REVERSIBLE FORMATION OF FATTY ACID ESTERS OF BUDESONIDE, AN ANTIASTHMA GLUCOCORTICOID, IN HUMAN LUNG AND LIVER MICROSOMES

ANDERS TUNEK, KARIN SJÖDIN, AND GÖSTA HALLSTRÖM
Preclinical Research and Development, Astra Draco AB

(Received May 8, 1997; accepted July 17, 1997)

ABSTRACT:
Microsomes from human lung and liver catalyze the formation of fatty acid esters of budesonide, a glucocorticoid used for inhalation treatment of asthma. The conjugation was dependent on coenzyme A and ATP. Addition of free fatty acids to the incubations affected the pattern of metabolites, but ester formation was observed also without such addition. Budesonide oleate, palmitate, linoleate, palmitoleate, and arachidonate were identified as metabolites. The fatty acid conjugates of budesonide were shown to be substrates for lipase in vitro, thus budesonide is regainable from the conjugates.

Budesonide is an antiinflammatory glucocorticoid used against asthma as a dry powder inhalation formulation. Its clinical properties (1, 2) and its pharmacokinetics and metabolism (3, 4) have been extensively investigated. Recently, in a study using a rat trachea superfusion model (5), a new route of metabolism for budesonide was discovered. It was noticed that a small fraction of a budesonide dose was retained by the tracheal tissue and only slowly released on extended superfusion with blank buffer. Surprisingly, liquid chromatography (LC) of ethanol extracts from the tracheas showed that unchanged budesonide comprised only a minor part of the radioactivity retained in the tissue. Instead, some very lipophilic metabolite(s) dominated the chromatograms. Subsequently, formation of metabolites with similar LC-migration properties was observed when budesonide was added to a number of biological systems, including human lung and liver microsomes.

The present study was performed to study the formation of lipophilic metabolites of budesonide in human liver and lung microsomes, to define partly the biochemistry of the process, and to identify the metabolite(s).

Materials and Methods
Chemicals. Budesonide ((22RS)-16α,17α-butyldenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione) (fig. 1A) was obtained from Astra Pharmaceutical Productions, Södertälje, Sweden. [3H]Budesonide (fig. 1B) (radiochemical purity 98.7%, chemical purity 99.0%, specific activity 0.62 TBq/mmol, stock solution 40.2 MBq/ml ethanol), [2H8]budesonide (fig. 1C), budesonide 21-oleate, budesonide 21-palmitoleate, and budesonide 21-palmitate were synthesized at the Department of Medicinal Chemistry, Astra Draco AB, Lund, Sweden.

The data suggest that an equilibrium between budesonide and these pharmacologically inactive lipoidal conjugates will be established in tissues at repeated exposure to budesonide. Since the fatty acid conjugates most likely will be retained intracellularly for a longer time than unchanged budesonide, the duration of tissue exposure to budesonide will depend partly on the rate of lipase-catalyzed hydrolysis of the conjugates. The findings in this study provide a possible explanation for the efficacy of budesonide in mild asthmatics also when inhaled once daily.
immediately mixed with 100 μl of ethanol. The ethanol eluate was evaporated under a stream of N2 at 2 ml of 70% ethanol followed. The fatty acid conjugates were then eluted with 2 ml of 70% ethanol before the sample was applied. A washing step with a 60-min incubation at 37°C, the reaction was stopped by adding ethanol to a final concentration of 70%. The sample was kept on ice for a few minutes before being centrifuged (4 min at 15,000 g). The concentration of ethanol in the incubations was 0.5%. Aliquots of 150 μl were withdrawn at 0, 1, 3, 5, and 10 min and immediately mixed with 100 μl of ethanol. The samples were kept on ice for a few minutes before being centrifuged at 15,000g for 4 min. 200 μl of the supernatant was injected into LC system 3, and budesonide was quantified.

Chromatography. LC system used for conjugate formation experiments (LC system 1). The chromatography equipment used was from Varian (pump 9012, injector 9100) and controlled by Varian LC Star System. The column was a Li Chro CART 125–4 (RP-18), 125 mm, 5 μm. The samples were chromatographed isocratically with acetonitrile/methanol/25 mM phosphoric acid (46/46/8). The flow rate was 0.8 ml/min. Fractions were collected (1 min/fraction during 50 min), and radioactivity was determined by liquid scintillation counting.

