Use of an Inhibitor To Identify Members of the Hormone-Sensitive Lipase Family†

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ABSTRACT: Hormone-sensitive lipase (HSL) contributes importantly to the mobilization of fatty acids from the triacylglycerols stored in adipocytes, which provide the main source of energy in mammals. On the basis of amino acid sequence alignments and three-dimensional structures, this enzyme was previously found to be a suitable template for defining a family of serine carboxylester hydrolases. In this study, the HSL family members are characterized rather on the basis of their inhibition by 5-methoxy-3-(4-phenoxyphenyl)-3*H*-[1,3,4]oxadiazol-2-one (compound 7600). This compound inhibits mammalian HSL as well as other HSL family members, such as EST2 from the thermophilic eubacterium *Alicyclobacillus acidocaldarius* and AFEST from the hyperthermophilic archaeon *Archaeoglobus fulgidus*. Various carboxylester hydrolases that are not members of the HSL family were found not to be inhibited by compound 7600 under the same experimental conditions. These include nonlipolytic hydrolases such as *Torpedo californica* acetylcholinesterase and pig liver esterase, as well as lipolytic hydrolases such as human pancreatic lipase, dog gastric lipase, *Thermomyces lanuginosus* lipase, and *Bacillus subtilis* LipA. When vinyl esters were used as substrates, the residual activity of HSL, AFEST, and EST2 decreased with an increase in compound 7600 concentration in the incubation mixture. The inhibitor concentration at which the enzyme activity decreased to 50% after incubation for 5 min was 70, 20, and 15 nM with HSL, AFEST, and EST2, respectively. Treating EST2 and AFEST with the inhibitor resulted in an increase in the molecular mass, as established by performing matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis. This increase in the molecular mass, which corresponds approximately to the molecular mass of the inhibitor, indicates that a covalent enzyme−inhibitor complex has been formed. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry analysis of a trypsin digest of AFEST treated with the inhibitor or not treated showed the occurrence of an increase in the molecular masses of the "GESAGG"-containing peptide, which is compatible with the formation of a covalent complex with the inhibitor.

The superfamily of carboxylester hydrolases includes ubiquitous enzymes of considerable physiological significance with a wide range of substrate specificities (*1*). Among the members of the carboxylester hydrolase superfamily, we previously distinguished between the subclass of lipolytic carboxylester hydrolases (lipases, acting on lipids) and nonlipolytic carboxylester hydrolases (those not acting on lipids) (*2*). On the basis of amino acid sequence homology, the superfamily of carboxylester hydrolases has been subdivided into three groups, C, L, and H, which stand for carboxylesterases, lipases, and hormone-sensitive lipases (HSL),[1] respectively (*3*, *4*). Members of the H group show a remarkable level of similarity with mammalian HSL at the sequence and structural level and are known as the HSL family (*4*−*9*).

HSL is thought to play a crucial role in the mobilization of free fatty acid (FFA) from the triacylglycerols (TAG) stored in adipocytes (for a review, see ref *10*). In vivo, HSL is activated by phosphorylation via cAMP-dependent kinase in response to various lipolytic hormones such as catecholamines. FFA stored in the form of TAG in adipose tissues are the main source of energy in mammals. High plasma FFA levels impair insulin signaling processes and have been thought to be associated with insulin resistance and type 2 diabetes mellitus (*11*). Haemmerle et al. (*12*) have reported

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[1] Abbreviations: AchE, *Torpedo californica* acetylcholinesterase; AFEST, esterase from hyperthermophilic archaeon *Archaeoglobus fulgidus*; BSA, bovine serum albumin; DAG, diacylglycerol; DGL, dog gastric lipase; E600, diethyl *p*-nitrophenyl phosphate; EST2, esterase from the thermophilic eubacterium *Alicyclobacillus acidocaldarius*; FFA, free fatty acid(s); HPL, human pancreatic lipase; HSL, hormone-sensitive lipase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SELDI-TOF, surface-enhanced laser desorption ionization time-of-flight; PLEst, pig liver esterase; TAG, triacylglycerol; THL, tetrahydrolipstatin; TLL, *Thermomyces lanuginosus* lipase.

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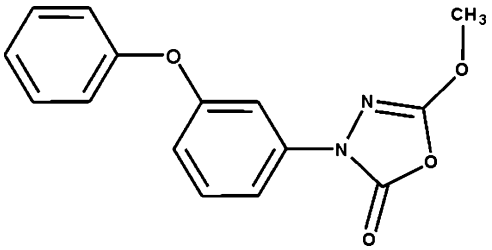

FIGURE 1: Chemical structure of 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-one (compound 7600).

that HSL deficiency in mice results in the accumulation of diacylglycerol (DAG) in various tissues. An adipose TAG lipase (ATGL) was identified (*13*) and proposed to be responsible for the initial step in TAG catabolism. HSL and ATGL may coordinately catabolize the TAG stored in mammalian adipose tissues (*13*).

The results of various biochemical studies (*14*−*16*) have suggested that HSL is composed of two domains, the N-terminal domain, which may interact with the adipocyte lipid-binding protein (*17*), and the C-terminal domain, which contains the catalytic site. A stretch of ∼150 amino acids named the regulatory module, which contains the phosphorylation sites, is located in the C-terminal domain of the HSL (*14*, *16*). It was recently reported (*18*) that the C-terminal portion of the HSL regulatory module appears to be involved in the lipid binding pocket. HSL is a lipolytic enzyme with a broad substrate specificity, which hydrolyzes long-chain TAG, DAG, and monoacylglycerol (MAG), as well as cholesteryl esters and retinyl esters (*10*, *19*).

