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Stress during Lactation Affects Fatty Acid Amide Hydrolase Protein Expression in Adipose Tissue and Liver of Adult Mice

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Abstract

CIntroduction: Early stress alters the endo-cannabinoid system of tissues involved in energy homeostasis, leading to long term consequences associated to hormonal and metabolic disruptions associated to overweight/obesity and insulin resistance. We have previously reported a decreased Anandamide (AEA)-hydrolysing activity in liver and epididymal fat of adult mice previously subjected to stress during lactation, a result that may indicate reduced activity of the enzyme fatty acid amide hydrolase present in both tissues (FAAH; able to hydrolyse the endocannabinoid AEA).

Objective: Since at present it is unknown whether early stress affects FAAH gene and/or protein expression in liver and adipose tissue during adulthood, we have performed studies with the aim of evaluating these parameters.

Methods: Male mice pups were subjected to a soft nociceptive stress during the first 21 days of life (whole lactation period) with a subcutaneous injection of saline solution in the back, and sacrificed when adults (130 days old) to extract liver and epididymal fat.

Results: Data obtained from real time PCR demonstrate that stress during lactation do not affect FAAH mRNA expression either in liver or epididymal adipose tissue of adult animals; however, Western Blot analyses indicates that FAAH protein amount was decreased by 35 and 65% in liver and epididymal fat, respectively.

Conclusion: Present results demonstrate that nociceptive stress during lactation leads to decreased FAAH protein expression in liver and epididymal fat of adult mice, which may be associated to the low AEA- hydrolysing activity previously reported by us in both tissues. Low levels and activity of FAAH should result in increased availability of AEA, leading to a sustained activity of type 1 cannabinoid receptors associated to undesirable cellular and physiological consequences.

Keywords: FAAH; Stress; Anandamide; CB_1R; Programming; Endocannabinoid System; Lactation

Introduction

Adverse health consequences due to inappropriate environmental perinatal conditions may become evident during adulthood as a result of the metabolic plasticity of mammalian organisms [1-3]. Specifically, studies performed by us and others in a CD-1 mice model have shown that pups repeatedly subjected to a mild nociceptive stress during lactation (NS-mice) present overweight in adulthood, in addition to an increased amount of epididymal fat and altered levels of glucose, insulin, lipids, leptin, corticosterone and adrenocorticotrophic hormone [4,5].

Several types of stress stimuli trigger endocannabinoids (endogenous lipid compounds formed from dietary fat, such as arachidonic and linoleic acids) production and the subsequent activation of type 1 cannabinoid receptors (CB_1R) in a time- and site-specific manner in the brain, including the negative fast-feedback actions of glucocorticoids [6,7]. Thus, CB_1R plays an important function in hypothalamus-pituitary-adrenal axis regulation. Since CB_1R are also present in several peripheral tissues involved in energy homeostasis, it might be also possible a role for these receptors in those stress-related metabolic alterations already reported. In fact, our previous study demonstrated that adult overweight and metabolic perturbations found in NS-mice were reversed by treatment with SR141716A, an antagonist/reverse agonist of CB_1R, suggesting a role for CB_1R in these alterations [5]. Interestingly, NS-mice had a reduced anandamide (AEA)-hydrolysing activity in liver and epididymal fat, a result indicating a low Fatty Acid Amide Hydrolyse Activity (FAAH), the enzyme able to specifically hydrolyse the endocannabinoid AEA. This fact should result in greater amounts and availability of AEA able to induce a sustained activity of CB_1R present in those tissues [5,8]. In this regard, it has been recently shown that lack of FAAH activity in $FAAH^{-/-}$ mice promotes energy storage through increased levels of AEA in different tissues, indicating a crucial role for FAAH in energy homeostasis [9]. Moreover, the importance of FAAH in human obesity has been associated to a mutation responsible for a decreased expression and activity of this enzyme, leading to increased levels of circulating AEA [10,11].

Since FAAH is a key component of the endocannabinoid system involved in energy metabolism, and with the aim to elucidate mechanisms involved in adult metabolic perturbations due to early stress; here we performed additional studies to evaluate FAAH mRNA and protein expression in liver and epididymal fat of NS-mice to clearly establish that the reduced AEA- hydrolysing activity previously found [5] is mainly due to FAAH activity.

