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Chemiluminescence and antioxidant levels during peroxisome proliferation by fenofibrate

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Abstract

Fenofibrate, the hypolipidemic drug and peroxisome proliferator, was given to mice (0.23% w/w in the diet) during 1–3 weeks and H_2O_2 and TBARS steady state concentrations, liver chemiluminescence and antioxidant levels were measured. Administration of fenofibrate during 2 weeks induced an increase of 89% in H_2O_2 steady state concentration. Spontaneous chemiluminescence was decreased by 57% during fenofibrate treatment, while no significant effect was observed on TBARS concentration. Hydroperoxide-initiated chemiluminescence was decreased by 56% after 15 days of fenofibrate treatment, probably due to an increase in endogenous antioxidant levels. Total and oxidized glutathione increased gradually after fenofibrate administration, obtaining maximal increases of 67% and 58% respectively, after 22 days of treatment. An increase of 55% was found in ubiquinol levels in treated mice, as compared with the controls. α-tocopherol content was decreased by 51% in the liver of fenofibrate-treated mice. According to our findings, the high rate of H_2O_2 production associated with peroxisome proliferation, would not lead to an increase in lipid peroxidation. This can be explained by the presence of high levels of ubiquinols, which act as an antioxidant. The increased production of H_2O_2, would lead to DNA damage directly, and not through lipid peroxidation processes.

Keywords: Peroxisome proliferation; Fenofibrate; Chemiluminescence; Antioxidant

1. Introduction

Hypolipidemic agents are known to produce hepatic peroxisome proliferation and induction of peroxisomal H_2O_2-producing oxidases in rats and mice [1,2]. Increased levels of hydrogen peroxide can lead to DNA damage and eventually to the development of carcinogenesis, as suggested by Reddy et al. [3,4]. Among the synthetic peroxisome proliferators, the largest group consists of the amphipathic carboxylates, such as Wy-14,643, benzafibrate, ciprofibrate, nafenopin, fenofibrate, and clofibric acid. Fenofibrate, an ester, is thought to be hydrolyzed to the amphipathic carboxylic acid in cells. These compounds are strong to moderate peroxisome proliferators and hepatocarcinogens in rodents [3].

We have previously characterized a model of peroxisome proliferation induced by fenofibrate in which we observed hepatomegaly, increased activities of

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acyl-CoA oxidase, catalase and urate oxidase, and increased H_2O_2 steady state concentrations in the liver of treated mice [5].

It has been postulated that peroxisome proliferators-induced carcinogenicity would be related to biologically active products of the proliferated peroxisomes rather than to a direct action of the compounds themselves or their metabolites [6]. The hepatocarcinogenic process due to long term administration of peroxisome proliferators (PPs) would be a consequence of the metabolic imbalance resulting from increased peroxisomal enzyme activities and oxidative stress [7]. H_2O_2 induces increases in intracellular free Ca^{2+}, activates Ca^{2+}/calmodulin-dependent protein kinase, and a kinase that phosphorylates the ribosomal protein S6. S6 phosphorylation would be involved in the stimulation of protein synthesis and the acquisition of cell growth competence [8]. In addition, oxidants might stimulate the phosphotransferase activity of protein kinase C (PKC) and increase the expression of the growth-competence-related proto-oncogenes c-fos, c-jun and c-myc. Activation of some of these proto-oncogenes has been observed in the liver of rodents treated with PPs [9].

Since PPs do not directly activate the nuclear-located peroxisome proliferator activated receptor (PPAR), an alternate hypothesis suggests that metabolites of these drugs act directly via signal-transduction pathways. Acyl-CoA derivatives of some PPs have been found to greatly increase the activity of PKC [10]. Phosphorylation/dephosphorylation of key proteins is essential to the control of cell growth, differentiation and proliferation. Orellana et al. have shown that PPs-activated PKC could influence the regulation of the epidermal-growth-factor system which is involved in processes of cellular differentiation and proliferation, and tumorigenesis [11].

