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Quantitative RT-PCR Comparison of the Urea and Nitric Oxide Cycle Gene Transcripts in Adult Human Tissues

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Abstract

The urea cycle and nitric oxide cycle play significant roles in complex biochemical and physiologic reactions. These cycles have distinct biochemical goals including the clearance of waste nitrogen; the production of the intermediates ornithine, citrulline, and arginine for the urea cycle; and the production of nitric oxide for the nitric oxide pathway. Despite their disparate functions, the two pathways share two enzymes, argininosuccinic acid synthase and argininosuccinic acid lyase, and a transporter, citrin. Studying the gene expression of these enzymes is paramount in understanding these complex biochemical pathways. Here, we examine the expression of genes involved in the urea cycle and the nitric oxide cycle in a panel of eleven different tissue samples obtained from individual adults without known inborn errors of metabolism. In this study, the pattern of co-expressed enzymes provides a global view of the metabolic activity of the urea and nitric oxide cycles in human tissues. Our results show that these transcripts are differentially expressed in different tissues. The pattern of co-expressed enzymes provides a global view of the metabolic activity of the urea and nitric oxide cycles in human tissues. Using the co-expression profiles, we discovered that the combination of expression of enzyme transcripts as detected in our study, might serve to fulfill specific physiologic function(s) in tissue including urea production/nitrogen removal, arginine/citrulline production, nitric oxide production, and ornithine production. Our study reveals the importance of studying not only the expression profile of an enzyme of interest, but also studying the expression profiles of the other enzymes involved in a particular pathway so as to better understand the context of expression. The tissue patterns we observed highlight the variety of important functions they conduct and provide insight into many of the clinical observations from their disruption.

Keywords

expression; RNA; liver; qRT-PCR; arginine; citrulline; urea cycle; nitric oxide

Introduction

The urea cycle is a complex series of biochemical reactions that produce urea from ammonia (Figure 1) ¹ and is highly conserved in all mammalian species ^{2,3}. It is the only known metabolic pathway capable of converting nitrogen derived from protein intake and/or protein

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degradation into urea. Although several tissues express some urea cycle enzymes, only the liver has the full metabolic capacity of detoxifying ammonia to urea. The biochemical reactions of the urea cycle also produce three physiologically important metabolites: ornithine, citrulline, and arginine. These metabolites are the substrates of a number of physiological biochemical reactions including new protein production, polyamine production, and the creation of nitric oxide through the nitric oxide cycle. The urea cycle and the nitric oxide cycle are linked in that these two pathways share the use of two enzymes, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) and a transporter (citrin).

The complete nitric oxide cycle involves the activity of the enzymes ASS and ASL as well as one of the three nitric oxide synthases (endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)). Additionally, the nitric oxide cycle uses the transporter citrin to import aspartate which condenses with citrulline (generated by ASS and ASL) to produce fumarate, which returns to the Krebs cycle. Nitric oxide production is tightly regulated at both the transcriptional and post-translational level^{4–8}. eNOS is primarily involved in the regulation of vascular tone and is produced by vascular endothelial cells. iNOS is rapidly upregulated in inflammatory and infectious conditions and is thought to be involved in defenses against pathogens. nNOS is most noted for production in neuronal tissue where it serves as a signaling molecule however, the full extent of its expression is still unknown. The expression of the nitric oxide synthases in combination with enzymes of the urea cycle is critical in determining the global metabolic potential of the urea and nitric oxide cycles in different human tissues. This is especially important in diseases where urea cycle and nitric oxide cycle function may be impaired.

Moderate to severe genetic defects in any of the genes that code for urea cycle enzymes result in a urea cycle disorder (UCD). Previous studies from our laboratory have shown that, in addition to causing UCDs, a quantitative change in urea cycle enzyme expression and function can affect nitric oxide production by limiting substrate availability^{9,10}. The broad and complex physiological functions of the enzymes shared by these two pathways present challenges in a variety of clinical conditions ranging from UCDs to post-cardiac surgery pulmonary hypertension, necrotizing enterocolitis, persistent pulmonary hypertension of the newborn, and bone marrow transplant related hepatic veno-occlusive disease^{11–15}.

