Identification of an Insulin-regulated Lysophospholipase with Homology to Neuropathy Target Esterase

Received for publication, November 26, 2007. Published, JBC Papers in Press, December 17, 2007, DOI 10.1074/jbc.M709598200

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Neuropathy target esterase (NTE) is a member of the family of patatin domain-containing proteins and exhibits phospholipase activity in brain and cultured cells. NTE was originally identified as target enzyme for organophosphorus compounds that cause a delayed paralyzing syndrome with degeneration of nerve axons. Here we show that the structurally related murine protein NTE-related esterase (NRE) is a potent lysophospholipase. The enzyme efficiently hydrolyzes sn-1 esters in lysophosphatidylcholine and lysophosphatidic acid. No lipase activity was observed when triacylglycerols, cholesteryl esters, retinyl esters, phosphatidylcholine, or monoacylglycerol were used as substrates. Although NTE is predominately expressed in the nervous system, we found the highest NRE mRNA levels in testes, skeletal muscle, cardiac muscle, and adipose tissue. Induction of NRE mRNA concentrations in these tissues during fasting suggested a nutritional regulation of enzyme expression and, in accordance with this observation, insulin reduced NRE mRNA levels in a dose-dependent manner in 3T3-L1 adipocytes. A green fluorescent protein-NRE fusion protein colocalized to the endoplasmic reticulum and lipid droplets. Thus, NRE is a previously unrecognized ER- and lipid droplet-associated lysophospholipase. Regulation of enzyme expression by the nutritional status and insulin suggests a role of NRE in the catabolism of lipid precursors and/or mediators that affect energy metabolism in mammals.

Patatin domain (Pfam01734)-containing enzymes comprise a large number of proteins from bacteria to men and can be divided by bootstrap techniques into six families (1). The patatin domain is characterized by comprising a canonical \( \alpha/\beta \) hydrolase fold and a GXSGX motif, both common structural features of lipases. The eponym of patatin domain-containing proteins is the potato tuber storage protein patatin (2), a non-specific acyl-hydrolase capable of hydrolyzing phospholipids (PL),\(^2\) monoacylglycerol (MG), and diacylglycerol (DG) substrates. Patatin has a Ser-Asp catalytic dyad instead of the classical catalytic triad observed in most lipid hydrolases. Patatin domain-containing proteins were found in bacteria, fungi, plants, as well as animals from flies to mammals. The family of mammalian patatin domain-containing proteins was officially named “patatin-like phospholipase domain-containing” (PNPLA) and comprises nine members divided into three subgroups (3). The first group PNPLA1–5 includes patatin-like phospholipase containing 1 (PNPLA1), adipose triglyceride lipase (ATGL, also called desnutrin, TTS2.2, PNPLA2, and iPLA2z), adiponutrin (PNPLA3), GS2 (PNPLA4), and GS2-like (PNPLA5). Members of this subfamily were shown to act as triacylglycerol lipases (ATGL, GS-2, and adiponutrin) (4–7) and retinylester lipases (GS-2) (8). Weak phospholipase activity has been demonstrated for ATGL, GS-2, and adiponutrin (5). To date, deletion mutants in mice only have been generated for ATGL (9). ATGL-deficient animals exhibited a major defect in the catabolism of stored TG in adipose and non-adipose tissues resulting in a multisystemic TG accumulation, obesity, cardiac dysfunction, and premature death. The second group of proteins includes two phospholipases, PLA2G6 (PNPLA9), iPLA2y (PNPLA8), and a pseudogene (PNPLA10) (3). The third subgroup of the patatin domain superfamily is comprised of neuropathy target esterase (NTE, PNPLA6) and NTE-related esterase (NRE, NTE-like, PNPLA7) (3, 10).

The NTE family has a distinct evolutionary origin from that of the other mammalian patatin-like lipase subfamilies that is consistent with the different domain architecture of NTE family members compared with other PNPLAs and its closer relation to bacterial patatin-like proteins (1, 11). NTE possesses phospholipase activity for lysophosphatidylcholine (LPC) and, possibly, phosphatidylcholine (PC) (12–14). The enzyme was originally identified as the target of organophosphates (OPs) inducing delayed neuropathy (OPIDN) in vertebrates (15, 16). OPIDN is characterized by the degeneration of long axons in

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\(^2\) The abbreviations used are: PL, phospholipid; ATX, autotaxin; BAT, brown adipose tissue; CE, cholesteryl ester; CM, cardiac muscle; ER, endoplasmic reticulum; FFA, free fatty acid; GFP, green fluorescent protein; LD, lipid droplets; LPA, lysophosphatic acid; LPC, lysophosphatidylcholine; LysoPL, lysophospholipid; MG, monoacylglycerol; NRE, neuropathy target esterase-related esterase; NTE, neuropathy target esterase; OP, organophosphate; PC, phosphatidylcholine; PNPLA, patatin-like phospholipase domain-containing; RE, retinyl ester; SM, skeletal muscle; TG, triacylglycerol; WAT, white adipose tissue; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GPCR, G-protein-coupled receptor.
the spinal cord and peripheral nerves (16). Symptoms include lack of muscular coordination, weakness, and paralysis of the lower limbs. Sensitive species include human, pig, sheep, cat, dog, and chicken whereas mice and rats appear refractory to OPIDN (16). Deletion of the nte gene in mice leads to death of the embryo by mid-gestation (17, 18) a phenotype that was attributed to failed placental development and impaired vacuologenesis (18). Tissue-specific deletion of NTE in murine brain provoked severe neuropathologic symptoms in the hippocampus, thalamus, and cerebellum and led to disruption of the provoked severe neuropathologic symptoms in the hippocampus, thalamus, and cerebellum and led to disruption of the neurogenesis (18). Tissue-specific deletion of NTE in murine brain was shown to contribute to the increased production and accumulation of GPC in mammalian renal cells.

