methods

Sensitive assay for hormone-sensitive lipase using NBD-labeled monoacylglycerol to detect low activities in rat adipocytes

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Abstract The recent finding that *p*-nitrobenzofurazan (NBD)-FA is incorporated into and released from the acylglycerols of isolated rat adipocytes in an insulin-sensitive manner [G. Müller, H. Jordan, C. Jung, H. Kleine, and S. Petry. 2003. *Biochimie.* 85: 1245–1246] suggests that NBD-FA-labeled acylglycerols are cleaved by rat adipocyte hormone-sensitive lipase (HSL) in vivo. In the present study, we developed a continuous, sensitive in vitro lipase assay using a monoacylglycerol (MAG) containing NBD (NBD-MAG). NBD-MAG was found to provide an efficient substrate for rat adipocyte and human recombinant HSL. Ultrasonic treatment applied in the presence of phospholipids leads to the incorporation of NBD-MAG into the phospholipid liposomes and to a concomitant change of its spectrophotometric properties. The enzymatic release of NBD-FA and its dissociation from the carrier liposomes is accompanied by the recovery of the original spectrophotometric characteristics. The rate of lipolysis was monitored by measuring the increase in optical density at 481 nm, which was found to be linear with time and linearly proportional to the amount of lipase added. To assess the specific activity of recombinant HSL, we determined the molar extinction coefficient of NBD-FA under the assay conditions. This convenient assay procedure based on NBD-MAG should facilitate the search for small molecule HSL inhibitors.— Petry, S., Y. Ben Ali, H. Chahinian, H. Jordan, H. Kleine, G. Müller, F. Carrière, and A. Abousalham. **Sensitive assay for hormone-sensitive lipase using NBD-labeled monoacylglycerol to detect low activities in rat adipocytes. *J. Lipid Res.* 2005.** 46: **603–614.**

Supplementary key words *p*-nitrobenzofurazan • liposomes • NBD-fatty acid

Lipases (EC 3.1.1.3) play a key role in human lipid metabolism, as they degrade dietary as well as stored lipids and thus initiate and regulate the release of free fatty acids into the serum. Lipases are therefore promising targets for the development of drugs in the field of obesity, diabetes, and atherosclerosis. Hormone-sensitive lipase (HSL) in particular is thought to play an important role in the mobilization of fatty acids from the triacylglycerols (TAGs) stored in adipocytes (for review, see 1), providing the main source of energy in mammals. In vivo, HSL is activated by phosphorylation via cAMP-dependent kinase in response to various lipolytic hormones such as catecholamines. The phosphorylation of HSL leads to its translocation from the cytoplasm to the lipid droplet (2). Insulin acts as an antilipolytic hormone by phosphorylating and activating phosphodiesterase 3B, which hydrolyzes cAMP and thus reduces the hydrolysis of TAG (3). In addition to adipocytes, HSL is expressed in other tissues (4), including skeletal muscle, heart, brain, pancreatic β cells, adrenal gland, ovaries, testes, and macrophages (1).

Because neutral lipases are water-soluble enzymes hydrolyzing insoluble long-chain TAG substrates and some phospholipids to a variable extent, the cleavage reaction has to occur at the lipid-water interface (5–7). The mechanisms involved in the enzymatic lipolysis depend strongly on the mode of organization of the lipid substrate in interfacial structures such as monolayers, micelles, liposomal dispersions, and oil-in-water emulsions. Lipases interact with these lipid complexes, or "supersubstrates," via hydrophobic domains that are exposed upon contact as the result of a substrate-induced conformational change, which sometimes has been called "interfacial activation"

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Abbreviations: DAG, diacylglycerol; DOG, 1,2-dioleoylglycerol; EtOAc, ethyl acetate; HPL, human pancreatic lipase; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MeOH, methanol; NBD, *p*-nitrobenzofurazan; OD, optical density; PC, phosphatidylcholine; PI, phosphatidylinositol; R_f, relative mobility; TAG, triacylglycerol; TOG, trioleoylglycerol.

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(8, 9). The two-dimensional nature of this lipase reaction does not obey Michaelis-Menten kinetics and depends critically on the quality of the interface (7–9). Obtaining accurate (i.e., substrate-specific) measurements of lipase activity as well as developing reliable lipase assay systems require taking these unique features into account.

We published a critical review describing the various lipase detection and assay methods available (10). Generally speaking, these methods can be classified in two groups: chemical methods, in which the amount of substrate disappearing or the amount of product released is measured; and physical methods, which are based on the changes with time in a given physical property, such as the conductivity, turbidity, or interfacial tension during the lipolytic reaction (10). In addition, we have developed a continuous lipase assay using naturally occurring fluorescent TAG isolated from *Parinari glaberrium* (11). Synthetic octadeca-9,11,13,15-tetronic-3-hydroxy-octadecyloxypropylester, a 1-acyl-2-alkyl glycerol from parinaric acid, is a diacylglycerol (DAG) analog that provides an efficient substrate for HSL. But its pronounced sensitivity to oxidation precludes its use under routine conditions (S. Petry, H. Jordan, H. Kleine, and N. Tennagels, unpublished results). An alternative ultraviolet spectrophotometric assay based on the use of TAG from *Aleutris fordii* seeds, which is less sensitive to oxidation, was recently introduced by our group (12).

Various fluorogenic substrates have been used to measure lipase activity (for review, see 13). It has been established that fluorophores such as BODIPY (14), rhodamine (15), and pyrene (16) incorporated into lipase substrates do not interfere with the cleavage of these substrates by lipolytic enzymes. In general, the chromophore should be as small as possible and should be hydrophobic to ensure optimum interactions with the lipase. In addition, the chromophore group should not interact with colored compounds and should be insensitive to oxidation. For these reasons, we selected the *p*-nitrobenzofurazan (NBD) moiety as a fluorescence label. For instance, the NBD group has been used previously as a fluorophore in discontinuous phospholipase A_2 assays (17, 18). We have also previously reported that NBD-FA is taken up by adipocytes and incorporated into acylglycerol in an insulin-sensitive manner and that NBD-FA is released from the NBD-FA-labeled acylglycerols upon challenging the adipocytes with catecholamines, which shows that NBD-modified fatty acid and lipid precursors/derivatives generally are accepted as substrates by lipid-handling enzymes (e.g., acyltransferases, HSL) (19). Starting with NBD-FA, we synthesized a water-insoluble lipase substrate, monoacylglycerol (NBD-MAG) in the form of mixed phospholipid liposomes, which constitutes a sensitive substrate for HSL as well as for other lipases tested.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PC), soybean phosphatidylinositol (PI), BSA, acetylcholinesterase, butyrylcholinesterase, pig liver esterase, 4-methylumbelliferyl butyrate, and 4-methylumbelliferyl palmitate were obtained from Sigma-Aldrich Fine Chemicals. 12-Aminododecanoic acid (compound 1) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (compound 2) were obtained from Fluka (Seelze, Germany). Collagenase (type I, 250 U/mg; Worthington) was provided by Biochrom (Berlin, Germany); male Wistar rats (220–250 g, fed ad libitum) were delivered from the Aventis Pharma animal breeding station (Kastengrund, Germany); Si-60 silica gel plates were purchased from Merck (Darmstadt, Germany). Nonidet-P40 and protease inhibitor cocktails were from Roche Diagnostics. Heparin-Sepharose CL-6B was obtained from Pharmacia-Biotech (Freiburg, Germany). ProBond Nickel-Chelating Resin was from Invitrogen Life Technologies. Porcine colipase devoid of phospholipase contamination was purified by J. de Caro (Enzymology at Interfaces and Physiology of Lipolysis, Marseille, France). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Synthesis of NBD-MAG

For the synthesis of NBD-FA (compound 3), sodium methanolate solution (14.5 ml, 76 mmol) was added under stirring to a solution of compound 1 (18 g, 83.7 mmol) in methanol (MeOH; 300 ml). After being incubated for 5 min, the reaction mixture became clear and a solution of compound 2 (15 g, 75 mmol) in MeOH (300 ml) was added. The reaction mixture, which immediately became dark, was stirred for 18 h at 25°C. Methanolic HCl (1 M, 100 ml, 100 mmol) was then added and the solvent was distilled off in vacuo. The residue was taken up in MeOH and filtered through silica gel. The filtrate was concentrated to dryness, and after being solubilized it was purified by flash chromatography [1:1 toluene-ethyl acetate (EtOAc)]. Compound 3 was obtained in the form of a red solid (26.4 g, 93%). Relative mobility (R_f): 0.16 (1:1 toluene-EtOAc). ^{1}H-NMR (250 MHz, $CDCl_3$): δ 8.5 (d, 1 H, ArH), 6.35 (m, 1 H, NH), 6.18 (d, 1 H, ArH), 3.48 (dt, 2 H, CH_2NH_2), 2.36 (t, 2 H, CH_2COOH), 1.9–1.2 (m, 18 H, 9 CH_2). MS (electrospray ionization-MS): 379.2 (M+1).

