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Reversible alteration of hepatic messenger RNA species for peroxisomal and non-peroxisomal proteins induced by the hypolipidaemic drug Wy-14,643

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Extensive peroxisomal proliferation in the hepatic parenchymal cells was observed when male rats were given a diet containing 0.1% Wy-14,643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid}, a potent lipid-decreasing drug. This drug also caused a marked increase in the concentrations of the mRNA species coding for four proteins with M_r 77000, 61000, 43000 and 31000, and a similar decrease in the concentrations of three mRNA species coding for proteins of M_r 25000, 24000 and 19000. Specific immunoprecipitation studies identified the proteins of M_r 19000, 43000 and 77000 as α_{2u}-globulin, 3-ketoacyl-CoA thiolase (EC 2.3.1.16) and enoyl-CoA hydratase (EC 4.2.1.17) respectively. Comparisons of the M_r values suggest that the 61000- and 31000-M_r proteins may be equivalent to two additional peroxisomal enzymes, namely catalase (M_r 61000) and uricase (M_r 31000). The identity of the mRNA species coding for the 25000- and 24000-M_r proteins is at present unknown.

Several structurally unrelated hypolipidaemic drugs and industrial plasticizers such as clofibrate (ethyl *p*-chlorophenoxyisobutyrate), tibric acid [2-chloro-5-(dimethylpiperidinosulphonyl)benzoic acid], di-(2-ethylhexyl) phthalate, methyl clofenapate {methyl 2-[4-(*p*-chlorophenyl)phenoxy]-2-methylpropionate} and Wy-14,643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid} cause hepatomegaly and marked proliferation of peroxisomes in liver cells (Hess *et al.*, 1965; Svoboda *et al.*, 1967; Reddy & Krishnakantha, 1975; Moody & Reddy, 1978*a*). The drug-induced proliferation of peroxisomes is characteristically associated with an increase in the total activity, as well as in the specific activity, of catalase and peroxisomal enzymes that are involved in β-oxidation of fatty acids (Reddy & Krisnakantha, 1975; Lazarow & de Duve, 1976; Osumi & Hashimoto, 1978; Reddy *et al.*, 1982*a*). Since the hypolipidaemic property of these agents is always associated with peroxisome proliferation (Reddy *et al.*, 1982*a*), it is believed that increased peroxisomal β-oxidation of fatty acids is responsible for decreasing the blood lipids (Lazarow, 1977, 1978). Peroxisomal β-oxidation enzymes which are distinct from those in the mitochondrial system (Cooper & Beevers, 1969; Lazarow, 1978; Hashimoto, 1982) include FAD-linked acyl-CoA oxidase (M_r 139000, consisting of three polypeptides of M_r 71900, 51700 and 20500) that transfers electrons to O_2 to form H_2O_2, a bifunctional enoyl-CoA hydratase–3-hydroxyacyl-CoA dehydrogenase (single-chain molecule, M_r 78000) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (M_r 89000, with two identical subunits of M_r 44500) (Cooper & Beevers, 1969; Lazarow, 1978; Osumi & Hashimoto, 1979; Hashimoto, 1982).

It has also been shown that, with long-term administration, peroxisome-proliferating agents induce hepatocellular carcinomas in mice and rats (Reddy *et al.*, 1976, 1980). Although these drugs are not mutagenic by themselves (Warren *et al.*, 1980), they seem to cause extensive accumulation of lipofuscin in parenchymal cells (Reddy *et al.*, 1982*b*), thus suggesting the possibility that free radicals produced in the peroxisomes can cause DNA damage and membrane alteration, thereby triggering neoplastic transformation of hepatocytes. To gain an insight into the mechanism of such aberrant effects of these apparently useful drugs, we have examined the alterations in the total hepatic mRNA species in rats under the influence of one of the most potent hypolipidaemic peroxisome proliferators, Wy-14,643 (Reddy & Krishnakantha, 1975). In the present paper we report that Wy-14,643 causes changes in the hepatic concentrations of mRNA species coding for both peroxisomal and non-peroxisomal proteins. Some of the mRNA species whose concentrations are markedly

Abbreviation used: SDS, sodium dodecyl sulphate.

increased by this drug code for the peroxisomal enzymes involved in fatty acid β-oxidation.

