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Short Communication

Chocolate consumption modulates cytokine production in healthy individuals

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ABSTRACT

Epidemiological studies suggest that chocolate increases the incidence and severity of acne. Here we demonstrate that chocolate consumption primes human blood mononuclear cells from volunteers to release more interleukin-1β (IL-1β) and IL-10 upon stimulation with *Propionibacterium acne* or *Staphylcoccus aureus*, the two microorganisms involved in the pathogenesis of acne. In contrast, production of the Th17-derived cytokine IL-22 was inhibited by chocolate. Modulation of inflammation could represent an important mechanism through which chocolate consumption influences acne.

1. Introduction

Acne vulgaris, a skin infection mainly caused by *Propionibacterium acnes* (*P. acnes*), has different incidences in various populations [1]. In addition to genetic differences, diet has been proposed to play a role in this phenomenon, and recent studies suggested that chocolate consumption might worsen acne in adolescents [2,3]. However, the mechanisms that drive the effects of chocolate on acne are not known. Chocolate contains a large number of flavonoids that have been shown to have important antioxidant properties [4], and thus provide beneficial effects on vascular diseases such as hypertension and atherosclerosis [5]. Moreover, chocolate flavonoids have been shown to have modulatory effects on inflammation and cytokine production [6,7], as well as on intracellular reactive oxygen species [8].

From a mechanistic point of view, the effect of chocolate on acne may be mediated through a direct effect on *P. acnes* growth, or it may indirectly influence acne through the modulation of the inflammation induced by *P. acnes*. This latter effect of chocolate has been shown when cells were stimulated with purified microbial ligands [4,7], yet it is not known whether chocolate would have the same effect if cells are stimulated by *P. acnes* or other microorganisms that often colonize the skin of acne patients such as *Staphylococcus aureus (S. aureus)*.

In the present study we investigated the mechanisms through which chocolate may influence the development of acne. We studied the effect of chocolate on the growth of *P. acnes*, and we investigated whether chocolate had modulatory effects on cytokine production stimulated by *P. acnes* or *S. aureus*.

2. Materials and methods

2.1. In vitro production of cytokines by peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by differential centrifugation with Ficoll-Paque (Amersham Biosciences) from blood collected from seven healthy volunteers, after written informed consent (approved by Ethical Committee of Arnhem–Nijmegen Region). 5×10^5 of PBMCs in 100 μL culture medium were stimulated with 100 μL of the culture medium RPMI-1640 supplemented with glutamax, 0.02 mM sodium pyruvate, 100 U/mL Penicillin, 100 μg/mL Streptomycin, as negative control (all from Gibco, Paisley, UK). In addition, cells were stimulated with heat-killed *P. acnes* or *S. aureus* 10^7/mL or 10^6/mL (prepared from clinical strains).

In two separate sets of experiments, the cells were stimulated with microbial stimuli without or with a combination of 2 μg/mL OmniCoA flavonoid preparation (OmniCoA, Ajinomoto OmniChem, Louvain-la-Neuve, Belgium) using two different protocols: either simultaneous stimulation with ligands and flavonoids, or in a subsequent manner with 24 h preincubation with flavonoids, followed by stimulation with microbial stimuli (*P. acnes* or *S aureus*). After 24 h stimulation, the TNF and IL-1β concentrations in the supernatants were measured using specific ELISA (Sanquin, Amsterdam).

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2.2. In vivo effects of chocolate consumption on cytokine production

Blood samples were collected by venipuncture from seven healthy volunteers, after written informed consent, before and after the consumption of 50 grams of chocolate (Milka, Switzerland, containing 30% of cocoa solids) for four days. Before the start of the study, the volunteers had not consumed chocolate for at least one week. The PBMCs were stimulated with RPMI, *P. acnes* 10^7/mL or 10^6/mL, or *S. aureus* 10^7/mL. ELISAs were performed on supernatants after 24 h (TNFα and IL-1β), 48 h (IFNγ and IL-10) and 7 days of stimulation (IL-17 and IL-22). The time points after which each cytokine was measured was chosen based on extensive previous experiments in our laboratory [9]. IFNγ, IL-10, IL-17 and IL-22 ELISA kits were purchased from R&D Systems (Minneapolis, USA).

