Reduced cellular expression and activity of the P129T mutant of human fatty acid amide hydrolase: evidence for a link between defects in the endocannabinoid system and problem drug use

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Fatty acid amide hydrolase (FAAH) inactivates the endogenous cannabinoid (endocannabinoid) anandamide and related lipid transmitters in vivo. A single nucleotide polymorphism (SNP) in the human FAAH gene (385C to A) has recently been described that, in homozygous form, is over-represented in subjects with problem drug use. This SNP, which converts a conserved proline residue in FAAH to threonine (P129T), suggests a potential role for the FAAH–endocannabinoid system in regulating addictive behavior. Nonetheless, the impact of the 385A mutation on the biochemical and cellular function of FAAH remains unknown. Here, we report that T-lymphocytes isolated from patients homozygous for the P129T-FAAH variant express less than half of the FAAH protein and activity observed in wild-type (WT) lymphocytes. Transfected COS-7 cells also expressed significantly lower levels of P129T-FAAH compared with WT-FAAH, indicating that the aberrant expression of the former protein is not a cell type-specific phenomenon. A comparison of the transcription/translation efficiencies and cellular stabilities of WT- and P129T-FAAH proteins revealed that the reduced expression of the mutant enzyme is due to a post-translational mechanism that precedes productive folding. These findings indicate that the natural 385A SNP in the human FAAH gene produces a mutant enzyme with reduced cellular stability, thus fortifying a potential link between functional abnormalities in the endocannabinoid system and drug abuse and dependence.

INTRODUCTION

Fatty acid amide hydrolase (FAAH) is a mammalian integral membrane enzyme that terminates the activity of a large class of endogenous signaling lipids termed the fatty acid amides (1–3). Representative fatty acid amides degraded by FAAH include the endogenous cannabinoid N-arachidonoyl ethanolamine (anandamide) (4), the anti-inflammatory substance N-palmitoyl ethanolamine (5) and the sleep-inducing lipid 9Z-octadecenamide (oleamide) (6). The central role that FAAH plays in regulating fatty acid amide signaling in vivo has been revealed by studies of rodents in which this enzyme has been genetically and/or chemically inactivated. For example, mice with a targeted disruption of the FAAH gene [FAAH(−/−) mice] possess dramatically elevated endogenous levels of fatty acid amides in the central nervous system (7,8), resulting in a cannabinoid receptor 1 (CB1)-dependent reduction in pain sensation (7,9). Similarly, administration of FAAH inhibitors to rodents causes a significant increase in brain levels of fatty acid amides that correlates with CB1-mediated anxiolytic and analgesic effects in these animals (10).

Thus, animal studies indicate that FAAH serves as the primary catabolic regulator of fatty acid amide signaling in the nervous system and, therefore, may play an important role in modulating a variety of neurobehavioral processes.

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To probe potential functional links among FAAH, the endocannabinoid system and diseases of the human nervous system, we recently conducted a search for mutations in the human FAAH gene in subjects with neurobehavioral disorders. These studies identified a single nucleotide polymorphism (SNP) in the FAAH gene (385C to A) that in homozygous form was significantly associated with both street drug use and problem drug and alcohol use (11). This mutation converts a conserved proline residue P129 to threonine, suggesting that it may produce a functional change in FAAH and, by extension, the endocannabinoid signaling system. Nonetheless, whether subjects with the 385A polymorphism express a FAAH protein with abnormal properties remains unknown. Here, we compare the expression and activity of the wild-type (WT) and P129T human FAAH proteins in both peripheral T-lymphocytes and transfected cells. These studies reveal that the P129T-FAAH variant is constitutively expressed at lower levels than WT-FAAH due to differential post-translational regulation, thus providing the first biochemical evidence that subjects with the 385A mutation possess functional alterations in the endocannabinoid signaling system.

