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Contribution of proteolysis and de novo synthesis to alanine production in diabetic rat skeletal muscle: a $^{15}N/^{1}H$ nuclear magnetic resonance study

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Summary To assess the role of leucine as a precursor of alanine α-amino nitrogen in skeletal muscle during diabetes, extensor digitorum longus muscles from control (*n* = 7 experiments) and streptozotocin-diabetic rats (*n* = 8 experiments) were isolated and superfused with [^{15}N]leucine (3 mmol/l) in the presence of glucose (10 mmol/l) for 2 h. Muscle perchloric acid extraction was performed at the end of superfusion in order to quantify newly synthesized alanine by $^{15}N/^{1}H$ nuclear magnetic resonance. Release of [^{15}N]alanine in the superfusion medium was also measured. The pool of newly synthesized [^{15}N]alanine was significantly increased (~ 40 %) in extensor digitorum longus muscles from streptozotocin-diabetic rats. Whereas a significant enhancement of total alanine release from muscle was induced by diabetes (20 %), only a slight increase in [^{15}N]alanine release was detectable under our experimental conditions. Consequently, we conclude that streptozotocin-diabetes in growing rats induces in skeletal muscle: 1) an increase in nitrogen exchange between leucine and alanine leading to newly synthesized [^{15}N]alanine; and 2) an increase of total alanine release from muscle originating from both proteolysis and de novo synthesis. [Diabetologia (1997) 40: 1159–1165]

Keywords Leucine, nitrogen transfer, rat skeletal muscle, experimental diabetes, gas chromatography-mass spectrometry.

Although glutamine has recently emerged as a major source of new carbon for gluconeogenesis, alanine is still considered to be an important glucose precursor in humans [1–4]. The mechanisms of altered alanine turnover rates during diabetes, however, are not completely understood. Alanine release is increased from rat muscle in experimental diabetes [5–7] and the interorgan flux of this amino acid is slightly elevated in spite of decreased plasma alanine in diabetic humans [8, 9]. From these data, skeletal muscle and splanchnic bed have been determined as the major sites of alanine release and uptake, respectively. However, to our knowledge, it has not been clearly demonstrated whether alanine release from muscle in diabetes originates from the increased protein catabolism – a known consequence of insulin deficiency [10] – or from de novo alanine synthesis.

At the whole body level, leucine was found to be a major nitrogen donor for de novo alanine synthesis in dogs [11] and postabsorptive humans [12, 13], but this role remains poorly documented during diabetes mellitus. Leucine is known to be transaminated and (at least in part) oxidized in skeletal muscle and the whole body. Leucine oxidation is enhanced both in insulin-dependent diabetic humans [1, 9] and in rat experimental diabetes [14, 15]. Moreover, a recent study suggests that, in whole body and leg tissues in type 1 diabetic patients, a substantial acceleration in

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Abbreviations: NMR, Nuclear magnetic resonance; ^{15}N, nitrogen 15; ^{1}H, proton; $^{15}N/^{1}H$, indirect detection of nitrogen 15 by the intermediary of proton; STZ, streptozotocin; PCA, perchloric acid; EDL, extensor digitorum longus; GC-MS, gas chromatography-mass spectrometry; R_T, total alanine; R_L, [^{15}N]alanine.

leucine transamination occurs in insulin-deprived states unless the cause is known [16]. The use of [^{15}N]leucine appears to be a good probe to assess directly the capacity of muscle to synthesize alanine de novo as a consequence of leucine transamination.

The aims of the present study therefore were to determine during experimental diabetes: 1) whether alanine synthesis is increased in rat skeletal muscle; 2) whether leucine nitrogen plays a significant role in this potential synthesis and, finally; 3) whether or not alanine released from muscle is newly synthesized. [^{15}N] alanine synthesis was investigated by ^{15}N/^{1}H nuclear magnetic resonance (NMR) in [^{15}N] leucine-superfused extensor digitorum longus (EDL) muscles from control and streptozotocin (STZ)-diabetic rats. This method has been previously used to measure leucine transamination in rat muscle homogenates [17]. Measurement of the enrichment of alanine released in the superfusion medium was performed using gas chromatography-mass spectrometry (GC-MS) analysis.