In contrast to NTE, neither the enzymatic properties nor the physiologic role of NRE has been studied so far. Here, we show that murine NRE is a potent lysophospholipase that is expressed predominantly in adipose tissue, muscle, and testis. Regulation of NRE by the nutritional status and by insulin suggests a role of this enzyme in energy metabolism.

EXPERIMENTAL PROCEDURES

Materials—Mipafox was purchased from PolyCarbon Industries (Devens, MA), and paraoxon from Chem Service (West Chester, PA). Diisopropyl fluorophosphate (DFP), p-nitrophenyl valerate, lysophosphatidylcholine (LPC; 1-palmitoyl-sn-glycero-3-phosphocholine), phosphatidylcholine (PC; 1,2-diacyl-sn-glycero-3-phosphocholine), 1(rac)-oleoyl glycerol, triolein, all-trans-retinol, 4-dimethylamino-pyridine, and dicyclocarbodiimide were from Sigma-Aldrich (Taufkirchen, Germany). [Dioleoyl-1-14C]phosphatidylcholine was purchased from American Radiolabeled Chemicals (St. Louis, MO). Glycerol tri[9,10(n)-3H]oleate was from Amersham Biosciences (GE Healthcare, Piscataway, NJ). Lysophosphatic acid (LPA; 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Roche Applied Sciences GmbH (Vienna, Austria). ER-Tracker Red™ dye and Bodipy® 558/568 C12 were purchased from Invitrogen Corp. (Carlsbad, CA).

Preparation of retinyl and cholesteryl esters was performed according to Boechzelt et al. (23) using [9,10(n)-3H]palmitic acid (2 mCi, 0.033 μmol (Amersham Biosciences)) and [9,10-3H]oleic acid (Amersham Biosciences) as radiolabel, respectively. All reagents used were of “per analysis” grade.

Animals—Adult male C57BL/6 mice between 12 and 16 weeks of age were maintained on a regular light-dark cycle (14 h light, 10 h dark) and fed a standard laboratory chow diet (4.5% wt/wt fat). Tissue samples were collected from fed (ad libitum access to food and water overnight) or fasted (food was removed for 16 h) animals between 9 and 10 AM.

cDNA Cloning of Recombinant-tagged Proteins—Total RNA was isolated from mouse tissue using the TRizol® Reagent procedure according to the manufacturer’s instructions (Invitrogen). Poly(A)+ RNA was isolated from liver and brain total RNA using the Oligotex® mRNA Mini Kit (Qiagen GmbH, Hilden, Germany). The mRNA was transcribed into first-strand cDNA using SuperScript™ Reverse Transcriptase protocol from Invitrogen. Second-strand cDNA was obtained on a first-strand template by adding Escherichia coli DNA ligase buffer, E. coli DNA ligase, E. coli DNA polymerase (New England Biolabs Inc., Beverly, MA), and dNTPs (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to the mixture (total 100 μl) and incubation at 16 °C for 3 h. Subsequently, T4 DNA polymerase (New England Biolabs) was added, and the mixture was further incubated for 20 min at 16 °C to obtain blunt-end cDNA.

Full-length coding sequences of NRE and NTE were amplified by polymerase chain reaction (PCR) using liver or brain cDNA as template, respectively. Primers were designed according to the sequence of the murine NRE gene (GenBank™ accession: NM_146251) and the murine NTE gene (GenBank™ accession: NM_015801), respectively. The following primers were used: NRE forward primer, 5′-CGAATTCCGAGCAGTCCCAGTCC-3′; NRE reverse primer, 5′-GCTCGAGTCAG-GAGGATGTTCCAGTCTT-3′; NTE forward primer, 5′-GAGCAGTCAG-GGAAGGATGTTCCAGTCTT-3′; NTE reverse primer, 5′-GATCTAGACTAGCDGGCACTGTCAGTGCT-3′. PCR reactions were performed in a total volume of 30 μl and contained 10 ng of cDNA, 10 pmol of primers, 10 nmol of dNTPs, 1 unit of Phusion™ High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland), and 6 μl of Phusion™ HF buffer. The PCR cycling profile included initial incubation at 98 °C for 2 min and 25 cycles of denaturation (98 °C, 30 s), primer annealing (60 °C, 40 s), and elongation (72 °C, 4 min). The amplified cDNAs of NRE and NTE were purified by agarose gel electrophoresis and subsequent elution by centrifugation using Ultrafree-MC 0.45 μm centrifugal filter devices (Millipore, Billerica, MA). The purified cDNAs were ligated into pcDNA4/HisMax vectors (Invitrogen) between EcoRI/Xhol and NotI/Xbal sites, respectively. A control pcDNA4/HisMax vector expressing β-galactosidase (LacZ) was provided by the manufacturer (Invitrogen). For generating an NRE-GFP fusion construct full-length NRE coding sequence was amplified using the pcDNA4/Hismax-NRE as template and the primers: forward, 5′-ATCTCGAGCTGAGCAGCCAGTCTGCCAGTCCCAGTCC-3′; reverse, 5′-TCTAGACTGAGCAGCCAGTCTGCCAGTCCCAGTCC-3′. The PCR mixture contained 10 ng of pcDNA4/Hismax-NRE, 10 pmol of primers, 10 nmol of dNTPs, 1 unit of Phusion™ High-Fidelity DNA Polymerase, and 6 μl of Phusion™ HF buffer in a total volume of 30 μl. The cycling profile, with the exception of primer annealing (63 °C, 30 s), and cDNA purification were the same as mentioned above. The purified NRE cDNA was fused to a GFP gene by cloning it into the Xhol/EcoRI sites of the pEGFP-C1 vector (Takara Bio Inc., Otsu, Japan). The resulting fusion construct encoded GFP at the N terminus of NRE. P. Glynn (24) (MRC
Toxicology Unit, University of Leicester, LE1 9HN, UK) provided a full-length human NTE (hNTE) carrying a GFP tag at the C terminus.

