JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Hydroxytyrosol Attenuates Peripheral Neuropathy in Streptozotocin-Induced Diabetes in Rats

Giuseppe Ristagno,[†] Francesca Fumagalli,[†] Carla Porretta-Serapiglia,[‡] Alessandro Orrù,[†] Chiara Cassina,[†] Marzia Pesaresi,[§] Serge Masson,[†] Luciano Villanova,[∥] Alessandro Merendino,[∥] Azzurra Villanova,[∥] Luigi Cervo,[†] Giuseppe Lauria,[‡] Roberto Latini,*[,†] and Roberto Bianchi[‡]

[†]Mario Negri Institute for Pharmacological Research, via La Masa 19, 20156 Milan, Italy

[‡]Carlo Besta Foundation IRCCS National Neurological Institute, via Celoria 11, 20133 Milan, Italy

[§]University of Pharmacological Sciences, via Balzaretti 9, 20133 Milan, Italy

[∥]Lachifarma Srl, S.S. 16 Adriatica, I-73010 Zollino, Lecce, Italy

ABSTRACT: Peripheral neuropathy is one of the most frequent and severe complications of diabetes. Hydroxytyrosol (HT), the major antioxidant polyphenolic compound of olive oil, has been investigated as a new potential treatment to counteract the progression of peripheral diabetic neuropathy in rats. An established model of streptozotocin-induced diabetes has been used. After confirmation of hyperglycemia, diabetic and nondiabetic animals were randomized to receive either a low dose or a high dose of HT, or the corresponding vehicle, for 6 weeks. At the end of the 6-week period of treatment, HT blunted plasma thiobarbituric acid-reactive substances increase ($p < 0.05$) and significantly reduced nerve conduction velocity ($p < 0.05$) and thermal nociception impairment in diabetic rats ($p < 0.05$). Sciatic nerve Na^+, K^+-ATPase activity reduction was also abolished by HT ($p < 0.05$). The present study provides evidence of the therapeutic potential of the natural substance hydroxytyrosol in the early stage of diabetic neuropathy.

KEYWORDS: *diabetic neuropathy, hydroxytyrosol, nerve conduction velocity, oxidative stress*

INTRODUCTION

Diabetes is a chronic metabolic disorder caused by complete or relative insufficiency of insulin secretion due to autoimmune destruction of the insulin-producing pancreatic beta cells. The subsequent chronic blood glucose elevation observed in diabetic patients eventually leads, directly or indirectly, to long-term complications of the disease, which are the major causes of morbidity and mortality.[1] Among these, diabetic neuropathy occurs in more than 50% of patients[1] and affects nerve fibers of peripheral nervous system. The patients often present with loss of feeling and numbness in their feet, hands, and legs, which may be accompanied by excessive sensitivity to thermal and nociceptive stimuli or may perceive normal stimuli as painful.[2] Diabetic peripheral neuropathy is also characterized by neuroanatomical changes, including decreases in nerve conduction velocity (NCV) and altered activity of the nervous fiber enzyme Na^+, K^+-ATPase, responsible for the maintenance of the potential difference throughout the nerve membranes.[3,4]

The role of oxidative stress in the pathogenesis of diabetic neuropathy has been considered as one of the leading mechanisms. In the nervous system, hyperglycemia, in fact, is responsible for increased production of free radicals, and lipid peroxidation, as well as decreased endogenous antioxidant level, and reduction of antioxidant enzyme activities.[5,6] These mechanisms, together with a concurrent decreased neurotrophic support, lead to damage in axons and myelin sheaths of peripheral nerves.[7] New therapeutic strategies directed to reduce the oxidative damage played by hyperglycemia have been therefore considered to ameliorate diabetic neuropathy. More specifically, recent scientific interest has been focused on potentials of polyphenolic substances, the predominant micronutrients of olive oil, that are endowed with interesting biological activities, including antioxidant and anti-inflammatory effects.[8,9]

Hydroxytyrosol (3,4-dihydroxyphenylethanol) (HT) is one of the major polyphenolic compounds of olive oil, ranging between 0.005 and 0.8% by weight. Earlier reports on HT have been mainly focused on demonstration of its remarkable antioxidant actions. Thus, it has been shown that HT promotes healing properties,[10] is a potent scavenger of superoxide anion and hydroxyl radical,[11] protects human erythrocytes from oxidative damage induced by hydrogen peroxide,[12] effectively counteracts the cytotoxic effects of reactive oxygen species in human intestinal epithelial cells,[13] regulates mitochondrial dynamic remodeling, and enhances antioxidant defenses in exercise-induced muscle and mitochondrial dysfunction.[14] The natural substance HT has the advantage to have the strongest antioxidant effects among the phenolic compounds, and since it is extracted from the olive mill waste, it has low cost and is largely available.[9] The present study was therefore designed to assess, for the first time, the capability of the HT rich extract to play a role as a new potential therapeutic compound to counteract the progression of peripheral neuropathy. We hypothesized that chronic treatment with HT, initiated at the early stage of diabetic neuropathy, would ameliorate the peripheral neuropathy in a model of streptozotocin (STZ)-induced diabetes in the rat.

Received: December 1, 2011
Revised: May 17, 2012
Accepted: May 18, 2012
Published: May 18, 2012

dx.doi.org/10.1021/jf2049323 | *J. Agric. Food Chem.* 2012, 60, 5859−5865

MATERIALS AND METHODS

Chemicals. Hydroxytyrosol was extracted from a natural source, *Olea europaea* L., applying an innovative patented method (Patents No. US7427358B2, US2009/0023815A1, EP1623960B1). The HT was supplied by Lachifarma Pharmaceutical Company, Zollino (LE), Italy. Pentobarbital, STZ, sucrose, EGTA, EDTA, glutathione, phosphoric acid, thiobarbituric acid, *n*-butanol, and 1,1,3,3-tetraethoxypropane standards were purchased from Sigma-Aldrich, St. Louis, MO.