RESULTS

Analysis of FAAH expression and activity in circulating T-lymphocytes from human subjects with WT and 385A/385A FAAH genes

The 385C to A polymorphism in the human FAAH gene associated with problem drug use converts a proline residue P129 to threonine. This amino acid, which resides on a surface loop on the cytoplasmic face of FAAH (Fig. 1A) (12), is completely conserved among all of the mammalian FAAH proteins identified to date (Fig. 1B) (13), suggesting that it may be important for the structure and/or function of the enzyme. However, in initial studies, the purified WT- and P129T-FAAH proteins were found to exhibit similar catalytic properties and structural stability, with the only detectable difference being a slightly faster rate of trypsin proteolysis for the P129T mutant (11). Thus, the potential impact, if any, of the P129T mutation on the function of human FAAH in vivo has remained obscure. To address this question, we isolated peripheral circulating T-lymphocytes from WT and 385A/385A human subjects and measured FAAH expression and activity in these cells. Interestingly, the 385A/385A lymphocytes expressed less than half of the FAAH activity found in the WT lymphocytes (Fig. 2A). Thus, in both human T-lymphocytes and transfected cells, the P129T-FAAH mutant was expressed at significantly lower levels than WT-FAAH. In contrast, transfected cells were found to express equivalent levels of WT- and P129T-FAAH mRNA (Fig. 3D), indicating that the reduced expression of the P129T-FAAH protein was due to a post-transcriptional and/or post-translational mechanism.

The reduced cellular expression of P129T-FAAH is due to a post-translational mechanism

To examine whether the reduced expression of P129T-FAAH was due to a defect in the translation of the mRNA encoding this mutant protein, we compared the levels of WT- and P129T-FAAH proteins produced by in vitro translation using a rabbit reticulocyte extract supplemented with dog microsomes. Equivalent levels of both proteins were generated in this assay (Fig. 4), indicating that the reduced expression of P129T-FAAH was not due to a defect in translation. The reduced expression of P129T-FAAH could be due to an enhanced rate of cellular turnover. Potentially consistent with this idea, P129T-FAAH was found previously to show enhanced sensitivity to trypsin proteolysis in vitro (11). To compare the relative cellular stabilities of WT- and P129T-FAAH, we measured their respective half-lives by pulse-chase metabolic labeling techniques. Myc-tagged versions of WT- and P129T-FAAH proteins were transiently transfected into COS-7 cells, and, after ~30 h, the culture medium was replaced with methionine-free medium supplemented with [35S]methionine (pulse step). After 2 h, the medium was exchanged once more with medium containing unlabeled methionine (chase step). After various times of incubation (6–48 h), cells were harvested and solubilized in phosphate-buffered saline (PBS) with 1% Triton X-100. [35S]labeled FAAH proteins were immunoprecipitated from solubilized cell extracts using anti-myc antibodies, separated by SDS–PAGE and quantified by phosphorimaging (Fig. 5A). Notably, although P129T-FAAH was again expressed at significantly lower levels than WT-FAAH (Fig. 5B, inset, 0 h time point), both proteins showed equivalent rates of cellular turnover (Fig. 5B; estimated half-lives of 12 h for WT- and P129T-FAAH). These data indicate that the reduced expression of P129T-FAAH is not due to differences in the rates of cellular turnover for these enzymes.

Evaluating the role of the proteasome in controlling WT- and P129T-FAAH expression

Given that WT- and P129T-FAAH proteins showed similar cellular half-lives, we next considered the possibility that the reduced expression levels of the latter enzyme may...
be due to rapid proteolytic degradation during or immediately following translation. In this regard, the proteasome has been shown to degrade certain proteins in a co- (16,17) or rapid post-translational (18) manner, especially if these proteins show aberrant folding rates (18). We therefore tested the role of the proteasome in regulating the cellular expression of WT- and P129T-FAAH by preincubating transfected cells with the proteasome inhibitor lactacystin (20 μM) for 4 h prior to pulsing with 35S-labeled methionine for 2 h and analysis of FAAH proteins by anti-myc-immunoprecipitation as described earlier. Lactacystin did not alter the levels of 35S-labeled WT- and P129T-FAAH proteins (Fig. 5C). Similar results were obtained with an additional proteasome inhibitor (MG-115; data not shown). These data indicate that the lower cellular expression of the P129T-FAAH mutant was not due to proteasome-mediated degradation.