In this system, unchanged steroids eluted with the solvent front. Approximate retention times for steroid fatty acid esters were as follows: budesonide oleate, 35–36 min; budesonide palmitoleate, 21 min; budesonide palmitate, 35–36 min.

LC system used for LC-MS (LC system 2). Two Pharmacia LKB 2150 pumps, controlled by Autochrom software via a CIM114 interface, and a Valco manual injector (50-μl loop) were used on line with the mass spectrometer. Analytical column: Applied Biosystems Brownlee Spheri-5 RP-8, 30 × 2.1 mm, 5 μm. For the mobile phase gradient, an isocratic phase (1 min) of 82% methanol (aq., v/v) with 50 mM sodium acetate was followed by 9 min of linear gradient to 91% methanol with 50 mM sodium acetate. After a 2-min isocratic period at 91% methanol, the system was returned to initial values. The flow rate was 0.20 ml/min.

Unchanged steroids eluted with the front in this system. The retention times for the various steroid fatty acid esters are seen in fig. 6.

LC system used for hydrolysis experiments (LC system 3). The chromatography equipment used was as follows: Waters M510 pumps, WISP 712 (autoinjector), Waters automated gradient controller M680, Waters data module 740 (integrator), and a Metric diode array detector model 1000s set at 245 nm. For tuning and calibration of the mass analyzer, background cluster ions (CH₃COONa)₃(CH₃COO⁻) from the mobile phase were used. The capillary and tube lens voltages of the ESI interface were optimized using reference budesonide oleate. Full scan negative ion mass spectra were recorded (mass range 400 – 850 in 2 sec) scanning Q1. MS/MS was performed with Ar gas (1.5 mtorr, 0.2 Pa) in the collision cell (Q2) and a collision energy of 15 eV. Product ion spectra of selected [M+CH₂COO⁻] ions (MAC⁻) were recorded.

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\text{Fig. 1. Structural formulas of budesonide (A, with some of the numbering indicated), } ^3\text{H}-\text{budesonide (B), and } ^3\text{H}_8-\text{budesonide (C).}
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\[^{[3\text{H}]\text{budesonide, keeping the same initial concentration (100 } \mu\text{M}) of total glucocorticoid. No external fatty acids were added to these incubations. After a 60-min incubation at } 37{ }^\circ\text{C, the reaction was stopped by adding ethanol to a final concentration of 70%. The sample was kept on ice for a few minutes before being centrifuged (4 min at 15,000g). The lipophilic metabolites (4–8% of the total budesonide content) and the remaining budesonide were separated on a BondElut solid phase extraction column (C18, 1 ml, Varian). The BondElut column was first activated with 2 ml of ethanol and then equilibrated with 2 ml of 70% ethanol before the sample was applied. A washing step with 2 ml of 70% ethanol followed. The fatty acid conjugates were then eluted with 1 ml of ethanol. The ethanol eluate was evaporated under a stream of N₂ at room temperature. The residue was dissolved in 50 μl of 90% methanol and centrifuged prior to analysis by LC-MS (10–15 μl injected).

Hydrolysis experiments. The porcine pancreas lipase and the bovine pancreas cholesterol esterase were dissolved in 0.1 M K₂HPO₄ buffer, pH 7.5, at a concentration of 0.25 mg/ml and 0.0125 mg/ml, respectively. In the case of cholesterol esterase, the incubation buffer also contained 10 mM taurocholate. The incubation mixtures were temperated for 5 min with gentle shaking in a water bath at 37°C. The incubation was started by adding the substrate (budesonide oleate or budesonide palmitoleate dissolved in ethanol) at an initial concentration of 5 μM. The concentration of ethanol in the incubations was 0.5%. Aliquots of 150 μl were withdrawn at 0, 1, 3, 5, and 10 min and immediately mixed with 100 μl of ethanol. The samples were kept on ice for a few minutes before being centrifuged at 15,000g for 4 min. 200 μl of the supernatant was injected into LC system 3, and budesonide was quantified.