Animals. Sixty-six male Sprague−Dawley rats (Harlan, Italy) weighing 180−200 g were used for the study. Animals were housed two per cage in a temperature (21 ± 1 °C) and humidity (60%) controlled environment. The light schedule was 12 h light and 12 h dark, light on at 7:00 a.m. All experimental procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare no. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Induction of Diabetes and Experimental Treatments. An established model of STZ-induced diabetes in the rat has been utilized, which develops neuropathy within 2 weeks from the STZ injection.[2] The model shares a number of features with human type 1 diabetic neuropathy at the functional and biochemical levels, such as decreased NCV together with reductions in Na^+, K^+-ATPase activity,[3] and early neurological dysfunction appearance, including altered thermal and mechanical nociceptive thresholds.[2,15]

Diabetes was induced in 42 rats fasted overnight by a single intraperitoneal injection of 60 mg/kg of STZ, dissolved in sodium citrate saline buffer (pH 4.5).[15] The other 24 rats were injected with vehicle and served as nondiabetic controls. Hyperglycemia was confirmed by measuring glycosuria 72 h after STZ injection, using Keto-Diabur test 5000 strips (Roche Diagnostics Spa, Italy). Only animals with glycosuria >5% were classified as diabetic and included in the study. Animals were then randomized to receive either a low dose (10 mg/kg/day) or a high dose (100 mg/kg/day) of HT or vehicle, according to the following groups of treatment: (1) STZ-rats treated with low dose HT, 10 mg/kg/day (n = 12); (2) STZ-rats treated with high dose HT, 100 mg/kg/day (n = 12); (3) STZ-rats treated with vehicle (n = 12); (4) nondiabetic control rats treated with low dose HT, 10 mg/kg/day (n = 8); (5) nondiabetic control rats treated with high dose HT, 100 mg/kg/day (n = 8); and (6) nondiabetic control rats treated with vehicle (n = 8).

Starting the fourth day after STZ or vehicle injection, the daily dose of HT or vehicle was administered by 2 gavages of half dose each, at 9:00 a.m. and 5:00 pm, respectively. The treatment was continued for 6 weeks. Animals were then euthanized by a lethal dose (150 mg/kg) of pentobarbital intraperitoneally injected, and plasma and sciatic nerves were collected to perform further analysis. The sacrifice was scheduled to occur within 2 h from the last HT or vehicle administration.

During the experiment, body weight of the rats was measured daily to monitor their weight gain. Hyperglycemia was confirmed in each rat by measuring tail vein blood glucose level using a Glucomen tester (Menarini, Italy), 15 days after STZ injection. A mean plasma glucose level above 300 mg/dL defined hyperglycemia. In addition, glycosuria was measured again 20 days after the onset of treatment, together with ketonuria.

Nerve Conduction Velocity. Tail nerve conduction velocity was assessed by a Neuropack S1 electromyograph (model MED 9400 A/K, Nihon Kohden, Japan), as previously described.[15] Briefly, recording ring electrodes were placed distally in the tail. The stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak, stimulus duration 100 ms, filter 1 Hz-5 MHz), and NCV was calculated. All the neurophysiological studies were performed under standard conditions in a temperature-controlled room adjacent to the animal housing room.

NCV was assessed at baseline, before STZ or vehicle injection, and two, four, and six weeks after the beginning of the treatment.

Thermal Nociceptive Threshold. The nociceptive threshold to radiant heat was quantified using the hot plate paw withdrawal test, as previously described.[15] Briefly, a 40 cm high Plexiglas cylinder was suspended over the hot plate (Ugo Basile) and the temperature was maintained at 50 ± 0.2 °C. Paw withdrawal latency was defined as the time between placing the rat on the hot plate and the time of withdrawal, or licking of hind paw, or discomfort manifested by the animal.

The test was performed at baseline, before STZ or vehicle injection, and two, four, and six weeks after the beginning of the treatment. Each animal was tested twice, separated by a 30 min rest interval, and the values were averaged.

Mechanical Nociceptive Tolerance. Mechanical nociceptive tolerance (Randall−Selitto paw withdrawal test) was assessed using an electromechanical analgesia meter.[16] This instrument generates a linearly increasing mechanical force applied directly to the dorsal surface of the rat hind paw via a cone-shaped plunger. The results represent the maximal pressure (expressed in grams) tolerated by the animals. During testing, the rats were held by hand. The test was performed at baseline, before STZ or vehicle injection, and two, four, and six weeks after the beginning of the treatment, 24 h after the hot plate test. Each animal was tested twice, separated by a 30 min rest interval, and the values were averaged.

Na^+, K^+-ATPase Activity. At the end of the six week period of treatment, tibial stumps of the sciatic nerves were collected, desheathed, immediately frozen on dry ice, and stored at −80 °C. For ATPase measurement, nerve specimens were thawed and homogenized in a glass−glass homogenizer (Elvejehm Potter; DISA, Milan, Italy) at 4 °C in 0.8 mL of chilled solution containing 0.25 mol/L sucrose, 1.25 mmol/L EGTA, and 10 mmol/L Tris, pH 7.5. Composite, Na^+, K^+-ATPase, and Mg^{2+}ATPase activities were determined spectrophotometrically at 340 nm (Ultrospec 2100 pro; Amersham-Biosciences, Cambridge, U.K.) by the coupled-enzyme assay, which continuously monitored NADH oxidation. Na^+, K^+-ATPase was defined as ouabain inhibitable activity per 3 mmol/L, final concentration. Protein content was determined with a microplate assay protocol (DC Protein; BioRad, Milan, Italy).[17]

Plasma Thiobarbituric Acid-Reactive Substances. At the end of the six week period of treatment, plasma was collected and EDTA and glutathione were added at 1.34 and 0.65 mmol/L final concentrations respectively. Thiobarbituric acid-reactive substance (TBARS) levels were determined, as an index of reactive oxygen species production, by previously described protocols.[18,19] Briefly, 100 μL of plasma was boiled in 0.6 mL of 1% (w/v) phosphoric acid and 0.2 mL of thiobarbituric acid (0.42 mmol/L) for 45 min. The cooled mixture was extracted by agitation with 1.2 mL of *n*-butanol and separated by centrifugation (10−20 min at 1500*g*). The upper phase was measured fluorimetrically (Infinite M200; Tecan, Milan, Italy) at excitation wavelength 532 nm and emission wavelength 553 nm. The calibration curve was prepared with 1,1,3,3-tetraethoxypropane standards 0 to 1.64 μmol/mL final concentration.

