ACUTE PHASE AND TRANSPORT PROTEIN SYNTHESIS IN SIMULATED INFECTION IN UNDERNOURISHED MEN USING UNIFORMLY LABELLED *SPIRULINA PLATENSIS* (PAPER 2)

P.J. REEDS[1], A. KURPAD[2], A. OPEKUN[1], F. JAHOOR[1], W.W. WONG[1], P.D. KLEIN[1]

[1]USDA/ARS Children's Nutrition Research Centre,
Department of Paediatrics, Baylor College of Medicine, Houston, Texas
[2]St. John's University Medical School, Bangalore, India.

Abstract

Although it has been known for many years that injury and infection lead to body nitrogen loss, the reason has remained obscure. In this paper, we develop the argument that the processes that are activated during infection demand the provision of specific amino acids which have to be supplied from body protein. In particular, we show that the positive acute phase proteins are very rich in the aromatic amino acids and the exaggerated use of these amino acids (phenylalanine, tryptophan and tyrosine) in acute phase protein synthesis lead to an endogenous "amino acid imbalance" which restricts the use of other amino acids for tissue protein synthesis. Minimally invasive protocols, involving the administration of ^{15}N and ^{13}C-labelled amino acids for studying whole body nitrogen turnover, amino acid oxidation and plasma protein synthesis are described.

1. BACKGROUND

It has been known for at least 60 years that accidental injury, surgical trauma and infection lead to a significant loss of body nitrogen [1]. Although the anorexia that often accompanies these insults will in itself lead to negative nitrogen balance, the nitrogen loss (approx. 220 mgN/kg/d; [1,2]) exceeds that to be expected under fasting conditions. It is generally accepted that much of the nitrogen mobilized derives from the skeletal muscle protein mass. This might be expected given the dominating influence of this protein pool on whole body protein. However, following infection or injury, mechanisms are activated that lead to depletion of muscle protein. These changes are presumably induced by the combined actions of the cytokines (e.g. interleukin-I, tumour necrosis factor-α) and the so-called stress hormones (the glucocorticoids, glucagon and epinephrine). Unfortunately the protein metabolic changes that initiate the loss of muscle protein remain poorly characterized and whether infection or trauma lowers the rate of muscle proteins synthesis remains controversial [3,4]. However there are also derangements in the membrane transport systems that are responsible for the maintenance of the large intracellular-extracellular glutamine concentration gradient [4] so that despite the activation in glutamine synthase induced by increased glucocorticoid levels traumatic conditions are associated with a reduction in the concentration of glutamine. Conditions associated with body nitrogen loss are also accompanied by exaggerated excretion of taurine [5] and creatine [6], observations that support the notion of significant derangements in muscle membrane function [7].

It seems a reasonable presumption that the mobilization of muscle protein confers an adaptive advantage to the organism and it is equally reasonable to suppose that the amino acids released are used in support of other processes that are activated as part of the individual's defense mechanisms. The central questions are:

(1) What are the processes associated with immune activation and trauma responses that demand the provision of amino acids ?

(2) Why does this lead to net nitrogen loss from the body ? In other words, why is the mobilized nitrogen utilized with such a low efficiency ?

Three factors appear to be important:

(1) Under conditions of infection, the proliferative response of the cells of the immune system will consume amino acids for both protein and nucleotide synthesis. The quantitative impact of this burst of metabolic activity has not been established in any detail.

(2) Much attention has also focused on the changes in glutamine metabolism that occur after a variety of stresses. It is clear that despite increased glutamine synthesis and efflux from skeletal muscle, muscle and plasma glutamine concentrations fall. This appears to be because glutamine utilization by the splanchnic tissues increases to an even greater extent [7] than glutamine synthesis by the peripheral tissues. Unfortunately the pathways that consume glutamine under these circumstances remain incompletely established. However, significant amounts of glutamine might be used in the synthesis of arginine ultimately destined for nitric oxide synthesis [8] and in the synthesis of glutathione [9]. The synthesis of the former is a critical factor in the regulation of macrophage activity [10] and hence it is a key regulator of the activation of host defenses. The latter factor is of critical importance in the maintenance of peroxidative protection under conditions of immune activation.