To synthesize 2,3-dihydroxypropyl 12-(7-nitrobenzo[1,2,3]oxadiazol-4-ylamino)dodecanoate (compound 4), a solution of compound 1 (12 g, 31.7 mmol) and 2,3-epoxypropanol (50 ml) in isopropanol (50 ml) was stirred at 50°C for 16 h. The solvent was distilled off in vacuo, and the residue was dried at 0.01 torr and then purified by flash chromatography (diisopropyl ether, ether, EtOAc). Compound 4 was obtained as a red oil (10.3 g, 71.8%). R_f: 0.18 (1:1 toluene-EtOAc); R_f: 0.5 (30:5:1 CH_2Cl_2-MeOH-NH_3), which crystallized from EtOAc-diethyl ether. ^{1}H-NMR (250 MHz, $CDCl_3$): δ 8.5 (d, 1H, ArH), 6.35 (m, 1 H, NH), 6.18 (d, 1 H, ArH), 4.19 (dd, 2 H, H-1, H-1′), 3.94 (m, 1 H, H-2), 3.65 (dd, 2 H, H-3, H-3′), 3.48 (dt, 2 H, CH_2NH_2), 2.35 (t, 2 H, CH_2COOH), 1.8 (m, 2 H, CH_2), 1.6 (m, 2 H, CH_2), 1.27–1.15 (m, 14 H, 7 CH_2). MS (electrospray ionization-MS): 453.4 (M+1).

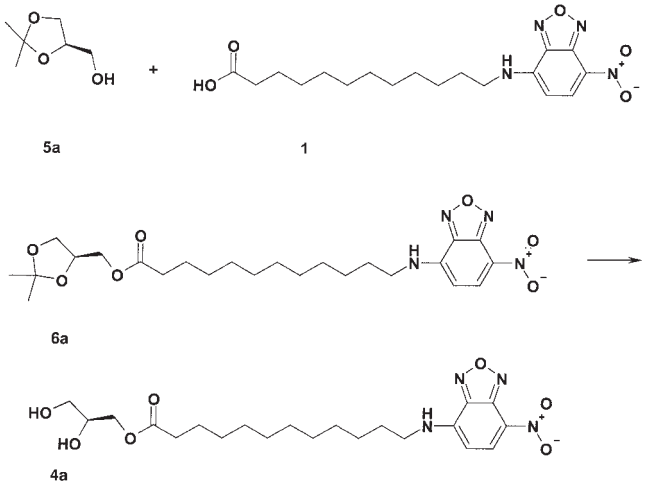
To synthesize (*S*)-2,2-dimethyl[1,3]dioxolan-4-ylmethyl 12-(7-nitrobenzo[1,2,5]oxadiazol-4-ylamino)dodecanoate (compound 5a), a solution of compound 1 (60 mg, 159 μmol) in CH_2Cl_2 (2 ml) was treated with dicyclohexylcarbodiimide (160 mg, 770 μmol) and stirred at 25°C for 30 min. A solution of (*R*)-(2,2-dimethyl[1,3]dioxolan-4-yl)methanol (100 mg, 760 μmol) and dimethylaminopyridine (94 mg, 770 μmol) in CH_2Cl_2 (2 ml) was then added, and the mixture was stirred for 4 h at 25°C. The solvent was distilled off in vacuo, and the residue was then purified by flash chromatography (15:1 toluene-EtOAc). Compound 5a was obtained in the form of a yellow fluorescent oil (46 mg, 58%). R_f: 0.29 (4:1 toluene-EtOAc). ^{1}H-NMR ($CDCl_3$): δ 8.5 (d, 1 H, aromatic), 6.2 (m, 1 H, NH), 6.16 (d, 1 H, aromatic), 4.31 (m, 1 H), 4.1 (m, 3 H), 3.73 (dd, 1 H), 3.48 (dt, 2 H, CH_2NH_2), 2.35 (t, 2 H, CO-CH_2), 2.0–1.2 (m, 18 H, 9 CH_2), 1.42 (s, 3 H, CMe_2), 1.37 (s, 3 H, CMe_2).

(*R*)-2,2-Dimethyl[1,3]dioxolan-4-ylmethyl 12-(7-nitrobenzo

[1,2,5]oxadiazol-4-ylamino)dodecanoate (compound 5b) was synthesized as described for compound 5a, but starting with (*S*)-(2,2-dimethyl[1,3]dioxolan-4-yl)methanol and NBD-FA (compound 3). The protected compound 5b was obtained in the form of a yellow fluorescent oil (51.4 mg, 65%). R_f: 0.29 (4:1 toluene-EtOAc). ^{1}H-NMR ($CDCl_3$): δ 8.5 (d, 1 H, ArH), 6.2 (m, 1 H, NH), 6.16 (d, 1 H, ArH), 4.31 (m, 1 H), 4.1 (m, 3 H), 3.73 (dd, 1 H), 3.48 (dt, 2 H, CH_2NH_2), 2.32 (t, 2 H, CO-CH_2), 2.0–1.2 (m, 18 H, 9 CH_2), 1.42 (s, 3 H, CMe_2), 1.37 (s, 3 H, CMe_2).

To synthesize (*S*)-2,3-dihydroxypropyl 12-(7-nitrobenzo[1,2,5]oxadiazol-4-ylamino)dodecanoate (compound 4a) and (*R*)-2,3-dihydroxypropyl 12-(7-nitrobenzo[1,2,5]oxadiazol-4-ylamino)dodecanoate (compound 4b), methanolic HCl (1 M, 200 μl) was added separately to a 13.9 mg (28.2 μmol) solution of compound 4a or a 17.8 mg (36.1 μmol) solution of compound 4b in 25 ml of MeOH. The mixture was stirred for 1.5 h at 25°C. The solvent was distilled off in vacuo, and the residue was purified by flash chromatography (2:1, 1:1 toluene-EtOAc). The fatty acylesters, compounds 4a and 4b, were obtained in yields of 10.5 mg (82%) and 9.3 mg (57%), respectively. ^{1}H-NMR ($CDCl_3$) data and mass spectra were identical to those obtained for compound 4.

The NBD-MAG enantiomers were synthesized starting with D- and L-1,2-*O*-isopropylideneglycerol, which was esterified with NBD-FA (1) by dicyclohexylcarbodiimide activation. The protective group was removed using 1 N methanolic HCl. (*R*)-(2,2-Dimethyl[1,3]dioxolan-4-yl)methanol (5a) was the starting material for synthesizing 12-(7-nitrobenzofurazan-4-ylamino)-dodecanoic acid (*S*)-2,3-dihydroxy-propyl ester (4a), and (*S*)-(2,2-dimethyl[1,3]dioxolan-4-yl)methanol (5b) was the starting material for the corresponding *R*-enantiomer 12-(7-nitrobenzofurazan-4-ylamino)-dodecanoic acid (*R*)-2,3-dihydroxy-propyl ester (4b).



