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THE INDUCTION OF LIVER PEROXISOMAL PROLIFERATION BY β,β′-METHYL-SUBSTITUTED HEXADECANEDIOIC ACID (MEDICA 16)

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Abstract—Treatment of rats by β,β′-methyl-substituted hexadecanedioic acid (MEDICA 16) resulted in a dose- and time-dependent increase in liver peroxisomal enoyl-CoA hydratase and cyanide-insensitive palmitoyl-CoA oxidation with a concomitant increase in the volume density of peroxisomes as determined by morphometry. The induced peroxisomal proliferation was sustained as long as treatment was maintained and was accompanied by an increase in liver weight.

Incubation of cultured rat hepatocytes in the presence of MEDICA 16 added to the culture medium resulted in a dose-dependent increase in peroxisomal β-oxidation activities with a concomitant elevation of the volume density of peroxisomes. The induction of peroxisomal proliferation by MEDICA 16 in culture could be prevented in the presence of carnitine palmitoyltransferase inhibitors added to the culture medium, e.g. 2-bromopalmitate, 2-tetradecylglycidic acid or 2-[5-(4-chlorophenyl)-pentyl]oxirane-2-carboxylate.

The induction of liver peroxisomes by MEDICA 16 conforms to the previously defined requirement for an amphipathic carboxylate in initiating peroxisomal proliferation. The prevention of peroxisomal proliferation by carnitine acyltransferase inhibitors may implicate the involvement of this acyltransferase in the induction of peroxisomal proliferation by xenobiotic or native amphipathic carboxylates.

β,β′-Methyl-substituted dicarboxylic acids (MEDICA) of C_{14}–C_{18} chain length (HOOC–CH_2–$C(CH_3)_2$–$(CH_2)_n$–$C(CH_3)_2$–CH_2–COOH) have been recently reported to act as hypolipidemic effectors with MEDICA 16 ($n = 10$) being the most potent of the series [1, 2]. The hypolipidemic effect of MEDICA 16 in the rat under conditions of fat-free carbohydrate-rich feeding could be accounted for by a reversible dose-dependent inhibition of liver ATP-citrate lyase [1, 3] with a concomitant inhibition of the incorporation of ^3H_2O or acetate into liver esterified fatty acids and 3-β-hydroxysterols. The hypolipidemic effect in the rat under conditions of a balanced diet which allows for the production of lipoproteins from exogenous fatty acids and cholesterol could be accounted for by an enhanced plasma catabolism of the triacylglycerols-rich lipoproteins mediated by pronounced decrease in total plasma apo C-III [2, 4].

In the light of the marked hypolipidemic effect of MEDICA 16 and the apparent mutual relationship between hypolipidemic drugs and peroxisomal proliferators in rodents [5], it became of interest to evaluate the capacity of MEDICA 16 as a peroxisomal proliferator both in the rat *in vivo* and in cultured rat hepatocytes. The induction of peroxisomal proliferation by MEDICA 16 was evaluated here by following respective peroxisomal β-oxidation enzymes as well as by quantitative morphometric evaluation of the volume density of liver peroxisomes determined by a recently developed television-based automatic image analysis [6, 7].

MATERIALS AND METHODS

Male and female albino rats weighing 150–200 g were fed *ad libitum* with a laboratory chow diet containing MEDICA 16 as indicated. MEDICA 16 was administered by adding the finely powdered drug to the diet for the specified time periods. Dosage was expressed as percent (w/w) of the administered diet.

Cultured rat hepatocytes were prepared according to Berry and Friend [8] with modifications as previously reported [9]. Following plating, the cultured cells were grown in Dulbecco's modified Eagle medium containing 15% fetal calf serum, 1 mU/ml of insulin, 10 μg/ml of hydrocortisone, 50 μg/ml of streptomycin sulfate and 50 μg/ml of penicillin G. MEDICA 16 was added as specified.

