Effects of Fraxetin on Glutathione Redox Status
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We have evaluated the effects of an oral treatment of mice with fraxetin (25 mg/kg for 30 days) on the glutathione system (GSH, GSSG, and GSSG/GSH ratio as stress index), glutathione reductase (GR) and glutathione peroxidase (GPx) in liver supernatants from male C57BL/6j mice (18-month old). A significant antioxidant effect in vivo was found under this treatment by a decrease in the GSSG/GSH ratio and an increased activity of GR compared with the control mice. GSSG rate and GSSG/GSH ratio were correlated with the decline of GPx activity. Our results of increased GR activity could be considered as a supercompensation in glutathione redox status that involves a decrease in the accumulation of GSSG, as well as, in GSSG/GSH ratio. Finally, we suggest that this possible mechanism of supercompensation could lead to an enhancement in the average life span.

Introduction
In the last few years, a wide spectrum of natural phenolic compounds has been screened as potential reactive oxygen species (ROS) scavengers, since this is a possible approach in the therapy of ROS-mediated cell and tissue damage (Payá et al., 1993; Payá et al., 1994; Montesinos et al., 1995). Fraxetin belongs to the coumarin group, phenolic antioxidants characterized by a benzopyrane nucleus and wide distribution in Nature (Murray et al., 1982; Egan et al., 1990; Kruedener et al., 1995). Fraxetin (6-Methoxy-7,8-dihydroxycoumarin) has low toxicity in mammals and presents a wide range of pharmacological actions. Therefore, it has been described as a potent inhibitor of superoxide generation in activated neutrophils (Payá et al., 1993) as well as an inhibitor of microsomal lipid peroxidation (Payá et al., 1992a).

Glutathione, together with glutathione peroxidases (GPx), glutathione reductase (GR), and the ancillary NADPH supplying reactions, form a key defense system against oxidative stress and ROS damage in the cell. The impaired reducing potential of the cell in terms of added bioconversion of oxidized (GSSG) to reduced glutathione (GSH) has been implicated in the pathogenesis of a number of degenerative conditions and disease states (Richie, 1992). If the GSH cellular status is manipulated by pharmacological and nutritional correction, the life span of organisms is expected to be changed (Vogt and Richie, 1993).

In this paper, we have evaluated the effects of an oral treatment with fraxetin (for 30 days) on the glutathione system (GSH, GSSG, and GSSG/GSH ratio as stress index), glutathione reductase (GR) and glutathione peroxidase (GPx) in liver supernatants from male C57BL/6j mice aged 18 months under normal conditions. The present study was intended to evidence an influence of fraxetin on the average life span, consistent with the data provided from previous references on butylated hydroxytoluene (BHT) treatment and other phenols which allow to establish a correlation between the glutathione redox status and the average life span.

Materials and Methods
Drugs
Fraxetin was purchased from Aldrich Chemical Co. (Milwaukee, WI, U. S. A.). All other chemicals were of the highest purity commercially available.

Animals and experimental design
Male C57BL/6J mice of 18 months of age were obtained from IFFA CREDDO (Lyon, France).

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The animals were maintained in a well-controlled environment: natural lighting conditions at a temperature of 22 ± 3 °C. They were fed with the normal laboratory diet as pellets and water *ad libitum*. The animals were used after an acclimatization period of at least 15 days to the laboratory environment. The mice were randomized in groups of 12 each and divided into two groups of treatment. One group served as control only receiving the vehicle and the other group was given fraxetin. Each treated mouse received daily 25 mg/kg of fraxetin for 30 consecutive days. For dosing, fraxetin was suspended in Tween 20 (10%, v/v in water) and was orally administered with an intra-gastric cannula. At the end of treatment, mice were decapitated and liver samples were immediately dissected and stored at -80 °C until analysis.

**Glutathione assays**

Liver tissue samples were homogenized in glass homogenizers in 20 volumes of cold 5% trichloroacetic acid containing 0.01 M HCl. This solution was previously deoxygenated by bubbling it with N₂. The homogenates were centrifuged at 3200 xg during 5 min under a N₂ atmosphere. The supernatants were divided in two aliquots. One was used for the measurements of total glutathione. To the second aliquot 4 μl of 2-vinylpyridine were added per each 0.1 ml of supernatant. This procedure derived GSH and at the same time neutralized the samples (Griffith, 1980). After at least 1 h in the presence of 2-vinylpyridine GSSG was measured. Total glutathione and GSSG were assayed (Tietze, 1969) by following the change in absorbance at 412 nm in the presence of 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.21 mM NADPH and 0.5 IU of GR per ml of assay mixture in 50 mM phosphate buffer pH 7.4, at 25°C. GSH values were obtained after subtracting GSSG from total glutathione. GSH and GSSG values were corrected for spontaneous reaction in absence of sample. The GSSG/GSH ratio was calculated as an estimator (indicator, marker or parameter) of tissue oxidative stress.

