Induction and peroxisomal appearance of gulonolactone oxidase upon clofibrate treatment in mouse liver

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Received 25 August 1999

Abstract Various antihyperlipemic peroxisome proliferators are known to be carcinogenic in rodents but not in human, other primates and guinea pig, which species lost their ability to synthesize ascorbate due to mutations in the *gulonolactone oxidase* gene. Ascorbate synthesis is accompanied by H_2O_2 production, consequently its induction can be potentially harmful; therefore, the in vivo effect of the peroxisome proliferator clofibrate was investigated on gulonolactone oxidase expression in mouse liver. Liver weights and peroxisomal protein contents were increased upon clofibrate treatment. Elevated plasma ascorbate concentrations were found in clofibrate-treated mice due to the higher microsomal gulonolactone oxidase activities. Remarkable gulonolactone oxidase activity appeared in the peroxisomal fraction upon the treatment. Increased activity of the enzyme was associated with an elevation of its mRNA level. According to the present results the evolutionary loss of gulonolactone oxidase may contribute to the explanation of the missing carcinogenic effect of peroxisome proliferators in humans.

Key words: Clofibrate; Peroxisome; Gulonolactone oxidase; H_2O_2; Ascorbate

1. Introduction

Many human cancers can be caused by exposure to non-genotoxic carcinogens. Non-genotoxic carcinogens stimulate cell transformation indirectly, since they are not converted to electrophilic derivatives. Among the most widespread non-genotoxic carcinogens there is a group of compounds collectively referred to as peroxisome proliferators. Peroxisome proliferators are a diverse class of chemicals, including the lipid and cholesterol lowering fibrate drugs (e.g. clofibrate) or endogenously occurring compounds (e.g. dehydroepiandrosterone, leukotriene B_4) [1]. Short-term administration of peroxisome proliferators results in a marked increase in the number and size of peroxisomes and an increase in liver size, while long-term exposure to peroxisome proliferators results in hepatocellular carcinomas [2,3]. A sustained increase in the amount of peroxisomes induces hepatic oxidative stress, since peroxisome proliferators cause a significant increase in the levels of the H_2O_2-generating peroxisomal fatty acid β-oxidation system in the liver [4]. Recent evidence indicates the presence of 8-hydroxydeoxyguanosine in DNA of rat liver chronically treated with peroxisome proliferators, suggesting a possible basis for carcinogenesis of this class of carcinogens [5–7].

However, there are remarkable species differences in response to peroxisome proliferators. Mice, rats, hamsters and rabbits are responsive to these agents and in these species hepatocellular adenomas and carcinomas develop upon long-term administration of fibrates [8–10]. In contrast, guinea pigs, humans, and non-human primates including rhesus monkeys exhibit peroxisome proliferation and enzyme induction, albeit to a lesser degree than in rodents, but show no evidence of carcinogenesis after chronic exposure to fibrate drugs [8–10]. These species have another common attribute besides the resistance against the carcinogenic effect of fibrates: they are unable to synthesize ascorbate due to multiple mutations in the L-*gulono-γ-lactone oxidase* gene [11]. Gulonolactone oxidase is the ultimate enzyme of hepatic ascorbate formation in ascorbate-synthesizing species. This flavoenzyme is an integral membrane protein in the endoplasmic reticulum of the liver [11]. In the course of its action equimolar amounts of O_2 and gulonolactone are consumed, and equimolar amounts of ascorbate and H_2O_2 are produced [12,13]. The regulation of its expression is poorly characterized. The mechanism by which humans, primates and guinea pigs are resistant to the carcinogenic effect of peroxisome proliferators is not known. It is supposed that the insensitivity of these species to the carcinogenic effect of fibrates may be related to the evolutionary loss of H_2O_2-producing gulonolactone oxidase. The question arises whether gulonolactone oxidase is inducible by peroxisome proliferators in ascorbate-synthesizing species. Therefore, the effect of clofibrate on gulonolactone oxidase mRNA expression and activity both in microsomal and peroxisomal fractions was investigated in murine liver.

