Mouse system-N amino acid transporter, mNAT3, expressed in hepatocytes and regulated by insulin-activated and phosphoinositide 3-kinase-dependent signalling

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Amino acid transporters are essential for normal cell function and physiology. In the present study, we report the identification and functional and regulatory characterization of a mouse system-N amino acid transporter, mNAT3. Expression of mNAT3 in *Xenopus* oocytes revealed that the strongest transport activities were preferred for L-alanine. In addition, mNAT3 is an Na⁺- and pH-dependent low-affinity transporter and it partially tolerates substitution of Na⁺ by Li⁺. mNAT3 has been found to be expressed predominantly in the liver, where it is localized to the plasma membrane of hepatocytes, with the strongest expression in those cells adjacent to the central vein, decreasing gradually towards the portal tract. Treatment of mouse hepatocyte-like H2.35 cells with insulin led to a significant increase in the expression of mNAT3, and this stimulation was associated

closely with an increase in the uptake of L-alanine. Interestingly, this insulin-induced stimulatory effect on mNAT3 expression was attenuated by the phosphoinositide 3-kinase inhibitor LY294002, but not by the mitogen-activated protein kinase inhibitor PD98059, although both kinases were fully activated by insulin. The results suggest that insulin-mediated regulation of mNAT3 is likely to be mediated through a phosphoinositide 3-kinase-dependent signalling pathway. The unique expression pattern and insulin-mediated regulatory properties of mNAT3 suggest that this transporter may play an important role in liver physiology.

Key words: amino acid transporter, insulin signalling, liver, mNAT3, phosphoinositide 3-kinase.

INTRODUCTION

Amino acid transporters play an essential role in a variety of cellular processes, including uptake of nutrients, energy and chemical metabolism, detoxification and specifically the nitrogen metabolism in the liver [1,2]. Mammalian amino acid transporters identified to date belong to a variety of gene families including CAT, A, N and EAAT [3–5]. Many of the gene families identified so far consist of multiple members that are expressed in a tissue-specific manner. Recently, we identified and characterized three system-N amino acid transporters, mNAT, mNAT2 and hNAT3; the first two are predominantly expressed in mouse liver and retina, whereas the last mentioned is predominantly expressed in human liver [6–8]. Orthologous genes of mNATs have been identified by our laboratory and also by other research groups; human g17 and rat SN1 [6,9,10] are orthologues of mNAT; rat GlnT [11] is an orthologue of mNAT2; and rat system-A transporter ATA3 [12] is an orthologue of hNAT3 and human ATA3 [13]. Both systems, N and A, mostly convey L-glutamine, L-histidine and L-alanine across cell membranes. Transport of glutamine and alanine is essential for control of nitrogen metabolism in the liver [2,14]. In the liver, glutamine is involved in the detoxification of ammonia and in the production of urea [15]. Alanine directly participates in the nitrogen metabolism between liver and muscle for glycolysis and in the removal of ammonia

from muscle. In addition, we have observed a graded distribution of mNAT expression from the central vein to the portal tract [6].

Previous studies have shown that activities of system-N and -A transport are regulated by growth factors and hormones (including insulin in some tissues), substrate availability, cell swelling, starvation, etc. [16–20]. In addition, activities of system-N and -A transport are up-regulated in the liver during prolonged fasting and corticosteroid treatment, becoming the rate-determining step in intrahepatic metabolic processes [17]. Although previous studies have shown that activities of system-N and -A transport appear to be regulated by physiological factors, the precise regulatory mechanism at the molecular level is unknown due to the lack of information regarding the identity of transporter proteins. The recent progress in molecular cloning permits investigation of the function and regulation of these transporters in close detail.

In the present study, we report the molecular identification and functional characterization of a mouse system-N transporter, mNAT3. mNAT3 was found to be predominantly expressed in mouse liver hepatocytes, with the strongest expression in those cells adjacent to the central vein. More importantly, the expression and function of mNAT3 were stimulated by insulin. This stimulatory effect appears to be mediated through the phosphoinositide 3-kinase (PI 3-kinase) signalling pathway. Its specific expression in the liver, selective transport properties and

Abbreviations used: GST, glutathione S-transferase; MAP, mitogen-activated protein; MeAIB, *N*-(methylamino)isobutyric acid; mNAT, mouse system-N amino acid transporter; ORF, open reading frame; PI 3-kinase, phosphoinositide 3-kinase; RT, reverse transcriptase.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB055004.

specific regulation by insulin suggest that this transporter plays a key role in liver physiology and function.

MATERIALS AND METHODS

Materials

mMESSAGE mMACHINE used for *in vitro* transcription was obtained from Ambion (Austin, TX, U.S.A.). The Marathon cDNA Amplification kit and mRNA Separator kit were purchased from Clontech (Palo Alto, CA, U.S.A.). Tri Reagent was obtained from Molecular Research Center (Cincinnati, OH, U.S.A.). ³H-labelled L-alanine, L-glutamine, L-glutamate, L-histidine and L-lysine were purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). Restriction endonucleases were purchased from New England Biolabs (Revere, MA, U.S.A.). Protease inhibitor cocktail tablets were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Nitrocellulose membrane was purchased from Schleicher and Schuell (Keene, NH, U.S.A.) and nylon transfer membrane Hybond H⁺ was from Oncor (Gaithersburg, MD, U.S.A.). Protein and RNA standard mixture, oligo(dT) primer and Superscript II reverse transcriptase (RT) were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Micro bicinchoninic acid Protein Reagent Assay kit was from Pierce (Rockford, IL, U.S.A.). X-OMAT AR films were from Eastman Kodak (Rochester, NY, U.S.A.). Paraformaldehyde (16% stock solution) was from Electron Microscopy Sciences (Fort Washington, PA, U.S.A.). Tissue-Tek™ OCT compound was from Miles Scientific (Naperville, IL, U.S.A.). Antibodies against phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr²⁰²/Tyr²⁰⁴), p44/42 MAP kinase, phospho-Akt (Thr³⁰⁸) and Akt were from Cell Signaling Technology (Beverly, MA, U.S.A.). All other chemicals were obtained from either Sigma (St. Louis, MO, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.).

