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# Olive Phenol Hydroxytyrosol Prevents Passive Smoking–Induced Oxidative Stress

Francesco Visioli, PhD; Claudio Galli, MD; Elena Plasmati, MS; Serena Viappiani, MS; Alicia Hernandez, PhD; Claudio Colombo, MS; Angelo Sala, PhD

**Background**—Oxidative stress is involved in the onset of several degenerative disorders, and epidemiological studies indicate that a high intake of dietary antioxidants, as in the case of the Mediterranean basin, is protective. Olive mill waste waters (OMWWs) are a byproduct of olive oil production rich in phenolic antioxidants, such as hydroxytyrosol. We tested the effects of a low dose of an OMWW extract in a model of sidestream smoke–induced oxidative stress in rats by evaluating the urinary excretion of 8-iso-prostaglandin (PG)  $F_{2\alpha}$  (iPF $_{2\alpha}$ -III).

**Methods and Results**—An OMWW extract (5 mg/kg, providing 414  $\mu$ g/kg of hydroxytyrosol) was administered to rats daily for 4 days, during which time the animals were exposed to sidestream smoke for 20 minutes once a day. Daily urines were collected, and the urinary excretion of 8-iso-PGF $_{2\alpha}$  was evaluated as an index of oxidative stress–induced in vivo lipid peroxidation. The exposure of rats to passive smoking increased the urinary excretion of 8-iso-PGF $_{2\alpha}$  by  $44\pm 4.2\%$  at 48 hours and by  $55\pm 10\%$  at 96 hours. Treatment with the OMWW extract was able to completely prevent the increase at 48 hours and resulted in lower increments ( $34\pm 18\%$  versus  $55\pm 10\%$ ) of 8-iso-PGF $_{2\alpha}$  excretion at 96 hours.

**Conclusions**—A low dose of hydroxytyrosol, administered through OMWW, reduces the consequences of sidestream smoke–induced oxidative stress in rats. (*Circulation*. 2000;102:2169-2171.)

**Key Words:** hydroxytyrosol ■ isoprostanes ■ atherosclerosis ■ smoking ■ lipids

Evidence of the involvement of oxidative stress, resulting in increased lipid peroxidation, in the onset of several diseases is accumulating<sup>1</sup>; epidemiological studies indicate negative correlations between the dietary intake of antioxidants and the incidence of such diseases.<sup>2</sup> Appropriate in vivo biomarkers of lipid peroxidation and oxidative stress are scarce, but increased plasma levels and urinary excretion of  $F_2$  isoprostanes (a family of prostaglandin [PG]  $F_{2\alpha}$  isomers that is produced from arachidonic acid via free radical–catalyzed mechanisms) are thought to be reflective of enhanced oxidative stress.<sup>3</sup>

In the Mediterranean area, where the diet is rich in fruits and vegetables and where olive oil is the principal source of fat, the incidence of coronary heart disease is lowest.<sup>4</sup> Olive oil is obtained by pressing of the olive fruit, which contains a variety of “minor constituents,” most of which are phenolic in nature, that give extra virgin olive oil its particular taste. Such compounds, eg, hydroxytyrosol, have been shown to possess interesting biological activities in vitro.<sup>5</sup> During olive oil production, large volumes of water are generated and subsequently discarded; this “waste water” contains notable amounts of relatively polar phenolic compounds, including hydroxytyrosol, whose antioxidant activities have been thoroughly investigated.<sup>5,6</sup>

Cigarette smoke induces oxidative stress, as reflected by increased circulating levels and urinary excretion of  $F_2$ -isoprostanes.<sup>7</sup> It is noteworthy that passive smoking also increases the risk of heart disease and that experimental evidence indicates that it significantly impairs endothelial function and accelerates atherosclerosis in animals.<sup>8,9</sup> It is conceivable that some of the noxious effects of second-hand smoke are due to an enhanced cellular exposure to reactive oxygen species, leading to the formation of lipid peroxidation products, such as isoprostanes, for which various deleterious biological activities have been proposed.<sup>3</sup>

The present study sought to investigate the effects of an olive mill waste water (OMWW) preparation, of which hydroxytyrosol was the only bioactive component, on the urinary excretion of 8-iso-PGF $_{2\alpha}$  (iPF $_{2\alpha}$ -III) in rats exposed to sidestream smoke.