Other Biochemical Analyses. At the end of the six week period of treatment, plasma glucose concentration was measured by enzymatic assay with glucose oxidase (Chema Diagnostica, Monsano, Italy). Plasma aspartate aminotransferase activity (AST or GOT) was determined by kinetic spectrophotometric assay (Chema Diagnostica, Monsano, Italy). Plasma creatinine concentration was measured by modified Jaffè method (Chema Diagnostica, Monsano, Italy). Plasma high sensitivity cardiac troponin T concentration was assessed with an electrochemiluminescence assay (ECLIA, Elecsys 2010 analyzer, Roche Diagnostics, Germany). Plasmatic AST/GOT, creatinine, and troponin T were assessed to identify the presence of any possible toxic effects of HT on liver, kidney, and heart, respectively.

Statistical Analysis. Data were analyzed by two-way analysis of variance (ANOVA), with treatment and disease as independent variables, followed by the Student−Newman−Keuls post hoc test. Data are reported as mean ± SEM. A 2 tail p value <0.05 was considered as statistical significant. All analyses were performed by StatView 5 (SAS Institute Inc.).

Table 1. Baseline Values Assessed in the Initial 66 Rats, Prior to Randomization[a]

body weight, g	216 ± 1
nerve conduction velocity, m/s	23.7 ± 0.5
hot plate, sec	16.5 ± 0.7
Randall−Selitto, g	113.9 ± 2.8

[a]Values are reported as mean ± SEM.

RESULTS

Overall Baseline Values Are Reported in Table 1. Among the 42 rats injected with STZ, seven were excluded from the study. Six of them, in fact, did not develop hyperglycemia, and one died after injection. Two additional STZ-injected rats died during the following six week period of treatment.

Changes in Body Weight and Plasma Glucose. Rats injected with STZ exhibited a marked hyperglycemia and no gain of body weight at the end of the 6 weeks ($p < 0.01$ vs nondiabetic rats, Table 2). HT administration, at either low or high dose, had no effect on plasma glucose level and body weight in diabetic rats ($p = 0.61$ and $p = 0.537$, respectively, Table 2).

Effects of STZ-Induced Diabetes and HT Treatment on NCV. During the 6 week period of treatment, the nondiabetic rats showed the expected increase in NCV already described in similar models, from 24.6 m/s to 31.7 m/s, due to the final maturation of the peripheral nerves occurring in these young adult rats. STZ-induced diabetes, instead, significantly affected NCV ($F = 87.29$, $p < 0.0001$). At the end of the 6 week period, vehicle-treated diabetic rats had 32% lower NCV than vehicle-treated nondiabetic ones (Figure 1, $p < 0.05$).

HT treatment significantly reduced the NCV impairment in diabetic rats ($F = 7.55$, $p = 0.0007$). Animals receiving low dose HT, in fact, presented 28% greater NCV compared to those treated with vehicle, 27.7 m/s vs 21.7 m/s ($p < 0.05$). However, in these animals the NCV remained significantly lower compared to that measured in the nondiabetic ones (Figure 1). Animals receiving high dose HT also presented a 29% greater NCV compared to the vehicle-treated diabetic rats, 27.9 m/s vs 21.7 m/s ($p < 0.05$). Again, the NCV in these rats was significantly lower than that in the nondiabetic ones (Figure 1). HT treatments did not show any effect on the NCV values in the nondiabetic rats.

Effects of STZ-Induced Diabetes and HT Treatment on Thermal Nociceptive Threshold. STZ-induced diabetes significantly affected thermal nociceptive threshold ($F = 36.02$, $p < 0.0001$). At the end of the 6 week period, diabetic rats had 111% higher thermal nociceptive threshold than nondiabetic controls, 20 s vs 9.5 s (Figure 2, $p < 0.05$).

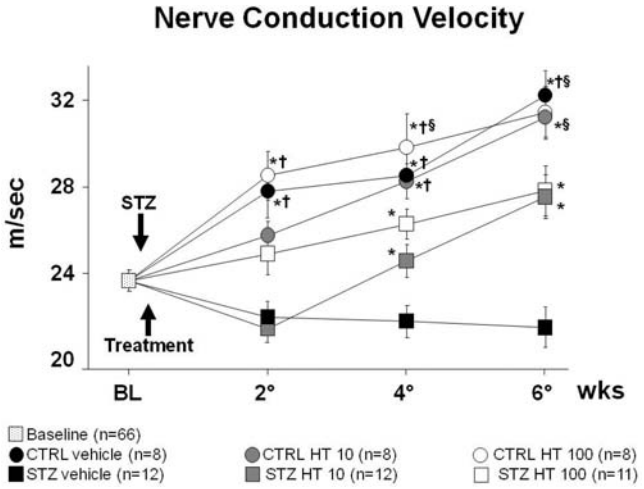

Figure 1. Nerve conduction velocity. Measurements were performed in diabetic (STZ) and nondiabetic control (CTRL) rats, at baseline (BL), prior to STZ injection, and during the 6 weeks (wks) of treatment with either vehicle or hydroxytyrosol (HT) at 10 or 100 mg/kg. One rat in the STZ HT 10 group and one in the STZ HT 100 group died during the six week period. Data are reported as mean ± SEM. * $p < 0.05$ vs STZ vehicle group; † $p < 0.05$ vs STZ HT 10 group; § $p < 0.05$ vs STZ HT 100 group.