Given the complex roles of the urea cycle and nitric oxide cycle enzymes, we hypothesized that we would be able to detect differences in gene expression in different tissues and that specific enzymes would be co-expressed together, reflecting the overall function of these enzymes in a specific tissue. To test our hypothesis, we investigated mRNA transcript expression using quantitative polymerase chain reaction (qRT-PCR) of the main enzymes of the urea and nitric oxide cycles, including CPSI, NAGs, OTC, ASS, ASL, ARG1, ARG2, CITRIN, ORNT1, eNOS, iNOS and nNOS, in a panel of normal human tissues. Our results showed that we were able to measure quantitatively, urea and nitric oxide cycle enzyme expression in each tissue. Furthermore, we were able to group co-expressed genes in order to determine a global view of the function of these enzymes in a particular tissue. This study provides an interesting view of tissue differences in the available transcripts for the different genes. In diseases where urea and/or nitric oxide metabolism is impaired, our results show that it is important to examine not only the expression of each individual enzyme but the context of the co-expression of enzyme groupings in different tissues.

Experimental Procedures

RNA From Human Tissue

Total human RNA from normal tissue samples from small intestine, ileum, liver, pancreas, kidney, brain, testis, skeletal muscle, spleen, heart, and lung were obtained from Stratagene and Chemicon. The RNA was isolated from two healthy deceased donors using a modified

guanidinium thiocyanate method (www.stratagene.com). RNA was tested by spectroscopy to verify concentration and purity.

cDNA Preparation

cDNA was prepared using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA was synthesized in a 20- μ l mixture containing 1 μ g of DNase (Invitrogen) treated total RNA from each tissue sample. Reverse transcription conditions were performed as suggested by the manufacturer for # cycles of 10 min at 25 °C, 120 min at 37 °C and 5 sec at 85 °C. A negative control consisted of a sample lacking reverse transcriptase.

Primers and Probes

qRT-PCR was performed using the following Taqman Gene Expression Assays ordered from Applied Biosystems: CPSI (Hs00919483_m1), NAGs (Hs00400246_m1), OTC (Hs00166892_m1), ASS (Hs00540723_m1), ASL (Hs00163695_m1), ARG1 (Hs00968979_m1), ARG2 (Hs00265750_m1), CITRIN (Hs00185185_m1), ORNT1 (Hs01111100_m1), eNOS (Hs00167166_m1), iNOS (Hs00167257_m1), nNOS (Hs00167223_m1), GAPDH (4333764F), B-actin (ACTB) (4333762F).

Relative Expression of mRNA Transcripts by qRT-PCR

qRT-PCR analyses for *CPSI*, *NAGs*, *OTC*, *ASS*, *ASL*, *ARG1*, *ARG2*, *CITRIN*, *ORNT1*, *eNOS*, *iNOS*, *nNOS*, *GAPDH* and *B-actin* mRNAs were performed using the ABI PRISM 7700 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager (PE Applied Biosystems, Inc., Foster City, CA). PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) using 3 μ l of diluted (1:3) cDNA, 200 nmol/L of probe, and 300 nmol/L of primers in a 10- μ l final reaction mixture. Each of the 50 PCR cycles consisted of 15 seconds of denaturation at 95°C and hybridization of probe and primers for 1 minute at 60°C. Each reaction was carried out in triplicate using the same RNA source in at least three separate reverse transcriptase reactions for quality control and redundancy (at least nine qRT-PCRs per gene).

