INTRODUCTION

Parkinson’s disease is a progressive neurodegenerative disorder that clinically presents itself with motor neuron deficits that include shuffling gait, rigidity, and tremor [1]. The pathological component of Parkinson’s disease is a degeneration of neurons that project from the substantia nigra to the striatum, resulting in a reduction in striatal dopamine. Motor loss is proportional to the resultant dopamine deficiency associated with the disease [2].

In addition to understanding the neuronal cell loss, there is mounting interest in the role of neuroinflammation in Parkinson’s disease. Neuroinflammation, resulting from uncontrolled microglial activation, appears to contribute to the neuronal damage seen in Parkinson’s disease. The discovery of major histocompatibility complex positive reactive microglia in the Parkinsonian substantia nigra (3), and the detection of elevated cytokines in the spinal fluid of Parkinson’s disease patients (4), and in the substantia nigra of post-mortem parkinsonian brains (5) strongly indicate that inflammation plays a key role in the pathology of Parkinson’s disease (6).

Reactive microglia cells have been implicated in the production of reactive oxygen species (ROS). Gial cells in the parkinsonian substantia nigra have increased levels of inducible nitric oxide synthase (iNOS), the enzyme that converts L-arginine to reactive NO (7-9). Gial-derived reactive NO can react with superoxide anion leading to the formation of peroxynitrite, a potent oxidizing and cytotoxic agent (10). Although the cause of dopaminergic cell death is as yet unknown, the result is a depletion of dopamine at the striatum and a corresponding decrease in motor function. Remarkably, oxidation alone induces sequential molecular events: ROS increase, activation of JNK MAP kinases, activation of the PITSRE kinase, p110, by both Caspase-1 and Caspase-3-like activities which lead to apoptotic cell death (11). To compensate for the dopamine loss associated with Parkinson’s disease, a variety of compounds have been tested and some have been approved for therapeutic use. The use of an effective nutritional product with a high safety margin would be an ideal therapeutic agent considering the duration of therapy. We have recently shown that hydrolyzed olive vegetable water (Hidrox®) (6), a proprietary formulation of olive polyphenols rich in hydroxytyrosol, inhibits the production of pro-inflammatory cytokines in an in vitro model of neuroinflammation. In contrast olive oil, the principal phenolic in olive leaf, was significantly less effective at inhibiting cytokine production. These results suggest that the anti-inflammatory activity of hydroxytyrosol may impact neuroinflammation in Parkinson’s and other neurodegenerative diseases.
protect cells from oxidative stress associated with metabolic fluxes (16). Previous studies have demonstrated that HT inhibits the production of Tumour Necrosis Factor–alpha (TNF-α), a key inflammatory cytokine (8). For example, in lipopolysaccharide (LPS)–treated BALB/c mice, treatment with 125mg FD Hidrox®, containing approximately 3 percent HT, reduced serum TNF-α by 95 percent.

Here, we evaluate the effects of hydrolysed olive vegetation water (liquid HIDROX®) on inflammatory cytokine production by activated astrocytes and microglia in an in vitro model of neuroinflammation. We also compare the activity of oleuropein, the principal phenolic component of olive leaf, with Hidrox®.

METHODS

Hidrox® preparation
Manzanilla olive fruits were harvested in Northern California during the month of November and processed within 48 hours. The olives were first de-pitted mechanically, and then the olive pulp was pressed to yield a slurry mixture including olive oil, vegetation water and solids. Separation of solids from the liquid fraction was achieved by a three phase centrifugation (decanter – Model 483 Alfa Laval). The liquid (oil and aqueous fractions) were also separated during the three phase centrifugation and collected separately. The olive vegetation water (OVW) was treated immediately with citric acid (1 percent) and stored for six months at room temperature prior to filtration from debris and analytical characterization (referred hereafter as liquid Hidrox®).