Lipase sources

The recombinant human HSL was expressed and purified from insect cells as described by Ben Ali et al. (20). The recombinant human pancreatic lipase (HPL) was expressed and purified from insect cells as described by Thirstrup et al. (21). Purified *Thermomyces lanuginosus* lipase was a generous gift from S. Patkar (Novo Nordisk). Lipoprotein lipase (affinity purified) from bovine milk, defatted BSA (fraction V), and phospholipase A_2 from human pancreas were purchased from Sigma (Deisenhofen, Germany).

Preparation of rat adipocyte extract

Adipocytes were isolated from epididymal fat pads of Wistar rats by performing digestion with collagenase and subsequent separation steps from undigested tissue using a nylon web, using previously published procedures (19, 22). Cells obtained from 10 rats were washed three times with 50 ml each of homogenization buffer (25 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, and 10 μg/ml each of leupeptin, antipain, and pepstatin) by flotation (500 *g*, 2 min, 25°C, swing-out rotor), suspended in 10 ml of homogenization buffer, and then homogenized by performing 10 strokes at 1,500 rpm in a loose-fitting Teflon-in-glass homogenizer (15°C). The homogenate was centrifuged for 10 min at 3,000 *g* at 4°C. The infranatant below the fat layer was aspirated and recentrifuged. This procedure was repeated three times to completely remove the residual lipid left at the top after the centrifugation. The final infranatant was centrifuged for 45 min at 48,000 *g* at 4°C. The resulting fat-free supernatant was mixed with 1 g of heparin-Sepharose (washed five times with 25 mM Tris-HCl, pH 7.4, and 150 mM NaCl), incubated at 4°C for 1 h (under head-to-end rotation of the vial), and then centrifuged for 10 min at 1,000 *g* at 4°C. The supernatant was adjusted to pH 5.2 and incubated for 30 min at 4°C. The precipitates were collected by centrifugation (25,000 *g*, 10 min, 4°C), suspended in 2.5 ml of 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 70 mM NaCl, 13% sucrose, and 10 μg/ml each of leupeptin, antipain, and pepstatin, and finally dialyzed (20 h, 4°C) against 3 × 500 ml of 25 mM Tris-HCl, pH 7.4, 50% glycerol, 1 mM EDTA, 1 mM DTT, and 10 μg/ml each of leupeptin, antipain, and pepstatin. Adipocyte extract was frozen in liquid N_2 and stored at −70°C for up to 4 weeks.

This procedure considerably decreases the 2-MAG lipase levels (during the acid precipitation step) and the removal of 70% of the LPL (during the heparin-Sepharose step) from the adipocyte extract, as shown by our experimental data (unpublished results).

Lipase assay

To prepare the NBD-MAG substrate, 41.5 μl of a PC solution (6 mg/ml in chloroform), 83.5 μl of a PI solution (6 mg/ml in chloroform), and 100 μl of a NBD-MAG solution (10 mg/ml in chloroform) were added to plastic scintillation vials and dried

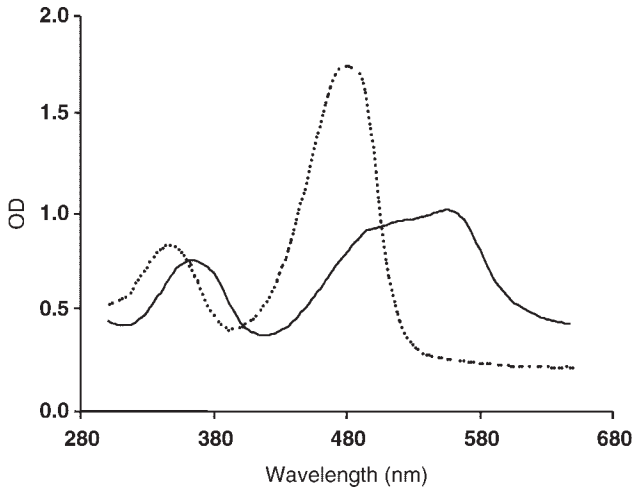


Fig. 1. Absorption spectra of *p*-nitrobenzofurazan (NBD)-FA (dashed line; 0.16 mM final concentration) and NBD-monoacylglycerol (MAG; solid line; 0.16 mM final concentration), both incorporated into phosphatidylcholine/phosphatidylinositol (PC/PI; molar ratio 1:3) liposomes, recorded in 20 mM sodium acetate (pH 6.0) and 150 mM NaCl. The absorption spectra of NBD-MAG were determined after sonication in the presence of PC/PI (1:3). OD, optical density.

over a stream of N_2. This NBD-MAG substrate incorporated into phospholipid liposomes can be stored ready for use for up to 3 days at 4°C without any significant loss of cleavage efficiency. The compound itself is crystalline and can be stored for longer than 1 year without any detectable degradation (purity after 2 years is >95%). The dried lipids were then resuspended in 20 ml of 25 mM Tris-HCl buffer, pH 7.4, and 150 mM NaCl and then subjected to an ultrasonic treatment in an ice bath using a Branson Sonifier (type II, standard microtip; 2 × 1 min at setting 2 followed by 2 × 1 min at setting 4 with 1 min intervals). During the ultrasonic treatment, the substrate suspension shifted from yellow (maximum absorbance at 481 nm) to pink (maximum absorbance at 550 nm) (**Fig. 1**). This suspension was used after a period of 15 min (minimum) to 2 h (maximum). To start the assay procedure, 180 μl of NBD-MAG substrate solution were warmed to 30°C and supplemented with either 30 μl of an adipocyte extract (appropriately diluted with 25 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.1% BSA) or 20 μl of recombinant human HSL in the wells of 96-well microtiter plates. The optical density (OD) at 481 nm was recorded continuously at regular intervals (from 1 to 30 min) using a microplate scanning spectrophotometer (PowerWave; Bio-Tek Instruments). Buffer alone was used in the control experiments.

Alternatively, the products generated in the reaction mixture were analyzed by TLC. For this purpose, 200 μl of the reaction mixture was transferred into 2 ml reaction vials and supplemented with 1.3 ml of MeOH-chloroform-heptane (10:9:7, v/v/v) and then with 0.4 ml of 0.1 M HCl. After intense vortexing, phase separation was initiated by centrifugation (800 *g*, 20 min, 25°C), and 200 μl aliquots of the lower organic phase were removed, dried under a vacuum (SpeedVac evaporator), and suspended in 50 μl of tetrahydrofuran. Five to 10 μl samples were separated by performing TLC on silica gel Si-60 plates using diethylether-petrol ether-acetic acid (78:22:1, v/v/v) as the elution solvent system. In a pure lane, authentic NBD-FA was run and used as a marker. In some experiments, the amount of NBD-FA acid released was assessed by fluorescence imaging using a PhosphorImager (Molecular Dynamics; Storm 860 and ImageQuant software) with an excitation wavelength of 460 nm and an emission wavelength of 540–550 nm.

The specific activity of recombinant human HSL was calculated from the steady-state reaction rate (ΔOD/min) using a molar extinction coefficient of 6,700 M^{-1} at pH 6.0 (see Results and Discussion). The specific activity was expressed in international units per milligram of purified lipase. One international unit corresponds to 1 μmol of fatty acid released per minute under the assay conditions.

Determination of the molar extinction coefficients of NBD-MAG and NBD-FA

The OD at time 0 ($OD_{t=0}$) can be expressed using the following formula:

$$OD_{t=0} = \varepsilon_s [S]_0 \qquad (Eq.\ 1)$$

where ε_s is the substrate (NBD-MAG) molar extinction coefficient and $[S]_0$ is the concentration of NBD-MAG at time 0.

The OD measured at time t (OD_t) was determined using the following formula:

$$OD_t = \varepsilon_s [S]_t + \varepsilon_p [P]_t \qquad (Eq.\ 2)$$

where ε_p is the molar extinction coefficient of the product (NBD-FA), $[S]_t$ is the concentration of NBD-MAG at time t, and $[P]_t$ is the concentration of the reaction product (NBD-FA) at time t.