Peroxisomal enoyl-CoA hydratase and palmitoyl-CoA oxidation (cyanide-insensitive) activities in culture were determined in triplicate as previously described [9]. The two respective activities in rat liver homogenates were determined by homogenizing the liver in 4 vol. of 0.25 M sucrose–0.1% ethanol followed by centrifuging the homogenate at 700 *g* for

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10 min and measuring the respective activities in the supernatant. Heat labile peroxisomal enoyl-CoA hydratase was assayed spectrophotometrically as previously described [10]. Cyanide-insensitive palmitoyl-CoA oxidation was determined according to Mannaerts [11].

Peroxisomal proliferation in culture was evaluated morphometrically in cultured rat hepatocytes which were incubated for 48 hr in the presence of MEDICA 16 as indicated, fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), stained by 3,3'-diaminobenzidine [12] and further processed by a modified *in situ* embedding technique [13]. Liver peroxisomal proliferation *in vivo* was evaluated morphometrically in livers fixed by perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The vibratome sections of the fixed liver tissues were stained by diaminobenzidine [12], postfixed in 1% osmium tetroxide and embedded in Epon 812. Morphological results were documented using a Zeiss Ultraphot II light microscope or a Philips 301 electron microscope. Morphometric analysis was performed directly on 1 μm semithin sections by scanning 20–50 randomly selected fields using a television-based image analysis system (TAS, Leitz, Wetzlar, F.R.G.) as described recently by Beier and Fahimi [6, 7]. The stereological parameters were based on definitions given by Weibel [14].

Dulbecco's modified Eagle medium and fetal calf serum were from Grand Island Biological Co. (Grand Island, NY). Tissue culture dishes were precoated with collagen as described by [15]. 2-Bromopalmitate was from Fluka. 2-[5-(4-Chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) and 2-tetradecylglycidic acid were kindly provided by Byk Gulden (Konstanz, Germany) and McNeil Pharmaceuticals (Springhouse, PA), respectively. MEDICA 16 was synthesized as previously described [1].

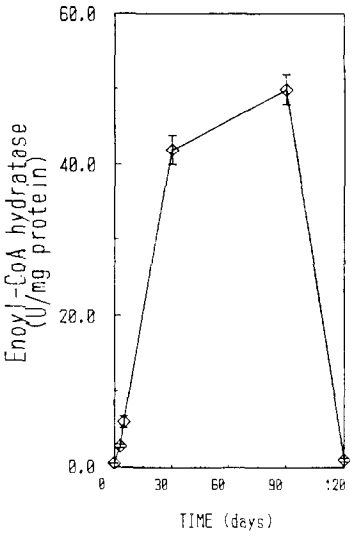

Fig. 1. The induction of liver peroxisomal enoyl-CoA hydratase *in vivo*: time curve. Male rats were treated with 0.25% (w/w) MEDICA 16 for the specified time periods. Following 90 days of treatment the drug was suspended. Mean ± SD (N = 4).

RESULTS

In vivo *experiments*

Treatment of male rats by MEDICA 16 in the diet resulted in a progressive increase in peroxisomal enoyl-CoA hydratase which was already evident on the second day of treatment and increased up to 100-fold following 30 days of treatment (Fig. 1). The induced activity was sustained as long as treatment was maintained and returned to pretreatment values upon eliminating the drug (Fig. 1). The induction of peroxisomal palmitoyl-CoA oxidation and enoyl-CoA hydratase was dose dependent (Fig. 2). Female rats were less sensitive to MEDICA 16 treatment than male rats. Thus, the maximal peroxisomal enoyl-CoA hydratase activity induced in female rats following 90 days of treatment with 0.25% (w/w) MEDICA 16 amounted to 12.0 ± 1.1 U/mg protein as compared to 50.0 ± 4.1 U/mg protein in male rats. The higher response of male rats to MEDICA 16 is similar to that previously reported for the induction of liver peroxisomal proliferation by fibrates [16].

