Metabolic pathways implicated in the kinetic impairment of muscle glutamine homeostasis in adult and old glucocorticoid-treated rats

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MUSCLE IS THE MAIN ORGAN affected by aging. From the age of 60, muscle mass decreases, which impairs the physical autonomy of old people by reducing their capacity for everyday acts (16). Moreover, aging is frequently accompanied by chronic pathologies (28), which amplify muscular atrophy (24). This sarcopenia is characterized by an alteration of the number, the size, and the proportion of various constitutive fibers (15). In addition, muscular atrophy leads to a reduction of the contribution of this tissue to the whole body protein turnover (20% in old people vs. 30% in adults). During stress, profound alterations of amino acids (AAs) and protein metabolism occur in muscle (8). This tissue is particularly important, because it is the main source of AAs, particularly of glutamine (Gln) for splanchnic tissues, the kidneys, and the immune system (27). Consequently, the reduced mass and contribution of muscle to body protein metabolism could be implicated in the inability of old people to adapt to stressful situations. Although the biochemical basis of muscle atrophy remains incompletely understood, we have hypothesized that muscle Gln homeostasis may be impaired. Indeed, this AA plays a key role in the metabolic response to stress (3) because of its numerous properties: precursor of purine and pyrimidine bases, role in acid-base homeostasis (13), and regulator of protein turnover by stimulating proteosynthesis (4) and inhibiting proteolysis (17). Gln also stimulates the immune system (18), which is impaired during aging. Muscle Gln homeostasis is the result of two main processes: first, its production, which depends primarily on de novo synthesis by Gln synthetase and, to a minor extent, on net muscle protein breakdown; second, its disappearance, which results from muscle Gln release.

In a previous study (22) on the kinetic impairment of muscle Gln metabolism in response to dexamethasone (DEX) with aging, it was observed that muscle Gln synthetase activity increased earlier, whereas muscle Gln concentration decreased later in old rats than in adults. However, there are no data concerning the pool of Gln coming from net muscle protein breakdown or concerning the flow of Gln released by the muscle. Thus the aim of the present study was to characterize by a kinetic approach the various components involved in Gln homeostasis in muscle from adult and old glucocorticoid-treated rats.

MATERIALS AND METHODS

Animals and experimental design. Male Sprague-Dawley rats (n = 143), supplied by Ifa Credo (a Charles River company in L’Arbresle, France), were 24 mo old (n = 67) or 3 mo old (n = 76). After their arrival in our animal facilities, the rats were maintained on a standard diet from UAR (A04; UAR, Epinay/Orges, France) containing 17% plant proteins, 3% fat, 59% carbohydrate, and 21% water, as well as fibers, vitamins, and minerals, and water ad libitum. The total caloric content of the diet was 2,900 kcal/kg. The animals were kept in a controlled environment (constant temperature 24°C, and a 12:12-h light-dark cycle). After 10 days of acclimatization in standard cages and 5 days in individual metabolic cages, the adult rats were divided into seven groups: A3 (n = 8), A5 (n = 15), A7 (n = 13), A3 pair-fed (PF; n = 8), ASPF (n = 8) or A7PF (n = 8), and one healthy control group fed ad libitum (AL; n = 16). Elderly rats were also divided into seven groups: E3 (n = 8), E5 (n = 9), E7 (n = 13), E3PF (n = 8), ESPF (n = 8), ESFP (n = 8), E7PF (n = 7) and AL (n = 14). Experimental groups A3 and E3, A5 and E5, A7 and E7 received 1.50 mg/kg body wt of a DEX solution (4 mg/ml) once a day by intraperitoneal injection for 3, 5, or 7 days, respectively. Finally, net muscle protein breakdown increased only in old DEX-treated rats (day 7). In conclusion, the impairment of muscle glutamine metabolism is due to a combination of an increase in glutamine production and a delayed increase in glutamine release.