$[P]_t$ and $[S]_t$, therefore, could be expressed as $Xp_t\ [S]_0$ and $(1 - Xp_t)\ [S]_0$, respectively, where Xp_t is the reaction progress coefficient ($0 < Xp_t < 1$).

The variation of the OD at 481 nm ($\Delta OD_{481\ nm}$) is given by the following equation:

$$\Delta OD_{481\ nm} = (Eq.\ 1) - (Eq.\ 2) = (\varepsilon_p - \varepsilon_s)\ [S]_0\ Xp_t \qquad (Eq.\ 3)$$

ε_p and ε_s were determined using the linear part of the Beer-Lambert law in a 3 ml quartz cuvette containing 0.16 mM pure NBD-FA and after being incorporated under the same experimental lipase assay conditions into PC and PI liposomes, respectively. The absorbance spectra were recorded at wavelengths between 280 and 680 nm (Fig. 1) using a Uvikon 860 spectrophotometer (Kontron Instruments). Because the length of the optical path of the quartz cuvette (1 cm) and that of the microtiter plates (~0.6 cm) were different, a correction factor of 1.66 was applied when using the microtiter plate.

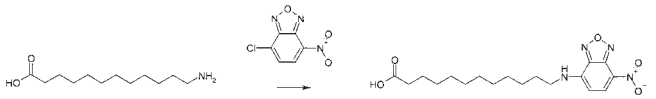
Miscellaneous

HSL activity was measured at pH values ranging from 3.0 to 9.0. The buffers used were 50 mM sodium acetate (pH 4.0–6.0), 50 mM Tris (pH 6.0–8.0), and 50 mM glycine (pH 8.0–10.0). Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL) with BSA as the standard.

RESULTS AND DISCUSSION

NBD-MAG synthesis

Previous studies (1, 5, 6, 23–30) have shown that HSL, LPL, pancreatic lipase, endothelial lipase, and hepatic lipase all hydrolyze long-chain TAG, 1,2/1,3-DAG, and 1/3-MAG, although with varying efficiencies. By contrast, 2-MAG is assumed to be cleaved specifically by a 2-MAG lipase (31) but may also rapidly isomerize to 1/3-MAG, thus leading to the complete degradation of TAG into fatty acids and glycerol by the above mentioned lipases. The possibilities of chemical versus enzyme-catalyzed isomerization of 1,2-dioleoylglycerol (DOG) and 2-oleoylglycerol have been previously discussed (32). With a view to exploring versatile substrates that can be used to assay most of the neutral lipases known to date, we decided to analyze a variety of long-chain TAG, DAG, and MAG derivatives esterified in the *sn*-1 position with NBD-labeled dodecanoic acid. This NBD-FA (compound 3) was synthesized by reacting compound 1 with compound 2 in MeOH, which gave a good yield.

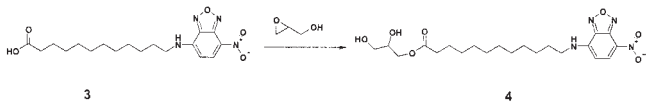


1 2 3

To synthesize NBD-MAG (compound 4) in the form of a racemic mixture, compound 3 was converted with a good yield by reacting it with 2,3-epoxypropanol using a similar procedure to that described by Ali and Bittman (33).

Several derivatives were prepared by acylating or alkylating the remaining hydroxyl groups. These NBD-labeled acylglycerols tested as potential lipase substrates showed variable levels of efficiency, with the rat HSL present in ad-

ipocyte extracts as well as with recombinant human HSL, upon being presented in emulsified droplets and subsequently analyzed by TLC and fluorescent imaging (S. Petry, H. Kleine, H. Jordan, N. Tennagels, and G. Müller, unpublished results).



3 4

Absorption spectra of NBD-MAG

The production of mixed liposomes consisting of phospholipids with intercalated NBD-MAG (see Materials and Methods) was accompanied by a color shift from yellow (maximum absorption at 481 nm) to pink (maximum absorption at 525–550 nm; Fig. 1). This was presumably attributable to the formation of charge transfer complexes between dimers or oligomers of the fluorophores when tightly packed in parallel within the phospholipid fatty acyl chains of the liposomes. By contrast, under the same experimental conditions, TAG or DAG harboring one or more NBD-FA did not exhibit any change in fluorescence or color (S. Petry, H. Kleine, H. Jordan, N. Tennagels, and G. Müller, unpublished results), probably because the hydrophilic/lipophilic balance of the NBD-MAG is favorable to its incorporation into phospholipid liposomes, unlike that of DAG- and TAG-containing NBD moieties.

Mixed phospholipid liposomes prepared from mixtures of PI and PC with higher levels of PI versus PC proved to be particularly efficient in terms of the NBD-MAG hydrolysis rate compared with liposomes consisting of either PC or PI alone (data not shown) or lyso-PC or DOG (**Fig. 2**). This result is in agreement with a previous finding that adding PC to the sonicated lipid droplets or adding PC-specific phospholipase C to intact lipid droplets reduced or stimulated, respectively, the hydrolysis of TAG by HSL in vitro(34). However, the NBD-MAG hydrolysis rate was significantly lower with droplets than with mixed phospholipid liposomes (Fig. 2). This difference can be attributed to the competition between NBD-MAG and the trioleoylglycerol (TOG) diffusing from the droplet core into the phospholipid surface monolayer, where cleavage by droplet-associated neutral lipases presumably occurs. Consequently, mixed liposomes with PI/PC at 3:1 were used as the standard substrate in the following experiments.

Kinetic recordings during NBD-MAG hydrolysis by recombinant human HSL

NBD-MAG hydrolysis was studied using the purified recombinant human HSL. The absorption spectrum of the reaction medium was recorded every minute for 10 min. **Figure 3A** shows the changes occurring upon lipolysis in the optical absorption, with an isobestic point at 510 nm and the maximum changes occurring in the 400–580 nm range. From the spectra presented in Fig. 3A, the OD did not vary significantly from 300 to 380 nm. However, the OD increased with time from 430 to 481 nm and decreased from 481 to 580 nm. These variations were clearly qualitatively correlated with the gradual release of the NBD-FA (absorbing in the 430–510 nm wavelength range) and the disappearance of the NBD-MAG (absorbing in the 510–550 nm wavelength range). From the spectra presented in Fig. 3A, differential spectra (ΔOD as a function of wavelength) were computed at each recording time (Fig. 3B), and an isobestic point was observed at 510 nm. It can be clearly seen here that the maximum OD variations occurred at either 481 nm ($\Delta OD = 0.04$) or 550 nm ($\Delta OD = -0.019$). One can thus monitor NBD-MAG hydrolysis by HSL by measuring the changes in the OD at either 481 or 550 nm. A typical example of the kinetic recordings obtained at 481 nm is shown in the inset in Fig. 3B, where a linear increase in the ΔOD can be seen to have occurred with time for at least 10 min.

Effects of the NBD-MAG concentration and the amount of recombinant human HSL on steady-state reaction rates

Mixed liposomes were prepared with initial NBD-MAG concentrations ranging from 0 to 0.3 mg/ml, and their

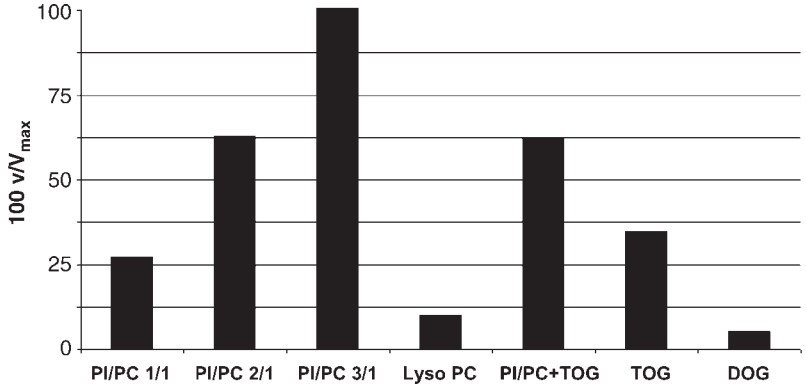


Fig. 2. Effect of liposome composition on hydrolysis rates of NBD-MAG. Liposomes consisting of NBD-MAG with various mixtures of PI and PC as indicated or with lyso-PC or 1,2-dioleoylglycerol (DOG) or droplets consisting of NBD-FA and trioleoylglycerol (TOG; 1.5 mg/ml final concentration) without or with PI/PC (3:1) as indicated were incubated (37°C) with 20 μg of rat adipocyte extract. Activity is expressed as a percentage of the maximum activity (100 v/V_m).