Materials and methods

Animals. Female Wistar rats (Iffa Credo, L'Arbresle, France), weighing 55 ± 5 g were fasted overnight and then injected intraperitoneally with either physiological saline ($n = 44$) or streptozotocin (100 mg/kg body weight in 0.01 mol/l citrate buffer, pH 4.5) ($n = 48$) at 10.00 hours. Animals were maintained in individual cages in a temperature-controlled room (22 ± 1 °C) with a 12.12-h light-dark cycle. During the 3 days after injection, they were given free access to food (standard rat chow) and water. Glycosuria was monitored daily by Clinistix (Bayer Diagnostics Ames, Basingstoke, Hampshire, England) in order to operationally define them as diabetic rats (the consistent presence of glycosuria) for experimental procedures. Urine pH was measured the day of the superfusion experiment. Another group of rats were maintained under standard conditions: hindlimb muscles from these animals were pooled and used for blank perchloric acid (PCA) extracts for the NMR calibration curve.

Muscle superfusion. In order to directly and continuously monitor intermediary metabolism in skeletal muscle from growing rats in real time by NMR, a muscle superfusion system (8 EDL muscles superfused with 50 ml of oxygenated buffer in each experiment) has been previously developed [18]. Superfusion experiments were always carried out from 11.00 hours to 13.00 hours, 3 days after streptozotocin (STZ) injection. Rats were anaesthetized (Pentobarbital, 45 μg/g body weight, i. p.) and their EDL muscles were dissected. EDL muscles were continuously superfused with Krebs-Henseleit buffer containing bovine serum albumin (0.13 %), glucose (10 mmol/l), [^{15}N]leucine (3 mmol/l) and 2-oxoglutarate (3 mmol/l) for 2 h. It was verified by ^{31}P NMR, as previously reported [18], that muscles from both control and STZ-diabetic rats were metabolically stable for more than 2 h, as indicated by lack of change in the peak area of phosphorus metabolites (ATP, PCr) and in muscle pH. At the end of superfusion, the muscles were promptly freeze-clamped and stored in liquid N_2 pending ^{15}N/^{1}H NMR measurements. Superfusion medium was frozen and stored at –20 °C until GC-MS and amino acid analysis. At the end of the muscle dissection, arterial blood was drawn from the heart. A small quantity of blood was saved for determination of blood pH and, after deproteinization with perchloric acid, for measurement of glucose. Rats were killed after surgery by a pentobarbital overdose.

Determination of [^{15}N] alanine level synthesized in EDL muscle from [^{15}N] leucine by ^{15}N/^{1}H NMR. Muscle PCA extracts were prepared as previously described [18]. The neutralized extracts were lyophilized. The resulting lyophilized powder was dissolved in 300 μl of D_2O containing 3 mmol/l EDTA to remove paramagnetic ions present in the sample and 2 μmol ml of [^{15}N]valine as internal standard. Before NMR analysis, the pH of the sample was measured and adjusted to the designated pH (8.5–9) and the sample placed in a 5 mm NMR tube. The same protocol was used for blank PCA extracts of the calibration curve.

^{1}H and ^{15}N NMR spectra were obtained using a BRUKER AM400 spectrometer (BRUKER/Spectrospin, Wissembourg, France) at 400 and 40.5 MHz, respectively. The heteronuclear spin-echo experiments were run in reverse mode using a QNP 5 mm probehead with heteronuclear decoupling as previously reported [17] except that [^{15}N] alanine synthesized instead of [^{15}N]leucine was detected in the same spectrum as the [^{15}N] valine peak used as internal standard. Detection of ^{15}N labels via three bonds ^{1}H-^{15}N coupling was carried out using the following experiment:

