Detection of Adiponectin in Cerebrospinal Fluid in Humans

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ABSTRACT

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 non-selected patients. Adiponectin was detected by immunoblot in CSF and was quantified in CSF and serum by ELISA. CSF adiponectin positively correlated to systemic levels and the CSF/serum adiponectin ratio 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 C-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 is approximately 0.1% of the serum concentration and therefore is below the 0.5% expected in the CSF due to the residual leakage of an undisturbed BBB/BCB. Taken together, the present study shows 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 efficient 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.

Key terms: Adiponectin, blood brain barrier, inflammation
INTRODUCTION

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 weight 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 decrease 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. 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, however, identified adiponectin in human CSF at a 1,000-fold lower concentration compared to serum (3, 4). AdipoR1 and AdipoR2 protein 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 gender difference or correlation with insulin resistance (3) and the trimeric isoform was the predominant form identified in human CSF (4).

Taken together, recent publications report contradictory findings concerning the abundance of adiponectin in human CSF. In the current study the presence of adiponectin in human CSF was demonstrated by immunoblot and ELISA. In addition, using recombinant rat adiponectin with a C-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 known from 41 patients and the mean BMI was 26.1 ± 5.0 kg/m². Mean age was 52 ± 15.5 years. Patients with the following diagnoses were included: multiple sclerosis/optic neuritis (17), other CNS inflammatory disorders (7), non-inflammatory 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 was diagnosed in 12 patients and normal barrier function with elevated IgG 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 3 patients as no serum controls were available. Experimental procedures were performed with the informed patient's consent 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 C-terminal FLAG-tag (20 μg/animal) was injected into the tail vein of 5 rats. After 3 h rats were sacrificed with carbon dioxide and CSF was obtained by suboccipital punctation. 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 was from Axxora (Grünberg, Germany). The ELISA to determine rat albumin was from Natutec (Frankfurt aM, 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 4 (RBE4).* RBE4 cells, a kind gift of F. Roux (11), were cultivated in DMEM/F12 supplemented with 10% FCS, 1 ng/ml 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. Trans Endothelial 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-polyacrylamide gel electrophoresis as recently described (1).

*FLAG-tag adiponectin ELISA.* Plasma and CSF were loaded over night on the anti-FLAG HS M2 96-well plate from Sigma (Deisenhofen, Germany). After washing with PBS-T, 100 μl of the biotinylated secondary rabbit anti-rat adiponectin antibody from BioCat was dispensed to each well and incubated for 3 h at RT. 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 value ± standard deviation (SPSS 12.0). Statistical differences were analysed by Student’s t-test and a value of p < 0.05 was regarded
as statistically significant. The Pearson's correlation was calculated using the SPSS 12.0 software.
RESULTS

Adiponectin in CSF and serum of the patients. Mean adiponectin in CSF was $3.2 \pm 1.9$ ng/ml and $4.4 \pm 2.8$ 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 analysed by non-reducing and reducing SDS-PAGE. Under non-reducing conditions plasma and CSF adiponectin mainly formed complexes with a molecular weight of 150 to 180 kDa whereas under reducing conditions lower molecular weight forms became visible (Fig. 1B).

Adiponectin in CSF of patients with a normal BBB/BCB function (group 1) was $3.0 \pm 1.8$ ng/ml, in CSF of patients with an impaired BBB/BCB and normal IgG it was $4.0 \pm 1.2$ ng/ml (group 2, $p = 0.07$ compared to group 1) and in CSF of patients with an undisturbed BBB/BCB and elevated IgG it was $2.1 \pm 0.4$ ng/ml (group 3, $p = 0.0007$ compared to group 2 and $p = 0.09$ compared to group 1) (Fig. 1C). Serum adiponectin was not significantly different in the 3 groups (group 1: $4.9 \pm 3.3$ µg/ml; group 2: $3.1 \pm 1.4$ µg/ml; group 3: $3.6 \pm 1.7$ µg/ml). CSF/serum adiponectin ratio was $0.0007 \pm 0.3$ in group 0.0014 ± 0.5 in group 2 ($p = 0.000002$ compared to group 1) and $0.0007 \pm 0.4$ in group 3 ($p = 0.5$ compared to group 1 and $p = 0.004$ compared to group 2) (Fig. 1D). The CSF/albumin ratio for group 1 was $0.0046 \pm 1.5$, for group 2 $0.0096 \pm 1.5$ and for group 3 $0.005 \pm 1.7$ and was significantly elevated in group 2 when compared to group 1 and 3 ($p = 0.0001$ for the 2 comparisons).

Systemic adiponectin in females was $4.8 \pm 3.1$ µg/ml and in males $3.4 \pm 1.9$ µg/ml ($p = 0.03$) and CSF adiponectin in females was $3.5 \pm 1.2$ ng/ml and in males $2.7 \pm 1.2$ ($p = 0.04$). Neither systemic ($r = -0.138$, $p = 0.384$) nor CSF adiponectin ($r = 0.02$, $p = 0.9$) negatively correlated to the body mass index (BMI). There was a positive correlation between systemic and CSF adiponectin ($r = 0.44$, $p = 0.001$) when all patients were analysed and the correlation was $r = 0.53$, $p = 0.0001$ when only patients with an undisturbed BBB/BCB (group 1 and 3) were analysed (Fig. 1E). The CSF/serum adiponectin ratio 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 group 1 and 3 were analysed (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 5 adults (Fig. 2A). AdipoR1 and AdipoR2 mRNA were amplified by specific primers from total brain mRNA of all donors (Fig. 2B). To get more quantitative data, the expression of the adiponectin receptors 1 and 2 was investigated by real-time RT-PCR in the mRNA isolated from whole brain samples and relative mRNA expression of AdipoR1 and AdipoR2 was \( 1.7 \pm 1.6 \) and \( 3.2 \pm 1.5 \), respectively, after normalization to \( \beta \)-actin (Fig. 2C).

