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Induction of the Adipose Differentiation-Related Protein in Liver of Etomoxir-Treated Rats

Sandra Steiner,*,[1] Daniel Wahl,* Bonnie L. K. Mangold,* Rodney Robison,* Jos Raymackers,† Lydie Meheus,† N. Leigh Anderson,‡ and André Cordier*

**Drug Safety Assessment, Toxicology, Sandoz Pharma Ltd, Basel, Switzerland; Sandoz Research Institute, East Hanover, New Jersey; †Protein Analysis Division, Innogenetics Ltd., Ghent, Belgium and ‡Large Scale Biology Corporation, Rockville, Maryland*

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The effects of etomoxir, an irreversible carnitine palmitoyltransferase I inhibitor, on the liver protein pattern and on liver morphology were examined by two-dimensional gel electrophoresis in female Sprague-Dawley rats treated with 125 mg/kg/day etomoxir for 28 days. In livers of treated animals a protein spot was found which was not present in controls. The spot was identified by internal amino acid sequence analysis as the adipose differentiation-related protein (ADRP). The expression of ADRP in liver is a novel finding as the protein has been described previously as adipocyte-specific. Additionally we found histopathologic evidence of lipid accumulation in the livers of etomoxir rats. The data show that for each treated rat there was a good correlation between ADRP levels and degree of lipid droplet formation. This observation may suggest a potential relationship between drug-induced expression of ADRP in liver and lipid accumulation.

Etomoxir has been shown to be a potent hypoglycemic agent in laboratory animals and humans (1, 2). Its pharmacological action is based on the irreversible inhibition of carnitine palmitoyltransferase I (CPT I), an enzyme located on the inner surface of the outer mitochondrial membrane. CPT I is involved in the transport of long-chain fatty acids into mitochondria and is considered a rate-limiting step of mitochondrial long-chain free fatty acid oxidation (3, 4). Etomoxir has been shown to inhibit long-chain fatty acid oxidation in liver and extrahepatic tissues such as heart and skeletal muscle. Secondarily and as a consequence of the inhibition of fatty acid oxidation, etomoxir increases glucose oxidation and decreases gluconeogenesis (1). Etomoxir-mediated impaired utilization and, hence, increase in intracellular levels of long-chain fatty acids (5) have been observed to induce peroxisomal beta-oxidation (6, 7) and accumulation of lipids in liver and heart (8).

The purpose of this study was to examine the effects of etomoxir on the rat liver protein pattern using two-dimensional protein gel electrophoresis combined with amino acid sequence analysis. The investigation was focused on the identification of proteins which were either induced or lost in the patterns of treated rats when compared to the controls. Similar studies have been undertaken previously to investigate the liver effects of drugs such as cyclosporine A (9) or methapyrilene (10). The effects on the liver protein pattern were compared to the treatment-related histopathological changes found in the liver of the animals.

We demonstrate that etomoxir induces the expression of the adipose differentiation-related protein (ADRP) in liver, a protein previously thought to be adipose tissue specific. Particular features of this effect suggest its relation to the drug-induced lipid accumulation in this organ.

MATERIALS AND METHODS

Animal Treatment and Sample Preparation.

Female Sprague Dawley rats [Charles River CD-Sprague-Dawley derived; Crl:CD (SD)BR], five to six weeks old and weighing 85 to 110 g on arrival were purchased from Charles River Laboratories (Kingston, New York). After acclima-

[1] To whom correspondence should be addressed. Fax: +41-61-4696565. email: steiner@dstox.pharma.sandoz.ch.

Abbreviations: ADRP, adipose differentiation-related protein; CPT I, carnitine palmitoyltransferase I; H&E, hematoxylin and eosin; ORO, Oil Red O; 2-D, two-dimensional; IEF, isoelectric focusing.