(3) Our research is concentrating on a third major pathway that consumes amino acids under conditions of infection and trauma. A uniform response to injury or infection is a rapid switch [11] in hepatic protein metabolism away from the synthesis of nutrient transport proteins, such as albumin, the apolipoproteins, prealbumin, retinol binding protein etc., towards the synthesis of a set of proteins termed collectively the positive acute-phase reactants [12,13]. Waterlow [14] has calculated that the quantities of protein that are synthesized during the peak of this response could amount to 1.2 g protein/kg per d - a value that represents a significant proportion of whole body protein synthesis. Given the anorexia that usually accompanies injury and infection, the amino acids necessary for the synthesis of these proteins come largely from the existing protein mass of the body, especially muscle.

It is important to recognize that the utilization of amino acids within the pathways of arginine, glutathione, lymphocyte and acute phase protein synthesis should not *a priori* lead to excessive amino acid nitrogen loss from the body because in strict terms these

processes involve the anabolic utilization of amino acids. Thus even though the rate of turnover of the acute phase proteins, has not, to our knowledge been measured in man, protein turnover in and of itself does not lead to amino acid loss because the amino acids released by proteolysis are recycled. The fact remains though that net nitrogen loss from the body does occur and hence net amino acid catabolism and oxidation are increased.

Recent work has established clearly that nutritional status has an important bearing on the ability of the liver to mount an adequate acute phase response [15,16] and Grimble's work [17] has suggested strongly that under conditions of immune activation cysteine appears to play a key role in the amino acid metabolism of the body. While it is reasonable to argue that changes in glutathione metabolism have an important bearing on cysteine metabolism under these circumstances, we also wondered whether the major acute phase proteins were generally rich in cysteine. Table I shows the gross amino acid composition of the major human positive acute phase reactants as calculated from their primary amino acid sequences [18]. The results show that these proteins in fact have low concentrations of cysteine. However the results reveal that four of these proteins (C-reactive, amyloid A, haptoglobin and α_1-antitrypsin) contain a high amount of phenylalanine, 5 of the proteins (C-reactive, fibrinogen, α_1-acid glycoprotein, haptoglobin and amyloid A) contain a high amount of tryptophan and 3 of the proteins (α_1-acid glycoprotein, haptoglobin and amyloid A) contain high amounts of tyrosine. If muscle is the major source of amino acids fuelling the acute phase protein response, then the results in table I and the calculations in tables II and III suggest that approximately 2 g of muscle protein must be mobilized to support the aromatic amino acid demands of an acute phase response totalling 1 g/kg/d. Because of the imbalance in aromatic amino acid content between the acute phase and muscle proteins, of the 2 g mobilized, approximately 0.7 g (130 mgN) is in effect wasted because the excess amino acids cannot be recycled back into a protein pool.

Thus the main hypothesis guiding the two projects described in this paper is that a significant part of the nitrogen loss associated with a variety of stresses represents an imbalance between the amino acid composition of the source (muscle protein) and that of the product (mixed acute phase proteins). Based on this thesis we predict a quantitative relationship between the magnitude of the acute phase reaction, the total synthesis rate of this set of proteins and the magnitude of the nitrogen loss. In order to test this hypothesis we are developing protocols based on experimental infection (immunization). These studies will examine the magnitude of changes in leucine and body nitrogen metabolism following measles vaccination in infants (in Houston) and the interaction between nutritional status and the acute phase response to typhoid vaccination in adults (in Bangalore).

2. METHODS

The two protocols that we are developing and are currently carrying out as pilot studies address two sides of the same question - the degree to which infection affects amino acid catabolism and the degree to which undernutrition alters this response. Both are using as a controllable model of immune activation - the body's response to vaccination. The two vaccines (measles/mumps/rubella in Houston and typhoid in Bangalore) are known to cause demonstrable clinical changes in the recipients. These

include in a number of cases fever and we believe that they will be effective methods of increasing, in a predictable way, acute phase protein synthesis.

2.1 "The use of measles vaccination as a model of immune activation"

In this protocol we are addressing two related issues.

(1) Whether vaccination induces an increase in leucine oxidation and whole body nitrogen flux.

(2) Whether the changes in leucine and nitrogen metabolism relate to fever (and increased CO_2 production) or whether they relate more closely to the timing and magnitude of the acute phase response.

To examine these questions we have elected to investigate the usefulness of a minimally invasive protocol that uses orally administered $^{15}N/^{13}C$ leucine and $^{2}H_2{}^{18}O$ water and relies on breath and urine samples to sample the metabolic pools. Three blood samples (2 ml each) are taken so that changes in acute phase proteins and plasma albumin can be monitored. It is in essence a double end-product method in which changes in leucine and nitrogen metabolism are inferred from changes in the labelling of their excretory products, CO_2 and urinary urea/ammonia.

