Caloric restriction prevents oxidative damage induced by the carcinogen clofibrate in mouse liver

Bin Qu[a], Barry Halliwell[a,*], Choon Nam Ong[b], Bee Lan Lee[b], Qiu-Tian Li[a]

[a] *Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*
[b] *Department of Community, Occupational and Family Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

Received 26 January 2000; received in revised form 11 April 2000

Edited by Veli-Pekka Lehto

Abstract Long-term caloric restriction in rodents is known to decrease levels of oxidative damage, which may contribute to an 'anti-ageing' effect. We show here that a shorter period (10 months) of caloric restriction had only small effects on levels of oxidative DNA and protein damage in the livers of mice, but completely attenuated increased oxidative damage caused by the carcinogen clofibrate. Since clofibrate is thought to exert its actions by increasing oxidative damage, our data suggest that 10 months of caloric restriction can increase the resistance of tissues to agents inducing oxidative stress. This may be an important factor in explaining how caloric restriction decreases cancer incidence.

Key words: Clofibrate; Cancer; Peroxisome proliferator; Protein carbonyl; 8-Hydroxy-2′-deoxyguanosine

1. Introduction

Reactive oxygen species such as superoxide radical, hydrogen peroxide and hydroxyl radical are thought to play an important role in the process of ageing in animals [1–4]. Indeed, levels of oxidative damage to proteins (usually measured as protein carbonyls) and to DNA have been reported to rise with age in some human and other animal tissues [1–7]. Long-term caloric restriction in rodents has been shown to extend maximum lifespan and to decrease levels of oxidative damage to DNA and proteins in older animals, providing further evidence for a role of reactive oxygen species in the ageing process [3,8–12]. These decreases in oxidative damage have mostly been described in brain and skeletal muscle and do not appear to be related to elevations of antioxidant defence enzymes [2,8–12]. For example, in one study [12] of mice caloric restriction decreased levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a commonly-used biomarker of oxidative DNA damage [13,14], in nuclear DNA from brain and skeletal muscle.

Cancer can arise from the actions of endogenously-generated reactive oxygen species [1,3] and also from DNA damage by exogenous carcinogens, some of which appear to act, in whole or in part, by causing oxidative DNA damage [1,15,16]. In the present paper, we show that caloric restriction for a period of 10 months, too short to alter endogenous levels of oxidative damage significantly, could nevertheless attenuate oxidative damage caused by the hepatocarcinogen clofibrate. The mechanism of the action of clofibrate is thought to involve peroxisome proliferation leading to increased oxidative damage which in turn facilitates cancer development [15,16].

2. Materials and methods

2.1. Animals

Weanling male ARC Swiss Albino Outbred mice were obtained from the animal center of the National University of Singapore. The animal room was operated on a 12 h light and 12 h dark cycle and constant temperature and humidity. All mice were fed diet A (Table 1) ad libitum for 2 weeks and then the mice were divided into two groups. The control group (AL) was fed diet A ad libitum for 10 months. The caloric restriction group was fed diet B at 60% of the mean caloric intake of control group for 10 months. The amount of food ingested by ad libitum mice was measured twice a week. Diet B was adjusted so that the intake of vitamins and minerals was comparable in both groups (Table 1). At the end of 10 months feeding, every group was subdivided into two groups. All four groups were allowed to take food (diet A) ad libitum. However, two groups were given clofibrate-treated pellets while the other two received control pellets. Clofibrate was dissolved in acetone (0.5 g/100 ml for 100 g food) and food pellets (diet A) were immersed in this solution for 1 h. The solvent was then completely evaporated by fan in air so that all the clofibrate remained in the pellets (0.5 g/100 g of food). Food from the same batch treated identically with acetone alone was used in control diets. Clofibrate had no effect on food intake as assessed by the weight of pellets consumed and by the gain of weight of the animals. From the weight of food consumed, it was calculated that the mice consumed 50 mg of clofibrate per day per animal. The numbers of mice used in each experiment are specified in the figure legends.