The induction of liver peroxisomal enzyme activities by MEDICA 16 *in vivo* was accompanied by a marked proliferation of peroxisomes (Fig. 3) and a respective increase in their volume density. Thus, following 3 days of treatment by 0.25% (w/w) of MEDICA 16 the volume density increased threefold as compared to nontreated rats. The proliferation of liver peroxisomes was evenly distributed in liver lobules with some variation between individual hepatocytes (Fig. 3B). Ultrastructural examination

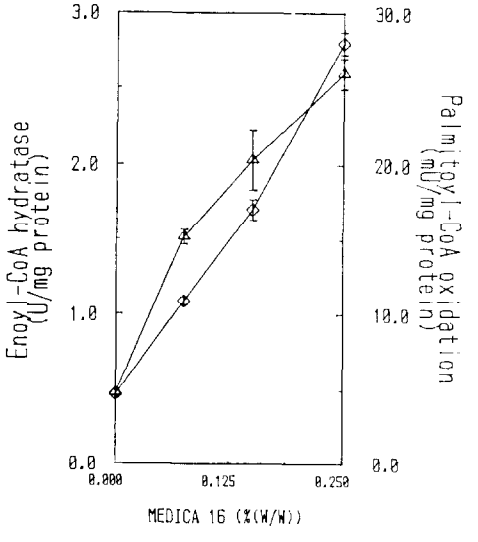

Fig. 2. The induction of liver peroxisomal enoyl-CoA hydratase and cyanide-insensitive palmitoyl-CoA oxidation by MEDICA 16 *in vivo*: dose curve. Male rats were treated for 3 days with MEDICA 16 (% w/w) added to the diet as indicated. Heat-labile enoyl-CoA hydratase (⧳). CN-insensitive palmitoyl-CoA oxidation (⧲). Mean + SD (N = 5).

