Antioxidant and anti-inflammatory properties of a Cucumis melo LC. extract rich in superoxide dismutase activity

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Abstract

The present study was conducted to evaluate in vitro and in vivo the antioxidant and anti-inflammatory properties of a cantaloupe melon (Cucumis melo LC., Cucurbitaceae) extract (CME) selected for its high superoxide dismutase activity. Peritoneal macrophages were pre-activated in vitro with 300 IU of interferon-\(\gamma\) (IFN-\(\gamma\)) and were then challenged in culture with IgG1/anti-IgG1 immune complexes (IgG1/IC) in presence of various CME extracts. The subsequent production of free radicals (superoxide anion, nitric oxide, and peroxynitrite) and of pro- (TNF-\(\alpha\)) and anti- (IL-10) inflammatory cytokines was evaluated. The CME inhibited in a dose-dependent manner the production of superoxide anion with a maximal effect at 100 \(\mu\)g/ml. This inhibitory effect of CME appeared to be closely linked to the SOD activity because it was dramatically decreased after heat inactivation of the SOD activity (HI-CME). In addition, the CME inhibited the production of peroxynitrite strengthening the antioxidant properties of this CME rich in SOD activity. The production of the pro- and anti-inflammatory cytokines, namely TNF-\(\alpha\) and IL-10, being conditioned by the redox status of macrophages we also evaluated the effect of CME and HI-CME on the IgG1/IC-induced cytokine production. When the SOD activity was present in the CME it promoted the IgG1/IC-induced production of IL-10 instead of TNF-\(\alpha\). These data demonstrated that, in addition to its antioxidant properties, the anti-inflammatory properties of the CME extract were principally related to its capacity to induce the production of IL-10 by peritoneal macrophages. The particular properties of wheat gliadin (Triticum vulgare, Poaceae) for the oral delivery of functional proteins led us to test it in a new nutraceutical formula based on its combination with the CME thus monitoring the SOD activity release during the gastro-intestinal digestive process. In these experiments C57BL/6 mice were supplemented orally everyday during 28 days with: (1) the placebo, (2) the CME extract alone, (3) the gliadin, (4) the CME/gliadin combination, or (5) the HI-CME/gliadin combination (SOD inactivated). At the end of the supplementation period all the animals were injected intra-peritoneal (i.p.) with the pro-inflammatory cytokine IFN-\(\gamma\) (300 IU) and peritoneal macrophages were harvested 24 h after to test their capacities to produce free radicals, TNF-\(\alpha\) and IL-10 after triggering with IgG1/IC. We demonstrated that animals supplemented during 28 days with the CME/gliadin combination were protected against the pro-inflammatory properties of IFN-\(\gamma\) while the other products were inefficient. These data did not only indicate that the SOD activity is important for the antioxidant and anti-inflammatory properties of the CME extract, but also demonstrated that when the SOD activity is preserved during the digestive process by its combination with wheat gliadin it is possible to elicit in vivo the pharmacological effects of this antioxidant enzyme.

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1. Introduction

Several classes of antioxidant dietary compounds have been suggested to present health benefits, and there are evidences that consumption of these products leads to a reduction of the expression of various pro-inflammatory and/or oxidative stress biomarkers (Halliwell, 2002; Peng et al., 2000; Jacob et al., 2003). The active principles in these vegetable extracts are principally water soluble or lipophilic antioxidant molecules. Indeed, most of these plant extracts contain various amounts of Vitamin E, Vitamin C, \(\beta\)-carotene, and other flavonoids (Aruoma, 1994; Aruoma,
2. Material and methods

2.1. Animals, chemicals, and reagents

C57BL/6 mice (6–8 weeks) were purchased from IFFA-Credo (Orleans, France). Dulbecco’s modified Eagle’s medium (DMEM) containing 1-glutamine and 4.5 g/l glucose was obtained from Sigma Chemical Co. (St Louis, MO, USA), as were all reagents not otherwise noted. Recombinant murine interferon-γ (IFN-γ) was purchased from Immungene (Los Angeles, CA). The melon extract isolated from the Cucumis melo LC. pulp (US Patent 5,747,043) revealed an SOD activity of 100 IU(NBT)/mg of powder which is 5–7 times more than in the classical “melon charentais.” All tissues and culture reagents and Cucumis melo extracts (CME) were assayed for endotoxin contamination by the Limulus lysate test (E-Toxate, Sigma) and were found to be less than 10 pg/ml. Heat-inactivated SOD in the CME (HI-CME) was obtained after warming the extract at 56 °C during 30 min. The content in other antioxidants in HI-CME was verified and remained unaffected after warming (data not shown).

