A BRIEF COMMUNICATION

Type I Diabetes Affects Skeletal Muscle Glutamine Uptake in a Fiber-Specific Manner

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Skeletal muscle serves as the body's major glutamine repository, and releases glutamine at enhanced rates during diabetes, but whether all muscles are equally affected is unknown. System N^m activity mediates most trans-sarcolemmal glutamine movement, and although two System N (SN) isoforms have been identified (SN1/sodium-coupled neutral amino acid transporter or System N and A transporters [SNAT]-3; and SN2/SNAT5), their expression in skeletal muscle remains controversial. Here, the impact of Type I diabetes on glutamine uptake and System N transporter expression were examined in fast- and slow-twitch skeletal muscle from spontaneously diabetic (BB/Wor-DP) rats. Net glutamine uptake in fast-twitch fibers was decreased 75%–95%, but enhanced more than 2-fold in slow-twitch muscle from diabetic animals relative to nondiabetic controls. Both *SNAT3* and *SNAT5* mRNA were expressed in both muscle fiber types and their abundance was unaffected by diabetes. This represents the first report of differential fiber-specific effects of diabetes on skeletal muscle glutamine transport and the co-expression of distinct System N transporters in skeletal muscle.
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Key words: SNAT3; SNAT5; sarcolemma; amino acid transport; diabetes; muscle

Glutamine transport both into and out of skeletal muscle has received considerable attention because of its recognized role in supplying this "conditionally essential" amino acid for key physiologic processes during catabolic states, and in regulating muscle carbohydrate metabolism, protein synthesis, and proteolysis rates largely through its effects on cell volume (1). For example, accelerated release of glutamine from skeletal muscle during diabetes has been well documented (2), and is thought to contribute to the negative nitrogen balance and heightened gluconeogenesis characteristic of this state. Augmented muscle glutamine release during catabolic states is well established, but the possibility has been raised that glutamine may be differentially lost from fast-twitch muscle (3). To further examine this possibility, the first aim of the study was to compare the effects of diabetes on net glutamine uptake in both fast-twitch and slow-twitch muscle fibers.

The transport activity that mediates the majority of bidirectional transmembrane glutamine movement in skeletal muscle is System N^m (4), named after a very similar activity, termed System N, in liver (5). These unique Na^+-dependent transport activities are characterized by narrow substrate specificities (glutamine, histidine, and, to a lesser extent, asparagine) and tolerance of Li^+-for-Na^+ substitution in driving uptake of their substrates. In 1999 and 2001, two distinct transporters with properties of System N were identified by expression cloning from rat-brain cDNA libraries, and designated SN1 and SN2, respectively (6, 7). Both belong to the SLC38 amino acid transporter family, which also includes the System A transporters. The nomenclature for the family members has recently been amended to "SNAT#" for sodium-coupled neutral amino acid transporter or System N and A transporters (8). As such, SN1 is now designated as SNAT3, whereas SN2 is SNAT5, according to the order in which they were isolated

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and designated within the SLC38 family. The expression of both SN1/SNAT3 and SN2/SNAT5 has been unequivocally demonstrated in brain and liver, two tissues with active glutamine cycles, but much controversy remains regarding their expression in skeletal muscle. The only report thus far of System N transporter expression in skeletal muscle tissue was provided by the isolation of an *SN1* cDNA from a rat muscle library (9). However, two other studies reported no detectable *SN1/SNAT3* or *SN2/SNAT5* mRNA expression in rat skeletal muscle (6, 7), raising the question of what transporter, alone or in combination, accounts for the System N^m activity chronicled in skeletal muscle. To partially resolve this issue, the second aim of this study was to definitively assess SNAT3 and SNAT5 expression in skeletal muscle.

Materials and Methods

All radiochemicals were obtained from Perkin-Elmer (Boston, MA). Chemicals and unlabeled glutamine were from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein detection reagents were purchased from Pierce Chemicals (Rockford, IL) and supplies and chemicals for scintillation spectrophotometry were from Packard Instruments (Meriden, CT).