Cloning of mNAT3 cDNA

The RT-PCR approach was utilized to identify additional member(s) of the mouse system-N transporter family. RNAs were isolated from various mouse tissues by Tri Reagent. To obtain the cDNA sequence, polyadenylated RNA was isolated through an mRNA separator kit. mRNA was converted into single-stranded cDNA by oligo(dT) and Superscript II RT. Several primer pairs were designed based on the similarity between conserved regions of the system-N amino acid transporters, mNAT (AF159856), SN1 (AF273025), g17 (U49082), mNAT2 (AF184240) and hNAT3 (AF193836). After more than 30 cycles of PCR amplification at the annealing temperature of 50 °C, one of the primer pairs (sense primer 5'-ATGGATCCCATGGAAGTGAAG-3'; antisense primer 5'-CAGTGTCTTCA-TTAGCAAATTG-3') generated a 0.14 kb DNA fragment from mouse liver cDNA. After sequencing [DNA Sequencing Facility, University of Texas Health Science Center at San Antonio (UTHSCSA)], the fragment showed similarity with hNAT3 and its corresponding orthologue. A mouse liver cDNA was prepared using the Marathon cDNA kit according to the manufacturer's instructions. Based on the sequence obtained from this 0.14 kb DNA fragment, two unique primers were designed: 5'-GGAG-TTTTCCATGCCGGTGTAGCT-3' to amplify the additional 5' sequence and 5'-CGCGGGGACAGTATTCAGGACAGC-3' to amplify the additional 3' sequence of this new gene. Subsequently, based on the newly generated additional sequence, six specific primers were used to generate a complete open reading frame (ORF) sequence of mNAT3.

Subcloning and preparation of cRNA for microinjection in *Xenopus laevis* oocytes

The cDNA was synthesized by PCR and subcloned into the *Xenopus* expression vector containing the 5'- and 3'-flanking sequences of the *Xenopus* β globin gene as described previously [21]. The primer pairs used to amplify the entire ORF of mNAT3 were designed with restriction sites *Bam*HI at the 5'-end and *Hind*III at the 3'-end: a sense primer containing a restriction site for *Bam*HI and a start codon 5'-ATGCGGATCCCAAATGG-ACCCCATGGAAGT-3' and an antisense primer including the 3'-end coding sequence and a stop codon: 5'-ATCGAA-GCTTGATTAGTGGTGGTGGGATTCG-3'. PCR products were purified and digested with *Bam*HI and *Hind*III before subcloning into the *Xenopus* expression vector. The constructs were confirmed by sequencing. The plasmids were linearized with *Not*I and cRNA was transcribed *in vitro* by T7 RNA polymerase using mMESSAGE mMACHINE. Capped cRNA was extracted with phenol/chloroform, precipitated with ethanol as described in [21], dissolved in diethyl pyrocarbonate-treated water at a concentration of 1.5–2.0 μ g/ μ l, and stored at –80 °C before use for microinjection into *Xenopus* oocytes.

Antibody preparation

Rabbit anti-mNAT3 IgG antibody was produced using a glutathione S-transferase (GST)-tagged fusion protein as described in [22]. A DNA fragment encoding amino acids 1–59, which is distinguished from mNAT, mNAT2 and their corresponding orthologues, was produced by PCR using an mNAT3 DNA clone as a template (sense primer 5'-GCGGGTATCCATGGA-CCCATGGAAGT-3'; antisense primer 5'-GCGGAATTC-AACCCATTTGTCAGGAAGT-3'). This fragment was inserted into the expression vector, pGEX-2T. The recombinant fusion protein was expressed in *Escherichia coli*, induced by isopropyl β -D-thiogalactoside, and isolated with GST beads. The purified fusion protein was used to raise polyclonal antiserum in a rabbit (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA, U.S.A.). The antiserum generated was affinity-purified by passage through two Sepharose CL-4B columns, GST-conjugated and GST-mNAT3 fusion protein-conjugated respectively.

Immunofluorescence staining and confocal laser microscopy

The immunofluorescence detection of mNAT3 was performed as described in [23]. Oocytes and mouse liver tissues were fixed in 2% (w/v) paraformaldehyde for 2 h, incubated overnight in 30% (w/v) sucrose in PBS at 4 °C, embedded in OCT and then frozen in liquid nitrogen. Frozen sections (10 μ m thickness) were fixed in acetone at –20 °C for 5 min. H2.35 cells were fixed in 2% paraformaldehyde for 30 min. Fixed sections of oocytes, liver and cells were incubated with blocking solution [2% (v/v) normal goat serum, 0.25% Triton X-100, 2% (w/v) fish skin gelatin and 1% BSA in PBS] for 30 min and were then incubated at 4 °C overnight with affinity-purified anti-mNAT3 antibody (1:300 dilution in blocking solution). The primary antibodies were labelled for 2 h at room temperature (22 °C) by FITC-conjugated goat anti-rabbit immunoglobulin (1:500 dilution). The specimens were analysed using a confocal laser scanning microscope (model: Fluoview; Olympus, Tokyo, Japan) with excitation at 488 nm by an argon laser.

Transport assay in *Xenopus* oocytes and H2.35 cells

The transport studies using *Xenopus* oocytes were performed based on our previous work [6,8]. Briefly, *X. laevis* oocytes were injected using 50 nl of synthetic cRNA or diethyl pyrocarbonate–

water as a control and were cultured for 2–3 days at 18 °C. Oocytes, in groups of 5–10/assay, were rinsed briefly in uptake buffer (2 mM KCl/1 mM MgCl₂/1 mM CaCl₂/10 mM Hepes/50 mM Tris) in the presence of 100 mM NaCl (Na⁺ buffer). These oocytes were transferred into a 24-well culture dish containing 2 ml of uptake buffer and were incubated for 2 min at room temperature. Amino acid transport activities were measured by incubating oocytes in 0.5 ml of uptake buffer in the presence of 50 or 500 µM L-amino acids plus 2 µCi of ³H-labelled L-amino acids as tracers for the indicated time periods. Before uptake experiments, preliminary experiments were conducted with the transport measured at various time points. The uptake rate after 30 min incubation is closest to the maximal retention of radiolabelled amino acids inside oocytes compared with any other time points tested. The oocytes were then washed four times in cold uptake buffer and lysed in 200 µl of 2% (w/v) SDS. The radioactivity accumulated by each oocyte was measured with a liquid-scintillation counter (Beckman, Fullerton, CA, U.S.A.) in 10 ml of scintillation solution.