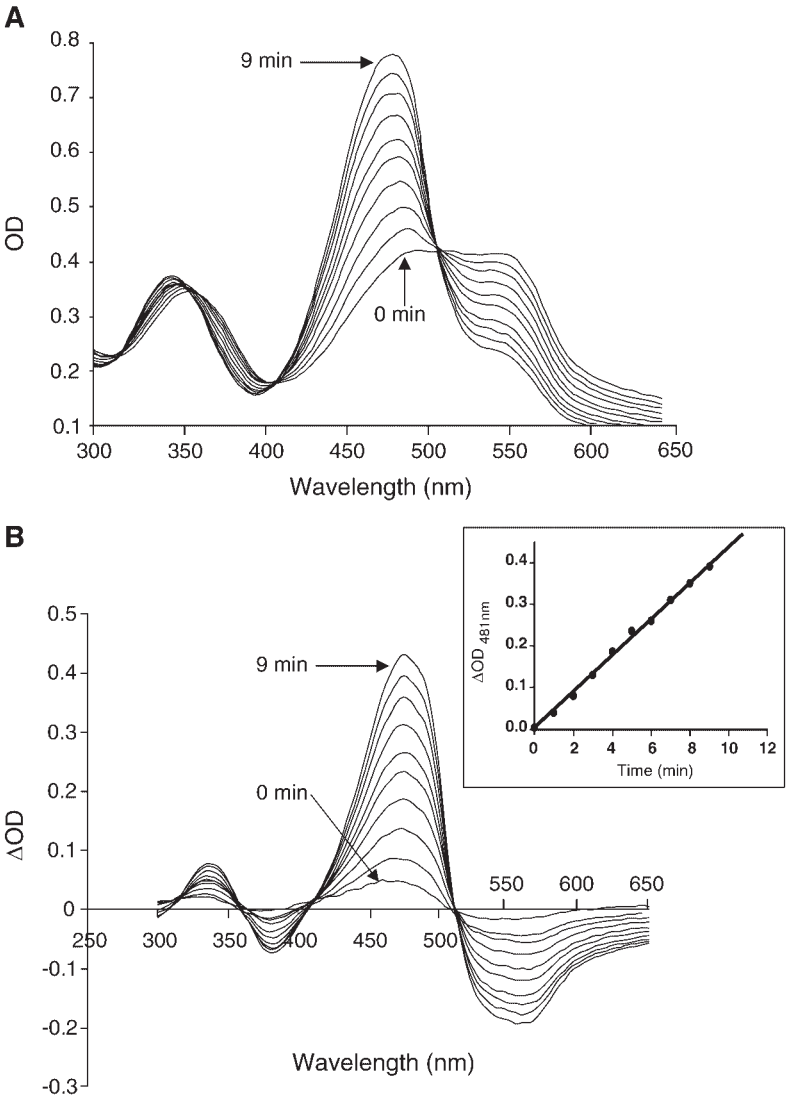


Fig. 3. Kinetic absorption spectra upon NBD-MAG hydrolysis by purified recombinant human hormone-sensitive lipase (HSL). A: The HSL was added (33 nM final concentration) to the NBD-MAG incorporated into PC/PI liposomes (see Materials and Methods). The absorption spectra were determined every 1 min for 9 min. B: Kinetic recording of the hydrolysis of NBD-MAG upon incubation with HSL (33 nM final concentration). The inset shows hydrolysis rates [change in optical density (ΔOD_{481nm}/min)] calculated from the differential spectrum obtained from B by subtracting the initial absorption spectrum at time 0 from the spectrum recorded at the reaction times indicated.

lipolysis was tested using recombinant human HSL. The steady-state reaction rates estimated from the slope of the variations with time in the OD at 481 nm were plotted versus the NBD-MAG concentration (**Fig. 4**). The reaction rates increased with the NBD-MAG concentrations from 0 to ~0.15 mM and then reached a plateau at higher NBD-MAG concentrations. Based on these results, a final NBD-MAG concentration of 0.16 mM was selected for further kinetic experiments.

Using NBD-MAG at a final concentration of 0.16 mM incorporated into PC/PI liposomes, we recorded the increase with time in the OD at 481 nm after injecting variable amounts of recombinant human HSL (final concentrations ranging from 0 to 7 nM). A typical linear kinetic recording obtained during 5 min is shown in **Fig. 5A**. The steady-state reaction rates were plotted as a function of increasing amounts of HSL (Fig. 5B). The kinetic curves were found to be linearly proportional to the amount of recombinant human HSL used (Fig. 5B). The sensitivity limit was found to be ~100 pM of pure recombinant human HSL.

Effects of pH on the recombinant human HSL and assessment of its specific activity

The specific activity of recombinant human HSL was assessed using the molar extinction coefficient $\Delta\varepsilon$ ($\varepsilon_p - \varepsilon_s$) (see Materials and Methods). **Table 1** shows ε_p and ε_s as well as $\Delta\varepsilon$ at pH values ranging from 4 to 10. Under these conditions, the specific HSL activity is maximum at pH 6.0 and reaches a value of 5 U/mg (**Fig. 6**). The enzyme activity is low in the pH 7.0–10 range. It is worth noting that in the acidic pH range (pH 4.0–5.0), the HSL activity is still ~50% of the maximum activity observed at pH 6.0. The relative rat HSL activity was reported to be 1:10:4 against ^{3}H-labeled TAG, DAG, and 1/3-MAG, respectively (35). Using these substrates emulsified with phospholipids (PC/PI, 1:3, w/w), the rat HSL showed a broad pH optimum of ~7, but this activity declined quickly and disappeared below pH 5 (35).

For the sake of comparison, the specific activity of other lipases was determined using NBD-MAG incorporated into PC/PI liposomes. The specific activities of *Thermomyces lanuginosus* lipase and HPL were found to be 3- and 2.4 U/mg, respectively. LPL (from bovine milk) exhibited sig-

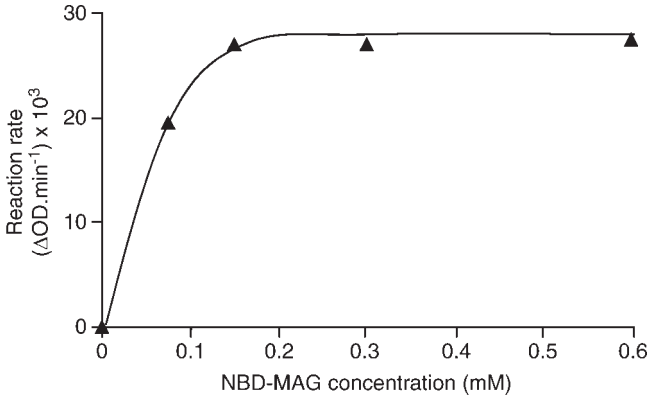


Fig. 4. Effects of the initial NBD-MAG concentration on the catalytic activity of HSL. The steady-state reaction rates at various initial NBD-MAG (incorporated into PC/PI liposomes) concentrations were determined by recording the increase in OD at 481 nm. Each experimental point corresponds to the average of two kinetic recordings performed with HSL (33 nM final concentration).

nificant enzymatic activity (~70% of the activity observed with HSL), whereas bacterial PC-specific phospholipase (from *Bacillus cereus*) and human pancreatic phospholipase A_2 were inactive (data not shown). These differences

