Consequences of age-related splanchnic sequestration of leucine on interorgan glutamine metabolism in old rats

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Dietary leucine (Leu) serves as a nitrogen donor for de novo glutamine (Gln) synthesis in muscle. However, aging is characterized by an increase in the splanchnic extraction of Leu (SPELeu), i.e., splanchnic sequestration (SSLeu), which may affect muscle Gln metabolism and its subsequent homeostasis at the whole-body level. The aim of the work was to assess the effect of age-related SSLeu on Gln metabolism in the muscle, gut, liver, kidney, and Gln exchanges among these organs during fed conditions. Young-adult (3-mo-old) or aged (24-mo-old), male Sprague-Dawley rats were studied during fed conditions. Young-adult (3-mo-old) or aged (24-mo-old), male Sprague-Dawley rats were studied during fed conditions. Young-adult (3-mo-old) or aged (24-mo-old), male Sprague-Dawley rats were studied during fed conditions. Young-adult (3-mo-old) or aged (24-mo-old), male Sprague-Dawley rats were studied during fed conditions.
housed individually in standard cages, allowing an acclimatization period of 15 days before the experiment.

The rats were fed a standard chow ad libitum (AO4; UAR, Villemoisson sur Orge, France), and their food intake was recorded daily over the acclimatization period. Water was provided ad libitum. Rats were subjected to reversed, 12-h light-dark cycle periods (light from 8 PM to 8 AM), and room temperature was maintained at 22°C.

The experiments on animals were supervised by a senior researcher (Dr. Christophe Moinard) of the Laboratory of Biological Nutrition, who possesses authorization (authorization no. 75522) to perform experiments on animals, according to the French regulations on ethics in experimental research and thus has the ability to review the ethical aspects of research protocols. Dr. Moinard has reviewed and approved the present study research protocol and allowed the experiment to be carried out.

**Experimental Protocol**

After an overnight fast, the rats were anesthetized by isoflurane inhalation (4.5%, 2 liters O2/min), followed by a subcutaneous injection of buprenorphine (Temgesic; 1 mg/kg body wt) to eliminate pain. Anesthesia was maintained via continuous inhalation of isoflurane (2.5%, 1.5 liters O2/min). During surgery, body temperature was kept at 37°C, using a temperature controller and heating device (Minerve, Esternay, France). The duodenum (two catheters), jugular vein (two catheters), mesenteric vein, abdominal aorta, carotid artery, portal vein, hepatic vein, right renal vein, and inferior vena cava were catheterized using a 25-gauge needle, held in a silastic tube (Silastic Medical Grade Tubing, 0.51 mm inside diameter, 0.94 mm outside diameter; Dow Corning, Midland, MI), and glued with cyanoacrylate (Cyanolit 201) (33).

**Para-aminohippuric acid infusion protocol.** The nontoxic probe para-aminohippuric acid (PAH) (Sigma-Aldrich, L’Isle D’Abeau, France) was used for plasma flow measurements (39). To allow blood flow measurements across the intestine, liver, kidneys, and hindquarters, a primed (450 nmol/rat and infused continuously, intravenously, at 37,215, 8,940, and 17,880 nmol/h per rat, respectively.

**Blood sampling and processing.** We performed pilot studies (data not shown) to validate that steady-state conditions required to measure protein turnover were obtained from 45 min onward after the start of the nutrition infusion. Therefore, time 60 min (T60) was chosen for blood sampling. Blood was collected on ice from the carotid artery (arterial blood), portal vein, hepatic vein, renal vein, and inferior vena cava (venous blood) into heparinized cups (Sarstedt, Orsay, France). The amount of blood taken was 500 μl/sample (2.5 ml in total), which equated to about 9.3% and 4.5% of circulating volume in young-adult and old rats, respectively (assuming that 8% of total body weight is blood).

For PAH determinations, 50 μl plasma was added to 250 μl of 6% trichloroacetic acid solution, mixed thoroughly for deproteinization, and then centrifuged. For determination of AA concentrations and tracer-tracer ratio (TTR), 80 μl plasma was added to 13 mg of 50% solid 5'-sulfosalicylic acid, then vortexed, frozen in liquid nitrogen, and stored at −80°C until analysis.

**Body-Composition Evaluation**

At T60, after blood sampling, the rats were killed and dissected to separate and weigh the carcass [carcass weight (CW)], which reflects the fat-free mass.

**Sample Analysis**

Plasma PAH was determined spectrophotometrically on a Cobas Mira S system (Roche Diagnostics, Hoffmann La Roche, Basel, Switzerland) by a standard enzymatic method (6).

