VPA response in SMA is suppressed by the fatty acid translocase CD36

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Functional loss of SMN1 causes proximal spinal muscular atrophy (SMA), the most common genetic condition accounting for infant lethality. Hence, the hypomorphic copy gene SMN2 is the only resource of functional SMN protein in SMA patients and influences SMA severity in a dose-dependent manner. Consequently, current therapeutic approaches focus on SMN2. Histone deacetylase inhibitors (HDACi), such as the short chain fatty acid VPA (valproic acid), ameliorate the SMA phenotype by activating the SMN2 expression. By analyzing blood SMN2 expression in 16 VPA-treated SMA patients, about one-third of individuals were identified as positive responders presenting increased SMN2 transcript levels. In 66% of enrolled patients, a concordant response was detected in the respective fibroblasts. Most importantly, by taking the detour of reprogramming SMA patients’ fibroblasts, we showed that the VPA response was maintained even in GABAergic neurons derived from induced pluripotent stem cells (iPS) cells. Differential expression microarray analysis revealed a complete lack of response to VPA in non-responders, which was associated with an increased expression of the fatty acid translocase CD36. The pivotal role of CD36 as the cause of non-responsiveness was proven in various in vitro approaches. Most importantly, knockdown of CD36 in SMA fibroblasts converted non- into pos-responders. In summary, the concordant response from blood to the central nervous system (CNS) to VPA may allow selection of pos-responders prior to therapy. Increased CD36 expression accounts for VPA non-responsiveness. These findings may be essential not only for SMA but also for other diseases such as epilepsy or migraine frequently treated with VPA.

INTRODUCTION

The group of proximal spinal muscular atrophies—combined as spinal muscular atrophy (SMA)—represents one of the most prevalent autosomal recessive disorders in humans. Onset and severity of the disease vary remarkably between patients and represent a continuous phenotypic spectrum. Based on the highest individually achieved motor function (1), the SMA phenotype has been subdivided into three different types termed SMA type I (severest form) to III (juvenile form). The characteristic feature common to all types of proximal SMA is a progressive degeneration of α-motor neurons in the anterior horns of the spinal cord. The estimated incidence is 1 in 6000 live births, while the carrier frequency varies between 1:25 and 1:105 (2,3). In >96% of all SMA patients, the SMN1 gene (survival motor neuron 1, OMIM 600354)—encoding the essential SMN protein—is homozygously deleted. In the remainder of the patients, SMN1 mutations hamper proper SMN function (4,5). As a result of a primate exclusive duplication of the genomic region harboring SMN1, a variable number of copy genes, termed SMN2, are present in the human genome (6,7). Yet, due to a synonymous variation in exon 7 altering splicing, each SMN2 copy

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produces merely 10% functional full-length protein (8). Although not capable of compensating for the loss of the SMN1 gene, SMN2 is the only resource of SMN protein in SMA patients. As such, it influences SMA disease severity in a dose-dependent manner. While, e.g. most severely affected type I patients usually carry two SMN2 copies, three copies are most often found in patients with intermediate SMA (type II) and four copies in mild SMA (type III) (2).

To date, there has been no cure available for SMA. However, among the prospective therapeutics for SMA, the histone deacetylase inhibitor (HDACi) valproic acid (VPA) advanced the most and entered phase II clinical trials (9–17). Mechanistically, VPA activates SMN2 by promoter hyperacetylation and thereby increases the SMN amount generated from each SMN2 locus.

Several clinical trials already investigated the potential of VPA as a SMA therapeutic (11–14,18). However, the results were inconsistent. In the initial pilot study using blood SMN2 transcripts as the only readout, a positive effect was noted in one-third of the patients (9). Subsequent open label clinical trials with type II and III/IV SMA patients assessing their motor abilities demonstrated significant improvements, particularly in the younger type II patients (<5 years) (11,14,18). The motor improvements in young SMA patients were confirmed in a double-blind, randomized, placebo-controlled clinical trial in type II and III SMA patients; however, no significant effect was found in the group of older individuals (12,13).

In summary, the accomplished clinical trials demonstrated that VPA is only beneficial to a restricted subset of SMA patients. These individuals show indeed the desired SMN2 activation in blood, while others do not or even exhibit the opposite (9,16). The underlying factors influencing the individual response were unknown. Several important questions arose from these observations. First, is it feasible to extrapolate from circulating blood monocyte SMN2 transcripts to a second, easily available specimen such as skin? Second, is such extrapolation even valid to the central nervous system (CNS)? And, most importantly, what is the pivotal factor influencing VPA response?