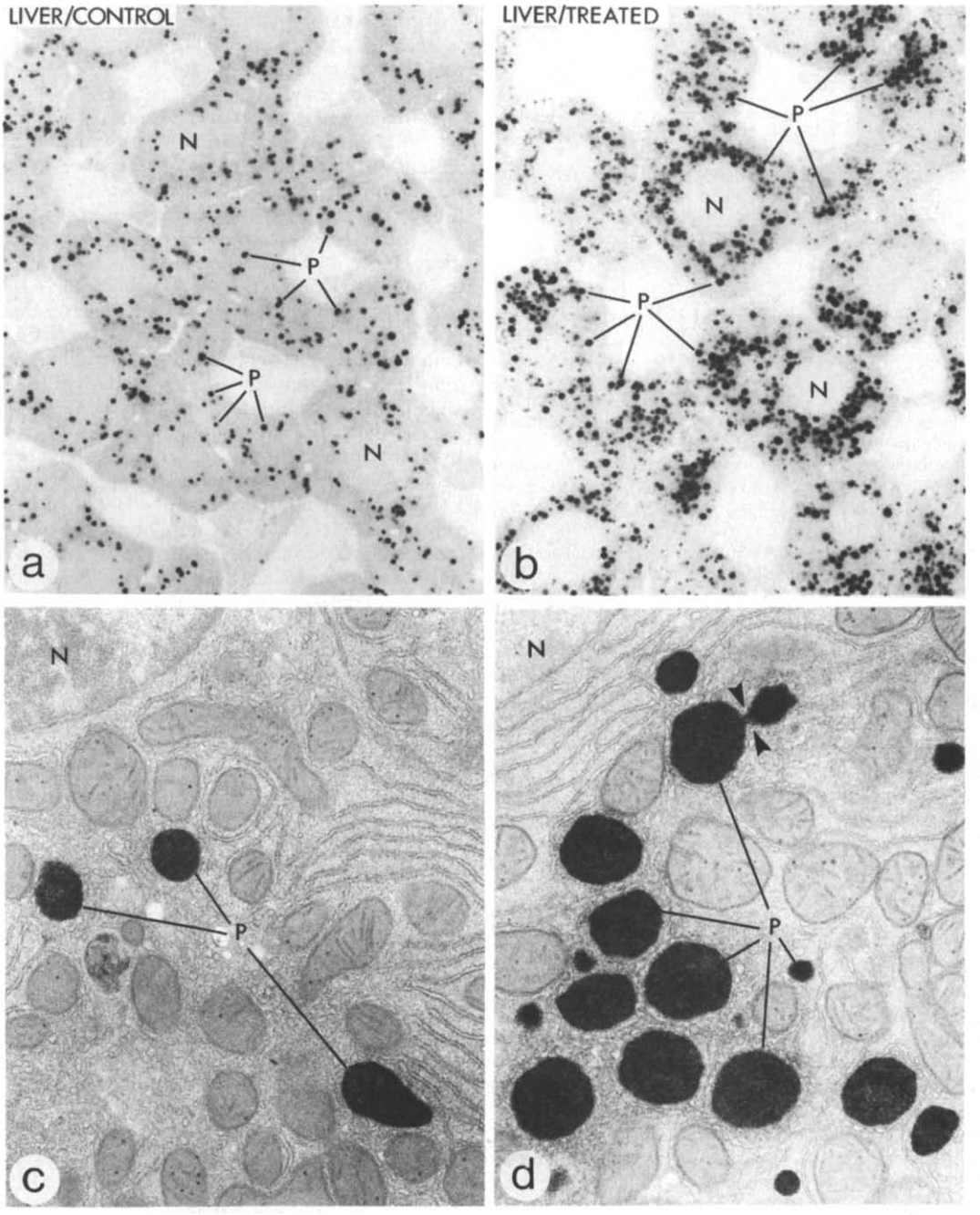

Fig. 3. The induction of liver peroxisomal proliferation by MEDICA 16 *in vivo*. Male rats were treated with 0.25% (w/w) MEDICA 16 for 3 days (b, d) or left untreated (a, c). The liver was processed for light microscopy (a, b; magnification ×1260) and electron microscopy (c, d; magnification ×19,500) as described in Materials and Methods. P—peroxisomes; N—nuclei. Note the formation of a cluster of large and small peroxisomes with an occasional continuity between two adjacent particles (arrowheads).

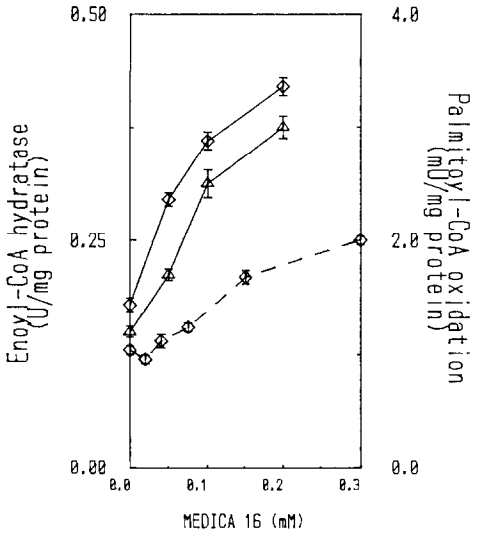

Fig. 4. The induction of peroxisomal enoyl-CoA hydratase and palmitoyl-CoA oxidation by MEDICA 16 in cultured rat hepatocytes. Cultured rat hepatocytes were prepared as described in Materials and Methods and incubated for 48 hr with MEDICA 16 as indicated in the presence (- - - -) or absence (———) of 0.015 mM of fatty acid free albumin. Heat-labile enoyl-CoA hydratase (⧗). CN-insensitive palmitoyl-CoA oxidation (⧗). Mean ± SD for three individual culture plates.

revealed a few abnormal peroxisomes having tubular matrical inclusions (not shown).

The induction of peroxisomal proliferation by chronic treatment with 0.25% (w/w) MEDICA 16 *in vivo* was accompanied by an increase in liver weight which became evident in 5 days of treatment, and amounted to 50% increase in weight following 2–4 weeks of treatment.

Culture experiments

The induction of peroxisomal enzyme activities by MEDICA 16 was also observed in cultured rat hepatocytes incubated in the presence of the drug (Fig. 4). The concentration of MEDICA 16 required for half of the maximal induction of the two peroxisomal marker activities amounted to 0.07 ± 0.02 mM of MEDICA 16. The addition of albumin reduced the efficacy of MEDICA 16 as a peroxisomal proliferator in culture, thus indicating that the free dioic acid is presumably the immediate transported ligand. The maximal induction observed was, however, independent of the presence of albumin.