## Methods

Male Sprague-Dawley rats (220 to 250 g), purchased from Charles River (Calco, Italy), were housed in the local animal care facility and were given free access to food and water.

OMWW extracts were prepared from freshly picked olives as previously described.<sup>6</sup> High-performance liquid chromatographic analysis<sup>6</sup> revealed that the OMWW extract used in the present investigation contained 26.7% (wt/wt) phenolic compounds, of

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which 31% (as evaluated by comparison with an authentic standard) was hydroxytyrosol. The individual components were separated and collected, and their antioxidant capacity was evaluated in a model of copper sulfate-oxidized LDL.<sup>10</sup> The results demonstrated that only hydroxytyrosol was able to inhibit LDL oxidation (not shown).

The extract was dissolved in ethanol and was administered to the rats by gavage between 9:00 and 9:30 AM (5% ethanol, ie, 50  $\mu$ L/rat). Control groups were given 5% ethanol in water.

### Experimental Protocols

Animals were randomly assigned to 2 experimental groups. The first group (n=6) was given the OMWW extract (5 mg/kg, once a day, ie, 414  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  d<sup>-1</sup> of hydroxytyrosol), and the second (n=6) the vehicle alone. Rats received the treatments for 2 days, after which they were exposed daily for 4 days to sidestream smoke as described below, 15 minutes after receiving the compounds under investigation. Every other day (days 0, 2, and 4 after beginning of smoking), rats were placed in metabolic cages, with free access to food and tap water, for the collection of urines. Twenty-four-hour urines were collected in the morning and stored at -80°C.

### Exposure of Rats to Passive Smoke

Rats were placed in a sealed Plexiglas box that was filled with smoke from 1 cigarette (nicotine 1.2 mg, tar 12 mg); the animals were kept in the smoke-filled chamber for 1 exposure of 20 minutes' duration. The average CO volume in the box during the smoke exposure was 0.26 $\pm$ 0.05%, as measured by a CO detector tube (AuerGesellschaft GmbH).<sup>11</sup>

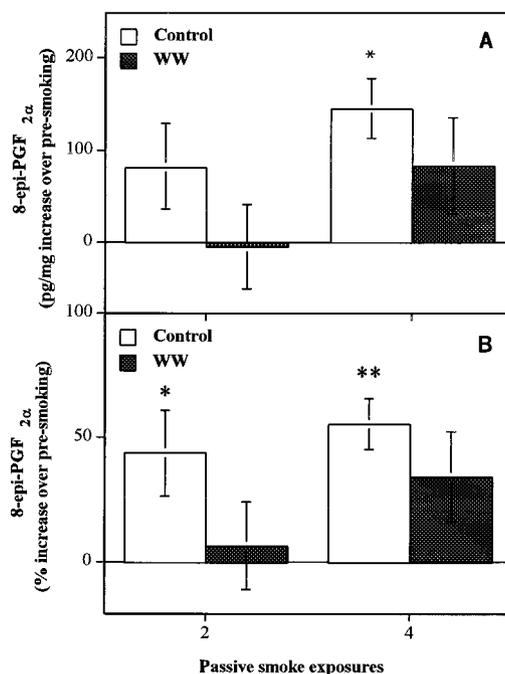
At the end of the treatment, the rats were returned either to their cage or to a metabolic cage for the collection of 24-hour urine.

### Evaluation of 8-iso-PGF<sub>2 $\alpha$</sub> Urinary Excretion

Urinary 8-iso-PGF<sub>2 $\alpha$</sub>  was purified by a double-extraction method according to Wang et al,<sup>12</sup> with modifications,<sup>13</sup> followed by quantification by an enzyme immunoassay. Acetylcholine esterase tracer was obtained from Cayman Chemical, and the specific antibody was kindly provided by the late Dr J. Maclouf (Hopital Lariboisiere, Paris). Urinary creatinine concentrations were measured with a commercial kit (Sigma Chemical Co). Results were corrected for the recovery of radiolabeled tracer and expressed as pg/mg creatinine. Unless stated otherwise, data were expressed as mean $\pm$ SEM of 6 observations, and comparisons were performed by use of the Wilcoxon rank sum test.

