i-ACES Vol. 1 No. 1 (2014)
Inquiry in ACES: An Undergraduate Research Journal
Special Edition: Research Apprentice Program 2014
College of Agricultural, Consumer and Environmental Sciences
University of Illinois at Urbana-Champaign

i-ACES
{inquiry-ACES: Highlights of Undergraduate Research in ACES}

Amino Acid Limitation Induces the Amino Acid Response (AAR) Pathway in a Time-and Species-Specific Manner

Heather Anderson[1], Huan Wang[2], Qian Li[3], Yuan-Xiang Pan[2,3], and Hong Chen[2,3*]
[1]Research Apprentice Program student
[2]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
[3]Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
[*]Corresponding author: Hong Chen, PhD, 472 Bevier Hall, MC 182, 905 S Goodwin Ave., Urbana, IL 61801, Tel.: 217-244-6160; Fax: 217-265-0925; Email: hongchen@illinois.edu.

ARTICLE INFO

Article history:
Received 31 July 2014
Accepted 29 September 2014

Keywords:
Amino acid limitation, AAR, human, rat, hepatoma, HepG2

ABSTRACT

Amino acids are critical to life and play central roles both as building blocks of proteins and as intermediates in metabolism. The amino acid response (AAR) pathway is activated by lacking of amino acids (i.e. amino acid starvation). We hypothesized that the activation of AAR pathway might occur in a time-dependent manner in hepatoma cells, which might induce restriction of protein production from DNA. Real-time RT-PCR was used to investigate mRNA expressions during this process. Our results showed that expression of *Atf3*, *Chop*, and *Asns* genes were induced significantly by amino acid deprivation in a time- and species- dependent manner.

INTRODUCTION

Amino acids are involved in various processes in both healthy and pathological states (Perta-Kajan et al. 2007; Suryawan et al. 2008) and their role as signaling molecules that regulate gene expression has received considerable interest in recent years (Hu et al. 2008; Liao et al. 2008). Amino acid availability influences a variety of regulatory processes including transcription, mRNA maturation, translation, mRNA turnover, and autophagy, and, as a consequence, amino acids impose an integrative effect on cellular metabolism as a whole (Kilberg et al. 2005). Limiting one or all of the amino acids in vivo or in vitro is an experimental model that is used extensively to characterize the cellular transcriptional response to nutritional stress and an ever-increasing number of amino acid regulated genes have been identified (Kilberg et al. 2005). If amino acid starvation could induce AAR pathway in a timely dependent manner in H4IIEC3 and HepG2 cells, then it could reduce restricting deviations of the conduit from DNA to proteins.

Amino acid starvation is a deficiency in amino acids and the limitation deviates steps in the parameter in the pathway from DNA to RNA to protein. Activation of the amino acid response (AAR) pathway in response to amino acid limitation occurs largely through signal transduction and mediated through altered transcriptional activity of specific genes, including *Asns* (Barbosa-Tessmann et al., 2000), *Atf3* (Pan, Chen, Siu, & Kilberg, 2003), and *Chop* (Abcouwer, Schwarz, & Meguid, 1999; Bruhat et al., 1997). Initiation of the AAR pathway requires phosphorylation of the eukaryotic translation initiation factor 2alpha (eIF2alpha) by GCN2 kinase leading to the decrease of global protein synthesis (Lu, Jousse, et al., 2004). At the same time, ATF4 protein synthesis increases by the modulation of short upstream open reading frames (uORFs) within the 5'- leader of Atf4 mRNA (Harding et al., 2000; Lu, Harding, & Ron,

2004; Vattem & Wek, 2004). As a consequence of elevated ATF4 protein expression, multiple stress-induced genes are activated, including genes containing an amino acid response element (AARE) (Harding et al., 2003). C/EBP-ATF response element (CARE) refers to a genomic sequence that contains a half-site for C/EBP family members and a half-site for ATF member binding within several target genes, including Chop (Fawcett, Martindale, Guyton, Hai, & Holbrook, 1999).