Diabetes Model. The spontaneously diabetic (genetically Type I diabetic) Bio-Breeding Worcester (BB-Wor/DP; Ref. 10) and control BB-Wor/DR rats (200–300 g) were obtained from Biomedical Research Models (Worcester, MA). Animals were housed in the Saint Louis University School of Medicine Animal Research Facility and had *ad libitum* access to chow and water. All procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee. Glycosuria was assessed through urinalysis with Keto-Diastix Reagent Strips from Fisher Scientific (Pittsburgh, PA). To minimize nutritional influences on experimental results, all animals were subjected to an overnight fast before surgery the following morning.

Glutamine Uptake Measurements in Isolated Skeletal Muscle. Animals were anesthetized with 5 mg/100 g body weight of sodium pentobarbital. The soleus (slow twitch), flexor digitorum brevis (fast twitch), and epitrochlearis (fast twitch) muscles were isolated and removed. These muscles were chosen for their fiber-specific composition and thin anatomical profiles (surface area to volume ratio), facilitating efficient transmembrane solute movement while minimizing diffusion barriers. Soleus muscles were split longitudinally before assays to obtain two strips that further enhanced intramuscular substrate diffusion. Excised muscles were incubated at 35°C in Dubnoff shakers in 25-ml Erlenmeyer flasks containing 2 ml of Na^+-containing Krebs Ringer bicarbonate (KRB) incubation medium, as previously described (11), and were gassed with 5% CO_2/95% O_2 throughout the experiment. Muscles were allowed to recover for 60 mins after dissection. After the recovery period, muscles were washed in 2 ml of Na^+-free Krebs Ringer bicarbonate buffer (Choline KRB) at 30°C for 10 mins. The transport assay was initiated when muscles were transferred to 25-ml Erlenmeyer flasks containing 3 μCi/ml of L-[^{3}H] glutamine in 2 ml of either Na^+-containing KRB or Na^+-free KRB for 10 mins at 30°C. This time point was chosen based on preliminary experiments that optimized isotopic signal within the linear range of uptake. Unlabeled glutamine was present in both the Na^+-containing and Na^+-free KRB buffer at 50 μ*M*, and assays were performed in the presence of 10 m*M* α-(methylamino)-isobutyric acid to minimize potential System A contributions to System N^m activity. After the transport incubations, muscles were quickly removed from the flasks and placed on a vacuum filter apparatus, rinsed rapidly three times with ice-cold PBS, then clamp-frozen in liquid nitrogen and homogenized in 1 ml of 0.3 *M* perchloric acid. The homogenate was assessed for protein content by the BCA method, and subsequently centrifuged. The supernatant (0.1 ml) was neutralized with 10 μl of 3 *M* NaOH and added to 1 ml of Microscint-20 for the determination of transported radioactivity by a scintillation spectrophotometry (Top Count; Packard Instruments). The rate of amino acid transport was calculated from the counts per minute (cpm) per sample, the specific activity of the uptake mix (in cpm/nmol) and normalized to cellular protein content with a Microsoft Excel spreadsheet. Transport values obtained in the absence of Na^+ (Na^+-independent uptake and nonsaturable components) were subtracted from the values obtained in the presence of Na^+ to yield the Na^+-dependent transport rates, reported in units of nmol/mg protein/10 mins. It should be noted that under the conditions used, net glutamine accumulation over the 10-min incubation represents both transport and anabolic metabolism (tRNA charging and protein synthesis), because skeletal muscle does not appreciably catabolize glutamine but mostly stores or produces it *de novo* via glutamine synthetase (12).

***SNAT3/5* mRNA Expression.** Total RNA isolated from soleus (slow), triceps (fast), and quadriceps (mixed) muscle were resolved by electrophoresis in 1% agarose gels (20 μg/lane) and transferred to a nylon membrane (Osmonics Inc., Westborough, MA). After ultraviolet cross-linking, blots were soaked in 0.1% sodium dodecylsulfate (SDS) and subjected to a 2-hr prehybridization at 68°C (for [α-^{32}P] uridine triphosphate–generated riboprobes) or 42°C (for [α-^{32}P] deoxyadenosine triphosphate–generated DNA probes) in hybridization buffer (Ultra-Hyb; Ambion Inc., Austin, TX), followed by hybridization overnight at the same temperature with 2×10^6 disintegrations per minute (dpm)/ml radiolabeled probe. The cDNAs used in this study were rat SN1 (in pBluescriptSK(+), kindly provided by Dr. Robert Edwards) and rat SN2 (in pSPORT1, kindly provided by Dr. Vadivel Ganapathy). Mouse β-actin cDNA was obtained from Ambion. Radiolabeled RNA probes were generated using SP6 RNA polymerase (MAXI-script; Ambion). Radiolabeled DNA