2. Materials and methods

2.1. Materials

Alamethicin, clofibrate, UDP-glucuronic acid (sodium salt), α,α′-dipyridyl, L-gulono-γ-lactone, 4-morpholinopropanesulfonic acid (MOPS) and Percoll were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade purity or better. μBondapak C_{18} HPLC column (average particle size 10 μm, 300×4 mm I.D.) was obtained from MZ-Analysentechnik (Mainz, Germany). Protein assay solution and 3MM filter papers were purchased from Bio-Rad Laboratories Ltd. (Budapest, Hungary). Hybond-N Nylon membrane and and Hyperfilm were provided by Amersham International (Amersham, UK). α-^{32}P-Labeled dCTP was obtained from Institute of Isotopes Ltd. (Budapest, Hungary). High Prime DNA labeling kit was purchased from Boehringer Mannheim (Mannheim, Germany).

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PII: S0014-5793(99)01184-9

2.2. Treatment of mice

Animals involved in these study received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health, USA. Sexually immature (4–6-week-old) male mice of the C57BL/6 (B6) strain were provided by Charles River Ltd. (Budapest, Hungary) and were housed with free access to standard food in a rigidly controlled animal room of the Department of Medical Chemistry, Semmelweis University of Medicine (Budapest, Hungary). The inducing compound clofibrate suspended in isotonic saline (220 mg/kg body weight) was administered daily by gastric intubation for 1 week before the mice were killed. The control group of animals received isotonic saline. The daily dose of clofibrate was approximately as much as consumed by the animals in the case of clofibrate-containing diets applied in other studies [4].

2.3. Preparation of liver microsomes and peroxisomes

Liver microsomal vesicles were prepared from B6 male mice as previously described [14]. Microsomes were resuspended at a concentration of 40–60 mg of protein/ml in 20 mM MOPS (pH 7.2) containing 100 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$. Hepatic peroxisomal vesicles were purified from the light mitochondrial fraction (λ fraction) of liver homogenates [15]. This fraction, enriched in lysosomes and peroxisomes, was subfractionated by isopycnic centrifugation in iso-osmotic self-generating Percoll gradients as previously described [16]. Microsomal and peroxisomal suspensions were frozen and maintained under liquid nitrogen until used.

2.4. RNA extraction and Northern blotting with GLO probe

Total RNA was extracted from the liver of each mouse by the acid guanidinium thiocyanate method [17]. The isolation of a rat GLO cDNA was described previously [18]. A pUC19 fragment of the cDNA, designated 15L [19], was kindly provided by Dr. Morimitsu Nishikimi (Wakayama Medical College, Department of Biochemistry, Wakayama, Japan). 15L and β-actin cDNA fragments were denatured and labelled with [α-^{32}P]dCTP using the High Prime DNA labelling kit from Boehringer Mannheim. Then these were used to probe the Northern blots. Northern blotting of RNA samples was performed using essentially the method described by Sambrook et al. [20].

2.5. Measurement of enzyme activities

For the measurement of *p*-nitrophenyl UDP-glucuronosyltransferase activities microsomal and peroxisomal vesicles were permeabilized by alamethicin using its optimally activating concentration (0.05 mg/mg protein). Permeabilized vesicles were incubated in the presence of 5 mM UDP-glucuronic acid and 500 μM *p*-nitrophenyl for 30 min at 37°C. Incubations were terminated by the addition of trichloroacetic acid (TCA; 5% final concentration). The aglycone content of the alkalinized deproteinized supernatants was measured spectrophotometrically as previously described [21]. Plasma ascorbate contents were measured by reverse phase HPLC following specific sample preparation as described earlier [22]. For the determination of gulonolactone oxidase activities intact microsomal and peroxisomal vesicles (usually 1 mg protein/ml) were incubated in the presence of 5 mM gulonolactone for 30 min at 37°C. Incubations were terminated by the addition of 0.05 volume of 100% TCA. Ascorbate content was measured from deproteinized supernatants by the method of Omaye et al. [23], based on the reduction of Fe^{3+} with the oxidation of ascorbate and the subsequent determination of the Fe^{2+}-α,α′-dipyridyl complex. Catalase activities were measured according to [24].

2.6. Miscellaneous

Protein concentration of microsomes and peroxisomes was determined using Bio-Rad protein assay solution with bovine serum albumin as standard according to manufacturer's instructions. All data are expressed as means ± S.E.M. Statistical analysis was carried out using Student's *t*-test.