*Adiponectin "diffusion" analysed in rat brain endothelial cells.* Rat brain endothelial (RBE4) cells were grown to maximum confluence and rat serum with 6 \( \mu \)g/ml adiponectin was diluted 1:10 in medium and added to the upper well and medium alone in the lower well. 1.7 \( \pm 0.1 \) ng/ml adiponectin was detected in the lower well after 30 min of cultivation and increased to 2.3 \( \pm 0.3 \) ng/ml after 1 h and to 8.3 \( \pm 0.5 \) ng/ml after 1.5 h. No further increase was observed when the medium was collected 2 h, 2.5 h 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 and adiponectin determined 3 h later in the lower wells steadily increased and the mean permeability was 1.7 \( \pm 0.2 \) %. Rat albumin was simultaneously determined and 4.4 \( \pm 1.0 \) % were detected at the basolateral side. Western blot analysis could demonstrate expression of the adiponectin receptor 1 and 2 in RBE4 (not shown).

*Diffusion of systemic adiponectin to the CSF in rats.* CSF and serum adiponectin were also determined in 6 rats and serum levels were 3.2 \( \pm 0.5 \) \( \mu \)g/ml and CSF levels 2.5 \( \pm 1.4 \) ng/ml and therefore was 1,629 \( \pm 630 \) 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 (5) were injected into the tail vein of 5 rats respectively. Human adiponectin was used to distinguish endogenous from injected adiponectin by an ELISA that specifically detects human adiponectin. Plasma was collected after 1 h, 2 h and 3 h and CSF after 3 h and 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 are 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 C-terminal FLAG-tag (20 μg for each animal) was injected into the tail vein of 5 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 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 about 1,200-fold lower when compared to the serum.
DISCUSSION

High levels of adiponectin circulate in serum of humans (13), however, controversy exists whether adiponectin crosses the BCB and/or BBB to exert central effects. In the current investigation adiponectin was detected in human CSF in a variety of patients even in the absence of BCB and BBB disturbance. In the current investigation adiponectin levels in CSF were found about 1,000-fold lower when compared to systemic levels and this is in accordance with two very recent reports where CSF and serum adiponectin were analysed in healthy probands (3, 4). 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 is higher in females than in males in our study group and in the mice studied by Qi et al. (9), a gender difference was not identified by Kos et al. (3). The gender 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 when compared to 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 the extracellular pathways from the circulation. This is 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 ng/ml concentrations exerts any effects. The adiponectin receptors AdipoR1 and AdipoR2 are 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 NPY (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 not recognized 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 fibroblast growth factor (FGF-2) and already 5 ng/ml adiponectin inhibit the binding of FGF-2 to its receptor (15). FGF-2 in the CSF induces 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 decreases body weight and the amounts of recombinant protein applied 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 immunoblot and only the hexamer and the trimer were found in CSF (4) whereas the HMW form was also detected in the corresponding serum samples. In the current study immunoblot was performed and only complexes with a molecular weight that may resemble hexameric adiponectin were detected. However this analysis was only done with a limited number of samples and a very simple technology and therefore no conclusions on adiponectin isoforms from our experiments can be drawn.

Two studies failed to detect systemic applied 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 is 1,600-fold lower when compared to serum and therefore the physiologic situation is more similar to humans with a 1,000-fold reduction than to mice with a 100-fold reduced level. Using 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, however, at a rate lower than albumin. CSF concentrations in humans make up only 0.1% of systemic levels and additional studies have to 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 weight adiponectin does not cross the BBB/BCB. Functional studies have to show the relevance of these low amounts of adiponectin in human CSF, which may be associated with regenerative processes.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Adiponectin in human CSF and serum. (A) Adiponectin levels in CSF (ng/ml) and serum (μg/ml) as determined by ELISA in samples of 52 patients. (B) CSF and serum (S) (1,000-fold dilution) were separated by SDS-PAGE without or with β-ME and the presence of adiponectin was analysed by immunoblot. (C) Adiponectin in CSF of patients with a normal BBB/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 group 1, group 2 and in group 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 circle, group 2: solid grey circles, group 3: solid circles).

Fig. 2. Adiponectin and adiponectin receptor mRNA in total human brain. (A) Agarose gel electrophoresis of RT-PCR amplification products of adiponectin mRNA from total brain RNA (1 to 5 of 5 different patients) and adipose tissue. (B) Agarose gel of RT-PCR amplification products of AdipoR1 and 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 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 h, 2 h and 3 h. (C) Rat adiponectin was injected intravenously into 5 rats and determined in plasma by ELISA after 1 h, 2 h and 3 h. (D) Rat adiponectin with a C-terminal FLAG-tag was injected into the tail vein of rats and
was determined in CSF and serum by immunoblotting after 3 h. (E) Rat adiponectin with a C-terminal FLAG-tag was injected into the tail vein of 5 rats and was determined in CSF of injected (APM) and control animals by ELISA.
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Adiponectin ng/ml

0 h 1 h 2 h 3 h

CSF, col., 3h

Serum, col., 3h

APM, 3h

Control

APM

Adiponectin ng/ml

0 200 400 600 800 1,000 1,200

0 1 h 2 h 3 h

Adiponectin ng/ml

0 300 600 900 1,200 1,500 1,800

0 2 4 6 8 10

Control

APM

Adiponectin ng/ml

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

Control

APM