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tisation for one week animals were randomly assigned to two groups of six rats and administered by gavage either vehicle or 125 mg/kg/day (+)-etomoxir, sodium salt, suspended in a mixture of 2% veegum and 2% methocel E5, for 28 days. The animals were killed on days 29-30 via axillary exsanguination under Ketaset/Rompun anesthesia. Liver samples (150 mg of the left apical lobe) were collected and frozen immediately in liquid nitrogen. Liver samples were homogenized in eight volumes of 9M urea, 4% cholamidopropyldimethylammoniopropanesulfonate (CHAPS), 1% dithiothreitol (DTT) and 2% carrier ampholytes (Pharmacia pH 8-10.5) using a 1 ml Wheaton glass homogenizer. The homogenates were centrifuged at 420,000 × g at 18°C for 12 min (Beckman TL100 ultracentrifuge; TLA 100.3 rotor; 100,000 rpm). The supernatant was removed, divided into four aliquots and stored at −80°C until analysis. A portion of the liver was collected and fixed in 10% neutral-buffered formalin. Three liver sections from each animal were stained with hematoxylin and eosin (H&E). In addition, a fixed-frozen section of liver from each animal was treated with Oil Red O (ORO). The sections were examined by light microscopy.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Analytical two-dimensional (2-D) electrophoresis. Sample proteins were resolved by 2-D electrophoresis using the 20 × 25 cm ISO-DALT 2-D gel system operating with 20 gels per batch as described by Anderson et al. (11). First dimension isoelectric focusing (IEF) gels were prepared using a standardized batch of ampholytes (BDH pH 4-8), each loaded with 10 μl of tissue sample, and run overnight for 33,000 volt-hours. An Angelique computer-controlled gradient casting system was used to prepare second dimension SDS gradient slab gels in which the top 5% of the gel was 9% polyacrylamide and the lower 95% of the gel varied linearly from 9% to 18%. First dimension tube gels were loaded directly onto the slab gels using an equilibration buffer with a blue tracking dye and were held in place by polyester fabric wedgies (Wedgies). Slab gels were run overnight at 150 V at 10°C and were taken out the next morning when the blue tracking dye reached the bottom of the gel. Following SDS electrophoresis, the slab gels were fixed overnight in 3 liters of 50% ethanol / 3% phosphoric acid and then washed three times for 30 min in 4 liters of cold tap water. They were transferred to 3 liters of 34% methanol / 17% ammonium sulfate / 3% phosphoric acid for one hour, and after the addition of one gram powdered Coomassie Blue G-250 the gels were stained for three days to achieve equilibrium intensity.

Micropreparative two-dimensional electrophoresis. In order to collect enough material for amino acid sequencing the above gel running protocol was slightly modified, to increase both the number of target spots as well as the amount of protein per spot. Each IEF gel was loaded with 15 μl solubilized liver sample from rats treated with CPT I inhibitor and run overnight as described. The basic ends of two IEF gels were then loaded side by side onto each slab gel, consequently, the number of IEF gels run was two times the number of slab gels (40 and 20 respectively). Following SDS-electrophoresis, fixation and staining as described above, the target spots were excised precisely from each gel for protein sequencing, resulting in two spots per slab gel.

Data Analysis. Stained slab gels were digitized in orange light using an Ektron 1412 CCD scanner with an optical density maximum of 1.0 and a resolution of 127 μm. The digitized 2048 × 2048 pixel images were archived on a DAT tape, and a gray scale graphic using a Seikosha videoprinter was prepared from each gel image as a hard-copy backup. The raw images were processed using the Kepler software system (LSB Corp., Rockville, Maryland) with the procedure PROC008 to yield lists of spot position, shape and density for each detected spot. The liver patterns obtained from the analytical two-dimensional electrophoresis were assembled in two experimental groups using Kepler's experiment definition database. To search for treatment-related protein abundance changes, the patterns were inspected by eye using the Kepler montage display format. Statistical evaluations of the abundance changes were performed using the Student's t-test. Bargraphs representing spot abundance data and spotmaps showing the location of spots within the master pattern, were prepared and printed as postscript on a DEClaser 2200. The spot maps were displayed in the standard orientation showing high molecular mass at the top and acidic proteins to the left.

Peptide Mapping and Microsequencing. Excised spot 1399 from 47 Coomassie Blue stained slab gels was submitted to proteolytic digestion in polyacrylamide gel according to the method of Rosenfeld et al. (12). Briefly, the excised gel pieces were washed with 40 ml of water for 2 hours. To remove most of the staining, they were transferred to a mixture of 40% acetone, 10% triethylamine and 5% acetic acid in water pH 6.4 and heavily shaken for half an hour (orbital shaker, 300 rpm.). They were then washed twice for one hour in 40 ml of water and incubated for half an hour in 50% acetonitrile. The solution was removed and the gel pieces were air dried for two hours. Digestion of protein spots was performed in an Eppendorf orbital mixer using a solution of 3 μg trypsin in 300 μl 100mM Tris-HCl pH 8.2/ 10% acetonitrile. On each dried gel piece 10 μl of the digestion solution were spotted and pieces were incubated for 20 hours at 37°C. The peptides were extracted twice for half an hour with 300 μl 60% acetonitrile / 0.1% trifluoroacetic acid in the Eppendorf orbital mixer at 37°C. The pooled extracts were vacuum-dried, resolubilized in 20 μl 20% acetic acid and stored at −20°C until analysis. 2D-spot derived tryptic peptides were diluted with 380 μl 0.1% trifluoroacetic acid and separated on a reverse phase column (C4 Vydac, 2.1 × 250 mm, Hesperia, CA) using a 140B Solvent Delivery System (Perkin-Elmer, Foster City, CA) and eluted with a gradient (7% to 70%) of acetonitrile in 0.1% trifluoroacetic acid. The column outlet was directly connected to a 1000S diode array detector (Perkin-Elmer), and peptide fractions were collected manually in Eppendorf tubes. Purified peptides were sequenced using a pulsed liquid model 477A sequencer equipped with an on-line 120 phenylthiohydantoin analyzer (Perkin-Elmer).