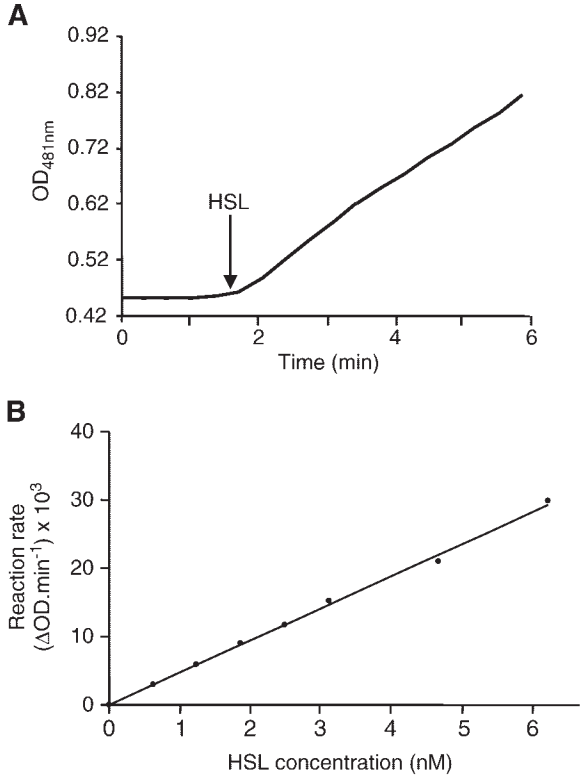


Fig. 5. A: Typical kinetic recording of the hydrolysis of NBD-MAG upon incubation with HSL. The 180 μl reaction medium containing NBD-MAG substrate (see Materials and Methods) was incubated at 30°C in the wells of 96-well microtiter plates. HSL (33 nM final concentration) was added after recording the background for 2 min. The OD at 481 nm was recorded continuously. B: The steady-state reaction rates were plotted as a function of the final HSL concentration.

TABLE 1. Substrate [*p*-nitrobenzofurazan-monoacylglycerol (NBD-MAG)] molar extinction coefficient (ε_s), product (NBD-FA) molar extinction coefficient (ε_p), and $\Delta\varepsilon$ ($\varepsilon_p - \varepsilon_s$) were determined (see Materials and Methods) at various pH values

pH	ε_s	ε_p	$\Delta\varepsilon$
		M^{-1}	
4	4,200	5,200	1,000
5	4,200	6,800	2,600
6	4,900	11,600	6,700
7	5,000	29,600	24,600
8	5,300	29,800	24,500
9	5,300	28,400	23,100
10	4,500	17,000	12,500

between the activities toward NBD-MAG, which were confirmed by TLC analysis of the NBD-FA released (data not shown), confirm the specificity of the NBD-MAG assay for neutral lipases (vs. phospholipases) as well as its optimization as far as substrate presentation is concerned in the case of HSL and LPL (vs. pancreatic and microbial lipases). The failure of phospholipases to promote a color shift demonstrates that the NBD-MAG assay specifically monitors the hydrolytic cleavage of NBD-MAG and not the disintegration of the mixed phospholipid liposomes per se.

The specificity of the assay was analyzed using nonlipolytic enzymes such as acetylcholinesterase, butyrylcholinesterase, and pig liver esterase. Acetylcholinesterase and butyrylcholinesterase did not hydrolyze NBD-MAG to any significant extent, and the fact that pig liver esterase showed only a very moderate level of activity (up to 0.02 U/mg) slightly above the background activity confirmed the selectivity of the assay for lipases versus esterases that are not lipases as well as the lipidic nature of the NBD-MAG substrate. In addition, the activity of HSL was also measured using other fluorogenic substrates, such as 4-methylumbelliferyl esters. The rate of hydrolysis by HSL was found to be only ~13 and 2 mU/mg on 4-methylumbelliferyl butyrate and 4-methylumbelliferyl palmitate, respectively. On the other hand, we have performed an ultraviolet spectrophotometric assay based on the use of TAG from *Aleutris fordii* seeds, but HSL showed only low

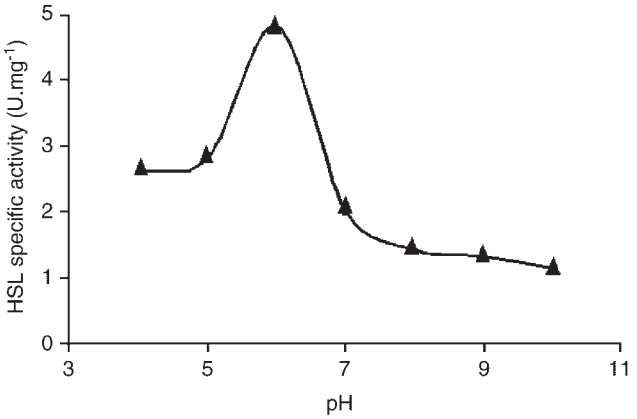


Fig. 6. Specific activity of HSL as a function of pH. NBD-MAG was incorporated into PC/PI liposomes by sonication in various buffers in the pH range from 3.0 to 9.0.

specific activity (60 mU/mg), whereas that obtained using an NBD-MAG substrate was 5,000 mU/mg.

Hydrolysis of NBD-MAG by rat adipocyte extract

NBD-MAG hydrolysis was also studied using rat adipocyte extract. Upon adding adipocyte extract enriched with HSL and devoid of LPL (see below), the reaction rate increased in a time-dependent manner, as reflected in the increase in the absorbance observed at 481 nm (**Fig. 7A**). Analysis of the total reaction mixture by TLC and fluorescent imaging showed that NBD-MAG was hydrolyzed, yielding free NBD-FA (Fig. 7B). The kinetic recordings were linear for up to 75 min with 40 μg of rat adipocyte extract. The signal-to-noise ratios were ~50 at the end of the linear phases, which is sufficient for high-throughput formats.

Extracts from adipocytes treated with isoproterenol for the activation of HSL exhibited maximally a 2.5-fold higher catalytic rate than extracts from untreated cells (Fig. 7C). This moderate activation is compatible with the previous finding that phosphorylation of HSL increases its activity toward acylglycerol only moderately (TAG) or not at all (MAG) when assayed in vitro (1, 30, 35).

Increasing the final assay concentration of BSA from 0.05% to 2% did not significantly affect the catalytic rates