**GR and GPx activities**

Liver tissue samples were homogenized in glass homogenizers in 20 volumes of cold 50 mM phosphate buffer (pH 7.4). The homogenates were sonicated at 38 watts for 30 s and centrifuged at 3200 xg for 20 min at 4°C and the supernatants were immediately used for enzymatic assays.

A part of the supernatants was dialyzed overnight the 50 mM phosphate buffer (pH 7.4, 4°C) and was used to measure GR activity. GR was assayed in liver supernatants (Pérez-Campo et al., 1990) by following NADPH oxidation at 340 nm in presence of 1 mM GSH, 0.2 mM NADPH and 1.5 mM cumene hydroperoxide as substrate in 50 mM phosphate buffer (pH 7.4). To inhibit possible remnant catalase activity after the freezing of the samples, 4 mM sodium azide was added to the reaction mixture. The enzymatic reaction was carried out at 37°C.

GR and GPx activities were corrected for spontaneous reaction in the absence of enzyme. Protein concentrations were measured by the Biuret method.

**Statistics**

All results are expressed as means ± SE. Newman-Keuls test of multiple comparison was applied to determine the difference between any two groups of mice for each parameter evaluated. P values lower than 0.05 were selected to be significant.

**Results**

The results show that hepatic GSH levels did not change under the treatment with fraxetin, whereas GSSG levels were significantly lower in fraxetin mice than in control group. The GSSG/GSH ratio was also significantly lower in treated mice than in control group (P < 0.05). GR activity was enhanced significantly by the fraxetin treatment (P < 0.05). The increase of this enzyme activity was not reflected by elevated GSH levels in treated mice. Total hepatic GPx activity was lower in liver who underwent fraxetin treatment than in those of control mice, although it was not statistically significant. Data were indicated in Table I.
Table I. Effects of fraxetin treatment on the redox status glutathione from liver from male mice C57BL/6J (18-month old).

<table>
<thead>
<tr>
<th>Oral treatment</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSSG/GSH</th>
<th>GR</th>
<th>GPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>3.61 ± 0.41</td>
<td>0.11 ± 0.01</td>
<td>0.031 ± 0.003</td>
<td>17.18 ± 1.66</td>
<td>416.49 ± 38.52</td>
</tr>
<tr>
<td>Fraxetin</td>
<td>3.45 ± 0.39</td>
<td>0.09 ± 0.01*</td>
<td>0.028 ± 0.002*</td>
<td>19.51 ± 1.71*</td>
<td>324.48 ± 37.21</td>
</tr>
</tbody>
</table>

GSH and GSSG values are expressed in μmol GSH equivalents per gram of tissue. GSSG/GSH are non-dimensional values. GR and total GPx activities are expressed in nmol -1 NADPH·min -1·mg protein -1. Asterisks represent significant differences to control group, * p < 0.05. N = 12 per mean.

Our results of GR activity obtained with this fraxetin treatment showed that the increased GR activity was not accompanied by significant changes of GSH levels. However, GSSG rate and GSSG/GSH ratio were lower in liver who underwent the fraxetin treatment than in those of control mice. The changes experienced in GSSG levels and this GSSG/GSH quotient (Pérez-Campo et al., 1990) correlated with the increase experienced by the GR activity and the decline suffered by the GPx activity (although it was not statistically significant in treated mice). Thus, this diminished peroxidase activity involves a decreased GSH oxidation (Sastre et al., 1992), as shown here.

Discussion

Changes in GSH levels are not decisive to determine a potential antioxidant effect of a compound. Furthermore, the GSSG/GSH ratio evaluated which is widely regarded as an indicator of the redox status of tissue (Sohal et al., 1990) expresses more suitably the antioxidant effect of fraxetin.

The induction of GR activity has also been proven with fraxetin in mice of 12-month old (Martín-Aragón et al., 1996) and with other antioxidants like butylated hydroxyanisole, propyl gallate, alfa-tocopherol (Khanna et al., 1992), and lobenzarit (disodium 4-chloro-2,2-iminodibenzoate) (Armesto et al., 1993).

It is plausible that large quantities of stable free radicals of fraxetin produced by hydrogen abstraction could provide GR activity induction resulting in an elevation of GSH or a decline of GSSG levels, as reported for BHT (Cha and Heine, 1982). Moreover, a favourable electronic distribution of fraxetin has been described: (6-Me-
increased production of peroxides involves an increase in GSH consumption (GSH oxidation) via GPx (Sastre et al., 1992).

Many studies have shown that addition of one of a number of different antioxidants in the diet can increase the average life span (Heidrick et al., 1984). These increases are attributed largely to inhibition by antioxidants of aging associated with the environment and diseases (Harman, 1993; Yu, 1994). These findings could support our data in order to suggest a correlation between the glutathione redox status (to respond to ROS-mediated diseases) achieved under the fraxetin treatment in mice, and the average life expectancy. Thus, finally, this possible mechanism of supercompensation in glutathione status could lead to an enhancement in the average life span, as described in mice under BHT diet (Clapp et al., 1979).

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