^{1}H 90 °–τ –180 °–τ – Acquire
^{15}N τ–90 °–τ

The response of ^{15}N coupled β-protons in heteronuclear ^{15}N/^{1}H spin echo NMR is dependent on the coupling constant ($^{3}J^{15}N$-^{1}H) via τ ($\tau = (1/(2 \times J\ ^{15}N\text{-}^{1}H))$; this coupling constant is different for alanine (3 Hz) and valine (3.5 Hz). By choosing a τ value equal to 166.7 ms, alanine and valine can be detected together with a correct signal to noise. Quantitation of [^{15}N]alanine resonance was realized from a standard curve obtained by spiking blank PCA extracts with known concentrations of [^{15}N]alanine and [^{15}N]valine. The following calibration curve has been established: [Ala]/[Val] (ratio of concentrations) = 4.495 × (Ala/Val) (ratio of NMR areas) + 19.98 ($n = 4$); $r = 0.998$). It should be pointed out that quantification by such a method was previously validated by usual GC-MS [19]. Resonance areas were determined on NMR spectra after computerized curve-fitting analysis (trade mark NMRi). Chemical shifts were expressed with respect to residual water which was assigned to a chemical shift of 4.8 ppm relative to 4,4-dimethyl 4-silapentane sodium sulfonate (DSS).

Determination of total intramuscular alanine pool. Because all superfused EDL muscles were necessary to make PCA extracts for NMR studies, some additional superfusion experiments ($n = 4$ in each group (control or diabetic)) were performed to determine levels of total alanine in 2 h superfused EDL muscle from control and diabetic rats. These concentrations were determined on PCA extracts (as described above) by amino acid analysis (Alpha Plus; LKB Biochrom LTD, Cambridge, England) using norleucine as internal standard.

Determination of alanine level and enrichment in muscle superfusion medium. The enrichment of alanine in the superfusion medium was measured after tert-butyldimethylsilyl derivatization and GC-MS analysis (Nermag R10–10C; Delsi-Nermag, Argenteuil, France) as previously described for threonine [20] except that the deproteinization of the medium (4 ml) was performed with an equal volume of perchloric acid (4 %) instead of sulfosalicyclic acid. The ion peaks at mass to electron charge

ratio (m/e) 260 and 261 were measured by electron impact selective ion monitoring. Alanine concentration was determined in the superfusion medium (4 ml) after PCA deproteinization, desalting and amino acid analysis using norleucine as internal standard.

Biochemical analysis. Plasma glucose was measured with a Beckman Glucose analyser (Beckman Instruments, Palo Alto, Calif., USA). Tyrosine level into the superfusion medium was assayed as previously described [19]. ATP, PCr and lactate were assayed enzymatically in freeze-dried tissue following muscle deproteinization as previously described [18].

Calculations. Because both total and [^{15}N]alanine remained stable after the first hour of incubation, alanine levels measured at the end of 2 h superfusion experiments were assessed under metabolic steady-state conditions.

The [^{15}N]alanine release (R_L) from muscle (labelled alanine) was estimated by

$$R_L = E \times R_T \quad (1)$$

where E is the α-amino ^{15}N enrichment and R_T the concentration of total alanine measured (by GC-MS and amino acid analysis, respectively) in alanine released in the medium superfusion at the end of 2 h superfusion with [^{15}N]leucine.

Alanine released in the medium of superfusion has two inflow components to its flux: release from protein breakdown and from alanine de novo synthesis, respectively. The fraction (%) of alanine originating from protein breakdown (B_{Ala}) was estimated by

$$B_{Ala} = ((R_T - R_L)/R_T) \times 100 \quad (2)$$

where (R_T-R_L) consists in the release of unlabelled alanine. The fraction of alanine synthesized from [^{15}N]leucine (D_{Ala}) and released in the medium of superfusion was estimated as follows

$$D_{Ala} = (R_L/R_T) \times 100 \quad (3)$$

Statistical analysis. Each superfusion experiment was repeated 7 to 8 times. It should be pointed that, in each of the 7 to 8 experiments EDL muscles from 4 different rats were necessary for each NMR superfusion experiment. Additional experiments to measure the alanine pool in muscle used $n = 4$ rats per group (control or diabetic). Values are means ± SEM. Data were compared using an unpaired *t*-test.