3. Results

3.1. The effect of clofibrate on microsomal and peroxisomal gulonolactone oxidase activities

The effect of clofibrate treatment on microsomal and peroxisomal gulonolactone oxidase activities was investigated in mice. There was no difference in terminal body weight between control and treated animals after 1 week of clofibrate administration (Table 1). According to earlier observations [2], in clofibrate-treated animals liver weights and relative liver weights were significantly higher compared to control mice. Relative microsomal protein content was not affected by clofibrate, while relative peroxisomal protein content showed an approximately 2.5-fold increase indicating the proliferation of peroxisomes. At the same time plasma ascorbate concentration exhibited a more than two-fold elevation in clofibrate-treated mice (Table 1). It was supposed that the increase in plasma ascorbate concentration was due to the stimulated synthesis of ascorbate in the liver. The initial ascorbate contents in both microsomes and peroxisomes were higher in clofibrate-treated mice compared to controls (Table 1). The activity of microsomal gulonolactone oxidase was increased approximately 8.5-fold by clofibrate and simultaneously the appearance of peroxisomal gulonolactone oxidase activity was observed (Table 1). In control mouse liver only 0.1% of the total gulonolactone oxidase activity was confined to the

Table 1
The effect of clofibrate treatment on microsomal and peroxisomal enzyme activities

	Control	Clofibrate-treated
Body weight (g)	22.7 ± 0.2	19.9 ± 3.4
Liver weight (mg)	1005 ± 51	1319 ± 38*
Relative liver weight (g liver/kg body weight)	41.1 ± 2.0	56.7 ± 0.9*
Relative peroxisomal protein content (μg/g liver)	482 ± 64	1201 ± 98*
Relative microsomal protein content (μg/g liver)	6819 ± 1199	6934 ± 226
Plasma ascorbate concentration (μM)	66.4 ± 4.2	135.2 ± 15.6*
Microsomal *p*-nitrophenyl UGT activity (nmol/min/mg protein)	29.58 ± 1.02	27.68 ± 0.60
Microsomal catalase activity (μmol/min/mg protein)	0.48 ± 0.14	0.53 ± 0.09
Microsomal ascorbate content (nmol/mg protein)	15.2 ± 1.3	36.7 ± 4.6*
Microsomal gulonolactone oxidase activity (nmol/min/liver)	11.8 ± 2.0	101.0 ± 8.7*
Distribution of gulonolactone oxidase activity (%)	99.9	92.2
Peroxisomal catalase activity (μmol/min/mg protein)	50.3 ± 9.2	114.3 ± 5.2*
Peroxisomal *p*-nitrophenyl UGT activity (nmol/min/mg protein)	0.07 ± 0.03	0.05 ± 0.01
Peroxisomal ascorbate content (nmol/mg protein)	4.64 ± 0.90	12.88 ± 2.43**
Peroxisomal gulonolactone oxidase activity (nmol/min/liver)	0.017 ± 0.002	8.59 ± 1.54*
Distribution of gulonolactone oxidase activity (%)	0.1	7.8

Data are expressed as means ± S.E.M. from 4–7 individual animals of each experimental group. Significant differences from the corresponding controls: $^{**}P < 0.05$, $^{*}P < 0.01$. Distribution of gulonolactone oxidase activities between microsomal and peroxisomal fractions was calculated on the basis of the sum of these two values regarding it as the total (100%).

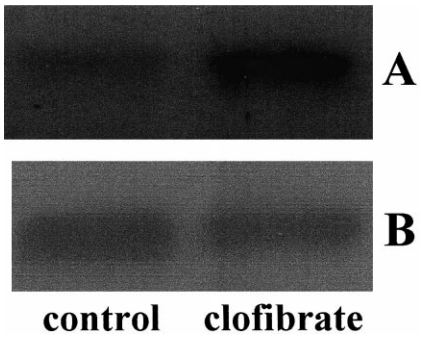

Fig. 1. The effect of clofibrate treatment on gulonolactone oxidase mRNA level in the liver of mice. Total RNAs (30 μg/sample) isolated from control and clofibrate-treated mice were subjected to electrophoresis in agarose gel containing formaldehyde followed by transfer to Hybond-N Nylon membrane as detailed in Section 2. The membrane was hybridized with [α-^{32}P]dCTP-labelled GLO (A) and β-actin (B) cDNA probes then autoradiography was used to visualize mRNAs. Northern blot analysis with GLO and β-actin probe was carried out with each RNA preparation from each animal involved in this experiment and a typical result is presented.

peroxisomal fraction while in clofibrate-treated liver almost 8% of the total activity was localized to them (Table 1).