Materials and Methods

Animals

Procedures were first analyzed and approved by the Bioethics' Committee for Animal Experimentation of the Instituto de Nutrición

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y Tecnología de los Alimentos, Universidad de Chile. Santiago, Chile. Synchronously pregnant female CD-1 mice were kept in the animal house under normal conditions of humidity and temperature (22-24ºC), on a 12:12 h light-dark cycle (light on at 7.00 AM and then off at 19.00 PM) with free access to purified tap water and food. A normal diet of 4 Kcal/g, equivalent to 2.8 assimilated Kcal/g (Champion Co, Santiago, Chile), was used during the study. Perinatal procedures were carried out as previously described [4,5]. After birth, a total of 12 male pups were utilized; in this way, 6 male pups showing homogeneous weights were randomly selected and assigned to a substitute mother to receive cross lactation. Thus, 2 substitute mothers with their respective 6 pups were accommodated in 2 different cages. Animals were then assigned to constitute one of the following groups:

1) Stressed, nociceptive stimulated mice (NS-mice): during the whole lactation, pups (6) were daily removed from the home cages and subcutaneously injected with a sterile saline solution (1 µl/g body weight) with a microsyringe in the back.

2) Control mice: pups (6) were only daily removed from the home cages, for a short period of time.

After lactation animals from both groups were separated from their mothers and groups of three animals were placed in separated new cages to avoid isolation-induced stress. Therefore, 6 animals per group are grown quietly into adulthood without any additional manipulation. Twice a week animals were placed in clean cages, with new bedding, purified deionized water and the standard diet already described.

Finally, adult 130 days old mice were sacrificed as already described, [5], liver and epididymal fat pads removed, quickly frozen in liquid nitrogen and then stored at -80ºC until use. Subsequently, tissues (liver and epididymal fat pads) from 6 or 4 different animals per group were utilized for real time PCR and western blots experiments respectively.

RNA isolation and real time PCR

Total mRNA was extracted from 80-100 mg of frozen liver and epididymal fat, using Trizol (Invitrogen, USA) and the RNeasy Lipid Tissue Mini kit (Qiagen, USA) respectively. Isolated mRNA was resuspended in nuclease-free water, quantified and stored at -80°C. Extracted RNA samples were used for cDNA synthesis with 0.5 µg oligo(deoxythimidine) primer using M-MLV reverse transcriptase (Promega Corporation, USA) according to manufacturer's instructions. Treatments of samples in the absence of reverse transcriptase to asses for DNA contamination were used as controls.

Messenger RNA levels were assessed by real-time PCR using an Eco™ Real-Time PCR System (Illumina, California, USA) with KAPA SYBR FAST qPCR kit Master Mix Universal (2x). The reaction was performed in a final volume of 10 µl, following the manufacturer's protocol. Paired oligonucleotides (Integrated DNA Technology, USA) for amplification of FAAH, β-actin and GAPDH genes were designed using Vector NTI Advance 11 software (Invitrogen, USA). Amplification of 18S rRNA and YWHAZ genes (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) were carried out with primers previously described [12,13], and synthesized by Integrated DNA Technologies (CA, USA)

For analysis of mRNA expression a threshold cycle (C_t value) was obtained for each amplification curve, and the $\Delta\Delta C_t$ method was applied [14]. β-actin, GAPDH, 18S rRNA and YWHAZ were used as housekeeping genes for normalization of real time PCR obtained data. The normalization factor was obtained, based on the geometrical mean of the Ct values of housekeeping genes for both tissues. All samples were quantified in duplicate. Primers and annealing temperatures used for each gene are listed in Table 1.

Western blotting

Liver (100 mg) and epididymal fat (200 mg) from control and NS-mice, were homogenized in 0.5-1 ml of 25 mM Tris-HCl buffer pH 7.6 containing 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS and 50-100 µl of a protease inhibitor cocktail. Total protein homogenate from liver and epididymal fat (12 and 40 ug respectively) was separated on a 10% SDS-polyacrylamide. Gel was transferred overnight at 4°C to PVDF membranes, and then blocked with 5% powdered non-fat milk in TTBS buffer during 1 h at room temperature. Subsequently, membranes were incubated with 1:500 dilution of FAAH Polyclonal Antibody # 101600 (Cayman Chemical Co, USA) during 1 h at room temperature. Corresponding protein band was visualized by chemiluminescence. Negative controls were performed with the peptide designed to specifically block FAAH antibody binding (#301600, Cayman Chemical Co, USA)

Statistical Analysis

Results are expressed as mean ± SEM. Real time PCR results and band intensities from western blots were compared by the non-parametric Mann-Whitney U test. Significant differences were considered at $p<0.05$.

