**Dark chocolate attenuates intracellular pro-inflammatory reactivity to acute
psychosocial stress in men: a randomized controlled trial**

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Abstract

Flavanol-rich dark chocolate consumption relates to lower risk of cardiovascular mortality, but underlying mechanisms are elusive. We investigated the effect of acute dark chocolate consumption on inflammatory measures before and after stress.

Healthy men, aged 20-50 years, were randomly assigned to a single intake of either 50g of flavanol-rich dark chocolate (n=31) or 50 g of optically identical flavanol-free placebo-chocolate (n=34). Two hours after chocolate intake, both groups underwent the 15-min Trier Social Stress Test. We measured DNA-binding-activity of the pro-inflammatory transcription factor NF- κ B (NF- κ B-BA) in peripheral blood mononuclear cells, as well as plasma and whole blood mRNA-levels of the pro-inflammatory cytokines IL-1 β and IL-6, and the anti-inflammatory cytokine IL-10, prior to chocolate intake before and several times after stress cessation. We also repeatedly measured the flavanol epicatechin and the stress hormones epinephrine and cortisol in plasma and saliva, respectively.

Compared to the placebo-chocolate-group, the dark-chocolate-group revealed a marginal increase in IL-10 mRNA prior to stress ($p=.065$), and a significantly blunted stress reactivity of NF- κ B-BA, IL-1 β mRNA, and IL-6 mRNA ($p's \leq .036$) with higher epicatechin levels relating to lower pro-inflammatory stress reactivity ($p's \leq .033$). Stress hormone changes to stress were controlled. None of the other measures showed a significant chocolate effect ($p's \geq .19$).

Our findings indicate that acute flavanol-rich dark chocolate exerts anti-inflammatory effects both by increasing mRNA expression of the anti-inflammatory cytokine IL-10 and by attenuating the intracellular pro-inflammatory stress response. This mechanism may add to beneficial effects of dark chocolate on cardiovascular health.

Keywords: Dark chocolate, stress reactivity, TSST, inflammation, NF- κ B, cytokines, mRNA, IL-1 β , IL-6, IL-10

1. Introduction

Epidemiological studies provide compelling evidence that consumption of dark chocolate relates to lower risk of cardiovascular mortality due to the chocolate's high content of cocoa flavanols (Di Castelnuovo et al., 2012; Ding et al., 2006; Katz et al., 2011; van Dam et al., 2013). Beneficial effects of dark chocolate or cocoa flavanol administration have been reported on non-inflammatory biological risk factors for cardiovascular disease (CVD) (Di Castelnuovo et al., 2012; Hooper et al., 2008; Ried et al., 2012; Shrime et al., 2011; Tokede et al., 2011) but also on inflammatory CVD risk factors such as pro-inflammatory cytokines (Khan et al., 2014).

In vitro studies showed that human immune cells incubated with cocoa flavanols for 8-72 hours exert anti-inflammatory effects through *inhibiting* mRNA expression and secretion of pro-inflammatory cytokines, including interleukin [IL]-1 β , IL-6, and tumor necrosis factor [TNF]- α (Mao et al., 2000a; Mao et al., 2000c; Mao et al., 2002; Wu et al., 2009) and *stimulating* mRNA expression and secretion of anti-inflammatory cytokines (Mao et al., 2000a; Mao et al., 2000b). A molecular mechanism proposed to underlie anti-inflammatory cocoa flavanol effects involves inhibition of the pro-inflammatory transcription factor nuclear factor- κ B (NF- κ B) at different levels in the activation pathway by flavanols (Fraga and Oteiza, 2011). Notably, activated NF- κ B promotes the expression of numerous genes, including genes encoding the pro-inflammatory cytokines IL-1 β and IL-6 (Pahl, 1999). In contrast to in vitro studies, human in vivo studies examining cocoa flavanol effects on inflammatory measures yielded inconsistent results. While a few studies found decreased plasma levels of inflammatory markers such as IL-1 β (Sarria et al., 2014), IL-6 (Stote et al., 2012), and C-reactive protein (Stote et al., 2012; Tzounis et al., 2011) after regular cocoa product consumption, others did not find significant changes (Di Renzo et al., 2013; Mathur et al., 2002; Monagas et al., 2009; Muniyappa et al., 2008). Notably, the conflicting results

between in vitro and in vivo studies might relate to conjugation of flavanol monomers after absorption, affecting biological activity of flavanols (Thilakarathna and Rupasinghe, 2013). To our knowledge, no human study has investigated effects of acute cocoa flavanol administration on inflammatory plasma cytokines, and only one study explored flavanol effects on inflammatory markers at the intracellular level. In that study, 6h after a single-intake of 40g of cocoa-powder, NF- κ B activation had significantly decreased in peripheral blood mononuclear cells (PBMCs) of healthy participants compared to baseline (Vazquez-Agell et al., 2013).