The purity of microsomal and peroxisomal preparations was also checked by measuring activities of the typical marker enzymes in these fractions. The activity of a microsomal marker enzyme, *p*-nitrophenyl UDP-glucuronosyltransferase, was not changed by clofibrate. Microsomes contained minimal peroxisomal contamination, because catalase activities in this fraction were lower than in peroxisomes by an order of magnitude (Table 1). On the other hand, the high catalase activities and the very low *p*-nitrophenyl UDP-glucuronosyltransferase activities in peroxisomes were indicative of the purity of peroxisomal preparations (Table 1). According to earlier findings, a modest elevation in catalase activity could be observed after clofibrate treatment, but at the same time microsomal catalase activity was not changed (Table 1).

3.2. *The effect of clofibrate treatment on hepatic gulonolactone oxidase mRNA level*

To determine whether the enhanced gulonolactone oxidase activity in clofibrate-treated mice occurs at the mRNA level, total RNA was isolated from livers of both control and clofibrate-treated animals. RNA samples were subjected to Northern hybridization with gulonolactone oxidase cDNA probe as described. The autoradiograph showed an elevation of the amount of gulonolactone oxidase mRNA in the liver of mice treated with clofibrate (Fig. 1).

4. Discussion

In the present study we demonstrated that gulonolactone oxidase is induced in the endoplasmic reticulum of murine liver by the peroxisome proliferator clofibrate at the level of mRNA (Fig. 1). Moreover, gulonolactone oxidase activity appears in the proliferated peroxisomes. The upregulation of gulonolactone oxidase could be due to the enhanced transcription from its gene. There are several genes encoding peroxisomal fatty acid metabolizing enzymes which are inducible by peroxisome proliferators [4]. Peroxisome proliferation is mediated by peroxisome proliferator-activated receptor-α (PPARα) [3]. Sequence analysis of the mouse gulonolactone oxidase promoter is needed to demonstrate the involvement of PPARα in the mechanism of induction.

Peroxisome proliferation is presumed to cause gene mutations indirectly by increasing intracellular H_2O_2 [5–7]. In rodent liver, peroxisome proliferators induce peroxisomal and mitochondrial fatty acid β-oxidation simultaneously with the H_2O_2-generating enzyme acyl-CoA oxidase but only modestly induce catalase [1]. In addition, the activity of the other H_2O_2-degrading enzyme, the cytoplasmic glutathione peroxidase, is often depressed following long-term administration of peroxisome proliferators [1]. Thus, excess H_2O_2 could potentially escape the peroxisomes and react with cellular macromolecules [1]. H_2O_2 can also activate several transcription factors acting as a signal molecule or by redox mechanisms [25]. The difference between humans and rodents in the expression of PPARα may explain partly the species differences in the carcinogenic response to peroxisome proliferators. The evolutionary loss of gulonolactone oxidase can give another, perhaps more appropriate explanation for this difference. Gulonolactone oxidase may be regarded as an ectopic oxidase which generates H_2O_2 in the endoplasmic reticulum rather than in the usual place, in peroxisomes. The induced gulonolactone oxidase produces two metabolites: ascorbate and H_2O_2. On the one hand the formation of ascorbate after clofibrate treatment is a necessary process: (i) ascorbate is required for carnitine biosynthesis [26], thus it can promote the transport of acyl-CoA groups into the mitochondria and peroxisomes for the peroxisome proliferator-induced fatty acid catabolizing enzymes; (ii) α-oxidation of fatty acids is confined to peroxisomes working at the highest rate in the presence of ascorbate [27]. On the other hand, H_2O_2 can disturb the cell cycle control, since there is no positive correlation between the activities of H_2O_2-producing and H_2O_2-eliminating enzymes after clofibrate treatment [1]. In the lack of gulonolactone oxidase the intracellular formation of hazardous H_2O_2 is decreased, lowering the possibility of carcinogenesis after the exposure to peroxisome proliferators. In the light of this conclusion the evolutionary loss of gulonolactone oxidase activity in humans, primates and guinea pigs could be advantageous because the carcinogenic side effect of peroxisome proliferation, a physiological process, could be prevented.