HT treatment significantly reduced the thermal nociceptive threshold impairment in diabetic rats ($F = 4.37$, $p = 0.0141$). At the end of the 6 weeks of treatment, administration of low dose HT resulted in 34% lower threshold compared to vehicle, 13.2 s vs 20 s ($p < 0.05$), while high dose HT resulted in 20% reduction, 16.1 s vs 20 s ($p > 0.05$ vs low dose HT and vehicle treated diabetic rats, Figure 2). By the end of the treatment period, the thermal nociceptive threshold in the diabetic rats that received HT was not significantly different compared to that in the nondiabetic animals (Figure 2). HT treatment did not have any effects on the thermal nociceptive threshold in the nondiabetic rats.

Effects of STZ-Induced Diabetes and HT Treatment on Mechanical Nociceptive Threshold. STZ-induced diabetes significantly affected the mechanical nociception ($F = 87.29$, $p < 0.0001$). At the end of the six weeks of treatment, the threshold was reduced in each group of diabetic animals compared to the nondiabetic ones, 62.7 ± 2.5 g vs 132.4 ± 4.2 g. HT treatment had no effects on mechanical nociceptive threshold ($F = 7.55$, $p = 0.38$). At the end of the 6 weeks of treatment, there were no significant differences in the values of Randall−Selitto test among vehicle, low dose, and high dose HT treated diabetic rats, which

Table 2. Body Weights and Plasma Glucose[a]

	CTRL			STZ		
	vehicle $n = 8$	HT 10 $n = 8$	HT 100 $n = 8$	vehicle $n = 12$	HT 10 $n = 12$	HT 100 $n = 11$
			Body Weight, g			
2 weeks	295 ± 4	298 ± 4	300 ± 4	231 ± 6[b]	222 ± 5[b]	216 ± 8[b]
4 weeks	358 ± 5	361 ± 4	361 ± 4	228 ± 8[b]	232 ± 9[b]	217 ± 7[b]
6 weeks	392 ± 7	398 ± 6	396 ± 6	224 ± 8[b]	229 ± 10[b]	214 ± 9[b]
			Plasma Glucose, mg/dL			
6 weeks	158 ± 7	150 ± 4	154 ± 4	970 ± 60[b]	952 ± 31[b]	888 ± 61[b]

[a]CTRL, nondiabetic control rat; STZ, streptozotocin induced diabetic rat. One rat in the STZ HT 10 group and one in the STZ HT 100 group died during the six week period. Values are reported as mean ± SEM. [b]$p < 0.01$ vs the CTRL groups.

dx.doi.org/10.1021/jf2049323 | J. Agric. Food Chem. 2012, 60, 5859−5865

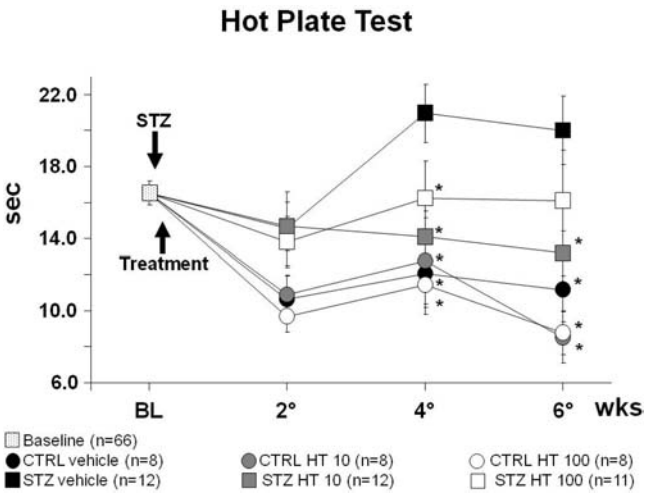

Figure 2. Hot plate test. Measurements were performed in diabetic (STZ) and nondiabetic control (CTRL) rats, at baseline (BL), prior to STZ injection, and during the 6 weeks (wks) of treatment with either vehicle or hydroxytyrosol (HT) at 10 or 100 mg/kg. One rat in the STZ HT 10 group and one in the STZ HT 100 group died during the six week period. Data are reported as mean ± SEM. * $p < 0.05$ vs STZ vehicle group.

were 56.5 ± 3.2, 64.3 ± 3.9, and 68.5 ± 5.5 g, respectively. HT treatment did not have any effects on mechanical nociceptive threshold in the nondiabetic rats, in which the values were 126.9 ± 7.4, 140 ± 8, and 130.3 ± 6.8 g, respectively for the vehicle, low dose, and high dose HT treatment group.

Effects of STZ-Induced Diabetes and HT Treatment on Na^+, K^+-ATPase Activity. STZ-induced diabetes significantly reduced sciatic nerve Na^+, K^+-ATPase activity ($F = 4.94$, $p = 0.03$), while HT treatment abolished this impairment ($F = 4.4$, $p = 0.0173$). At the end of the 6 week period of treatment, the Na^+, K^+-ATPase activity in the diabetic rats was 35% lower than that in nondiabetic ones ($p < 0.05$, Figure 3). Treatment with both low and high dose HT completely prevented the loss of the enzymatic activity ($p > 0.05$ vs nondiabetic rats and $p < 0.05$ vs STZ-induced diabetic rats treated with vehicle, Figure 3). HT treatments did not have any effects on the Na^+, K^+-ATPase activity in the nondiabetic rats.

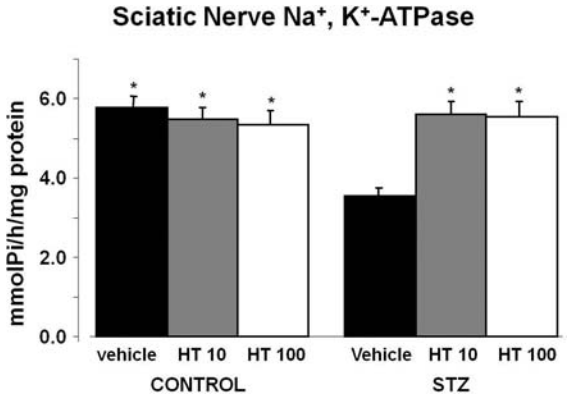

Figure 3. Sciatic nerve Na^+, K^+-ATPase. Measurements were performed in diabetic (STZ) and nondiabetic control rats, at the end of the 6 weeks of treatment with either vehicle or hydroxytyrosol (HT) at 10 or 100 mg/kg. Data are reported as mean ± SEM. * $p < 0.05$ vs STZ vehicle group.