The induction of peroxisomal enzyme activities in culture was accompanied by a dose dependent increase in the number and the volume density of peroxisomes (Figs 5 and 6).

As reported recently for bezafibrate [17], the induction of peroxisomal proliferation by MEDICA 16 in cultured rat hepatocytes could be prevented in a dose-dependent manner by carnitine acyltransferase inhibitors such as 2-bromopalmitate, 2-tetradecylglycidic acid or POCA (Fig. 7). 2-Bromopalmitate was the most effective of the three inhibitors employed, having an apparent ED_{50} of 5 μM.

DISCUSSION

Peroxisomal proliferation by MEDICA 16 was verified here *in vivo* as well as in cultured rat hepatocytes incubated in the presence of the added drug. Under both experimental conditions the overall effect consisted of time- and dose-dependent induction of peroxisomal β-oxidation enzyme activities with a concomitant increase in the volume density of peroxisomes. Since the changes in enzyme activities induced by hypolipidemic compounds depend on the peroxisomal marker enzyme employed [9, 18], the morphometric verification constitutes a sensitive technique by which the overall proliferatory potential of various drugs may be examined and compared independently of the specific marker enzyme employed. By the application of a modified *in situ* embedding technique, including a DAB-incubation step for the visualization of peroxisomes, stereological data can be obtained directly and automatically from semithin sections of Epon embedded cells. Additional information on possible pathological cellular alterations can be obtained by conventional light- and electron microscopy, using the same embedded material. Thus, in agreement with previous reports [6, 7, 16, 19], morphological studies and morphometric analysis constitute a valuable supplement to biochemical investigations in experiments concerned with the proliferation of peroxisomes in cell culture systems and *in vivo* [16, 20].

Peroxisomal proliferation by MEDICA 16 is similar in nature to that previously reported for other peroxisome proliferating agents such as fibrates [21, 22], phthalate esters [23] and others [5]. The simplicity of MEDICA 16 structure may, however, help in defining the minimal structural constraint for a peroxisomal proliferator. Indeed, all these substances appear to consist of a carboxylic function carried on an hydrophobic backbone to yield an amphipathic carboxylate. The carboxylic function may either be present initially as in the case of MEDICA 16, fibrates, WY-14,643 [5], RMI-14,514 [24], or may be derived by metabolic oxidation of respective alcohols or aldehydes [25]. The free carboxylic function or a derivative thereof is presumably directly involved in the inductive process while the nature of the hydrophobic backbone may be assumed to determine the respective efficacy. It is worth noting that similarly to fibrates [26], MEDICA 16 was also found to serve as substrate for the rat liver microsomal long chain fatty acyl-CoA synthase with the formation of the respective mono CoA thioester (R. Hertz, unpublished).

The induction of peroxisomal enzyme activities by native long chain fatty acids [9] conforms to the requirement for an amphipathic carboxylate, and could be responsible for the *in vivo* induction of peroxisomal proliferation by fat rich diets [27] or under conditions of diabetes and severe starvation [28, 29]. The relatively high concentrations required in culture or *in vivo* and the relatively lower rates of proliferation observed as compared with those initiated by xenobiotic amphipathic carboxylates