Results

Real time PCR and Western blotting for FAAH

Messenger RNA levels were quantified by real time PCR has already described. In Figure 1 can be observed that no differences were found in FAAH mRNA expression either in liver or epididymal fat when comparing control with NS-mice. Conversely, western blot analysis indicated that FAAH protein expression from both tissues was significantly decreased in NS-mice Figure 2. In this case, for densitometric quantification purposes, β-actin was used as loading control and results were expressed as relative percentage of control mice. Expression of FAAH protein was decreased by about 35 and 65% in liver and epididymal fat respectively. No bands were observed, when adipose tissue and liver proteins were incubated with the corresponding blocking peptide together with the specific antibody. Interestingly, these values are closely related to the extent of AEA-hydrolysing activity inhibition previously reported in both tissues from NS-mice [5], where it should be considered the presence of non-specific amidase or esterase activities able to hydrolyse AEA. In addition, we have determined here that FAAH contributes with 77.1 ± 3.3 and 72.7 ± 3.6% of the total AEA-hydrolysing activity in tissues homogenates from control and stressed animals, respectively (n=5; no significant differences). These values were obtained by performing the enzyme assays in the presence of 1,5 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), a compound able to specifically inhibit FAAH activity.

Discussion

Present results demonstrate that adult mice previously subjected to nociceptive stimulation during lactation (NS-mice), have decreased FAAH protein expression but unchanged mRNA levels in tissues involved in energy homeostasis, such as liver and epididymal adipose tissue. In this regard, previous reports have shown that early NS causes overweight together with a disrupted hormonal and metabolic profile in adult mice, including increased amounts of visceral fat [4,5]. This abnormal condition was reversed by treatment with SR141716A, a CB_1R antagonist/reverse agonist, suggesting involvement of overactive

Gene	Primers Sequence	Annealing Temperature
FAAH	Fw. 5'-gtggatagcctggcattgtg-3' Rev. 5'-gagtgggcatggtgtagttg-3'	64°C
β-actin	Fw. 5'-ccgtaaagacctctatgcca-3' Rev. 5'-aagaaagggtgtaaaacgca-3'	58°C
GAPDH	Fw. 5'-aggtcggtgtgaacggatttg-3' Rev. 5'-tgtagaccatgtagttgaggtca-3'	65°C
18S	Probe. 5'-/6FAM/accggcgcaagacggaccaga /36TAMSp/-3' Fw. 5'-cgccgctagaggtgaaattc-3' Rev.5'-cattcttggcaaatgctttcg-3'	60°C
YWHAZ	Fw. 5'-acttttggtacattgtggcttcaa-3' Rev. 5'-ccgccaggacaaaccagtat-3'	57°C

Table 1: Primers Sequence and Annealing Temperatures for FAAH and Housekeeping Genes Utilized in Real Time PCR.

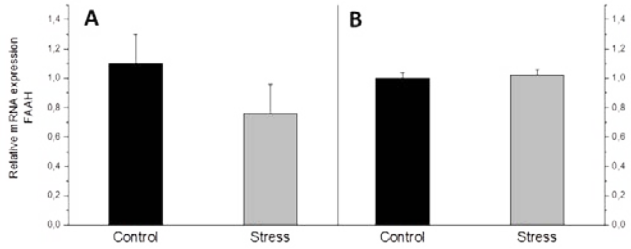

Figure 1: Real Time PCR expression of FAAH mRNA in epididymal fat (A) and liver (B) of adult control and NS-mice. Quantification of FAAH mRNA was performed by the ΔΔCt method. β-actin, GAPDH, 18S rRNA and YWHAZ were used as housekeeping genes for normalization of real time PCR obtained data. All samples were quantified in duplicate. Data shown are the mean ± SEM of values obtained in tissues from five independent animals of each experimental group. No significant differences were observed.

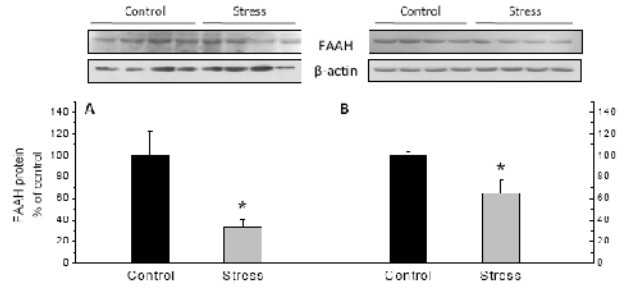

Figure 2: Western blot analysis of FAAH. Relative levels of FAAH protein (63 kDa) present in epididymal fat (A) and liver (B) of adult control and NS-mice. Proteins were resolved by SDS¬-PAGE, blotted and immune detected with a polyclonal antibody using an ECL system. For densitometric quantification purposes, β-actin (42 kDa) was used as loading control. Data represent the mean ± SEM of values obtained in tissues from four independent animal of each group.*p<0.05 (Mann-Whitney U test).