### Results

The urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  under basal conditions averaged 264 $\pm$ 82 pg/mg creatinine (mean $\pm$ SD, n=12) and was not significantly different between controls and OMWW-treated rats. Levels of 8-iso-PGF<sub>2 $\alpha$</sub>  in urines of nontreated rats after 2 exposures to sidestream smoke rose from 237 $\pm$ 34 to 319 $\pm$ 35 pg/mg creatinine (n=6), ie, an increase of 82 $\pm$ 46 pg/mg creatinine (Figure 1A), or 44 $\pm$ 4.2% (Figure 1B,  $P$ <0.05), over presmoking levels. Treatment with the OMWW extract completely prevented this increase, with urinary excretion averaging 291 $\pm$ 32 pg/mg creatinine before and 287 $\pm$ 66 pg/mg creatinine after 2 exposures to sidestream smoke (n=6), ie, a decrease of 4 $\pm$ 45 pg/mg creatinine (Figure 1A), or 6.8 $\pm$ 4.3% (Figure 1B),  $P$ =NS versus presmoking values. When control rats were exposed to sidestream smoke 4 times, the urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  was 375 $\pm$ 58 pg/mg creatinine, ie, an increase of 145 $\pm$ 33 pg/mg creatinine (Figure 1A,  $P$ <0.05), or 55 $\pm$ 10% (Figure 1B,  $P$ <0.01), over presmoking levels. Antioxidant treatment attenuated this increase to 84 $\pm$ 51 pg/mg creatinine (Figure 1A,  $P$ =NS), ie, 34 $\pm$ 18% (Figure 1B,  $P$ =NS), over



**Figure 1.** Passive smoking and urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  (iPF<sub>2 $\alpha$</sub> -III) in rats. Increase in urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  is expressed either as pg/mg creatinine (A) or as percent increase over presmoking values (B). \* $P$ <0.05, \*\* $P$ <0.01, unpaired data analysis, Wilcoxon rank sum test. WW indicates OMWW.

presmoking levels, resulting in a mean 8-iso-PGF<sub>2 $\alpha$</sub>  excretion in this group of 375 $\pm$ 51 pg/mg creatinine (n=6).

### Discussion

The data reported in this article demonstrate that an OMWW extract, the main antioxidant component of which is hydroxytyrosol, decreases oxidative stress in rats exposed to sidestream smoke.

Passive smoking is known to induce several effects normally associated with active smoking. For example, we have previously observed an increase in bronchial reactivity in animals exposed to passive smoking,<sup>11</sup> and we are now reporting that repeated exposure of rats to sidestream smoke results in a significant elevation of urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  (iPF<sub>2 $\alpha$</sub> -III), a reliable marker of oxidative stress in vivo.<sup>3</sup> Furthermore, enhanced urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub>  has been reported in smokers as well as in different pathologies associated with increased levels of oxidative stress.<sup>7,14</sup> In particular, an increase in 8-iso-PGF<sub>2 $\alpha$</sub>  urinary excretion has been reported in patients suffering from chronic obstructive pulmonary disease, in whom a significant correlation with inflammation and respiratory functions was also observed.<sup>15</sup> The increase in the urinary excretion of 8-iso-PGF<sub>2 $\alpha$</sub> , evaluated semiquantitatively by immunoassay, after 2 and 4 days of passive smoking in rats reported here suggests that enhanced oxidative stress represents an immediate-early feature of the inflammatory response associated with lung exposure to smoke components, although we cannot rule out the possibility that other, cyclooxygenase-derived, compounds might be detected by this assay and contribute to the high interindividual variability observed.

It is noteworthy that the OMWW extract used in this study contained 8.3% hydroxytyrosol as the active antioxidant compound and therefore, the actual dose of hydroxytyrosol administered to the laboratory animals was only  $414 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ . In a recent study using apoE-deficient mice, vitamin E decreased isoprostane, ie,  $\text{iPF}_{2\alpha}\text{-VI}$ , levels at an estimated dose of  $\approx 270 \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ <sup>16</sup>; although experimental conditions were different in the 2 studies, our results suggest that the hydroxytyrosol present in OMWW extracts is very effective in decreasing the oxidative stress associated with passive cigarette smoke. The amphiphilic nature of this compound, as opposed to the lipophilic characteristics of vitamin E, might provide an additional protection against oxidative processes that take place in biological systems at the water-lipid interface.

Evidence of a dose-dependent absorption of hydroxytyrosol in humans was recently obtained in our laboratory<sup>17</sup>; the present investigation indicates that hydroxytyrosol retains its antioxidant activity in vivo, and additional work is needed to evaluate its potential activity in humans.

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