0 ± 1.2 ng/ml (group 2, P = 0.07 compared with group 1); and in CSF of patients with an undisturbed BBB/BCB and elevated IgG, it was 2.1 ± 0.4 ng/ml (group 3, P = 0.0007 compared with group 2, and P = 0.09 compared with group 1) (Fig. 1C). Serum adiponectin was not significantly different in the three groups (group 1, 4.9 ± 3.3 μg/ml; group 2, 3.1 ± 1.4 μg/ml; group 3, 3.6 ± 1.7 μg/ml). CSF/serum adiponectin ratio (×10⁻³) was 0.7 ± 0.3 in group 1, 1.4 ± 0.5 in group 2 (P = 0.000002 compared with group 1), and 0.7 ± 0.4 in group 3 (P = 0.5 compared with group 1, and P = 0.004 compared with group 2) (Fig. 1D). The CSF/albumin ratio (×10⁻³) for group 1 was 4.6 ± 1.5, for group 2 was 9.6 ± 1.5, and for group 3 was 5.0 ± 1.7 and was significantly elevated in group 2 compared with groups 1 and 3 (P = 0.0001 for the 2 comparisons).
Systemic adiponectin in females was 4.8 ± 3.1 μg/ml and in males 3.4 ± 1.9 μg/ml (P = 0.03), and CSF adiponectin in females was 3.5 ± 1.2 ng/ml and in males 2.7 ± 1.2 (P = 0.04). Neither systemic (r = -0.138, P = 0.384) nor CSF adiponectin (r = 0.02, P = 0.9) was negatively correlated to BMI. There was a positive correlation between systemic and CSF adiponectin (r = 0.44, P = 0.001) when all patients were analyzed, and the correlation was r = 0.53 and P = 0.0001 when only patients with an undisturbed BBB/BCB (groups 1 and 3) were analyzed (Fig. 1E). The CSF/serum adiponectin ratio was correlated to the CSF/serum albumin ratio (r = 0.76, P = 0.0001) (Fig. 1F). There was a positive correlation of CSF adiponectin with the number of leukocytes in the CSF (r = 0.45, P = 0.002), and this correlation was r = 0.48 when only patients from groups 1 and 3 were analyzed (not shown).

Adiponectin and adiponectin receptor mRNA in whole brain. Whereas adiponectin mRNA was highly abundant in adipose tissue, it was not detected in mRNA isolated from total brain of five adults (Fig. 2A). AdipoR1 and AdipoR2 mRNA was amplified by specific primers from total brain mRNA of all donors (Fig. 2B). To get more quantitative data, the expression of AdipoR1 and AdipoR2 was investigated by real-time RT-PCR in the mRNA isolated from whole brain samples, and relative mRNA expression of AdipoR1 and AdipoR2 was 1.7 ± 1.6 and 3.2 ± 1.5, respectively, after normalization to β-actin (Fig. 2C).

Adiponectin “diffusion” analyzed in rat brain endothelial cells. Rat brain endothelial (RBE4) cells were grown to maximum confluence, and rat serum with 6 μg/ml adiponectin was diluted 1:10 in medium and added to the upper well, with medium alone in the lower well; 1.7 ± 0.1 ng/ml adiponectin were detected in the lower well after 30 min of cultivation and increased to 2.3 ± 0.3 ng/ml after 1 h and to 8.3 ± 0.5 ng/ml after 1.5 h. No further increase was observed when the medium was collected 2, 2.5, and 3 h later (Fig. 3A). Recombinant human adiponectin was also added to the upper wells, and similar results were obtained (not shown). Rat serum diluted with medium 20-fold, 10-fold, 5-fold, and 2.5-fold was added to the apical side; adiponectin determined 3 h later in the lower wells steadily increased, and the mean permeability was 1.7 ± 0.2%. Rat albumin was simultaneously determined, and 4.4 ± 1.0% was detected at the basolateral side. Western blot analysis could demonstrate expression of AdipoR1 and AdipoR2 in RBE4 (not shown).

Fig. 2. Adiponectin and adiponectin receptor mRNA in total human brain. A: agarose gel electrophoresis of RT-PCR amplification products of adiponectin (APM) mRNA from total brain RNA (1–5 of 5 different patients) and adipose tissue. B: agarose gel of RT-PCR amplification products of adiponectin receptor-1 (AdipoR1) and adiponectin receptor-2 (AdipoR2) mRNA from total brain RNA. C: AdipoR1 and AdipoR2 mRNA in brain as determined by LightCycler RT-PCR. Values were normalized to β-actin expression of the same RNA sample.