RESULTS

About one-third of SMA Patients are VPA Pos-responders

In an initial clinical trial with VPA in 20 SMA patients using circulating blood monocyte SMN2 transcripts as readout, we have been able to show that about one-third of the patients are pos-responders (FL-SMN2†), while in the remainder of the patients the FL-SMN2 levels remained constant or even declined (non-responders) (9). To prove that this is indeed a reproducible observation and not a random finding, we analyzed another 16 SMA patients currently undergoing experimental VPA therapy. Again, circulating SMN2 transcripts were quantified to assess VPA response. We identified five pos-responders and eleven non-responders (Supplementary Material, Table S1). Of note, for three of the pos-responders, attenuation in the VPA response was observed, i.e. SMN2 levels returned to baseline during the course of time. In summary, these results are comparable with those of our previous study (9) and confirmed that about one-third of patients are VPA pos-responders.

Response to VPA treatment is concordant across blood, fibroblasts and GABAergic neurons

Although the finding that about one-third of SMA patients are pos-responders was verified, it remained disputable whether blood data allow inference of VPA response in other tissues. Therefore, primary fibroblast lines were established from skin biopsies of 15 VPA-treated SMA patients. Triplicates of each culture were treated with VPA and SMN levels assessed by semiquantitative western blotting. Initially, some of the fibroblast lines were treated with VPA doses between 5 and 500 μM as published previously (10). However, during the course of the experiments, VPA concentrations were adapted since higher concentrations provided more robust results (Supplementary Material, Table S2).

A concordant response between blood and fibroblasts was recorded in about 66% across all analyzed cell lines (Fig. 1 and Supplementary Material, Table S2). Within the group of concordant pos-respondor fibroblast lines (ML67, ML72 and ML89), SMN levels were increased by 30–120% on average. As expected, no notable change in SMN expression was detected for six and decreased levels for two non-responders. Of note, increased SMN2 expression was recorded unexpectedly for three non-responders (ML62, ML96 and ML94). All discordant fibroblast lines were excluded from subsequent experiments.

While the fibroblast data indicated a mostly concordant response, it remained elusive whether this allows inference to the CNS. Since it is not feasible to sample neurons from SMA patients, we took the detour of generating iPSCs (induced pluripotent stem cells) from SMA fibroblast and differentiating them into neurons. Using the classical four-factor approach (19), iPSCs were generated from a pos- responder (HKG1, ML17) and a non-responder (HKG4, ML73) fibroblast cell line. Following extensive validation and characterization regarding their pluripotency (Supplementary Material, Figs S1 to S4), these iPSCs were differentiated into GABAergic neurons (Fig. 2A and B) via the intermediate step of long-term self-renewing rosette-type neuroepithelial stem cells (lt-NES) (20). GABAergic neurons were selected for two reasons: first, they are the primarily affected population of neurons in epilepsy patients, who represent the majority of VPA-treated individuals worldwide. Second, differentiation into motor neurons would be desirable, but the efficiency of this process is too low to allow proper assessment of VPA response. qRT-PCR analysis revealed increased SMN2 expression in pos-responder HKG1 (ML17) under VPA treatment, but showed no change in non-responder HKG4 (ML73) neurons (Fig. 2C). Since GABA release is typically stimulated by VPA application (21), we also quantified GABA levels in the cell culture supernatant of these cultures. Accordingly, a nearly 2.5-fold increase in GABA release upon VPA application was recorded for the pos-responder HKG1. Moreover, the opposite was found for the non-responder HKG4 (Fig. 2D).

Taken together, these data strongly suggest that the response to VPA treatment is concordant among blood, fibroblasts and CNS neurons.
VPA does not trigger any response in non-responders

Next, we focused on understanding which factor prevents a positive treatment outcome. Therefore, all 10 concordant fibroblast lines were analyzed in more detail. To increase the power of our subsequent analysis, we included the well-characterized pos-responder fibroblast lines (ML5, ML16 and ML17) (10,22,23). Unfortunately, no blood data from the respective donors were available.