2.3. Statistical analysis

Cytokine production was compared between the groups using a paired Student *t*-test (Excell software). Data are presented as individual values before and after chocolate consumption. *p*-Values lower than 0.05 were considered statistically significant.

3. Results

3.1. In vitro effects of chocolate flavonoids on cytokine production

The TNFα and IL-1β production induced by *P. acnes* or *S. aureus* in PBMCs was not influenced by simultaneous incubation with chocolate flavonoids (not shown). Interestingly however, 24 h preincubation of PBMCs with chocolate flavonoids, followed by stimulation with microbial ligands, significantly primed the cells for TNF and IL-1β production secondary to stimulation with *S. aureus* or *P. acnes* (Fig. 1). *S. aureus*-induced IL-10 production was also primed twofold (64 vs 30 pg/mL) when PBMCs were preincubated with flavonoids, while *P-acnes*-induced IL-10 was not influenced. Chocolate flavonoids had no effects on IFNγ production (not shown). These data argue that chronic exposure of immune cells to chocolate can prime their production of proinflammatory cytokines.

3.2. In vivo effects of chocolate consumption on cytokine production

In order to assess the relevance of these effects in vivo, cytokine production was assessed in cells isolated from seven healthy volunteers before and after consumption of chocolate. Although chocolate had no effect on TNFα production induced by low concentrations of *P. acnes* (10^6/mL), a tendency towards a higher TNFα production stimulated by *P. acnes* 10^7/mL was apparent after chocolate consumption (Fig. 2a). More strikingly, after stimulation of PBMCs with *P. acnes* (10^6/mL), there was a significant 2-fold increase in IL-1-production after chocolate consumption ($p = 0.03$, Fig. 2b).

No significant differences were measured in the production of lymphocyte-derived cytokines IL-10, IFNγ, IL-17 and IL-22 (Fig. 2c and d, and not shown). In contrast, we documented a significant 16-fold increase of IL-10-production after stimulation of cells with *S. aureus* after chocolate consumption ($p = 0.0007$, Fig. 2e). Moreover, chocolate consumption decreased by 34% the IL-22-production after stimulating the PBMCs with *S. aureus* ($p = 0.08$, Fig. 2f). Finally, TNFα, IL-1β, IFNγ and IL-17-production induced by *S. aureus* was not modulated by the chocolate consumption (data not shown).

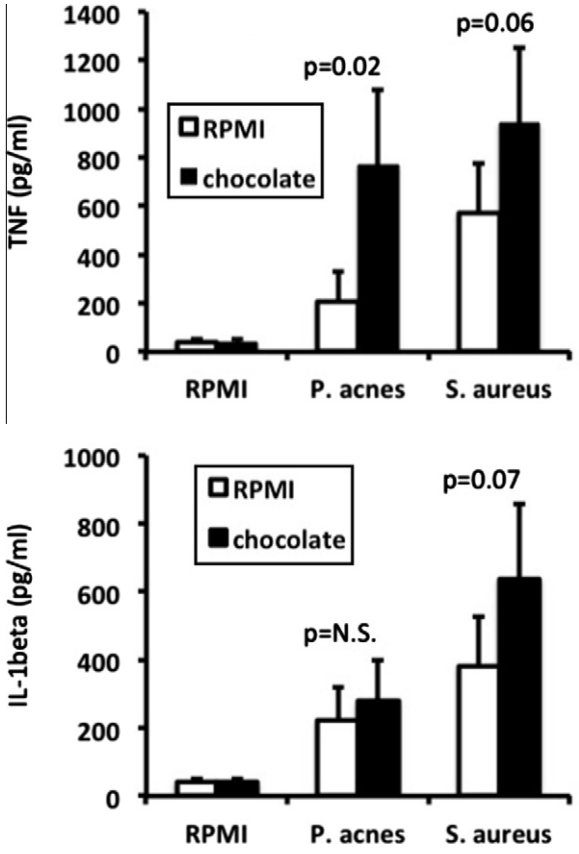

Fig. 1. In vitro effects of chocolate consumption on cytokine production. After 24 h preincubation of PBMCs with culture medium (RPMI) or chocolate flavonoids (chocolate), cells were stimulated for an additional 24 h with *P. acnes* (10^7 colony forming units-CFU/mL) or *S. aureus* (10^7 CFU/mL). After 24 h, the TNFα and IL-1 concentrations were measured in the supernatants. $n = 7$, $^*p$-values calculated by paired Student *t*-test.