Peroxisome proliferators may cause oxidation of membrane fatty acids and lipid peroxidation [12,13]. Goel et al. observed accumulation of lipofuscin – an end product of free-radical-induced oxidative polymerization reactions – in liver during hepatocarcinogenesis by peroxisome proliferators [14].

On the other hand, it has also been described that some peroxisome proliferators such as clofibrate, and phthalates produce an increase in the levels of ubiquinols in several tissues, including rat liver [15]. Ubiquinols are the only liposoluble endogenous antioxidants in animal tissues; they inhibit lipid peroxidation and thus protect against oxidative damage [16].

In this study, we continued the evaluation of the previously presented model [5], through: (a) lipid peroxidation measurements, such as in vivo spontaneous chemiluminescence and thiobarbituric acid reactive substances, (b) hydroperoxide-initiated chemiluminescence, (c) antioxidant levels, and (d) histologic studies.

2. Materials and methods

2.1. Animals and treatment

Female Swiss mice (25–30 g) were obtained from School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. Fenofibrate dissolved in ethanol was incorporated into the feed, and the solvent was evaporated to provide a final concentration of 0.23% w/w. Animals were fed the treated diet for 1, 2 or 3 weeks. Control mice were fed normal diet subjected to the same procedure but without the drug.

2.2. Preparation of mouse liver homogenates

Animals were sacrificed by cervical dislocation. Mouse liver was excised and immediately placed in saline solution at 0–2°C and homogenized in a medium consisting of 120 mM KCl and 30 mM potassium buffer (pH 7.4) at a ratio of 1 g of organ to 9 ml of solution, and centrifuged at $600 \times g$ for 10 min at 0–2°C, to discard nuclei and cellular debris. The supernatant, a suspension of preserved organelles, was termed 'homogenate'.

2.3. Hydrogen peroxide steady state concentration

Steady state concentration of H_2O_2 was measured in the incubation medium of liver slices after reaching diffusion equilibrium. Tissue slices 0.1 mm thick were incubated 10 min in 120 mM NaCl, 30 mM phosphate buffer (pH 7.4) at 30°C and at a tissue/medium ratio of 1/20. Samples of the incubation medium were diluted 1/2.5 with 100 mM phosphate buffer (pH 7.4) containing 2.8 U/ml horseradish peroxidase and 40 μM p-hydroxyphenylacetic acid as hydrogen donor and fluorescence inten-

sity was measured at 317–414 nm. H_2O_2 concentration was determined by a standard calibration curve and calculated by subtracting the value of a sample treated with 0.1 μM catalase from the value of an untreated sample [5].

2.4. In situ liver chemiluminescence

Control and treated mice were anesthetized with urethane i.p. (1.5 g/kg of weight). The liver surface was exposed by laparotomy and chemiluminescence was measured with a Johnson Foundation photon-counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA) [17]. Chemiluminescence was expressed in counts per second per unit of liver surface (cps/cm^2).

2.5. Thiobarbituric acid reactive substances (TBARS)

TBARS were determined in mouse liver homogenates immediately after homogenate preparation by a fluorescent method [18] with modifications [19], based on the 2-thiobarbituric acid reaction. After the extraction with *n*-butanol, the fluorescence was measured at 515 nm excitation and 555 nm emission. The values were expressed as nmol TBARS/g tissue. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane.

2.6. Hydroperoxide-initiated chemiluminescence of liver homogenates

The homogenates were diluted to a final concentration of 1 mg of protein/ml in the same buffer, 3 mM *tert*-butyl hydroperoxide was added and chemiluminescence was measured at 30°C in a Packard Tri-Carb Model 3320 scintillation counter in the out-of-coincidence mode, as previously described [20]. The emission was expressed in counts/min · mg of protein. Protein content was assayed by the method of Lowry et al. using bovine serum albumin as standard [21].