Effects of STZ-Induced Diabetes and HT Treatment on Plasma TBARS. STZ-induced diabetes significantly increased plasma TBARS ($F = 84.65$, $p < 0.0001$). Vehicle-treated diabetic rats had plasma levels of TBARS doubled compared to those measured in the vehicle-treated nondiabetic ones, 1.50 nmol/mL MDA vs 0.75 nmol/mL MDA ($p < 0.05$, Figure 4). HT treatment

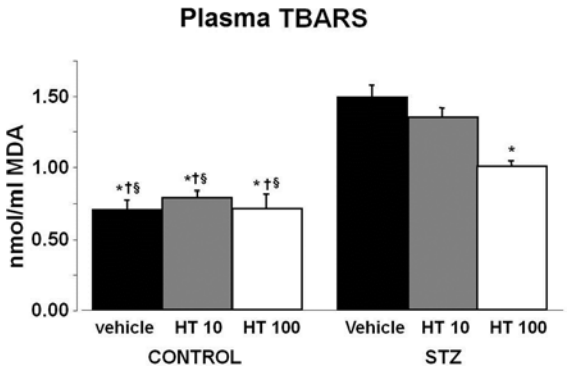

Figure 4. Plasma thiobarbituric acid-reactive substances (TBARS). Measurements were performed in diabetic (STZ) and nondiabetic control rats, at the end of the 6 weeks of treatment with either vehicle or hydroxytyrosol (HT) at 10 or 100 mg/kg. Data are reported as mean ± SEM. * $p < 0.05$ vs STZ vehicle group. † $p < 0.05$ vs STZ HT 10 group; § $p < 0.05$ vs STZ HT 100 group.

significantly blunted the plasma TBARS increases ($F = 6.08$, $p = 0.0043$). However, plasma TBARS were significantly lower only in diabetic rats treated with high dose HT compared to those treated with vehicle, 1.01 nmol/mL vs 1.50 nmol/mL ($p < 0.05$, Figure 4). Moreover, the plasma level of TBARS in these animals remained significantly higher compared to that assessed in the nondiabetic ones ($p < 0.05$, Figure 4). HT treatments did not have any effects on plasma level of TBARS in the nondiabetic rats.

DISCUSSION

Peripheral neuropathy is one of the most frequent and potentially severe complications of diabetes, leading to pain, skin ulcers, muscle weakness, and overall impairment of the patient's quality of life.[3] The present study provides evidence of the therapeutic potential of hydroxytyrosol in the early stage of diabetic neuropathy, in a model of type 1 diabetes. HT, in a dose dependent manner, significantly reduced the marked increase in plasma thiobarbituric acid-reactive substances measured in STZ-diabetic rats, demonstrating the clear in vivo antioxidant effects of this natural compound. Two parameters, mainly related to the structure/function of large myelinated fibers, namely, nerve conduction velocity and sciatic nerve Na^+, K^+-ATPase activity, were reduced in STZ-treated animals by 33 and 38%, respectively. By the end of the six weeks of treatment, NCV was only 14% lower in HT treated diabetic animals compared to the nondiabetic ones, while the decrease in the Na^+, K^+-ATPase activity was completely abolished. During the six week period of treatment, hind paw thermal response latencies progressively increased in diabetic rats. HT treatment, however, prevented this increase. These beneficial effects were independent of hyperglycemia, as this variable was not affected by the HT administration.

Current treatment of diabetic neuropathy relies on the control of glycemic, oxidative stress, and neural and vascular risk factors.[5] Hyperglycemia causes oxidative stress in the peripheral nervous system that can promote the development of diabetic neuro-

Table 3. Biochemical Plasma Analyses Assessed for Safety[a]

	CTRL			STZ		
	vehicle $n = 8$	HT 10 $n = 8$	HT 100 $n = 8$	vehicle $n = 12$	HT 10 $n = 12$	HT 100 $n = 11$
			Plasma Creatinine, mg/dL			
6 weeks	0.57 ± 0.02	0.58 ± 0.03	0.59 ± 0.01	0.64 ± 0.07	0.58 ± 0.07	0.63 ± 0.03
			Plasma hs-cTnT, ng/L			
6 weeks	5.47 ± 1.09	5.26 ± 0.52	8.89 ± 3.98	8.72 ± 1.99	9.99 ± 2.54	10.71 ± 2.1
			Plasma GOT/AST, U/L			
6 weeks	100 ± 21	111 ± 22	142 ± 16	94 ± 6	108 ± 21	118 ± 27

[a]CTRL, nondiabetic control rat; STZ, streptozotocin induced diabetic rat; hs-cTnT, high sensitive troponin T; GOT/AST, aspartate aminotransferase activity. One rat in the STZ HT 10 group and one in the STZ HT 100 group died during the six week period. Values are reported as mean ± SEM.

pathy.[5,20] Several mechanisms, including auto-oxidative glycosylation, formation of advanced glycation end products, and increased polyol pathway activity, contribute to the increased oxidative stress.[5,20] An antioxidant-based therapy might be therefore promising as adjunctive strategy to counteract the negative effects of oxidative stress. Thus, many of the complications of diabetes have been diminished, but not totally reversed, upon supplementation with dietary antioxidants, such as flavonoids and polyphenols.[21] Among these compounds, HT showed the strongest antioxidant effects in in vitro studies.[22,23] HT comes from the hydrolysis of oleuropein and is found in great quantities in the remains from oil processing, such as pomace olive oil, olive-mill wastewater, or the rinse waters, from which it can be recovered with ad hoc techniques.[9,24] The absorption of HT has been reported to be rapid, with maximum plasma concentration being reached 5−10 min after ingestion. Plasma concentrations up to 3.26 μg/mL have been reached 10 min after oral administration of 10 mg/kg HT to rats.[25] The time required for HT and its metabolites to be completely eliminated in the urine has been reported to be roughly 5 h in rats.[26] Based on these data, we chose the 10 mg/kg as the starting lower dose, and we empirically decided to administered a 10-fold higher dose in order to cover a wide range, such to discover the presence of both therapeutic and toxic effects of HT after a chronic daily administration.

