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CHANGES IN THE LIVER PROTEIN PATTERN OF FEMALE WISTAR RATS TREATED WITH THE HYPOGLYCEMIC AGENT SDZ PGU 693

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Summary

SDZ PGU 693 acts as a hypoglycemic agent by stimulating glucose utilisation in insulin-sensitive peripheral tissues, such as skeletal muscle and fat. In a 28 day toxicity study the compound was found to induce hepatocellular hypertrophy in Wistar rats treated with 300 mg/kg/day. To gain insights into the pathomechanism of these alterations, aliquots of liver samples from control and treated female Wistar rats were separated by two-dimensional protein gel electrophoresis and the digitized images of the protein patterns were searched for protein abundance changes. Significant treatment-related quantitative changes ($P < 0.001$) were found in 29 liver proteins. Major increases were observed in several microsomal proteins, including NADPH cytochrome P-450 reductase, cytochrome b5 and serine protease inhibitor. The changes in the cytochrome related enzymes, both known co-factors of the P-450 enzyme system, strongly suggest that SDZ PGU 693 induces microsomal proliferation and induction of the P-450 enzyme system. Decreases were observed in a series of mitochondrial proteins, such as F_1ATPase-δ subunit and ornithine aminotransferase precursor as well as in several cytosolic proteins such as the liver fatty acid binding protein, arylsulfotransferase and the senescence marker protein-30. The changes in F_1ATPase-δ subunit and liver fatty acid binding protein together suggest a down-regulation of the mitochondrial liver fatty acid metabolism, likely reflecting the pharmacological action of the compound. These results show that SDZ PGU 693 produces a complex pattern of gene expression changes which give insights into the molecular mechanisms of both its pharmacological action and a toxic response.

Key Words: SDZ PGU 693, hypoglycemic agent, liver, toxicity, 2D-electrophoresis, proteome pattern

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Non-insulin-dependent diabetes mellitus (NIDDM) is a chronic, progressive metabolic disease characterized by hyperglycemia, which is almost always accompanied by hyperlipidemia and insulin resistance. A major factor in the impaired ability to dispose of an oral glucose load is resistance to the peripheral biological effects of insulin. Such peripheral insulin resistance is primarily manifested as a defect in glucose uptake and storage in skeletal muscle (1, 2). The impaired ability of insulin to clear glucose from the blood leads to a compensatory hyperinsulinemia, worsening insulin resistance and, eventually, resulting in hyperglycaemia.

SDZ PGU 693 ((-)-trans-2S[(chlorophenoxy)methyl]-7a-(3, 4-dichlorophenyl)-2, 3, 7, 7a-tetrahydropyrrolol[2, 1-b]oxazol-5(6H)-one) was intended to be developed as an orally active agent for the treatment of NIDDM. The compound improves whole body glucose disposal by stimulating glucose utilization in insulin-sensitive peripheral tissues such as skeletal muscle and fat. In rat toxicity studies the liver was the major target for toxicity and induction of centrilobular hepatocellular hypertrophy was found in both male and female animals. To obtain insights into the mechanisms leading to these adverse effects two-dimensional protein gel electrophoresis (2DE) was performed with liver homogenates from control and treated animals. Previous studies performed in our laboratory showed very similar liver proteome patterns for male and female Wistar rats (3) and in this study only the samples from female control rats and female rats treated with 300 mg/kg/day SDZ PGU 693 for 28 days were analysed for protein abundance changes. SDZ PGU 693 was found to produce a complex set of changes in the liver protein pattern. Major increases in protein abundance were observed in microsomal proteins and several mitochondrial proteins were found to be decreased. The changes in the microsomal proteins suggest the compound being an inducer of the P-450 enzyme system. The alterations in the mitochondrial proteins most likely reflect an impairment of the liver fatty acid metabolism which may be related to the pharmacological action of the compound.