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DEX-treated groups (A3, A5, A7 and E3, E5, E7, respectively) and were injected with an iso-osmotic solution of 0.9% NaCl. The AL group received no treatment and were fed ad libitum throughout the study. These rats were killed on the same day as the rats from the A5PF and ESPF groups (day 5). For statistical analysis, healthy rats (AL) in each population were randomly assigned to two subgroups: A0 (n = 9) and A0PF (n = 7) in adults; E0 (n = 7) and E0PF (n = 7) in old rats. The experiment was conducted in three successive series, each series including a balanced number of each group and each subgroup. Because mortality appeared in the E7 group (see results) during the first series, numbers of rats in the A7 and E7 groups in the third series were adjusted to reach an adequate number of rats for analysis in these groups. Overall, there was no difference among results from the three series (data not shown); therefore, the data were pooled for analysis. Body weight and food intake were recorded daily from day 0 to day 7. Twenty-four hours after the last DEX or NaCl injection, the rats were anesthetized and killed by beheading. Animal care and experimentation complied with the rules of our institution, and two of our team (M.-P. Vasson and L. Cynober) are authorized by the French Ministry of Agriculture and Forestry to use animal models of stress.

**Plasma Gln Concentrations**

After the beheading of animals, mixed blood was collected from the jugular vein and carotid artery and was withdrawn in heparinized tubes to measure concentrations of free AAs. Blood was centrifuged (+4°C, 4,500 g, 10 min), and the plasma was deproteinized with sulfosalicylic acid (50 mg/ml). The supernatant was stored at −80°C for no longer than 15 days before the plasma AA analysis, which was performed by ion exchange chromatography with an AA autoanalyzer (model 6300; Beckman, Palo Alto, CA). The results of our participation in ERNDIM (the European Quality Control Scheme) indicate the accuracy of our AA determinations, in particular for Gln. Results are expressed in micromoles per liter.

**Muscle Determinations**

Because of the small muscle mass in both adult and old rats, it was impossible to study the muscle Gln homeostasis (production, release, and concentration) in a single muscle. Consequently, two types of muscles [epitrochlears and extensor digitorum longus (EDL)] were analyzed, because their fiber composition is roughly similar (i.e., rich in type II fibers).

**Muscle Gln concentration.** Left epitrochlears muscles were removed, weighed, frozen in liquid nitrogen, and stored at −80°C. After grinding in trichloroacetic acid, muscle Gln concentrations were determined by ion exchange chromatography as described in Plasma Gln Concentrations.

**Muscle Gln production: Gln synthetase activity.** Muscles of the hindlimbs (EDL) were rapidly removed, weighed, frozen in liquid nitrogen, and used to measure Gln synthetase activity with a colorimetric method previously described (21).

**Net protein breakdown.** Right epitrochlears muscles were dissected intact for incubation. Net protein breakdown was measured according to Baracos and Goldberg (2) with minor differences. Briefly, muscles were preincubated flacid (7) for 30 min in Krebs-Henseleit buffer (in mM: 119 NaCl, 4.8 KCl, 25 NaHCO₃, 1 CaCl₂, 1.24 NaH₂PO₄, and 1.25 MgSO₄, pH 7.4) supplemented with 5 mM glucose, 2 mM HEPES, and 100 U/l insulin (Novo Nordisk, Bagsvaerd, Denmark) and were saturated with a 95% O₂-5% CO₂ gas mixture. Muscles were then transferred into fresh medium of the same composition. Flacid incubated muscles were removed from the medium after 120 min of incubation, and the medium was frozen in liquid nitrogen until tyrosine (Tyr) analysis. Because Tyr is neither synthesized nor degraded by muscle, the release of this AA from muscle into the incubation medium directly reflects the net protein breakdown (2). The incubation medium was deproteinized with sulfosalicylic acid (50 mg/ml), and Tyr concentration was determined fluorometrically by combination with nitrophenol (30).

**Net muscle Gln release.** Right epitrochlears muscles were incubated as we have described. The incubation medium was deproteinized with sulfosalicylic acid (50 mg/ml), and Gln concentration, which directly reflects the net muscle Gln release, was measured by ion exchange chromatography (see Plasma Gln Concentrations).