Differentiation of 3T3-L1 Cells and Insulin Treatment—
3T3-L1 cells (ecacc, Salisbury, UK) were seeded in 100-mm dishes (Greiner Bio-one, Frickenhausen, Germany) and grown to 2 days postconfluence in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal calf serum (FCS, Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified air (89–91% saturation) and 5% CO2. Differentiation was induced by changing the medium to DMEM containing 10% FCS, antibiotics, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 0.4 μg/ml dexamethasone (Sigma), and 10 μg/ml bovine insulin (Sigma). After 48 h the medium was replaced by DMEM containing 10% FCS, antibiotics, and 10 μg/ml insulin (day 0 of differentiation). After another 48 h of incubation and every 2 days thereafter, the medium was changed to DMEM containing 10% FCS, antibiotics, and 0.5 μg/ml insulin. For studying insulin regulation of mRNA expression, fully differentiated adipocytes (10 days after induction) were incubated for 14 h in serum-free DMEM containing 2% defatted BSA and insulin at the doses indicated.

Cultivation and Transient Transfection of OP9 Cells—
OP9 cells were a generous gift from Dr. Toru Nakano (25). These bone marrow-derived mouse stromal cells can be differentiated into adipocytes accumulating TG and expressing adipocyte late marker proteins (26). Cells were cultivated in Minimum Essential Medium (MEM) (Invitrogen) with 20% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified air (89–91% saturation) and 5% CO2. For transfection, OP9 cells were seeded at a density of 150,000 cells per well on 24 × 24 mm coverslips positioned at the bottom of 6-well plates and cultivated overnight. OP9 cells were transiently transfected using 4 μg of DNA and 12 μl of Optifect reagent (Invitrogen) according to the manufacturer’s instructions. After 4–5 h, the transfection medium was replaced by MEMα with 20% FCS, 2 mM l-glutamine, antibiotics, 500 μM sodium olate, 2.7% defatted BSA, and 1 μg/ml bovine insulin to induce differentiation. For fluorescence microscopy analyses, OP9 cells were cultivated in differentiation medium for 60 h.

Cultivation and Transient Transfection of COS7 Cells—
COS7 cells (ecacc) were maintained in DMEM containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified air (89–91% saturation) and 5% CO2. The day before transfection COS7 cells were seeded in 6 well plates at a density of 150,000 cells per well and cultivated overnight. For fluorescence microscopy analyses cells were seeded on 24 × 24 mm coverslips positioned at the bottom of the wells. Cells were transfected with 1 μg of DNA and 4 μl of Metafectene (Biontex GmbH, Munich, Germany), according to the manufacturer’s instructions. After 4 h, the transfection medium was replaced by DMEM containing 10% FCS and antibiotics. 24 h after transfection, cells were collected for preparation of cell extracts. For simultaneous localization of NRE and lipid droplets by fluorescence microscopy COS7 cells were cultivated for additional 16 h with DMEM containing 10% FCS, antibiotics, 500 μM sodium olate, and 2% defatted BSA.

Preparation of Cell Extracts—Two days after transfection COS7 cells were collected by trypsinization and washed twice with phosphate-buffered saline (0.01 M phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.3). Cells were disrupted on ice in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml pepstatin, pH 7.0) by sonication (Vironson 475, Virtis, Gardiner, NJ). Nuclei and cell debris were removed by centrifugation at 1,000 × g at 4 °C for 5 min. The supernatant was centrifuged at 100,000 × g at 4 °C for 1 h to obtain membrane (pellet) and cytosolic (supernatant) fractions. The membrane fractions were resuspended in lysis buffer.

For anion exchange chromatography cell extracts were prepared in lysis buffer containing high detergent concentration (20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1 mM EDTA, 20 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml pepstatin). Cells were disrupted by repeated sonication alternated with incubation on ice for 10 min to enhance solubilization. Nuclei and cell debris were removed by centrifugation at 1,000 × g at 4 °C for 5 min, mitochondria by centrifugation at 10,000 × g at 4 °C for 20 min. The supernatant was centrifuged at 60,000 × g at 4 °C for 1 h to obtain the microsomal fraction (pellet) and the cytosolic fraction (supernatant). The cytosolic fraction was used for anion exchange chromatography.

Western Blot Analysis—Cell extracts or fractions (50 μl) obtained by anion exchange chromatography were boiled with SDS-PAGE sample buffer (0.2 M Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 8% SDS, 40% glycerol, bromphenol blue), and proteins were separated by 7.5% or 10% SDS-PAGE using the Laemmli discontinuous buffer system (27). Subsequently, proteins were electro-transferred onto a polyvinylidene fluoride transfer membrane (Pall Life Sciences, Pensacola, FL). Nonspecific protein binding sites were blocked by incubation of the membrane with 5% blocking grade milk powder (Carl Roth) in Tris/NaCl/Tween 20. Specific His-tagged proteins were detected with a mouse anti-His monoclonal antibody (Hisα, Amersham Biosciences) at a dilution of 1:5,000 in blocking solution. Following incubation, the blots were washed three times in Tris/NaCl/Tween 20 for 10 min, incubated with horse-radish peroxidase-conjugated sheep anti-mouse antibody (Amersham Biosciences) at a dilution of 1:10,000, and developed by enhanced chemiluminescence detection (ECL plus, Amersham Biosciences).

Anion Exchange Chromatography—The cytosolic fractions of cell extracts containing His-tagged NRE or the negative control LacZ were loaded onto a Q-Sepharose column (HiTrap, 1 ml, Amersham Biosciences) equilibrated with 5 ml of start buffer (20 mM Tris-HCl, pH 8.0, 0.01% Triton X-100). The matrix was washed with the same buffer to remove unbound protein. Proteins were eluted with an increasing linear gradient (15 ml) of 0 to 100% NaCl. Before starting the gradient fractions of 1 ml were collected, during the gradient fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min using the Äkta prime protein purification system (Amersham Biosciences). The fractions were analyzed by Western blotting and examined for lyso-phosphatidylcholine hydrolase activity.