Methods

1. Animal Treatment Protocol

Female Wistar Hannover rats (HsdBRL:WH; Harlan Sprague Dawley, Inc, Dublin, Virginia), 6 weeks of age and weighing 107-121 g were used. The animals were housed individually in suspended stainless steel cages in an environmentally controlled room and were fed with a Lab Diet™ Certified Rodent Diet 5002 (PMI Feeds, Richmond, Indiana) and tap water *ad libitum*. Two groups of ten rats each received 300 mg/kg/day of SDZ PGU 693 by gavage for four weeks or the vehicle solution (SDZ PGU 693 Microemulsion Preconcentrate) for an identical period. The animals were sacrificed with carbon dioxide gas on the day following the last treatment. Liver samples (150 mg of the left apical lobe) were collected and frozen immediately in liquid nitrogen and kept at -80°C.

2. Sample and Tissue Preparation

The samples were homogenized with a 1 ml Wheaton glass homogenizer in eight volumes of 9M urea, 4% Nonidet P-40, 1% dithiothreitol (DTT) and 2% carrier ampholytes pH 8-10.5 (Pharmacia, Uppsala, Sweden). The homogenates were centrifuged at 420,000 x g at 18°C for 12 min (TL100 ultracentrifuge, TLA 100.3 rotor, 100,000 rpm, Beckman Instruments, Palo Alto, CA). 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). The sections were examined by light microscopy.

3. Two-dimensional polyacrylamide gel electrophoresis

Sample proteins were resolved using the 20 x 25 cm ISO-DALT® 2-D system (4). First dimensional isoelectric focusing (IEF) gels were prepared using a standardized batch of pH 4-8 carrier ampholytes (BDH, Poole, UK). 10 µl of solubilized sample were applied to each gel, and the gels were run for about 34,000 volt-hours using a progressively increasing voltage with a high-voltage programmable power supply. An Angelique™ computer-controlled gradient-casting system (Large Scale Biology Corporation, Rockville, MD, USA) was used to prepare the second-dimension sodium dodecyl sulfate (SDS) polyacrylamide gradient slab gels, in which the top 5% of each gel was 11%T acrylamide, and the lower 95% of the gel varied linearly from 11% to 19%T. The IEF 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™, Large Scale Biology Corporation, Rockville, MD, USA) to avoid the use of hot agarose. Second-dimensional slab gels were run overnight at 160 V in cooled DALT tanks (10°C) with buffer circulation and were taken out when the 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.

4. Quantitative Computer Analysis

4.1. Scanning and Image Analysis

Stained slab gels were digitized in red light at 133 micron resolution, using an Eikonix 1412 scanner and analysed as described previously (5). Each 2-D gel was processed using the Kepler® software system with procedure PROC008b to yield a spotlist giving position, shape and density information for each detected spot. This procedure makes use of digital filtering, mathematical morphology techniques and digital masking to remove background, and uses full two-dimensional least-squares optimization to refine the parameters of a 2-D gaussian shape for each spot. Processing parameters and file locations were stored in a relational database, while various log files detailing operation of the automatic analysis software were archived with the reduced data. The computed resolution and level of gaussian convergence of each gel were inspected and archived for quality control purposes.

4.2. Assembly of Data from Multiple Gels

An experiment package was constructed using the Kepler® experiment definition database to assemble groups of 2-D patterns corresponding to the groups of treated and control animals. In order to increase the statistical power of the experiment, duplicate gels were included for each sample. The 2-D patterns were matched to the appropriate "master" 2-D pattern (Wistarliv1), thereby providing linkage to the existing rodent protein 2-D databases through a translation table to F344MST3 (6), the Fischer 344 master pattern. Subsequently, an automatic program matched additional spots to the master pattern using as a basis the manual landmark data entered. The groups of gels making up an experiment were scaled together (to eliminate quantitative differences due to gel loading or staining differences) by a linear procedure based on all matched spots in the control group. All gels in the experiment were then scaled together by setting the summed abundance of the selected spots equal to a constant (linear) scaling.