In addition to HSL, the HSL family includes human liver arylacetamide deacylase (*20*) and various microbial carboxylester hydrolases such as Brefeldin A esterase (BFAE) from *Bacillus subtilis* (*7*), acetyl-hydrolase from *Streptomyces viridochromogenes* (*21*), lipase 2 from *Moraxella* TA144 (*22*), Rv1399c from *Mycobacterium tuberculosis* (*23*), heroin esterase from *Rhodococcus* sp. strain H1 (HerE) (*24*), and the thermophilic esterases from *Alicyclobacillus acidocaldarius* (EST2) (*8*, *25*) and *Archaeoglobus fulgidus* (AFEST) (*9*). The three-dimensional (3D) structures of HerE (*24*), BFAE (*7*), EST2 (*8*), and AFEST (*9*) have been determined. They all include a common topological α/*β*-hydrolase fold, which is also observed in lipases (*26*), consisting of a central parallel *β*-sheet core surrounded on both sides by α-helices containing the catalytic triad (Ser,Asp/Glu,His).

All the 3D structures of the members of the HSL family published so far (*7*−*9*, *24*) show the presence of two separate helical regions external to the α/*β*-core, forming a cap covering the active site. However, this putative cap appears to be structurally different from the lid occurring in various lipases (*18*). On the basis of kinetic studies with recombinant human HSL, we recently reported (*2*) that this enzyme was inhibited by serine esterase inhibitors such as phenylmethanesulfonyl fluoride and diethyl *p*-nitrophenyl phosphate (E600) in the absence of detergent. These results suggested that the catalytic serine of HSL is directly accessible to inhibitors (*2*).

We recently (*27*) carried out a comparative study on the substrate specificity and kinetic properties of enzymes belonging to the HSL family. The fact that the same substrate specificity was observed with HSL, EST2, and AFEST using short-chain acyl esters confirms that the HSL catalytic domain constitutes the basic structural characteristic common to the members of this protein family. However, the substrate specificity of HSL on long-chain acyl ester and its apparent K_m during the hydrolysis of short-chain acyl esters clearly distinguish HSL from the other HSL family members. A high-throughput screening procedure was used (*28*) to identify various classes of HSL inhibitors. Oxadiazolone is one of the most interesting classes, and 5-methoxy-3-(4-phenoxyphenyl)-3*H*-[1,3,4]oxadiazol-2-one (compound 7600, the structure of which is shown in Figure 1) was selected since it was found to be the most potent members of this class. Oxadiazolones with aliphatic moieties on N3 have been reported (*29*) to be acetylcholinesterase (AchE) inhibitors as well as potent insecticides. The aim of this study was to investigate the selective inhibition exerted on three members of the HSL family (HSL, EST2, and AFEST) by compound 7600. For the sake of comparison, we investigated the kinetics of the inhibition exerted on these three members of the HSL family, as well as on other nonlipolytic and lipolytic carboxylester hydrolases, by tetrahydrolipstatin (Orlistat, THL), a known digestive lipase inhibitor, and E600, a classical serine esterase inhibitor.

EXPERIMENTAL PROCEDURES

Reagents. 5-methoxy-3-(4-phenoxy-phenyl)-3H-[1,3,4]oxadiazol-2-one [compound 7600, molecular mass of 284 Da (Figure 1)] was synthesized as described previously (*30*). Tributyroylglycerol, dioleoylglycerol, vinyl butyrate, cholesterol oleate, E600, bovine serum albumin (BSA), and sodium taurodeoxycholate (NaTDC) were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). THL was from Hoffmann-La-Roche Ltd. (Basel, Switzerland). All other chemicals and solvents were of reagent quality or better and were obtained from local suppliers.

Proteins. Recombinant human HSL was expressed and purified from baculovirus-infected insect cells as described previously (*2*). The purified EST2 (*25*) and AFEST (*31*) were produced at the Institute of Protein Biochemistry (Naples, Italy). *Torpedo californica* AchE and pig liver esterase (PLEst) were obtained from Sigma-Aldrich Fine Chemicals. Recombinant human pancreatic lipase (HPL) was expressed and purified from insect cells as described by Thirstrup et al. (*32*). Recombinant dog gastric lipase (DGL) was provided by Meristem Therapeutics (Clermont-Ferrand, France). Recombinant *Thermomyces lanuginosus* lipase (TLL) was kindly provided by S. Patkar (Novo Nordisk, Copenhagen, Denmark). *B. subtilis* LipA was a gift from W. Quax (Groningen, The Netherlands). Porcine colipase was purified by J. De Caro (EIPL-CNRS, Marseille, France). The protein concentrations were determined using Bradford's procedure (*33*), with Bio-Rad Dye Reagent and BSA as the standard.

Enzymatic Activity Measurements. Enzymatic activity was assayed by measuring the amount of free fatty acid (FFA) released from mechanically stirred acylglycerol or vinyl ester solutions or emulsions, using 0.1 N NaOH with a pH-stat (TTT80 radiometer) adjusted to a fixed end point value. When olive oil, dioleoylglycerol, or cholesterol oleate was used as the substrate, gum arabic was used as an emulsifier as previously described (*2*). Each assay was performed in a thermostated (37 °C) vessel containing 0.25 mM Tris-HCl buffer and 150 mM NaCl. The HPL activity was measured

in the presence of porcine colipase at a colipase:lipase molar ratio of 5. The specific activities are expressed here in international units (IU) per milligram of enzyme. One unit corresponds to 1 μmol of fatty acid released per minute.