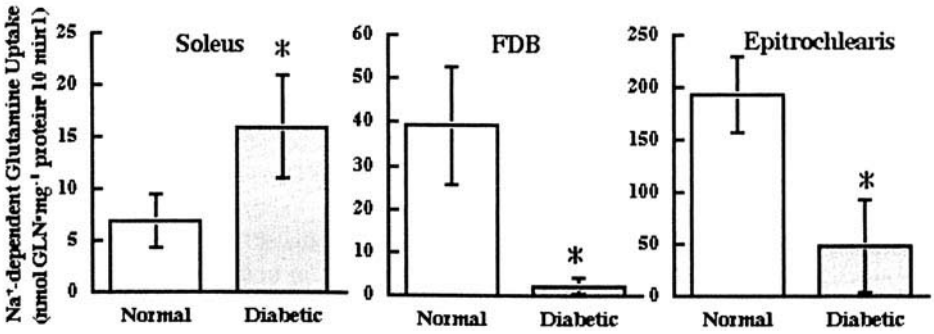

Figure 1. Sodium-dependent glutamine uptake rates in fast- and slow-twitch muscles from normal and diabetic rats. Radiotracer uptake assays were performed to determine Na^+-dependent glutamine uptake rates in slow twitch (soleus) and fast twitch (flexor digitorum brevis and epitrochlearis) muscle fibers from nondiabetic and diabetic rats, as described in the Materials and Methods section. Rates are reported in units of nmol glutamine/mg protein/10 mins,and represent the average ± SD of values from three (diabetic) or four (nondiabetic) animals. *$P < 0.010$ vs. nondiabetic values.

probes were generated via random hexamer priming using the Megaprime kit (Amersham, Piscataway, NJ). Membranes were washed three times in 1× SSPE (composed of sodium chloride, sodium phosphate, and EDTA) + 0.1% SDS at 42°C for 10 mins and three times in 0.1× SSPE + 0.1% SDS at 68°C for 10 mins and exposed to Fuji x-ray film at −80°C with intensifying screens. Blots initially assessed for SNAT3 or SNAT5 expression were subsequently hybridized with a β-actin probe for normalization. Band intensities were quantified using a Kodak EDAS 290 system with one-dimensional (1-D) imaging software (Eastman Kodak, New Haven, CT).

Reverse-transcriptase polymerase chain reaction (RT-PCR) reactions were performed on 100 ng of sample RNA with the one-step SuperScript II RT/Platinum Taq system (Invitrogen, Carlsbad, CA). The identities of RT-PCR–generated cDNAs were confirmed by restriction endonuclease analysis. The *SN1/SNAT3* RT-PCR product was digested with *Sph*1, and yielded two bands at the expected 78 base pairs (bp) and 224 bp. The *SN2/SNAT5* RT-PCR product was digested with *Tsp*1, yielding two bands of the expected 195 bp and 125 bp. Primers used for RT-PCR were: SN1/SNAT3, sense 5′-AGATACCCCGACAGACAGAGAT-3′ and antisense 5′-AAGCAGGAACAGGAAGAGGA-3′, amplifying bp 102–403 of the corresponding cDNA; SN2/SNAT5, sense 5′-ATGGAAATGCAGGAACCAAA-3′ and antisense 5′-AATTTAGGTGACACTATAGAGAGGTGGATAGAGTAAGAAG-3′ (SP6 site underlined), amplifying bp 116–415 of the corresponding cDNA.

The DNA sequences for *SNAT3* and *SNAT5* from a rat quadriceps muscle were obtained using the sense primers in an automated sequencing reaction (Applied Biosystems Model 3100; PNACL laboratory at Washington University School of Medicine, St. Louis, MO). Resulting sequences were aligned with the known *SNAT3* and *SNAT5* cDNA sequences using the ClustalW sequence analysis tool (http://www.ebi.ac.uk/clustalw/) from the European Bioinformatics Institute.