Data Analysis

The cycle threshold (Ct) values were determined using the software package for qRT-PCR analysis provided by ABI (PE Applied Biosystems, Inc., Foster City, CA). The Ct value reflects the PCR cycle number at which the fluorescence generated within the reaction exceeds the threshold (background fluorescence) and is inversely proportional to the amount of transcript present in a sample. Thus, the larger the Ct value the less detectable message is present to act as template. While a Ct value for no transcript cannot be determined in these samples, the value for extremely low or single copy presence is set at a Ct value of ≥ 34 (ABI publication cms-042380), which is based upon data from a large number of genes and reference curves. The quality and reproducibility of the reactions was determined by calculating the standard deviation of the Ct value on at least three reactions. A standard deviation value ≤ 0.250 indicates a 95% probability of detecting a two-fold dilution or increase in template (1 Ct). Since this experiment did not compare changes in RNA from a basal state in the tissues, a Delta Delta Ct was not calculated. The Ct values for the control transcripts (GAPDH and B-actin) are shown as a demonstration of integrity of the RNA. Since these control genes vary in expression between tissues, they were not used for standardization between RNA sources. Changes in cycle count do not reflect linear changes; therefore, we have tried to avoid further mathematical manipulation of the data. For graphical display and ease of understanding, we subtracted the Ct value from 34 (cutoff for no template) which presents the samples with the most starting template (lowest Cts) as the largest numbers. We do not compare Ct values between genes, since the relative efficiency of the qRT-PCR reactions for each gene makes such data difficult

to interpret. For graphical display of the Ct results in the pathway format, the line thickness for each transcript is reflective of the $(34 - Ct)$ value for each transcript in each tissue.

Results and Discussion

As a central hub of basic biochemistry and physiology, the expression of urea and nitric oxide cycle enzymes will affect many clinical disease states^{11–16}. We were able to amplify mRNA transcripts coding for each gene in the urea and nitric oxide cycles, including the transporters ORNT1 and citrin, from eleven different human tissues. A graphical representation of the Ct values for each gene is presented in Figure 2. The Ct values and standard deviations for each gene were consistent across multiple reverse transcriptions and qRT-PCRs (Table 1). To better visualize the patterns of co-expressed genes, we generated a graphic of the urea and nitric oxide cycles and assigned the $(34 - Ct)$ value to the line thickness for each gene in each tissue (Figure 3). Dotted lines indicate no significant transcript detection. The gene expression patterns show trends that are consistent with the traditional physiologic roles associated with these specific tissues (Figures 2 and 3). The RNA samples studied are from whole organ preparations and as such, represent a global view of metabolic activity, and do not reflect gene expression at the cellular level. The expression patterns measured in this study are not necessarily reflective of protein levels in each tissue. Although the regulation of protein abundance is not completely determined by mRNA expression, differences in metabolic activity have been correlated with the mRNA levels of many genes. Therefore, these co-expressed genes may affect molecular protein production and physiologic functions. Examples of co-expression affecting physiologic expression are described below:

Urea Production

We detected the expression of the full complement of urea cycle enzymes only in the liver tissue. More specifically, we observed marked elevations of CPSI, NAGS, OTC, ASS, ASL, ARG1, CITRIN, and ORNT1 transcripts with minimal increase in the ARG2 transcript. The small intestine expressed all of the enzymes except for ARG1. This may explain why despite the majority of the urea cycle enzymes being present in the small intestine and ileum, these tissues are not able to compensate for the ammonia clearance capacity lost from chronic liver disease, such as cirrhosis.

Arginine and Citrulline Production

Arginine and citrulline production are crucial for the function of both the urea and nitric oxide cycle. Arginine serves as a substrate in both cycles. We observed elevations of CPSI, NAGS, OTC, ASS, ASL, and CITRIN transcripts with minimal increases in ARG1 and ORNT1 transcripts. This expression of this complement of enzymes would be sufficient to carry nitrogen through the upper and middle urea cycle to generate citrulline or arginine. These intermediates may be exported to other tissues for protein synthesis, nitric oxide production, or polyamine production. We observed this pattern in the small intestine and the ileum, which is consistent with the clinical need to provide supplemental arginine and citrulline in patients with urea cycle disorders even after liver transplantation^{17, 18}. Studies in animals have shown that the small intestine is a net exporter of citrulline. Citrulline can be converted to arginine, either in the kidney or the small intestine itself, where we have observed sufficient amounts of ASS and ASL transcripts¹⁹. The co-expression of this complement of enzymes would supply substrates for numerous biochemical reactions (NO production, polyamine production, protein production, etc.).