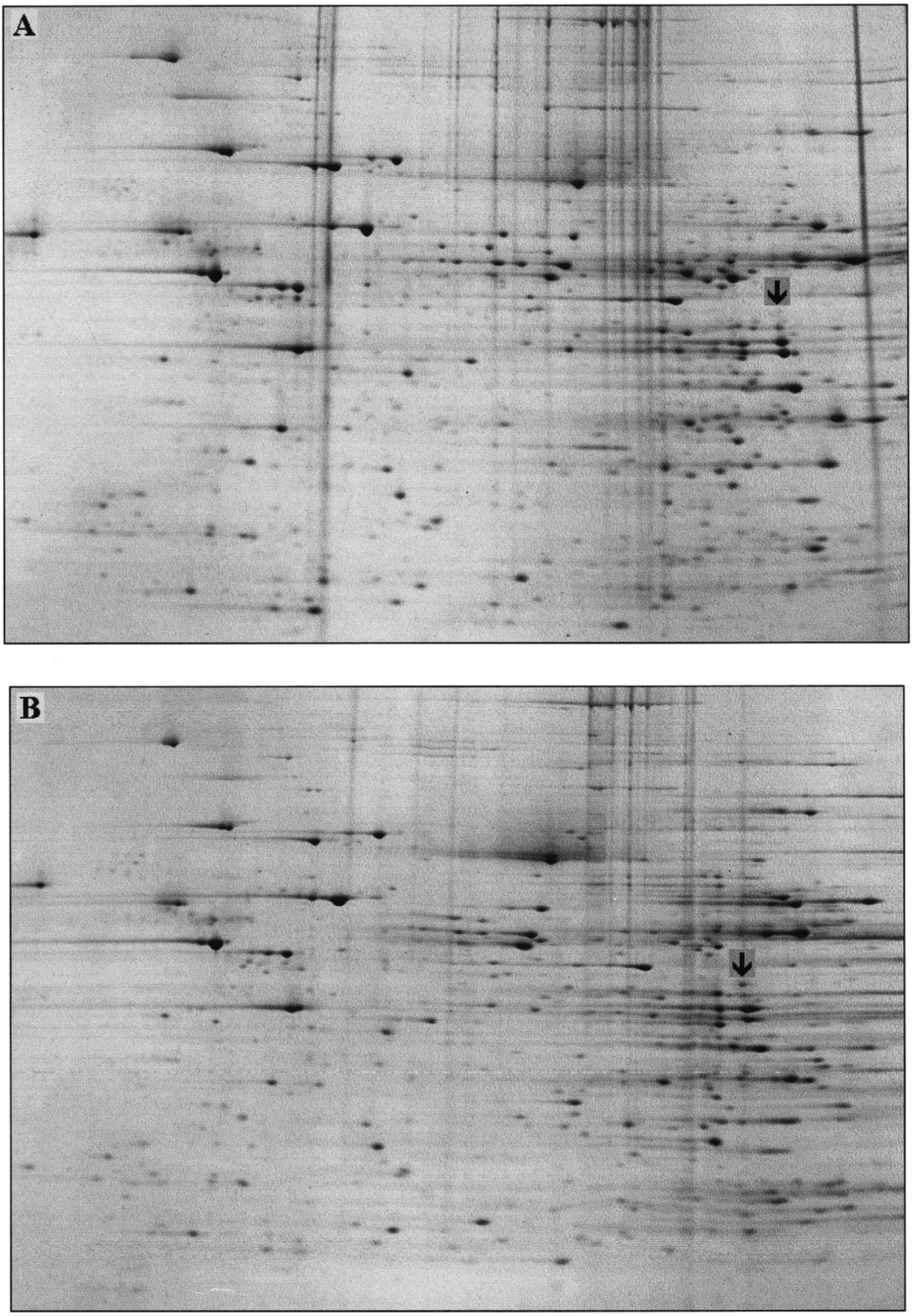

FIG. 1. Section of a 2-D liver protein pattern from (A) a control and (B) an etomoxir-treated rat. The arrow indicates the protein spot induced by etomoxir.