2.2. Peritoneal macrophages preparation

Peritoneal cells were isolated from C57BL/6 mice after washing the peritoneal cavity with 5 ml of physiological water. The cells were washed twice and seeded at densities of 5–6 × 10⁵ cells/cm² on petri dishes (100 × 15 mm) in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 µg/ml; RPMI–FBS). Peritoneal macrophages were allowed to adhere for 2–3 h in 5% CO₂ humidified atmosphere. Then, the non-adherent cells were removed by washing twice with 10 ml pre-warmed medium and dishes were incubated for 10 min at 4 °C. The supernatants were then carefully removed and discarded and the plates were washed once with pre-warmed Dulbecco’s phosphate buffered saline (PBS; GIBCO). Cold PBS (15 ml) containing 1.5% FBS (PBS–FBS) was first added followed by 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages removed by 10 washes using a 10 ml syringe. The viability of the resulting cells was assessed by Trypan blue exclusion and the rate of macrophages determined after cytoplasmic staining with acridine orange and examination using a fluorescence microscope. Cell preparations were >95% viable and contained >95% macrophages.

2.3. Macrophage culture and activation

Peritoneal macrophages were maintained in DMEM containing 3.7 g/l of sodium bicarbonate supplemented with 10% of fetal calf serum (Bioproducts, France) and 1% of non-essential amino acids, in a humidified 37°C atmosphere with 10% CO₂. For the in vitro activation, macrophages...
were seeded at \(10^6\) cells/ml, 100 \(\mu\)l/well in 96-well tissue culture plates (Becton Dickinson, Grenoble, France). Plates were incubated for approximately 1–2 h prior to activation to allow adherence of macrophages. Macrophages were then activated during 48 h with IFN-\(\gamma\) (300 IU/ml) and then challenged with IgG1 IC complexes (anti-IgG1 20 \(\mu\)g and IgG1 100 \(\mu\)g) as previously described (Vouldoukis et al., 2000) in the presence or in the absence of different doses of CME or HI-CME. The production of TNF-\(\alpha\), IL-10, and nitrite were checked in the cell-free supernatant after 24 h while only after 2 h for the cellular production of superoxide anion and peroxynitrites.

2.4. Preparation of the CME/gliadin combination

Considering their hydrophobic nature, wheat gliadin biopolymers (Calderon de la Barca et al., 1996) were evaluated to improve the oral bioavailability of drugs (Regnault et al., 1996; Ezepeleta et al., 1999). In the present study, we used these hydrophobic gliadin biopolymers (Triticum vulgare) to preserve the SOD activity present in the CME during the digestive process (Stella V et al., 1995) and to activate the intestinal mucosal immune system (Fassano et al., 2000).

The CME/gliadin combinations were done as already described for many other drug delivery systems (Stella et al., 1995; Ezepeleta et al., 1999; Mauguet et al., 2002). Briefly, CME (100 IU/mg) preparations were mixed with gliadin in a 40% hydro-alcoholic solution, in such a ratio that the SOD activity in the final product is 1 IU/(NBT)/mg. The global SOD activity contained in the CME/gliadin combination was checked after dissolution of the product in acetic acid as already described (Stella et al., 1995). Briefly, the SOD activity was measured in the solubilized CME/gliadin combination by the reduction of nitroblue tetrazolium NBT on 10% of polyacrylamide gel as previously described (Beauchamp and Fridovich, 1971) using the CME product as internal reference.

2.5. Oral supplementation with free CME, gliadin, or the CME/gliadin preparations

C57/BL-6 mice of 6–8 weeks old (25–30 g) were divided into five groups (\(n = 10\)) receiving, respectively, by oral force feeding during 28 days: (1) the placebo in control, (2) the CME (0.05 mg equivalent of 5 IU/NBT of SOD), (3) the gliadin (1 mg), (4) the gliadin/CME combination (5 mg equivalent to 5 IU/NBT of SOD), and (5) the gliadin/Hi-CME combination (5 mg) inactivated for the SOD activity. Throughout the 28 days of supplementation, daily food intake and weight gain were recorded. At the end of the supplementation period animals were injected intra-peritoneal (i.p.) with 300 IU of IFN-\(\gamma\). The peritoneal macrophages were then harvested after 24 h to be challenged ex vivo with IgG1 IC immune complexes as previously described (Vouldoukis et al., 2000). The production of TNF-\(\alpha\), IL-10, and nitrite were checked in the cell-free supernatant after 24 h while only after 2 h for the cellular production of superoxide anion and peroxynitrites.