Materials and methods

Animals and drug treatment

Male F344 rats (approx. 200g body wt.) were obtained from Charles River Breeding Laboratories, North Wilmington, MA, U.S.A. They were housed in an air-conditioned room with a controlled cycle of light and darkness. Six rats were given Wy-14,643 at 0.1% (w/w) in the diet for 8 weeks. After this period, three rats were killed for the extraction of hepatic mRNA. The other three rats were withdrawn from the drug for 10 days before they were killed for mRNA extraction. Control animals were given the same chow without Wy-14,643.

Isolation of mRNA and its cell-free translation in rabbit reticulocyte lysates

Total hepatic nucleic acid was extracted by the phenol/SDS procedure (Rosenfeld *et al.*, 1972) and poly(A)-containing RNA was isolated by affinity chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972). The mRNA was translated *in vitro* in micrococcal-nuclease-treated rabbit reticulocyte lysates by the method of Pelham & Jackson (1976). Typically, $0.2A_{260}$ unit of poly(A)-containing RNA and 500μCi of [^{35}S]methionine (approx. 1000Ci/mmol; Amersham)/ml were included in 30μl of reaction mixture, and incubation was for 60min at 30°C.

Total translation products of hepatic mRNA were separated on a 16%-polyacrylamide slab gel in the presence of 0.1% SDS (Laemmli, 1970). Quantitative immunoprecipitation of α_{2u}-globulin, 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase was performed by the procedure described by Roy *et al.* (1977). The immunoprecipitates were dissolved in the sample buffer of Laemmli (1970) and electrophoresed on 16%-polyacrylamide slab gels in the presence of 0.1% SDS. A mixture of ^{14}C-labelled proteins of known M_r (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) were run with every gel, and the M_r values of the unknown proteins were calculated on the basis of their electrophoretic mobilities relative to these known marker proteins. After electrophoresis, slab gels were fixed with methanol/acetic acid/water (4:1:5, by vol.), dried and autoradiographed by using Kodak X-ray film. The autoradiograms of the immunoprecipitated proteins were quantified by scanning and integration of the area under the respective peaks.

Determination of the cytoplasmic content of α_{2u}-globulin

Preparation of rat liver cytosol and its radioimmunoassay for α_{2u}-globulin have been described (Roy, 1977; Chatterjee *et al.*, 1983).

Ultrastructural analysis of the liver

Small segments of liver from normal, Wy-14,643-treated and drug-withdrawn rats were fixed in 2.5% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.4, for 1h, post-fixed in 1% OsO_4 buffered to pH7.4 with 0.1M-sym-collidine and processed for electron microscopy. Thin sections cut on an LKB ultramicrotome with a diamond knife were examined in a JEOL 100-CX electron microscope.

Results and discussion

Electron-microscopic examination of livers of drug-treated rats showed extensive proliferation of peroxisomes (Fig. 1*b*) as compared with normal liver (Fig. 1*a*). Withdrawal of the drug from the diet restored the morphological appearance to almost normal within 10 days (Fig. 1*c*). Previous studies (Reddy & Krishnakantha, 1975; Lazarow, 1977; Moody & Reddy, 1978*b*; Lalwani *et al.*, 1981; Reddy *et al.*, 1981) showed that Wy-14,643 causes a 2-fold increase in hepatic catalase activity and several-fold increases in the activities of hepatic carnitine acetyltransferase and the peroxisomal fatty acid β-oxidation system.