4. Discussions

In the present study we investigated the immunomodulatory effects of chocolate on cytokine production induced by *P. acnes* and *S. aureus*, two microorganisms involved in the pathogenesis and complications of acne. No direct effects of chocolate could be seen on the growth of *P. acnes* (data not shown). In contrast, chocolate had stimulatory effects of proinflammatory cytokines such as TNF and IL-1β induced by *P. acnes*. Interestingly, chocolate increased production of the anti-inflammatory cytokine IL-10 induced by *S. aureus*, while decreasing the release of IL-22.

In a direct stimulation assay in vitro, we have not observed significant effects of chocolate flavonoids on *P. acnes*-induced cytokines. In contrast however, preincubation with chocolate flavonoids primed immune cells to produce higher amounts of proinflammatory cytokines upon secondary stimulation with bacterial ligands such as LPS. The studies that investigated the effects of chocolate on inflammation until now have used standard stimuli of mononuclear cells, such as lipopolysaccharide (LPS) or phytohemaglutinin [4,7,10]. However, these stimuli are less relevant for a well-defined clinical condition such as acne, which is induced mainly by *P. acnes*. Moreover, in vitro studies of the inflammatory reaction cannot be easily extrapolated to explain the effects of chocolate in humans, and therefore in vivo studies are needed. In our study performed in human healthy individuals, volunteers have abstained from chocolate consumption for one week before the start of the study. At the beginning of the study (before chocolate was administered) and after 4 days consumption of 50 g of