Results

Animal characteristics. Table 1 summarizes the initial body weights and growth rates of control and STZ-treated rats, as well as measurements made at the time of superfusion experiments. STZ-treated rats were markedly hyperglycaemic and gained no weight, whereas controls gained approximately 7 g/day. Mass of the EDL muscle from STZ-diabetic rats was significantly lower than that of controls. In contrast, there was no significant difference between 2 h superfused EDL muscles from control and STZ-diabetic rats with regard to the content of ATP, PCr (5.4 ± 0.2 and 23.0 ± 0.6 μmol/g wet weight respectively; pooled means ± SEM) and pH measured by ^{31}P NMR (~ 7 for 2 h superfusion).

Table 1. Characteristics of control and streptozotocin (STZ)-diabetic rats

	Control ($n = 44$)	Diabetic ($n = 48$)
Initial body weight (g)	51 ± 2	50 ± 2
Growth rate (g/day)	7.3 ± 0.4	2.0 ± 0.5[a]
EDL muscle weight (mg)	31 ± 1	27 ± 1[a]
Plasma glucose (mmol/l)	6.3 ± 0.6	23.0 ± 1.0[a]
Blood pH	7.48 ± 0.02	7.51 ± 0.02
Urine pH	5.64 ± 0.07	5.23 ± 0.16

Values are presented as means ± SEM; [a] $p < 0.05$ significantly different from control rats. Initial body weight was determined at time of streptozotocin injection. EDL muscle weights and biochemical measurements were made at the time of superfusion experiments

Measurement of alanine synthesized in 2 h superfused EDL muscles from control and STZ-diabetic rats by $^{15}N/^{1}H$ NMR. Figure 1A shows a typical ^{15}N/^{1}H NMR spectrum of PCA extract of EDL muscles from diabetic rats after 2 h of [^{15}N]leucine superfusion. In order to assign the alanine resonance under our experimental conditions without ambiguity, we spiked blank PCA extract with pure [^{15}N]alanine and recorded the spectrum under the same conditions as the experimental PCA extract of EDL muscle (not shown). We have previously verified that no resonance appeared in the ^{15}N/^{1}H NMR spectrum of blank PCA extract from skeletal muscle. By comparing experimental and standard spectra, the complex resonance observed in the region between 1.43 and 1.59 ppm was assigned to ^{15}N coupled methyl protons of alanine. The same resonance was detected in EDL muscles from control rats (Fig. 2). This result demonstrates that the ^{15}N from leucine was incorporated into α-nitrogen atom of alanine in EDL muscles whatever the group of rats (control and diabetic). The Cβ proton resonance of valine observed at 2.15 and 1.92 ppm in Figures 1A and 2 was used, as previously [17, 19], as internal standard in order to check calibration of [^{15}N]alanine. It should be pointed out that [^{15}N] leucine did not resonate in spectra obtained by ^{15}N/^{1}H spin echo experiment with the chosen τ value under our experimental conditions. The presence of two peaks in alanine NMR signal (a major one at 1.59 ppm and a minor one at 1.43 ppm) in experimental and standard PCA extracts (Figs. 1A, 2 and 3A) may be explained by the presence of alanine on two different states. By adding EDTA in excess to the blank PCA spiked with alanine, the resonance at 1.43 ppm disappeared (Fig. 3B) indicating that this resonance was due to the presence of a complex structure between 2 molecules of alanine and a double-charged cation, for example Mg^{++}, present in the PCA extract. A standard curve of alanine in relation to valine was established that took into account the multiple resonance of this amino acid (peaks (4) and (5) in Figs. 1 and 2). Analysis of peak areas in