Using qPCR (quantitative PCR, also known as real-time (RT-PCR)) relative mRNA expressions can be detected in specific samples. Experiments were designed to test whether the AAR pathway is affected in the treatment. Amino acid starvation could induce the AAR pathway in a timely dependent manner in H4IIEC3 and HepG2 cells. Each gene that was used for each PCR test was specifically chosen because they are believed to have a direct effect on the AAR pathway and were used in the investigation in the activation of the pathway. The downstream of AAR pathway were investigated to confirm the activation of this pathway. qPCR investigates the specific gene expression by pre-designed primers. mRNA samples were reverse transcribed to cDNA. Then, the cDNA was examined using a substance that contains fluorophore, which is SYBR Green. SYBR Green binds to double stranded DNA in order to detect the products of PCR. Inside the PCR machine are sensors inside the thermal cycler that measure the brightness of the fluorescence that the sample gives off. This permits the measurement of the rate of generation during each cycle.

The purpose of the present study was to investigate the time-specificity of activation of the AAR pathway and comparison between human and rat hepatoma models. Unraveling these mechanisms provides valuable insight into the understanding of amino acid limitation in different species and period for future investigations.

LITERATURE REVIEW

In the study conducted by Pan et al. in 2003, they studied the genes ASNS and ATF3 and how amino acid deprivation induces the expression of multiple activating transcriptions for mRNA. This experiment performed is similar in the fact that it uses the same genes and it studies the effects of amino acid deprivation. This study is different than the one mentioned because the affects of amino acid starvation on the amino acid response pathway were observed. (Pan et al, 2003) In a study published in 2004, the genes tested were ATF4, ATF3, and C/EBP protein. The study observes their expression level just like this study. It also tests the time of deprivation after amino acid limitation, which is also similar. This study observes slightly different genes i.e. ASNS and CHOP. (Chen et al, 2004).

METHODOLOGY

Cell culture and treatments

The human and rat hepatoma cell lines HepG2 and H4IIEC3 were purchased from ATCC (Manassas, VA). Minimum essential medium (MEM) and amino acid free medium (-AA) were manufactured by the Cell Media Facility at the University of Illinois. Unless otherwise mentioned, all general chemicals and laboratory supplies were obtained from Fisher Scientific. Cell culture ware was purchased from Sarstedt (Newton, NC). HepG2 and H4IIEC3 cells were cultured in MEM, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (ABAM) at 37 °C in a humid incubator with 5% CO2. All experiments were performed using cells with 2-6 passages.

For testing the effects of amino acid limitation time course, HepG2 and H4IIEC3 cells were treated in triplicate with total amino acid free media for 0, 4, 8, 12 and 24 hours, and cell samples were collected for mRNA expression.

Quantitative real-time PCR

Following amino acid deprivation treatment, cells were harvested in TriReagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. mRNA concentration was measured by SmartSpec Plus spectrophotometer (BioRad laboratories Inc, Irvine, CA) at 260 nm. Total mRNA (2 μg) was used for cDNA synthesis using High Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA) in a DNA 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The program was as follows: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s. After the reaction, 25 ng of samples were used for quantitative real-time PCR and gene expression levels were determined using 2x Perfecta SYBR Green fast master mix (Quanta BioSciences) in a 7300 real-time

PCR system (Applied Biosystems, Foster City, CA). Primer sequences for the experiment were designed by Vector NTI software (Invitrogen Corporation, Carlsbad, CA) and primers were synthesized by Integrated DNA Technologies. The sequences of the primers used are in Table 1. The reaction was as follows: 95 °C for 15 min to activate Taq polymerase followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. After amplification, dissociation curves were acquired by stepwise increases from 55 °C to 95 °C to ensure a specific product was amplified in the reaction. Standard curves with slope of -3.10±0.20 and R2≥0.99 were accepted. Human L7a was used as an internal control to normalize the raw data.

FINDINGS

Figure 1

Atf3 mRNA Expressions of the AAR pathway in HepG2 cells.

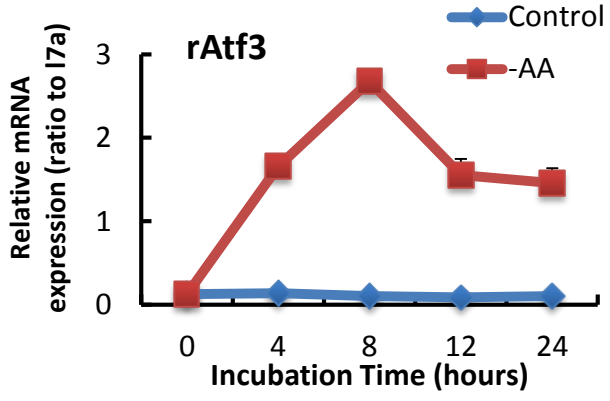


Figure 2

Atf3 mRNA Expressions of the AAR pathway in H4IIEC3 cells.