Initially, we tested the effect of VPA on SMN2 promoter hyperacetylation in pos- and non-responders by ChIP analysis. As expected, acetylation was increased by 20–100% in pos-responders, whereas no change or even decreased levels were detected in non-responder fibroblast lines (Fig. 3A). This lack of promoter induction offers a good explanation why SMN protein is not increased by VPA treatment. To test whether this is a VPA-specific observation, SMA fibroblasts were treated with other HDACi known to activate SMN2 gene expression. Similar to VPA, sodium butyrate, another short-chain fatty acid HDACi, did not trigger any response in non-responders, while LBH589 (hydroxamic acid) and phenyl butyrate (aromatic acid) increased the SMN levels expectedly (Fig. 3B). This suggested that the effect of VPA is not specifically suppressed but rather prohibited by a more general mechanism. To determine whether this is occurring on the level of HDACs, protein kinase A (PKA) activity as a HDAC-independent read-out of VPA treatment was assessed (24). Within the group of pos-responders, PKA activity was VPA-dose dependently reduced to approximately 50% (Fig. 3C). In contrast, no such effect was observed in the non-responders. These data together with the aforementioned ChIP assays confirmed that in non-responders, the effect of VPA is

Figure 1. VPA treatment of SMA fibroblasts identifies pos- and non-responders. (A) Schematic overview of the VPA treatment of SMA patients’ fibroblasts. Depicted are SMN protein levels as mean ± SEM after treatment with increasing VPA concentrations for 16 h (n = 3 for each data point). Dashed line represents mock SMN levels set as 100%. ML67, ML72 and ML89 were considered as concordant pos-responders, while all non-responders except ML62, ML96 and ML94 were concordant. Statistical significances and details on how fibroblast lines were categorized are given in the Supplementary Material, Table S2. Furthermore, SMN2 transcript levels in the blood of the respective patient under VPA treatment are summarized in Supplementary Material, Table S1. (B) Representative western blot illustrating the variable response to VPA in fibroblasts. The bar chart gives the mean protein levels ± SEM of three independent experiments for all three fibroblast lines. ML67 is a pos-responder, while ML105 is a non-responder with constant SMN levels and ML73 is a non-responder with decreased SMN levels (*P < 0.05, **P < 0.01, ***P < 0.001).
not HDAC specifically suppressed, but rather on a more general level.

Transcriptome profiling identifies CD36 as a candidate for VPA non-responsiveness

To identify the pivotal factor(s) preventing a positive response to VPA treatment, we performed transcriptome-wide differential expression analysis of mock and VPA-treated SMA fibroblasts. Applying initially relatively loose criteria (Supplementary Material, Fig. S5), we reduced the number of potential candidates by increasing the stringency of the analysis: First, Benjamini–Hochberg correction for multiple testing was applied. Second, all transcripts not showing at least a 1.0 log2FC expression difference were excluded. Applying these criteria, no single transcript was differentially expressed upon VPA treatment in non-responders, whereas in the pos-responders only ANKRD1 met these requirements (Fig. 4A). Interestingly, eight genes were detected as significantly differentially expressed between mock-treated pos- and non-responders, presumably representing an initial set of genes regulating the response to VPA (Fig. 4A and Supplementary Material, Table S3).

Based on the extensive data mining of public databases, the focus was laid on CD36 in the subsequent experiments (Table 1). In the context that short-chain fatty acid HDACi failed to trigger any response in non-responders, the involvement of CD36—a fatty acid translocase (25)—was striking. CD36 was expressed in non-responders at 5-fold higher RNA levels (Fig. 4B), while the differences in protein levels were less pronounced than expected (Fig. 4C). Since CD36 undergoes post-translational modifications (26), certain portions of CD36 protein might not have been properly detected.

CD36 suppresses VPA response

Initially, we tested whether CD36 is capable of suppressing the response to VPA. For this purpose, CD36- or green fluorescent protein (GFP)-transfected HEK293 cells were treated with VPA. Semiquantitative western blotting demonstrated that CD36 indeed prevented SMN induction by VPA application (Fig. 5A). While in GFP-transfected cells, SMN was increased 1.4-fold, no change in SMN protein levels was recorded for the CD36-transfected HEK293 cells (Fig. 5B). In line with this, also the effect of VPA on SMN2 promoter acetylation was strikingly reduced in CD36-transfected HEK293.
cells (Fig. 5C). This clearly demonstrated that CD36 overexpression suppresses the inhibitory effect of VPA on HDACs. Based on the function of CD36 as a fatty acid translocase (25,27,28), an altered transport of VPA across the plasma membrane in fibroblasts is suggestive. However, no direct effect of CD36 on either VPA import or export could be detected (Supplementary Material, Fig. S6).