Inhibition of HSL, EST2, and AFEST by Compound 7600. The enzyme−inhibitor incubation method was used (*34*) to test, in aqueous medium and in the absence of substrate, whether any direct interactions may occur between the lipase and the inhibitor (*34*). Recombinant human HSL (0.2 mg/mL, final concentration of 2.3 μM) was dissolved in Na_2HPO_4 buffer (pH 7.5) containing 0.1 M NaCl, 20% glycerol, 3 μM β-mercaptoethanol, and 0.1% Nonidet-P. EST2 (0.1 mg/mL, final concentration of 2.9 μM) and AFEST (0.14 mg/mL^{-1}, final concentration of 4 μM) were dissolved in Tris-HCl buffer (pH 7.5). Compound 7600 (10 mM) was dissolved in dimethyl sulfoxide (DMSO). An aliquot of each of the enzymes mentioned above was preincubated at 25 °C with compound 7600 at an enzyme:inhibitor molar ratio of 1:10 (final DMSO concentration ranged from 2 to 3%). The residual enzyme activity was then measured at various incubation times, using solutions (in the case of EST2 and AFEST) or emulsions (in the case of HSL) of vinyl butyrate as the substrate (see above). Alternatively, each of the enzymes cited above was preincubated at various enzyme:inhibitor molar ratios for 5 min at 25 °C, and the residual enzyme activity was measured to determine the concentration of compound 7600 which reduced the enzyme activity to 50% of its initial value (IC_{50}). Control experiments were performed in the absence of inhibitor and with the same concentration of DMSO. It is worth noticing that DMSO at a final concentration of less than 5% has no effect on the enzyme activity. For the sake of comparison, the inhibitory properties of compound 7600 were also tested with AchE, PLEst, LipA, HPL, DGL, and TLL under the same experimental conditions. Inhibition of HPL, DGL, and TLL by compound 7600 requires the presence of NaTDC. At values above its critical micellar concentration, NaTDC forms mixed micelles with the inhibitor, thus inducing the opening of the lid covering the active site of these lipolytic enzymes.

Inhibition of HSL, EST2, and AFEST by THL or E600. Recombinant human HSL [0.2 mg/mL in Na_2HPO_4 buffer (pH 7.5) containing 0.1 M NaCl, 20% glycerol, 3 μM β-mercaptoethanol, and 0.1% Nonidet-P], AFEST [0.14 mg/mL in Tris-HCl buffer (pH 7.5)], or EST2 [0.1 mg/mL in Tris-HCl buffer (pH 7.5)] was preincubated at 25 °C with E600 (4 mM in an ethanolic solution) or THL (0.4 mM in an ethanolic solution) at an enzyme:inhibitor molar ratio of 1:20 for 30 min. In both cases, the final concentration of ethanol was 3%. The residual enzyme activity was then measured using solutions (in the case of EST2 and AFEST) or emulsions (in the case of HSL) of vinyl butyrate as the substrate. For the sake of comparison, the inhibitory effects of E600 or THL were also tested with AchE, PLEst, LipA, HPL, DGL, and TLL, under the same experimental conditions. Control experiments were performed in the absence of inhibitor.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry Analysis. MALDI-TOF mass spectrometry was performed on a Voyager DE-RP device (Perseptives Biosystems). A solution of sinapinic acid in water and acetonitrile (40:60, v/v) was used as a matrix. Enzymes were preincubated for 5 min at 25 °C with compound 7600 (10 mM in DMSO) or THL (10 mM in ethanol) at an enzyme:inhibitor molar ratio of 1:100. Blank experiments were performed without compound 7600 or THL. One microliter of nontreated or treated enzyme solution was mixed on the plate target with an equal volume of the above matrix, and the spot was allowed to air-dry. Ions were accelerated with an extraction voltage of 25 kV. Spectra were obtained by performing 256 successive laser shots. It is worth noting that when proteins are being studied, an error of less than 0.5% can be expected to occur with MALDI-TOF analysis.

Surface-Enhanced Laser Desorption Ionization-TOF (SELDI-TOF) Mass Spectrometry Analysis. The AFEST protein was preincubated for 5 min at 25 °C with compound 7600 (10 mM in DMSO) at an enzyme:inhibitor molar ratio of 1:100. Labeled and unlabeled AFEST (3.5 μg each) were fractionated via SDS−PAGE, and after mass-compatible silver staining, bands were excised from the gel and treated with trypsin gold (mass spectrometry grade) as follows. Gel pieces were incubated (twice for 10 min each) in 0.4 mL of 0.1 M ammonium bicarbonate (pH 8) on a shaker at room temperature, washed with 0.5 mL of 50% acetonitrile in 0.1 M ammonium bicarbonate (pH 8), and dehydrated by being incubated (for 15 min) in 50 μL of 100% acetonitrile and then dried on a SpeedVac (15 min) after solvent removal.

Gel pieces were incubated in 10−40 μL of 0.01 μg/μL trypsin in 25 mM ammonium bicarbonate (pH 8) for 16 h at 37 °C. Aliquots of 1−2 μL were spotted onto an NP 20 ProteinChip array (Ciphergen Biosystem Inc.), overloaded with 1 μL of α-cyano-4-hydroxycinnamic acid solution (20% saturation in 25% acetonitrile, 0.25% TFA), and read on a SELDI-TOF Personal Model Series 4000 device (Ciphergen Biosystem Inc.). Calibration was performed with the All-in-1 peptide standard (range of 1000−7000; Ciphergen Biosystem Inc.). Protein identification was performed by carrying out a peptide mass search with Profound (http://bioinformatics.genomicsolutions.com/service/prowl/).

RESULTS

Inhibition of HSL, EST2, and AFEST by Compound 7600. The specific activity of HSL was measured on emulsified vinyl butyrate and found to be 150 units/mg. The specific activities of EST2 and AFEST on molecular solutions of vinyl butyrate were 3366 and 355 units/mg, respectively. The specific activities of HSL, EST2, and AFEST were also measured using olive oil, dioleoylglycerol, and cholesterol oleate emulsified with gum arabic. No lipolytic activity was detected with EST2 and AFEST. However, the specific activity of HSL was found to be around 4, 30, and 20 units/mg using emulsified olive oil, dioleoylglycerol, and cholesterol oleate, respectively.

To test the effects of compound 7600 on the catalytic activity of the enzymes mentioned above, the inhibitor was preincubated with HSL, EST2, or AFEST at an enzyme:inhibitor molar ratio of 1:10 and the residual activity was measured as a function of the incubation time (Figure 2A). The residual activity of HSL decreased rapidly after incubation for a few seconds with compound 7600 and reached a plateau value at around 10−20% in the case of HSL, 10% in the case of EST2, and 5% in the case of AFEST after incubation for approximately 20 min (Figure 2A).