Analysis of phenolic composition
Following sample preparation, liquid Hidrox® was briefly centrifuged at 10,000 rpm for 5 minutes to pellet particulate matter. The cleared supernatant was mixed 1:1 with Solvent A (10 percent methanol, 3 percent acetic acid in HPLC-grade ddH2O) and analysed by HPLC. The HPLC analysis (Figure 1) was performed on a Beckman-Coulter 125NM series system consisting of a 125 NM series pump, a 166 NMP series detector and an analytical Ultrasphere reverse phase column (C-18; 150 x 4.6 mm id). Separation was achieved by an elution gradient using an initial composition of Solvent A 99.5%/Solvent B (100 percent methanol) 0.5 percent for 20 minutes, followed by increasing Solvent B to 100 percent over 15 minutes. Data was collected and analysed using Beckman 32 Karat Software. Compound identification was confirmed by analysing retention times and absorption of standards at 5 different wavelengths (220, 254, 280, 320 and 340 nm).

Cell culture and cytokine production
For these experiments, astrocytes and microglia were isolated from C57Bl/6 male mice as described (17). Briefly, cortices were isolated from neonatal C57Bl1/6 mice, meninges were removed and cells were dissociated. This procedure was approved by Institutional Animal Care and Use Committee (Parkinson’s Institute). After passage through 100 pin mesh filter, cells were washed in DMEM (containing 5 percent fetal calf serum, 5 percent horse serum and 1 percent penicillin/ streptomycin), and were plated and cultured at 37°C in 5 percent CO2 atmosphere. When confluent (10 days), microglia were detached from the astrocyte monolayer by rotation. Cultured supernatant containing detached microglia was re-plated in multi-well plates. Plates were returned to the incubator and microglia cells were allowed to attach for 1 h prior to stimulation. The remaining astrocyte monolayer was removed by trypsinization. Astrocytes were washed, re-suspended and re-plated in multi-well plates. Serum was withdrawn prior to stimulation. Triplicate cultures were stimulated with bacterial lipopolysaccharide (LPS; 100 ng/ml) (E. coli; serotype 0111:134; Sigma, St. Louis, MO), LPS plus dexamethasone (10 mM) (Sigma), LPS plus Hidrox® at dilutions of 1:20, 1:100, and 1:200 (effective polyphenol concentration is equal to 5 g/L) or LPS plus oleuropein at dilutions of 1:20: and 1:200 (effective polyphenol concentration is equal to 10 mg/ml). After 6 h, culture supernatants were assayed for IL-6 (ng/mL) and TNF-α (ng/L), and cell lysates were assayed for IL-1b (µg/g) by ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis
The results are expressed as mean +/- SEM of at least 3 independent measures. Data were analysed by one-way ANOVA to determine significant differences among the groups, followed by Dunnett’s post-test to compare each of the test conditions with LPS-treated cells. Differences were considered significant when P < 0.05. Data analyses were performed using Prism software (Graphpad).

RESULTS

We began these studies to address the question of whether hydrolysed olive vegetation water (liquid Hidrox®) , which we had previously demonstrated to have potent free radical scavenging activity, also had anti-inflammatory activity on activated microglia and astrocytes, cells that have been implicated in the propagation of neuronal damage associated with Parkinson’s disease.

HPLC Analysis
HPLC analysis of liquid Hidrox® shows a major peak with a retention time of ~6 min that corresponds to HT. Additional peaks at ~11 min and ~30 min correspond to tyrosol and oleuropein, the secoiridoid glucoside of HT and the major polyphenol in olive leaf, respectively (Figure 1).

Hydrolysed olive water decreases cytokine production in astrocytes and microglia
Initial experiments were performed to determine the anti-inflammatory activities of liquid Hidrox® (CR-21) and oleuropein on CNS immuno-effector cells. LPS (100µg/L) had a robust immuno-stimulatory effect on microglia, stimulating TNF-α, IL-6 and IL-1B production (Figure 2). Liquid Hidrox® significantly inhibited LPS-induced cytokine products at a concentration of total polyphenols as low as 3mg/L. In contrast, oleuropein, at a concentration of 50mg/L polyphenols was ineffective and only exhibited a maximal inhibition of LPS-induced cytokine production in microglia at a concentration of 500mg/L. Cytokine induction in LPS-treated astrocytes was reduced as
compared to microglia cells. However, liquid Hidrox® again was much more effective at inhibiting production and/or secretion of all three cytokines (Figure 3) when compared with oleuropein in these cells.