2.2. Subcellular fractionation

The mice were killed by cervical dislocation after 14 days on the clofibrate (or control) diets and the livers were immediately excised and sliced in sucrose I buffer (0.25 M sucrose, 5 mM HEPES and 0.5 mM EGTA, pH 7.5) on ice. Excess blood was rinsed off with this buffer and the tissue cut up into small pieces in the same buffer. It was then homogenized on ice using six strokes of a manually-operated loose plunger (Wheaton 55 ml glass homogenizer, USA). The homogenate was spun at $1000\times g$ for 10 min at 4°C. Tissue paper was used to remove the fat layer present on the surface and the supernatant was centrifuged in the same manner twice more. The final supernatant was collected and recentrifuged at $10\,000\times g$ for 10 min at 4°C to obtain the mitochondrial pellet, which was then washed with sucrose II solution (0.25 M sucrose and 5 mM HEPES, pH 7.5) and resuspended in this solution. The mitochondria were purified from this fraction using sucrose gradients [17]. Nuclear fractions were obtained by centrifugation of liver homogenates at $1000\times g$ for 20 min and then further purified by centrifugation through 1.75 M sucrose for 1 h at $40\,000\times g$ [18].

*Corresponding author. Fax: (65)-779-1453.
E-mail: bchbh@nus.edu.sg

PII: S0014-5793(00)01506-4

2.3. Biochemical assays

Peroxisomal β-oxidation was determined by direct assay of acyl-CoA oxidase activity using decanoyl-CoA as substrate [19]. Protein carbonyls, a general assay of oxidative protein damage, were measured by a reaction with dinitrophenylhydrazine (DNPH) [20]. The nuclear fractions were homogenized in 1 ml buffer (100 mM KH_2PO_4–K_2HPO_4, 0.1% digitonin, pH 7.4) containing 100 μl of protease inhibitor cocktail (5 μg/ml aprotinin, 5 μg/ml leupeptin, 7 μg/ml pepstatin A and 10 mM EDTA) and 80 μg of phenylmethanesulfonyl fluoride (dissolved in ethanol). The homogenized fractions were left at room temperature for 15 min and then centrifuged at $2800\times g$ for 10 min. The supernatant was removed and incubated at room temperature for 15 min with streptomycin sulfate solution (final concentration 1%, w/v) to allow the DNA to precipitate. After centrifugation at $2800\times g$ for 10 min, the supernatant was collected for nuclear protein carbonyl determination. Purified mitochondrial fractions were used to determine mitochondrial protein carbonyls after incubation with streptomycin sulfate [20]. The purified nuclear or mitochondrial fractions were incubated with either 10 mM DNPH in 2 M HCl or with 2 M HCl alone for 1 h. Protein was then precipitated by adding an equal volume of 20% (w/v) trichloroacetic acid and allowing to stand for 15 min. The protein was spun down at $3400\times g$, and the pellets were washed three times with an ethyl acetate:ethanol mixture (1:1, v/v) to remove excess DNPH. The final protein pellet was dissolved in 6 M guanidinium hydrochloride and the absorbance was measured at 370 nm using 22 000/M/cm as the molar extinction coefficient of carbonyls. The protein concentration was calculated from the A_{280} of the HCl samples. The protein concentrations for other assays were determined by the method of Lowry et al. [21], with bovine serum albumin as standard.

2.4. Assay of oxidative DNA damage

Nuclear DNA was extracted according to Gupta [22] except that butylated hydroxytoluene was added [23]. Mitochondria prepared with sucrose gradients [17] were used to isolate mitochondrial DNA as described by Birnboim and Doly [24] and Palva and Palva [25]. Mitochondrial DNA (10–20 μg) and nuclear DNA (100 μg) were digested with DNase I, nuclease P1 and alkaline phosphatase [26,27]. The high-performance liquid chromatography system used for 8-OHdG measurement consisted of a Gilson 811 pump (Gilson, Villers-Le-Bel, France), a Whatman Partisphere-5 C18 column (Whatman, Clifton, NJ, USA), a Gilson 234 sample injector, a Gilson 832 temperature regulator and a Shimadzu model CR-5A integrator (Shimadzu, Kyoto, Japan). A Hewlett-Packard 1050 UV detector (254 nm) (Waldbronn, Germany) connected to a Hewlett-Packard 1049 electrochemical detector in series was used to monitor deoxyguanosine and 8-OHdG, respectively [27]. The mobile phase consisted of 10 mM $NH_4H_2PO_4$,10 mM KCl, 1 mM EDTA and 5% methanol (pH 7.4) at a flow rate of 1 ml/min.