Finally, as a reciprocal experiment to the CD36 overexpression, we also performed a knockdown of CD36 in non-responder fibroblasts to test whether this overcomes VPA non-responsiveness. While in fibroblasts transfected with a control siRNA, VPA treatment did not affect SMN2 expression, SMN levels were increased by 20–50% when VPA treatment was combined with a knockdown of CD36 (Fig. 5D and E).

In summary, these data prove that CD36 is indeed one of the determinants of VPA non-responsiveness and might become a valuable target to turn non- into pos-responders.

DISCUSSION

Proper drug evaluation for human diseases is labor intensive, but without doubt the most critical part in the introduction of experimental drugs to routine clinics. In contrast to other drugs currently evaluated for the use in SMA, VPA has already passed all requirements for application in humans, since it has long been a recognized therapy of epilepsy (29). Nevertheless, the efficacy of VPA for the treatment of SMA still has to be conclusively shown (9,11,12).

In contrast to other diseases for which VPA is indicated, its impact in SMA could possibly be monitored by measuring SMN2 transcripts and protein. Indeed, a debate is ongoing on whether circulating blood cell SMN2 messenger RNA is an appropriate surrogate marker for the therapeutic success of SMA drugs (9,11,30–32). Histological parameters such as neuromuscular junction maturation or motor neuron degeneration may be appropriate in animal models (33,34), but are not feasible in humans. Motor function tests in SMA patients can be a powerful tool to assess disease progression, but are complex and strongly dependent on the stage of disease progression (35). By applying SMN2 transcripts as a surrogate marker, roughly one-third of the enrolled patients exhibited a positive response, while the remainder either showed no or a negative response to VPA. Although such an observation is not uncommon in pharmacology (and sometimes referred to as the ‘rule of thirds’), only little attention has been paid to the molecular basis of drug non-responsiveness in the field of SMA (11,17,18). However, with a growing list of potential SMA candidate drugs, biomarkers are of utmost importance to predict the treatment outcome and to allow the stratification of patients in clinical trials.

Drug response often differs among tissues (36). Therefore, it was essential to verify that the employed fibroblasts are indeed an accurate surrogate to anticipate CNS response. During the past years, iPS cells generated from fibroblasts have emerged as a versatile source of diverse cell types—among them CNS neurons (37,38). Although the introduction of viral transgenes is still considered problematic, extensive validation pipelines, as they were applied in this study, mostly exclude the occurrence of artifacts (39). We conclusively demonstrate that the
response to VPA is concordant among peripheral blood cells, fibroblasts and the CNS neurons. Although only two iPSC clones were incorporated in this study, this finding is crucial for SMA drug research, since it suggests that fibroblasts are an appropriate specimen for drug evaluation. Moreover, the treatment of fibroblasts with VPA may help identifying pos-responders prior to therapy, which might be considered in terms of outcome and costs for future clinical trials.

Using differential transcriptome microarray analysis, CD36 was identified as the crucial factor controlling the VPA response in SMA fibroblasts. Within this analysis, the non-responders completely lacked response to VPA, while in the pos-responders—like in studies with other HDACi (40)—a reasonable number of transcripts were identified. CD36 is involved in diverse cellular processes (41,42), among these transport of fatty acids across the plasma membrane (25,27). Although suggestive, we could not find any hint that the suppression of VPA response by CD36 is a matter of VPA transport. Nevertheless, since VPA non-responders also failed to respond to sodium butyrate, while...
other HDACi showed full effect, supposable a yet unidentified downstream factor is triggered by increased CD36 levels. One consequence of such a factor could, among others, be an altered fatty acid metabolism. Since the knockdown of CD36 effectively overcame VPA non-responsiveness, the relation between this putative factor and CD36 must be quite tight.

In summary, we aimed to discover the underlying cause of VPA non-responsiveness and by this an appropriate biomarker to identify those patients who will benefit the most from VPA treatment. Our data suggest that CD36 may be such a biomarker for VPA response. Furthermore, targeting CD36 may help to overcome VPA non-responsiveness—not only in SMA but also in, e.g. epilepsy patients, who are the majority of patients treated with VPA.

MATERIALS AND METHODS

Treatment of SMA patients with VPA and patients sampling

Detailed procedures are given in the supplementary material. In brief, two blood samples were taken to establish baseline $SMN2$ expression, while another two to five samples were taken under VPA treatment. Following RNA extraction, $SMN2$ expression was analyzed by qRT-PCR as described previously (9). Fibroblast lines were established from SMA patients’ skin biopsies. This study has been approved by the ethical committee of the University Hospital of Cologne.