5. Protein Spot Identification

5.1. Comparison with the Fischer 344 Rat Liver Protein Pattern

The "master" 2-D pattern used in this study (Wistarliv1) is linked to the Large Scale Biology Corporation rodent protein 2-D database through a translation table to the Fischer 344 rat master pattern. The automatic translation capability between the proteins of the two master patterns allows to cross-reference spots identified in the Fischer rat liver pattern to the Wistar rat liver pattern.

5.2. Peptide Mapping and Microsequencing

High to medium abundant spots changed by the treatment but not yet identified in the Fischer 344 master pattern were excised from the Coomassie Blue stained slab gels and submitted to proteolytic digestion in polyacrylamide gel (PAG) according to the method of Rosenfeld et al. (1992) (7). 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 two times 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 was spotted and pieces were incubated for 20 hours at 37°C. The peptides were extracted two times 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 x 250 mm, Hesperia, CA, USA) using a 140B Solvent Delivery System (Perkin-Elmer, Foster City, CA, USA) 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).

Results

A set of 29 liver proteins were reliably increased or decreased as a result of treatment with SDZ PGU 693, based on application of the two-tailed Student's *t*-test ($P < 0.001$). An overview of the protein spots affected by the treatment showing their position in the gel and the degree and significance of their change is shown in the VKPL™ arrow plot display in Fig. 1. From the spots altered with SDZ PGU 693 seven could be identified by cross-reference to the Fischer 344 rat master pattern (MSNs 175, 251, 87, 196, 55, 117, 227) and two were identified by sequence analysis (MSNs 214, 252). From spot 214 three peptides were sequenced and all contained sequences from rat ornithine aminotransferase and from spot 252 two peptides were sequenced which contained sequences of a rat serine protease inhibitor.

Interestingly, all protein spots assigned to the microsomal fraction of the liver (5) were increased and all spots assigned to mitochondrial proteins were decreased (Table I). Microsomal proteins showing 2- to 4-fold inductions by the treatment include NADPH cytochrome P-450 reductase (master spot number (MSN) 175 and 251), cytochrome b5 (MSN 87) and serine protease inhibitor (MSN 252). Mitochondrial proteins decreased by 25 to 60 % include F_1ATPase-δ subunit (MSN 196) and ornithine aminotransferase precursor (MSN 214) and the unidentified proteins with

MSNs 286, 395 and 426. The cytosolic proteins modified by the treatment showed a mixed pattern of increases and decreases. Senescence marker protein-30 (MSN 55), arylsulfotransferase (MSN 117), liver fatty-acid binding protein (MSN 227) and the unidentified proteins with MSNs 113, 178 and 282 were decreased by 15 to 45% when compared to the controls. 1.5- to 2.5-fold increases were observed in a series of non-identified proteins with MSNs 240, 266, 276, 292, 347 and 401. In the group of proteins with unknown location decreases were found in the spots with MSNs 91, 315, 327, 824 and 1343 and increases in the spots with MSNs 105, 186 and 839 (Table II).

Light microscopic examination of the liver of SDZ PGU 693 treated rats revealed diffuse, mild centrilobular hepatocellular hypertrophy in all animals. The enlarged hepatocytes had a generally eosinophilic cytoplasm. This cytologic change was not seen in control rats.