The specificity of mNAT3-mediated amino acid uptake was examined using an amino acid competition assay. The uptake of L-alanine at concentrations of 50 µM and 1 mM was measured in the presence of 5 mM of 20 non-radioactive L-amino acids and *N*-(methylamino)isobutyric acid (MeAIB). The Na⁺ dependence and Li⁺ tolerance of L-alanine transport by mNAT3 were investigated using Na⁺ buffer, choline⁺ buffer or Li⁺ buffer. The effects of extracellular pH on L-alanine uptake mediated by mNAT3 were investigated over the pH range 6.5–8.5 by adjusting the Na⁺ buffer with Tris base or hydrochloric acid as described previously [24,25]. All experiments were repeated at least three times and the data collected are presented as means ± S.E.M.

For analysis of the uptake of L-alanine in H2.35 cells, cells were treated in the presence or absence of 0.5 µg/ml insulin for 24 h, followed by rinsing three times with uptake buffer (150 mM NaCl/3 mM KCl/2 mM CaCl₂/0.8 mM MgCl₂/5 mM glucose/10 mM Hepes; pH 7.4). These cells were incubated in uptake buffer for an additional 10 min, transferred to uptake buffer containing 50 µM ³H-labelled L-alanine for 5 min and then washed three times with cold PBS. The cells were lysed in lysis buffer (2% SDS in PBS), and 200 µl of cell lysates was transferred to the scintillation solution and the radioactivity of the cells was measured.

Northern-blot analysis

Northern blots were performed as described in [22]. Total RNAs were extracted from different tissues of adult mice and H2.35 cells using Tri Reagent according to the manufacturer's instructions. RNA (30 µg) was loaded, separated by agarose-gel electrophoresis with formaldehyde, and transferred to a nylon membrane. The membrane was hybridized at 45 °C for 12 h in a hybridization solution containing 50% (v/v) formamide and ³²P-labelled cDNA probe corresponding to nucleotides 160–330 of mNAT3. The probed membrane was washed in a high-stringency solution of 0.1 × SSC and 0.1% SDS at 63 °C for 1 h.

Membrane protein preparation and immunoblot analysis

Crude membrane extracts were prepared from liver H2.35 cells and neuroblastoma-like N2A cells as described previously [6,26,27]. The lysates from liver H2.35 and N2A cells were centrifuged at 100 000 *g* at 4 °C for 30 min. The membrane pellet was collected and the protein concentration was determined using the Micro

bicinchoninic acid Protein Reagent Assay kit. Protein (20 µg) was loaded, separated by SDS/PAGE (10% gel) and transferred to a nitrocellulose membrane. The membranes were blotted with antibodies raised against mNAT3 (1:300 dilution) and β-actin (1:5000) for samples from liver H2.35 and N2A cells and with antibodies raised against phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴, 1:2000) and phospho-Akt (Thr³⁰⁸, 1:5000) for samples from H2.35 cells. The membranes blotted with antibodies against phospho-MAP kinase and phospho-Akt were extensively washed and reblotted with p44/42 MAP kinase antibody (1:1000) and Akt antibody (1:1000) respectively. The primary antibodies were detected using secondary anti-rabbit antiserum (1:5000) for mNAT3, MAP kinase, Akt and phospho-Akt or anti-mouse antiserum (1:5000) for β-actin and phospho-MAP kinase. These secondary antibodies were conjugated with either peroxidase or alkaline phosphatase and were detected using either chemiluminescence reagent kit (ECL[®]) or alkaline phosphatase colour-metric reaction respectively. The membranes treated with ECL[®] were exposed to X-OMAT AR films and detected by fluorography.

Statistic analysis

Data were analysed using one-way ANOVA and Student–Newman–Keuls multiple comparison test with the Instat Biostatistic program (GraphPAD Software for Science, San Diego, CA, U.S.A.). Data are presented as the means ± S.E.M. for more than three determinations. Asterisks indicate the degree of significant differences when compared with the controls (***P* < 0.005).

RESULTS

Molecular cloning of mNAT3 and sequence analysis

We used PCR-homology cloning to identify member(s) of system-N transporters. cDNAs from various mouse tissues were screened using conserved primer pairs. One fragment of 0.14 kb was obtained from liver tissue. This fragment showed 86 and 92% nucleotide identity with hNAT3 nucleotides 188–225 and 299–324 respectively and 82% nucleotide identity with rat ATA3 nucleotides 188–324. Further cloning using primers derived from this PCR fragment sequence generated a complete ORF of the new gene, which encodes 547 amino acid residues (GenBank[®] Nucleotide Sequence Database accession no. AB055004) with a predicted molecular mass of 60.6 kDa. This new protein shares 55 and 44% amino acid similarity with mNAT (AF159856) [6] and mNAT2 (AF184240) [7] respectively. However, the similarity of this protein with hNAT3 (AF193836) [8,13] and its rat orthologue, ATA3 (AF295535) [12], are much higher, being 87 and 91% respectively. The results of sequence analysis suggest that this protein is the mouse orthologue of hNAT3 and ATA3, and is therefore termed as mNAT3. The nucleotide and amino acid sequences of mNAT3 are shown in Figure 1. The first seven transmembrane domains are highly conserved between mNAT family members, whereas the N-terminal region prior to the first transmembrane domain is highly diverse. Based on hydrophobicity analysis (PSORT version 6, Nakai file server; TMPRED, Swiss, EMBNET; BCM Search Launcher), mNAT3, like other members of the mNAT family, is a plasma membrane protein (certainty = 69.6%) with a high probability of having ten transmembrane domains. As the deduced sequence lacks a signal peptide, the long N- and the short C-terminal tails are predicted to be in the cytoplasm (high probability score).