**Statistical Analysis**

Data are presented as means ± SE. For parameters measured daily in live animals, an analysis of variance (ANOVA) with repeated measurement and with one factor (effect of treatment) was performed for the A7 and E7 groups. For parameters measured after the animals were killed, a two-way ANOVA followed by a Newman-Keuls test was performed in adult or old rats to discriminate among effects of DEX (D), duration of treatment (T), and their interaction (D × T). Because the two-way ANOVA requires two groups of rats at each time (one treated group and one control group), healthy ad libitum-fed rats (i.e., AL group) were randomly assigned to two subgroups: A0 and A0PF in adults; E0 and E0PF in old rats. When the effect of duration of treatment was significant (P < 0.05), treated groups were compared with healthy control rats (AL). Thus A3, A5, A7 and E3, E5, E7 groups were compared with A0 and E0, respectively; A3PF, A5PF, A7PF and E3PF, E5PF and E7PF were compared with A0PF and E0PF, respectively. When the effect of DEX was significant (P < 0.05), treated groups (A3, A5, A7, E3, E5, and E7) were compared with control pair-fed rats (respectively, A3PF, A5PF, A7PF, E3PF, E5PF, and E7PF). In these cases, the specific effect of DEX was studied and the effect of anorexia discarded. Muscle Gln release was analyzed with a two-way ANOVA, with changes in muscle Gln concentration as analysis of covariate. A direct statistical comparison of values between adult and old rats could not be performed, because baseline values between these two populations were different for several parameters.

PCSM software (Deltasoft, Grenoble, France) was used. Values of P < 0.05 were considered as significant.

**RESULTS**

**Characteristics of Animals**

All the adult rats survived glucocorticoid treatment. In aged rats, overall mortality rate was 10.4% and concerned the E7 group. In this latter group, mortality rate was 54%; most died after 5 days of glucocorticoid treatment (n = 1 at day 5, n = 2 at day 6, and n = 3 at day 7), except one who died at day 2. The final number of rats in the E7 group submitted to analysis was n = 6.

DEX induced anorexia from day 3 in adult rats and from day 6 in old animals. In adult rats, food intake improved after day 4 (Fig. 1).

At the beginning of the experiment (day 0), body weights of adult and old rats were, respectively, 369 ± 4 g (AL group: 354 ± 4 g, pair-fed group: 364 ± 3 g, and glucocorticoid-treated group: 379 ± 7 g) and 690 ± 12 g (AL group: 681 ± 23 g, pair-fed group: 697 ± 23 g, and glucocorticoid-treated group: 689 ± 14 g). The daily rate of body weight change was 2.2 ± 0.4 g for adult rats and −1.9 ± 1.2 g for old rats belonging to the AL group. Both adult and old glucocorticoid-treated rats lost body weight (Table 1). Most of this effect was
induced specifically by DEX (P < 0.05 between DEX-treated rats and pair-fed animals).

**Plasma Gln Concentration**

DEX treatment specifically decreased plasma Gln concentration (in μmol/l) from day 5 in adults [A5: 502 ± 25 vs. A5PF: 671 ± 29; A7: 519 ± 43 vs. A7PF: 728 ± 20; significant effect of DEX (D); P = 0.006] and from day 7 in old rats (E7: 561 ± 31 vs. E7PF: 715 ± 28; significant effect of D: P = 0.003).

**Muscle Parameters**

**Muscle mass.** An atrophy of EDL for 5 days of treatment by glucocorticoid was observed in adult treated rats (Table 2). No similar change was noted in EDL from aged rats. In epitrochlearis, atrophy occurred in old rats owing to the specific catabolic effect of DEX after 7 days of treatment by glucocorticoids.

**Muscle Gln concentrations.** In epitrochlearis, Gln concentrations decreased from 3 and 5 days of treatment by glucocorticoids in adult and old rats, respectively (Fig. 2).

**Table 1. Body weight loss in adult and old glucocorticoid-treated rats**

<table>
<thead>
<tr>
<th>Treatment Time, days</th>
<th>3 Mo</th>
<th>24 Mo</th>
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<tbody>
<tr>
<td></td>
<td>Pair fed</td>
<td>DEX</td>
</tr>
<tr>
<td>D3</td>
<td>2.4±2.8</td>
<td>-10.7±2.6†</td>
</tr>
<tr>
<td>D5</td>
<td>-6.4±1.7</td>
<td>-18.2±1.1†</td>
</tr>
<tr>
<td>D7</td>
<td>-4.3±2.8</td>
<td>-23.9±1.2†</td>
</tr>
<tr>
<td>Treatment/time effect</td>
<td>D, T, D × T</td>
<td>D, T, D × T</td>
</tr>
</tbody>
</table>