Assays for Lipid Hydrolase Activities—For determination of triacylglycerol, cholesteryl ester, and retinyl ester hydrolase activity.
activities in cytosolic extracts of transfected COS7 cells, 0.1 ml of 1,000 \times g supernatant (100 \mu g of protein) were mixed with 0.1 ml of radiolabeled substrate and incubated in a water bath at 37 °C for 60 min. For the determination of phosphatidylincholine hydrolase activities 0.1 ml of the membrane fraction (100,000 \times g pellet, 100 \mu g of protein) of COS7 cells were used. The reaction was terminated by the addition of 3.25 ml of methanol/ chloroform/heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5 and samples were vigorously mixed for extraction. After centrifugation (800 \times g, 20 min) radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting. Lipid substrates were prepared in 50 mM potassium phosphate buffer, pH 7.0 and 2.5% defatted BSA and contained 33 nmol of glycerol tril[9,10(n)-3H]oleate 40,000 cpm/nmol, 20 nmol/assay of cholesterol ester (cholesterol [9,10-3H]oleate, 50,000 cpm/nmol), 20 nmol/assay retinyl palmitate (9,10(n)-3H]palmitate, 50,000 cpm/nmol) or 20 nmol/assay phosphatidylcholine (dioleoyl-1-14C]phosphatidylcholine, 35,000 cpm/nmol). All lipid substrates were prepared by sonication on ice (Virstonc 475, Virtis).

MG hydrolase activities in lysates of transfected COS7 cells were determined by incubating 10 \mu g of total membrane fraction (100,000 \times g pellet) with 1 mM 1(rac)-oleoyl glycerol complexed with equimolar defatted BSA in 50 mM potassium phosphate buffer, pH 7.4 in a total volume of 0.1 ml. The mixture was incubated in a water bath at 37 °C for 20 min. The reaction was stopped by the addition of 0.1 ml of CHCl3 and samples were vigorously mixed for extraction. After centrifugation at 20,000 \times g for 1 min the glycerol concentration in the upper aqueous phase was determined using the Free Glycerol Reagent (Bio-Rad 1-14C]phosphatidylcholine, 35,000 cpm/nmol). All lipid substrates were prepared by sonication on ice (Virstonc 475, Virtis).

MG hydrolase activities in lysates of transfected COS7 cells were determined by incubating 10 \mu g of total membrane fraction (100,000 \times g pellet) with 1 mM 1(rac)-oleoyl glycerol complexed with equimolar defatted BSA in 50 mM potassium phosphate buffer, pH 7.4 in a total volume of 0.1 ml. The mixture was incubated in a water bath at 37 °C for 20 min. The reaction was stopped by the addition of 0.1 ml of CHCl3 and samples were vigorously mixed for extraction. After centrifugation at 20,000 \times g for 1 min the glycerol concentration in the upper aqueous phase was determined using the Free Glycerol Reagent and the Glycerol Standard Solution from Sigma according to the manufacturer’s instructions.

The hydrolysis of lysophospholipids was determined according to Zacccheo et al. (12) with slight modifications. Microsomal proteins (50 – 80 \mu g; 100,000 \times g pellet) were incubated with 1.5 mM lysophosphatidylcholine (1-palmitoyl-sn-glycero-3-phosphocholine) or lysophosphatidic acid (1-palmitoyl-2-hydroxy-sn-glycero-3-phospho acid) in 0.1 ml of 50 mM sodium phosphate, pH 7.8, containing 0.5 mM EDTA, 300 mM NaCl, and 2.4 mM CHAPS for 20 – 60 min at 37 °C in a water bath. Reactions were stopped by chilling the assay mixture on ice. Fatty acids liberated were determined using a commercially available assay kit (WAKO Chemicals Gmbh, Neuss, Germany).

For analysis of anion exchange chromatography fractions (20 to 37), 0.05 ml of each fraction were incubated with 3 mM lysophosphatidylcholine in 0.1 ml of 20 mM Tris–HCl, pH 8.0 for 60 min at 37 °C and chilled on ice to stop the reaction. Liberated fatty acids were determined as described above.

**p-Nitrophenyl Valerate Esterase Assay—**p-Nitrophenyl valerate hydrolysis was determined in a microtiter plate using 5 \mu g of microsomal protein (100,000 \times g pellet) in 100 \mu l of Tris–HCl (50 mM, pH 8.0, 0.2 mM EDTA) containing either no inhibitor (control) or various concentrations of the organophosphorus inhibitors diisopropyl fluorophosphate, mipafox, or paraaxon. Preincubations with these inhibitors were performed at 37 °C for 30 min. Subsequently, 100 \mu l of 3 mM p-nitrophenyl valerate in 50 mM Tris–HCl pH 8.0, containing 0.2 mM EDTA and 0.03% Triton X-100, were added. The absorbance of released p-nitrophenol was measured at 405 nm at room temperature over a time period of 15–30 min. The molar extinction coefficient of p-nitrophenol (e405 nm) used for the calculation of enzymatic activity was 18,300 M\(^{-1}\) cm\(^{-1}\).