**DISCUSSION**

The CB1 receptor is the primary site of action in the nervous system for Δ9-tetrahydrocannabinol, the psychoactive component of marijuana (19). The CB1 receptor, along with its endogenous lipid ligands anandamide and 2-arachidonoyl glycerol and their respective biosynthetic and degradative enzymes, form the endocannabinoid system (20,21). Recent evidence suggests that the endocannabinoid system plays an important role in drug addiction and dependence, not only as it relates to marijuana (22), but also other drugs of abuse (23–25). For example, mice with a targeted disruption in the CB1 receptor exhibit reduced withdrawal to morphine (26), suggesting functional crosstalk between the endogenous opioid and cannabinoid systems in neural pathways that mediate addiction. Nonetheless, whether genetic alterations in the human endocannabinoid system contribute to drug abuse and addiction remains unknown. Recently, we reported the identification of a SNP, C385 to A, in the gene for the principal anandamide-degrading enzyme FAAH that, in homozygous form, was significantly over-represented in subjects with problem drug and/or alcohol use (11). The C385A mutation converts a conserved proline residue (P129) to threonine, suggesting that it may impact the biochemical and cellular functions of FAAH. Here, we have examined this possibility by comparing the levels and activity of FAAH in cells expressing WT- and P129T-variants of this enzyme.

First, we examined the FAAH expression in peripheral T-lymphocytes isolated from WT or C385A/C385A human subjects and found that the latter samples possessed less than half of the FAAH protein and activity of WT samples. COS-7 cells transfected with the P129T-FAAH cDNA also showed lower FAAH levels when compared with cells transfected with the WT-FAAH cDNA, indicating that the defective expression of the P129T-FAAH mutant was not a cell type-specific phenomenon. Northern blotting revealed that WT- and P129T-FAAH transfected cells possessed similar quantities of FAAH mRNA, suggesting that the lower expression levels of the P129T-FAAH protein were caused by a post-transcriptional or post-translational mechanism. Considering further that similar levels of the WT- and P129T-FAAH protein were produced by an in vitro translation system, it seems unlikely that a defect in the translational efficiency of the P129T-FAAH mRNA accounted for the reduced cellular expression of this FAAH variant.

We next compared the half-lives of the WT- and P129T-FAAH proteins by metabolic labeling to determine whether a faster cellular turnover rate might be responsible for the reduced steady-state expression levels of the latter protein. However, the WT- and P129T-FAAH proteins were found to display equivalent rates of cellular degradation, with both proteins exhibiting half-lives of ~12 h in transfected COS-7 cells. These results, which to our knowledge represent the first analysis of the cellular turnover of FAAH, indicate that this enzyme and its P129T variant are relatively stable proteins that display medium to long cellular half-lives.

Collectively, the studies described in this manuscript suggest that the reduced cellular expression levels of P129T-FAAH are caused by a post-translational mechanism that precedes the productive folding of this enzyme. For example, P129T-FAAH may show inefficient rates of folding compared with WT-FAAH, which could make this mutant enzyme more susceptible to rapid degradation by cellular proteases. In this model, the P129T-FAAH enzyme, once properly folded, would show equivalent stability to WT-FAAH, consistent with the similar rates of cellular turnover observed for these enzymes. Although our preliminary efforts to rescue the defective expression of P129T-FAAH by incubating transfected

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**Figure 1.** Location of the P129 residue in FAAH. (A) Structure of a FAAH homodimer as determined by X-ray crystallography (12). Residue 129 is located on a loop near the predicted cytosolic face of the enzyme. The active site-bound inhibitor methoxy-arachidonyl phosphonate is depicted with van der Waal’s surface rendering in yellow. (B) Sequences of mammalian FAAH proteins identified to date from residues 120–139, highlighting the total conservation of P129.
cells with the proteasome inhibitors have failed to elevate the levels of this protein, it is possible that its degradation is mediated by other proteases that are insensitive to these inhibitors. In this regard, proteasome-independent pathways for degradation have been described for several intracellular proteins (28–30).