Chromatography. LC system used for LC-MS (LC system 2). Two Pharmacia LKB 2150 pumps, controlled by Autochrom software via a CIM114 interface, and a Valco manual injector (50-μl loop) were used on line with the mass spectrometer. Analytical column: Applied Biosystems Brownlee Spheri-5 RP-8, 30 × 2.1 mm, 5 μm. For the mobile phase gradient, an isocratic phase (1 min) of 82% methanol (aq., v/v) with 50 mM sodium acetate was followed by 9 min of linear gradient to 91% methanol with 50 mM sodium acetate. After a 2-min isocratic period at 91% methanol, the system was returned to initial values. The flow rate was 0.20 ml/min.

Unchanged steroids eluted with the front in this system. The retention times for the various steroid fatty acid esters are seen in fig. 6.

Mass Spectrometry (MS). A Finnigan TSQ700 mass spectrometer was used with a Finnigan electrospray ionization (ESI) interface for the LC system (LC system 2). Instrument control and data processing were performed on a DECstation 5000/125 with Finnigan ICL and ICIS II software (versions 7.6 and 7.4). Interface variables were as follows: spray voltage, 4 kV; sheath gas (N₂), 80 psi; auxiliary gas (N₂), 8 units; capillary temperature, 220°C.

For tuning and calibration of the mass analyzer, background cluster ions (CH₃COONa)₃(CH₃COO⁻) from the mobile phase were used. The capillary and tube lens voltages of the ESI interface were optimized using reference budesonide oleate. Full scan negative ion mass spectra were recorded (mass range 400 – 850 in 2 sec) scanning Q1. MS/MS was performed with Ar gas (1.5 mtorr, 0.2 Pa) in the collision cell (Q2) and a collision energy of 15 eV. Product ion spectra of selected [M+CH₂COO⁻] ions (MAC⁻) were recorded.

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\text{Fig. 2. Radiochromatograms obtained after incubation of tritiated budesonide (100 } \mu\text{M) with human lung microsomes (1 mg protein/ml) for 1 hr.}
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Conjugation: Dependence on CoA and ATP. Budesonide (100 \( \mu \text{M} \)) was incubated for 60 min with human lung microsomes with and without CoA and ATP. The radiochromatograms are shown in fig. 2. Lipophilic metabolites were formed, eluting in at least one major and two minor peaks in LC system 1. The formation was dependent on CoA and ATP. Of the reference compounds available, budesonide-21-oleate and budesonide-21-palmitate would comigrate with the major peak (fractions 31–38), while budesonide-21-palmitoleate would migrate among the minor peaks (fractions 18–23). Unchanged budesonide elutes with the first five to seven fractions in this chromatography system. When using human liver microsomes, the chromatograms (cf. fig. 4) qualitatively looked very similar to those obtained with lung microsomes. Cytosolic fractions did not produce these lipophilic metabolites.

Conjugation: Time Courses. Budesonide (100 \( \mu \text{M} \)) was incubated for up to 60 min with human liver and lung microsomes in the presence of CoA and ATP. The main fractions of lipophilic metabolites (sum of fractions 31–38; cf. fig. 2) were taken as measure of conjugate formation. The results are shown in fig. 3. Both lung and liver catalyzed formation of the conjugates. The results presented in the figure are derived from only one microsome preparation from each organ. Thus, no conclusion about the relative capacity of the two organs should be made.

Conjugation: Effects of Fatty Acids. Budesonide (100 \( \mu \text{M} \)) was incubated with human lung and liver microsomes in the presence of ATP and CoA, and the incubations were also supplemented with oleic acid or palmitoleic acid. The results are shown in fig. 4. The different microsomes responded somewhat differently to the fatty acids. In lung microsomes, oleic acid almost abolished formation of metabolites eluting in fractions 18–23 (fractions covering retention time for budesonide-21-palmitoleate) and also reduced by around 50% the amount of metabolites eluting in fractions 31–38 (fractions covering retention times for budesonide-21-oleate and budesonide-21-palmitate). Palmitoleic acid added to lung microsomes prevented formation of metabolites eluting in fractions 31–38 and increased the amount of metabolites with its maximum in fractions 18–23. In liver microsomes, oleic acid increased formation of metabolites eluting in fractions 31–38 almost 3-fold and reduced amounts of metabolites in fractions 18–23. Palmitoleic acid added to liver microsomes inhibited formation of metabolites eluting in fractions 31–38 and increased the amounts of metabolites in fractions 18–23.