**Northern Blot Analysis—**Total RNA was extracted from murine tissues or 3T3-L1 cells using TRIzol® Reagent (Invitrogen) according to the manufacturer’s instructions. RNA was separated on a denaturing agarose gel and blotted onto a nylon membrane (Hybond-N+, Amersham Biosciences) according to the protocol of Amersham Biosciences. Transfer of RNA onto the nylon membrane was performed by vacuum blotting (Bio-Rad 785) at 5 mm Hg negative pressure and 10 × SSC (3 mM NaCl, 0.3 mM sodium citrate, pH 7.0) as blotting buffer. After blotting overnight, RNA was covalently fixed to the nylon membrane by UV-cross-linking (120 ml/cm\(^2\))

DNA probes were labeled by random priming using the Prime-a-Gene Labeling Kit (Promega, Madison, WI). 12.5 ng of DNA in a volume of 10 \mu l were denatured at 99 °C for 2 min and cooled on ice for 1 min. After brief centrifugation (2000 × g, 30 s) 5 \mu l of 5 × labeling buffer, 1 \mu l of 10 mM dNTPs (dATP, dTTP, dGTP), 1 \mu l of BSA (10 mg/ml), 2.5 units of Klenow polymerase (New England Biolabs), and 25 \muCi of [\(\alpha-32P\)]dTCTP (Amersham Biosciences) were added. The incubation was performed at room temperature for at least 2 h. Labeled probes were purified and separated from unincorporated nucleotides by gel filtration using Sephadex G-50 DNA Grade F (Amersham Biosciences). Prior to hybridization the probe was denatured at 99 °C for 5 min, chilled on ice, and briefly centrifuged.

Membranes were prehybridized by incubation for 4 to 6 h at 65 °C in hybridization solution (~100 \mu l/cm\(^2\), 250 mM sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA). A denatured radiolabeled probe was added to the solution and the incubation was performed at 65 °C overnight. The membrane was washed according to the following protocol: 2 × SSC, 0.1% SDS shortly at room temperature; 1 × SSC, 0.1% SDS for 20 min at 65 °C; 0.5 × SSC, 0.1% SDS for 20 min at 65 °C. Radioactivity was visualized by exposing the membrane to a \(32P\)-imaging screen (Amersham Biosciences) and scanning with a Phosphorimager (Storm 860, Amersham Biosciences).

**Determination of Protein Concentrations—**The protein concentration of cell lysates was determined with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, CA) according to the manufacturer’s instructions using BSA as standard.

**Live Cell Imaging—**COS7 and OP9 cells cultivated on 24 × 24-mm coverslips were transiently transfected with GFP-tagged NRE (see above). Endoplasmic reticulum (ER) was stained with 4 \muM ER Tracker Red\(^TM\) for 15 min at 37 °C. Lipid droplets were stained with 2 \muM Bodipy\(^R\) 558/568 C\(_{12}\) (dodecanic acid) for 30 min at 37 °C. After staining, the cells were washed twice with phosphate-buffered saline.

For microscopy, coverslips and attached cells were mounted on standard microscope slides. Microscopy was performed using a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) with spectral detection and a Leica 63× water immersion objective (HCX PL APO W Corr CS, 1.2 NA). GFP fluorescence was excited at 488 nm and detected in the
Insulin-regulated Lyso phospholipase

A

| Protein | Domain Summary |
|---------|----------------|
| NRE     | CAP_ED CAP_ED CAP_ED Patat.In |
| NTE     | CAP_ED CAP_ED CAP_ED Patat.In |
| hNTE    | CAP_ED CAP_ED CAP_ED Patat.In |

60% 61% 96%

B

| Protein | Sequence Characteristics |
|---------|--------------------------|
| NTE     | LVLGLGSSGAGCSCSIVGLVXAEAGVPVLGSITLSGFTGLYAEERASPSRTHQRKRAE |
| hNTE    | LVLGLGSSGAGCSCSIVGLVXAEAGVPVLGSITLSGFTGLYAEERASPSRTHQRKRAE |
| NRE     | KSMTSVLEFVLDLTVPTVFTMSFTGAFNRSIHVRFDQKQIDLWLFYFVDITIDATSAMR |

Homologous—Based on alignment analysis, murine NRE and NTE, and human hNTE shows the highly homologous C-terminal patatin domain as well as three CAP_ED domains upstream from the patatin domain. CAP_ED: effector domain of the CAP family of transcription factors, potential cyclic nucleotide monophosphate binding site. Identities between the proteins based on the amino acid level are indicated in percent next to the arrows. A, amino acid sequences within the patatin domains of murine NRE and NTE, as well as human hNTE were compared using ClustalW. Alignment scores: 75 between murine NRE and NTE, 76 between murine NRE and human hNTE, 99 between murine NTE and human hNTE.

**FIGURE 1.** Domain comparison of murine NRE (GenBank™ accession: NP_666363), murine NTE (GenBank™ accession: NP_056616), and human NTE (GenBank™ accession: NP_006693). A, NCBI conserved domain summary of murine NRE and NTE, and human hNTE shows the highly homologous C-terminal patatin domain as well as three CAP_ED domains upstream from the patatin domain. CAP_ED: effector domain of the CAP family of transcription factors, potential cyclic nucleotide monophosphate binding site. Identities between the proteins based on the amino acid level are indicated in percent next to the arrows. B, amino acid sequences within the patatin domains of murine NRE and NTE, as well as human hNTE were compared using ClustalW. Alignment scores: 75 between murine NRE and NTE, 76 between murine NRE and human hNTE, 99 between murine NTE and human hNTE. Red, small and hydrophobic (including aromatic-Y); blue, acidic; magenta, basic; green, hydroxyl and amine and basic-Q. Consensus symbols: asterisk, amino acids are identical in all sequences; colon, conserved substitutions; dot, semi-conserved substitutions.

**RESULTS**

The Domain Architectures of NRE and NTE Are Highly Homologous—Based on alignment analysis, murine NRE shares 60% amino acid sequence identity with murine NTE and 61% identity with human hNTE, whereas the amino acid sequences between murine NTE and human hNTE are 95% identical (Fig. 1A). The proteins share highest homology within the patatin domain located at the C-terminal region (75% identity between murine NRE and NTE, Fig. 1B). The patatin domain of both murine NRE and NTE comprises a “predicted esterase of the alpha/beta hydrolase fold” domain (COG1752), as well as a His-tagged proteins, NRE is a more potent lysophospholipase than NTE. Both NRE and NTE did not exhibit measurable phospholipase activity against PC (Fig. 2B). In contrast to its potent lysophospholipase activity, NRE was unable to hydrolyze substrates containing TG, CE, RE, or MG (Fig. 2, C and D).