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1 gctgaattctaggtctgcttccacatcttgaatgttttta
tccccatgaacagagtgttctgagcgtcaacacaaagtgaagagcctcgagctgaaggcgtgtgtattatttgcactgaagactcttt
tctgtggacaggaagggtgtgaggtcaaattggaccccatggaactgaaaagactctttctgtggacaggaagggtgtgaggtcaa
161 atggaccccatggaactgaacaacgtcagcatcgaaccgacggagacagctgcagcgggacagtattcaggacagctacaccggcatg
M D P M E L N V S I E P D G D S C S G D S I Q D S Y T G M
251 gaaaactccgacaaggacgcatgaacagccaatttgcataagatgccgaaagtcagaagttcctgacaaaatgggttttaggggaag
E N S D K D A M N S Q F A N E D A E S Q K F L T N G F L G K
341 aagaagctagccgattacgcggatgagcatcacctggaatgacttcctttggaatgtcctcatttaacctgagcaacgcatcatgggc
K K L A D Y A D E H H P G M T S F G M S S F N L S N A I M G
431 agtgggatcttaggtgtcctatgccatggccaacacccggatcctcttttataatcatgctgcttactgtggcaatactctcgctc
S G I L G L S Y A M A N T G I I L F I I M L L T V A I L S L
521 tactcgttccaccttttgcgaagacagccaaggaaggaggtctctaatctatgaaaaattgggcgagaagcatttggatggcctggg
Y S V H L L L K T A K E G G S L I Y E K L G E K A F G W P G
611 aaaattggagccttctctattacaatgcagaacatggagccatcagactacctcttcatcattagtagcaactgacctgaagta
K I G A F I S I T M Q N I G A M S S Y L F I I K Y E L P E V
701 atcagagcattcatgggacttgaagaaaacactgggaatggtacctcaacggcaactacctcgtcttatttgttccgtgggagatcatc
I R K M Q T V S N I S I S G M L V M Y L L A A L F G Y L S F
791 ctcccgctctctctcttaaaatttaggtacctggctacaccagtggtatttctctcctcgtcatggtgttttctcgtcagtggtgtg
L P L S L L K N L G Y L G Y T S G F S L S C M V F F V S V V
881 atttcaaaaaattccaaattccctgcctctgctgctgtgacacacaacgaaatctgacgttcaacaacacacttccgattcac
I Y K F Q L P C P L P A L D H N N G N L T F N N T L P I H
971 atgatctcgtgcctaatgactcggagagctcgggtgtgaacttcatgatggattacgctcaccacaaccagctggggtggatgagaag
M I S L P N D S E S S G V N F M M D Y A H H N P A G L D E K
1061 caggtcgcagggcctcttcacagcaacggcgtggagtagcaagccagggtgctgagaaatgccaaacaaatactttgtgttcaattcc
Q V A G V A G S N G V E Y E A Q A G A E K C Q P K Y F V F N S F
1151 cggacggcctatgcaatcccaatcctggcttttgccttttgcctgccaccctgaggtccttcccatctacagcgagcttaagatcgatcc
R T A Y A I P I L A F A F V C H P E V L P I Y S E L K D R S
1241 cgcagaagatgcagacgggtgtccaacatttccatctcaggcatgtcgtcatgtaccttcttgcggccctctttggttatctgagcttc
R R K M Q T V S N I S I S G M L V M Y L L A A L F G Y L S F
1331 tacggggacgttgaagacgagctgctgcatgcttacagcaaggtctacacatttgatacggctcttctcatggtgcgctggcagtcctg
Y G D V E D E L L H A Y S K V Y T F D T A L L M V R L A V L
1421 gtggcagtgacactgacgtgcccctcgtgctgttcccgatccgtacttcggtgatcacactgctgtttccaaggaaacccctcagctgg
V A V T L T V P I V L F P I R T S V I T L L F P R K P F S W
1511 ctgaagcatttcgggatcgctgcaatcatcatcgactcaacaacatcctggtcacctcgtgctaccatcaaatatcatcttggattc
L K H F G I A A I I I A L N N I L V I L V P T I K Y I F G F
1601 ataggggcttcttccactatgctgatttttcttccggtgcgttttatctcaagctcgtcaagaaagaaccttaagatcaccc
I G A S A T M L I F I L P A A F Y L K L V K K E F G Y L S F
1691 cagaagattggggctttgttcttcttctgtgactggaattattttcatgatgggaagcatggcgctcattatactcgactggatctacaac
Q K I G A L V F L V T G I I F M M G S M A L I I L D W I Y N
1781 ccgccaatcccaatcaccactaatccccggggagacgcgtctccactggaacagctgaaattgtctgaaggacatttttagtgtcttga
P P N P N H H *
1871 ttgggatgttagtctgaggaattagcaagattccaaagcgtttttctagctctatcatgggatacttgtggaagagaaaattatggggt
1961 ttgttgggaatggttttgttgggaatggtgaaggatgcattaaaaattctgtggcacacatttta

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Figure 1 Nucleotide and deduced amino acid sequences of mNAT3

A portion of a cDNA fragment was sequenced on both strands. The ORF of mNAT3 starting at nucleotide 161 and ending at nucleotide 1804 (underlined) encodes a protein with 547 amino acids. The derived amino acid sequence of mNAT3 with single-letter code is shown on the lower line. * indicates the stop codon.

mNAT3 preferentially transports L-alanine, L-histidine and L-glutamine

To determine the function of mNAT3, we expressed mNAT3 in *Xenopus* oocytes, which is one of the model systems often used to study the properties of transporters [6,28,29]. Using an affinity-purified mNAT3 antibody, expression of mNAT3 was detected by immunofluorescence analysis as early as 24 h after cRNA injection. Three days after injection, mNAT3 protein was mainly localized in the oocyte plasma membrane, whereas positive signals were not detectable with an affinity-purified preimmune antibody (Figure 2A). The anti-mNAT3 antibody did not detect positive signals in oocytes injected with water (results not shown).

The uptake of ^3H -labelled L-alanine, L-glutamine, L-glutamate, L-histidine and L-lysine, representing groups of zwitterionic, anionic and cationic amino acids, was measured using two different concentrations of the substrates in *Xenopus* oocyte system. Compared with control water-injected oocytes, oocytes expressing mNAT3 exhibited uptake of L-alanine most strongly, followed by L-histidine and L-glutamine. A modest increase in L-lysine uptake was observed, whereas there was no significant

increase in L-glutamate uptake (Figure 2B). These results demonstrate that the most favourable substrate for mNAT3 is L-alanine followed by L-histidine and L-glutamine. mNAT3 has a transporting substrate selectivity similar to the other system-N transporters, mNAT, SN1 and mNAT2 [6,7,9], and the transporting pattern is almost identical with its orthologues hNAT3 and ATA3 [8,12].