An additional potential mechanism involved in the link between dark chocolate and CVD risk may relate to psychosocial stress. Psychosocial stress is increasingly recognized as an independent CVD risk factor (Kivimaki et al., 2012; Rosengren et al., 2004; Rozanski, 2014). Chronic but also acute psychosocial stress can trigger acute coronary events (ACS) in vulnerable persons supposedly by increasing immune responses such as inflammatory activity (Steptoe and Brydon, 2009; Steptoe and Kivimaki, 2012, 2013). The inflammatory stress response comprises stress-induced transient increases in circulating inflammatory markers that may promote plaque disruption, thrombus formation, and ventricular electrical instability (Steptoe and Brydon, 2009). Notably, the most reliable inflammatory changes in response to psychosocial stress comprise NF- κ B activation (Bierhaus et al., 2003; Kuebler et al., 2015; Pace et al., 2006; Wolf et al., 2009) and increases of the NF- κ B-regulated pro-inflammatory cytokines IL-1 β and IL-6, both at the mRNA level and in plasma (Kuebler et al., 2015; McInnis et al., 2015; Steptoe et al., 2007b).

Given that acute psychosocial stress is capable of inducing increases in inflammatory activity, and, given the anti-inflammatory effects of cocoa flavanols, flavanol-rich dark chocolate may attenuate the inflammatory stress response. To date, buffering effects of flavanol consumption on physiological responses to acute psychosocial stress in humans have

been shown for endocrine and prothrombotic measures (Steptoe et al., 2007a; von Kanel et al., 2014; Wirtz et al., 2014), but effects on inflammatory stress responses have not yet been investigated.

Therefore, our randomized placebo-controlled study investigates for the first time whether acute dark chocolate consumption affects intra- and extracellular inflammatory responses to acute psychosocial stress in healthy men, and whether potential effects relate to flavanol plasma concentration. Because human studies examining effects of chocolate or cocoa flavanols on inflammatory activity are sparse and show contradictory results (Di Renzo et al., 2013; Mathur et al., 2002; Monagas et al., 2009; Muniyappa et al., 2008; Sarria et al., 2014; Stote et al., 2012; Tzounis et al., 2011), we also explored non-stress effects of acute dark chocolate consumption on intra- and extracellular inflammatory measures. We repeatedly assessed mRNA and plasma levels of the pro-inflammatory cytokines IL-1 β and IL-6 and of the anti-inflammatory cytokine IL-10, in addition to DNA binding-activity of NF- κ B (NF- κ B-BA) up to 120min after stress cessation. We hypothesized that one serving of flavanol-rich dark chocolate would reduce NF- κ B-BA stress reactivity, as well as stress reactivity of mRNA and plasma levels of the pro-inflammatory cytokines IL-1 β and IL-6. We further hypothesized that higher plasma levels of the flavanol epicatechin before stress would relate to lower pro-inflammatory stress reactivity.

2. Material and methods

2.1. Participants

This study is part of larger project assessing the effects of dark chocolate consumption on psychobiological stress reactivity in healthy men (von Kanel et al., 2014; Wirtz et al., 2014). We intentionally recruited 68 healthy, medication-free, non-smoking men (20-50years) who reported to never or only occasionally consume dark chocolate and who did not report frequent consumption of flavanol-containing food or beverages such as black tea, apples, or

red wine. Participants with any self-reported acute or chronic somatic or psychiatric disorder or regular dark chocolate consumption were not eligible for the study. Explicit exclusion criteria were: alcohol abuse and illicit drug use, any heart disease, varicosis, and thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, hypertension, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, HIV, cancer, chronic pain, sleep disturbances, thyroid disease, and current infectious diseases. If the personal history was not conclusive, the participants' primary care physician was contacted for clarification.

