Detection of adiponectin in cerebrospinal fluid in humans

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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 albumin ratio. Furthermore, 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. Moreover, disturbed function of the blood-brain barrier (BBB) was associated with type 2 diabetes mellitus, showing that higher order structures are important for the biological function of adiponectin (14).
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 expressed in insect cells was purified as recently described (5). Rat adiponectin ELISA was from BioCat (Heidelberg, Germany), and rat adiponectin was from Axxora (Gruenberg, Germany). The ELISA to detect human adiponectin was from R&D Systems (Minneapolis, MN, USA). Recombinant human adiponectin, polyclonal adiponectin antibody, and ELISA to detect human adiponectin were from R&D Systems (Minneapolis, MN, USA). Recombinant human adiponectin was from BioCat (Heidelberg, Germany), and rat adiponectin was from Axxora (Gruenberg, Germany). The ELISA to detect human adiponectin was from R&D Systems (Minneapolis, MN, USA). Recombinant human adiponectin, polyclonal adiponectin antibody, and ELISA to detect human adiponectin were from R&D Systems (Minneapolis, MN, USA). Recombinant human adiponectin was from BioCat (Heidelberg, Germany), and rat adiponectin was from Axxora (Gruenberg, Germany). The ELISA to detect human adiponectin was from R&D Systems (Minneapolis, MN, USA).

**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).

![Image](http://ajpendo.physiology.org/DownloadedFrom)
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 ng/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% was detected at the basolateral side. Western blot analysis could demonstrate expression of AdipoR1 and AdipoR2 in RBE4 (not shown).

**Diffusion of systemic adiponectin to the CSF in rats.** CSF and serum adiponectin were also determined in six rats: serum levels were 3.2 ± 0.5 μg/ml, and CSF levels were 2.5 ± 1.4 ng/ml; therefore, adiponectin was 1,629 ± 630-fold higher in serum. To investigate whether adiponectin from plasma enters...
the CSF, 20 \( \mu g \) 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 \( \pm \) 719 ng/ml after 1 h, 108 \( \pm \) 33 ng/ml after 2 h, and 75 \( \pm \) 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 \( \mu g \) for each animal) was injected into the tail vein of five rats. Plasma was collected after 1 h, and 1,157 \( \pm \) 36 ng/ml recombinant adiponectin were detected; after 2 h, adiponectin decreased to 866 \( \pm \) 111 ng/ml (\( P = 0.006 \)), and 3 h later, it was 820 \( \pm \) 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 \( \pm \) 0.06 ng/ml were found in CSF 3 h after injection into the tail vein (Fig. 3E), a level \( \sim \)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 \( \sim \)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 \( \mu g/ml \) (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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References


