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Cacao extracts suppress tryptophan degradation of mitogen-stimulated peripheral blood mononuclear cells

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ABSTRACT

Ethnopharmacological relevance: The fruits of *Theobroma cacao* L. (Sterculiaceae) have been used as food and a remedy for more than 4000 years. Today, about 100 therapeutic applications of cacao are described involving the gastrointestinal, nervous, cardiovascular and immune systems. Pro-inflammatory cytokine interferon-γ and related biochemical pathways like tryptophan degradation by indoleamine 2,3-dioxygenase and neopterin formation are closely associated with the pathogenesis of such disorders.
Aim of the study: To determine the anti-inflammatory effect of cacao extracts on interferon-γ and biochemical consequences in immunocompetent cells.
Materials and methods: Effects of aqueous or ethanolic extracts of cacao were examined on mitogen-induced human peripheral blood mononuclear cells (PBMC) of healthy donors and on lipopolysaccharide-stimulated myelomonocytic THP-1 cells. Antioxidant activity of extracts was determined by oxygen radical absorption capacity (ORAC) assay.
Results: In mitogen-stimulated PBMC, enhanced degradation of tryptophan, formation of neopterin and interferon-γ were almost completely suppressed by the cacao extracts at doses of ≥5 μg/mL. Cacao extracts had no effect on tryptophan degradation in lipopolysaccharide-stimulated THP-1 cells.
Conclusions: There is a significant suppressive effect of cacao extracts on pro-inflammatory pathways in activated T-cells. Particularly the influence on indoleamine 2,3-dioxygenase could relate to some of the beneficial health effects ascribed to cacao.

1. Introduction

Consumption of cacao or chocolate is very popular, from the ancient people of Olmec, Maya and Aztec cultures up to the present, and has been associated with regalement and a sense of delight. Especially the indigenous people of Central and South America still use the fruits of *Theobroma cacao* L. (Sterculiaceae) as a traditional medicine. Reviewing available literature concerning the historical use of cacao or chocolate for medicinal purposes revealed appetite stimulating, relaxing and also mood-enhancing effects as the most consistent applications (Dillinger et al., 2000). Recently, dark chocolate was also demonstrated to induce coronary vasodilation, to improve coronary vascular function, and to decrease platelet adhesion within short time after consumption. These beneficial effects seem to go along with a significant reduction of serum oxidative stress and were positively correlated with changes in serum epicatechin concentration (Buijsse et al., 2006; Flammer et al., 2007). For all these effects, the extent of cacao present in chocolate is considered to be of ample importance.

Cacao refers to cocoa powder derived from the beans of *Theobroma cacao* L. (Sterculiaceae) by grinding and removing the cocoa butter from the dark, bitter cocoa solids. Several *in vitro* and *in vivo* studies suggest that the active compounds in cocoa exhibit protective effects against conditions such as cardiovascular disease and cancer, diseases which are also associated with inflammation and impaired immune function (Kris-Etherton and Keen, 2002; Steinberg et al., 2003; Yamagishi et al., 2003; Ramljak et al., 2005; Jourdain et al., 2006). Cocoa compounds were shown to improve or normalize, e.g., eicosanoid production (Schramm et al., 2001; Noreen et al., 1998), platelet activation (Rein et al., 2000; Holt et al.,

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DMSO, dimethylsulfoxide; EGCG, Epigallocatechin-gallate; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; IDO, indoleamine 2,3-dioxygenase; IFN-(, interferon-(; IL-2, interleukin-2; kyn/trp, kynurenine to tryptophan ratio; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethyldiazol-2-yl]-2,5 diphenyl tetrazolium bromide; ORAC, oxygen radical absorption capacity; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; ROS, reactive oxygen species; TE, trolox equivalents; TLRs, toll like-receptors; TNF-α, tumor necrosis factor-α.

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2002; Pearson et al., 2002), nitric oxide-dependent activities (Fisher et al., 2003; Heiss et al., 2003), and cytokine production (Heiss et al., 2003; Mao et al., 2000, 2002a, 2003). Thus, cocoa-derived products have the potential to positively modulate the inflammatory status that characterizes several chronic diseases.

During Th-1 type immune response, activated cells release large amounts of cytokines such as interleukin-(IL)-2 or interferon-(IFN)-γ. Pro-inflammatory cytokine IFN-(is probably the most important multiplier of anti-microbial and anti-tumoral host defence producing a variety of physiological and cellular responses, e.g. induction of high amounts of anti-microbial and cytocidal reactive oxygen species (ROS) by macrophages and other cells (Nathan, 1986). ROS are capable of interfering with various redox-sensitive intracellular signal-transduction cascades involving, e.g. activation of nuclear factor-κB (Schreck et al., 1991; Asehnoune et al., 2004), which leads to the production of further pro-inflammatory cytokines such as tumor necrosis factor-(TNF)-α (Min et al., 2003). Consequently, accumulation of ROS further amplifies Th1-type immune response, and thus appears as a positive regulator in addition to pro-inflammatory Th1-type cytokines.