Identification of Lipophilic Conjugates. Electrospray ionization negative ion mass spectra of the reference compounds in the sodium acetate-fortified mobile phase (LC system 2), with the chosen interface conditions, showed mainly the acetate adduct ([M+CH\(_3\)COO\(^-\)], MAc\(^-\)) (fig. 5).

Fig. 3. Formation of lipophilic metabolites from budesonide in human liver and lung microsomes (1 mg protein/ml) in the presence of CoA and ATP.

Fig. 4. The influence of externally added fatty acids can be seen in the formation of lipophilic metabolites from budesonide in human lung microsomes (top) and human liver microsomes (bottom).
lung microsomal incubation extract. Fig. 7 shows a negative ion mass spectrum of the metabolically formed oleic acid conjugate. Qualitatively, similar results were obtained using human liver microsomes (not shown).

The product ion spectra of the acetate adducts yielded structural information (figs. 8 and 9). Fig. 8 shows the product ion spectra of a metabolite (budesonide oleate) and the corresponding reference compound. In addition to remaining parent ion, a fragment indicating the loss of the acetal moiety, a major fragment at m/z 339 and a minor fragment showing the anion of the carboxylic acid of the ester were seen. The m/z 339 ion is indicative of the steroid skeleton of budesonide, i.e. the ester as well as the acetal are lost (fig. 9).

Thus, the following compounds were identified as metabolites in liver as well as lung microsomes: budesonide esterified with palmitoleic acid, palmitic acid, linoleic acid, oleic acid, and arachidonic acid. The identity of the compounds was deduced from the molecular mass, the presence of a deuterium-labeled congener, the daughter ion spectra, and the chromatographic properties and verified by synthetic references, esterified in the 21-position, in the cases of oleate, palmitoleate, and palmitate. None of the esters appeared in extracts from incubations without the budesonide substrate. The LC-MS methodology used in the present study was not developed and validated for quantitative purposes; thus, no attempts were made in the MS analyses to determine quantities of the different conjugates.

Hydrolysis by Lipase. Budesonide oleate and budesonide palmitoleate were incubated with porcine pancreatic lipase and bovine pancreatic cholesterol esterase. Budesonide was formed during these incubations in a time-dependent manner (fig. 10). The budesonide fatty acid conjugates are thus lipase substrates. Similar experiments showed that porcine liver carboxyl esterase, on the other hand, did not degrade the steroid fatty acid esters tested (not shown).

Discussion

In the present study, we have shown that microsomes from human liver and lung are able to conjugate the antiinflammatory glucocorticoid budesonide with fatty acids. Moreover, the budesonide fatty acid esters were shown to be lipase substrates; thus, budesonide is regaining from the conjugates (summarized in fig. 11). The conjugation reaction was dependent on ATP and CoA, and various fatty acids (oleic acid, palmitic acid, palmitoleic acid, linoleic acid, and arachidonic acid).
donic acid) were utilized for the conjugation. Theoretically, budesonide contains two possible conjugation sites, the 11- and 21-hydroxyl groups. The conjugation apparently occurs on the 21-hydroxyl group because the metabolites match the synthetic reference 21-esters. We also found that fluticasone propionate does not undergo this metabolism (not shown). Fluticasone propionate lacks the hydroxyl at the 21-position but possesses the 11-hydroxyl. The 21-position is also known to be more accessible to chemical derivatization. For example, cortisol (7) and budesonide can be acetylated chemically on the 21-hydroxyl without affecting the more sterically hindered 11-hydroxyl group.

Fatty acid conjugation is well known to occur with cholesterol (8) and with several endogenous steroid hormones (9). Most well studied among the steroid hormones are the estrogens. Fatty acid ester formation has also been shown for xenobiotics like cannabino (10), halothanols (11), chlorinated phenols (12), and some others (reviewed in ref. 13). To our knowledge, this type of metabolism has hitherto not been demonstrated for steroid xenobiotics used in medical therapy.

The esterification of cholesterol is catalyzed by the ATP- and CoA-dependent microsomal acyl CoA:cholesterol acyl transferase.

**Fig. 8.** Product ion spectra of MAc• ions (m/z 753.5) from a human lung microsomal metabolite (top) and reference budesonide-21-oleate (bottom).

**Fig. 9.** Proposed structures of fragments recorded in product ion spectra of MAc• ions from metabolic fatty acid conjugates and reference esters of budesonide.

