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Modifications on Antioxidant Capacity and Lipid Peroxidation in Mice under Fraxetin Treatment

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Abstract

Fraxetin belongs to an extensive group of natural phenolic antioxidants. We have investigated the modifications in endogenous antioxidant capacity; superoxide dismutase (SOD), catalase (CAT), total and selenium-dependent glutathione peroxidases (GPx) and glutathione reductase (GR) and stress index; glutathione disulphide (GSSG)/reduced glutathione (GSH) ratio and thiobarbituric acid-reactive substances (TBARs) in liver and brain supernatants of C57BL/6J male 12-month-old mice under fraxetin treatment for 30 days.

Liver SOD and GPx (total and Se-dependent) activities were not significantly affected by fraxetin, whereas they were increased in the brain compared with control animals. GR activity increased significantly only in the liver of treated mice. Fraxetin treatment-related decreases were shown for GSSG/GSH ratio and rate of accumulation of TBARs (not significant in TBARs) in both tissues.

We concluded that the net effect of fraxetin treatment on endogenous antioxidant capacity suggests that this compound might provide an important resistance to, or protection against, free-radical-mediated events which contribute to degenerative diseases of ageing.

The susceptibility of the body to peroxidative damage is related to the balance between the pro-oxidant load and the adequacy of antioxidant defenses (Yu 1994). Decreasing the damage could be feasible by increasing the antioxidant concentration in tissues (Harman 1993). One approach may be to use therapeutic agents to augment the normal or diminished antioxidant defences against free-radical reactions leading to increased global antioxidant capacity (Harman 1993; Barja de Quiroga et al 1994). The most desirable substances for clinical use are compounds with antioxidant properties which are minor components of the diet (Egan et al 1990; Vladimirov et al 1991).

An extensive group of phenolic antioxidants, widely distributed in nature, is the coumarins (Murray et al 1982). Some of the pharmacological properties of coumarins have been related to their antioxidant ability and there has been considerable focus on the action of such compounds on oxidative damage (Vladimirov et al 1991; Payá et al 1993). The simple coumarins have low toxicity in mammals and present a wide range of pharmacological actions (Murray et al 1982; Egan et al 1990). Fraxetin (6-methoxy-7,8-dihydroxycoumarin) is a simple coumarin and has been demonstrated in in-vitro experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Payá et al 1992, 1993).

In this paper, we have determined the modifications in endogenous antioxidant capacity and lipid peroxidation in liver and brain supernatants of C57BL/6J male 12-month-old mice under chronic treatment with fraxetin. This experiment was performed to study the effects of fraxetin treatment in the protection against endogenous free radicals continuously produced in tissue of animals under normal physiological conditions. The antioxidant capacity examined in liver and brain supernatants includes the following markers: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (total and selenium (Se)-dependent, GPx and Se-GPx), glutathione reductase (GR) activities, and glutathione (reduced and disulphide, GSH and GSSG) concentration. Finally, two different estimators of tissue oxidative stress were determined in both tissues: GSSG/GSH ratio and TBARs (thiobarbituric acid-reactive substances).

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Materials and Methods

Drugs

Fraxetin was purchased from Janssen Chimica. All other reagents were of the highest available grade.

Animals and treatment

C57BL/6J male 12-month-old mice were procured from Iffa-Creddo (Lyon, France) and maintained under natural lighting conditions in a temperature controlled (22 ± 3°C) room and acclimatized to laboratory environment for a minimum of 15 days before experimentation. Animals were segregated into two groups of 12 each: group a, control; and group b, experimental. All mice received a standard pelleted diet and had free access to food and water. The experimental group was treated orally for 30 days with fraxetin ($25\,mg\,kg^{-1}$ body weight day^{-1}) suspended in 10% (v/v) Tween 20 aqueous solution. Selection of dosage and timing of treatment were based upon the LD50 of fraxetin and upon earlier reports including studies of other antioxidants (Comfort et al 1971; Branen 1975; Boadi et al 1991; Khanna et al 1992). Control mice only received the vehicle of fraxetin. At the end of treatment, all animals were sacrificed by decapitation 24 h after the last administration. Liver and brain samples were imme-

diately removed and stored at −80°C until analysis. The liver was selected because it is one of the tissues showing a high rate of free radical generation. The brain is also important as it continuously generates large amounts of free radicals from mitochondrial oxidative activity and catecholamine catabolism, and at the same time contains high levels of polyunsaturated fatty acids, the preferred targets of free radical damage in cell (Pérez-Campo et al 1993).

Biochemical assays

Tissues were homogenized in 50 mM phosphate buffer (pH 7·4) (the liver at 50 mg mL^{-1} and the brain at 25 mg mL^{-1}) and centrifuged at 3200 g for 20 min (4°C) to measure enzymes and tissue peroxidation. For glutathione assays, tissue samples were homogenized in 5% (w/v) trichloroacetic acid with 0·01 M HCl and centrifuged for 5 min (4°C) in an N_2 atmosphere.