To determine the substrate specificity of mNAT3, a competition assay in the presence of all 20 L-amino acids and the system-A-specific substrate MeAIB [3] was performed (Figure 2C). As the most optimal substrate for mNAT3, L-alanine was selected as a model for the competition assay. In this assay, the rate of L-alanine uptake was determined in oocytes injected with mNAT3 cRNA in the presence of 5 mM non-radioactive-labelled 20 L-amino acids and MeAIB. The uptake of L-alanine at 50 μM was significantly inhibited, listed in order of inhibitory potency, by the following amino acids: L-alanine > L-histidine > L-serine > L-glycine > L-asparagine > L-methionine. When 1 mM L-alanine was used in the uptake assay in the presence of 5 mM non-radioactive-labelled 20 L-amino acids and MeAIB, the blocking of L-alanine uptake was less significant, although the

Figure 2 mNAT3 expression, amino acid transport and competition analyses in *Xenopus* oocytes

(A) Immunofluorescence of mNAT3. Frozen sections of *X. laevis* oocytes 3 days after injection with mNAT3 cRNA were immunolabelled with an affinity-purified anti-mNAT3 antibody or preimmune antibody and were examined by confocal microscopy at a scanning interval section of 0.5 μm. Scale bar = 30 μm. Abs, antibodies. (B) Amino acid uptake rates in oocytes expressing mNAT3 against control, non-expressing oocytes. L-Alanine, L-glutamine, L-glutamate, L-histidine and L-lysine uptake rates were measured in uptake buffer containing 50 and 500 μM ^3H -labelled L-amino acids and incubated for 30 min ($n = 7$). (C) Competitive inhibition by 20 L-amino acids and MeAIB on the uptake of L-alanine. The inhibition of 50 μM and 1 mM L-alanine uptake in oocytes expressing mNAT3 by 5 mM of 20 L-amino acids as well as MeAIB (Me). The percentage values after subtracting the controls injected with water are presented as 50 μM or 1 mM L-alanine uptake, defined as 100% ($n = 6$). A*, L-alanine uptake in the absence of non-labelled amino acids. All data are presented as means $\pm$ S.E.M.

pattern of the inhibition was sustained. The system A-specific substrate MeAIB was unable to block the transport of L-alanine significantly, suggesting that mNAT3 is a system-N amino acid transporter and is not a system-A transporter.

mNAT3 is an Na^+ - and pH-dependent low-affinity transporter

To characterize the transport properties of mNAT3, we examined the Na^+ dependence and tolerance for Li^+ substitution of mNAT3 (Figure 3A). L-Alanine uptake into oocytes expressing mNAT3 was significantly greater in the presence of Na^+ buffer when compared with the uptake in the absence of Na^+ . Replacement of Na^+ by Li^+ partially recovered the stimulatory effect by Na^+ . Choline ion substitution for Na^+ led to a significant decrease in L-alanine uptake, although there was 20% transport activity remaining. The 20% uptake rate observed in the absence of Na^+ could be due to the residual levels of Na^+ leaking from the injected oocytes or the transporting activity of another system activated by the presence of mNAT3. To confirm further the Na^+ dependence of the transport activities of mNAT3, alanine uptake experiments with various concentrations of Na^+ were performed (Figure 3B). The increase in uptake rate is associated with an increase in Na^+ concentration and reached a maximum at concentrations higher than 100 mM. These results demonstrate

that L-alanine uptake mediated by mNAT3 is Na^+ -dependent and partially tolerates replacement of Na^+ by Li^+ , which is consistent with the properties exhibited by system-N transporters as described previously [6,30].

Extracellular pH over the range between 6.5 and 8.5 affected L-alanine uptake mediated by mNAT3. The results showed that uptake of L-alanine exhibited a pH dependence increasing from low to high pH (Figure 3C). To analyse the transport rate and substrate affinity of mNAT3, the concentration dependence of L-alanine uptake mediated by mNAT3 was investigated (Figure 3D). At a lower concentration, the rate of uptake was an incremental function of concentration, whereas at higher concentrations, uptake was close to saturation. This observation indicates that L-alanine uptake in oocytes expressing mNAT3 behaved like a carrier-mediated transporter. The V_{max} determined is $22 \pm 7.1 \text{ pmol}^{-1} \cdot \text{min}^{-1} \cdot \text{oocyte}^{-1}$ and the K_m value is $1.6 \pm 0.3 \text{ mM}$. Compared with other mNATs analysed in *Xenopus* oocytes [6,7], mNAT3 has a comparable substrate affinity K_m , indicating a low-affinity transporter.

mNAT3 is predominantly expressed in the liver

To elucidate the tissue-specific expression of mNAT3, Northern blots of equivalent amounts of RNA isolated from various

Figure 3 Na⁺, pH and concentration dependence analyses of mNAT3

(A) Effect of Na⁺ on the uptake of L-alanine mediated by mNAT3. Uptake of 50 μ M L-[³H]alanine was measured either in the presence of Na⁺ or in the presence of Li⁺ buffer or choline⁺ buffer to replace Na⁺ buffer ($n = 5$). (B) Na⁺ dependence of L-alanine uptake. Uptake of L-alanine was measured in the presence of 0, 15, 30, 60, 90, 120 and 150 mM Na⁺ ($n = 5$). (C) pH dependence of L-alanine uptake. Uptake of 50 μ M L-[³H]alanine was measured with Na⁺ buffer under the indicated pH values ($n = 5$). (D) Concentration dependence of L-alanine uptake. Net uptake of L-alanine was measured at different concentrations (0–8 mM) of L-alanine in Na⁺ buffer at pH 7.5. $K_m = 1.6 \pm 0.3$ mM and $V_{max} = 22 \pm 7.1$ pmol⁻¹·min⁻¹·oocyte⁻¹ for L-alanine ($n = 6$). All data are presented as means $\pm$ S.E.M.

Figure 4 Northern-blot analyses of tissue-specific expression patterns of mNAT3

Northern blots with equal amounts of total RNAs from different mouse tissues were hybridized with [³²P]DNA probes of mNAT3 and β -actin under highly stringent conditions. Molecular size standards are indicated on the left.

tissues were probed with labelled mNAT3 and β -actin DNA fragments under highly stringent conditions (Figure 4). Two bands of size approx. 4 and 2 kb were detected in the mouse liver. The intactness of the β -actin band shown on the blots (Figure 4, lower panel) and the proper intensity ratio of 28 and 18 S rRNA (results not shown) excluded the possibility that the two bands detected by the mNAT3 probe were caused by the degradation of RNA. Trace levels of mNAT3 expression were detected in muscle, whereas no signals were detected in heart, kidney, brain, lung, small intestine, spleen and thymus.