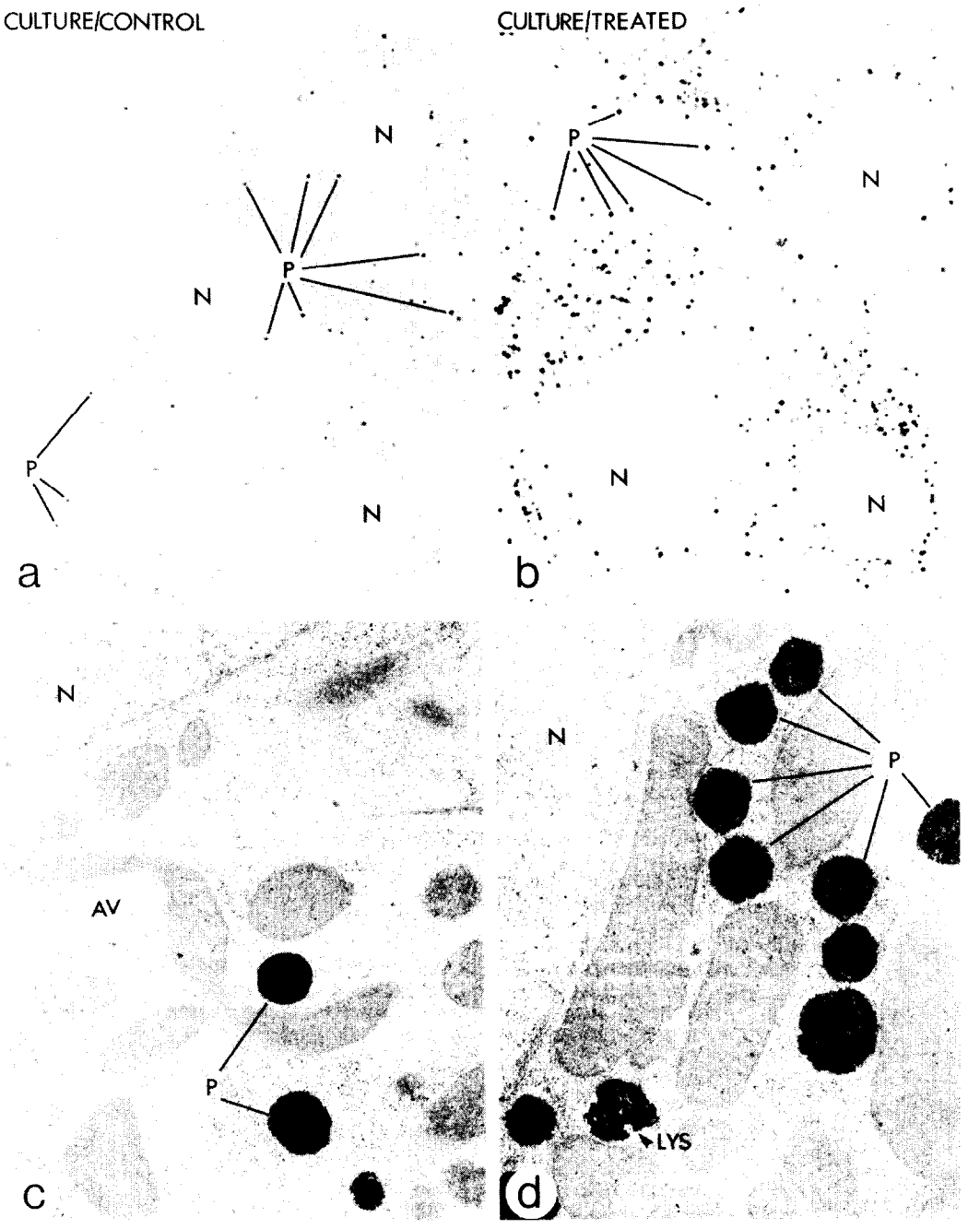

Fig. 5. The induction of peroxisomal proliferation by MEDICA 16 in cultured rat hepatocytes. Cultured rat hepatocytes were prepared as described in Materials and Methods and were incubated for 48 hr in the presence (b, d) or absence (a, c) of 10^{-5} M of MEDICA 16 added to the culture medium. The cultures were embedded *in situ* and were further processed for light microscopy (a, b; magnification ×1180) and electron miscroscopy (c, d; magnification ×19,500) as described in Materials and Methods. AV—autophagic vacuoles; Lys—lysosomes; N—nuclei; P—peroxisomes. Note the marked proliferation of peroxisomes in treated hepatocytes (b, d).