We have developed a proprietary process to produce a HT-rich formulation (liquid Hidrox®) using 1 percent citric acid. Acid hydrolysis releases HT from its natural precursors, oleuropein and verbascoside, and this formulation has strong antioxidant and anti-inflammatory activities. This process eliminates tyrosol and other proteinaceous compounds found in olive seed. This hydrolysed aqueous fraction (liquid Hidrox®, Hidrox® seed. This hydrolysed aqueous fraction (liquid Hidrox®) has demonstrated a solid safety profile in animals, with no toxicity at levels up to 2000 mg/kg (12) (expressed as FD Hidrox®). HT, the major simple phenolic component of liquid Hidrox®, is also a metabolite of dopamine. In vivo, HT is produced from the oxidative deamination of dopamine by monoamine oxidase to 3,4-dihydroxyphenylacetaldheyde (DOPAL). The subsequent reduction of DOPAL to HT occurs via reduction of DOPAL by aldose reductase. DOPAL is a highly unstable and reactive aldehyde that interacts with sulphydryl groups to disrupt and denature proteins. Interestingly, DOPAL and HT accumulate in PC12 cells in response to metabolic stress; however, while DOPAL is toxic to differentiated PC12 cells, HT is not, suggesting that its role may be one of cytoprotection (21).

Some naturally-occurring polyphenols have been shown to possess anti-inflammatory activity in addition to their anti-oxidant activity. For example, resveratrol, a polyphenol present in red wines and some vegetables has been shown to inhibit production of O2^- in activated neutrophils (22), to suppress TNF-induced activation of cytokines in endothelial cells (23), to suppress TNF-induced apoptosis in a variety of cell lines (24), and to prevent hypoxia-induced IL-6 production in glial cells (25). Catechins, the principal polyphenol in green tea, have been shown to inhibit IL-1β -induced IL-8 production and cell surface adhesion molecule expression in a cell model of gastric inflammation (26). Chronic gastric inflammation induced by Helicobacter pylori has been proposed to be a causative pathway in the carcinogenesis of stomach cancer (26), suggesting a role for catechins in the prevention of stomach cancer (27).

In the brain, the presence of on-going oxidative stress (28) and of active inflammatory processes in the substantia nigra of parkinsonian brains and in animal models of Parkinson’s disease (29) make Parkinson’s disease an ideal target disease for therapeutic strategies that combine antioxidants and anti-inflammatory agents (30). In fact, consumption of flavonoid-rich blueberries or strawberries (flavonoids are one type of flavonoids present in the foods of this type of diet. For example oleurosides and ligstrosides in olives, lycopene in tomatoes and anthocyanins in grapes all contribute by virtue of their anti-oxidant and free radical scavenging activities. Olive fruit, in particular, are healthy by virtue of their combination of oil and their high anti-oxidant and free radical content. Olive oil is rich in oleic acid, a monounsaturated fatty acid that is considered a “good” fatty acid not only because of its lack of saturation and thus its ability to resist free radicals, but also because it has been shown, when consumed, to change the ratio of HDL to LDL (19).

Olive phenolics and HT in particular have some of the highest antioxidant activities of any known natural antioxidant. Olive phenolics have been shown to inhibit LDL oxidation; inhibit platelet aggregation; scavenge superoxide and other ROS; scavenge hypochlorous acid; inhibit neutrophils respiratory burst; and increase plasma antioxidant capacity (20). HT itself has been shown to inhibit free radical generation; scavenge reactive oxygen and nitrogen; inhibit smoking-induced oxidative stress in rats; and increase plasma antioxidant capacity (15, 20).
inhibiting LPS-induced cytokine production in both microglia and activated astrocytes. Microglia and astrocytes are sources of potential cytotoxic compounds that can aggravate neuronal injury during degenerative processes such as Parkinson’s disease. Hidrox® was effective at a concentration of 250ng/g in inhibiting production of TNF-a and IL-1b in microglia, and also showed efficacy; albeit at a higher concentration (2500ng/g) in inhibiting production of all 3 cytokines in activated astrocytes. Oleuropein, the principal phenolic in olive leaf, was much less effective at inhibiting cytokine production in both cell types. Thus, the anti-inflammatory capacity of Hidrox® for astrocytes and microglia may impact neuroinflammation in Parkinson’s disease and other neurodegenerative diseases with an inflammatory component.

REFERENCES AND NOTES