The Ethics Committee of the State of Zurich, Switzerland, formally approved the research protocol of our mechanistic study. The study was carried out in accordance with the Declaration of Helsinki principles. Recruitment was carried out with the aid of the Swiss Red Cross of the State of Zurich and through advertisements. All participants provided written informed consent before participating.

2.2. Study design and psychosocial stress procedure

We used a placebo-controlled single-blind between-subjects design. Participants and the TSST panel were blind to the study group assignment of a participant, whereas the study coordinator responsible for the age-matching procedure was not. Eligible participants were assigned to either the experimental dark-chocolate-group or the placebo control group and informed that they would receive dark chocolate with either higher or lower cocoa content. To age-match the two experimental groups a first person was randomly assigned to one group, whereas a second person of similar age (± 3 years) was assigned to the respective other group.

Participants abstained for 48h prior to the experimental session from strenuous physical activity and from consumption of polyphenol containing foods and beverages, including dark chocolate, red wine, black tea, and apples. They also abstained from

consuming milk and milk-containing products as well as caffeinated products and alcohol starting on the evening before the experimental day. Food and drinks other than water were not allowed in the 2h before reporting to the lab.

Participants reported to the lab of the Psychological Institute of the University of Zurich at 9:50am where they received a standardized breakfast consisting of 1 roll, 20g margarine, 30g honey, and water at 10:00h. A venous catheter was inserted into the non-dominant arm at 10:45h followed 45min later by the first blood and saliva sampling with subsequent administration of 50g of dark or placebo-chocolate required to be consumed instantly. Based on previous observations on the kinetics of plasma flavanol increases following oral intake of dark chocolate, participants underwent the psychosocial stressor 2h after study chocolate ingestion, when plasma flavanol levels are expected to peak (Flammer et al., 2007; Rein et al., 2000). We presumed that dark chocolate would induce significantly elevated plasma epicatechin levels during the entire stress experiment because flavanol plasma levels were previously found to be still elevated 6h following oral administration of dark chocolate (Rein et al., 2000). We applied the standard protocol of the widely used Trier Social Stress Test (TSST), which combines a short introduction phase followed by a 5-min preparation phase, a 5-min mock job interview, and a 5-min mental arithmetic task in front of an audience (Kirschbaum et al., 1993). The TSST evokes reliable increases in stress hormones and inflammatory measures (Kirschbaum et al., 1993; Steptoe et al., 2007b). After the TSST, participants remained seated in a quiet room.

Blood samples for NF- κ B binding activity measurements were obtained before chocolate consumption, immediately before the TSST, as well as 10, 60, and 120 min after stress cessation. Blood samples for mRNA gene expression measurements of IL-1 β , IL-6, and IL-10 were obtained before chocolate consumption, immediately before the TSST, as well as 60 and 120 min thereafter. For determination of plasma IL-1 β , IL-6, and IL-10 levels, blood

samples were obtained before chocolate consumption, immediately before the TSST, as well as 60, 90, and 120 min after stress cessation. Blood samples for epicatechin analyses were obtained before chocolate consumption, immediately before the TSST, and 120 min thereafter. For this part of the project, we determined stress increases in epinephrine from blood samples obtained before chocolate consumption, immediately before and after the TSST, and in cortisol from saliva samples collected before chocolate consumption, immediately before and 10 min after the TSST. Blood pressure (BP) was measured under resting conditions immediately before catheter insertion and immediately before chocolate administration by Omron sphygmomanometry and mean arterial blood pressure (MAP) was calculated by the formula $(2/3 \times \text{diastolic BP}) + (1/3 \times \text{systolic BP})$.

At the end of blood and saliva sampling, participants were debriefed and compensated with 175 Swiss Francs for their participation.