To further confirm that NRE has lysophospholipase activity, the His-tagged protein was partially purified by ion exchange chromatography (Fig. 3, A and B) and hydrolysis of lysophosphatidylcholine was determined in the collected fractions (Fig. 3C). By using lysis buffer containing 1% Triton X-100 as detergent, NRE was solubilized from the membrane fraction of COS7 cells, subjected to anion exchange chromatography, and eluted by increasing NaCl concentrations in the elution buffer (Fig. 3A). The presence of NRE protein and the enzymatic activity in chromatography fractions was examined by Western blotting analyses and hydrolase activity assays, respectively (Fig. 3, B and C). Lysophospholipase activity co-eluted perfectly with NRE protein whereas no NRE protein or lysophospholipase activity was detected in the chromatography fractions examined by Western blotting analyses and hydrolase activity assays, respectively (Fig. 3, B and C). Lysophospholipase activity was detected in the chromatography fractions after separation of lysates expressing LacZ (not shown).

The presence of putative cyclic nucleotide binding sites at the N terminus of NRE (Fig. 1A) suggests a possible regulation by GXSXG site with a putative active serine typical for numerous lipases and esterases. Upstream of the patatin domain, NRE and NTE contain three CAP_ED domains (cd00038). The CAP_ED domain is responsible for the binding of cAMP or cGMP in proteins of the catabolite gene activator protein (CAP) family of transcription factors. In addition, this domain shows homology to the regulatory subunit of protein kinase A.

NRE Exhibits Lipolytic Activity against LPC and LPA—NTE was reported to hydrolyze LPC in vitro (14) and in vivo (13). Thus, we examined the potential hydrolytic activity of NRE upon lysophospholipids (LysPLs) and compared it to that of NTE. Lipolytic activities were determined by incubating lysates of COS7 cells overexpressing NRE, NTE, or LacZ with radioactive substrates containing LPC or LPA. Fig. 2A shows that NRE efficiently hydrolyzed both LysPL substrates resulting in a 4.8-fold (LPC) and 3.5-fold (LPA) increase in the release of fatty acids compared with the LacZ control. NTE overexpression caused a 2.2-fold (LPC) and 1.7-fold (LPA) increase in released fatty acids compared with LacZ. Thus, when normalized to the expression level of the range between 500 and 535 nm. ER-Tracker Red™ fluorescence was excited at 543 nm and detected in the range from 600 to 650 nm. Bodipy® 558/568 C12 fluorescence was excited at 543 nm and detected in the range between 550 and 650 nm. Fluorescence emission of GFP and ER Tracker Red™ as well as of GFP and Bodipy® 558/568 C12 were detected simultaneously.

**Statistical Analysis**—All data are expressed as means ± S.D. Statistical significance was determined by Student’s unpaired t-test (two-tailed). Group differences were considered statistically significant for p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)
cAMP, a second messenger that is involved in lipid and energy metabolism. Thus, we investigated whether addition of cAMP to the assay mixture affects NRE lysophospholipase activity upon LPC. We did not detect an influence of cAMP on NRE lysophospholipase activity (not shown).

**NRE and NTE Exhibit Different Sensitivities to Organophosphorus Inhibitors**—Phenyl valerate has been widely used as artificial substrate to determine NTE esterase activity. In this assay, NTE activity was defined as phenyl valerate esterase activity that is sensitive to the neuropathic compound mipafox but insensitive to the non-neuropathic compound paraoxon (28). We used the related compound p-nitrophenyl valerate to determine the esterase activity of NRE and its sensitivity to organophosphates. Lysates of COS7 cells overexpressing NRE were able to hydrolyze p-nitrophenyl valerate. Based on the expression levels of the His-tagged proteins, the esterase activity of NTE was 3.3-fold higher than that of NRE (Fig. 4A). Both proteins attained maximum activity at ~1 mM of substrate concentration. The examination of p-nitrophenyl valerate esterase activities at different pH revealed a similar pH dependence for NTE and NRE with a slightly alkaline pH optimum (Fig. 4B).

In good agreement with previous studies (29), the phenyl valerate esterase activity of human hNTE is inhibited by mipafox and DFP at inhibitor concentrations below 50 μM, but is not influenced by the non-neuropathic OP paraoxon. Human hNTE exhibited IC\(_{50}\) values for mipafox and DFP at concentrations of 33.5 and at 0.6 μM, respectively. Murine NTE was also inhibited by DFP at an IC\(_{50}\) of 21.6 μM but in contrast to human hNTE we did not observe an inhibition of murine NTE esterase activity by mipafox (IC\(_{50}\) > 10³ μM). This finding might explain the relatively low sensitivity of rodents to neuropathic OPs compared with other species (16). Murine NRE inhibition was observed at an IC\(_{50}\) of 3.1 μM for DFP and, similarly to murine NTE, the enzyme was rather insensitive to mipafox (IC\(_{50}\) ~ 290 μM). Paraoxon had no effect on human hNTE, murine NTE, or murine NRE esterase activities.

**NRE Is Regulated by the Nutritional Status and Insulin**—The tissue-specific expression pattern of NRE was compared by Northern blotting analysis in fed and fasted states (Fig. 5A). NRE mRNA (~4 kb) was predominantly found in tissues involved in energy metabolism including skeletal muscle (SM), cardiac muscle (CM), as well as brown (BAT) and white adipose tissue (WAT). NRE mRNA levels in brain, liver, spleen, and lung were comparatively low. Interestingly, a larger transcript of NRE was found in testis (~5 kb).

mRNA expression increased substantially during fasting in adipose tissue, muscle, and testis, indicating that NRE is nutritionally regulated (Fig. 5A). To examine whether the regulation of NRE by feeding/fasting is mediated by insulin, NRE mRNA levels were determined in 3T3-L1 adipocytes (Fig. 5B). Already undifferentiated 3T3-L1 preadipocytes exhibited detectable NRE mRNA levels. Upon differentiation into adipocytes, NRE mRNA concentrations increased and reached a maximum at 6 days after differentiation. In comparison, the highly adipose specific mRNA for adiponutrin was undetectable in preadipocytes and highly expressed in cells that were differentiated for at least 4 days. When differentiated 3T3-L1 cells were cultivated in media supplemented with increasing concentrations of insulin, NRE mRNA levels decreased significantly at insulin concentrations exceeding 100 pg/ml and reached a minimum at insulin levels of 10 ng/ml (Fig. 5C). In contrast, insulin markedly induced the mRNA expression for adiponutrin at levels above 1 ng/ml confirming previous observation that feeding and high insulin levels upregulate adiponutrin expression (30).