Plasma AA concentrations and TTRs were measured using a fully automated liquid chromatography-mass spectrometry system (Thermoquest LCQ, Veenendaal, The Netherlands) (43) after precolumn derivatization with fluorenylmethyloxycarbonyl chloride (44).

**Calculations**

**Whole-body rate of appearance.** Whole-body rate of appearance (WbRa): 1/TTR.

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**Table 1. Amino acid composition of the mixture infused into the rat duodenum**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>1,336</td>
</tr>
<tr>
<td>Leucine</td>
<td>2,612</td>
</tr>
<tr>
<td>Lysine</td>
<td>1,712</td>
</tr>
<tr>
<td>Methionine</td>
<td>1,174</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1,364</td>
</tr>
<tr>
<td>Threonine</td>
<td>1,155</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>306</td>
</tr>
<tr>
<td>Valine</td>
<td>1,496</td>
</tr>
<tr>
<td>Alanine</td>
<td>3,652</td>
</tr>
<tr>
<td>Arginine</td>
<td>2,155</td>
</tr>
<tr>
<td>Asparagine</td>
<td>564</td>
</tr>
<tr>
<td>Cysteine</td>
<td>413</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1,421</td>
</tr>
<tr>
<td>Glutamate</td>
<td>850</td>
</tr>
<tr>
<td>Glycine</td>
<td>3,067</td>
</tr>
<tr>
<td>Histidine</td>
<td>645</td>
</tr>
<tr>
<td>Ornithine</td>
<td>240</td>
</tr>
<tr>
<td>Proline</td>
<td>2,391</td>
</tr>
<tr>
<td>Serine</td>
<td>714</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>55</td>
</tr>
</tbody>
</table>

5,311 nmol/rat and infused continuously, intravenously, at 37,215, 8,940, and 17,880 nmol/h per rat, respectively.
**Leu splanchnic extraction.** SPELeu represents the fraction (as a percentage) of ingested AA, taken up by the gut and liver during its first pass, and was calculated as follows

\[
\text{SPELeu} = 100\% \times \left\{1 - \left(\frac{\text{WbRa} [\text{C}]\text{Leu}}{\text{WbRa} [\text{H}]\text{Leu}}\right)\right\} \tag{I}
\]

Note that in this calculation, the enrichment of the chosen precursor pool (arterial or venous) falls out of the equation and thus will not affect the SPELeu calculation.

**Organ balance tracer measurements.** Flux across organs: plasma flow across the portal drained viscera (PDV), liver, kidneys, and hindquarters was calculated using the PAH-based, indicator-dilution technique, as described previously (36).

Tissue AA turnover was calculated using a two-compartment model, with the venous TTR used as proxy for the intracellular TTR (2). Fluxes were calculated by multiplying plasma venous-arterial concentration difference by mean PDV plasma flow. Splanchnic organ AA flux was calculated by multiplying hepatic vein-arterial concentration differences by hepatic plasma flow. Liver AA fluxes were calculated by subtracting PDV flux from splanchnic flux. Renal AA fluxes were calculated by multiplying renal venous-arterial concentration differences by mean renal plasma flow. Hindquarter AA fluxes were calculated by multiplying inferior vena cava-arterial concentration differences by mean hindquarter plasma flow.

Flux (F) represents the net balance of an AA. Fluxes were expressed in nmol-100 g⁻¹ CW-⁻¹ to take into account the age-related changes in body composition. A positive flux denotes a net release, and a negative flux reflects a net uptake.

Tracer net balance (nb), utilization (U), and production (P) rate (nmol-100 g⁻¹ CW⁻¹ min⁻¹), across the PDV, splanchnic region, kidney, and hindquarters, were calculated for [5-¹⁵N]Gln

\[
\text{nb} = \text{plasma flow} \times \left\{\left[(\text{Arterial}) \times \text{TTR}_{a}\right] - \left[(\text{Venous}) \times \text{TTR}_{v}\right]\right\} \tag{2}
\]

where TTRₐ and TTRₐ are the TTR of Gln in arterial plasma and venous plasma, respectively. Use of Gln across organs was calculated as

\[
\text{U} = \text{nb} / \text{TTR}_{v} \tag{3}
\]

Production of Gln across organ was calculated as

\[
\text{P} = \text{U} + \text{nb} \tag{4}
\]

The rats were studied during fed conditions, and the total production rate of a substrate measured across the PDV was a combination of the endogenous production and output of substrate absorbed by the gut from the nutrition infused into the duodenum that was not retained for utilization (5).

Gln endogenous production in the PDV was calculated as

\[
\text{Endogenous} \ P = P - P_{ND} \tag{5}
\]

where \(P_{ND}\) is derived from the duodenal nutrition infusion (I_{NUTR}) and is not metabolized and not disposed of

\[
P_{ND} = I_{NUTR} - U \tag{6}
\]

In RESULTS and DISCUSSION, the PDV will be referred to as gut, and the hindquarter will be referred to as muscle.