2.3. Dark chocolate and placebo-chocolate

The dark chocolate was based on a commercially available dark chocolate (“Noir 72%”, Chocolat Frey) with a 72% cocoa content produced with a batch of carefully processed cocoa powder to keep the polyphenol content the highest possible (approximately 20% over average). One serving of 50g contained 281kcal from 13.5g of sugar and 22.8g of fat with a final epicatechin content of 125mg. The placebo-chocolate was an initially white flavanol-free chocolate that was dyed with food colors in order to match the color and appearance of the dark chocolate. Moreover, the placebo-chocolate was flavored by a nutritional chemist from Chocolat Frey in order to best match the specific slightly bitter taste of the dark chocolate. One serving of 50g of the flavanol-free placebo-chocolate contained 310.5kcal (13g of sugar and 27g of fat) and 0mg epicatechin.

Both chocolate types were produced as 100 g bars that were packed identically into the commercially available “Noir 72%” wrappings. A small hidden letter on the backside of each chocolate wrapping indicated whether the contained bar consisted of dark or placebo chocolate. Both chocolate types looked otherwise optically identical with and without wrapping.

2.4. Biochemical analyses

NF-κB binding-activity to DNA. We used an adapted version of the Nuclear-Extract-Kit (ActiveMotif,LaHulpe,Belgium) in combination with the TransAM NFκB p65 Transcriptionfactor-Assay-Kit (ActiveMotif,LaHulpe,Belgium) as described elsewhere (Kuebler et al., 2015).

Messenger RNA gene expression. Venous blood was drawn in PAXgene Blood RNA Tubes (PreAnalytiX GmbH,Hombrechtikon,Switzerland) and stored at -80°C until analysis. Total RNA purification was performed using the PAXgene-Blood-RNA-Kit (PreAnalytiX GmbH,Hombrechtikon,Switzerland), followed by reverse transcription with HighCapacity RNA-to-cDNA-Kit (AppliedBiosystems,Zug,Switzerland). The quantitative real-time (RT)-PCR was conducted using TaqMan Fast Advanced-MasterMix (AppliedBiosystems, Zug,Switzerland) on a 7900HT Fast Real-Time-PCR-System (AppliedBiosystems). mRNA was measured using the following TaqMan Gene-Expression-Assays (AppliedBiosystems, Zug,Switzerland): Hs01555410_m1 for IL-1β, Hs00985639_m1 for IL-6, Hs00961622_m1 for IL-10, and Hs99999905_m1 for GAPDH. GAPDH was amplified as reference gene. Gene expression relative to the pre-chocolate baseline was calculated by using the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008).

Plasma cytokines. For quantification of the cytokines IL-1β, IL-6, and IL-10, venous blood was drawn in EDTA-coated monovettes (Sarstedt,Numbrecht,Germany), and

immediately centrifuged for 10min at 2000g and 4°C. Obtained plasma was stored at -80°C until analysis. Plasma cytokine levels were determined with a high sensitivity sandwich immunoassay (MesoScaleDiscovery (MSD),Rockville,USA) with intra- and inter-assay coefficients of variance (CVs) of 4.1% and 7.7% for IL-1 β , 4.5% and 7.3% for IL-6, and 3.7% and 10.1% for IL-10. Detection limits were 0.041pg/ml for IL-1 β , 0.117pg/ml for IL-6, and 0.046pg/ml for IL-10. Values below sensitivity were substituted with half the detection limit where appropriate. Notably, IL-1 β levels were very low and without substitution of levels below the detection limit we could only obtain complete and measurable IL-1 β levels in 14 participants from the dark-chocolate-group and in 14 participants from the placebo group. Four participants in the dark-chocolate-group and two participants in the placebo group were without any detectable IL-1 β levels. Therefore, IL-1 β plasma results are to be interpreted with caution.

Plasma epinephrine and plasma epicatechin. Blood was drawn into EDTA-coated monovettes (ethylenediaminetetraacetic acid; Sarstedt, Numbrecht, Germany), and immediately centrifuged for 10 min at 2,000g and 4°C; plasma was stored at -80°C until analysis. Plasma epinephrine, and epicatechin levels were determined by means of high-pressure liquid chromatography (HPLC) and electrochemical detection after liquid-liquid extraction (Ehrenreich et al., 1997; Kotani et al., 2003) in the “Laboratory for Stress Monitoring” (Göttingen, Germany). While epinephrine was quantified after liquid-liquid extraction (Ehrenreich et al., 1997), conjugated epicatechin in plasma was hydrolysed enzymatically using beta glucuronidase and sulfatase, and subsequently extracted with ethyl acetate (Kotani et al., 2003). With this method, epicatechin concentrations reflect the sum of epicatechin metabolites in plasma. The lower limit of detection was 10 pg/ml for epinephrine and 5 ng/ml for epicatechin. Inter- and intra-assay coefficients of variance (CVs) were < 5 %. Undetectable values were replaced by half the lower limit of detection (Hornunga and Reed, 1990).