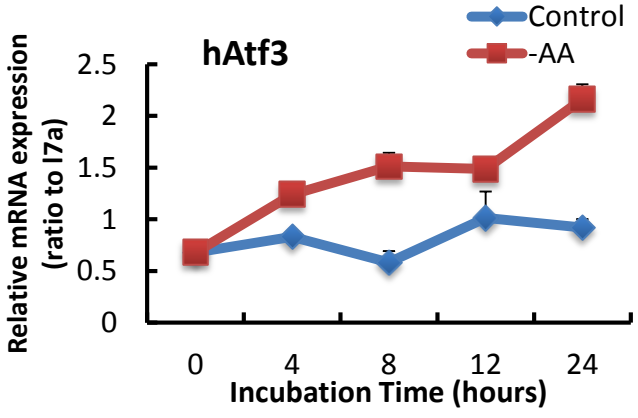


Figure 3

Chop mRNA Expressions of the AAR pathway in HepG2 cells.

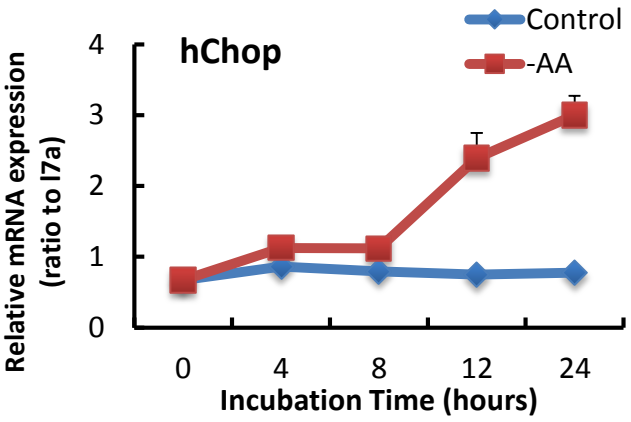


Figure 4

Chop mRNA Expressions of the AAR pathway in H4IIEC3 cells.

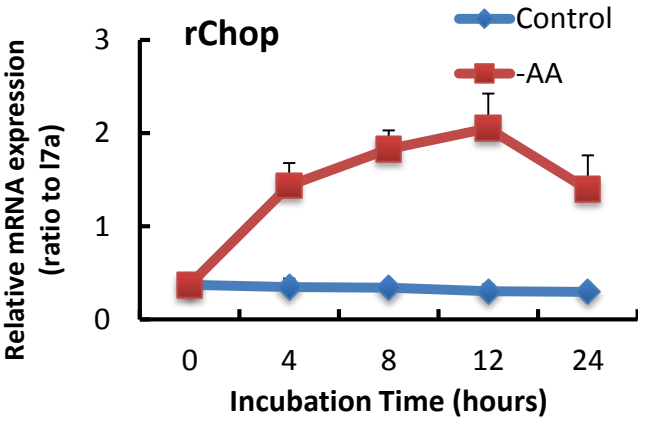


Figure 5

Asns mRNA Expressions of the AAR pathway in HepG2 cells.