**Whole-body Gln homeostasis during fed conditions.** Gln homeostasis is reflected by whole-body Gln flux, which takes into account Gln flux across organs that produce it (muscles) and Gln flux across organs that use it (gut and kidneys). Hindquarters represent one-half of the total muscle mass in rats. Thus total Gln release from muscle is obtained by doubling the quantity of Gln released by the hindquarters, and Gln homeostasis can be calculated using the following formula

\[
\text{Gln homeostasis} = 2 \times \text{Gln hindquarters} + \text{Gln PDV} \tag{7}
\]

As the liver Gln flux is in balance during fed conditions (45), liver flux is not included in this calculation.

**Statistical Analysis**

Data are presented as means ± SE. The Student’s t-test was used to evaluate differences between adult and old rats. Statistical analysis was run on StatView 5.0 software, and the level of significance was set at \(P < 0.05\). Between-parameter correlations were analyzed using the Z-test.

**RESULTS**

**Rat Body Weight and Food Intake**

Total body weight was higher in old rats than in adults (adult: 333 ± 9 g; old: 694 ± 46 g, \(P < 0.05\)). Food intake was not affected by aging (adult: 28 ± 1 g; old: 27 ± 2 g food/day).

**Leu Metabolism**

First-pass SPELeu was doubled in old rats (adult: 24 ± 10%; old: 56 ± 5%, \(P < 0.01\)). Leu arterial plasma level was lower in old rats than in adults (\(P = 0.055\); Fig. 1B). Leu plasma level was correlated to muscle Leu uptake in muscle by the Z-test of correlation. CW, carcass weight.

**Gln Metabolism**

Plasma Gln levels were lower in old rats than in adults (Fig. 2B). In the intestine and the kidney, Gln uptake and use

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**Fig. 1.** Effect of aging on (A) leucine (Leu) plasma level in periphery, (B) Leu uptake in muscle, and (C) their relationship. Open bars, adult rat; closed bars, old rat. A negative flux value reflects net uptake, whereas a positive flux value reflects net release. Values are expressed as means ± SE. *\(P < 0.05\) vs. adult group (Student’s t-test). Correlation between Leu plasma level and Leu uptake in muscle was analyzed by the Z-test of correlation. CW, carcass weight.
were lower in old rats, whereas Gln production was similar between groups (Fig. 2A). In muscle in old rats, Gln release and production were lower, whereas use was unaffected by aging. There was no age-related effect on liver Gln metabolism (Fig. 2A). Whole-body Gln flux or homeostasis was maintained in old and adult rats (adult: 526 ± 766; old: 943 ± 1,270 nmol·100 g−1 CW·min−1) during fed conditions.

Gln production in muscle was negatively correlated with SPELeu (r = −0.501, P < 0.01; Fig. 2C) and Leu uptake (r = 0.418, P < 0.05).

**DISCUSSION**

We demonstrated that in old rats during fed conditions, Gln homeostasis is maintained, despite large changes in Gln interorgan exchange. We show that a reduced muscle Gln release is accompanied by a reduced uptake of Gln by the gut and kidney. In addition, we observed that this change of Gln transport between tissues is likely related to the sLeu. Not of note, splanchnic sequestration involved most AA, as demonstrated in a previous study carried out under the same conditions (21).

Here, muscle Gln production was observably lower in old rats compared with adult rats. Minet-Quinard et al. (28) have shown that Gln synthetase (GS) activity is similar in adult and old rats studied under physiological conditions. Therefore, the reduced muscle Gln production in healthy old rats is probably not related to impairment in GS activity but more likely to decreased precursor levels.

Rats were studied during fed conditions, which is a key requisite for assessing the SSLeu phenomenon. In this situation, dietary Leu serves as a nitrogen donor for de novo muscle Gln synthesis. In line with that observation, after ingestion of a protein meal that contains Leu or parenteral infusion of Leu, increased Gln release from muscle is observed (16). Our observation of an age-related doubling in dietary SPELeu in old rats is comparable with the one made in elderly humans (4). In addition, we demonstrate that SSLeu is negatively correlated with muscle Gln production and that Leu uptake by muscle is correlated with muscle Gln release. Furthermore, Leu uptake by muscle is correlated with Leu arterial plasma level. Taken together, these results strongly support the fact that SSLeu and the consecutive decrease in the peripheral availability of dietary Leu could be responsible for the lower muscle Gln synthesis and release that we observed in old rats. A limitation of our study is that we did not measure the contribution of dietary Leu to muscle Gln production.