Nitric Oxide Metabolism

Nitric oxide has many physiologic functions involving the cardiovascular, immune, and neurological systems. The expression of eNOS, iNOS, or nNOS was detected in all tissues

except testis and kidney. The liver, ileum, small intestine, and brain stem had elevated ASS and ASL transcripts coupled with co-expression of one of the NOS enzymes, thus providing these tissues with the capability to generate nitric oxide. The heart, lung, spleen, and pancreas had elevated ASS or ASL transcripts, but not both. It is known that the heart, lung and spleen are nitric oxide producers so our results imply that these tissues obtain arginine substrate by importing it or that the nitric oxide cycle in these tissues is regulated above the level ASS and ASL gene expression.

Polyamine Production, Urea Production, or Arginine Competition

Arginase enzymes are capable of producing ornithine for the production of polyamines. Therefore, we would expect that the co-expression of ORNT1 and either ARG1 or ARG2 without elevation of the transcripts from the first part of the cycle (CPSI, OTC, NAGS) would permit polyamine production. We detected high levels of ARG2 and ASL transcripts in the kidney, moderate expression of ASS and ORNT1 transcripts and low levels of eNOS and nNOS transcripts. We suggest that the expression of this specific complement of enzymes in the kidney may be involved with functional osmolarity during filtration. It should be noted that we did not detect ARG1 transcript in the kidney in our study, which contradicts early reports of ARG1 presence in kidney²⁰. The lung, heart, and spleen had elevated expression of ARG1 but lacked expression of the enzymes from the upper portion of the urea cycle (CPSI, OTC, NAGS). The expression of ARG1 in these tissues may provide a balance for the elevated amounts of NOS transcripts seen in these tissues by scavenging arginine. This mechanism has been proposed as a regulation of NOS by a number of studies^{21–26}. Further studies examining metabolite processing coupled with enzyme expression at the cellular level is required to understand better these co-expression patterns.

As hypothesized, we were able to distinguish co-expression patterns for the enzymes of the urea and nitric oxide cycles in a panel of human tissues. Overall, these results suggest that there are at least three functional patterns for co-expressed enzymes including the production of urea, the production of citrulline/arginine and ornithine, and the production of nitric oxide for signaling uses. At a broader level, and with the discovery that the number of human genes is less than predicted by function, the co-expression of the enzymes in these pathways exemplifies an economical use of genetic code. Our co-expression data also suggests that these enzymes may share common regulation at the transcriptional level. While the double duty of many of the enzymes in this pathway is efficient, it also makes these pathways biologically vulnerable. Thus, ammonia clearance, arginine/citrulline production, nitric oxide metabolism, creatine production, polyamine production, and urea production are all vulnerable to genetic defects in this system. This may explain the high degree of evolutionary conservation seen in these genes, and the serious effects of genetic defects in any of these genes³. For example, in patients with rare inborn errors of urea cycle function, we have observed complications beyond those explained by straightforward hyperammonemia and lack of arginine production^{27–33}. The intertwining nature of the urea and nitric oxide cycles in multiple tissues may be one of the reasons for this^{12–14, 32, 34, 35}. As these studies progress, transcriptional regulation, tissue specificity, and the microgeography of the full complement of enzymes must be considered in patients with classic urea cycle defects. Future studies will examine the change in the relationships of these genes under stress or stimulus conditions. The results of this study indicate the need for a change in the way we consider classic inborn errors of urea cycle metabolism and the degree of complexity they present.

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List of Abbreviations

UCD	Urea cycle disorder
qRT-PCR	quantitative real-time polymerase chain reaction
Ct	cycle threshold
mRNA	messenger ribonucleic acid
cDNA	complementary deoxyribonucleic acid
NO	nitric oxide
ASS	argininosuccinate synthetase
ASL	argininosuccinate lyase
CPSI	carbamoyl phosphate synthetase
NAGs	n-acetyl glutamate synthase
OTC	ornithine transcarbamoylase
ARGI	arginase I
ARGII	arginase II
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase

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Figure 1.

Schematic representation of the urea and nitric oxide cycles.