3. Results

3.1. General lack of effect of caloric restriction on oxidative damage in liver

The dietary restriction regime used decreased the caloric intake of the mice by 40%, but the intake of vitamins and minerals was maintained constant by adjusting the composition of the diets. As expected, caloric restriction led to a decrease in the body weight of the mice relative to ad libitum fed animals even after a return to a normal diet for 2 weeks

Table 1
The composition of the diets used in this study

Dietary components	Diet A (g/100 g)	Diet B (g/100 g)
Casein	21	21
DL-Methionine	0.15	0.15
Sucrose	15	15
Dextrin	43.65	38.85
Corn oil	10	10
Mineral mix	5	8.34
Vitamin mix	2	3.33
Choline chloride	0.2	0.33
Solka-Floc	3	3
Total (g)	100	100

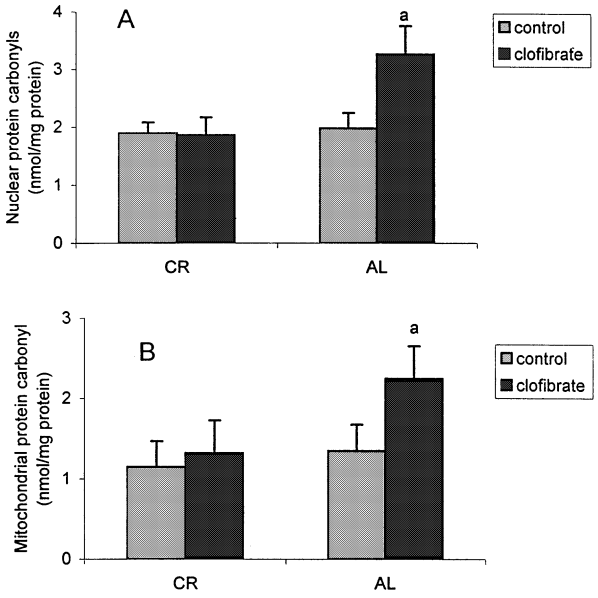

Fig. 1. Ten month caloric-restricted (CR) and ad libitum fed (AL) mice were treated with clofibrate for 14 days. Protein carbonyl levels in liver nuclear (A) and mitochondrial (B) fractions are presented as means ± S.D. The numbers of animals are: CR control group, 7; CR clofibrate, 9; AL control, 6, and AL clofibrate, 7. (a) $P<0.005$ for clofibrate-treated animals compared to control in AL group.

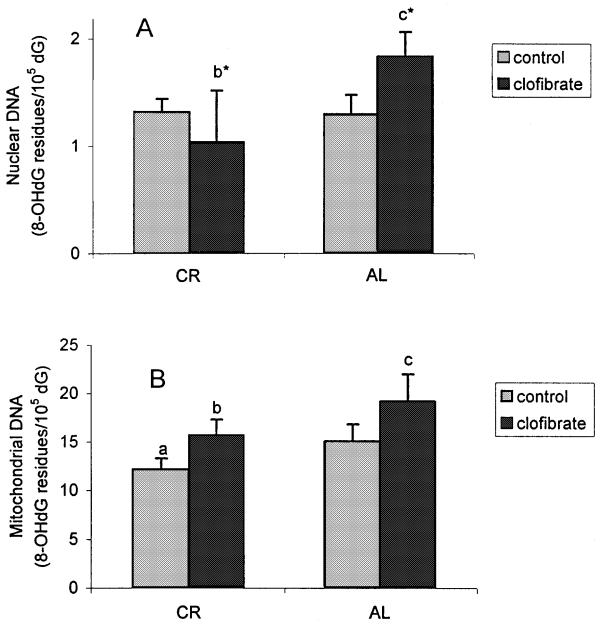

Fig. 2. Ten month caloric-restricted (CR) and ad libitum fed (AL) mice were treated with clofibrate for 14 days. 8-OHdG levels in liver nuclear and mitochondrial fractions are presented as means ± S.D. The number of samples are: CR control group, 7; CR clofibrate, 9; AL control, 6, and AL clofibrate, 7. (a) $P<0.05$ for CR compared to AL in control group. (b) $P<0.05$; (b*) $P<0.01$ for CR compared to AL in clofibrate-treated group. (c) $P<0.05$; (c*) $P<0.01$ compared to control in AL group.