Expression of mNAT3 in liver hepatocytes surrounding the central vein

The molecular mass predicted by the mNAT3 clone is approx. 60.6 kDa. Western-blot analysis with an affinity-purified mNAT3-specific antibody revealed an immunoreactive protein band at 80 kDa in liver samples (Figure 5A, lane 2, arrow). The specificity of this protein band was further ascertained by the lack of detected immunoreactivity by probing with preimmune serum (Figure 5A, lane 1) or by preincubating the mNAT3 antibody with the mNAT3 antigen (Figure 5A, lane 3). Furthermore, an antibody for a non-related protein, connexin 43, did not compete for the binding of the mNAT3 antigen (results not shown). Expression of mNAT3 was detected in the liver using confocal microscopy. Immunofluorescence detection with antibody specific to mNAT3 demonstrated its localization to hepatocytes (Figure 5B). Localization of mNAT3 appeared to be

Figure 5 Expression and localization of mNAT3 in the liver

(A) Western-blot detection of mNAT3 expression in the mouse liver. Crude membranes isolated from the liver were separated by SDS/PAGE (10% gel) and immunoblotted with 1:300 dilution of affinity-purified preimmune (lane 1), anti-mNAT3 (lane 2) antibodies and anti-mNAT3 pretreated with mNAT3 antigen (lane 3). Molecular-mass markers are indicated on the left. (B) Immunofluorescence detection localized mNAT3 to the plasma membrane of hepatocytes surrounding the central vein (V) (a). The expression diminishes gradually towards the portal tract (T) (b). Scale bar: 20 μ m (a), 200 μ m (b).

Figure 6 Expression of mNAT3 in mouse hepatocyte-like cells

(A) Northern blots with RNAs from H2.35 cells (lane 1) and mouse liver (lane 2) were hybridized with [32 P]DNA probe of mNAT3 and β -actin under highly stringent conditions. Molecular size standards are indicated on the left. (B) Immunoblot of crude membranes isolated from H2.35 (lane 1) and N2A (lane 2) cells was labelled with 1:300 dilution of affinity-purified anti-mNAT3 antibody. Molecular-mass markers are indicated on the left. (C) Immunofluorescence detection of mNAT3. Hepatocyte-like cells were immunolabelled with affinity-purified anti-mNAT3 antibody (1:300 dilution) and the expression of mNAT3 was examined by fluorescence confocal microscopy (b). Phase-contrast image is shown in (a). Arrowheads point to the expression of mNAT3 at the cell borders.

basolateral in the plasma membrane of hepatocytes surrounding the central vein (Figure 5B, panel a). There is a graded decreased distribution of mNAT3 expression from the central vein to the portal tract (Figure 5B, panel b). This specific distribution of mNAT3 in liver is consistent with a function of this protein in amino acid transport [31].

Insulin induced an increase in the expression of mNAT3 and the uptake of L-alanine in hepatocyte-like H2.35 cells

The expression of mNAT3 in the form of mRNA and protein was detected in hepatocyte-like H2.35 cells (Figures 6A and 6B). The 4 kb form of mRNA is more dominant in H2.35 cells (Figure 6A, lane 1) when compared with that from the liver (Figure 6A, lane 2). An 80 kDa migrating protein band was detected with anti-mNAT3 antibody in hepatocyte-like H2.35 cells (Figure 6B, lane 1), whereas no expression of mNAT3 protein was observed in neuroblastoma-like N2A cells (Figure 6B, lane 2). Immunofluorescence of confocal microscopy of the cells shows that mNAT3 was localized at the plasma membrane (Figure 6C, panel b, arrowheads).

Treatment with insulin increased the expression level of mNAT3, and this increase was directly associated with the concentration of insulin applied (Figure 7A). This effect was observed even at 0.5 $\mu\text{g/ml}$ insulin (Figure 7A, lanes 1 and 2). Moreover, the insulin-induced stimulation of mNAT3 expression was dependent on the length of the treatment period. Compared with the expression of the housekeeping protein β -actin, a significant increase in mNAT3 expression was observed after 4 h of insulin treatment, becoming more profound at 8 h and sustaining up to 24 h of treatment (Figure 7B). The increase in the amount of mNAT3 during the treatment period of 8–24 h was less significant. This implies that a longer period of insulin treatment may not stimulate or stimulates less mNAT3 biosynthesis and the existing intracellular protein pool of mNAT3 is stabilized. Insulin also stimulated the uptake of L-alanine in H2.35 cells. Under the various insulin-treatment periods, the uptake of L-alanine was gradually increased (Figure 7C). A significant up-regulation of the transport was observed 4 h after the treatment and the up-regulation continued up to 24 h. An increase in L-alanine uptake was not caused by increased cell numbers, since, compared with control, the total number of cells was not altered under identical treatments of insulin (results not shown). Together, these results suggest that the up-regulation of mNAT3 expression by insulin will probably lead to an increase in L-alanine uptake in hepatocytes.

Regulation of the expression of mNAT3 by insulin–PI 3-kinase signalling

The insulin signalling cascade consists of two major downstream pathways: the MAP kinase pathway and the PI 3-kinase pathway [32]. To determine which pathway is responsible for the stimulatory effect of insulin, we took advantage of two well-characterized specific inhibitors: PD98059 for the MAP kinase pathway and LY294002 for the PI 3-kinase pathway [32]. The expression of Akt, a downstream effector of PI 3-kinase, and MAP kinase in H2.35 cells was ascertained through the immunoblots with their corresponding antibodies (Figure 8A). The activation of MAP kinase and Akt kinase exhibited through the phosphorylation on certain amino acid residues was determined using antibodies raised against phospho-MAP kinase (Thr²⁰²/Tyr²⁰⁴) and phospho-Akt kinase (Thr³⁰⁸) (Figure 8A). Compared with the non-treated control (Figure 8A, lane 1), the activation of both kinases was detected on treatment with insulin (Figure 8A, lanes 4). MAP kinase activation was inhibited with PD98059