2.7. Glutathione content

Total and oxidized glutathione were determined after 8, 15 and 22 days of fenofibrate treatment. The sum of the reduced and oxidized forms of glutathione was determined using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), NADPH and glutathione reductase in a kinetic assay at 412 nm. Oxidized glutathione (GSSG) was determined using NADPH and glutathione reductase, at 340 nm. Glutathione concentration was expressed in μmol/g organ [22]. Reduced glutathione (GSH) content was calculated based on the measured values of total glutathione and GSSG.

2.8. Lipid-soluble antioxidants

Liver homogenates were extracted with 4 ml of hexane and then centrifuged for 5 min at $1000 \times g$. The hexane layer was removed and evaporated to dryness under nitrogen. The residue was dissolved in methanol/ethanol 1/1 v/v and filtered through a 0.22 μm-pore membrane. For ubiquinols measurements, samples were reduced with $NaBH_4$ and then extracted into 4 ml of hexane following the steps described above. Measurements of α-tocopherol and ubiquinols in the methanol/ethanol extracts were made by HPLC on a 8-C reverse phase column with electrochemical detection [23]. The antioxidant concentrations were referred to commercial standards and expressed as nmol/g tissue [24].

2.9. Statistics

Results are expressed as mean values ± S.E.M. Student's t test was used to study significance of the differences between the means of pairs of groups. ANOVA and Tukey tests [25] were used to analyze the differences between mean values of more than two groups.

2.10. Histopathology

Samples of the liver of mice killed after 1, 2 and 3 weeks of treatment were fixed in a mixture of alcohol, acetic acid and formaldehyde and in formol-buffer [26], and embedded in Histoplast (Sistemas Analíticos, Buenos Aires). Five μm sections were stained with hematoxylin-eosin, PAS and PAS-diastase following standard procedures [26]. Frozen sections of the samples fixed in formol-buffer were stained with oil red O [27].

3. Results

3.1. H_2O_2 steady state concentration

The intracellular H_2O_2 steady state concentration was measured in the incubation medium of liver slices of control and fenofibrate-treated animals after reaching diffusion equilibrium. Administration of fenofibrate during 2 week induced an increase of 89% in H_2O_2 steady state concentration (control value: 0.09 ± 0.01 μM) (Fig. 1)

3.2. Spontaneous liver chemiluminescence

Spontaneous in situ organ photoemission is highly sensitive to characterize increased rates of lipoperoxidation. Spontaneous chemiluminescence was determined in the liver of control and treated mice after 15 days of treatment with fenofibrate. A decrease of 57% was observed in spontaneous chemiluminescence, as compared with the control photoemission (115 ± 18 cps/cm^2) (Fig. 2).

3.3. Thiobarbituric acid reactive substances (TBARS)

TBARS were determined in liver homogenates of control and treated mice after 8, 15 and 22 days of treatment. A slight decrease (15%) although non significant, was observed in 15 days fenofibrate-treated animals as compared with the controls (control value: 0.074 ± 0.004 μmol/g liver) (Fig. 2).

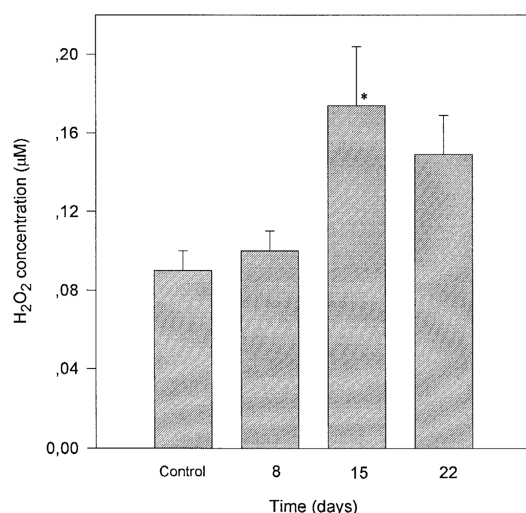

Fig. 1. Effect of fenofibrate administration on liver H_2O_2 steady state concentration. Bars represent mean values ± S.E.M., from six animals. * $P < 0.01$.