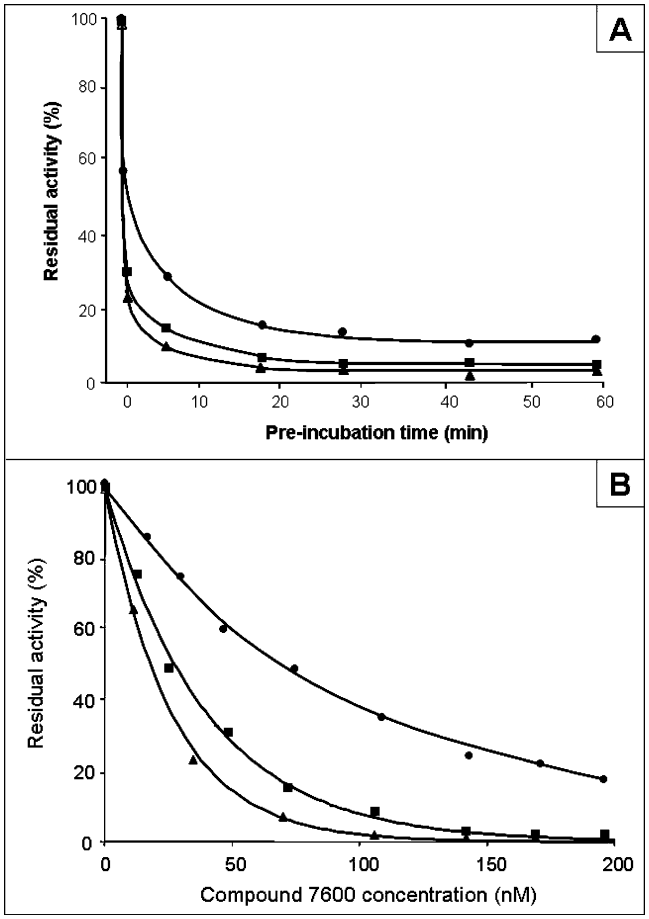

FIGURE 2: Residual activities of HSL, EST2, and AFEST, measured on vinyl butyrate using the pH-stat technique. (A) Residual activities of HSL (●), EST2 (▲), and AFEST (■) measured as a function of the incubation time at an enzyme:compound 7600 molar ratio of 1:10. (B) Effects of increasing concentrations of compound 7600 on the rate of hydrolysis of vinyl butyrate by HSL (●), EST2 (▲), and AFEST (■). Each of the latter enzymes was preincubated at various enzyme:compound 7600 molar ratios for 5 min at 25 °C. Kinetic assays were performed in a mechanically stirred thermostated (37 °C) vessel containing solutions or emulsions of vinyl butyrate in 0.25 mM Tris-HCl buffer and 150 mM NaCl.

The effects of increasing concentrations of compound 7600 on the residual activities of HSL, EST2, and AFEST are shown in Figure 2B. The residual activity of HSL, AFEST, and EST2 decreased with increasing concentrations of compound 7600 (Figure 2B). The IC_{50} values were found to be 70, 20, and 15 nM with HSL, AFEST, and EST2, respectively.

Effects of Compound 7600 on Other Carboxylester Hydrolase Activities. For the sake of comparison, the inhibitory effects of compound 7600 were tested under the same experimental conditions using lipolytic (LipA, HPL, DGL, and TLL) and nonlipolytic (AchE and PLEst) hydrolases which are not members of the HSL family. Each of the above-mentioned enzymes was preincubated with compound 7600 for 30 min at an enzyme:inhibitor molar ratio of 1:20. The residual activities were then measured on vinyl butyrate (in the case of HSL, AFEST, EST2, and PLEst), vinyl acetate (in the case of AchE), and tributyroylglycerol (in the case of LipA, HPL, TLL, and DGL),

The data obtained in these experiments are summarized in Table 1. The residual activities of LipA, HPL, DGL, TLL, AchE, and PLEst were found to be 80, 94, 91, 90, 90, and 87, respectively. These values obtained in the absence of NaTDC are similar to those obtained in the presence of NaTDC in the incubation medium (Table 1). Under the same experimental conditions, the catalytic activities of the members of the HSL family (HSL, EST2, and AFEST) were completely abolished (Table 1).

Table 1: Residual Activities of HSL, EST2, AFEST, AChE, PLEst, LipA, HPL (with a 5-fold molar excess of colipase), DGL, and TLL Measured on Vinyl Butyrate (in the case of HSL, AFEST, EST2, and PLEst), Vinyl Acetate (in the case of AchE), and Tributyroylglycerol (in the case of LipA, HPL, TLL, and DGL) Using the pH-stat Technique[a]

		residual activity (%)					
		7600		THL		E600	
	enzyme	without NaTDC	with NaTDC	without NaTDC	with NaTDC	without NaTDC	with NaTDC
H	HSL	**0**	**0**	**8**	**8**	**7**	**7**
	EST2	**0**	**0**	**7**	**7**	**0**	**0**
	AFEST	**0**	**0**	**5**	**5**	**0**	**0**
C	PLEst	87	87	**9**	**9**	**0**	**0**
	AChE	90	90	100	100	**0**	**0**
L	LipA	80	82	8	**7**	**6**	**6**
	HPL	94	82	66	**8**	90	**5**
	DGL	91	78	70	**7**	93	**10**
	TLL	90	80	55	**7**	96	**7**

[a] Each enzyme was preincubated with compound 7600, E600, or THL for 30 min at an enzyme:inhibitor molar ratio of 1:20. The experiments were carried out in the absence or presence of 4 mM NaTDC in the incubation medium. The residual activities of each enzyme were measured as described in Experimental Procedures. Results are expressed as means of at least two assays. The inhibitory effects exerted on carboxylester hydrolases (in the presence of NaTDC) by compound 7600, THL, and E600 are given in boldface type. Families H, C, and L of carboxylester hydrolases as previously decribed (*4*, *6*, *47*) are shown at the left.