Results and Discussion

Net Glutamine Uptake in Fast- and Slow-Twitch Skeletal Muscle. Fast-twitch fiber glutamine transport rates were obtained from the flexor digitorum brevis and epitrochlearis muscles, and slow-twitch fiber glutamine transport rates were obtained from soleus muscle (Fig. 1). In the fast-twitch flexor digitorum brevis muscle, Na^+-dependent glutamine transport rates were 2.1 ± 2.0 nmol/mg protein/10 mins in the diabetic rats ($n = 3$) and 39.2 ± 13.4 nmol/mg protein/10 mins in the control rats ($n = 4$; $P < 0.010$), representing a 95% decline in diabetes. A similar pattern was seen in the fast-twitch epitrochlearis muscle, in which, again, the Na^+-dependent uptake rates in the diabetic rats (48.0 ± 45.5 nmol/mg protein/10 mins; $n = 3$) were 75% lower ($P < 0.010$) than the rates seen in the control rats (193.7 ± 36.2 nmol/mg protein/10 mins; $n = 4$). In contrast, the slow-twitch soleus muscle displayed an opposite pattern, in which net Na^+-dependent glutamine uptake was lower in the muscle from the control rats (6.9 ± 2.6 nmol/mg protein/10 mins; $n = 4$) compared with the muscle from the diabetic rats (16.0 ± 5.0 nmol/mg protein/10 mins; $n = 3$; $P < 0.010$), representing a 2.3-fold increase in the diabetic animals. These results indicate that, in diabetes, net Na^+-dependent glutamine uptake is diminished in fast-twitch muscles, but is enhanced more than 2-fold in a slow-twitch muscle. These findings are significant because they represent the first report of differential fiber-specific responses in net muscle glutamine accumulation to diabetes, and imply that the net arterial-venous glutamine balance across any muscle during diabetes may depend on its relative composition of fast- and slow-twitch fibers.

Tissue and Muscle Fiber–Specific Expression of System N Isoforms. System N^m was first described in perfused rat hindlimb (4). Therefore, we chose to use the quadriceps as a representative skeletal muscle in which to first examine System N transporter expression levels. Northern blot analysis of total RNA from the liver and quadriceps muscle of both nondiabetic and diabetic rats confirmed expression of both a 2.4-kilobase (kb) *SN1/*

SNAT3 and a 2.6-kb *SN2/SNAT5* mRNA (Fig. 2); thus, skeletal muscle, similar to liver, expresses the larger of two *SN2* mRNA species (7). This is the first report of *SN2/SNAT5* expression in skeletal muscle. Diabetes did not influence muscle mRNA levels of either transporter, similar to previous results for *SN1* in liver (13). Because the quadriceps is a mixed muscle, further studies were performed to determine whether the expression of either transporter was specific to, or enhanced in, either fast- or slow-twitch muscle fibers. Northern analysis of total RNA isolated from the soleus (slow twitch) and triceps (fast twitch) muscle of normal and diabetic rats revealed that both the *SN1/SNAT3* and *SN2/SNAT5* mRNA were expressed at comparable levels in each muscle type (Fig. 3). Again, diabetes failed to influence the mRNA levels of either transporter in each muscle type. Hepatic expression of both *SN1/SNAT3* and *SN2/SNAT5* mRNA was more robust than in skeletal muscle and served as a positive control for these analyses (Figs. 2 and 3). It should be noted that although the smaller, thinner epitrochlearis and flexor digitorum brevis muscles were used to obtain fast-twitch fiber glutamine uptake rates in the activity studies (Fig. 1), RNA for the Northern blot (Fig. 3) and RT-PCR analyses (Fig. 4) was harvested from the larger, thicker fast-twitch triceps muscle to obtain sufficient amounts of RNA for analysis.