Salivary cortisol. Saliva samples for the measurement of salivary cortisol levels were collected using Salivette collection devices (Sarstedt, Rommelsdorf, Germany), which were stored at -20°C until analysis in the Biochemical Laboratory, Institute of Psychology, University of Zurich. Thawed saliva samples were centrifuged at 2000 g, yielding low-viscosity saliva. Salivary free cortisol concentrations were determined using a commercial chemiluminescence immunoassay with high sensitivity of 0.16ng/ml (LIA) (IBL Hamburg, Germany). Inter- and intra CVs were below 11.5% and 7.7%, respectively.

2.5. Statistical analyses

Data were analyzed using SPSSInc. version 20.0 for MacOSX (Chicago,IL,USA) and presented as mean $\pm$ SEM. All tests were two-tailed with the significance level set at $p\leq.05$ and the level of borderline significance set at $p\leq.10$.

A-priori sample size calculation using G*Power 3.1 defined an optimal sample size of $n=48$ to detect a conservatively expected small to medium effect size (i.e. $.15\leq f\leq.20$) varying between $f=0.17$ (4 repetitions, mRNA expression) and $f=0.16$ (5 repetitions, NF- κ B-BA/plasma cytokines) in general linear models with repeated measures given two groups with $\alpha=.05$ and a power of .80. (Effect size conventions; small: $f=.10$, medium: $f=.25$, large: $f=.40$).

For epinephrine and cortisol levels, stress change scores were determined by subtracting levels immediately before stress from peak stress response levels (i.e. from immediately before to 1min after stress for epinephrine levels [epinephrine-stress-change] and from immediately before to 10min after stress for cortisol levels [cortisol-stress-change]).

We used univariate analyses of variance (ANOVAs) to compare groups on subjects' characteristics and epicatechin and stress hormone baseline levels; univariate analyses of

covariance (ANCOVAs) were applied to test for group differences in stress hormone changes, covarying for levels determined before chocolate consumption (cf. Table 1).

To address whether chocolate consumption affects inflammatory measures, we used a three-step procedure. We first tested (step 1) for non-stress effects of dark chocolate consumption on inflammatory measures by calculating general linear models for repeated measures with group (dark chocolate vs. placebo-chocolate) as the independent variable and the two time points prior to stress as repeated dependent variables. Next (step 2), using general linear models for repeated measures, we tested whether the TSST induced significant inflammatory responses across both study groups and the five, four, and five time points, respectively, at which NF- κ B-BA, mRNA levels, and plasma cytokine levels were repeatedly measured. In the main analyses (step 3), we additionally entered group (dark chocolate vs. placebo chocolate) as the independent variable to test whether dark chocolate consumption relates to attenuated pro-inflammatory stress responses. Post hoc testing of significant effects from the main analyses comprised (1) re-analyses of the general linear models of the main analyses separately for each study group, and (2) AN(C)OVAs to test for group differences at each time point. Notably, we used non-collapsed and non-transformed NF- κ B-BA and mRNA data for post hoc testing for group differences at the time point before chocolate consumption. The cardiovascular risk factors age, BMI, and MAP were entered as a-priori defined potential confounders in all repeated measures group comparisons (Wirtz et al., 2008; Wirtz et al., 2004a; Wolf et al., 2009).

To test whether epicatechin levels before stress relate to changes in inflammatory measures prior to stress and/or to changes in inflammatory stress reactivity, we recalculated the above general linear models with repeated measures, whereby entering plasma epicatechin levels assessed immediately before stress as the independent variable instead of group. Next, we tested whether the hypothesized association between higher epicatechin levels and

attenuated inflammatory stress responses would be influenced by dark chocolate-induced alterations in epinephrine and cortisol stress reactivity by entering stress-induced epinephrine and cortisol change scores as covariates. We did so because we previously found dark chocolate to relate to lower epinephrine and cortisol stress reactivity, but not to norepinephrine stress reactivity (Wirtz et al., 2014).