Figure 7 Stimulatory effect of insulin on the expression of mNAT3 and the uptake of L-alanine in H2.35 cells

H2.35 cells were cultured in normal growth medium until they were confluent. Cells were incubated in serum-free medium for 24 h [39] before treatment with insulin. (A) Expression of mNAT3 in cells treated with various concentrations of insulin. H2.35 cell cultures were treated in the presence of insulin (lane 2, 0.5 $\mu\text{g/ml}$; lane 3, 1 $\mu\text{g/ml}$; lane 4, 2 $\mu\text{g/ml}$; lane 5, 4 $\mu\text{g/ml}$; lane 6, 8 $\mu\text{g/ml}$; lane 7, 16 $\mu\text{g/ml}$) and in the absence of insulin (lane 1) for 24 h. Immunoblots of crude membranes isolated from treated H2.35 cells were labelled with affinity-purified anti-mNAT3 antibody (1:300). (B) Expression of mNAT3 in cells treated with insulin for various time periods. H2.35 cell cultures were treated in the presence (lanes 1, 3, 5, 7 and 9) and absence (lanes 2, 4, 6, 8 and 10) of 0.5 $\mu\text{g/ml}$ insulin for 0 h (lanes 1 and 2), 4 h (lanes 3 and 4), 8 h (lanes 5 and 6), 16 h (lanes 7 and 8) and 24 h (lanes 9 and 10). The immunoblots of isolated membrane from treated H2.35 cells were labelled with affinity-purified anti-mNAT3 antibody (1:300) or monoclonal anti- β -actin (1:5000) antibody. Molecular-mass markers are indicated on the left. (C) The uptake of L-alanine by insulin treatment. H2.35 cells were pretreated with 0.5 $\mu\text{g/ml}$ insulin for 0, 4, 16 and 24 h, and the uptake analyses were performed for all treated cells under the identical length of the cultured period. The uptake of L-alanine was measured in uptake buffer containing 50 μM ^3H -labelled L-alanine for 5 min ($n = 3$). ** $P < 0.005$.

[Figure 8A, lane 3, blotted with antibodies for MAP kinase (MAPK^{Thr202/Tyr204})]. Treatment with the PI 3-kinase inhibitor LY294002 (lane 2) attenuated Akt activation (Figure 8A, lane 2, blotted with antibodies for Akt^{Thr308}). A similar inhibitory effect was also observed by another Akt inhibitor wortmannin (results not shown). Together, these results suggest that insulin activated MAP kinase and PI 3-kinase signalling in H2.35 cells and the inhibitors PD98059 and LY294002 can efficiently block the activation of each signalling pathway.

The potent inhibitors PD98059 and LY294002 were adopted to dissect which downstream pathway(s) of insulin-activated signalling was involved in the up-regulation of the expression of mNAT3 in H2.35 cells (Figure 8B). Consistent with the

Figure 8 LY294002 inhibited the stimulatory effect of insulin on mNAT3 expression

(A) Analysis of the activation of kinases and the effects of specific inhibitors. H2.35 cells were preincubated in the absence (lanes 1 and 4) and presence of the MAP kinase inhibitor PD98059 (50 μ M) (lane 3) or the PI 3-kinase inhibitor LY294002 (50 μ M) (lane 2) for 1 h, followed by treatment with (lanes 2–4) and without (lane 1) insulin (0.5 μ g/ml) for another 1 h. The treated cells were lysed and immunoblotted with antibodies against phospho-p44/42 MAP kinase (MAPK^{PThr202/Tyr204}) and phospho-Akt kinase (Akt^{PThr308}). The blotted membranes were stripped and reblotted with antibodies against p44/42 MAP kinase (MAPK) and Akt. (B) Expression of mNAT3 in cells treated with insulin and its signalling inhibitors. H2.35 cells were preincubated in the absence (lanes 1 and 2) and presence of LY294002 (50 μ M) (lane 3) or PD98059 (50 μ M) (lane 4) for 1 h, followed by treatment with (lanes 2–4) and without (lane 1) insulin for 24 h. Immunoblotting was performed with a 1:300 dilution of affinity-purified anti-mNAT3 antibody and a 1:5000 dilution of monoclonal anti- β -actin antibody. Molecular-mass markers are indicated on the left.

results shown in Figure 7, insulin induced an increase in mNAT3 expression (Figure 8B, compare lanes 1 and 2). This increased expression was blocked by the PI 3-kinase inhibitor LY294002 (Figure 8B, lane 3), but not by the MAP kinase inhibitor PD98059 (Figure 8B, lane 4). Inhibition of insulin-induced up-regulation of mNAT3 by the PI 3-kinase inhibitor wortmannin was also observed (results not shown). These results suggest that the insulin-stimulated up-regulation of mNAT3 in hepatocytes is likely to be mediated through the PI 3-kinase signalling pathway.

DISCUSSION

In the present study, we have identified and characterized the third member of the system-N amino acid transport family, mNAT3, expressed predominantly in the liver hepatocyte. Sequence and functional analyses show that mouse mNAT3 is related to the system-N transporters, namely mouse mNAT and mNAT2, and their orthologues [6–11,13,33]. The degree of amino acid similarity among mNAT3, mNAT and mNAT2 is 50–55%. The greater degree of divergence between mNAT3 and the other two mNATs is in the N-terminus of the transporter. Based on the predicted topology, mNAT3, like mNAT2, is a type 2 membrane protein with ten predicted transmembrane segments, placing the N- and C-termini in the cytoplasm. Among the system-N family members, mNAT3 and its orthologues are the largest in size, with 547 amino acids and a large second intracellular loop domain.

The substrate selectivity of mNAT3 conforms to that of the previously characterized system-N transport [3]. However, it is not sufficient to classify amino acid transporter systems solely on the basis of substrate selectivity, since other systems, such as

system A, can also mediate Na⁺-dependent L-alanine uptake [34]. An important feature used to distinguish between these two systems is the amino acid and its related substrate-transport competition assay. It has been generally accepted that inhibition by MeAIB and L-histidine can be used to define systems A and N respectively [3]. The activity of L-glutamine transport mediated by mNAT3 can be effectively inhibited by the system-N substrate L-histidine, but not by the system-A substrate MeAIB. Therefore we define mNAT3 as a system-N instead of system-A transporter. In the reported substrate transport assay of ATA3 in *Xenopus* oocytes, uptake of the system-A substrate MeAIB is less than 25-fold when compared with the uptake of L-alanine [12], which further supports our observation that the system-A substrate MeAIB is not preferred by mNAT3 and its orthologues. Moreover, unlike L-alanine, MeAIB fails to induce currents in ATA3-expressing oocytes [12]. The weak uptake of MeAIB is also reported in human ATA3-expressing HRPE cells [13]. These results suggest that the functional features of mNAT3 closely match those of the system-N transporters.