Table 2
Effect of clofibrate on animal weights, liver mass and acyl-CoA oxidase activity in diet-restricted mice

Treatment	Animal weight (g)	Liver weight (g)	Acyl-CoA oxidase activity (nmol/min/mg protein)
Ad libitum feeding ($n=6$)	48.5 ± 3.5	2.35 ± 0.11	3.1 ± 2.1
Ad libitum+clofibrate ($n=7$)	50.0 ± 8.7	3.17 ± 0.44[a]	6.7 ± 1.5[a]
Caloric restriction ($n=7$)	39.2 ± 2.9[a]	2.10 ± 0.39	1.4 ± 1.1
Caloric restriction+clofibrate ($n=9$)	36.0 ± 3.2[a]	3.00 ± 0.39[b]	8.4 ± 5.2[b]

[a] $P < 0.01$ compared to ad libitum fed.
[b] $P < 0.01$ compared to caloric restriction group.

(Table 2). The weight of the liver was slightly decreased, but not significantly ($P > 0.05$).

3.2. The response of the liver to clofibrate

Caloric restriction did not alter the response of the liver to clofibrate, in terms of increase in liver mass and peroxisome proliferation. Clofibrate administration produced similar percentage increases in liver mass (approximately 40%) in both normal and caloric-restricted animals (Table 2). Caloric restriction decreased the basal activity of peroxisomal β-oxidation in the mice (Table 2), but the rise in β-oxidation capacity in both ad libitum fed and caloric-restricted animals after clofibrate administration were not significantly different, although there was a greater standard deviation in the clofibrate-treated animals.

3.3. Oxidative damage induced by clofibrate is attenuated in caloric-restricted animals

Caloric restriction had no effect on the levels of oxidative protein damage, as measured by the carbonyl assay (a general assay of oxidative protein damage [2,28]), in either mitochondrial or nuclear fractions (both purified by gradient methods). Oxidative DNA damage was measured as levels of 8-OHdG, a well-established biomarker [13,14]. Again, caloric restriction had no effect on the levels of 8-OHdG in liver nuclei from the mice (Fig. 2A). Levels of 8-OHdG in mitochondrial DNA were higher than in nuclear DNA, as expected from previous work [3,29], and caloric restriction produced a small but significant (25%) drop in these levels (Fig. 2B). Measurements were made at 14 days of clofibrate administration since our previous time-course study showed that oxidative damage had reached maximum levels at that time [30].

Clofibrate administration raised levels of oxidative protein damage in both nuclear and mitochondrial fractions of ad libitum fed animals (Fig. 1). It also raised levels of 8-OHdG in both fractions. The increase in protein carbonyls in both fractions was prevented in the caloric-restricted animals, as was the rise in 8-OHdG in the nucleus. The rise in 8-OHdG in the mitochondria was also attenuated.

4. Discussion

In our experiment we calorically restricted mice for 10 months, a time period which had little effect on basal levels of oxidative DNA or protein damage in the liver, although levels of 8-OHdG in mitochondrial DNA were decreased somewhat. It is possible that changes in basal levels of oxidative damage in liver did occur during the caloric restriction but were reversed during the 2 week return to a normal diet. However, previous studies on caloric-restricted animals have shown that decreases in parameters of oxidative damage are more likely to be seen in post-mitotic tissues such as brain than in liver and also they generally only appear in mice calorie-restricted for periods longer than those used here [7–12].

Clofibrate treatment of ad libitum fed mice elevates levels of oxidative damage to proteins and DNA in both mitochondria and nucleus of the liver, consistent with previous reports [15,16,30]. Although the metabolic response of the liver to clofibrate did not appear to be substantially altered in the caloric-restricted animals, as assessed by changes in liver mass or peroxisomal β-oxidation activity, the oxidative damage normally caused by clofibrate in nucleus and mitochondria [15,16,30] was almost completely abolished. This might mean that the oxidative damage can be dissociated from the peroxisome proliferation, perhaps mitochondrial free radical generation is involved [30]. Alternatively, or in addition, it could mean that the system is better able to withstand oxidative damage, e.g. by up-regulation of antioxidant defence or repair systems. Hence, prior caloric restriction can apparently render the liver, and perhaps other tissues, more resistant to insults involving oxidative damage to DNA and protein, even when the animals have returned to a normal diet. This may help to explain why caloric restriction can delay cancer development in animals.