SOD was measured after 24 h of dialysis by following the inhibition of pyrogallol autoxidation at 420 nm (Marklund & Marklund 1974). SOD activity was expressed as percentage of pyrogallol autoxidation min^{-1} (mg protein)$^{-1}$. CAT was measured in Triton X-100 (1%, v/v)-treated supernatants by following H_2O_2 disappearance at 240 nm (Beers & Sizer 1952) and enzyme levels were expressed as substrate (μmol H_2O_2) transformed min^{-1} (mg protein)$^{-1}$. Total and Se-dependent GPx were determined following NADPH oxidation at 340 nm in the presence of excess GR, GSH, and cumene hydroperoxide (for total GPx) (Lawrence & Burk 1976) or hydrogen peroxide (for Se-dependent GPx) (Paglia & Valentine 1967). Both GPx activities were expressed as substrate (nmol NADPH) transformed min^{-1} (mg protein)$^{-1}$. GR activity was analysed following NADPH oxidation at 340 nm in presence of GSSG (Massey & Williams 1965) and expressed as substrate (nmol NADPH) transformed min^{-1} (mg protein)$^{-1}$. GR and both GPx activities were corrected for spontaneous reaction in the absence of biological sample. Glutathione was measured by the method of Tietze (1969) in the presence of 5,5′-dithiobis (2-nitrobenzoic acid), NADPH, and GR. GSSG was assayed after derivatization of GSH with 2-vinylpyridine (Griffith 1980). GSH and GSSG values were corrected for spontaneous reaction in the absence of biological sample. Concentrations of both GSH and GSSG were expressed as mM GSH equivalents (g tissue)$^{-1}$. The thiobarbituric acid (TBA) assay adapted to tissue extracts (Barja de Quiroga et al 1988) was performed to measure tissue peroxidation (TBARs) in supernatants from phosphate buffer homogenates. The results of lipid peroxidation were expressed as μM MDA (g tissue)$^{-1}$ (MDA, malonaldehyde). Protein concentration was determined by Biuret method.

Statistics

Data are presented as the mean ± s.e.m. The significance level of treatment effects was determined using one-way analysis of variance followed by the Newman–Keuls test to analyse the significance between paired groups. A P value of 0·05 or less was considered to be significant.

Results and Discussion

Liver SOD and GPx (total and Se-dependent) activity were not changed significantly by fraxetin treatment (data not shown), nevertheless, significant increases were found for brain levels in relation to control mice (Table 1). CAT activity in both the liver and the brain was not significantly affected by treatment (data not shown). GR activity was augmented in mice treated with fraxetin, only significantly for liver supernatants (Table 1).

GSH showed increases both in the liver and in the brain resulting from fraxetin treatment (Tables 2, 3). GSSG content of brain decreased very significantly from the control group to the fraxetin group (Table 3) and no significant differences among the two groups were found in liver (Table 2). GSSG/GSH ratio of liver and brain decreased significantly by fraxetin treatment (Tables 2, 3).

TBARs values were higher in brain than in liver. Fraxetin treatment diminished the rate of accumulation of TBARs in liver and brain, although not significantly (Table 4).

The effects of fraxetin studied here were obtained in intact animals. However, under normal physiological conditions, cellular homeostasis is incessantly challenged by stressors arising from both internal and external sources (Yu 1994). The endogenous antioxidants are therefore subject to homoeostatic control in animal tissues. Thus, exposure of animals to an oxidative stress in-vivo leads to compensatory induction of endogenous antioxidants (Sohal et al 1984; López-Torres et al 1993). It is then possible that addition of high amounts of an exogenous antioxidant will modulate endogenous antioxidants (Harman 1993). However, the results obtained here show that neither the antioxidant enzymes nor the levels of GSH are depressed by fraxetin treatment. Thus, in the liver, fraxetin caused increases in GR activity and decreases in GSSG/GSH ratio. In the brain, this phenolic compound increased SOD, total GPx and Se-GPx, and diminished GSSG/GSH ratio.

Survival data from manipulation of discrete endogenous antioxidants in animals (López-Torres et al 1993), suggest that treated animals exhibit a higher survival than control. The increases in SOD, GSH, and especially GR, that have been reported here, could therefore be considered as supercompensation. This induction may help to control hydroxyl

Table 1. Superoxide dismutase and glutathione peroxidase (total and Se-dependent) activities in brain supernatants and glutathione reductase activities in liver supernatants from control and fraxetin-treated mice.

	Activities in brain supernatant			in liver supernatant
	SOD (units(mg protein)$^{-1}$)	Total GPx (nmol NADPH min^{-1} (mg protein)$^{-1}$)	SeGPx (nmol NADPH min^{-1} (mg protein)$^{-1}$)	GR (nmol NADPH min^{-1} (mg protein)$^{-1}$)
Control	3·36 ± 0·34	23·60 ± 2·46	19·95 ± 1·95	23·13 ± 2·13
Fraxetin	4·24 ± 0·13*	31·22 ± 1·79*	25·01 ± 1·95*	25·34 ± 2·19

Values are means ± s.e.m., n = 12 animals per group. *Significantly different from respective control values ($P < 0·05$).