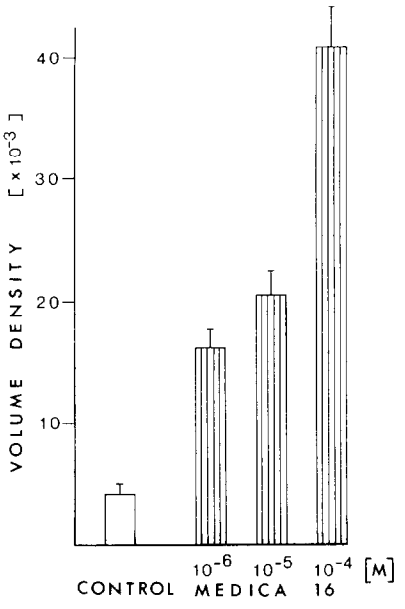

Fig. 6. The induction of peroxisomal proliferation by MEDICA 16 in cultured rat hepatocytes: morphometric analysis. Cultured rat hepatocytes were prepared as described in Materials and Methods and were incubated for 48 hr in the absence or presence of MEDICA 16 as indicated. The *in situ* fixed cultures were processed for light microscopy and morphometric analysis as described in Materials and Methods. The volume density of peroxisomes was defined as $A_{AP}/A_T.K$, where A_{AP} equals the area of peroxisomal profile on test area, R_T equals the test area and the correction factor (K) equals $1 + 3T/2D$ with T = section thickness and D = mean particle diameter [6, 14]. Mean ± SE $P < 0.05$ between all groups except for the two low concentrations of MEDICA 16 where $P < 0.1$.

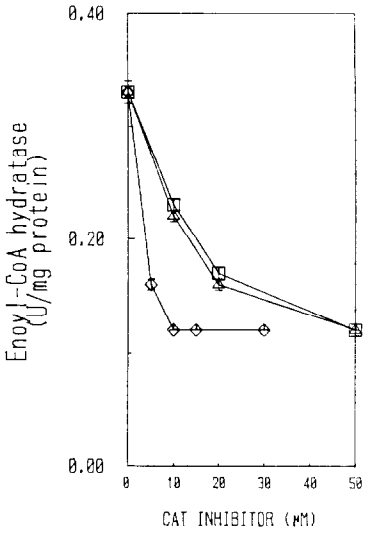

Fig. 7. Prevention of peroxisomal proliferation by carnitine acyltransferase inhibitors in culture. Cultured rat hepatocytes were prepared as described in Materials and Methods and incubated for 48 hr in the presence of 0.15 mM of MEDICA 16 and either 2-bromopalmitate (◇), POCA (△) or 2-tetradecylglycidic acid (□) at the indicated concentrations. Heat labile enoyl-CoA hydratase was determined as described in Materials and Methods. Mean ± SD for three individual culture plates. The hydratase activity in the absence of added MEDICA 16 amounted to 0.12 ± 0.01 U/mg protein and was unaffected by the three carnitine acyltransferase inhibitors employed.

could be due to the fast metabolic clearance of the native long chain fatty acids. Since MEDICA compounds may be considered as nonmetabolic analogues of long chain fatty acids which can neither be esterified into glycerol-3-phosphate nor β-oxidized due to their ω-carboxyl and β,β′-methyl-substitutions, respectively, their capacity as potent peroxisomal proliferators may help to elucidate the inherent peroxisome proliferative capacity of native long chain fatty acids.

The prevention of peroxisomal proliferation by inhibitors of carnitine acyltransferase was recently reported for bezafibrate [17] and confirmed here with MEDICA 16. The apparent requirement for the carnitine acyltransferase in the induction of peroxisomal proliferation by either bezafibrate or MEDICA 16 could not be accounted for by a putative requirement for the respective bezafibroylcarnitine or hexadecanedioylcarnitine derivatives. Thus, the formation of both carnitine derivatives from the respective CoA thioesters could not be detected using cell free preparations of the transferase under conditions where an activity approximately 0.2% of that observed with either octanoyl- or palmitoyl-CoA could still be observed ([17]; R. Hertz, unpublished]. Hence, the apparent involvement of the transferase could implicate the sequestration of mitochondrial CoA [30, 31] or a novel function of the transferase protein.

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