Available references are indicated by a + symbol.

**Fig. 10.** Formation of budesonide in incubations of budesonide-21-oleate (squares) or budesonide-21-palmitoleate (triangles) with porcine pancreas lipase (0.25 mg/ml, broken lines) or bovine pancreas cholesterol esterase (0.0125 mg/ml in the presence of 10 mM taurocholate, full lines).
(ACAT) (8) and possibly (at low taurocholate levels) by the pancreatic cholesterol esterase (14). Estrogen fatty acid conjugates are formed by enzyme(s) that some authors have termed acyl CoA: ester-di-17β acyltransferase (AEAT) (15). It has further been shown that several endogenous steroid hormones are esterified in rat plasma, probably by lecithin:cholesterol acyltransferase (16). Esterification of the same steroids in rat liver microsomes was dependent on CoA and ATP, but differences in the fatty acid composition of these esters compared with cholesterol esters, and lack of sensitivity to an ACAT inhibitor, suggested that perhaps some other enzyme than ACAT was involved (16). The enzymology of the esterification of the xenobiotics is not as well understood, but the reaction seems sometimes—but not always—dependent on ATP and CoA, suggesting other pathways beside ACAT or related enzymes (as discussed in ref. 13). The fact that the esterification of budesonide required ATP and CoA indicates that this type of steroid is conjugated by the ACAT/AEAT-type enzymes, but the exact identity of the enzyme responsible remains to be elucidated.

The purpose of the present paper was to identify the metabolites and to examine the biochemistry of the process. To obtain this, we chose to work with microsomes from human lung and liver because microsomes was the simplest system we could show that would form these metabolites (e.g., the cytosolic fractions were virtually devoid of activity). Other data we have collected suggest the formation of these conjugates in a wide variety of biological systems such as liver and lung microsomes from the rat, rat lung and trachea following inhalation of budesonide propionate in vivo, rabbit trachea in situ, human blood monocytes, CACO-2 cells (human colon carcinoma cell line), COS cells (monkey kidney cells), F2002 cells (human embryonic lung fibroblasts), J774 mouse macrophages, R1 cells (rat fibroblasts), primary cultures of human bronchial epithelial cells, and human lung tissue following inhalation of budesonide (parts of these data are presented in preliminary form in refs. 5 and 17; further reports are presently being prepared). In most of these cases, the conclusions are based on LC-migration similarities, and no MS identification of budesonide fatty acid esters has yet been obtained.

The results shown in fig. 4 show that microsomes from human lung and human liver responded somewhat differently to the addition of exogenous free fatty acids to the incubations. The reason for this is not clear. However, fatty acid conjugation is a multistep process involving, e.g., activation of fatty acids, and perhaps the rate determining step for budesonide ester formation may be different in microsomes from the two organs. Another reason may be that the peaks in the radiochromatograms contain more than one metabolite; the major peak contains at least the olate and the palmitate ester, while the minor peak(s) contains the palmitoleate ester but probably other components as well. This finding further suggests that in an intact cell with regulated supply of activated fatty acids to the acylating enzymes, the pattern of fatty acid esters may be different than in microsomes.

The fatty acid conjugation of budesonide will most likely attenuate budesonide’s acute pharmacological effects, since fatty acid conjugates of this type have negligible glucocorticoid activity per se (17, 18). However, as shown in this study, budesonide is regainable from the conjugates through the action of lipase. The fatty acid conjugates will most likely be retained intracellularly for a longer time than unchanged budesonide. Thus, it may be that the lipoidal conjugates will form an intracellular depot slowly releasing active steroid. The duration of tissue exposure to budesonide will depend partly on the rate of lipase-catalyzed hydrolysis of the conjugates. The conjugation will therefore prolong the duration of the pharmacological effects of budesonide. Investigations into the impact of the conjugation on duration of antiinflammatory effects in cell culture systems (17) and in rat airways in vivo (19) support these assumptions. The duration aspects are of particular interest because not all therapeutically used glucocorticoids can be conjugated in this way. As an example, fluticasone propionate lacks the 21-hydroxyl group and is not involved in this type of metabolism. It is tempting to speculate that the metabolic route of budesonide described in the present study explains why budesonide so efficiently can control mild asthma, even when dosed once daily (20).

References