RT-PCR analysis was performed on RNA from soleus, triceps, and quadriceps muscle using primer pairs specific for rat *SN1/SNAT3* and rat *SN2/SNAT5* (Fig. 4A). The expected product size was obtained for both *SN1/SNAT3* (302 bp) and *SN2/SNAT5* (320 bp) in each muscle type. Restriction digests of both PCR-generated cDNAs yielded the expected products, as described in the Materials and Methods section (data not shown). As a third confirmation, *SNAT3* and *SNAT5* RT-PCR products from a rat quadriceps muscle sample were sequenced and found to be 82% and 79% homologous to the reported DNA sequence for each isoform, respectively. The discordant (approximately 20% of each muscle PCR product) sequence was largely caused by subthreshold or ambiguous base readings on the chromatogram (Fig. 4B). Furthermore, the published sequence for *SNAT3* was only 42% homologous to the

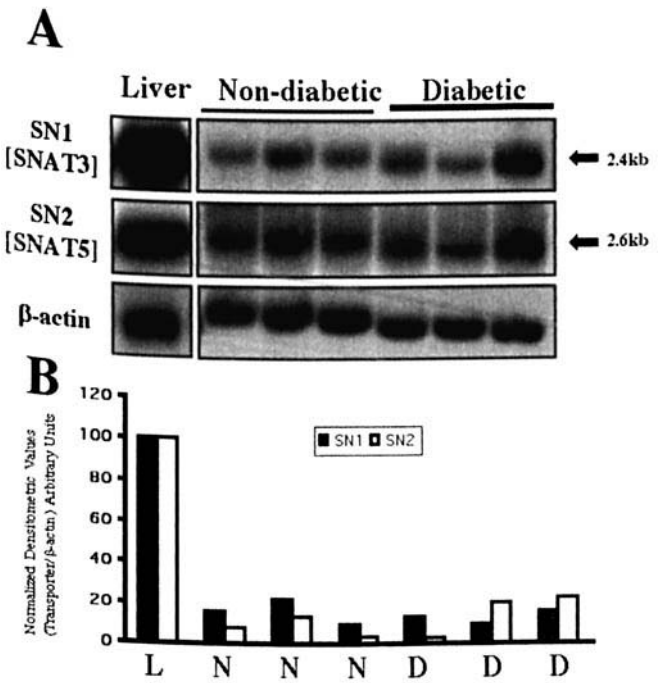

Figure 2. Expression of *SN1/SNAT3* and *SN2/SNAT5* in rat quadriceps. (A) Northern blot analysis was performed using riboprobes to assess *SN1/SNAT3* and *SN2/SNAT5* mRNA expression in quadriceps muscle from nondiabetic and diabetic rats. Levels of transporter mRNA from liver are shown for comparison. Exposure times were 24 hrs for *SN1/SNAT3*, 24 hrs for *SN2/SNAT5* and 5 hrs for β-actin. Data from one set of samples is shown; however, corroborative assays were performed on two separate blots with similar results. (B) Band intensities of each of the samples from (A) were quantified using the Kodak 1-D image analysis software and normalized to the corresponding β-actin mRNA levels in normal (N) or diabetic (D) animals. The values on the *y* axis are in arbitrary densitometric units, with values for liver set to 100.