Fig. 2 shows the electrophoretic distribution of the products of translation *in vitro* of the total hepatic mRNA obtained from normal, drug-treated and drug-withdrawn animals. These results show that treatment with Wy-14,643 causes both increase and decrease in the hepatic concentrations of several mRNA species compared with controls. The mRNA molecules coding for four proteins, with M_r 77000, 61000, 43000 and 31000 were markedly increased by Wy-14,643. Among the mRNA species whose hepatic concentrations declined significantly after the drug treatment were those coding for proteins of M_r 19000, 24000 and 25000. Withdrawal of the drug for 10 days not only restores the normal ultrastructural characteristics of the hepatocytes, but the pattern of hepatic mRNA also becomes almost indistinguishable from that of the normal control.

Three of the mRNA species whose hepatic concentrations show marked changes after treatment with Wy-14,643 were identified and quantified by specific immunoprecipitation (Fig. 3). The 19000-M_r protein was identified as α_{2u}-globulin, an androgen-dependent urinary protein, synthesized and secreted by the hepatic parenchymal cells (Roy, 1979). As determined by scanning of the electrophoretogram of the immunoprecipitate, treatment with the hypolipidaemic drug caused a 15-fold decrease in the hepatic concentration of α_{2u}-globulin mRNA. Results presented in Table 1 show that, similarly to its mRNA, the hepatic content of α_{2u}-globulin also decreased markedly (more than 10-fold) after treatment with Wy-14,643, and its concentration returned to normal after withdrawal of

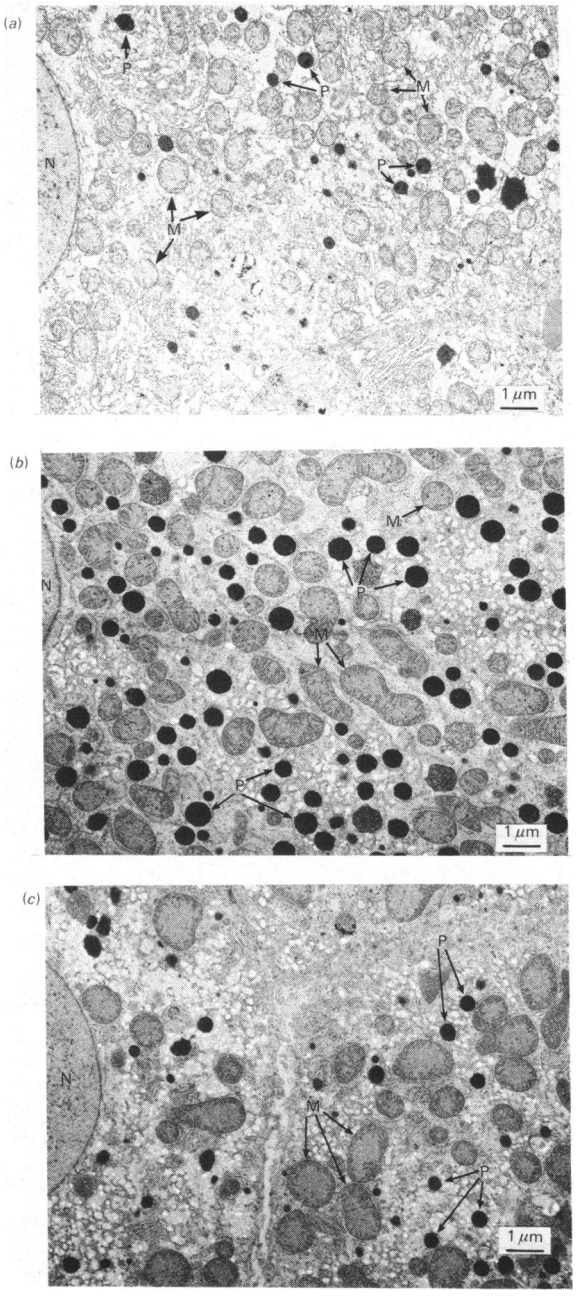

Fig. 1. *Reversible proliferation of peroxisomes in hepatic parenchymal cells with diet containing Wy-14,643*
The hypolipidaemic drug Wy-14,643 was administered in powdered chow at 0.1% for 8 weeks. Ultrathin sections of liver were stained with uranyl acetate and lead citrate, and examined in an electron microscope. Wy-14,643 feeding for 8 weeks (*b*) caused a marked increase in peroxisome population in liver cells compared with the control (*a*). Rats killed 10 days after withdrawal of the drug (*c*) show a relatively normal distribution of peroxisomes. Key: P, peroxisomes; M, mitochondria; N, nucleus.