**NRE Is Localized to the Endoplasmic Reticulum and Lipid Droplets**—To determine the intracellular localization of NRE, biochemical and histological analyses were performed. Lysates of transfected COS7 cells overexpressing His-tagged NRE or NTE were separated into membrane fraction (100,000 × g pellet) and a cytosolic fraction (100,000 × g supernatant) and subjected to Western blotting analysis. Both NRE and NTE were detected as 150 kDa proteins exclusively in the membrane fraction (Fig. 6A). Additionally, a heterologously expressed fusion protein of NRE and GFP localized to the ER of monkey kidney cells.
cells (COS7) and differentiated mouse adipose cells (OP9) (Fig. 6, upper two panels). The pictures captured by live cell imaging showed that NRE-GFP colocalizes with the ER marker and that NRE-GFP is distributed in a punctiform manner within the ER. These results indicated that NRE might be localized to ER-domains where lipid droplets are forming. Thus, we stained lipid droplets of COS7 and OP9 cells expressing NRE-GFP with a fluorescently labeled dodecanoic acid (C12:0). Microscopic images showed that the NRE-GFP fusion protein partially colocalizes with lipid droplets (Fig. 6, lower two panels).

**DISCUSSION**

LysoPLs have multiple functions in biological systems. They are intermediary products of lipid synthesis and degradation, structural components of cell membranes or bioactive molecules affecting a variety of signaling pathways. The major route by which LysoPLs are catabolized is considered to be degradation by lysophospholipases A (31). Here we show that murine NRE is a lysophospholipase A hydrolyzing LPC and LPA.

Mammalian lysophospholipases A can be divided into the two groups, high (>50 kDa) and low (<30 kDa) molecular mass enzymes. Among the latter group two enzymes have been cloned, lysophospholipase A I (31) and lysophospholipase A II (32), which are widely distributed in tissues. These serine-esterases employ a classic catalytic triad and specifically hydrolyze LysoPLs. The high molecular mass enzymes generally have other enzymatic activities besides lysophospholipase activity. Because their activities toward lipid substrates depend highly on the substrate presentation and assay conditions it remains unclear if all of these enzymes exhibit lysophospholipase activities in vivo. Members of the high molecular mass lysophospholipases A include hepatic lipase and intestinal lipase, group IV calcium-dependent cytosolic phospholipases A2, and calcium-independent phospholipases A2 (33). NTE has been identified as high molecular weight lysophospholipase A, and in this study we add NRE as a novel member to this group of enzymes.

Murine NRE shares 96% amino acid sequence identity with rat NRE (10) and both enzymes hydrolyze phenyl valerate (10). With NTE, murine NRE shares 60% amino acid sequence iden-
Although NRE and NTE exhibit high structural homology, the proteins differ in their tissue-specific expression profile. NTE is predominantly expressed in the nervous system (24, 34) and inactivation of the NTE gene in mice resulted in embryonic lethality due to placental failure and impaired vasculogenesis (18). Brain-specific deletion of NTE led to neurodegeneration characterized by disruption of the ER, vacuolation of nerve cell bodies, and abnormal reticular aggregates (19) indicating an essential function of NTE in phospholipid homeostasis. Murine NRE is mainly detected in target tissues of insulin involved in energy metabolism such as adipose tissue, skeletal muscle, cardiac muscle, or in testis, a tissue with a very high lipid turnover (35). The testis specific mRNA size is larger (~5 kb) than those in the other tissues (~4 kb). A tissue-specific size variation was previously reported for rat NRE (10). In addition to the 5-kb mRNA size, two additional NRE mRNA variants were detected in rat testis (4.0 and 2.6 kb). These smaller isoforms were not detectable in mouse testis. NRE mRNA levels are up-regulated in the fasted state and studies with 3T3-L1 adipocytes revealed that insulin decreases NRE expression in a dose-dependent manner. Together, these data suggest that the enzyme possesses a function in energy homeostasis. The presence of putative cyclic nucleotide binding sites at the N terminus of NRE implicates a possible regulation by cAMP, a second messenger that occupies a central position in the regulation of energy homeostasis. However, we failed to detect an influence of cAMP on NRE lysophospholipase activity in vitro.

Fluorescence labeling of the murine enzyme and cell fractionation experiments indicated that NRE is located at the ER and (small, early) lipid droplets suggesting a function of the enzyme in the degradation of intracellular LysoPLs. Most of the cellular LysoPLs are structural components of biological membranes and derive from the phospholipase A-mediated hydrolysis of membrane PLs. Their concentration is generally low.