RESULTS AND DISCUSSION

Inspection of the liver protein patterns revealed one spot which was present in relatively high amounts in five of six etomoxir-treated animals but was absent in the patterns of the controls. (Fig. 1). The abundance of the etomoxir-induced spot in each animal is shown in Fig. 2. It is currently unclear why the spot was not detected in all treated animals but it may well be that in one of the treated rats the protein is expressed to a level below detection. The new protein spot, which has not been detected previously in any rat liver patterns, was given the number 1399 in the Wistar liver 2-D map WISLIV1, and the number 1310 in the Fisher rat liver map F344MST3.

Spot 1399 was excised from the Coomassie Blue stained 2D-gels, a total of forty seven spots were pooled and submitted to proteolytic digestion. Two of the resulting peptides were sequenced with initial phenylthiohydantoin derivative yields of 7.5 and 4.5 pmol, respectively. A homology search showed that the first peptide LPILNQPTSEIVASAN differed in just one amino acid from the sequence of aa94-107 in the mouse adipose differentiation-related protein (ADRP) (13) in which aa107 is S. The second peptide SQETISQL?STV?LIEFA (? corresponds to the amino acid histidine which is not detectable at amounts below 5 pmol in this analysis system) showed 100% homology with the sequence of aa244-261 in mouse ADRP. From these data it can be assumed that the protein induced in the livers of etomoxir-treated rats is the rat homolog of mouse ADRP. The detection of ADRP in liver tissue is a novel observation since this protein was previously thought to be adipose tissue specific (13).

Light microscopic examination of the liver of etomoxir treated rats revealed pale and swollen hepatocytes with minimal to moderate diffuse cytoplasmic microvesicular vacuolation. Evaluation of the ORO stained sections confirmed increased fat in hepatocytes with diffuse moderate pan-lobular steatosis (two of six treated rats) and minimal to mild steatosis primarily in periportal regions (four of six treated rats). No steatosis was evident in control rats. Photomicrographs of H&E stained liver sections from a control and a treated animal is shown in Fig. 3. This finding is in agreement with previously published data from studies with etomoxir (8). Interestingly, the two animals with relatively high levels of steatosis in liver also showed high ADRP levels. These data

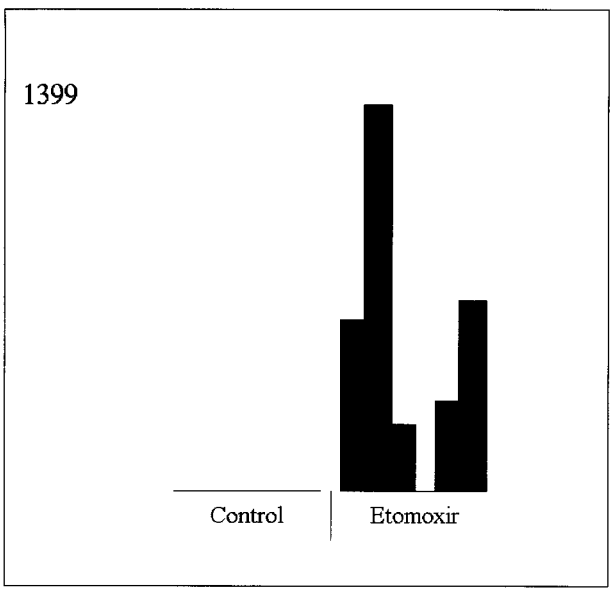

FIG. 2. Bar graphs showing the abundance of the etomoxir-induced spot in each animal. Each bar within a group represents the spot abundance in an individual animal.