In the substrate competition assay, although the inhibitory patterns of substrate inhibition persist, the inhibitory effect by 1 mM L-alanine is less significant when compared with the inhibition by 50 μ M L-alanine. A possible explanation is that the K_m value of other competing amino acids could be lower than that of L-alanine. Therefore amino acids (5 mM) 5-fold more competing than alanine (1 mM) may not be sufficient to block alanine transport. The inhibition by 5 mM unlabelled L-alanine is also less significant. This could be explained by the lower difference in the uptake rates between 1 and 5 mM L-alanine when compared with the difference in uptake rates between 50 and 5 mM substrate (see Figure 3D). Our substrate transport assay suggests that L-histidine is transported almost like glutamine, but

in the substrate competition assay, L-histidine acts much more effectively as an inhibitor. This phenomenon could be interpreted by the fact that sites on the transporter responsible for substrate translocation and for substrate binding and inhibition may be different. Although histidine displays substrate-transport capability similar to glutamine, it may have a stronger binding affinity when compared with glutamine, thus displaying stronger inhibition capability. Evidence for the discrepancy between substrate translocation and substrate inhibition has been reported previously for L-type amino acid transporter 1 [35], prostaglandin transporter PGT [36] and organic transporter rOCT1 [37], and different amino acid residues responsible for substrate translocation and binding have been identified [36].

Northern-blot analysis revealed the existence of two distinct mRNA species, of 2 and 4 kb. Similar migrating patterns of mRNAs are also observed for rat ATA3, but only the single form of mRNA is reported for human hNAT3 and ATA3 [8,12,13]. These two forms of mRNAs may reflect multiple transcription start sites, alternatively spliced isoforms or the existence of a relatively stable processing intermediate. Compared with liver samples, the 4 kb form of mRNA is more dominant in H2.35 cells, indicating that the ratio of the two mRNAs could be altered under different cell contexts as reported previously for the cationic amino acid transporter cat-1 gene [38]. The 60.6 kDa mass predicted by the DNA sequence (SWISS-PROT) is considerably smaller when compared with 80 kDa observed by SDS/PAGE from liver and H2.35 cell samples. The differences between the expected and observed protein bands could be due to post-translational modifications, such as glycosylation and phosphorylation. However, we failed to detect any conserved N-glycosylation consensus site in the predicted extracellular loop regions. Moreover, deglycosylation or dephosphorylation treatment did not alter the mNAT3 migrating patterns (results not shown), suggesting that it is unlikely that both types of modifications can account for these differences. The discrepancy between predicted and measured molecular mass can be attributed to the structural complexity of membrane proteins and the anomalous migration of the protein in SDS/PAGE. The migration on SDS/PAGE is reported to differ from predicted levels in mNAT and in other proteins with multiple transmembrane segments, such as the gap junction protein Cx45.6 [6,22].

Our results show for the first time that insulin at the molecular level regulates the expression and functions of a member of the mNAT family. We have identified the predominant expression of mNAT3 in liver and a weak expression in the muscle. mNAT3 is specifically localized in the basolateral membranes of hepatocytes and hepatocyte-like cells. Mouse H2.35 cells have been characterized to mimic closely primary hepatocytes with the expression of typical marker proteins and enzymes [39,40], therefore serving as an ideal model for hepatocytes *in vitro*. We found that the treatment with insulin up-regulated L-alanine uptake and mNAT3 expression in H2.35 cells, but had no effect on cell proliferation. Moreover, this process was mediated by a PI 3-kinase-dependent signalling pathway. A couple of previous studies, however, have shown that insulin induces the transport activities of system-A- and system-N-like substrates [16,19] and insulin-induced uptake was facilitated by the activation of PI 3-kinase [19]. Similarly, a recent study reveals that the activation of system-N transport in burn injury is mediated by PI 3-kinase signalling [41]. In contrast with these reports, stimulation of system-A transport by amino acid starvation in cultured fibroblasts requires extracellular-signal-regulated kinase ('ERK') 1/2 activation instead of PI 3-kinase activation [42]. Previous studies have implied that stimulation of system-A-like substrate transport via the MAP kinase pathway is mediated through a

slow mechanism dependent on overall protein synthesis and cell proliferation [43,44]. However, we found that cell number was not up-regulated by long-term insulin treatment, whereas the expression of mNAT3 was increased. Our finding is consistent in part with another report that insulin stimulates system-N activity in muscle cells by a mechanism that is characterized by its sensitivity to inhibition by cycloheximide, indicating the involvement of the biosynthesis of specific proteins [45]. Therefore it appears that the choice of different signalling transduction pathways depends on the type of stimulation and extracellular-signal-regulated kinase activation is not required for insulin-induced transport stimulation.

Based on the specific expression pattern, transport function and regulation by insulin, our results indicate that mNAT3 may be involved in the metabolism and physiological functions in the liver. A previous study [46] showed that, under the resting conditions, gluconeogenesis in isolated periportal hepatocytes is higher than in perivenous hepatocytes when incubated with L-alanine. However, the exercise results in a higher gluconeogenesis activity using the L-alanine substrate in perivenous hepatocytes than in periportal hepatocytes. Therefore the expression and function of mNAT3 in perivenous hepatocytes may be partially involved in the uptake of plasma alanine and glutamine to accommodate the activity of gluconeogenesis. Since insulin is known to inhibit gluconeogenesis in hepatocytes, the stimulation of the expression and function of mNAT3 by insulin is, thus, unlikely to be related to the activities of gluconeogenesis. Previous studies have shown that insulin can increase the overall mRNA translation and protein biosynthesis [47]. It is likely that the insulin-stimulated uptake activity of mNAT3 may assist in providing more free amino acids inside the cells to meet the needs of a corresponding increase in protein synthesis. Further studies will shed light on the function and the regulation of mNAT3 in the liver *in vivo*.

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