In our model, HT's antioxidant effects were confirmed by a dose-dependent decrease in plasma TBARS in the diabetic rats that received the compound daily, although levels equivalent to those measured in the nondiabetic rats were not achieved. As it might have been expected, the higher was the dose of administered antioxidant, namely, HT, the greater was the reduction in the biomarker of oxidative stress, plasma TBARS. Nevertheless, the protective action of HT against diabetic neuropathy has been consistently observed in each treated diabetic rat, i.e. improvements in NCV, thermal nociception, and sciatic nerve Na^+, K^+-ATPase activity. The multifactorial physiopathology of diabetic neuropathy, including ischemic, inflammatory, and trophic mechanisms, might explain this absence of further amelioration of the disease in the presence of an augmented antioxidant condition.

In a model of alloxan-induced diabetes, HT has been recently proved to be efficient in inhibiting hyperglycemia and oxidative stress induced by diabetes. More specifically, administration of purified HT to diabetic rats caused a 55% decrease in plasma glucose level and an increase in renal superoxide dismutase, catalase, and glutathione peroxidase activities in liver and kidney. A protective action against hepatic and renal toxicity in diabetic rats was clearly observed.[21] Hence, we now focused mainly on effects of HT on functional outcome of diabetic neuropathy, rather than investigating its already proven antioxidant actions. Indeed, HT significantly counteracted the axonal degeneration caused by STZ-induced diabetes at the behavioral, neurophysiological, and neuropathological level. In particular, at the end of the six weeks of treatment, tail NCV was significantly faster in diabetic rats daily treated with both low and high dose HT compared with vehicle-treated rats, reflecting the rescue of large sensory fiber function. Hot plate test that examines small sensory fibers, carrying nociceptive and thermal sensations, demonstrated similar results. However, the previously reported beneficial effects of HT on plasma glucose were minimal in our in vivo experiments.

Na^+, K^+-ATPase is responsible for the maintenance of chemical gradients in the axonal plasma membrane, and its low activity in peripheral nerves is considered as one of the potential mechanisms accounting for NCV slowing during hyperglycemia. The effects of diabetes on Na^+, K^+-ATPase isoenzyme activity in sciatic nerve membranes has been usually related to alterations in either protein subunit expression, enzyme kinetics, or both. More specifically, the reduction of Na^+, K^+-ATPase activity is not simply secondary to fiber loss, but quite likely contributes to the pathogenesis and self-maintenance of diabetic neuropathy in humans.[27] Pharmacological treatments preventing or restoring this activity are, therefore, potentially capable to protect or reverse the decreases in NCV.[15,28] In our model, HT significantly counteracted the diabetes-induced reduction of Na^+, K^+-ATPase activity. By the end of the 6 weeks of treatment the isoenzyme activity in diabetic rats receiving HT was equivalent to that observed in the nondiabetic animals. This protective effect on Na^+, K^+-ATPase, in agreement with other observations, was associated with improved NCV.[15,17,29] Interestingly, both the low and the high dose of HT completely prevented the loss of the Na^+, K^+-ATPase activity. Because improvements of NCV and thermal nociception were also equivalent in the two dose treatments, it may be extrapolated that a lower dose of 10 mg/kg of HT administered daily might have been most likely sufficient to ameliorate diabetic neuropathy in our animals, without additional benefits from a higher dose.

In our model, behavioral tests demonstrated that, 6 weeks after diabetes induction, the thermal response latency and mechanical withdrawal thresholds changed significantly. An increase in thermal nociceptor latency and a decrease in the mechanical threshold in diabetic rats were observed. However, no significant effect of HT administration was observed in mechanical thresholds in treated diabetic rats. Mechanical hyperalgesia is caused by a direct effect of hyperglycemia on the peripheral nervous system and consequent activation of polyol pathway in Schwann cells and endothelial cells in peripheral nerves. This leads to tissue sorbitol and fructose accumulation and a number of other metabolic abnormalities.[30] HT had no effects on hyper-

glycemia in our diabetic rats, and this might explain the absence of beneficial effects on mechanical hyperalgesia. By contrast, HT significantly reduced the diabetes-induced thermal hypoalgesia. Thermal nociceptive threshold was more than double in diabetic animals compared to the nondiabetic ones. This impairment was reduced by HT to approximately 34%. This HT's protective effect on thermal sensitivity impairment might be explained by protection against skin nerve fiber degeneration.[29]

Literature on HT toxicity is scarce. When a single dose of 2 g/kg HT was administered to rats, no toxic effects or macroscopic alterations in organs have been observed, except for a transitory piloerection.[26] In another study, a chronic daily oral administration to rats of 2 g/kg of olive-pulp extract, containing 50 to 70% of HT, caused only the presence of soft or liquid feces, but no other toxic manifestations.[31,32] Accordingly, we have treated our animals daily with doses of 10 mg/kg or 100 mg/kg HT for 6 weeks and we have not observed gross adverse effects in body weight and behaviors. Both diabetes and HT administration, at either low or high dose, had no effect on plasma creatinine (p = 0.34 and p = 0.79, respectively), GOT/AST (p = 0.23 and p = 0.84, respectively), and high sensitive cardiac troponin T levels (p = 0.48 and p = 0.83, respectively). Nevertheless, chronic treatment with high dose HT, 100 mg/kg, showed a trend toward increases in hepatic and cardiac biomarkers of organ injury in both diabetic and nondiabetic animals (Table 3).