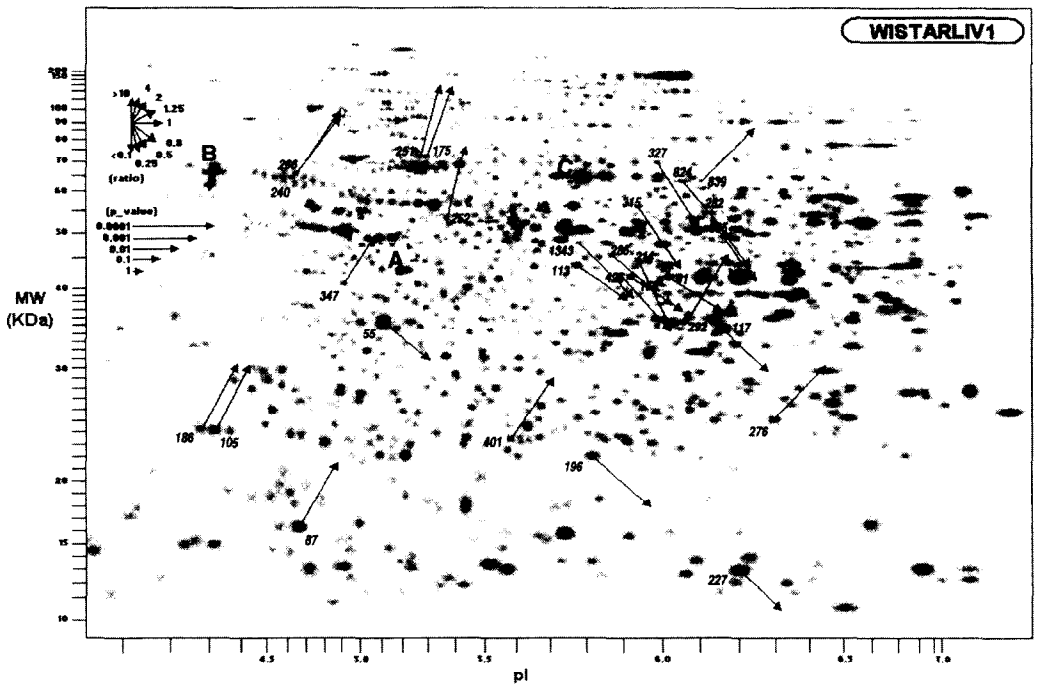

Fig. 1

Display of protein changes after treatment of female Wistar rats with 300 mg/kg/day SDZ PGU 693 for 28 days. The background picture is a representation of the 2-D protein pattern from Wistar rat liver (A: actin, B: calreticulin, C: albumin). Approximately 900 spots with a molecular weight range from approximately 15 to 200 kDa (Y-axis) and an isoelectric point range of approximately 4 - 6.5 are covered (X-axis). The 29 proteins showing treatment-related significant quantitative changes are indicated with arrows and numbers. The arrow angle indicates the relative magnitude and the direction of the change (abundance ratio treated/control), and the arrow length indicates *t*-test statistical significance of the difference. The identification of the following labeled liver spots are known: 251 and 175: NADPH cytochrome reductase; 87: cytochrome b5; 252: serine protease inhibitor; 55: senescence marker protein-30; 117: arylsulfotransferase; 196: F_1ATPase-δ subunit; 227: liver fatty acid binding protein; 214: ornithine aminotransferase.

TABLE I

Subcellular Location of the Changed Proteins

	Increased by treatement	Decreased by treatement
Microsomal	4	0
Mitochondrial	0	5
Cytosolic	6	6
Unknown location	3	5

TABLE II

Spot Abundance Data

		CONTROL		TREATED			
Master Number	Subcellular Location	Group Average Volume	CV	Group Average Volume	CV	*t*-test P-value	Treated vs. Control
55	Cytosolic	24772.0	36%	18199.7	24%	0.00092	0.73
87	Microsomal	37193.8	16%	76363.9	25%	<0.00001	2.05
91	Unknown	12323.0	21%	10117.7	19%	0.00080	0.82
105	Unknown	4809.9	34%	10835.5	23%	<0.00001	2.25
113	Cytosolic	7293.5	14%	5868.4	15%	0.00011	0.80
117	Cytosolic	13114.9	31%	9122.8	29%	0.00018	0.70
175	Microsomal	1245.2	27%	3796.0	17%	<0.00001	3.05
178	Cytosolic	6439.9	16%	5410.8	20%	0.00096	0.84
186	Unknown	2452.5	30%	5406.5	28%	0.00002	2.20
196	Mitochondrial	20100.6	17%	14700.6	11%	0.00001	0.73
214	Mitochondrial	12421.1	27%	5012.5	25%	<0.00001	0.40
227	Cytosolic	33686.7	26%	23116.2	24%	0.00021	0.69
240	Cytosolic	1110.0	25%	1891.5	31%	0.00007	1.70
251	Microsomal	556.0	36%	2088.8	34%	0.00001	3.76
252	Microsomal	1544.6	42%	6487.8	26%	<0.00001	4.20
266	Cytosolic	715.1	29%	1309.4	24%	0.00001	1.83
276	Cytosolic	10523.2	16%	15867.8	20%	0.00001	1.51
282	Cytosolic	2235.6	27%	1259.7	29%	0.00001	0.56
286	Mitochondrial	3263.5	15%	2407.1	18%	0.00001	0.74
292	Cytosolic	2563.5	32%	5014.1	24%	<0.00001	1.96
315	Unknown	2255.2	26%	1202.8	31%	<0.00001	0.53
327	Unknown	8470.1	33%	4376.4	26%	0.00001	0.52
347	Cytosolic	1121.3	36%	2211.8	37%	0.00014	1.97
395	Mitochondrial	23821.6	50%	13408.4	38%	0.00033	0.56
401	Cytosolic	3129.1	19%	5853.0	23%	<0.00001	1.87
426	Mitochondrial	2522.1	23%	1608.2	36%	0.00006	0.64
824	Unknown	9801.3	24%	6043.5	25%	0.00001	0.62
839	Unknown	827.1	29%	1236.4	20%	0.00004	1.49
1343	Unknown	2260.1	25%	1514.1	20%	0.00005	0.67