CB_1R in such alterations. Interestingly, we have previously found that AEA-hydrolysing activity of NS-mice (which include FAAH and non-specific *amidase* and esterase activities) was lowered by approximately 40 and 60% in membrane preparations of liver and epididymal fat pads respectively [5], a result that suggested to us a low FAAH activity in both tissues.

The precise mechanism by which FAAH protein expression is lowered without changes in mRNA levels remains to be elucidated. On one side, degradation of FAAH protein by the ubiquitin-proteasome system and or autophagic pathways could be accelerated in NS-mice [15,16]. Alternatively, several studies have shown a role for microRNAs in repressing protein translation without affecting mRNA levels [17]. Interestingly, restraint stress increases microRNAs expression in mice [18]. Further studies are necessary to know whether early NS has long term consequences in reprogramming degradation/autophagic systems and/or spatiotemporal levels of microRNAs in different tissues, and thus affecting FAAH protein expression and as a result leading to decreased enzymatic activity.

A key role for FAAH activity in energy homeostasis has been recently shown in FAAH-deficient ($FAAH^{(-/-)}$) mice. These $FAAH^{(-/-)}$ animals show increased body weight and total amount of adipose tissue together with increased levels of AEA in several tissues [9]. In this regard, Maccarrone et al. [8] have suggested an interesting model involving intracellular adiposomes as reservoirs for AEA accumulation in cells. This fact, may have nutritional relevance since endocannabinoids are derivatives of arachidonic acid (AA), which may have a direct dietary origin as AA or indirect from linoleic acid after Δ^6-desaturase activity. Therefore, a low FAAH activity could play a central role in increased trafficking and availability of this endocannabinoid to exert sustained autocrine/paracrine actions through CB_1R, and thus leading to metabolic alterations due to overactivity of these receptors. Since in our present and previous studies [5] have demonstrated a decreased FAAH protein expression and AEA-hydrolysing activity in liver and epididymal fat from NS-mice, it may be suggested that expected increased amounts of intracellular AEA could lead to overactivity of CB_1R in both tissues.

Overactive CB_1R in adipose tissue may increase lipoprotein lipase activity and as a result a higher availability of free fatty acids to be stored as triglycerides in adipocytes [19] thus giving account for the increased amount of epididymal fat found in adult NS-mice [4,5]. On the other hand, overactive hepatic CB_1R increase *de novo* synthesis of free fatty acids due to increased expression of lipogenic agents such as the transcription factor SREBP-1c and its target enzymes, acetyl coenzyme A carboxilase 1 (ACC1) and fatty acid synthase (FAS) [20]. Moreover, Osei-Hyiaman et al. [21] using a liver-specific CB_1R knockout mice model, demonstrated that hepatic CB_1R are required for development of diet-induced steatosis, altered serum lipids profile, and insulin and leptin resistance. With all these antecedents in mind, it is quite possible that overactive CB_1R due to a decreased FAAH activity may impact different metabolic pathways leading to a physiopathological systemic condition involving some characteristics of the metabolic syndrome [22]. Interestingly, a key role for FAAH in human obesity has been associated to a FAAH single mutation responsible for a decreased expression and activity of this enzyme [10,11]. In this way, low FAAH activity leading to increased chronic availability of AEA may have relevant long term consequences for health. In this regard, we have recently reported that administration of AEA during lactation induces overweight, fat accumulation and a marked state of insulin resistance, concomitant to an increased level of CB_1R expression in visceral adipose tissue of adult mice [23].

In conclusion, this and our previous study show that a mild nociceptive stress during lactation may modify programming and regulation of the peripheral endocannabinoid system evidenced as a decreased FAAH expression and activity in adipose tissue and liver. Increased availability of AEA in both tissues, suggests overactivity of CB_1R during the adult life leading to hormonal and metabolic alterations [22,24].

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Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards provided by the American Medical Veterinary Association on the care and use of laboratory animals [25]. In addition procedures were also approved by the Bioethical Committee for Animal Experimentation of the Institute of Nutrition and Food Technology, University of Chile. Santiago, Chile.

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