Fibroblast and HEK293 cell culture

Primary human fibroblast lines and HEK293 cells were cultured under standard conditions. For drug treatment, cells, seeded out the previous day ($2 \times 10^5$ cells/10 cm dish or $1 \times 10^5$ cells/six-well plate), were treated with the indicated compound under previously described conditions (10,22,43). VPA and sodium butyrate (both Sigma) were dissolved in dd$H_2$O, phenyl butyrate (Sigma) and LBH589 (Novartis) in DMSO.

iPSC generation and expansion

Using retroviruses harboring the four Yamanaka factors SOX2, OCT4, KLF4 and c-MYC (44), iPierian (South San Francisco, CA, USA) generated the iPSC (induced
pluripotent stem cells) lines HGK1 from the VPA pos- responser (ML17) and HGK4 from a non-responder (ML73). Stable iPSC lines were cultured on irradiated murine embryonic fibroblasts in knockdown-Dulbeco’s modified Eagle’s medium (DMEM) containing 20% knockout serum replacement, 1% nonessential amino acids, 1 mM l-glutamine, 0.1 mM β-mercaptoethanol and 6–10 ng/ml basic fibroblast growth factor (all from Life Technologies). Regular passaging was performed by lifting iPSC colonies with collagenase (Life Technologies) and careful titration in a 1:3 or 1:4 ratio every 3 to 4 days. A human embryonic stem cell (ESC)-derived cell line (a kind gift of L. Studer), termed Ctrl ESC, containing a bacterial artificial chromosome expressing GFP under the control of the mouse Hb9 promoter was used as a control in all experiments (45).

Generation of lt-NES

Long-term, self-renewing lt-NES were generated as described (20). In brief, iPSC colonies were detached by collagenase treatment and transferred to non-adhesive Petri dishes for embryoid body (EB) formation in DMEM/F12 containing 10% knockout serum replacement, 1% nonessential amino acids, 1 mM l-glutamine, 1% pyruvate and 0.1 mM β-mercaptoethanol. Medium was changed every other day, and at day 6 EBs were plated onto poly-l-ornithine/laminin (both Sigma)-coated tissue culture plates. Emergence of rosette-like neural structures was observed during the next days. Neural rosettes were carefully picked and cultured in suspension for 2 further days in DMEM/F12, 2 mM l-glutamine, 1.6 g/l glucose, 0.1 mg/ml penicillin/streptomycin and N2 supplement (1:100; Life Technologies). Finally, neural spheres were dissociated by trypsin and single cells seeded out on poly-l-ornithine/laminin-coated dishes. lt-NES were expanded in N2 medium supplemented with B27 (1 μM/l, Life Technologies), 10 ng/ml FGF2 and 10 ng/ml EGF (both from R&D systems). Cells were split at a ratio of 1:2–1:3 every 2 to 3 days by trypsin treatment.

Neuronal differentiation of lt-NES

Differentiation was induced by withdrawal of the growth factors FGF2 and EGF and further propagation in neurobasal medium supplemented with B27 (1:50, Life Technologies) and DMEM/F12 supplemented with N2 (1:100) mixed at a 1:1 ratio. Cyclic adenosine monophosphate (cAMP) (300 ng/ml) was added to the differentiation media (20).

Immunocytochemistry in iPSCs

Cells were fixed with 4% paraformaldehyde in 1 × phosphate buffered saline for 10 min at room temperature and blocked in 10% fetal calf serum (FCS) (Life Technologies) in Hank’s buffered salt solution (HBSS). Pluripotency-associated surface markers TRA-1–60, TRA–1–81, SSEA-3 were detected by incubating samples with primary antibodies overnight at 4°C. After washing three times with HBSS, the samples were treated with secondary antibodies for 1 h at room temperature, stained with 4’,6-diamidino-2-phenylindole and mounted with Mowiol 4–88 mounting solution (Carl Roth). For every other antibody, blocking solution was additionally supplemented with 0.1% Triton X-100 (Sigma).