Table 2. Liver glutathione system in control and fraxetin-treated mice.

	GSH (μmol g^{-1})	GSSG (μmol g^{-1})	GSSG/GSH
Control	3·55 ± 0·31	0·097 ± 0·009	0·027 ± 0·003
Fraxetin	4·38 ± 0·43*	0·087 ± 0·012	0·020 ± 0·002*

Values are means ± s.e.m., n = 12 animals per group. *Significantly different from control mice ($P < 0·01$). Concentrations of both GSH and GSSG were expressed as μM GSH equivalents (g of liver)$^{-1}$. The estimator of tissue oxidative stress, GSSG/GSH ratio, is a non-dimensional value.

Table 3. Brain glutathione system in control and fraxetin-treated mice.

	GSH (μmol g^{-1})	GSSG (μmol g^{-1})	GSSG/GSH
Control	0·242 ± 0·025	0·036 ± 0·003	0·149 ± 0·013
Fraxetin	0·257 ± 0·022*	0·022 ± 0·002*	0·086 ± 0·008*

Values are means ± s.e.m., n = 12 animals per group. *Significantly different from control mice ($P < 0·05$). Concentrations of both GSH and GSSG were expressed as μM GSH equivalents (g of brain)$^{-1}$. The estimator of tissue oxidative stress, GSSG/GSH ratio, is a non-dimensional value.

Table 4. Lipid peroxidation (TBARs) in liver and brain of control and fraxetin-treated mice.

	Liver TBARs (nM MDA g^{-1})	Brain TBARs (nM MDA g^{-1})
Control	256 ± 71	370 ± 85
Fraxetin	208 ± 75	317 ± 91

Values are means ± s.e.m., n = 12 animals per group. Results of lipid peroxidation were expressed as nM MDA (g tissue)$^{-1}$ (MDA, malonaldehyde). TBARs values were not significantly different between control and fraxetin-treated mice in both tissues.

radicals indirectly (due to superoxide scavenging by SOD or reduction of GSSG to GSH by GR) or directly (GSH is the major cellular sulphydryl compound which serves as both a nucleophile and an effective reductant by interacting with numerous electrophilic and oxidizing compounds) even in the presence of high hydrogen peroxide levels. The effect of fraxetin administration on tissue GSH concentration may be similar to the elevation caused in blood, brain, lung and liver GSH levels by suplementing diet with excesses of some naturally occurring antioxidant substances (Boadi et al 1991). Moreover, elevation of GPx activities in brain of fraxetin-treated mice can be estimated as positive effect of this treatment since an increased resistance of human endothelial cells against hyperoxia and a prevention of lipid peroxidation propagation of biological membranes were found to be associated with an increase in GPx activity (Michiels et al 1994).

A comparison of TBARs results indicates that the brain showed higher tissue peroxidation levels than the liver. This may be because the brain contains relatively high concentrations of easily peroxidizable fatty acids (Carney et al 1991). In addition, it is know that certain regions of the brain are highly enriched in iron, a metal that, in its free form, is catalytically involved in production of damaging oxygen free radical species (Carney et al 1991). Vulnerability of brain to oxidative stress induced by oxygen free radicals seems to be due to the fact that on one hand, the brain utilizes about one-fifth of the total oxygen demand of the body and on the other, that it is not particularly enriched, when compared with other organs, in any of the antioxidant enzymes. Relatively lower activity of these enzymes may be responsible in part for the vulnerability of this tissue (Nisticò et al 1992).

Although a causal relationship is not yet established there is an inverse correlation between the lower content of GSH and the higher level of peroxides (Yu 1994). Accordingly, in this study, we have found that fraxetin treatment increased GSH levels significantly and diminished lipid peroxidation in liver and brain (although not significantly). In addition, there is information suggesting that a global increase in antioxidant capacity can increase the resistance to factors causing early death, leading to an increase in mean life span. This would be consistent with epidemiological evidence supporting the concept that levels of endogenous antioxidants correlate with a lower incidence of cardiovascular diseases or cancer in human populations (López-Torres et al 1993; Warner 1994).

In accordance with these references, and as reported for some free radical inhibitors (Harman 1993), we conclude that the global effect of fraxetin on endogenous antioxidant capacity suggests that this phenolic compound might provide an important resistance to, or protection against, free-radical-mediated events which contribute to degenerative diseases of ageing (Ames et al 1993). Finally, in future investigations, we will try administering gradually increasing dosages of this phenolic compound to discover whether there is a broad and safe range of fraxetin supplementation between deficiency and optimal protection from oxidative damage in animals under normal nonstressful conditions.

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