At the analytical level, the method is based entirely on variants of gas isotope ratio mass spectrometry. Both $^{13}CO_2$ and urinary ^{15}N labelling will be measured in a Europa ANCA GIR machine, the former by introducing breath from vacutainers into the gas line, the latter by Dumas combustion of isolated dried samples. The enrichment of deuterium and ^{18}O will be measured using a VG SIRA-12 ar 3 Finnigan Delta E gas isotope ratio machines [19].

2.1.1 Expected results

In this study we expect to find a positive correlation between the timing and magnitude of the acute phase response and the rate of leucine catabolism. We also expect to find a reduction in the urinary ^{15}N enrichment during the acute phase response, signifying an increase in nitrogen flux. At this stage we have no *a priori* expectation with regards to ^{15}N excretion. It is possible that because of enhanced need for non-essential amino acid synthesis, associated with changes in glutamine synthesis and use, the proportional rise in ^{15}N excretion will be less than that of leucine carbon catabolism, signifying an important role of leucine as a nitrogen donor. On the other hand if there is an inherent inefficiency in the use of the amino acid mobilized from muscle (as implied by the changes in phenylalanine and tryptophan concentrations that accompany infection [2]) the proportion of ^{15}N dose excreted may alter to the same extent as leucine carbon oxidation rate.

To date we have completed one study in a child before and 24 h after measles vaccination. The proportion of ^{13}C- expired in CO_2 was increased substantially following the vaccination despite no change in the CO_2 synthesis rate as deduced from the $^{2}H_2{}^{18}O$.

	Percent dose excreted	CO_2 production (μmol/kg/min)\
Control study	13.2	266
24 h post vaccination	24.5	266

2.2 "A study of acute phase and transport protein synthesis in simulated infection in undernourished men"

In this protocol we are pursuing two main aims; one biological, the other technical. The biological question is :

(1) To what extent does prior nutritional status influence the degree to which a standard "infection" stimulates acute phase protein synthesis and suppresses transport protein synthesis ?

(2) Does the use of repeated oral administration of highly ^{15}N enriched protein provide a useful method for measuring plasma protein synthesis in man ?

This protocol is being carried out in well- and under-nourished Indian men and involves an analogue of the continuous infusion approach that has generated a large literature. The label - lyophilized ^{15}N-labelled spirulina algae - is administered at half hourly intervals over a 6 hour period. Blood samples are taken at 4 points between 3 and 6 hours of "infusion" and processed for the separation of albumin, isolated by an acid ethanol extraction and the "non-albumin" fraction. Separate samples are processed in Houston by GCMS to measure the isotopic enrichment of a number of amino acids in the hepatic secretory protein VLDL apoB-100. In previous studies employing intravenous infusion we and others have shown that these rapidly turning over proteins can be brought to isotopic equilibrium within a few hours and once at isotopic plateau provide a direct measure of the isotopic enrichment of the amino acids passing into hepatic protein synthesis. We believe that this approach has some advantages over conventional methods. First it seems to us that the use of a complete amino acid mixture as tracer will minimize bias in the data and, in particular, minimize uncertainties with respect to the isotopic enrichment of the protein synthetic precursor pool. Second, by applying gas isotope ratio mass spectrometry we can achieve a substantial increase in analytical sensitivity.

We have used a variant of this method in which the isolated amino acid fraction has been infused intravenously into infant pigs [20]. This shows highly satisfactory results with particularly good analytical precision.

2.2.1 Expected results

We anticipate that typhoid vaccination will, within 48 hours, activate acute phase protein synthesis. This will be reflected in an increase in the labelling of the "globulin" fraction of the plasma accompanied by a concomitant fall in that of albumin. This will, as in the children, be accompanied by an increase in nitrogen flux as measured by a reduction in the ^{15}N enrichment of urinary urea and ammonia. We anticipate that the magnitude of the increase in acute phase protein synthesis in the under-nourished subjects will be less than that of the well nourished but that the reduction in albumin synthesis will be at least as great. These results will be reflected in a smaller increase in whole body N flux and perhaps by a smaller increase in urinary nitrogen loss.

3. PLANS FOR FUTURE WORK

Our long term aims are two-fold.

(1) To identify whether there is a level of nutritional deficit below which host defenses become compromised. Animal results [15] suggest that, as far as

the acute phase response is concerned, the interaction between dietary protein intake and the response is a continuous one.

(2) To identify whether the degree of nitrogen loss can be ameliorated by diets that are supplemented with aromatic amino acids.