Body weight loss, expressed in %, is calculated by the difference between basal body weight at day 0 and body weight at day 3, day 5, or day 7. Values are means ± SE. A 2-way ANOVA was performed in adult or old rats to discriminate among effects of dexamethasone (DEX), D, duration of treatment (T), and their interaction (D × T). Significant effect (P < 0.001) is indicated by letters D, T, or D × T; NS, not significant; D3, D5, and D7, day 3, day 5, and day 7, respectively. Comparison of means was carried out with the Newman-Keuls test; *P < 0.01 vs. 0-day treatment, †P < 0.01 vs. pair-fed controls.
E674  EFFECT OF STRESS ON MUSCLE GLUTAMINE DURING AGING

Fig. 3. Gln synthetase activities in extensor digitorum longus (EDL) according to glucocorticoid impregnation time in adult and old rats. Gln synthetase activities were measured in EDL of adult or old rats treated for 3 (D3), 5 (D5), or 7 (D7) days by DEX (filled bars) or from adult or old control rats (open bars) receiving for 3 (D3), 5 (D5), or 7 (D7) days ip injection of NaCl solution and pair-fed with groups treated by DEX for 3, 5, or 7 days, respectively. Gln synthetase activities measured in healthy control rats (horizontal striped bars) can be considered as basal values and consequently have been represented as D0 values. At this time, 2 groups are represented which fit, respectively, to A0 and A0PF for adults and to E0 and E0PF for old rats. A 2-way ANOVA was performed in adult or old rats to discriminate among effects of D, T, and D x T. Significant effects of D, T and D x T were observed in adults (D: P = 0.001; T: P < 0.015; D x T: P = 0.045) and in old rats (D: P < 0.0001; T: P = 0.003; D x T: P = 0.002). Comparison of means was carried out with the Newman-Keuls test: * * * P < 0.01 vs. D0, † † P < 0.01 vs. pair-fed controls.

(3PF: 23.0 ± 3.6; A5PF: 28.0 ± 8.3; A7PF: 24.2 ± 5.4 vs. A0PF: 19.2 ± 9.2). In old animals, the rate of Gln release (in nmol·g⁻¹·min⁻¹) from epitrochlearis muscle was not modified by 3 days of treatment by glucocorticoid (E3: 18.3 ± 4.2 vs. E3PF: 18.2 ± 5.1, nonsignificant), but increased after 5 days of treatment (E5: 28.2 ± 8.9 vs. E5PF: 15.8 ± 1.5; E7: 28.2 ± 4.5 vs. E7PF: 16.9 ± 2.0, significant effect of D: P = 0.006).

DISCUSSION

Glucocorticoid administration induces a severe stress. In this context, it is not surprising that long-term (i.e., 7 days) DEX administration induced a marked mortality rate in rats belonging to the E7 group, even though this was not expected when we started the experiments. Two reasons may explain this high mortality rate. First, it could be primarily related to the hypermetabolic state. The latter is assessed by the greater body weight loss in old glucocorticoid-treated rats compared with pair-fed controls, which have the same food intake. It can be hypothesized that animals who died were in hypermetabolic state. This may have been more important than in rats surviving glucocorticoid treatment; perhaps the rats who died were unable to compensate for this hypermetabolic state. Measurement of metabolic rate would have been necessary to support these hypotheses. Second, this greater hypermetabolic state and the associated DEX-induced mortality could result from the development of infection (because DEX is known to be immunosuppressive) and/or the inability of the old organisms to provide sufficient amounts of Gln, which is an important source of energy in particular for immune cells during infection (26).

We have demonstrated that the kinetic response to DEX of components involved in muscle Gln homeostasis differs markedly in adult and old rats (Table 3). As previously shown (22), muscle Gln concentration decreased later in old glucocorticoid-treated rats (day 5) than in adults (day 3). Our findings clearly show that two mechanisms were implicated: on the one hand, muscle Gln production (i.e., Gln synthetase activity and net protein breakdown), which increased earlier in old rats than in adults; on the other hand, muscle Gln release, which occurred later in old rats than in adults. This suggests a coordinated response to glucocorticoid treatment to maintain the muscle Gln concentration at a normal level for as long as possible. Except for Gln release, muscle parameters in pair-fed rats remained unchanged, suggesting that this response is specific to glucocorticoids and relatively independent of anorexia induced by DEX.