2.6. TNF-\(\alpha\) and IL-10 measurements

At the end of the culture period the cell-free supernatants were kept at \(-80^\circ\)C in order to avoid TNF-\(\alpha\) degradation. TNF-\(\alpha\) and IL-10 levels were measured using ELISA Kits from British Biotechnology (England) and the threshold of detection were 10 pg/ml.

2.7. Assay for \(O_2^{•−}\), \(NO^{•−}\), \(H_2O_2\), and ONOO\(^{−}\) production by resting and activated cells

To assess the amount of NO produced, the stable end product of NO, \(NO_2^{•−}\) was measured using the Griess reaction as previously described (Vouldoukis et al., 1995; Dugas et al., 1996). The generation of \(O_2^{•−}\) was assayed by measuring the reduction of ferricytochrome C at 37 °C by adherent cells (McCord and Fridovich, 1969) pre-incubated in the presence of 150 \(\mu\)M ferricytochrome C. After two additional hours of incubation in the presence or in the absence of IgG1 IC the absorbance change was assayed at 550 nm in a spectrophotometer.

To measure ONOO\(^{−}\), production was evaluated after oxidation of dihydrodihydromine 123 to fluorescent rhodamine as previously described (Kooy et al., 1994). Briefly, 5 \(\times\) 10\(^5\) cells in 500 \(\mu\)l RPMI medium were pre-incubated for 6 h in the presence of 100 \(\mu\)M of dihydrodihydromine 123. The suspension was pre-warmed at 37 °C, before a 50 \(\mu\)l addition of IgG1 immune complexes (IgG1 IC) and fluorescence was then measured on a spectrophotometer (LS-50, Perkin-Elmer Corporation, Norwalk, CT) with excitation and emission wavelength of 500 and 536 nm, respectively, and excitation and emission slit widths of 2.5 and 3.0 nm, respectively, and 30 min after addition of IC. Cells (2 \(\times\) 10\(^6\)/ml) were measured under the different culture conditions.

2.8. Statistical analysis

Data shown represent the mean and standard deviation for triplicate or quadruplicate cultures in a representative experiment. All experiments were done at least four times with equivalent results. Mann–Withney U test was used for comparison of populations.

3. Results

3.1. Effect of the CME extract on the redox status of macrophages

The CME extract is a rich antioxidant nutritional extract that naturally contain a high SOD activity (an average
of 100 IU/NBT per mg of dry extract) but also catalase activity (10 IU/mg), residual Gpx activity and number of natural antioxidant quenching molecules (Table 1). Considering that antioxidants are essential for reducing oxidative stress (Halliwell et al., 1995; Yagi et al., 2002; Lii et al., 1998; Lee and Man-Fan Wan, 2000) and that heterologous SOD (antagonistic) may have some immunoregulatory properties (He et al., 2002; Mullerad et al., 2002), we evaluated in vitro the effect of crude CME on resident peritoneal macrophages of C57BL/6 mice. As shown in Fig. 1, the crude CME extract retaining its SOD activity inhibited in a dose-dependent manner the production of superoxide anion by IgG1 IC-stimulated macrophages, the maximal inhibitory effect being reached at 100 µg/ml of CME (equivalent to 101 IU/NBT of SOD activity). With the heat-inactivated CME (HI-CME, lacking its SOD activity) we observed a significant difference in the inhibitory effect. This suggested that even whether the other antioxidant products were able to reduce the production of superoxide anion by IgG1 IC-treated macrophages the SOD activity was essential to reduce oxidative stress.

Considering that the IgG1 IC-stimulation process is known to stimulate the NO*-dependent pathway we evaluated the effect of the two different extracts on the production of nitrogen species derived from the iNOS pathway (Bugas et al., 1995) and on the production of the highly pro-oxidant peroxynitrite that results from the chemical combination of NO* with O2**• (Ischiropoulos et al., 1992). As demonstrated in Table 2, both extracts were able to reduce the production of peroxynitrites without affecting significantly the NO* production but this inhibitory effect was more pronounced with CME extract when the SOD activity was maintained. Taken together these data suggested that even whether we cannot totally exclude the inhibitory effects of the different antioxidant products (enzyme and/or quencher molecules) present in the CME, the SOD activity is at least responsible for the suppressive effect of CME on IgG1 IC-induced production of superoxide and peroxynitrites by macrophages.