Several limitations have to be considered in the interpretations of our findings. A model of STZ-induced diabetes was employed, and therefore effects of HT on a model of type 2 diabetes, i.e. using genetically modified rats or fat-enriched diet, remains to be proven. Nevertheless, STZ-induced diabetes is a widely accepted diabetes type 1 experimental model based on the selective damage to endocrine beta-cells in rat pancreatic islets, related to activation of apoptosis. In our model we have obtained very high levels of plasma glucose, more than 900 mg/dL, indicating severe damage to the pancreatic endocrine beta-cells. However, severe hyperglycemia, from 600 mg/dL to more than 900 mg/dL, has been constantly reported in this rat model of diabetes.[2,33] Accordingly, a constant hyperglycemia is fundamental to induce diabetic neuropathy and the extent of peripheral nerve abnormalities is more pronounced if the hyperglycemia is not controlled.[33] In addition, these high levels of plasma glucose were similar in all the three groups of diabetic animals, but the effects of HT treatment on diabetic neuropathy have been clearly demonstrated. STZ-induced diabetes in rats is also associated with development of diabetic neuropathy well correlated with the clinical data reported in diabetic patients. In addition, the outcome measures used in our experimental model are available also for clinical studies, making our findings of potential interest to investigate the neuroprotective effect of this compound in patients.[34] Finally, the relationships between biochemical, functional, and neuroanatomical variables remain unsolved, as is the mechanism by which HT treatment prevents or delays the histological damage.

These limitations notwithstanding, the present study has clearly demonstrated the potential therapeutic effects of HT on diabetic neuropathy. Indeed, the daily treatment with HT reduced plasma TBARS and ameliorated the impairment in Na^+, K^+-ATPase activity, NCV, and thermal threshold, brought about by the diabetic state. The weekly measurement of NCV and thermal and mechanical nociception further reinforces the findings, because it highlights the progressive deterioration of functional variables of peripheral neuropathy in vehicle-treated animals, while a progressive amelioration was observed in HT-treated ones. It has to be recognized, however, that a total recovery from the experimental neuropathy was not achieved, nor could have been expected, due to the multiple factors playing concurrently in the progression and deterioration of the disease. These results therefore suggest that HT, a natural component of olive oil, may be a promising therapeutic adjunct for diabetic neuropathy in humans.

AUTHOR INFORMATION

Corresponding Author

*Department of Cardiovascular Research, Mario Negri Institute for Pharmacological Research, via La Masa 19, Milan 20156, Italy. Tel: +39 0239014454. Fax: +39 0233200049. E-mail: roberto.latini@marionegri.it.

Funding

This study was financially supported by Lachifarma Srl, Zollino (LE), Italy, which did not influence to any extent experiments, data analysis and drafting of manuscript.

Notes

Luciano Villanova, Alessandro Merendino, and Azzurra Villanova are employees of Lachifarma. All the other authors declare no conflict of interest.

ACKNOWLEDGMENTS

G.R. is a fellow of "Amiche del Mario Negri", Milan, Italy. All authors read and approved the final manuscript. We gratefully thank the summer student Anita Pintar, who was directly involved in the conduction of the in vivo experiments, and Dr. Tarcisio Vago (Ospedale Luigi Sacco, Milan, Italy) for assaying hs-cTnT.

ABBREVIATIONS USED

NCV, nerve conduction velocity; HT, hydroxytyrosol; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substances

REFERENCES

(1) Said, G. Diabetic neuropathy—a review. *Nat. Clin. Pract. Neurol.* **2007**, *3*, 331−340.

(2) Hoybergs, Y. M.; Meert, T. F. The effect of low-dose insulin on mechanical sensitivity and allodynia in type I diabetes neuropathy. *Neurosci. Lett.* **2007**, *417*, 149−154.

(3) Dobretsov, M.; Romanovsky, D.; Stimers, J. R. Early diabetic neuropathy: triggers and mechanisms. *World J. Gastroenterol.* **2007**, *13*, 175−191.

(4) Sima, A. A. F.; Prashar, A.; Nathaniel, V.; Bril, V.; Werb, M. R.; Greene, D. A. Overt diabetic neuropathy: Repair of axo-glial dysjunction and axonal atrophy by aldose reductase inhibition and its correlation to improvement in nerve conduction velocity. *Diabetic Med.* **1993**, *10*, 115−121.

(5) Vincent, A. M.; Russell, J. W.; Low, P.; Feldman, E. L. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr. Rev.* **2004**, *25*, 612−628.

(6) Jemai, H.; El Feki, A.; Sayadi, S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves an alloxan-diabetic rats. *J. Agric. Food. Chem.* **2009**, *57*, 8798−804.

(7) Vinik, A. I.; Park, T. S.; Stansberry, K. B.; Pittenger, G. L. Diabetic neuropathies. *Diabetologia* **2000**, *43*, 957−973.

(8) Stark, A. H.; Madar, Z. Olive oil as a functional food: epidemiology and nutritional approaches. *Nutr. Rev.* **2002**, *60*, 170−176.

(9) Obied, H. K.; Allen, M. S.; Bedgood, D. R.; Prenzler, P. D.; Robards, K.; Stockmann, R. Bioactivity and analysis of biophenols recovered from olive mill waste. *J. Agric. Food. Chem.* **2005**, *53*, 823−837.

(10) Zhang, X.; Cao, L.; Zhong, L. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human