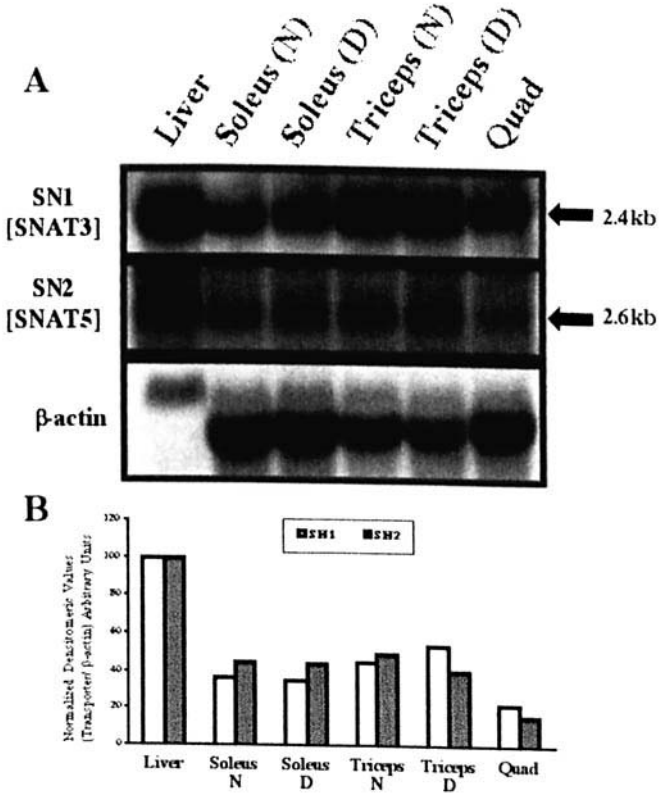

Figure 3. Expression of *SN1/SNAT3* and *SN2/SNAT5* in rat slow- and fast-twitch skeletal muscle. (A) Northern blot analysis was performed using random hexamer-generated radiolabeled DNA probes to assess *SN1/SNAT3* and *SN2/SNAT5* mRNA levels in fast twitch (triceps), slow twitch (soleus), and mixed (quadriceps) muscle from nondiabetic (N) or diabetic (D) animals. The *SN1/SNAT3* and *SN2/SNAT5* mRNA levels from liver are shown for comparison. Exposure times were 48 hrs for *SN1*, 48 hrs for *SN2*, and 9 hrs for β-actin. Riboprobes were used to assess β-*actin* levels. Results from one blot are shown, but similar results were obtained on two subsequent Northern analyses. (B) Band intensities from (A) were quantified using the Kodak 1-D image analysis software and normalized to β-actin mRNA levels. The values on the *y* axis are in arbitrary densitometric units, with values for liver set to 100.