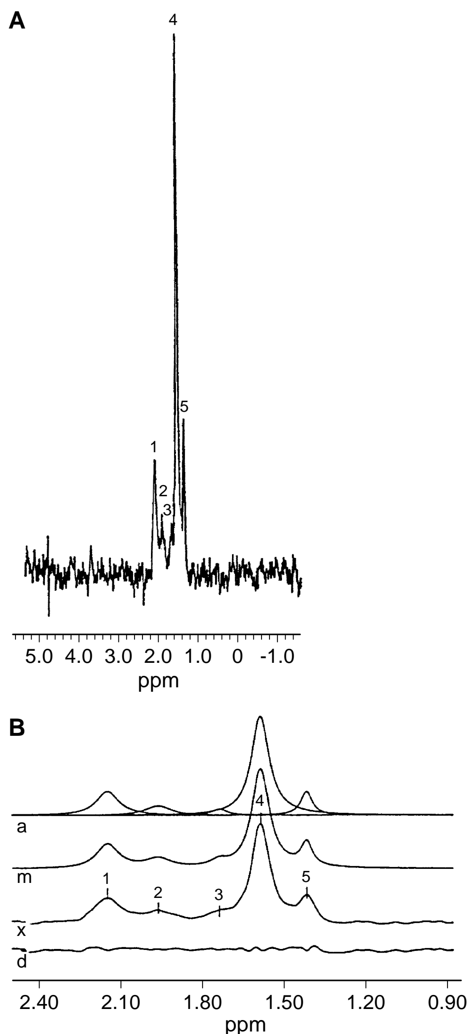

Fig. 1 (A) $^{15}N/^{1}H$ NMR spectrum of PCA extract of [^{15}N]leucine-superfused EDL muscles from diabetic rats (**B**) same spectrum treated by curve-fitting to determine peak areas (x: experimental spectrum; m: calculated spectrum; a: fitted peaks and d: difference spectrum). Acquisition parameters: 90° ^{1}H pulse width, 5 ms; 180° ^{1}H pulse width, 1800 μs; 90° ^{15}N pulse width, 18 μs; number of scans, 3200; delay τ, 166.7 ms; sweep width, 4000 Hz; line-broadnening 15 Hz. Peak identification: (1) and (2) valine at 2.15 and 1.92 ppm, (3) unknown resonance at 1.75 ppm, (4) free alanine at 1.59 ppm, (5) alanine in a complex structure at 1.43 ppm. Only peaks 1, 2, 4 and 5 were used for the quantification. This figure is of a representative experiment

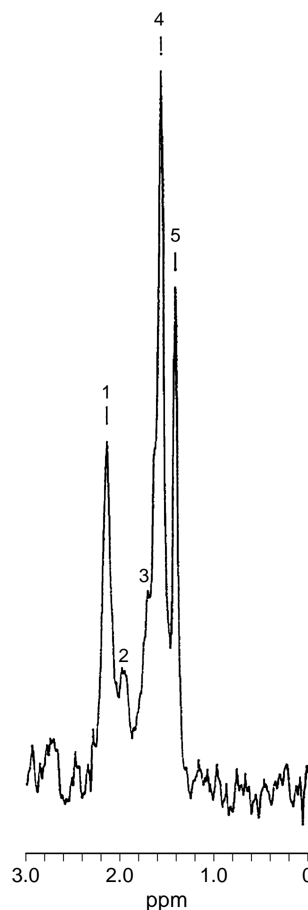

Fig. 2 $^{15}N/^{1}H$ NMR spectrum of PCA extract of [^{15}N]leucine-superfused EDL muscles from control rats. Peak identification: (1) and (2) valine at 2.15 and 1.92 ppm, (3) unknown resonance at 1.75 ppm, (4) free alanine at 1.59 ppm, (5) alanine in a complex structure at 1.43 ppm. Only peaks 1, 2, 4 and 5 were used for the quantification. This figure is of a representative experiment

spectra were carried out after curve fitting as reported in Figure 1B.

The size of newly synthesized [^{15}N]alanine pool in EDL muscles, calculated from $^{15}N/^{1}H$ NMR data is reported in the Table 2. There is a significant difference in these values between control and STZ-diabetic rats. STZ diabetes resulted in a 1.4-fold increase in de novo alanine synthesis from [^{15}N]leucine. In some experiments, we verified that total alanine level in EDL muscles is also increased by STZ diabetes (Table 2).