Inhibition of HSL, EST2, AFEST, and Other Carboxylesterases by THL and E600. For the sake of comparison, the inhibitory effects of other known carboxylester hydrolase inhibitors (E600 and THL) were studied with enzymes belonging to the HSL family (HSL, AFEST, and EST2) as well as other carboxylester hydrolases (AchE, PLEst, LipA, HPL, DGL, and TLL). As shown in Table 1, preincubating E600 with HSL, EST2, AFEST, AchE, PLEst, or LipA for 30 min at an enzyme:inhibitor molar ratio of 1:20 leads to nearly 100% inactivation of the enzyme, whereas HPL, DGL, and TLL activities were significantly abolished only in the presence of NaTDC (4 mM, final concentration) in the incubation medium (Table 1).

Under the same experimental conditions that are described above, THL reduced the activities of HSL, EST2, AFEST, PLEst, and LipA by ~90−95% in both the absence and the presence of NaTDC in the incubation medium. The same levels of inhibition were obtained with HPL, DGL, and TLL in the presence of NaTDC in the incubation medium (Table 1). In the absence of NaTDC, these lipolytic enzyme activities were reduced by THL to 55−70% of their initial values. It is worth noticing that the activity of AchE was not affected by THL in the absence or presence of NaTDC (Table 1).

Analysis of EST2− and AFEST−Compound 7600 Complexes with MALDI-TOF Mass Spectrometry. After preincubation of EST2 or AFEST for 10 min with compound 7600 (enzyme:compound 7600 molar ratio of 1:100) at 25 °C and

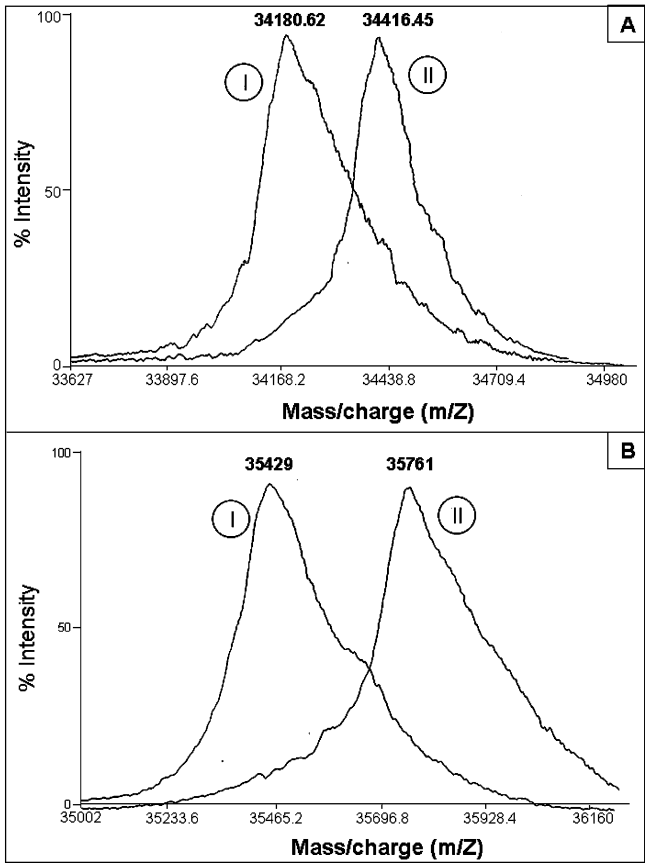

FIGURE 3: MALDI-TOF mass spectrometry analysis of nontreated EST2 (A, spectrum I) or AFEST (B, spectrum I) and compound 7600-treated EST2 (A, spectrum II) or AFEST (B, spectrum II) at an enzyme:compound 7600 molar ratio of 1:100. MALDI-TOF mass spectrometry analysis was conducted as described in Experimental Procedures.

pH 7.5, the catalytic activities of these enzymes were completely abolished. A sample was analyzed by MALDI-TOF mass spectrometry as described in Experimental Procedures. Figure 3A shows typical spectral recordings of nontreated EST2 (spectrum I) and EST2 treated with compound 7600 (spectrum II). As can be seen from these spectra, the molecular masses of EST2 alone and EST2 treated with the inhibitor were 34 180 and 34 416 Da, respectively (Figure 3A). It is worth noticing that a mass shift of around 236 Da was observed: this difference corresponds approximately to the molecular mass of compound 7600 (284 Da).

Under the same experimental conditions that are described above, the spectral recordings obtained with nontreated AFEST (spectrum I) and AFEST treated with compound 7600 (spectrum II) are shown in Figure 3B. A mass shift of around 338 Da was observed, which corresponds approximately to the molecular mass of compound 7600 (284 Da).

Figure 4 shows the spectra obtained before and after preincubation of AFEST for 10 min with THL (enzyme:THL molar ratio of 1:100) at 25 °C and pH 7.5. Spectrum I gives the molecular mass of untreated AFEST (35 413 Da), and spectrum II gives that of the AFEST treated with THL (35 963 Da). The shift of around 550 Da observed here was probably due to the covalent binding of THL (496 Da) to AFEST. Under the same experimental conditions, similar

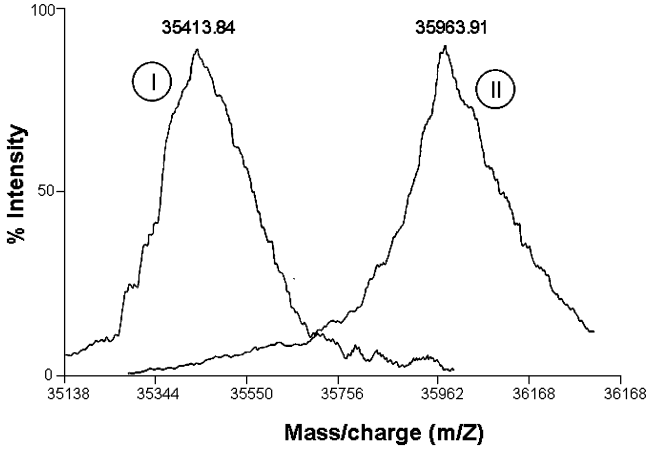

FIGURE 4: MALDI-TOF mass spectrometry analysis of nontreated (spectrum I) and THL-treated (spectrum II) AFEST at an AFEST:THL molar ratio of 1:100. The MALDI-TOF mass spectrometry analysis was conducted as described in Experimental Procedures.

results were obtained in the case of EST2 treated with THL (data not shown).