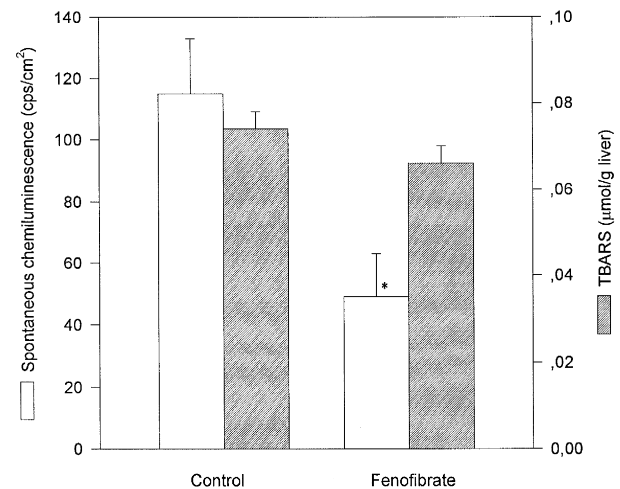

Fig. 2. Spontaneous chemiluminescence and TBARS concentration in control and fenofibrate-treated mice. Values are the mean from 4 animals and bars indicate S.E.M. * $P < 0.01$.

3.4. Hydroperoxide-initiated chemiluminescence

The homogenate chemiluminescence initiated by *tert*-butyl hydroperoxide was found 56% decreased in mice treated for 15 days with fenofibrate, as compared with control values ($18\,400 \pm 1140$ cpm/mg protein) (Table 1).

3.5. Glutathione content

Total and oxidized glutathione levels were measured in liver homogenates of control and treated mice after 8, 15 and 22 days of treatment. Total and oxidized glutathione increased gradually after fenofi-

Table 1
Hydroperoxide-initiated chemiluminescence and liposoluble antioxidant levels in mice treated with fenofibrate for 15 days and in control animals

Parameter	Control	Fenofibrate
Hydroperoxide-induced chemiluminescence (cpm/mg)	$18\,377 \pm 1\,138$	$8\,099 \pm 519$ *
α-tocopherol (nmol/g liver)	5.3 ± 0.2	2.6 ± 0.2 *
Ubiquinol-9 (nmol/g liver)	3.1 ± 0.1	4.8 ± 0.5 *

* $P < 0.0005$.

brate administration. Maximal values were obtained after 22 days of treatment, with increases of 67% and 58% in total and oxidized glutathione content respectively (control values for total glutathione: 8.3 ± 0.3 μmol/g liver; GSSG: 0.20 ± 0.01 μmol/g liver) (Fig. 3). An increase of 72% in GSH levels was observed after 22 days of treatment, as compared with control values (7.9 ± 0.3 μmol/g liver) (Fig. 3). The ratio [GSH]/[GSSG] was not affected by fenofibrate treatment (control ratio: 40 ± 3).

3.6. Lipid soluble antioxidants

Determinations of hepatic levels of α-tocopherol and ubiquinols were carried out in lipid extracts of control and 15 days-treated mice. α-tocopherol content was decreased by 51% after 15 days of treatment with fenofibrate (control value: 5.3 ± 0.2 nmol/g liver). An increase of 55% was found in ubiquinols levels in treated mice, as compared with the controls (3.1 ± 0.1 nmol/g liver) (Table 1).

3.7. Histopathology

The histologic study showed mild microvacuolar fatty changes in animals killed after 2 and 3 week of treatment (Fig. 4). Enlarged hepatocytes, increased eosinophilia of the cytoplasm and mild to moderate depletion of glycogen were also observed in fenofibrate-treated animals (figures not included).