The major role of glucocorticoids as a regulator of Gln synthetase activity has been well demonstrated in adult and old rats by Verdier et al. (29). Muscle Gln synthetase activity is stimulated earlier in old than in adult rats. These results are in line with those obtained previously by both ourselves (22) and others (20). The alteration of muscle fiber composition during aging (involvement of type II to type I fibers) (10) and the greater number of glucocorticoid receptors in type I fibers (9) could explain the greater sensitivity of Gln synthetase activity to glucocorticoids in old rats.

The second component that contributes to the appearance of free Gln in the muscle is net protein breakdown. However, physiologically the implication of this mechanism in muscle Gln homeostasis is not very significant because, according to Kuhn et al. (12), only 13% of Gln in the total free pool arises from muscle proteinolysis. The effects of glucocorticoids on protein turnover in muscles in models such as those used in the present study are controversial (31), and perhaps we missed a significant increase in net protein breakdown in DEX-treated rats.

Table 3. Summary of modifications of muscle and plasma Gln metabolism as a function of duration of treatment by glucocorticoids in adult and old rats

<table>
<thead>
<tr>
<th>Duration of DEX Treatment, day</th>
<th>Adult Rats</th>
<th>Old Rats</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Muscle Gln concentration</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>Gln synthetase activity</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Net protein catabolism</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Muscle Gln release</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>Plasma Gln concentrations</td>
<td>→</td>
<td>→</td>
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</tbody>
</table>

Modifications for all parameters are shown vs. pair-fed treatment except muscle Gln release, which in adults is shown vs. day 0 treatment. ↓ Significantly decreased; ↑ significantly increased; → no significant change.
muscles from adult rats. However, the important and relevant point is the comparison between muscles from adult and old rats, which were all treated in the same conditions. From this point of view, our results are in agreement with those observed in the same type of model by Dardevet et al. (5). The increase in protein breakdown occurred only in old animals treated for 7 days by glucocorticoids and reached the values observed in adults at basal state.

Concerning muscle Gln release, this parameter is increased in adults in both glucocorticoid-treated and pair-fed rats, suggesting that the decrease in food intake induced by glucocorticoids is responsible for this phenomenon. In old DEX-treated rats, the increase in muscle Gln release did not result from the decrease in food intake but merely from the direct effect of DEX. Parry-Billings et al. (25) demonstrated that muscle Gln release was lower in healthy old rats than in adult rats. This result does not appear to be supported by the present study (muscle Gln release in nmol g⁻¹ min⁻¹: A0: 16.9 ± 6.2; E0: 18.3 ± 5.4). In our catabolic conditions, increase in muscle Gln release occurred later in old rats than in adults. These results suggest that affinity or number of Gln transporters could deteriorate during physiological aging but also during DEX treatment in old rats, possibly in relation to an age-related alteration in hormonal control (19).

In conclusion, the simultaneous study of muscle Gln synthesis, net muscle protein breakdown, and muscle Gln release has for the first time provided new insights into the mechanisms possibly underlying muscle Gln alterations during aging, and it forms a coherent pattern. We have demonstrated in old glucocorticoid-treated rats that the muscle capacity to synthesize Gln occurred earlier in old rats (day 3) than in adults (day 7), whereas an increase in muscle Gln release occurred later in old rats (day 5) than in adults (day 3). The combination of these two phenomena during the first phase of stress response induced a preservation of muscle Gln concentration in old rats. During a later phase, depletion in plasma Gln occurred in old rats, whereas muscle Gln production and release increased. This depletion in plasma Gln is probably partly the result of decrease in food intake but may also suggest that consuming-organ requirements for this AA are increased.

The conventional view (26) of adaptation to stress is an increase in Gln release by muscles to support increased requirements by the liver, the kidneys, the intestine, and immune cells. As a consequence, muscle Gln synthesis is increased, but not sufficiently, and in turn, muscle free Gln drops, which may induce an increase in net muscle protein breakdown (11); however, this latter point is controversial (14) and not in line with results of the present study.

Our data suggest that the metabolic adaptation in response to a glucocorticoid may change during aging: muscle Gln synthesis is preserved in elderly glucocorticoid-treated rats, but interorgan flow of Gln, which is essential to maintain whole body homeostasis, seems to be altered. In particular, muscles from elderly DEX-treated rats exhibit a reduced ability to release Gln. Further studies are required to determine whether this alteration could be responsible for higher mortality in the E7 group, why this change of priority occurs, and what the consequences are in terms of central organ requirement in stressed, aged people.

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