Analysis of the AFEST−Compound 7600 Complex with SELDI-TOF Mass Spectrometry. Preincubating AFEST for 10 min with compound 7600 (enzyme:compound 7600 molar ratio of 1:100) at 25 °C and pH 7.5 leads to complete inactivation of the enzyme. A sample of the incubation medium was subjected to in-gel tryptic cleavage after SDS−PAGE analysis, and the peptides that were obtained were analyzed by SELDI-TOF mass spectrometry as described in Experimental Procedures. Results are given in Figure 5. The rate of sequence coverage for nontreated AFEST and AFEST treated with compound 7600 was found to be 35 and 43%, respectively. Peptide 154−174 (IFVGGDSAGGNLAAAVSIMAR) contains the Ser160 residue in the active site of the enzyme. The peak corresponding to this peptide (1978 Da) is present in the case of untreated AFEST but not in the case of AFEST treated with the inhibitor. In the latter case, a new peptide with a molecular mass of 2271 Da was detected. This molecular mass of 2271 Da likely corresponds to the peptide of 1978 Da plus the molecular mass of the inhibitor (284 Da) in the case of AFEST treated with compound 7600 (2262 Da).

DISCUSSION

The HSL family is an increasingly large group of proteins exhibiting structural similarities with the catalytic domain of HSL. Amino acid sequence alignment of EST2 (residues 81−283), AFEST (residues 87−286), and the catalytic domain of HSL (residues 349−724) showed the existence of ∼23% identical sequence among these three family members in a 201-amino acid stretch (Figure 6). This family of carboxylester hydrolases is characterized by the conserved HGGG consensus sequence (see Figure 6), which is involved in hydrogen bonding interactions promoting the stabilization of the oxyanion hole and in the process of catalysis (*7*, *8*, *35*).

The 3D structures of EST2, AFEST, BFAE, and the modeled catalytic domain of HSL were found to be superimposable (*36*). These results suggest that the 3D structures of these nonlipolytic carboxylester hydrolases (EST2, AFEST, and BFAE) are homologous to that of the catalytic

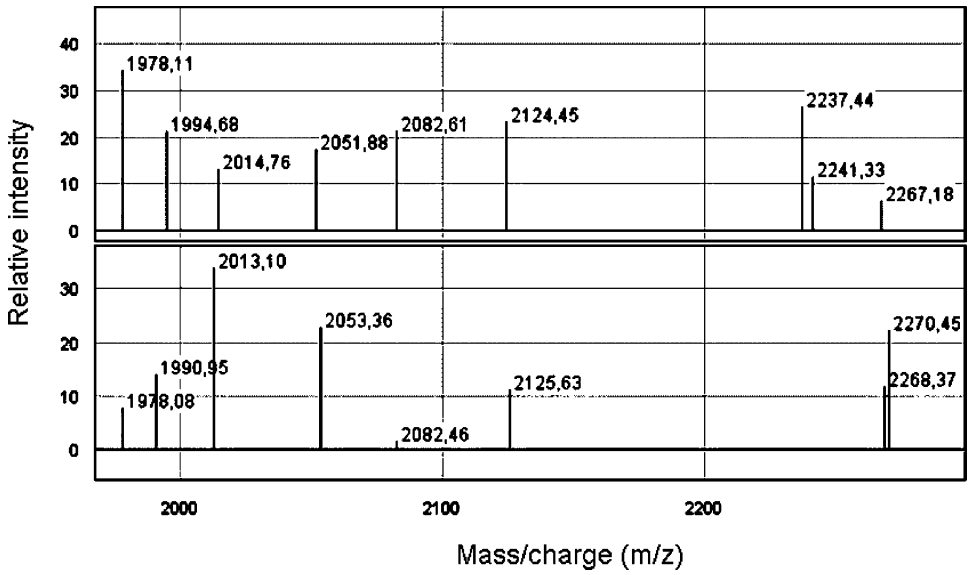

FIGURE 5: Tryptic peptide map of nontreated AFEST (top) and AFEST treated with compound 7600 (bottom). Zoom on the region of interest. The two spectra were normalized to the total current ion and aligned with the instrument software. The peak at 1978 Da corresponds to peptide 154−174 (IFVGGDSAGGNLAAAVSIMAR), while the peak at 2270 likely corresponds to the same peak plus compound 7600.

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EST2     81  HGGGWVVGDLETHDPVCRVLAKDGRAVVFSVDYRLAPEHKFPAAVEDAYDALQWIAERAADFHLDPARIAVGGDSAGGNL
AFEST    87  HGGGFVICSIESHDALCRRIARLSNSTVVSVDYRLAPEHKFPAAVYDCYDATKWVAENAEELRIDPSKIFVGGDSAGGNL
HSL     347  HGGGFVAQTSRSHEPYLKSWAQELGAPIISIDYSLAPEAPFPRALEECFFAYCWAIKHCALLGSTGERICLAGDSAGGNL
             ****:*    .:*:.  :  *:   : :.*:** ****  ** *: :.: *  *  :..  :     :* :.********

EST2    161  AAVTSILAKERGGPALAFQLLIYPSTGYDPAHPPASIEENAEGYLLTGGMSLWFLDQYLN--------------------
AFEST   167  AAAVSIMARDSGEDFIKHQILIYPVVNFVAPTPSLLEFG-EGLWILDQKIMSWFSEQYFS--------------------
HSL     427  CFTVALRAAAYGVRVPDGIMAAYPATMLQPAASPSRLLS-LMDPLLPLSVLSKCVSAYAGAKTEDHSNSDQKALGMMGLV
             . ..:: *   *       :  ** .   .. ..          :*   :     . * .