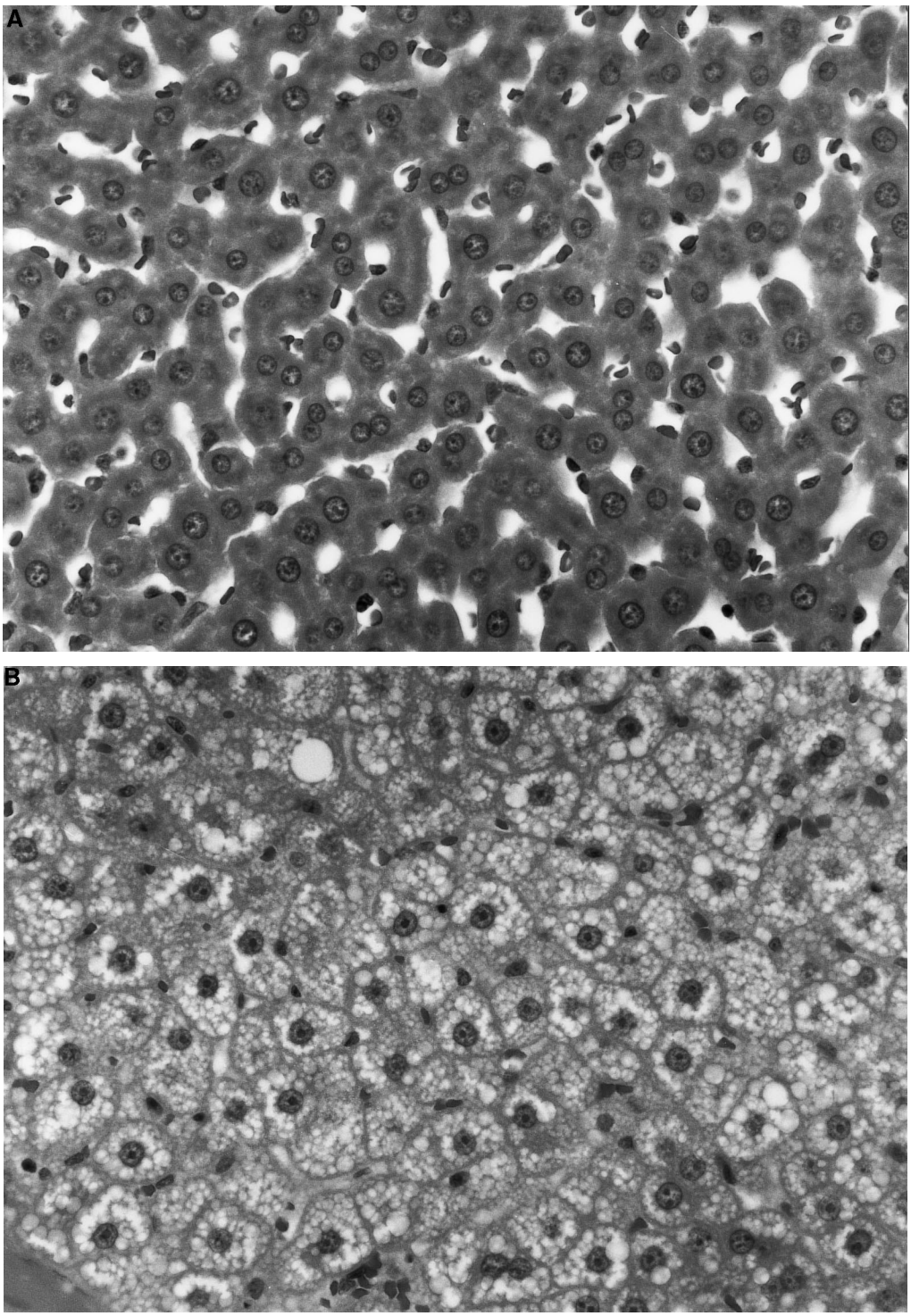

FIG. 3. Photomicrographs of H&E stained liver sections from (A) a control and (B) an etomoxir treated rat. Note marked accumulation of microvesicular fat in hepatocytes from the treated animal (× 600).

strongly suggest that the induction of ADRP in livers of etomoxir treated rats is related to the drug-mediated lipid accumulation in this organ. It has been reported that ADRP mRNA levels are rapidly and maximally induced after triggering preadipocyte differentiation (14); however, the exact functions of this membrane-associated protein are not known. Although the presence of ADRP in liver tissue has not been described before, it seems very likely that the membrane associated protein has a location similar to the closely related perilipin. Perilipin is a protein found exclusively at the surface of lipid storage droplets and was reported to have a significant sequence relationship with ADRP (approximately 65% similarity through 105 aa) (13). The alignment of our sequencing data with the known sequence of perilipin however, unambiguously showed that the etomoxir-induced protein is not perilipin.

In conclusion, we have shown for the first time that the expression of ADRP, a protein thought to be adipocyte-specific, can be induced in rat liver. The limited data available suggest that the expression of ADRP is related to increased lipid accumulation in liver. It is hypothesized that in livers of etomoxir-treated animals, ADRP may be associated with the surface of lipid droplets. Immunohistochemical localization of ADRP in liver tissue of etomoxir-treated animals may be an approach to test this hypothesis.

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