Discussion

Treatment of rats with the hypoglycemic agent SDZ PGU 693 was shown to induce hepatocellular hypertrophy. Not surprisingly, these treatment-related morphological changes were found to be associated with a whole set of alterations in the liver protein expression patterns of these animals. Although microsomal, mitochondrial and cytosolic proteins were affected by the compound to a similar extent, it was interesting that all proteins assigned to the microsomal fraction were strongly induced and all the spots assigned to the mitochondrial fraction were decreased by the treatment. SDZ PGU 693 was a strong inducer of the two microsomal proteins cytochrome b5 and NADPH cytochrome P-450 reductase, the latter being an enzyme required for electron transfer from NADP to cytochrome P-450 and to cytochrome b5 (8). Both proteins are known co-factors of the P-450 enzyme system, strongly suggesting that SDZ PGU 693 is an inducer of the P-450 isoenzymes. Most of the P-450 isoenzymes have a very basic p*I* and are therefore not resolved on the gel system used in this study, however it is expected that their abundance is also substantially increased by the treatment. Such a strong induction of the P-450 enzyme system must likely be associated with microsomal proliferation reflected as the hepatocellular hypertrophy observed in these animals.

There is good indication that SDZ PGU 693 down-regulates the mitochondrial liver fatty acid metabolism as reflected by the decrease in the cytosolic liver fatty-acid binding protein paralleled by a decrease in the F_1ATPase-δ subunit. The liver fatty acid binding protein binds free fatty acids in the cytoplasm and is considered an intracellular carrier for fatty acids in the hepatocyte (9). The F_1ATPase-δ subunit is one of the five chains of the enzymatic component (coupling factor F1) of the mitochondrial ATPase complex which drives the formation of ATP. Keeping in mind the effect of the compound on the glucose metabolism, it seems very likely that the perturbation of the fatty acid metabolism is closely related to the pharmacological action of SDZ PGU 693.

No obvious link to the pharmacological and toxicological action of the compound was found for the other proteins identified so far. Among those are the serine protease inhibitor, another microsomal protein strongly induced by the compound which belongs to a family of proteins that function to control the action of serine proteases in many diverse physiological processes (10). Induction of this protein has been observed previously after treatment with all-trans-N-4-hydroxiphenyl retinamide, a cancer prevention compound (11). The relevance of the induction of this protein is currently unclear. The same holds true for the ornithine aminotransferase, a mitochondrial matrix enzyme involved in protein catabolism and induced in liver by high dietary protein (12), for the arylsulfotransferase, a cytosolic protein catalyzing the formation of sulfamates (13) and for the senescence marker protein-30, a cytosolic calcium binding protein which decreases with aging, which were all decreased following treatment with SDZ PGU 693.

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