3. Results

3.1. Participant flow

Of the total of the 68 recruited participants, 65-completed the study. Of these 31 had been randomized to the dark-chocolate-group and 34 to the placebo-chocolate-group. Notably, based on a power analysis (see statistical analyses) and for reasons of limited funding, we measured intracellular variables (i.e. NF- κ B binding-activity and cytokine mRNA expression) in 48 out of 65 participants (i.e. 24-participants in each experimental group) starting after implementation of the respective assay. Due to technical problems with blood sampling and assaying, some data points were missing, yielding the following sample sizes for the respective measures (chocolate group vs. placebo group): NF- κ B binding-activity, 23vs.22; IL-1 β mRNA, 24vs.24; IL-6 mRNA, 24vs.19; IL-10 mRNA, 23vs.20; plasma IL-1 β , 30vs.33; plasma IL-6, 30vs.34; plasma IL-10, 30vs.34; plasma epicatechin, 30vs.34, plasma epinephrine, 31vs.33, salivary cortisol 31vs.34. In 35 participants (20vs.15) all major outcomes could be measured.

3.2. Group characteristics

Table 1 depicts the characteristics of the two study groups. As reported previously (von Kanel et al., 2014; Wirtz et al., 2014), plasma epicatechin levels before and 120min after stress were significantly higher in the dark-chocolate-group compared to the placebo-

chocolate-group. Epicatechin levels before dark chocolate consumption and epicatechin levels in the placebo-chocolate-group were below the detection limit of 5ng/ml. The groups did not significantly differ in age, BMI, and MAP.

3.3. Non-stress effects of chocolate consumption on inflammatory measures

Step 1: As compared to the placebo-chocolate-group, the dark-chocolate-group revealed a marginal increase in mRNA expression of the anti-inflammatory cytokine IL-10 from baseline to 2h after chocolate consumption, both without (interaction group-by-time $F[1.0/41.0]=3.66, p=.063, f=0.30$; Fig.1D) and with controlling for age, BMI, and MAP (interaction group-by-time $F[1.0/38.0]=3.61, p=.065, f=0.31$). There were no significant group differences in other inflammatory measures (interactions group-by-time: $p's \geq .19$). Controlling for the cardiovascular risk factors age, BMI, and MAP did not change these results (interactions group-by-time: $p's \geq .20$).

3.4. Stress reactivity of inflammatory measures in the dark-chocolate-group and the placebo-chocolate-group

Step 2: Across both study groups, we observed significant increases over time in all inflammatory parameters except IL-6 mRNA (main effects of time: NF- κ B-BA: $F[3.5/146.8]=3.07, p=.023, f=0.27$; IL-1 β mRNA: $F[2.5/116.4]=5.80, p=.002, f=0.35$; IL-10 mRNA: $F[2.7/113.7]=4.18, p=.010, f=0.32$); plasma IL-1 β : $F[3.3/201.8]=3.11, p=.024, f=0.22$; plasma IL-6: $F[1.8/112.0]=31.86, p<.001, f=0.71$); plasma IL-10: $F[2.3/142.5]=4.21, p=.013, f=0.26$; IL-6 mRNA: $p=.11$).

Step 3: The dark-chocolate-group revealed a significantly blunted stress reactivity of all pro-inflammatory cytoplasmic measures under study, i.e. NF- κ B-BA, IL-1 β mRNA, and IL-6 mRNA as compared to the placebo-chocolate-group (interactions group-by-time: NF-

κB-BA: $F[3.6/142.9]=3.10, p=.021, f=0.28$, Fig.1A; IL-1β mRNA: $F[2.7/124.7]=4.51, p=.006, f=0.31$, Fig.1B; IL-6 mRNA: $F[1.8/73.0]=3.71, p=.034, f=0.30$, Fig.1C). Controlling for age, BMI, and MAP did not change results (interactions group-by-time: NF-κB-BA: $F[3.9/142.5]=2.90, p=.026, f=0.28$; IL-1β mRNA: $F[2.9/123.2]=4.14, p=.009, f=0.31$; IL-6 mRNA: $F[2.0/76.0]=3.19, p=.047, f=0.29$). There were no significant group differences in stress reactivity of the anti-inflammatory cytoplasmic measure IL-10 mRNA and the plasma cytokines IL-1β, IL-6, and IL-10 (Table 2), both without ($p's \geq .23$) and with controlling for age, BMI, and MAP ($p's \geq .24$).