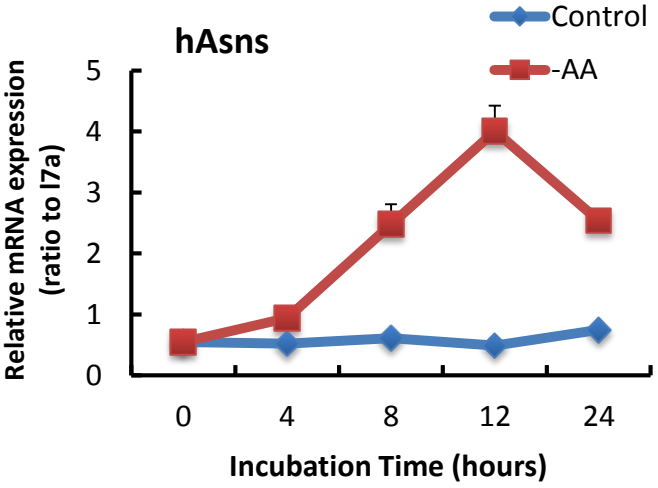
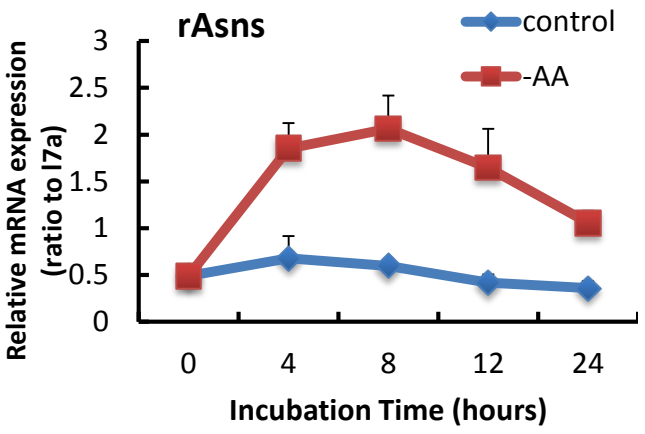


Figure 6
Asns mRNA Expressions of the AAR pathway in H4IIEC3 cells.



The mRNA expression levels were the highest in the sample with the hASNS primers. The only two samples that kept increasing over time were human samples. The rat samples decreased usually after 8 hours of incubation compared to a spike in the human sample after 12 hours. There was also a steadier incline in the first 4 hours of incubation with the amino acid free medium (-AA). The rat samples had a much larger expression rate within the first 4 hours. Most of the declines were very rapid and sudden for both the rat and human samples. At the end of 24 hours, every single sample still had a higher expression rate than what they started with. They also always had higher expression rates than the control, which shows that the –AA treatment does in fact increase the relative mRNA expression in these genes. The amplification due to the amino acid free medium is not only time sensitive, but also species sensitive. Typically after either 8 or 12 hours of incubation, the expressions level would decrease. The –AA treatment's expressions are exponentially higher than those for the control. Even at the lowest point, it produces higher expressions than the control's highest.

In humans, the mRNA expression levels of *Atf3*, *Chop* and *Asns* were induced significantly in a time-dependent manner. Interestingly, *Asns* dropped slightly after 12 hours incubation. In rat, *Atf3*, *Chop* and *Asns* decreased after 8 hours incubation, but still significantly induced compared to control. There was also a more steady induction in the first 4 hours of incubation with the amino acid free medium (-AA). In rat, the induction of the mRNA expressions of downstream AAR genes starts after first 4 hours of amino acid starvation. In conclusion, our results show that amino acid limitation significantly induces the mRNA expression levels of *Atf3*, *Chop* and *Asns* differently in human and rat, moreover, the induction is time sensitive.

Table 1
Primers used in PCR for analysis of gene expression of the AAR Pathway Genes.

Gene	Positions	Species	Sequence
L7a	+145F	human	TTTGGCATTGGACAGGACATCC
L7a	+208R	human	AGCGGGGCCATTTCACAAAG
L7a	+64F	rat	GAGGCCAAAAAGGTGGTCAATCC
L7a	+127R	rat	CCTGCCCAATGCCGAAGTTCT
ATF3	+6530F	human	ATACCTGTGATAGAACCCACCTCGG
ATF3	+6612R	human	TCTGCGCTGGAATCAGTCACTGT
ATF3	+688F	rat	AGABAGGBAACBATTBGCABGAGBCTABAGC
ATF3	+767R	rat	TGGBGGTBGGABAAABGGABGGABTTC
CHOPB	+595F	human	GAACGGCTCAAGCAGGAAATCG
CHOPB	+668R	human	ATTCGGTCAATCAGAGCTCGGC
CHOP	+636F	rat	CTCBTGABTCGBACCBGCABTGGBTCABG
CHOP	+731R	rat	TGGBCGTBGATBGGTBGCTBGGG
ASNS	+1290F	human	GCABGCTBGAABAGABAGCBCCABAGT
ASNS	+1351R	human	TGTCTTCCATGCCAATTGCA
ASNS	+11091F	rat	TGGBACTBCCABGCTBTGGBTTG
ASNS	+11157R	rat	AGABGCABTAGBGGCBACTBTGG

CONCLUSIONS

The induction of AAR pathway by amino acid deprivation is not only time sensitive, but also species sensitive. Specifically, the downstream of AAR pathway is significantly induced by amino acid deprivation after 4 hours of treatment and the increase last until 24 hours. The AAR pathway responded earlier in rat than human. Furthermore, the trend of induction dropped after 12 hours, but still significantly different from the control, which indicates the activity of the AAR pathway is time sensitive.