Determination of alanine, lactate and tyrosine release from control and STZ-diabetic rats. Whereas [^{15}N] alanine cannot be successfully detected by $^{15}N/^{1}H$ NMR due to its low abundance in the superfusion medium, we used GC-MS combined with amino acid analysis to determine the overall release of alanine and its ^{15}N enrichment. By calculation, we can infer the rate of [^{15}N]alanine release from muscle (see equation 1 and Table 2). Relative to control, STZ-diabetes resulted in a significant increase in total alanine release (20%). The value of [^{15}N]alanine release was only slightly higher in STZ-diabetic rats than in control; this difference was not statistically significant (Table 2).

In order to evaluate net protein degradation in superfused EDL muscles, the rate of tyrosine release into the superfusion medium was measured (123 ± 10 and 186 ± 12 nmol · g muscle^{-1} · 2 h^{-1}, in control and STZ-diabetic rats, respectively). Indeed, Pepato et al. [10], in the acute phase (1–3 days) of

Table 2. Effect of diabetes on synthesis and rate of release of alanine from [^{15}N]leucine-superfused EDL muscles

Group	Intramuscular pool (μmol/g wet weight muscle)		EDL rate of release (μmol · 2 h^{-1} · g^{-1})	
	Total alanine	[^{15}N]alanine	Total alanine (R_T)	[^{15}N]alanine (R_L)
Control	2.46 ± 0.01	0.79 ± 0.05	1.80 ± 0.07	0.74 ± 0.04
Diabetes	2.73 ± 0.14	1.10 ± 0.07[a]	2.15 ± 0.10[a]	0.88 ± 0.14

Values are given as means ± SEM (***n*** = 4 to 8, number of experiments/treatment group); [a] $p < 0.05$ significantly different from control rats. ^{15}N/^{1}H NMR, GC-MS and aminoacid measurements were made at the end of 2 h superfusion experiments

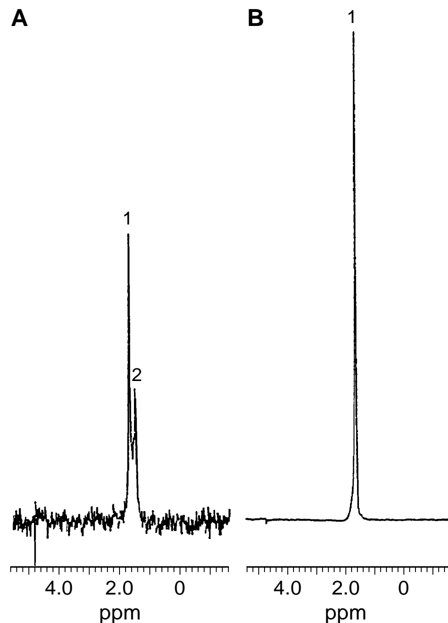

Fig. 3 **(A)** ^{15}N/^{1}H NMR spectrum of blank PCA extract spiked with [^{15}N]alanine (10 mmol/l), (**B**) same extract added with an excess of EDTA (10 mmol/l). Acquisition parameters: 90° ^{1}H pulse width, 5 ms; 180° ^{1}H pulse width 1.8 ms; 90° ^{15}N pulse width, 18 μs; number of scans, 160; delay τ, 166.7 ms; sweep width, 4000 Hz; line-broadening 15 Hz. Peak idenfication: (1) Free alanine at 1.59 ppm, (2) complex structure with alanine at 1.43 ppm

STZ-diabetes in young rats, reported that total protein breakdown in the EDL muscle increased by 50 % compared to the controls, although protein synthesis was also depressed. This value is in very good agreement with the increased rate of net tyrosine release measured in the present studies (51 %). Thus, we assumed that the increased net tyrosine release largely reflects increased proteolysis. In contrast, lactate release was not modified by diabetes (15.1 ± 1.9 and 15.9 ± 1.5 μmol · g muscle^{-1} · 2 h^{-1}) in control and STZ-diabetic rats, respectively.

Discussion

The present work describes, for the first time to our knowledge, the application of ^{15}N/^{1}H NMR spectroscopy for measuring de novo alanine synthesis directly in skeletal muscle. Based on the superfusion of EDL muscles with [^{15}N]leucine, this study confirms that leucine is a significant donor of α-amino N for the synthesis of alanine (Fig. 1), as previously estimated from whole body flux measurements in humans [12, 13] and in dogs [11]. This conclusion is in good agreement with a coupling between branched chain amino acid transaminase and alanine aminotransferase within the muscle [for reviews, see 14, 22]. Because exogenous glucose is included in the superfusion medium, the glucose-derived pyruvate necessarily provides the carbon skeleton for the newly synthesized alanine as previously reported [22–26].