Acknowledgements: We wish to express our gratitude to Dr. Morimitsu Nishikimi (Wakayama Medical College, Department of Biochemistry, Wakayama, Japan), for the rat GLO cDNA. This work was supported by OTKA (Országos Tudományos Kutatási Alap, F022495, T019907), Hungary, by the Ministries of Health (ETT 448) and Education, Hungary and by the Hungarian Academy of Sciences.

References

[1] Gonzalez, F.J., Peters, J.M. and Cattley, R.C. (1998) J. Natl. Cancer Inst. 90, 1702–1709.
[2] Peters, J.M., Cattley, R.C. and Gonzalez, F.J. (1997) Carcinogenesis 18, 2029–2033.
[3] Gonzalez, F.J. (1997) Biochimie 79, 139–144.
[4] Lee, S.S., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H. and Gonzalez, F.J. (1995) Mol. Cell. Biol. 15, 3012–3022.
[5] Reddy, J.K. and Rao, M.S. (1989) Mutat. Res. 214, 63–68.
[6] Cerutti, P.A. (1985) Science 227, 375–381.
[7] Ashby, J., Brady, A., Elcombe, C.R., Elliott, B.M., Ishmael, J.,

Odum, J., Tugwood, J.D., Kettle, S. and Purchase, I.F. (1994) Hum. Exp. Toxicol. 13, S1–117.
[8] Green, S., Tugwood, J.D. and Issemann, I. (1992) Toxicol. Lett. 64/65, 131–139.
[9] Watanabe, T., Horie, S., Yamada, J., Isaji, M., Nishigaki, T., Naito, J. and Suga, T. (1989) Biochem. Pharmacol. 38, 367–371.
[10] Tugwood, J.D., Holden, P.R., James, N.H., Prince, R.A. and Roberts, R.A. (1998) Arch. Toxicol. 72, 169–177.
[11] Bánhegyi, G., Braun, L., Csala, M., Puskás, F. and Mandl, J. (1997) Free Radical Biol. Med. 23, 793–803.
[12] Bánhegyi, G., Csala, M., Braun, L., Garzó, T. and Mandl, J. (1996) FEBS Lett. 381, 39–41.
[13] Chatterjee, I.B. (1970) Methods Enzymol. 18, 28–34.
[14] Coughtrie, M.W.H., Burchell, B., Shepherd, I.M. and Bend, J.R. (1987) Mol. Pharmacol. 31, 585–591.
[15] de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617.
[16] Verheyden, K., Fransen, M., Van Veldhoven, P.P. and Mannaerts, G.P. (1992) Biochim. Biophys. Acta 1109, 48–54.
[17] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[18] Koshizaka, T., Nishikimi, M., Ozawa, T. and Yagi, K. (1988) J. Biol. Chem. 263, 1619–1621.
[19] Nishikimi, M., Koshizaka, T., Ozawa, T. and Yagi, K. (1988) Arch. Biochem. Biophys. 267, 842–846.
[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., pp. 7.39–7.52, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[21] Bock, K.W., Burchell, B., Dutton, G.J., Hanninen, O., Mulder, O.J., Owens, I.S., Siest, G. and Tephly, T.R. (1983) Biochem. Pharmacol. 32, 953–953.
[22] Harapanhalli, R.S., Howell, R.W. and Rao, D.V. (1993) J. Chromatogr. 614, 233–243.
[23] Omaye, S.T., Turnbull, J.D. and Sauerberlich, H.E. (1979) Methods Enzymol. 62, 3–7.
[24] Bergmeyer, H.U. (Ed.) (1974) Methods of Enzymatic Analysis, Vol. 2, Verlag Chemie-Academic Press, New York.
[25] Hogg, N. (1998) Semin. Reprod. Endocrinol. 16, 241–248.
[26] Rebouche, C.J. (1991) Am. J. Clin. Nutr. 54, 1147s–1152s.
[27] Casteels, M., Croes, K., Van Veldhoven, P.P. and Mannaerts, G.P.J. (1997) Inherit. Metab. Dis. 20, 665–673.