In summary, we report the first evidence that the natural 385A SNP in the human FAAH gene associated with problem drug use produces a mutant enzyme (P129T-FAAH) with defective biochemical and cellular properties. Given that reduced levels of the P129T-FAAH enzyme were observed in both primary (human T-lymphocytes) and transfected (COS-7) cells, we suggest that defective expression may prove to be a general feature of this mutant protein, and, therefore, human subjects homozygous for the 385A mutation may possess constitutively lower levels of FAAH activity in both central and peripheral tissues. Although it is tempting to speculate that these subjects may display a corresponding increase in endocannabinoid signaling that contributes, at least in part, to their drug use problems, further studies are needed to address this important issue. Indeed, drug addiction and dependence are highly complex traits that are undoubtedly influenced by many genetic and environmental factors (31), and it is important to emphasize that the 385A SNP is also present in human subjects who do not display any apparent problems with drug use (11). Regardless, the finding that a common natural mutation in the human FAAH gene produces an enzyme with altered expression and activity highlights that significant functional variability exists in the endocannabinoid system in the human population. Future studies aimed at establishing links between alterations in endocannabinoid signaling and behavior should increase our understanding of the role that this important lipid transmitter system plays in human physiology and pathology.

MATERIALS AND METHODS

Research subjects

Anonymous blood donors who agreed with informed consent to participate in genetics research were genotyped for the FAAH 385 alleles by methods described previously (11). In a survey of 163 regular anonymous blood donors, 11 FAAH 385 A/A subjects were identified and paired with FAAH 385 C/C control subjects on the basis of matching age, gender, medication use and ethnicity for blinded assays in groups of 2–4. Whole blood samples (100 ml) were collected from each subject and peripheral T-lymphocytes were isolated as described later. This protocol was approved by the local institutional review board, and all subjects signed informed consent documents.

Negative T-lymphocyte isolation

Using sterile procedures in a laminar flow hood, 3.2 ml of room temperature Optiprep® solution (Greiner Bio-One) was added to 25 ml of EDTA-anticoagulated whole blood in sterile 50 ml tubes according to the manufacturer’s instructions. Peripheral blood mononuclear cells (PBMCs) were separated by layering 1 ml PBS containing 0.1% bovine serum albumin (BSA) on top and centrifuging at 1500 g for 30 min at 20°C with no brake. The layer containing PBMCs was then isolated, pelletted at 500 g for 10 min and depleted of platelets by washing three times in 15 ml of sterile PBS containing 0.1% BSA and centrifuging at 250 g for 5 min at 4°C. After PBMC preparation, cell counts averaged 4 × 10⁷ with no microscopically detected platelet contamination. Negative isolation of T-lymphocytes from PBMCs was
accomplished using the Dynal® T cell negative isolation kit (Dynal) according to the manufacturer’s instruction and produced a >90% pure and >95% viable T-lymphocyte population that was not exposed to antibodies or other activation or suppressing factors. T-lymphocyte counts at the completion of the negative isolation procedure averaged 1.2 × 10^7 cells. The T-lymphocyte pellet was stored on ice in sterile tubes for immediate analysis of FAAH enzyme activity.

Analysis of FAAH activity and expression levels in T-lymphocytes

T-lymphocyte pellets were resuspended in 1 ml PBS and pelleted by centrifugation for 5 min at 2800g. Cell pellets were then homogenized and sonicated in 200 μl of 50 mM Tris–HCl, pH 9.0, with 125 mM NaCl (buffer 1). Cell membrane pellets were isolated by centrifugation at 100 000 g for 45 min at 4°C, washed, homogenized in 95 μl of buffer 1 and assayed with 100 μM of 14C-oleamide for 60 min at 37°C. Reactions were analyzed as previously reported using a thin layer chromatography assay (27). Additionally, paired samples were analyzed by western blotting with anti-FAAH polyclonal antibodies, as previously described (27).

Analysis of FAAH activity and expression levels in the transfected COS-7 cells

Myc-tagged human WT- and P129T-FAAH constructs were generated by PCR using the following primers: 5′-GGAGTTTCCATGCTGACGAG-3′ and 5′-CCGCCTGACGGATGACTCTTCTTCC-3′. PCR products were subcloned into the pcDNA 3.1Myc-His B vector (Invitrogen) using Eco RI and Xho I restriction sites. COS-7 cells were transiently transfected with each construct by co-administration with cationic lipids (Lipofectamine, Invitrogen) following previously described methods (27). Substrate assays and western blotting with anti-FAAH antibodies were conducted as described in the previous section. Anti-myc western blots were performed with anti-myc monoclonal antibodies (Invitrogen; 1:5 000 dilution). FP-rhodamine labeling of WT- and P129T-FAAH activity was conducted by treating the transfected cell
membrane fractions (1 mg/ml protein) with FP-rhodamine (2 μM) for 1 h at room temperature prior to analysis by SDS–PAGE. Quantification of FP-rhodamine labeled FAAH proteins was accomplished by in-gel fluorescence scanning following previously described methods (15).