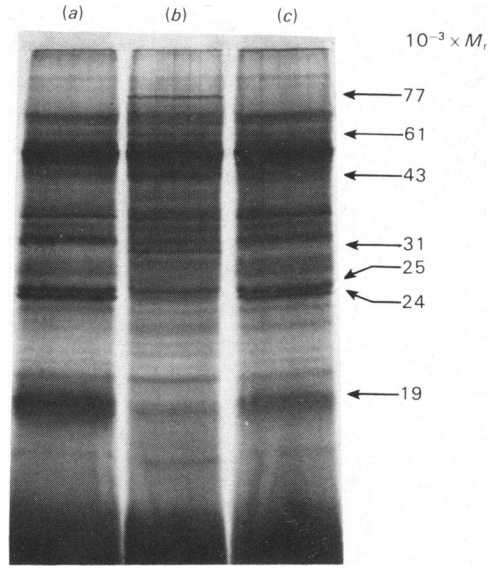

Fig. 2. *Electrophoretically separated translation products of the hepatic mRNA from the drug-treated and drug-withdrawn rats*
Poly(A)-containing hepatic mRNA was pooled from three rats and translated in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and the released peptide chains (10^6 c.p.m.) were subjected to SDS/polyacrylamide-slab-gel electrophoresis. The autoradiogram of the dried gel is shown. The protein bands whose concentrations show marked changes are indicated with arrows. (*a*) Control; (*b*) drug-treated; (*c*) drug withdrawn.

the drug. The mRNA species coding for two other proteins (M_r 24000 and 25000) whose concentrations were significantly decreased by the drug still remain to be identified. Among the mRNA species coding for peroxisomal enzymes whose syntheses are increased by the drug, immunoprecipitation studies with specific antibodies have identified the M_r-43000 protein as 3-ketoacyl-CoA thiolase and the M_r-77000 protein as enoyl-CoA hydratase. These two enzymes are involved in the β-oxidation of fatty acids in the peroxisomes (Furuta *et al.*, 1982). A comparison of their M_r values with those of the known peroxisomal enzymes indicates that the other two mRNA species (coding for M_r-31000 and -61000 proteins) whose concentrations are increased by the drug may represent uricase (M_r 31000) (Goldman & Blobel, 1978) and catalase (M_r 61000) (Robbi & Lazarow, 1978). However, unavailability of specific antisera against these two enzymes has prevented their positive identification.

The close correlation between the M_r values of the translation products of two mRNA species whose concentrations are increased under the influence of