3.2. Effect of the CME extract on the production of TNF-α and IL-10 by macrophages

The oxidative metabolism being intimately linked to the pro- and/or anti-inflammatory capacities of macrophages we evaluated the effect of the different CME extracts on the IgG1 IC-induced production of pro-inflammatory cytokine IFN-γ and IL-10 (anti-inflammatory cytokine) production by macrophages. Stimulation of IFN-γ-activated macrophages by IgG1 IC-induced a simultaneous production of TNF-α and of IL-10 (Fig. 2). The inverse correlation observed between TNF-α and IL-10 concentrations in these experiments suggests the existence of a physiological equilibrium between the production processes of these two cytokines.
In the presence of CME the production of TNF-α was significantly reduced ($P < 0.01$) whereas the IL-10 production was enhanced ($P < 0.01$) suggesting that the oxidative metabolism is likely involved in the different processes of cytokine productions (Fig. 3). Interestingly, in the same experiments the HI-CME reduced the production of TNF-α ($P < 0.05$) but did not significantly affect the production of IL-10 suggesting that the SOD activity in the CME extract is an important component to promote its anti-inflammatory properties.

3.3. In vivo anti-inflammatory properties of the CME extract combined or not to a wheat gliadin

The possible use of hydrophobic gliadin biopolymers not only as potent drug delivery system (Stella et al., 1995; Ezpeleta et al., 1999; Mauguet et al., 2002), but also as promoter of mucosal tolerance immunity (Rossi et al., 1999; Husby, 2000), led us to evaluate the anti-inflammatory effect of oral supplementations with the CME extract. Considering the relative activity of the SOD in the CME extract we have designed a 28 days long protocol of oral supplementation which correspond to the minimal period of treatment to obtain an in vivo anti-inflammatory effect on chronic diseases (Baret et al., 1984; Jadot et al., 1995). After this period of supplementation, resident peritoneal macrophages were harvested to evaluate their pro- and anti-inflammatory profiles. As shown in Fig. 4, after the CME/gliadin supplementation period the IgG1 IC-induced TNF-α (Fig. 4A) production in IFN-γ activated macrophages was significantly reduced ($P < 0.01$) whereas the production of IL-10 (Fig. 4B) was significantly enhanced ($P < 0.01$). When compared to the other per os treatment it was found that the non-protected CME extract was unable to affect the IgG1 IC-induced production of TNF-α (Fig. 4A) and of IL-10 (Fig. 4B), whereas the gliadin alone or the gliadin/HI-CME (without SOD activity) slightly ($P < 0.05$) enhanced the production of...
TNF-α (Fig. 4A) but did not affect the production of IL-10 (Fig. 4B).

In a second set of experiments we demonstrated that this protective effect of CME/gliadin supplementation was effective even after intra-peritoneal injection of the pro-inflammatory cytokine IFN-γ (300 IU). As demonstrated in Fig. 5, the IFN-γ injection not only induced the production of the pro-inflammatory cytokine TNF-α by IgG1 IC-activated macrophages but also promoted an important oxidative stress as revealed by the production of superoxide anion (Fig. 5A), nitric oxide (Fig. 5B), and peroxynitrite (Fig. 5C). In the same experiments only animals supplemented with CME/gliadin were protected against the pro-inflammatory and pro-oxidative effects of IFN-γ (Murata et al., 2002). Taken together these data confirmed that, even whether we cannot exclude the effect of the other antioxidant components, the SOD activity is essential for the anti-inflammatory effect of the CME. In addition, they also demonstrated that the binding abilities of gliadin polymers with epithelial intestinal cells allowed to obtain a new oral combination able to promote the anti-inflammatory properties of the CME extract thus defining a new class of antioxidant nutraceutical product, GliSODin® (Germano, 2001; Dugas, 2002).
4. Discussion

Intensive research was conducted over the last 10 years to promote the antioxidant nutritional medicine in the nutraceutical field (Lee et al., 2003; Dugas et al., 1999; Introna et al., 1997). Plant extracts with antioxidant activities were traditionally used to strengthen the natural immune defences and to promote host defence mechanisms (Panossian et al., 1999; Xiao et al., 1993). However, even whether these plant extracts are of interest as immune support it is always very difficult to clearly demonstrate in vivo their immune supporting effects. In the present work we demonstrated that Cucumis melo L.C. extracts rich in SOD activity are able to promote concomitantly antioxidant and anti-inflammatory properties likely by inducing the production of IL-10 by macrophages. The SOD activity in these extracts appeared to be important since the production of IL-10 was significantly reduced after heat inactivation of the enzymatic activity. These data fit with previous reports indicating that the anti-inflammatory properties of SOD were not only related to its enzymatic antioxidant property but also to the concomitant stimulation of the production of anti-inflammatory and immuno-regulatory molecules (Vouldoukis et al., 2000; Mullerad et al., 2002; He et al., 2002). Even whether we cannot exclude the effect of the other antioxidant molecules in the CME extracts we demonstrated that the SOD activity present in this extract was able to control the production of the redox-sensitive anti-inflammatory cytokine, IL-10 (Haddad and Fahlman, 2002). The antioxidant and anti-inflammatory effects of melon extracts have been already described (Murcia et al., 2001; Campanella et al., 2003) but little was known on the potent pharmacological effect of the SOD activity in these extracts (Palma et al., 1997; Sandalio et al., 1997). In the present report and according to the current literature on SOD (Torrens, 1988), we demonstrated that the SOD activity in the melon extract is essential for its pharmaco-protective effect.