Due to the limited sensitivity of the method which requires more than 800 nmol/g muscle, direct alanine synthesis kinetics cannot be continuously followed with success by ^{15}N/^{1}H NMR over the 2 h superfusion of EDL muscles; however, analysis of muscle PCA extracts has demonstrated the advantage of selectively detecting and quantifying the newly synthesized alanine pool within the muscle. In contrast, most studies on muscle in vitro [5, 23] give an indirect estimation of alanine synthesis from the increase of total alanine release proportional to the concentration of leucine added to the muscle incubation medium or to the hindquarter perfusion medium. Often these investigations did not clearly distinguish between the processes of synthesis and release of alanine by skeletal muscle; this may lead to confusion between the terms release and synthesis.

There is strong evidence that diabetes in the acute phase (1–3 days) causes a situation in which protein synthesis is markedly reduced, protein degradation is increased [10, 26] and the efflux of amino acids from skeletal muscle studied in vitro [5, 7] or in vivo [6, 16] is increased. The lack of gain in body weight, the atrophy of EDL muscles (13 %) and the increased total alanine and tyrosine release (20 and 50 % of controls, respectively) from superfused EDL muscles obtained in our experimental STZ-diabetic rats confirm these observations. The major finding of this study provides evidence that diabetes increases the nitrogen exchange between leucine and alanine leading to a 1.4 fold increased intramuscular pool of [^{15}N]alanine (Table 1). This result is consistent with an enhanced muscle capacity to oxidize leucine during diabetes, as previously reported in studies in vitro [14, 15] and in vivo in human legs [16]. It should be

pointed out that this study is the first direct and quantitative estimation of de novo α-amino alanine synthesis within skeletal muscle from STZ-diabetic rats. Until now, in vivo studies, using tracer methods with determinations solely in plasma [1, 7], failed to prove an increase of alanine turnover in spite of increased proteolysis in insulin-dependent diabetic humans.

The increased release of alanine observed in diabetes and fasting is most commonly attributed to the need for glucogenic substrates in these situations [5–7]. Although this study is in good agreement with these observations (see values of total alanine release in Table 2), it further demonstrates, by using a nitrogen 15 tracer method, that the newly synthesized alanine, from [^{15}N]leucine in the presence of glucose (10 mmol/l), does not totally explain the enhanced alanine release from EDL muscle of STZ-diabetes in growing rats. Leucine contributes approximately 41 % of alanine α-amino N released (D_{ala}) from diabetic muscles. It may be surprising that this nitrogen transfer from leucine to alanine is similar in control rats. Consequently, protein breakdown in alanine release amounts to approximately 59 % (B_{ala}) whatever the group of animals. These results are consistent with a concomitant increase in de novo synthesis of alanine and protein catabolism during diabetes explaining the difference in release of total alanine between STZ-diabetic and control rats observed in the present experiment (about 20 % rise in STZ-diabetes). These data are at variance with previous findings, suggesting that an increase in alanine release from muscle can only be explained by an increased protein catabolism [26]. The enhanced alanine release from muscle during STZ-diabetes may be explained, as previously reported [26], by a stimulation of system A amino acid transport. In addition to clearly distinguishing between alanine synthesis and release from muscle, the design of the present study allows for the determination of the origin of released alanine (either de novo synthesis or protein catabolism).

In conclusion, we have shown that ^{15}N/^{1}H NMR spectroscopy can be used to directly measure de novo alanine synthesis from [^{15}N]leucine in skeletal muscle studied ex vivo. We further observed that the response of alanine metabolism to diabetes in rat skeletal muscle in the presence of [^{15}N]leucine consists of: i) an enhanced de novo alanine synthesis, and ii) an increase of total alanine release from muscle derived from both proteolysis and de novo synthesis.

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