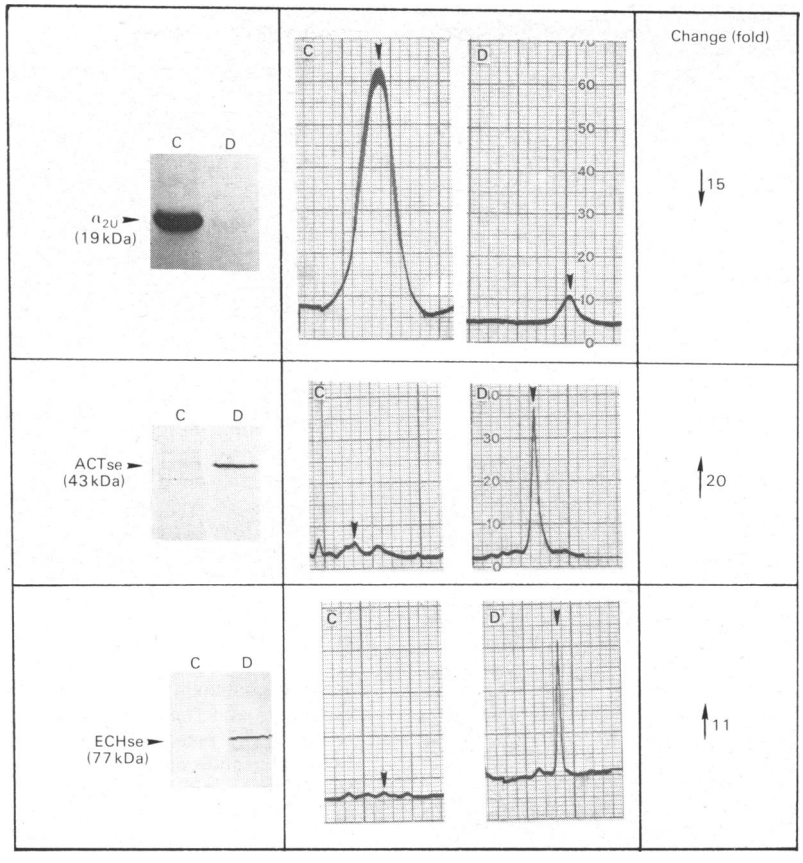

Fig. 3. *Immunochemical identification and determination of the translation products of mRNA species for α_{2u}-globulin, 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase within the translation products of total hepatic mRNA obtained from control (C) and drug-treated rats (D)*

The autoradiograms of the immunoprecipitated translation products were scanned and the area under the respective peak (arrowed) was used to compute the relative changes after drug treatment (fold decrease ↓; fold increase ↑). Immunoprecipitates from controls without mRNA failed to show any band on the autoradiograms. Abbreviations: α_{2u},α_{2u}-globulin (M_r 19000); ACTse, 3-ketoacyl-CoA thiolase (M_r 43000); ECHse, enoyl-CoA hydratase (M_r 77000).

Table 1. *Effect of Wy-14,643 on the hepatic concentration of α_{2u}-globulin in the male rat*

Hepatic cytosol preparations from animals with different treatments were assayed for α_{2u}-globulin by radioimmunoassay (Roy, 1977) and total protein (Lowry *et al.*, 1951). The results are means of the individual values shown in parentheses.

Treatment	Hepatic α_{2u}-globulin (ng/mg of hepatic protein)
Normal diet	1253 (1283, 1324, 1154)
Wy-14,643 for 8 weeks	118 (92, 112, 150)
Wy-14,643 for 8 weeks, followed by normal diet for 10 days	1119 (1112, 920, 1325)

Wy-14,643, and the immunological identification of the two of the translation products as 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase, suggest that this drug acts at a pretranslational level to stimulate the synthesis of the peroxisomal enzymes, some of which are involved in the β-oxidation of fatty acids. A similar increase in the mRNA species for three peroxisomal enzymes, 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and acyl-CoA thiolase, by the industrial plasticizer di-(2-ethylhexyl) phthalate, has been reported (Furuta *et al.*, 1982). Di-(2-ethylhexyl) phthalate, like Wy-14,643, also induces proliferation of peroxisomes (Moody & Reddy, 1978*a*) and enhances the peroxisomal fatty acid β-oxidation system (Osumi & Hashimoto, 1978). Therefore it seems reasonable to assume that the mechanism of hypolipidaemia after treatment with either of these drugs involves an increased accumulation of the mRNA species for the peroxisomal enzymes, leading to the proliferation of peroxisomes and enhanced degradation of fatty acids in the liver.