EST2         ------------------------------------------------------------------------------
AFEST        ------------------------------------------------------------------------------
HSL     506  RRDTALLLRDFRLGASSWLNSFLELSGRKSQKMSEPIAEPMRRSVSEAALAQPQGPLGTDSLKNLTLRDLSLRGNSETSS

EST2         ------------------------------------------------------------------------SLEELTHP
AFEST        ------------------------------------------------------------------------REEDKFN
HSL     586  DTPEMSLSAETLSPSTPSDVNFLLPPPEDAGEEAEAKNELSPMDRGLGVRAAFPEGFHPRRSSQGATQMPLYSSPIVKNP

EST2    229  WFSPVLYPD--LSGLPPAYIATAQYDPLRDVGKLYAEALNKAGVKVEIENFEDLIH  282
AFEST   233  PLASVIFAD--LENLPPALIITAEYDPLRDEGEVFGQMLRRAGVEASIVRYRGVLH  285
HSL     666  FMSPLLAPDSMLKSLPPVHIVACALDPMLDDSVMLARRLRNLGQPVTLRLVEDLPH  723
              ::.:: .*  *..***. * :.  **: * . : .. *.. *  . :   ..: *
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FIGURE 6: Alignment of amino acid sequences of HSL (residues 347−723), EST2 (residues 81−283), and AFEST (residues 87−286). The sequences were aligned using CLUSTALW (http://www2.igh.cnrs.fr/bin/clustalw-guess.cgi) and then adjusted manually to achieve maximum alignment. Single dots, double dots, and stars give the level of conservation, in decreasing order (stars give the most highly conserved residues). Catalytic triad residues are indicated with arrowheads.

domain of HSL and may constitute the minimum structural component required for the catalytic process to occur. To confirm this hypothesis, we recently (*27*) carried out a comparative study of the substrate specificity and kinetic properties of enzymes belonging to the HSL family. The fact that the same substrate specificity was observed with HSL, EST2, and AFEST using short-chain acyl esters confirms that the HSL catalytic domain is a minimum catalytic requirement in the members of this protein family. HSL is the only carboxylester hydrolase that belongs to the HSL family having clearly detectable lipolytic activity on long-chain TAG, DAG, MAG, and cholesterol ester substrates (*2*, *10*, *19*). When olive oil, dioleoylglycerol, or cholesterol oleate was used as the substrate, no lipolytic activity was found to occur with EST2 and AFEST. These findings, along with those obtained in our previous study (*27*), clearly distinguish HSL from the other HSL family members.

These data clearly show that compound 7600 specifically inhibits the HSL family members (HSL, EST2, and AFEST) tested in this study. The IC_{50} values were found to be 70, 20, and 15 nM with HSL, AFEST, and EST2, respectively. These results may indicate that this inhibitor reacts more efficiently with EST2 and AFEST than with HSL. No significant inhibitory effects were observed with other carboxylester hydrolases that are not members of the HSL family, including nonlipolytic hydrolases such as AchE and PLEst and lipolytic hydrolases such as HPL, DGL, LipA, and TLL (see Table 1). It was previously reported that HPL (*37*), porcine pancreatic lipase (*38*), and human gastric lipase (*37*) were inhibited by E600 only in the presence of a

detergent such as NaTDC. Similar results were obtained in the case of the inhibition exerted on HPL by THL (*39*). It has been established that the active Ser152 site in porcine pancreatic lipase (*40*) and in HPL (*41*) was covalently labeled with E600 and THL, respectively. It was suggested that bile salts at levels above their CMC may form mixed micelles, including the E600 inhibitor, thus inducing the opening of the lid on the active site of these lipolytic enzymes. Here we confirmed these findings in the case of HPL, DGL, and TLL (see Table 1).

Furthermore, in the case of HSL, EST2, and AFEST, the inhibitory effects exerted by compound 7600 were also observed in the absence of NaTDC. On the other hand, we noted that HSL as well as EST2 and AFEST is also inhibited by E600 in the absence of NaTDC (see Table 1). These results suggest that the catalytic serine of these HSL family members is reactive and readily accessible. It can therefore be concluded from both the data presented here and previous data (*2*) that the inhibitory effects exerted by E600, THL, and compound 7600 in the absence of detergent (see Table 1) could be used as an experimental criterion for predicting the absence of a lid domain in carboxylester hydrolases. In addition, using a water molecule as the probe, the area of AFEST and EST2 giving access to the catalytic Ser was calculated using Turbo-Frodo (*42*) and found to be 20 and 13 Å^2, respectively. These values are comparable to that obtained in the case of cutinase (4 Å^2), whereas the catalytic Ser of lipolytic carboxylester hydrolases (HPL and TLL) in the closed conformation was found to be totally inaccessible (accessible area of 0 Å^2). De Simone et al. (*43*) have previously speculated that the presence of a cap domain covering the EST2 active site might be structurally analogous to the lid domain of lipases (*43*). These authors further hypothesized on the basis of homology modeling with EST2 that the HSL region of P270−D308 may be involved in structural rearrangements similar to those observed in EST2 and perhaps controlling the accessibility to the enzyme's active site (*43*). However, the cap putatively present in these structures seems to be structurally different from the lid found to exist in several lipases. On the basis of the area accessible to the catalytic Ser and the inhibition kinetics of members of the HSL family (EST2, AFEST, and HSL), we clearly established that the catalytic serine of these HSL family members is readily accessible and confirmed that the 3D structure of HSL may therefore lack the lid domain covering the active site.