Fig. 3. Adiponectin crosses the BBB in vitro and in vivo. A: rat serum (10-fold diluted) was added to the upper wells of confluent rat brain endothelial cells (RBE4 cells) on Transwell plates, and adiponectin was determined in the lower wells. One representative result with 3 wells each of 2 independent experiments is shown. B: human adiponectin was injected intravenously into 5 rats and determined in plasma by ELISA after 1, 2, and 3 h. C: rat adiponectin was injected intravenously into 5 rats and determined in plasma by ELISA after 1, 2, and 3 h. D: rat adiponectin with a COOH-terminal FLAG tag was injected into the tail vein of rats and was determined in CSF and serum by immuno-blotting after 3 h. E: rat adiponectin with a COOH-terminal FL-AG tag was injected into the tail vein of 5 rats and was determined in CSF of injected (APM) and control animals by ELISA. *P < 0.05.
the CSF, 20 μg of human adiponectin (expressed in a mouse cell line forming isoforms larger than trimers; Ref. 5) were injected into the tail vein of five rats. Human adiponectin was used to distinguish endogenous from injected adiponectin by an ELISA that specifically detects human adiponectin. Plasma was collected after 1, 2, and 3 h and CSF after 3 h; human adiponectin assayed with an ELISA was 775 ± 719 ng/ml after 1 h, 108 ± 33 ng/ml after 2 h, and 75 ± 41 ng/ml after 3 h in serum (Fig. 3B). Taking into account a 1,600-fold lower CSF concentration, 47 pg/ml human adiponectin were expected in CSF. The detection limit of the ELISA is 40 pg/ml, and therefore human adiponectin in CSF may have been too low for this assay. The experiments were repeated using trimeric human adiponectin purified from insect cells (5), but similar results were obtained (not shown). Therefore, rat adiponectin with a COOH-terminal FLAG tag (20 μg for each animal) was injected into the tail vein of five rats. Plasma was collected after 1 h, and 1,157 ± 36 ng/ml recombinant adiponectin were detected; after 2 h, adiponectin decreased to 866 ± 111 ng/ml (P = 0.006), and 3 h later, it was 820 ± 93 ng/ml (Fig. 3C). CSF was collected after 3 h, and the recombinant adiponectin was detected by immunoblot using a FLAG tag antibody in plasma diluted 100-fold. A faint but clearly visible FLAG tag signal was detected in the CSF of injected rats but not the control CSF (Fig. 3D). Quantification of the FLAG-tagged adiponectin revealed that 0.72 ± 0.06 ng/ml were found in CSF 3 h after injection into the tail vein (Fig. 3E), a level ~1,200-fold lower compared with serum.

**DISCUSSION**

High levels of adiponectin circulate in the serum of humans (13); however, controversy exists as to whether adiponectin crosses the BCB and/or BBB to exert central effects. In the present investigation, adiponectin was detected in human CSF in a variety of patients even in the absence of BCB and BBB disturbance. In the present investigation, adiponectin levels in CSF were found to be ~1,000-fold lower compared with systemic levels, and this is in accordance with two very recent reports (3, 4) where CSF and serum adiponectin were analyzed in healthy probands. Whereas similar results were described in rodents (9), two recent studies failed to detect these unexpected low amounts of adiponectin in human CSF because they applied less sensitive ELISAs (7, 12). Whereas CSF adiponectin was higher in females than in males in our study group and in the mice studied by Qi et al. (9), a sex difference was not identified by Kos et al. (3). The sex difference is not very prominent, and the number of samples investigated by Kos et al. may have been too small to detect this difference.

CSF/serum adiponectin is significantly higher in patients with a disturbed compared with patients with an undisturbed BCB/BBB. A modest correlation of CSF to serum adiponectin and a considerable correlation of the adiponectin and albumin CSF/serum ratio indicate that adiponectin is derived by leakage via extracellular pathways from the circulation. This was supported by the finding that adiponectin mRNA is not expressed in the brain, a result also described by Spranger et al. (12). Nevertheless, CSF adiponectin is rather low, and future studies have to demonstrate whether adiponectin in nanograms-per-milliliter concentrations exerts any effects. The adiponectin receptors AdipoR1 and AdipoR2 were expressed in the neuronal cells in the hypothalamus, and AdipoR1 protein was detected in the anterior and posterior hypothalamus, whereas AdipoR2 was more abundant in neurons of the paraventricular nucleus that also stained positive for neuropeptide Y (3). The dissociation constant for high-affinity binding sites of AdipoR1 and AdipoR2 is 0.06 μg/ml (17), a concentration exceeding CSF amounts in humans. It may be suggested that, so far, unrecognized adiponectin receptors with a higher affinity are expressed in the brain or that adiponectin is concentrated at specific sites of the brain. In addition, adiponectin associates with growth factors like basic FGF (FGF-2), and already 5 ng/ml adiponectin inhibit the binding of FGF-2 to its receptor (15). FGF-2 in the CSF induced angiogenesis in transient cerebral ischemic damage (16). CSF adiponectin positively correlates to the number of leukocytes in the CSF, indicating a function of adiponectin in inflammatory and regenerative processes in the brain.

At least in mice, the injection of adiponectin in the lateral cerebral ventricle decreased body weight, and the amounts of recombinant protein administered were in the physiological range of mice. Recombinant wild type, the globular form, and the Cys39Ser mutant form were similarly effective in this study (9). Adiponectin isomers were investigated in human CSF by gel filtration chromatography and subsequent immunoblotting, and only the hexamer and the trimer were found in CSF (4), whereas the higher molecular mass form was also detected in the corresponding serum samples. In the present study, immunoblot was performed, and only complexes with a molecular mass resembling that of hexameric adiponectin were detected. However, this analysis was only done with a limited number of samples and very simple technology, and therefore no conclusions on adiponectin isoforms from our experiments can be drawn.

Two studies failed to detect systemically administered adiponectin in the CSF of mice (7, 12), and this may be in part explained by the relatively low amounts of recombinant adiponectin used. In vivo studies with RBE4 cells indicated that rat adiponectin crosses the cell layer most likely by leakage of the monolayer. CSF adiponectin in rats was 1,600-fold lower compared with that in serum, and therefore the physiological situation is more similar to humans with a 1,000-fold reduction than to mice with a 100-fold reduced level. With the use of recombinant rat adiponectin, it was demonstrated that adiponectin enters the CSF within 3 h after injection in the tail vein.

In conclusion, adiponectin from the blood circulation enters the CSF in rats and most likely in humans, at a rate lower than albumin, however. CSF concentrations in humans make up only 0.1% of systemic levels, and additional studies can investigate whether this can be explained by a rapid degradation of adiponectin in CSF or at least in part by the observation that high molecular mass adiponectin does not cross the BBB/BCB. Functional studies are needed to show the relevance of these low amounts of adiponectin in human CSF, which may be associated with regenerative processes.

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