Since Wy-14,643 itself is not mutagenic (Warren

et al., 1980) in the Ames test (Ames *et al.*, 1975), the basis of the carcinogenic effect of this drug is not clear. It is noteworthy that transformed hepatocytes and hepatoma cells do not synthesize α_{2u}-globulin, an androgen-dependent hepatic secretory protein (Motwani *et al.*, 1980). Treatment with Wy-14,643, however, did not significantly alter the circulating concentration of the androgen (serum testosterone; control, 6.5 μg/ml; drug-treated, 5.8 μg/ml). The significance of the Wy-14,643-mediated decrease in the synthesis of α_{2u}-globulin remains unclear. It will be of interest to determine whether other peroxisome proliferators also decrease the hepatic content of the mRNA species for α_{2u}-globulin, as well as for the M_r-24000 and -25000 proteins.

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References

Ames, B. N., McCann, J. & Yamasaki, E. (1975) *Mutat. Res.* **31**, 347–364

Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408–1412

Chatterjee, B., Demyan, W. F. & Roy, A. K. (1983) *J. Biol. Chem.* **258**, 688–692

Cooper, T. G. & Beevers, H. (1969) *J. Biol. Chem.* **244**, 3516–3520

Furuta, S., Hashimoto, T., Miura, S., Mor, M. & Tatilana, M. (1982) *Biochem. Biophys. Res. Commun.* **105**, 639–646

Goldman, B. M. & Blobel, G. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5066–5070

Hashimoto, T. (1982) *Ann. N.Y. Acad. Sci.* **386**, 5–12

Hess, R., Staubli, W. & Reiss, W. (1965) *Nature (London)* **208**, 856–858

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lalwani, N. D., Reddy, M. K., Mark, M. M. & Reddy, J. K. (1981) *Biochem. J.* **198**, 177–186

Lazarow, P. B. (1977) *Science* **197**, 580–581

Lazarow, P. B. (1978) *J. Biol. Chem.* **253**, 1522–1528

Lazarow, P. B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2043–2046

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Moody, D. E. & Reddy, J. K. (1978*a*) *Toxicol. Appl. Pharmacol.* **45**, 497–504

Moody, D. E. & Reddy, J. K. (1978*b*) *Am. J. Pathol.* **90**, 435–446

Motwani, N. M., Unakar, N. J. & Roy, A. K. (1980) *Endocrinology* **107**, 1606–1631

Osumi, T. & Hashimoto, T. (1978) *J. Biochem. (Tokyo)* **83**, 1361–1363

Osumi, T. & Hashimoto, T. (1979) *Biochem. Biophys. Res. Commun.* **89**, 580–584

Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256

Reddy, J. K. & Krishnakantha, T. P. (1975) *Science* **190**, 787–789

Reddy, J. K., Rao, M. S. & Moody, D. E. (1976) *Cancer Res.* **36**, 1211–1217

Reddy, J. K., Azarnoff, D. L. & Hignite, C. E. (1980) *Nature (London)* **283**, 397–398

Reddy, J. K., Warren, J. R., Reddy, M. K. & Lalwani, N. D. (1982*a*) *Ann. N.Y. Acad. Sci.* **386**, 81–110

Reddy, J. K., Lalwani, N. D., Reddy, M. K. & Qureshi, S. A. (1982*b*) *Cancer Res.* **42**, 259–266

Reddy, M. K., Qureshi, S. A., Hollenberg, P. F. & Reddy, J. K. (1981) *J. Cell Biol.* **89**, 406–417

Robbi, M. & Lazarow, P. B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4344–4348

Rosenfeld, G. C., Comstock, J. P., Means, A. R. & O'Malley, B. W. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1695–1703

Roy, A. K. (1977) *Eur. J. Biochem.* **73**, 537–543

Roy, A. K. (1979) *Biochem. Actions Horm.* **6**, 481–517

Roy, A. K., Dowbenko, D. J. & Schiop, M. J. (1977) *Biochem. J.* **164**, 91–97

Svoboda, D., Grady, H. & Azarnoff, D. (1967) *J. Cell Biol.* **35**, 127–152

Warren, J. R., Simmon, V. F. & Reddy, J. K. (1980) *Cancer Res.* **40**, 36–41