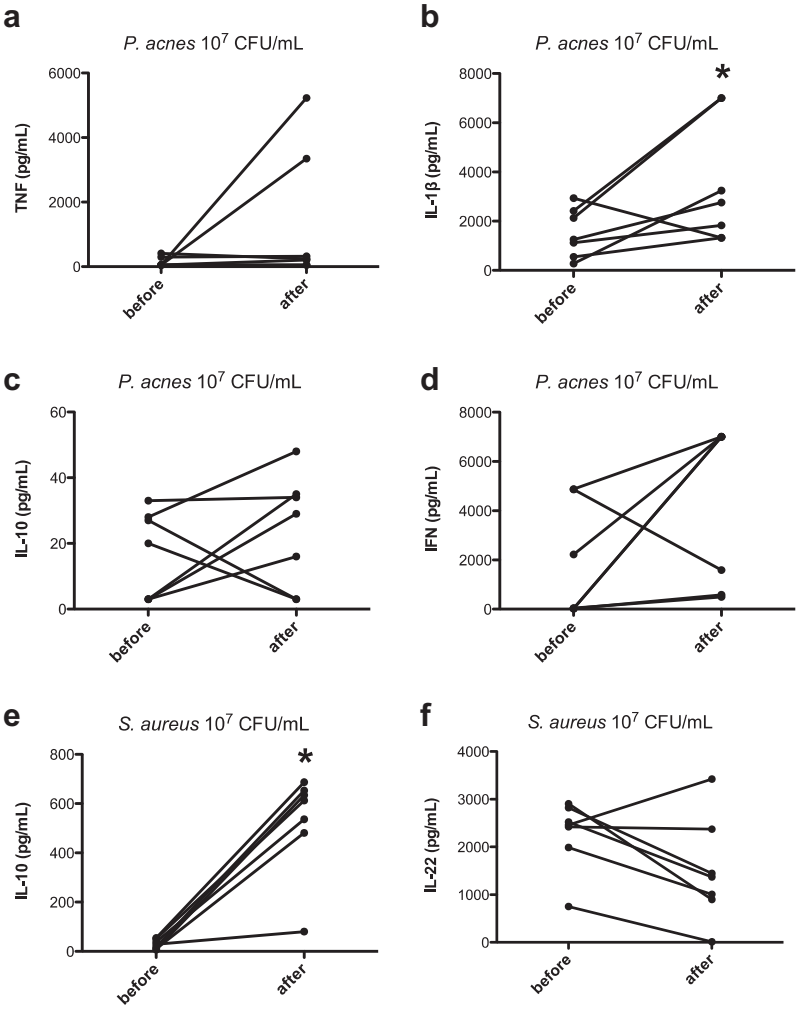

Fig. 2. In vivo effects of chocolate consumption on cytokine production. PBMCs were isolated from 7 human volunteers before and after a 4-day course of 50 g milk chocolate, and stimulated with 1×10^7 colony-forming units (CFU) *P. acnes* (a–d) or *S. aureus* (e and f). After 24 h, the TNFα and IL-1 concentrations were measured in the supernatants (a and b). IL-10 and IFNγ production was assessed after 48 h (c–e), while IL-22 production was assessed after 7 days (f). $^*p < 0.05$ calculated by paired Student *t*-test.

chocolate, PBMCs were isolated from the volunteers and *P. acnes*- and *S. aureus*-induced cytokine production was assessed. Chocolate consumption had clear effects on cytokine production induced by both microorganisms studied. After chocolate consumption, more IL-1β and TNFα was produced when cells were stimulated by *P. acnes*. This may indeed represent one of the mechanisms that could explain the effects of chocolate on acne, as overinflammation is an important component of the pathogenesis of acne. Antibiotic treatment that at the same time has antimicrobial and anti-inflammatory effects (e.g. minocycline) has been demonstrated to be the most effective in this condition [11].

On the other hand, chocolate consumption increased production of IL-10 when cells were stimulated with *S. aureus*, while the synthesis of IL-22 was decreased [12]. Because IL-10 is a modulatory cytokine that can decrease host defense against this microorganism, while IL-22 induces production of defensins from epithelial cells, chocolate consumption may facilitate suprainfection of acne lesions with *S. aureus*, thus worsening the skin lesions and delaying the recovery.

Several questions remain unanswered, and should be evaluated in future studies. One aspect concerns the precise component of chocolate responsible for its in vivo effects. Chocolate flavonoids have been suggested to exert immunomodulatory effects and our studies support this hypothesis. However, significant differences between the in vivo effect of chocolate and the in vitro effects of flavonoids suggest that additional components (e.g. fats, sugars) may be at work as well. Future studies should try to address whether fat-free chocolate would have the same effects as normal chocolate, or whether different types of chocolate (dark, milk, white) would exert similar effects. Another important aspect that remains untouched by the studies to date is to explore the molecular mechanisms responsible for the effects of chocolate at the cellular level.

An important aspect is represented by the potential relevance of our study to the epidemiological data suggesting beneficial effects of chocolate on cardiovascular diseases [13,14]. On the one hand, the increase in TNF and IL-1β production after chocolate consumption may be counterintuitive when considering these effects of chocolate. However, there is only an apparent discrepancy of our study with the data in cardiovascular studies, as one has to consider that the stimulations assays have been done in our study with *S. aureus* and *P. acnes*, stimuli relevant for acne, but not for atherosclerosis. On the other hand, the strong increase in IL-10 production induced by chocolate, albeit after stimulation with a non-related stimulus, may provide a possible explanation for the beneficial effects of chocolate in cardiovascular diseases. More studies are warranted on chocolate effects, using atherosclerosis-relevant stimuli.

In conclusion, in the present study we provide the proof-of-principle that chocolate consumption has the capacity to modulate the immune responses in vivo. This first in vivo investigation of chocolate effects on microbial-induced inflammation not only shows that indirect effects of chocolate on the immune response may partly explain its influence on acne, but also suggest that chocolate may influence antimicrobial host defenses in general. This first set of in vivo data should therefore stimulate further larger studies not only to decipher the mechanisms of chocolate effect on acne, but also its impact on other diseases in which chocolate has been suggested to act as a modulator, such as cardiovascular diseases.

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