Pulse-chase metabolic labeling of FAAH proteins

COS-7 cells were transiently transfected with the myc-tagged WT- and P129T-FAAH constructs and, after a 24 h incubation, cells were washed with PBS and treated with 5 ml of methionine-free, DMEM media containing 10% FCS and 80 μCi of Tran[35S]-labeled methionine (pulse) (MP Biomedicals). Following a 2 h incubation, cells were washed with PBS and treated with fresh DMEM containing 10% FCS and 100 μM methionine (chase). At appropriate time points, plates were washed with PBS, scraped and pelleted by centrifugation at 2800g spin for 5 min. Cell pellets were resuspended in 1 ml of PBS containing 1% Triton X-100 and complete protease inhibitor cocktail (Roche) sonicated and rotated at 4°C for 15 min. Samples were then centrifuged at 100,000 g for 45 min to remove unsolubilized material. The supernatant was then incubated with 50 μl of a 1:1 (v/v) suspension of c-myc agarose affinity gel (Sigma) in PBS and rotated at 4°C for 3 h. Beads were washed with a 1200g spin for 2 min and washed four times with 1 ml of PBS containing 0.1% Triton X-100. Bound protein was eluted from the beads using 100 μl of 0.2 M glycine, pH 2.7 and neutralized with 6 μl of 1.5 M Tris–HCl, pH 8.8. Samples were then separated by SDS–PAGE (14% acrylamide), transferred to nitrocellulose and radioactivity quantified using a Cyclone PhosphorImager (PerkinElmer Life Sciences).

Analysis of proteasomal contribution to FAAH degradation

Prior to metabolic labeling, the transfected COS-7 cells were preincubated for 4 h with the proteasome inhibitors lactacystin (20 or 100 μM; A.G. Scientific) or MG-115 (5 μM, Calbiochem). Cells were then washed with PBS and labeled with [35S]methionine containing DMEM, as described earlier, supplemented with the appropriate proteasome inhibitor. Cells were harvested immediately after the 2 h pulse labeling and FAAH proteins isolated and quantified as described earlier.

Northern blot analysis

RNA samples for northern blotting were isolated from the FAAH-transfected COS-7 cells. Cells were harvested in room temperature Trizol® buffer (Gibco) and total cellular RNA was isolated according to the manufacturer’s guidelines. Total RNA (10 μg) was fractionated by electrophoresis on a 1.1% agarose gel containing formaldehyde. The RNA was transferred to Protran™ nitrocellulose (Schleicher and Schuell), crosslinked to the membrane by exposure to UV light and prehybridized for 45 min in ULTRAhyb™ (Ambion). An 876 bp fragment of the FAAH cDNA (bp 712–1588) was labeled with [α-32P]dCTP (Amersham Biosciences) by random primer DNA labeling (Invitrogen), added to the prehybridization solution and incubated for 16 h. Blots were washed under high stringency conditions (65°C, 30 min) and radioactivity quantified using a Cyclone PhosphorImager (PerkinElmer Life Sciences). FAAH signals were normalized against 7S RNA signals as an internal standard.

In vitro transcription/translation

The WT- and P129T-proteins were expressed in vitro using the TNT® Quick Coupled Transcription/Translation Systems (Promega) in the presence of canine pancreatic microsomal membranes (Promega) and [35S]-labeled 1-methionine (MP Biomedicals) following the manufacturer’s guidelines. Circular FAAH cDNA in the pCDNA™ 3.1Myc-His B vector (Invitrogen) was used as the template for expression under the T7 promoter. [35S]-labeled translation products were separated by SDS–PAGE (14% acrylamide), transferred to nitrocellulose and quantified using a Cyclone PhosphorImager (PerkinElmer Life Sciences).

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