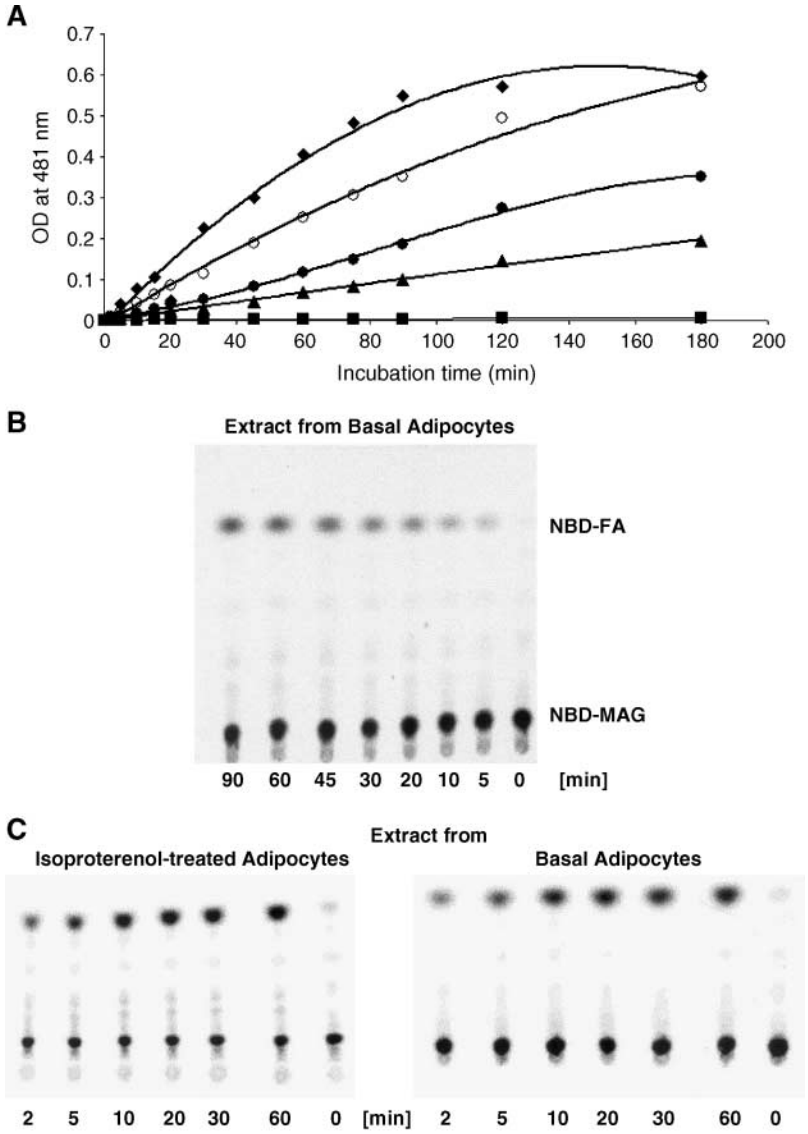


Fig. 7. Time course of cleavage of NBD-MAG. NBD-MAG incorporated into PC/PI liposomes was incubated (30°C) (see Materials and Methods) in the presence of 40 μg (diamonds), 20 μg (open circles), 10 μg (closed circles), and 5 μg (triangles) or in the absence (squares) of rat adipocyte extract prepared from basal (A, B) or isoproterenol-treated (1 μM final concentration; C) adipocytes. At the time points indicated, absorption was measured at 481 nm using a microtiter plate reader (n = 8, mean; A). Alternatively, portions of the incubation mixtures were extracted with methanol (MeOH)-chloroform-heptane (10:9:7, v/v/v) and then subjected to phase separation. The organic phase was dried, suspended in tetrahydrofurane, and analyzed by TLC and fluorescence imaging as described in Materials and Methods (n = 8, mean; B, C).

or the linearity of the hydrolytic reaction (data not shown). Binding of free NBD-FA to BSA may have abolished the feedback inhibition of HSL by the NBD-FA released, as previously reported to occur in the case of free fatty acids (36). Apparently, the NBD-FA generated is either quantitatively bound to (fatty acid binding) proteins contained in the adipocyte extract and/or does not leave the mixed phospholipid/NBD-MAG liposomes but remains associated in a "monomeric" state that does not support charge transfer. Formation of the mixed liposomes was sensitive to the presence of Triton X-100 (>0.05% final concentration) and sodium taurodeoxycholate but insensitive to DMSO up to 2.5% final concentration (data not shown). Importantly, NBD-MAG hydrolysis by rat adipocyte extracts (in the absence or presence of heat-treated rat serum) was not inhibited by 1 M NaCl or excess antiserum to bovine LPL (which cross-reacts with rat LPL). By contrast, the presence of 100 mM NaF, which completely blocks purified rat adipocyte HSL (30), reduced the release of fatty acids from both TOG and NBD-MAG by the rat adipocyte extract by 62–75% (data not shown). These data suggest that approximately two-thirds of the lipolytic activity of rat adipocyte extract measured using NBD-MAG incorporated into phospholipid liposomes is based on the *sn*-1/3-MAG lipase activity of HSL. The remaining portion may be attributable to a distinct MAG lipase expressed in rat adipocyte, which is resistant to NaF (31, 37). This statement was based on the fact that HSL, LPL, and NaF-resis-

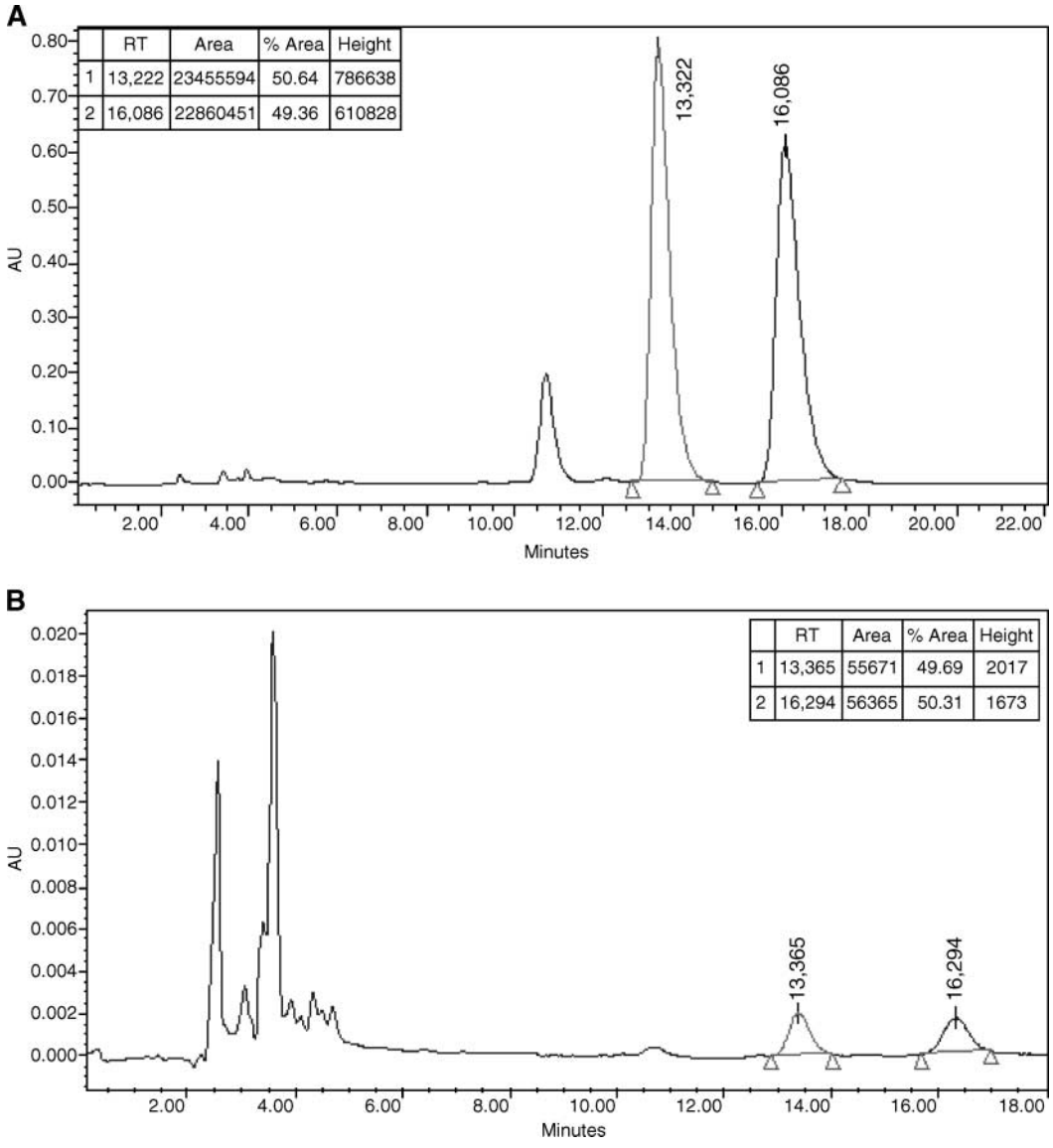


Fig. 8. Separation of enantiomers of a racemic mixture of NBD-MAG by chiral liquid chromatography using a Chiralpak AD-H/30, 250 × 4.6 column (ethanol-MeOH, 1:1) before (A) and after (B) half-maximal cleavage by rat adipocyte extract. The peaks were assigned after enantioselective synthesis of the pure enantiomers (see Results). Peak 1 (RT 13,365) and peak 2 correspond to (*R*)- and (*S*)-2,3-dihydroxypropyl 12-(7-nitrobenzo[1,2,5]oxadiazol-4-ylamino) (compounds 4b and 4a), respectively. AU, absorbance units; RT, retention time.