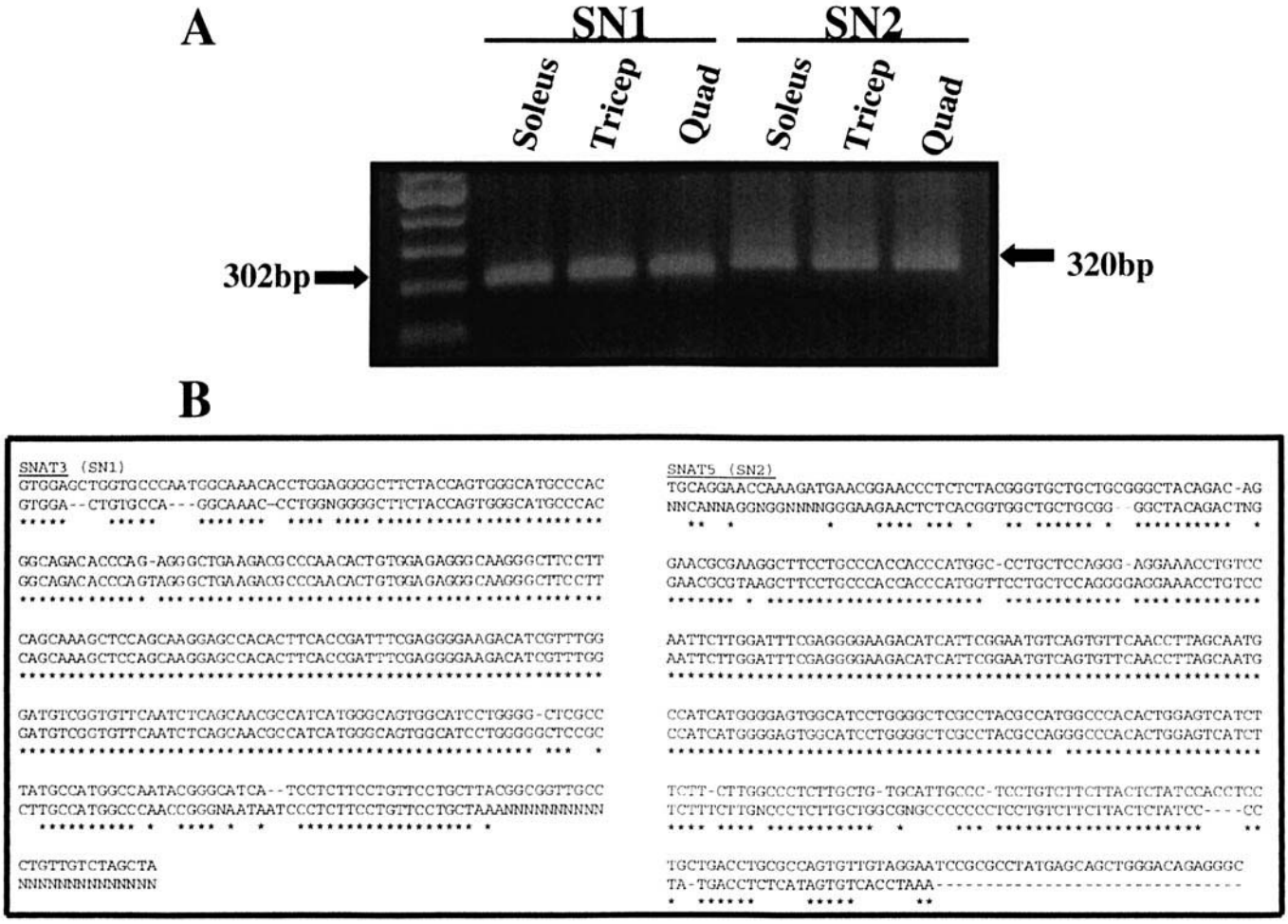

Figure 4. Confirmation of *SNAT3* and *SNAT5* cDNA in rat slow- and fast-twitch skeletal muscle by RT-PCR and DNA sequencing. (A) The presence of both *SN1* and *SN2* mRNA was assessed by RT-PCR performed on RNA isolated from slow twitch (soleus), fast twitch (triceps), and mixed fiber (quadriceps, quad) skeletal muscle from nondiabetic rats. The expected PCR product sizes were obtained when resolved and visualized on a 1% agarose gel. The identities of *SNAT3/SN1* and *SNAT5/SN2* were further confirmed by restriction enzyme digestion analyses of the PCR products (not shown). (B) The DNA sequences of both *SNAT3* and *SNAT5* cDNA isolated from rat quadriceps muscle were obtained via the same sense strand primers used in the RT-PCR (see Materials and Methods) in an automated sequence reaction. The *SNAT3* and *SNAT5* sequence results obtained (lower sequence) were 82% and 79% homologous to the established *SNAT3* and *SNAT5* cDNA sequences (upper sequence), respectively. An asterisk below a pair of nucleotides represents a match between the known sequence and the PCR-generated sequence.

SNAT5 PCR product, and, reciprocally, the established sequence for *SNAT5* was only 39% homologous to the *SNAT3* PCR product sequence, further confirming that both distinct isoforms of System N are expressed in rat skeletal muscle (Fig. 4B). In conclusion, contrary to previous reports of no detectable *SN1/SNAT3* or *SN2/SNAT5* mRNA expression in skeletal muscle (6, 7), the studies presented here unequivocally establish that rat skeletal muscle expresses both transporters.

Understanding the molecular underpinnings of enhanced muscle glutamine efflux during diabetes is important because this amino acid is used as a major gluconeogenic substrate, not only in liver, but especially in kidney and small intestine, whose quantitative contribution to global *de novo* glucose production is significant and becoming increasingly recognized (14, 15). Glutamine is also a key substrate in the hexosamine biosynthetic pathway, via the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Flux through GFAT (hexosamine biosynthesis) is hypothesized to serve as a glucose- and satiety-sensing pathway and to contribute to the development of peripheral insulin resistance characteristic of Type II diabetes (16, 17). Compared with other sarcolemmal amino acid transporters, System N^m–mediated glutamine uptake seems to be uniquely subject to significant insulin stimulation (2, 18), and the proposed role of System N^m in governing muscle protein economy and nitrogen balance during diabetes (2) and other catabolic states, such as sepsis and burn injury, provides compelling justification for further investigation into the transporters that comprise this activity, and their role in physiological regulation, as hypothesized by Rennie nearly 20 years ago (19).

Since their initial isolation in 1999 and 2001, studies on the biology and potential contributions of both SNAT3 and

SNAT5 to tissue-specific System N activity are just beginning to emerge (20). Likewise, the results presented here establish that SNAT3 and SNAT5 could largely comprise muscle System N^m activity, but the contribution of each to the observed diabetes-induced changes in fiber-specific glutamine uptake awaits investigations that are more detailed, including localization of the cognate proteins in individual muscle fibers once suitable antibodies are developed. As we enter the dawn of systems biology of tissue function, hopefully the results presented here will serve as a framework and springboard to future mechanistic studies on the role, regulation, and physiologic importance of each transporter in muscle nitrogen balance and glutamine economy during catabolic states, and in the differential response of slow- and fast-twitch muscle fiber glutamine transport to diabetes.

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