ACKNOWLEDGEMENTS

Heather Anderson would like to thank the members of the Nutrient-gene Interaction laboratory at the University of Illinois and especially her mentors, Huan Wang and Qian Li, the faculty mentors Dr. Yuan-Xiang

Pan and Dr. Hong Chen, and the Research Apprentice Program for allowing her to participate in this very educational experience in the Department of Food Science and Human Nutrition.

REFERENCES

Abcouwer, S. F., Schwarz, C., & Meguid, R. A. (1999). Glutamine deprivation induces the expression of GADD45 and GADD153 primarily by mRNA stabilization. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. J Biol Chem, 274(40), 28645-28651.

Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Siu, F., Schuster, S. M., Nick, H. S., & Kilberg, M. S. (2000). Activation of the human asparagine synthetase gene by the amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements. [Research Support, U.S. Gov't, P.H.S.]. J Biol Chem, 275(35), 26976-26985. doi: 10.1074/jbc.M000004200

Bruhat, A., Jousse, C., Wang, X. Z., Ron, D., Ferrara, M., & Fafournoux, P. (1997). Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. [Research Support, Non-U.S. Gov't]. J Biol Chem, 272(28), 17588-17593.

Chaveroux, C., Lambert-Langlais, S., & Cherasse, Y. (n.d.). Molecular mechanisms involved in the adaptation to amino acid limitation in mammals. Science Direct. http://dx.doi.org/10.1016/j.biochi.2010.02.020.

Chen, H., Pan, Y.-X., Dudenhausen, E. E., & Kilberg, M. S. (2004). Amino Acid Deprivation Induces the Transcription Rate of the Human Asparagine Synthetase Gene through a Timed Program of Expression and Promoter Binding of Nutrient-responsive Basic Region/Leucine Zipper Transcription Factors as Well as Localized Histone Acetylation. The Journal of Biological Chemistry. http://dx.doi.org/10.1074/jbc.M409173200.

Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T., & Holbrook, N. J. (1999). Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. Biochem J, 339 (Pt 1), 135-141.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., & Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. Mol Cell, 6(5), 1099-1108.

Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Ron, D. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. [Research Support, U.S. Gov't, P.H.S.]. Mol Cell, 11(3), 619-633.

Kilberg, M. S., Pan, Y.-X., Chen, H., & Leung-Pineda, V. (2013). Nutritional Control of Gene Expression: How Mammalian Cells Respond to Amino Acid Limitation*. National Institutes of Health. http://dx.doi.org/10.1146/annurev.nutr.24.012003.132145.

Lu, P. D., Harding, H. P., & Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. J Cell Biol, 167(1), 27-33. doi: 10.1083/jcb.200408003.

Lu, P. D., Jousse, C., Marciniak, S. J., Zhang, Y., Novoa, I., Scheuner, D., . . . Harding, H. P. (2004). Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. EMBO J, 23(1), 169-179. doi: 10.1038/sj.emboj.7600030.

Pan, Y.-X., Chen, H., Siu, F., & Kilberg, M. S (2003). Amino Acid Deprivation and Endoplasmic Reticulum Stress Induce Expression of Multiple Activating Transcription Factor-3 mRNA Species That, When Overexpressed in HepG2 Cells, Modulate Transcription by the Human Asparagine Synthetase Promoter. The Journal of Biological Chemistry. http://dx.doi.org/10.1074/jbc.M304574200.

Vattem, K. M., & Wek, R. C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. [Research Support, U.S. Gov't, P.H.S.]. Proc Natl Acad Sci U S A,

101(31), 11269-11274. doi: 10.1073/pnas.0400541-101.

This special edition of *iACES* is composed of articles written by high school students enrolled in the College of ACES Research Apprentice Program (RAP), University of Illinois. We gratefully acknowledge the many faculty, staff, and graduate student mentors who account for the success of RAP, its Director, Dr. Jesse Thompson, as well as its generous sponsors. Further appreciation extends to Emily Mayhew for her editorial assistance.