**FIGURE 5.** Northern blotting experiments showing NRE mRNA expression profiles. A, levels of NRE mRNA in tissues of fed and fasted C57BL/6 mice. Northern blotting was performed using total RNA of various mouse tissues in fed and fasted states and a probe for NRE mRNA that binds distal to the highly conserved patatin domain. The control probe hybridized with the mRNA of the ribosomal phosphoprotein P0. The ethidium bromide stain of the corresponding RNA agarose gel served as loading control (the bands of 28 S and 18 S rRNAs are indicated). BAT, white adipose tissue; BAT, brown adipose tissue; SM, skeletal muscle; CM, cardiac muscle. B, expression of NRE mRNA during adipocyte differentiation. 3T3-L1 preadipocytes were differentiated to adipocytes and total RNA was isolated at the time points indicated. Northern blotting was performed using the same probe as in A and B and the ethidium bromide stain of the 28 S rRNA served as loading control. C, regulation of NRE mRNA expression by insulin. 3T3-L1 adipocytes were incubated for 14 h in serum-free medium with various concentrations of insulin and total RNA was used for Northern blotting (the probe for NRE was the same as in A). The ethidium bromide stain of the 28 S rRNA served as loading control.

**FIGURE 6.** Localization of NRE in COS7 and OP9 cells. A, NRE and NTE were expressed in COS7 cells and cellular lysates (1,000 × g supernatant) were fractionated by centrifugation (100,000 × g) into membrane fraction (pellet) and cytosolic fraction (supernatant). Protein detection was performed by immunoblotting using an antibody against the N-terminal His tag. B, COS7 and OP9 cells expressing the heterologous NRE-GFP fusion protein (green) were incubated with ER-Tracker™ Red dye (red, COS7 and OP9 upper panels) to stain the ER or Bodipy® 558/568 C12 (red, COS7 and OP9 lower panels) to stain lipid droplets. Colocalization of NRE-GFP and the ER/lipid droplets (yellow) was visualized live by confocal laser scanning microscopy. Bars, 10 μm.
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(0.5–6% of total membrane lipid weight) and an accumulation of LysoPLs can distort membrane integrity, affect the activities of many membrane-bound enzymes, and may even cause cell lysis (33). Thus, LysoPL levels have to be tightly controlled to maintain cell survival and function. Due to their inverted cone shape, LysoPLs influence membrane curvature and, consequently, also the formation and fission of vesicles (36, 37). It was shown that increasing the ratio of LysoPL/PL in the outer leaflet of a membrane causes an outward curvature that at its most extreme leads to tubule formation (37). Phospholipases A2, which generate LysoPLs, play critical roles in multiple membrane trafficking events. NRE could be part of this membrane-modifying machinery by degrading LysoPLs produced by PLA2 action and the down-regulation of the enzyme by insulin suggests that NRE activity specifically counteracts processes initiated by insulin signaling.

A key event mediated by insulin signaling in adipose tissue is the stimulation of TG synthesis. LPA, the simplest naturally occurring LysoPL, is the first intermediary product of glycero phospholipid and TG synthesis and is generated by the glycerol-3-phosphate acyltransferase reaction. NRE catalyzes the opposite reaction producing glycerol-3-phosphate and FFAs. Thus, an insulin-mediated inhibition of NRE expression would favor the accumulation of LPA and lipid synthesis. Moreover, LPA binds and activates peroxisome proliferator-activated receptor γ (PPARγ) (38), a member of the nuclear hormone receptor superfamily essential for adipose differentiation and for the expression of fat-specific genes involved in lipid synthesis reactions. Thus, it can be speculated that inhibition of NRE by insulin could increase LPA levels and activate PPARγ. Alternatively, NRE could reduce cellular LPA levels and PPARγ activation under fasted conditions where lipid stores are mobilized for energy production.

Another conceivable physiological function of NRE involves the degradation of LysoPLs that accumulate after stimulus-induced release of arachidonic acid via phospholipase A2 activity. Arachidonic acid derived eicosanoids are potent bioactive mediators that regulate a variety of physiological and pathophysiological processes (39). Cleavage of the ester bond at the sn-1 position of LysoPLs by NRE might help to prevent the accumulation of intracellular LysoPLs for maintaining normal cellular functions.

In general, most of the signaling reactions mediated by LysoPLs characterized to date are extracellular events, which are initiated by their binding to G-protein-coupled receptors (GPCR) on the cell surface. LysoPLs function as extracellular signaling molecules in multiple biologic processes and also affect insulin metabolism (40) and adipocyte growth and function (41). LPC, the most prominent LysoPL, modulates the expression of a number of proteins like growth factors, mononuclear leukocyte adhesion molecules, and vasoprotective enzymes like nitric oxide synthase and cyclooxygenase-2 (33). It plays an etiologic role in atherosclerosis, is a major constituent of atherogenic lipoproteins (42), and exhibits proinflammatory functions including activation of macrophages (43) and expression of chemotactic factors in endothelial cells (44). Importantly, LPC is capable of inducing insulin secretion from pancreatic β-cells and, recently, the underlying mechanism via an orphan GPCR has been identified (40). LPA is also a multifunctional phospholipid messenger. LPA controls growth, motility, and differentiation (45) and evokes various biologic effects, including neurogenesis (46), vasculogenesis (47), and carcinogenesis (48). Moreover, LPA plays an important role in the paracrine control of preadipocyte proliferation (41). Whether NRE potentially reduces the level of LysoPLs (LPC or LPA) that can act on plasma membrane GPCRs by a paracrine/autocrine mechanism is currently unknown. It is interesting to note that autotaxin (ATX), a secreted lysophospholipase D, is responsible for the generation of LPA from LPC released from cells (41). Up-regulation of ATX expression during adipocyte differentiation and in genetic obesity suggests an involvement of this protein in adipogenesis and obesity-associated disorders. It is conceivable that NRE antagonizes ATX activity by removing both the substrate (LPC) and the reaction product (LPA) of this enzyme.

Taken together, we identified NRE as lysophospholipase expressed in insulin-sensitive tissues that is localized to the ER and (nascent) lipid droplets. NRE is up-regulated by fasting and down-regulated by insulin in 3T3-L1 adipocytes. The role of lysophospholipids in the regulation of adipocyte function and differentiation as well as the expression pattern, intracellular localization, and hormonal regulation of NRE indicate a role of this enzyme in energy metabolism.

Acknowledgment—We thank E. Zechner for critically reviewing the manuscript.

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