The following primary antibodies were employed: TRA-1-60 (1:500, Life Technologies), TRA-1-81 (1:500, Life Technologies), SSEA3 (1:80, gift from P. Andrews; ESTOOLS consortium), SOX2 (1:500, R&D Systems), PLZF (1:50, Calbiochem), ZO-1 (1:100, Zymed), βIIIl-tubulin (1:1,000, Covance), GAD65/67 (1:1,000, Chemicon), nestin (1:200, Millipore), AFP (1:100, Dako) and SMA (1:100, Dako). The secondary antibodies were Alexa488 anti-ms, Alexa555 anti-ms, Alexa488 anti-rb, Alexa555 anti-rb (all 1:1000, Life Technologies) and Cy3 anti-rat (1:300, Jackson/ Dianova). Images were acquired with an Axiovision MRn and processed using the Axiovision software (Zeiss).

Spontaneous differentiation of iPSCs

Undirected in vitro differentiation of mesoderm and endoderm occurred by transferring detached iPSC colonies to non-adhesive Petri dishes in knockdown-DMEM containing 20% knockout serum replacement, 1% nonessential amino acids and 1 mM l-glutamine to induce EB formation. After 1 week, EBs were plated on gelatine-coated dishes for further differentiation in DMEM containing 10% FCS, 1% pyruvate and 1% nonessential amino acids.

GABA-ELISA

Following GABAergic neuronal differentiation, neurons were treated daily with 500 μM VPA from day 17 onwards. Before VPA treatment and on day 25, the cell culture supernatant was collected and analyzed for GABA release. The concentration of GABA was determined by an enzyme-linked immunosorbent assay (ELISA) (Abnova) according to manufacturer’s instructions.

Chromatin immunoprecipitation (ChIP)

To determine SMN2 promoter acetylation, chromatin was isolated, precipitated and purified using the LowCell chip kit together with anti-acetyl H3K9 antibodies following the manufacturer’s instructions (Diagenode). Subsequent qRT-PCR of the SMN2 promoter region was performed on an ABI 7500 machine (Life Technologies) (22).

Determination of cAMP-dependent protein kinase assay

Quantification of in vitro PKA activity was performed using the PepTag assay for non-radioactive detection of PKA (Promega) according to the manufacturer’s instructions. Phosphorylated and non-phosphorylated dyes were separated by agarose gel electrophoresis and the dye intensity was determined using a microplate reader (Tecan).

Whole transcriptome analysis and quantitative real-time RT-PCR (qRT-PCR)

For expression analysis, total RNA from cells was isolated employing the RNeasy mini kit plus the RNase-free DNase I set and reversely transcribed using the QuantiTect Reverse
transcription kit (all Qiagen). HuGene1.0 ST v1 Gene Chips (Affymetrix) were used as outlined in the supplementary methods for the identification of expression differences between pos- and non-responders. Except for SMN2 transcripts (delineated in (9)), all qRT-PCRs were performed on an ABI 7500 machine using the Power SYBR green master mix (both Life Technologies). The respective primers and conditions are given in Supplementary Material, Table S4.

Western blot analysis

Whole cell lysates (WCLs) from cells were prepared with radioimmunoprecipitation assay buffer (Sigma-Aldrich). SDS-PAGE (Tris-HCl) and subsequent wet blot were performed following standard protocols. For CD36, WCLs from fibroblasts were prepared with NP40 buffer and deglycosylated overnight with PNGase F (NEB) according to the manufacturer’s instructions. The following primary antibodies were used: mouse mAb anti-β-actin (1:20 000, Sigma-Aldrich), mouse mAb anti-SMN (1:2000, BD Transduction laboratories) and mouse mAb anti-CD36 (1:300, Santa Cruz).

Transfection experiments

CD36 cDNA (corresponding to NM_001001547) was amplified from fibroblast RNA and cloned into the pcDNA3.1/V5-His TOPO vector (Life Technologies). The correct sequence was verified by Sanger sequencing. Transfection into HEK293 cells was carried out using Lipofectamin 2000 (Life Technologies). Primary human fibroblasts were transfected with plasmid DNA using a Nucleofector (Program U-20) together with the basic nucleofector kit for primary mammalian fibroblasts (both Lonza) according to the manufacturer’s guidelines. CD36 knockdown was achieved using a commercially available siRNA (Qiagen, r(GAA CCU AUU GAU GGA UUA ATT)dTdT) together with Dharmafect1 (Thermo Fisher) following the standard protocols.

Statistical analysis

Excel 2003 (Microsoft) and Sigma Plot 9.0 (Systat Software) were used to perform statistical analysis. The significant differences between datasets were identified using a two-sample two-tailed Student's t-test. Three levels of statistical significance were distinguished: *P < 0.05; **P < 0.01; ***P < 0.001.

Further materials and methods are given in the supplementary material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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