monocytic cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2009**, *379*, 581−586.
(11) Fragopoulou, E.; Nomikos, T.; Karantonis, H. C.; Apostolakis, C.; Pliakis, E.; Samiotaki, M.; Panayotou, G.; Antonopoulou, S. Biological activity of acetylated phenolic compounds. *J. Agric. Food. Chem.* **2007**, *55*, 80−89.
(12) Zhang, X.; Jiang, L.; Geng, C.; Yoshimura, H.; Zhong, L. Inhibition of acrylamide genotoxicity in human liver-derived HepG2 cells by the antioxidant hydroxytyrosol. *Chem. Biol. Interact.* **2008**, *176*, 173−178.
(13) Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl) ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *J. Nutr.* **1997**, *127*, 286−292.
(14) Feng, Z.; Bai, L.; Yan, J.; Li, Y.; Shen, W.; Wang, Y.; Wertz, K.; Weber, P.; Zhang, Y.; Chen, Y.; Liu, J. Mitochondrial dynamic remodeling: Regulatory effects of hydroxytyrosol. *Free Radical Biol. Med.* **2011**, *50*, 1437−1446.
(15) Bianchi, R.; Buyukakilli, B.; Brines, M.; Savino, C.; Cavaletti, G.; Oggioni, N.; Lauria, G.; Borgna, M.; Lombardi, R.; Cimen, B.; Comelekoglu, U.; Kanik, A.; Tataroglu, C.; Cerami, A.; Ghezzi, P. Erythropoietin both protects from and reverses experimental diabetic neuropathy. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 823−828.
(16) Randall, L. O.; Selitto, J. J. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* **1957**, *111*, 409−419.
(17) Bianchi, R.; Marini, P.; Merlini, S.; Fabris, M.; Triban, C.; Mussini, E.; Fiori, M. G. ATPase activity defects in alloxan-induced diabetic sciatic nerve recovered by ganglioside treatment. *Diabetes* **1988**, *37*, 1340−1345.
(18) Ha, H.; Endou, H. Lipid peroxidation in isolated rat nephron segments. *Am. J. Physiol.* **1992**, *263*, F201−F207.
(19) Pesaresi, M.; Giatti, S.; Cavaletti, G.; Abbiati, F.; Calabrese, D.; Bianchi, R.; Caruso, D.; Garcia-Segura, L. M.; Melcangi, R. C. Sex differences in the manifestation of peripheral diabetic neuropathy in gonadectomized rats: a correlation with the levels of neuroactive steroids in the sciatic nerve. *Exp. Neurol.* **2011**, *228*, 215−221.
(20) Sima, A. A. New insights into the metabolic and molecular basis for diabetic neuropathy. *Cell. Mol. Life Sci.* **2003**, *60*, 2445−2464.
(21) Hamden, K.; Allouche, N.; Damak, M.; Elfeki, A. Hypoglycemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste in vitro and in rats. *Chem.-Biol. Interact.* **2009**, *180*, 421−432.
(22) Pereira-Caro, G.; Sarriá, B.; Madrona, A.; Espartero, J. L.; Goya, L.; Bravo, L.; Mateos, R. Alkyl hydroxytyrosyl ethers show protective effects against oxidative stress in hepG2 cells. *J. Agric. Food. Chem.* **2011**, *59*, 5964−5976.
(23) Bouallagui, Z.; Bouaziz, M.; Lassoued, S.; Engasser, J. M.; Ghoul, M.; Sayadi, S. Hydroxytyrosol acyl esters: biosynthesis and activities. *Appl. Biochem. Biotechnol.* **2011**, *163*, 592−599.
(24) Sabatini, N. Recent patents in olive oil industry: New technologies for the recovery of phenols compounds from olive oil, olive oil industrial by-products and waste waters. *Recent Pat. Food, Nutr. Agric.* **2010**, *2*, 154−159.
(25) Bai, C.; Yan, X.; Takenaka, M.; Sekiya, K.; Nagata, T. Determination of synthetic hydroxytyrosol in rat plasma by GC−MS. *J. Agric. Food. Chem.* **1998**, *46*, 3998−4001.
(26) D'Angelo, S.; Manna, C.; Migliardi, V.; Mazzoni, O.; Morrica, P.; Capasso, G.; Pontoni, G.; Galletti, P.; Zappia, V. Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metab. Dispos.* **2001**, *29*, 1492−1498.
(27) Scarpini, E.; Bianchi, R.; Moggio, M.; Sciacco, M.; Fiori, M. G.; Scarlato, G. Decrease of nerve Na,K-ATPase activity in the pathogenesis of diabetic neuropathy. *J. Neurol. Sci.* **1993**, *120*, 159−67.
(28) Gerbi, A.; Sennoune, S.; Pierre, S.; Sampol, J.; Raccah, D.; Vague, P.; Maixent, J. M. Localization of Na,K-ATPase alpha/beta isoforms in rat sciatic nerves: effect of diabetes and fish oil treatment. *J. Neurochem.* **1999**, *73*, 719−726.
(29) Leonelli, E.; Bianchi, R.; Cavaletti, G.; Caruso, D.; Crippa, D.; Garcia-Segura, L. M.; Lauria, G.; Magnaghi, V.; Roglio, I.; Melcangi, R. C. Progesterone and its derivates are neuroprotective agents in experimental diabetic neuropathy: a multimodal analysis. *Neuroscience* **2007**, *144*, 1293−1304.
(30) Wuarin-Bierman, L.; Zahnd, G. R.; Kaufmann, F.; Burcklen, L.; Adler, J. Hyperalgesia in spontaneous and experimental animal models of diabetic neuropathy. *Diabetologia* **1987**, *30*, 653−658.
(31) Soni, M. G.; Burdock, G. A.; Christian, M. S.; Bitler, C. M.; Crea, R. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food Chem. Toxicol.* **2006**, *44*, 903−915.
(32) Christian, M. S.; Sharper, V. A.; Hoberman, A. M.; Seng, J. E.; Fu, L.; Covell, D.; Diener, R. M.; Bitler, C. M.; Crea, R. The toxicity profile of hydrolyzed aqueous olive pulp extract. *Drug Chem. Toxicol.* **2004**, *27*, 309−330.
(33) Cermenati, G.; Giatti, S.; Cavaletti, G.; Bianchi, R.; Maschi, O.; Pesaresi, M.; Abbiati, F.; Volonterio, A.; Saez, E.; Caruso, D.; Melcangi, R. C.; Mitro, N. Activation of the liver X receptor increases neuroactive steroid levels and protects from diabetes-induced peripheral neuropathy. *J. Neurosci.* **2010**, *30*, 11896−11901.
(34) Cavaletti, G.; Jann, S.; Pace, A.; Plasmati, R.; Siciliano, G.; Briani, C.; Cocito, D.; Padua, L.; Ghiglione, E.; Manicone, M.; Giussani, G. Italian NETox Group. Multi-center assessment of the Total Neuropathy Score for chemotherapy-induced peripheral neurotoxicity. *J. Peripher. Nerv. Syst.* **2006**, *11*, 135−141.