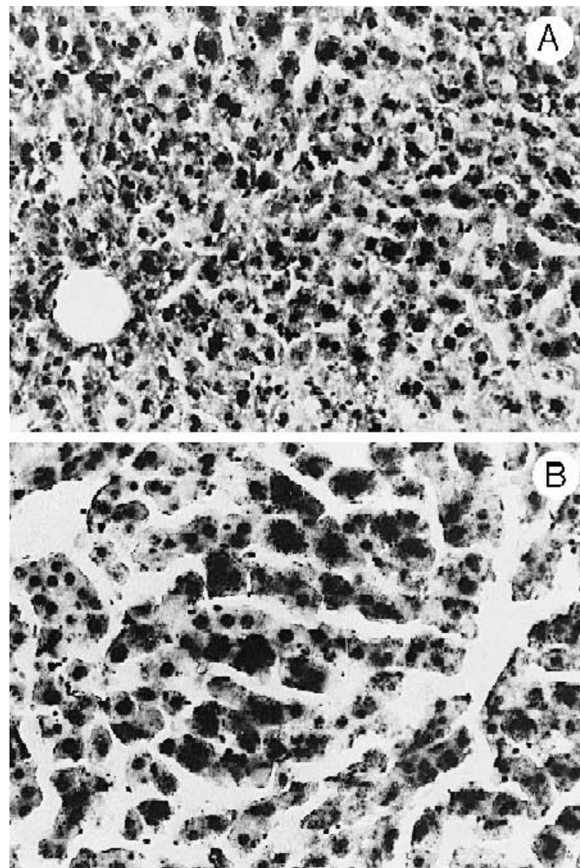

Fig. 4. (A) Liver of control mouse. Oil red O (×250). (B) Microvacuolar steatosis in the liver of a mouse treated with fenofibrate. Oil red O (×250).

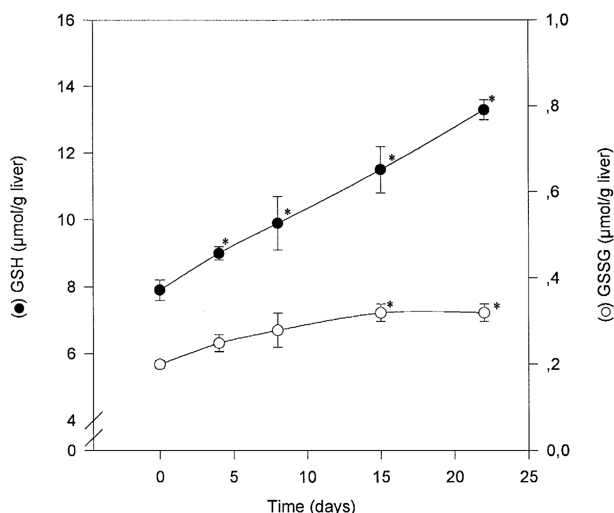

Fig. 3. Effect of fenofibrate treatment on reduced (●) and oxidized (○) glutathione. Symbols indicate mean values from 6 animals and bars indicate S.E.M. * $P < 0.01$.

4. Discussion

It has been postulated that liver carcinogenesis is associated to peroxisome proliferation generated by the oxidative stress resulting from the sustained increase in the peroxisomal generation of H_2O_2 [2]. Although the precise mechanism by which intracellular oxidative stress influences initiation and promotion of carcinogenesis remains unknown, it is generally accepted that H_2O_2 and other reactive oxygen species can cause DNA damage either directly or by initiating lipid peroxidation [14].