In response to stress, an age-related increase in Gln production and a delayed increase in Gln release were observed in an ex vivo model of incubated muscle (28, 29), and these alterations did not seem related to Gln synthase activity variations with age (27). Hence, the consequences of SSLeu on Gln muscle metabolism could be of particular importance in frail, elderly subjects, susceptible to contribute to their impaired response to stress situations (38).

The decrease in peripheral availability of dietary Leu may have other implications, including lower basal-muscle protein synthesis in elderly people. In fact, Leu is vital as a key metabolic regulator of muscle protein synthesis, triggering activation of the mammalian target of the rapamycin pathway. Several authors (31, 35) have postulated the existence of a metabolic regulator of muscle protein synthesis, suggesting that the two processes are related. Thus the decrease in postprandial Leu availability, due to an
age-related increase in SPELeu, may contribute to muscle anabolic resistance, thus leading, over time, to an inevitable, age-related decline in skeletal muscle mass. Finally, it is unlikely that the different Leu concentrations during fed conditions were due to different baseline Leu concentrations, since during fasted conditions, Leu plasma concentrations remain roughly similar in adults and old rats (17, 25).

We observed that arterial plasma Gln levels during fed conditions were lower in old rats than in adult rats (27). A lower postprandial AA concentration seems to be a general pattern in old rats compared with young adults (21). Homeostasis is achieved when the amount of Gln produced covers the need of the organs that use it. Plasma AA level is the sum of exchanges among organs, but this calculation only gives a static view. We chose to assess homeostasis in a dynamic state, i.e., during fed conditions, by taking into account the balance of Gln flux across organs that produce it and Gln flux across organs that use it. Although a nutrient’s arterial plasma level is often used as a marker of its homeostasis, this static view may lead to misinterpretation in a dynamic process, such as during fed condition (12, 15). Therefore, direct measurement of interorgan Gln exchanges provides a more accurate measurement of Gln homeostasis, which is achieved when the amount of Gln produced covers the needs of the organs using it. In healthy conditions, muscle is the main exporter of Gln (7, 15), whereas gut and kidney are among the main consumers (15). Liver can be a Gln producer or Gln consumer, depending on the global body needs and the acid-base status (37). During conditions of acute, chronic inflammation, some organs reduce Gln uptake (e.g., gut), whereas others start taking up Gln (e.g., liver). In the present work, we did not observe a net liver production or consumption of Gln, whatever the age of the rats. This result is in agreement with results of our previous experiments on isolated, perfused rat liver, which showed that liver Gln balance and liver Gln concentrations were not different in livers from old and adult rats fed a normal or a high-protein diet (20).

Although we observed an age-related decrease in Gln production by muscle, it was compensated by a decrease in Gln uptake by the gut and kidney, thus showing maintenance of Gln homeostasis in old rats.

An important, unresolved question is what is the driving force in Gln homeostasis? Data available in the literature (41, 42) suggest that Gln use by the gut does not control the rate of Gln release by muscle. In the present work, we observed that despite profound perturbation of Gln metabolism, Gln homeostasis was still maintained. Therefore, it may be suggested that in fed, old rats, gut and kidney adapt to the lower production and release of Gln by muscle by taking up and using less Gln. This idea is supported by the fact that Gln catabolism in the kidney is controlled by Gln availability (11). Remarkably, even if there are age-related alterations of interorgan exchanges of Gln, the homeostatic balance of this AA is maintained. However, this metabolic adaptation could not happen during metabolic stress, but the limited data about geriatric trauma patients available in the literature (19) will not make it possible to draw any conclusions.

One parameter that could influence the interpretation of these results is the choice of the control group, i.e., 3-mo-old rats. Indeed, it is possible that the highly anabolic phase at 3 mo of age may have overaccentuated the differences between old and young rats. Extrapolating rat age to humans remains a problematic issue, as discussed elegantly by Quinn (34). Of note, Obled and Arnal (32) did not find any major difference in protein metabolism between animals aged 3 mo and 6 mo old.

In conclusion, this study demonstrates that interorgan Gln metabolism is altered in old rats studied during fed conditions. The increase in SPELeu may be one of the possible causes of an age-related decline in muscle Gln production. The hypothesis that age-related perturbations in muscle Gln metabolism might have a deleterious effect during illness, when the gut and the immune system have a greater Gln need, warrants further research.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.E.P.D., L.C., and C.A. conception and design of research; M.J. performed experiments; M.J. analyzed data; M.J., N.E.P.D., L.C., and C.A. interpreted results of experiments; M.J. prepared figures; M.J. drafted manuscript; N.E.P.D., L.C., and C.A. edited and revised manuscript; M.J., N.E.P.D., L.C., and C.A. approved final version of manuscript.

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