On the assumption that the protocols give the expected results we plan two main developments. On the presumption that undernourished individuals will indeed show a blunted acute phase response we will then examine how rapidly nutritional supplementation restores the response to vaccination to "normal". Given the results obtained [16] we would expect to find that the full response is restored only when body composition (e.g. weight for height and/or body mass index) approaches normal. An obvious protocol would be to follow four cohorts and administer vaccines at different points during nutritional recovery. This may identify a degree of deficit below which the host defenses become clearly compromised.

In children, on the other hand, we will need to attempt the reverse. First, by showing that in relatively severely undernourished infants and children, the response to vaccination is blunted (a likely outcome) but, more importantly, to identify populations that present a variable nutritional status. Once again the aim is to identify whether there is a continuous relationship between nutrient status (as assessed by anthropometry) and immune competence.

We would plan to address the second of the aims initially in an animal model before passing to specific human supplementation trials. These will require a certainty of outcome because the design will be complex.

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TABLE I. GROSS AMINO ACID COMPOSITION OF POSITIVE ACUTE PHASE REACTANT PROTEINS AND SKELETAL MUSCLE PROTEIN (SMP)

	C-reactive	Fibrinogen	α_1-acid glycoprotein	α_1-antitrypsin	Haptoglobin	Amyloid A	SMP
Amino acid							
Leucine	91[1]	62	101	124	82	29	80
Isoleucine	54	32	48	49	47	29	48
Valine	77	48	46	59	84	8	54
Lysine	71	77	75	92	92	33	98
Histidine	16	27	17	37	38	35	51
Phenylalanine	105	46	64	83	30	103	40
Tyrosine	50	56	74	27	70	67	36
Tryptophan	42	35	30	11	32	45	13
Methionine	16	32	11	28	16	22	25
Cysteine	13	15	18	6	24	0	13
Arginine	36	84	52	23	28	116	69
Proline	44	48	34	41	44	34	48
Glycine	46	59	19	33	44	61	45
Serine	84	91	31	49	40	47	41
Alanine	31	29	36	43	54	106	59
ASX[2]	82	113	102	106	113	128	92
GLX[2]	112	119	173	136	115	87	145
Threonine	58	60	74	66	54	30	47

[1] Values are expressed as g. amino acid per kg protein and adjusted for water of hydrolysis.
[2] ASX = Aspartate + asparagine; GLX = Glutamate + glutamine.

Sequence information was obtained from Barker [21,22] within which the source references can be found.

TABLE II. AVERAGE MUSCLE EQUIVALENTS OF AMINO ACID IN ACUTE PHASE PROTEINS.

Leucine	1.02 ± 0.37
Isoleucine	0.91 ± 0.18
Valine	0.99 ± 0.35
Lysine	0.74 ± 0.20
Histidine	0.55 ± 0.18
Phenylalanine	1.71 ± 0.66
Tyrosine	1.59 ± 0.44
Tryptophan	2.50 ± 0.84
Threonine	1.21 ± 0.29
Methionine	0.83 ± 0.29
Cysteine	0.97 ± 0.60

TABLE III. ESTIMATE OF THE AMINO ACID NEEDS TO SUPPORT A "TYPICAL"[1] ACUTE PHASE PROTEIN RESPONSE EXPRESSED IN ABSOLUTE TERMS[2] OR IN TERMS OF MUSCLE PROTEIN EQUIVALENT[3], TOGETHER WITH AN ESTIMATE OF THE EXCESS AMINO NITROGEN RELEASE.

	Acute Phase response	Muscle protein equivalent	Excess release	
			Amino acid (mg)	Nitrogen (mg)
Amino acid				
Leucine	89	1090	72	8
Isoleucine	54	1120	41	4
Valine	67	1240	40	5
Lysine	90	920	104	20
Histidine	33	640	68	11
Phenylalanine	79	1980	0	0
Tyrosine	55	1530	16	1
Tryptophan	24	1850	2	< 1
Methionine	23	910	26	2
Cysteine	14	1080	12	1
Arginine	54	780	83	28
Proline	50	1320	25	3
Glycine	50	1110	39	7
Serine	70	1700	11	1
Alanine	51	860	66	10
ASX	121	1320	61	12
GLX	147	1010	140	15
Threonine	65	1380	28	4

[1] Assuming that the "typical" acute phase response consists of an increase (mg/kg) of C-reactive protein (250), fibrinogen (200), α_1-acid glycoprotein (50), α_1-antitrypsin (200), haptoglobin (50), amyloid A (100)

[2] mg amino acid/kg body weight per d.

[3] mg muscle protein/kg body weight