Acknowledgements: This work was funded by National University of Singapore Research Grants, RP920326 and RP3982345. B.Q. is the recipient of a National University of Singapore Research Scholarship.

References

[1] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, ch. 10, 3rd edn., Oxford University Press, Oxford, UK.
[2] Sohal, R.S. and Weindruch, R. (1996) Science 273, 59–63.
[3] Beckman, K.B. and Ames, B.N. (1998) Physiol. Rev. 78, 547–581.
[4] Wallace, D.C. (1992) Science 256, 628–632.
[5] Oliver, C.N., Ahn, B.W., Moerman, E.J., Goldstein, S. and Stadtman, E.R. (1987) J. Biol. Chem. 262, 5488–5491.
[6] Mecocci, P., Fano, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U. and Beal, M.F. (1999) Free Radic. Biol. Med. 26, 303–308.
[7] Yan, L.J. and Sohal, R.S. (1998) Proc. Natl. Acad. Sci. USA 95, 12896–12901.
[8] Aksenova, M.V., Aksenov, M.Y., Carney, J.M. and Butterfield, D.A. (1998) Mech. Ageing Dev. 100, 157–168.
[9] Leeuwenburgh, C., Wagner, P., Holloszy, J.O., Sohal, R.S. and Heinecke, J.W. (1997) Arch. Biochem. Biophys. 346, 74–80.
[10] Dubey, A., Forster, M.J., Lal, H. and Sohal, R.S. (1996) Arch. Biochem. Biophys. 333, 189–197.
[11] Sohal, R.S., Agarwal, S., Candas, M., Forster, M.J. and Lal, H. (1994) Mech. Ageing Dev. 76, 215–224.
[12] Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J. and Lal, H. (1994) Mech. Ageing Dev. 74, 121–133.
[13] Kasai, H. (1997) Mutat. Res. 387, 147–163.
[14] Halliwell, B. (1998) Free Radic. Res. 29, 469–486.
[15] Rao, M.S. and Reddy, J.K. (1987) Carcinogenesis 8, 631–636.

[16] Nilakantan, V., Spear, B.T. and Glauert, H.P. (1998) Free Radic. Biol. Med. 24, 1430–1436.
[17] Sausen, P.J., Lee, D.C., Rose, M.L. and Cattley, R.C. (1995) Carcinogenesis 16, 1795–1801.
[18] Pleshakova, O.V., Kutsyi, M.P., Sukharev, S.A., Sadovnikov, V.B. and Gaziev, A.I. (1998) Mech. Ageing Dev. 103, 45–55.
[19] Chu, C., Mao, L.F. and Schulz, H. (1994) Biochem. J. 302, 23–29.
[20] Alam, Z.I., Daniel, S.E., Lees, A.J., Marsden, D.C., Jenner, P. and Halliwell, B. (1997) J. Neurochem. 69, 1326–1329.
[21] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Gupta, R.C. (1984) Proc. Natl. Acad. Sci. USA 81, 6943–6947.
[23] Adachi, S., Zeisig, M. and Moller, L. (1995) Carcinogenesis 16, 253–258.
[24] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[25] Palva, T.K. and Palva, E.T. (1985) FEBS Lett. 192, 267–270.
[26] Cahill, A., Wang, X. and Hoek, J.B. (1997) Biochem. Biophys. Res. Commun. 235, 286–290.
[27] Shen, H.M., Chia, S.E., Ni, Z.Y., New, A.L., Lee, B.L. and Ong, C.N. (1997) Reprod. Toxicol. 11, 675–680.
[28] Berlett, B.S. and Stadtman, E.R. (1997) J. Biol. Chem. 272, 20313–20316.
[29] Richter, C. (1995) Int. J. Biochem. Cell Biol. 27, 647–653.
[30] Qu, B., Li, Q.T., Wong, K.P., Ong, C.N. and Halliwell, B. (1999) Free Radic. Biol. Med. 27, 1095–1102.