The following enzymes are involved (in parentheses is the acronym followed by the Enzyme Commission numbers): carbamoyl phosphate synthetase (CPSI) [EC 6.3.4.16](#), ornithine transcarbamoylase (OTC) [EC 2.1.3.3](#), argininosuccinate synthetase (ASS) [EC 6.3.4.5](#), argininosuccinate lyase (ASL) [EC 4.3.2.1](#), arginase I (ARG) [EC 3.5.3.1](#), endothelial nitric oxide (eNOS) [EC 1.14.13.39](#), inducible nitric oxide (iNOS) [1.14.13.39](#), neuronal nitric oxide (nNOS) [1.14.13.39](#), ORNT1 and citrin.

Figure 2.

CT values for transcripts expressed as (34 – CT) using 34 as a theoretical “no-expression” value for qRT-PCR. Genes are grouped by their position or function in the cycle. Tissues are roughly grouped by the functional patterns observed. All determinations were performed from at least three independently prepared RNA isolations.

Figure 3.

Pathway representation of relative expression levels by tissue. The line thicknesses reflect the (34-CT) levels for each gene with thicker lines denoting greater RNA transcript levels. Dotted lines indicate that the (34 - CT) value was 0 or less than 0 and that there was no significant amount of transcript.

Table 1

Raw Ct Values for Urea and Nitric Oxide Cycle Enzymes in Normal Human Tissue
Raw data results reporting the raw Ct Values +/- SD obtained from 5 independent quantitative real-time PCR experiments.

Data from qRT-PCR Assays												
OTC	ASS	ASL	ARG1	CITRIN	ORNT1	ARG2	eNOS	iNOS	nNOS	GAPDH	B-actin	
25.07±0.13	20.75±0.37	24.50±0.23	22.56±0.06	25.30±0.11	22.91±0.40	28.50±0.14	29.38±0.30	37.08±0.30	34.88±0.25	25.75±0.08	20.36±0.18	
25.42±0.30	23.91±0.53	25.53±0.27	39.53±0.41	27.23±0.40	29.95±0.47	24.32±0.48	32.27±0.44	30.64±0.36	32.36±0.25	22.88±0.35	19.11±0.47	
26.45±0.45	23.03±0.44	25.43±0.45	33.79±0.41	27.98±0.19	26.37±0.11	25.84±0.43	29.53±0.41	26.09±0.20	31.10±0.21	21.17±0.31	19.31±0.17	
31.86±0.08	23.44±0.47	27.61±0.41	34.94±0.48	27.03±0.13	25.36±0.27	26.47±0.16	28.31±0.35	34.36±0.44	29.04±0.33	20.16±0.13	19.62±0.20	
36.29±0.36	21.53±0.55	27.08±0.25	36.42±0.49	27.38±0.47	27.64±0.12	23.88±0.33	30.61±0.44	37.55±0.47	29.96±0.47	23.99±0.52	20.15±0.48	
36.12±0.14	27.19±0.23	29.08±0.12	31.69±0.10	26.37±0.11	29.47±0.16	27.11±0.36	29.45±0.27	32.61±0.39	27.59±0.43	23.83±0.20	20.22±0.07	
36.08±0.27	26.28±0.37	27.80±0.11	33.39±0.10	29.18±0.42	30.85±0.33	29.01±0.30	33.09±0.41	35.28±0.47	30.93±0.33	22.42±0.23	21.25±0.20	
36.95±0.36	26.67±0.43	29.70±0.45	35.16±0.41	32.99±0.55	28.18±0.03	27.65±0.24	33.23±0.32	38.08±0.44	24.54±0.53	21.41±0.48	22.27±0.35	
36.21±0.68	28.49±0.27	26.41±0.46	31.44±0.45	27.78±0.43	28.18±0.03	30.18±0.14	28.09±0.16	38.35±0.44	36.16±0.38	23.35±0.02	18.62±0.12	
33.30±0.11	22.54±0.26	26.36±0.40	27.97±0.03	27.24±0.40	28.51±0.28	25.56±0.14	28.01±0.32	37.39±0.36	34.27±0.33	17.81±0.06	19.70±0.24	
30.98±0.11	24.36±0.12	26.92±0.25	27.56±0.08	27.97±0.08	29.74±0.27	29.24±0.23	28.21±0.44	32.13±0.53	29.11±0.25	20.41±0.21	22.93±0.31	