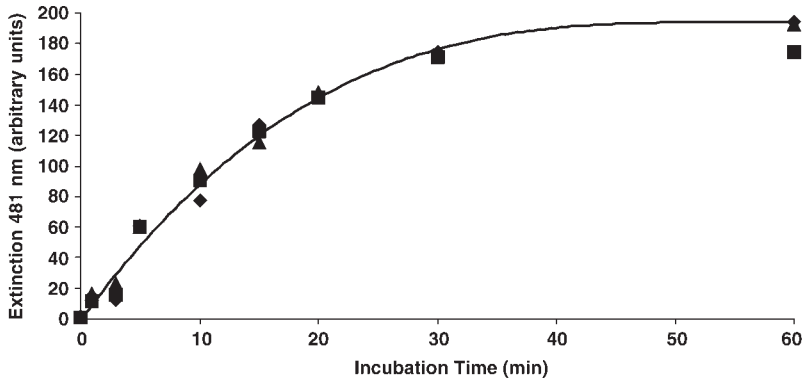


Fig. 9. Time course of cleavage of NBD-MAG enantiomers by rat adipocyte extract. NBD-MAG as racemic mixture (compound 4; diamonds) or the pure *S*-enantiomer (compound 4a; squares) or the pure *R*-enantiomer (compound 4b; triangles) was incorporated in PI/PC liposomes and incubated (60 min, 30°C) in the presence of adipocyte extract (40 μg protein). At the time points indicated, extinction was measured at 481 nm (n = 8, mean).

tant MAG lipase are the only 1/3-MAG lipases to have been identified at the molecular level to date. The possible existence of additional acylglycerol lipases (38–40) certainly cannot be ruled out on the basis of the lack of inhibition by NaF. This point needs to be studied in the future by assaying adipose extract derived from HSL knockout mice or immunodepletion of HSL in the presence of NaF. Nevertheless, the data available clearly show the validity of the present HSL assay, given its compatibility with pure recombinant enzyme (see above). Any contamination by LPL from adipose tissue vascular endothelial cells [sensitive to NaCl (5, 24)] was presumably negligible.

All in all, it can be concluded that the primary NBD-1/3-MAG is efficiently accepted by rat adipocyte HSL. It is noteworthy that 1/3-MAG, unlike 2-MAG, has been described as a very poor substrate for the adipocyte MAG lipase. The assay method presented in this study, therefore, has a pronounced specificity for HSL and can be used to monitor its activity on 1/3-MAG in comparison with that of other lipases preferably cleaving 2-MAG, such as the well-known 2-MAG lipase of rat adipose tissue. In addition, the activity of NaF-resistant 1/3-MAG lipase(s) other than HSL can also be monitored by means of this assay.

We next addressed the question of whether rat HSL may prefer one of the two enantiomers as a substrate. NBD-MAG was analyzed by performing chiral liquid chromatography using a Chiralpak AD-H/30 (250 × 4.6) column and ethanol/MeOH (1:1) as the eluent, which led to baseline separation of the two enantiomers (**Fig. 8A**). This system was then used to investigate the composition of enantiomers after approximately half-maximum cleavage of NBD-MAG by adipocyte extract (Fig. 8B). Preferential acceptance of one of the enantiomers as substrate by HSL would result in different amounts of enantiomers being left in the incubation mixture. However, analysis of the enantiomers showed the presence of both enantiomers in comparable amounts, which indicates that HSL did not differentiate between the two enantiomers (Fig. 8B). To assign the enantiomers to the two peaks and directly study the enantioselectivity of HSL, we synthesized the two enantiomers separately.

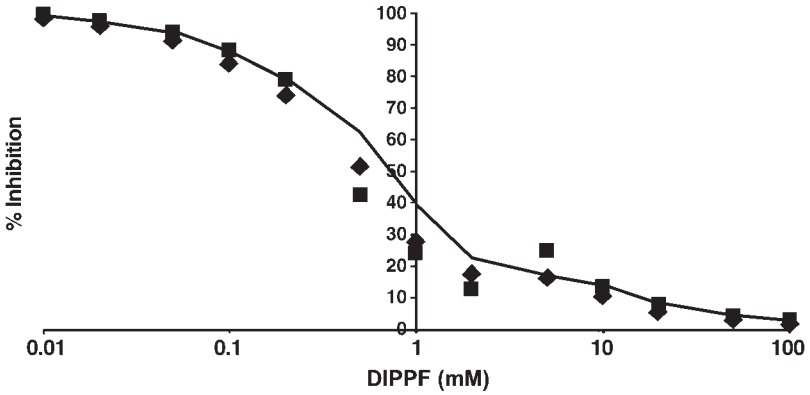


Fig. 10. Inhibition of lipolytic cleavage of NBD-MAG by diisopropyl phosphofluoridate (DIPPF). NBD-MAG incorporated in PI/PC liposomes was incubated (60 min, 30°C) with rat adipocyte extract (20 μg of protein) in the presence of increasing concentrations of diisopropyl phosphofluoridate. The increase in extinction at 481 nm and the amount of released NBD-FA in the organic phase after extraction with acidic chloroform-MeOH was determined by photometry (squares) and TLC analysis/fluorescence imaging (diamonds), respectively. Cleavage in the absence of inhibitor was set at 100% for both measurements (n = 4, mean).

These compounds were used to assign the two peaks to the enantiomers (Fig. 8). Peak 1 corresponds to the *R*-enantiomer (4b) and peak 2 to the *S*-enantiomer (4a). Both enantiomers were tested separately as substrates for HSL (**Fig. 9**). No significant differences between the kinetics of their cleavage or compared with racemic mixtures were detected. Therefore, HSL does not discriminate between the two distinct MAG enantiomers.

Finally, we studied the inhibition of cleavage of NBD-MAG by rat adipocyte HSL in the presence of an unspecific lipase inhibitor, diisopropyl phosphofluoridate, which reacts efficiently with serine residues at the active site of a number of esterases (41–43). Analysis of the cleavage of NBD-MAG (by measuring the color shifts or performing TLC analysis on the NBD-FA released) yielded typical sigmoidal inhibition curves with increasing concentrations of diisopropyl phosphofluoridate (**Fig. 10**). The IC_{50} did not differ significantly between the extinction measurements (0.8 mM) and the TLC data (1.1 mM) and were in good agreement with previously published data on the inhibition of rat HSL (43–45).

In conclusion, the lipase assay method described here is based on the release of NBD-FA from NBD-MAG incorporated into PC/PI liposomes and can be used either in a discontinuous procedure (extraction, TLC analysis, and fluorescence imaging) or, more conveniently, to perform continuous kinetic recordings (extinction measurements). This method is characterized by its high sensitivity, its versatility with regard to the type of neutral lipase assayed, and its relatively low sensitivity to the solvents and proteins present in the incubation mixture.

The special characteristics of the novel lipase substrate NBD-MAG, namely *i*) a much higher signal-to-noise ratio during cleavage than that observed with the widely used nonlipidic substrates such as 4-methylumbelliferyl esters (this study) and *p*-nitrophenylbutyrate (S. Petry, H. Kleine, H. Jordan, N. Tennagels, and G. Müller, unpublished results), *ii*) high stability in the presence of oxygen, *iii*) easy handling during routine use, and *iv*) storage in a form readily available for use in the assay in combination with its lipidic (although admittedly unphysiological) structure, make the corresponding lipase assay a sound compromise between feasibility and the reflection of a typical interfacial lipase reaction, which to our knowledge is not achieved with any of the other currently available methods of assay. These advantages will become apparent during the screening (high-throughput) and kinetic characterization of novel modulators of neutral lipases, namely human HSL and LPL, which are interesting drug targets for the treatment of non-insulin-dependent diabetes mellitus and obesity (see introduction). In addition, this method should provide a useful means of analyzing crude serum and tissue samples to assess the neutral lipase activity that seems to be expressed in most mammalian cell types (46).

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