Treating carboxylesterases belonging to the HSL family (EST2 and AFEST) with compound 7600 resulted in the complete inhibition of these enzymes and in a concomitant increase in the molecular mass, as shown by the MALDI-TOF mass spectrometry analysis data. This increase in the molecular mass corresponds approximately to the molecular mass of the inhibitor (see Figure 3), which suggests that the formation of a stoichiometric covalent enzyme−inhibitor complex has occurred. Furthermore, SELDI-TOF mass spectrometry analysis of a trypsin digest of untreated AFEST or AFEST treated with compound 7600 showed differences between the peptide signals which were compatible with the molecular masses of the "GESAGG"-containing peptide and its complex with the inhibitor. This process might involve a nucleophilic attack by the hydroxy group of the enzyme's catalytic Ser on the carbon atom of the carbonyl moiety of the inhibitor's oxadiazolone ring, leading to the formation of a covalent enzyme−inhibitor intermediate (Figure 7). It is also possible that the covalent enzyme−inhibitor intermediate may be further hydrolyzed, releasing an adduct and the active enzyme form. The deacylation reaction may be faster with HSL than with EST2 and AFEST, which is in line with the IC_{50} values obtained with HSL (70 nM), EST2 (20 nM), and AFEST (15 nM) (see Figure 2). On the basis of chemical modeling studies, Huang and Bushey (*29*) have previously suggested that the opening of the ring may result from a nonenzymatic nucleophilic attack on the carbonyl group of the oxadiazolone heterocycle. A reaction occurring between the active site serine of the enzyme and the methyl ester of compound 7600 might provide an alternative mechanism. In this case, the nucleophilic attack by the hydroxyl group of the enzyme's catalytic Ser on the C═N double bond of the oxadiazolone ring would lead to the release of the methanol group. However, this possibility is unlikely to occur. We investigated the resistance of compound 7600 to several nucleophiles such as butylamine and analyzed the products (data not shown). In all the cases that were tested, the oxadiazolone ring was opened, whereas the methoxy group remained intact. In addition, on the basis of the SELDI-TOF data (Figure 5), the possible occurrence of the methanol release mechanism can be ruled out. No peaks were observed which might have supported this suggestion.

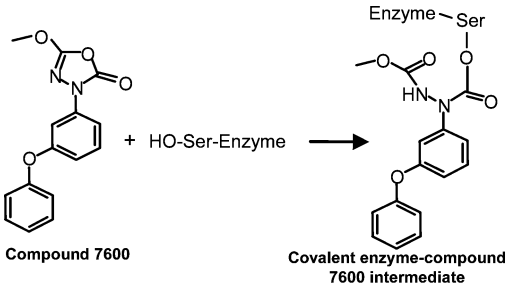

FIGURE 7: Mechanism possibly underlying the inhibition exerted on EST2, AFEST, or HSL by compound 7600. The acyl−enzyme intermediate is formed by the nucleophilic attack by the hydroxy group of the enzyme's catalytic Ser on the carbonyl atom of the carbonyl moiety of the inhibitor's oxadiazolone ring, leading to the formation of an enzyme-bound intermediate.

The formation of a covalent complex was also observed in the case of the inhibitory effects of THL exerted on EST2 (data not shown) or AFEST (see Figure 5). As reported during the inhibition of various lipases by THL (*41*, *44*, *45*), a transient acyl−enzyme intermediate is formed after the nucleophilic attack by the hydroxy group of the catalytic serine of EST2 or AFEST on the β-lactone ring of THL.

The formation of a covalent enzyme−compound 7600 complex described above in the case of EST2 and AFEST might also be expected to occur in that of HSL. However, no such covalent complex formation was detected here using MALDI- or SELDI-TOF mass spectrometry methods. On one hand, the high molecular mass of human recombinant HSL (88 000 Da) made it difficult to accurately determine by performing MALDI-TOF mass spectrometry analysis whether a shift in the molecular mass of around 284 Da (molecular mass of compound 7600) had occurred. On the other hand, the covalent HSL−compound 7600 complex may be rapidly deacylated during the incubation of the enzyme with the inhibitor, resulting in the reactivation of the enzyme

(data not shown). The latter hypothesis may explain why the modified HSL peptide containing the active site serine was not detected when SELDI-TOF mass spectrometry analysis was performed.

Comparisons between the nonlipolytic and lipolytic carboxylester hydrolase inhibition exerted by compound 7600, E600, and THL (see Table 1) indicated that compound 7600 is specific to the HSL family members that were tested (HSL, EST2, and AFEST), whereas THL and E600 significantly inhibit the HSL family members (HSL, EST2, and AFEST) as well as PLEst in the presence of NaTDC and lipolytic carboxylhydrolases (HPL, DGL, LipA, and TLL). It is worth noticing that AchE is inhibited by only E600, whether NaTDC is present. It seems likely that due to steric hindrance, the THL molecule and compound 7600 may not be able to reach the AchE active site gorge, in which the catalytic serine is deeply buried (*46*).

In conclusion, the HSL family was characterized here for the first time at the biochemical level in terms of the inhibition exerted on the members of this family by oxadiazolone (compound 7600). In addition, the inhibition of carboxylesterases belonging to the HSL family (EST2 and AFEST) resulted in a covalent modification of the active site serine and in a concomitant increase in the molecular mass, corresponding in size to the expected molecular mass of the inhibitor.

Studies of this kind targeting FFA metabolism may provide useful information about the fundamental causes of insulin resistance and type 2 diabetes and may thus lead to the development of new therapeutic methods. The data obtained in this study on the specific inhibition of HSL should therefore be of pharmacological interest and could be used to find means of reducing FFA levels and decreasing peripheral insulin resistance. Studies on the inhibition of HSL in adipocytes are now under way at our laboratory using in vivo models.

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