In addition, we demonstrated that the combination of CME with other vegetal extracts such as hydrophobic gliadin biopolymers, led to the development of a 100% vegetal product that promotes the oral pharmacology of SOD (Germano, 2001; Dugas, 2002). Indeed, we demonstrated that the per os supplementation by the CME/gliadin combination regulated the activation state of macrophages as indicated by their decreased production of the pro-inflammatory cytokine TNF-α and by their promoted production of the anti-inflammatory cytokine IL-10. Interestingly, the enzymatic activity of SOD is mandatory to obtain the production of IL-10 by the macrophages since either in vitro or in vivo the production of IL-10 was not observed when the SOD was heat inactivated, suggesting that the production of this anti-inflammatory cytokine was redox sensitive. Considering that antigenic SOD were able to promote the polarization of a Th1-dependent immune response (He et al., 2002; Mullerad et al., 2002; Vouldoukis et al., in press) we suggest that these enzymatically and antigenically active proteins concomitantly induce the production of the pro-oxidant cytokine INF-γ and the anti-inflammatory IL-10 protecting cells and tissues against the deleterious effects of free radicals (Kourilsky and Truffa-Bachi, 2001).

Taken together these data demonstrated for the first time that the Cucumis melo extract rich in SOD activity is able to promote, in vitro as well as in vivo, a protective innate immune response by increasing the production of the anti-inflammatory cytokine, IL-10. Such a mechanism not only provides new tools to evaluate the immunoregulatory properties of antioxidants, but also suggests that the dual antigenic versus antioxidant nature of SOD is mandatory to display the full pharmacological anti-inflammatory properties of this enzyme (Baret et al., 1984; Jadot et al., 1995; Tabatabai and Pugh, 1994). In addition, the development of a new natural vegetal combination to promote the oral administration of active peptides and proteins opens a great field of investigation not only in drug pharmacology but also in functional nutrition for limiting the risk of food allergy (Dugas et al., 2003). Indeed, most of the orally administered proteins and peptides were unable not only to resist to the digestive process but also to cross the epithelial cell layer and enter the intestinal mucosa and circulation. The natural capacity of the wheat gliadin biopolymers (Calderon de la Barca et al., 1996) to bind to intestinal mucosa (epithelial cells) and to immuno-competent cells (Farre-Castany et al., 1995) is also essential to preserve the SOD activity present in the CME extract in order to promote its delivery in the intestinal mucosa and to trigger the immune system.

Without excluding the effects of other components (antioxidant molecules present in the CME and/or gliadin) once absorbed in the intestinal mucosa, the antigenic melon SOD is able to promote locally the activation of the immune system and then to induce in cascade the activation of macrophages in the overall body. In that particular situation the antigenic SOD can be presented as a ying/yang immunoregulatory molecule. Indeed, the intrinsic antioxidant/anti-inflammatory properties of SOD limit the consequence of an uncontrolled activation of the immune system that could induce certain immuno-pathological situations (Lipscomb and Masten, 2002). This dual function of the antigenic SOD defines a new class of super-antioxidant molecules that not only present the classical antioxidant properties of such molecules but also stimulate a protective immune response (Germano, 2001; Enwonwu and Sanders, 2001). At the nutritional level, the development of this novel class of molecule is of importance because in the search of nutritional antioxidant efficacy the CME/gliadin combination (GliSODin®) provides useful tools for the validation of new health nutraceutical biomarkers (Griffiths et al., 2002; Lindsay and Astley, 2002; Potschman, 2003). In conclusion, our results provide strong evidence that in the present Cucumis melo extract, the SOD activity is essential to promote its antioxidant and anti-inflammatory properties. In addition, we demonstrated that, using vegetal hydrophobic biopolymers, it is possible to define a new
class of super-antioxidant nutraceuticals that promote the oral pharmacology of the SOD.

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