We had previously reported an increased H_2O_2 production together with increases in peroxisomal

acyl-CoA oxidase activity during peroxisome proliferation by fenofibrate treatment [5]. In the present study, increased H_2O_2 steady state concentrations were not correlated with increased rates of lipid peroxidation. In vivo chemiluminescence was significantly decreased by 57% in the liver of fenofibrate-treated animals and a slight decrease was observed in TBARS levels. Although both parameters might be useful to estimate lipid peroxidation, chemiluminescence seems to be more sensitive than TBARS. In vivo chemiluminescence offers a noninvasive and continuous monitoring of electronically excited species, mainly singlet oxygen, formed by the free radical chain oxidation of polyunsaturated fatty acids [28]. Our results are consistent with the observations of Huber et al. [29], that showed a decrease in TBARS after nafenopin treatment. However, lipid peroxidation (assessed by measuring the levels of conjugated dienes in lipid extracts) was enhanced in primary cultures of rat hepatocytes after incubation for 44 h with nafenopin [6]. Other studies on oxidative injury after various peroxisome proliferator administration have shown that TBARS were only slightly increased or even unchanged [30,31]. Goel et al. suggested that sustained injury to liver cells by long-term peroxisome proliferation may be necessary to induce peroxidative damage to membrane lipids [14].

Peroxisome-proliferating agents are also known to produce numerous alterations in hepatic lipid metabolism. Lipid accumulation was observed in the liver of fenofibrate-treated animals. As previously described by Makowska et al., intrahepatic accumulation of lipid causes an induction of cytochrome P450IVA1 [32]. Because of the specificity of P450IVA1 for fatty acids, this leads to an accumulation of ω-hydroxylated fatty acids, which are further oxidized in the cytosol to dicarboxylic acids (preferential substrates for the peroxisomal β-oxidation system), which then act as the proximal stimulus for peroxisomal proliferation (and hence H_2O_2 production).

Hydroperoxide-induced chemiluminescence measures the balance between pro-oxidants and antioxidants present in the tissue [33]. Hydroperoxide-induced chemiluminescence was decreased by fenofibrate treatment. This can be explained by the presence of high levels of endogenous antioxidants. Increased hydroperoxide-induced chemiluminescence was associated to antioxidant depletion in several experimental conditions, such as tumor-bearing animals [34] and mitoxantrone treatment [35]. An enhancement of the antioxidant response associated to peroxisome proliferation by fenofibrate, would lead to an inhibition of the emission.

In our model, glutathione and ubiquinols levels were increased by fenofibrate treatment. Gray et al. have suggested that treatments with peroxisome proliferators could lead to increased levels of oxidized glutathione in hepatocytes [36]. This increase could be due to the degradation by glutathione peroxidase of high levels of H_2O_2, coupled to GSH oxidation during peroxisome proliferation. Aberg et al. observed that peroxisome proliferators such as clofibrate and the plasticizer di(2-ethylhexyl)phthalate are highly efficient in increasing liver ubiquinone [15]. An early report indicated increased liver ubiquinone content in the mild oxidative stress of depancreatized rats [37]. Ubiquinol has a strong effect in inhibiting lipid peroxidation in biological systems, such as liposomes [23,38], microsomes [39] and low density lipoproteins [40]. It has been suggested that ubiquinol could act as an antioxidant, scavenging lipoperoxyl radicals and also regenerating tocopheril radicals to α-tocopherol [23,39,41].

α-Tocopherol levels were lower in the liver of fenofibrate-treated animals, as compared with control ones; it appears that α-tocopherol is highly consumed during peroxisome proliferation. Since α-tocopherol is normally incorporated into the liver by lipoproteins, an impairment of the lipoprotein uptake mechanisms by hypolipidemic drugs would result in a decrease in vitamin E levels.

Overproduction of hydrogen peroxide, resulting from increased peroxisomal enzyme activities would lead to the modulation of the expression of protooncogenes via signal transduction pathways. Carcinogenicity of these compounds would be related to active oxygen species produced by proliferated peroxisomes, and would not be a consequence of a direct action of the compound or its metabolites.

In summary, our results indicate that fenofibrate treatment increases the production of H_2O_2, but not stimulate lipid peroxidation, probably due to the presence of high levels of some endogenous antioxidants (ubiquinols, glutathione). We suggest that carcino-

genesis would arise from DNA damage produced by H_2O_2 and not through lipid peroxidation processes.

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