In human macrophages, T-cell derived IFN-γ induces also the enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenines (Wirleitner et al., 2003) and formation of the immune activation marker neopterin, via induction of the enzyme guanosine-triphosphate-(GTP)-cyclohydrolase (Fuchs et al., 1988). Increased tryptophan degradation and neopterin production develop in patients during diseases which are associated with Th1-type immune activation such as infections, autoimmune diseases, malignant disorders, and during allograft rejection episodes (Murr et al., 2002). Higher neopterin concentrations are also associated with increased cardiovascular risk and they parallel the course of neurodegenerative disorders such as Parkinson's disease and Alzheimer's dementia (Blasko et al., 2007). IDO plays a central role in the suppression of intracellular bacteria and viruses during an antimicrobial immune response, as ongoing tryptophan degradation limits protein biosynthesis due to deprivation of this essential amino acid (Pfefferkorn, 1986; Ozaki et al., 1988). More recently, it has been demonstrated *in vitro* that also T cell proliferation is inhibited efficiently by IDO (Munn et al., 1999; Frumento et al., 2002). In patients, accelerated tryptophan degradation was found to parallel, and even to predict, the future course of several clinical conditions, including HIV infection, malignancy and autoimmune syndromes such as rheumatoid arthritis (Schroecksnadel et al., 2006a,b; Murray, 2003).

The essential amino acid tryptophan is not only required for protein synthesis, but also acts as a precursor for the biosynthesis of the neurotransmitter 5-hydroxytryptamine (5-HT; serotonin), which appears to be strongly involved in the pathogenesis of mood disorders and depression (Young and Leyton, 2002). Accordingly, activation of IDO seems to represent a link between the immunological network and the pathogenesis of depression, when the availability of tryptophan limits serotonin biosynthesis (Widner et al., 2002; Russo et al., 2003; Dantzer et al., 2008). If cacao extracts were able to interfere with IDO activation, it would correspond nicely to the effect of cocoa to improve mood.

In an approach to evaluate the effects of commercially available cacao on the T-cell/macrophage interplay, we studied the influence of cacao extracted in water or ethanol (30%) on tryptophan degradation in peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA), which activates formation of pro-inflammatory cytokine IFN-γ (Nathan et al., 1983) and subsequently tryptophan degradation and neopterin production (Weiss et al., 1999). In addition, effects of cacao extracts were also tested on lipopolysaccharide (LPS)-stimulated myelomonocytic THP-1 cells, an appropriate model to study monocyte activation by another pro-inflammatory stimulus (Neurauter et al., 2003; Singh et al., 2005). To test for the antioxidant activity of cacao extracts, the Oxygen Radical Absorption Capacity (ORAC) assay was applied using fluorescein as a fluorescent probe (Ou et al., 2001).

2. Materials and methods

2.1. Chemicals

Ethanolic (30%) and aqueous extracts of cacao were prepared from commercially available pure (100%) powdered cacao, produced from Western Africa *Theobroma cacao* (L.) beans (Bensdorp powdered cacao, Kraft foods, Vienna, Austria) and sterile filtered for cell culture experiments, which according to the manufacturer contains 185 mg/g protein, 140 mg/g carbohydrates, of which 18 mg/g is sugar, 210 mg/g fat of which 130 mg/g are saturated fatty acids, 290 mg/g fiber and 0.1 mg/g sodium, and according to J. Lied 17.2 mg/g total phenolics, 0.96 mg/g epicatechin, 0.4 mg/g protocatechuic acid and 0.32 mg/g procyanidin (Lied, 2002). Epigallocatechin-gallate (EGCG), ascorbic acid and Trolox were purchased from Sigma–Aldrich (Vienna, Austria) dissolved in dimethylsulfoxide (DMSO) and stored at −80 °C. Fluorescein, disodium salt (Anaspec, San Jose, CA) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; Wako Chemicals, Germany) was dissolved in phosphate buffer (75 mmol/L; pH 7.4).

2.2. Isolation and stimulation of human PBMC and THP-1 cells

PBMC were isolated from whole blood obtained from healthy donors, of whom informed consent was obtained that their donated blood unit was used for scientific purposes if not otherwise used. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate buffered saline containing 0.2% EDTA [0.5 mmol/L]. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mmol/L glutamine (Serva, Heidelberg, Germany) and 0.1% of gentamicin (50 mg/mL, Bio-Whittaker, Walkersville, MD) in a humidified atmosphere containing 5% CO_2 for 48 h. This procedure was observed earlier to reveal best reproducible results when applied for testing of anti-inflammatory effects of compounds or drugs (Widner et al., 1997). Average tryptophan content in the supplemented RPMI 1640 medium was 31.5 μmol/L. For each of the four experiments run in duplicates, PBMC were freshly prepared.

Isolated PBMC were plated at a density of 1.5×10^6 cells/mL in supplemented RPMI 1640, preincubated for 30 min with or without cacao extracted in water or ethanol (30%) and stimulated or not with 10 μg/mL PHA for 48 h.

The myelomonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and was cultured in complete medium as described earlier (Neurauter et al., 2003). Cells were used from early passages and kept for <1.5 months. All THP-1 experiments were repeated at least twice and run in triplicates. The cells were regularly tested negative for mycoplasma.

2.3. Measurement of tryptophan, kynurenine, neopterin and interferon-γ concentrations

After incubation of cells for 48 h, supernatants were harvested by centrifugation and tryptophan and kynurenine concentrations