Post hoc testing of significant interaction effects revealed stress-induced increases in NF-κB-BA and IL-1β mRNA in the placebo-chocolate-group (main effects of time: NF-κB-BA: $F[3.4/63.64]=5.04, p=.002, f=0.51$; IL-1β mRNA: $F[2.6/58.8]=7.18, p=.001, f=0.56$) but not in the dark-chocolate-group ($p's \geq .39$). In terms of IL-6 mRNA, stress-induced increases were of borderline significance in the placebo-chocolate-group (main effect time IL-6 mRNA: $F[1.5/27.0]=3.3, p=.065, f=0.43$), and non-significant in the dark-chocolate-group ($p=.77$). As compared to the placebo-chocolate-group, the dark-chocolate-group showed significantly lower NF-κB-BA at 1min after stress ($F[3.4/41.0]=6.00, p=.019$), lower IL-1β mRNA levels at 60 min ($F[1.0/46.0]=5.49, p=.024$) and 120 min after stress ($F[1.0/46.0]=8.19, p=.006$), and lower IL-6 mRNA levels at 120 min after stress ($F[1.0/41.0]=4.89, p=.033$). Group differences in IL-6 mRNA levels at 60 min after stress were of borderline significance ($F[1.0/41.0]=3.88, p=.056$). The groups did not significantly differ from one another at any other time point including the time-point before chocolate consumption ($p's > .23$).

3.5. Associations between epicatechin levels assessed immediately before stress and inflammatory measures before stress

Epicatechin levels (assessed as the sum of epicatechin metabolites in plasma) prior to stress were not significantly associated with any inflammatory measure under study across both study groups ($p > .21$).

3.6. Associations between epicatechin levels assessed immediately before stress and subsequent inflammatory stress reactivity

In line with the previous group differences, higher epicatechin levels (assessed as the sum of epicatechin metabolites in plasma) prior to stress significantly related to lower stress reactivity of NF- κ B-BA, IL-1 β mRNA, and IL-6 mRNA across both study groups (interactions epicatechin-by-stress: NF- κ B-BA: $F[3.6/144.6]=2.94, p=.027, f=0.27$; IL-1 β mRNA: $F[2.7/121.2]=3.3, p=.027, f=0.27$; IL-6 mRNA: $F[1.8/71.7]=4.10, p=.024, f=0.32$). There were no significant associations between epicatechin levels and stress reactivity of IL-10 mRNA ($p=.74$), and plasma cytokines (IL-1 β : $p=.29$, IL-6: $p=.37$, IL-10: $p=.68$).

Controlling for epinephrine and cortisol stress change scores did not significantly change the interaction of epicatechin levels with stress reactivity of NF- κ B-BA ($p=.035$) and of IL-1 β mRNA ($p=.015$), whereas stress reactivity of IL-6 mRNA failed to reach significance ($p=.14$).

4. Discussion

This is the first human study to investigate the effects of acute flavanol-rich dark chocolate intake on the intra- and extracellular inflammatory response to acute psychosocial stress. In addition, we explored effects of acute dark chocolate intake prior to stress induction on intra- and extracellular inflammatory measures.

4.1. Stress reactivity effects of chocolate consumption

Regarding stress reactivity, we found a significantly blunted reactivity of both NF- κ B-BA in PBMCs and whole-blood mRNA expression of the pro-inflammatory cytokines IL-1 β and IL-6 after dark chocolate intake as compared to placebo-chocolate intake. Higher plasma levels of the cocoa flavanol epicatechin, which was determined as the sum of epicatechin metabolites, were directly related to lower stress reactivity of these pro-inflammatory measures. The dark chocolate effects were of medium to large effect size, independent of age, BMI, and MAP, and not due to differences in baseline levels because study groups did not significantly differ in NF- κ B-BA or mRNA expression levels at baseline (p 's>.53; data not shown). In contrast, we did not find stress reactivity of plasma cytokines or mRNA expression of the anti-inflammatory cytokine IL-10 to be significantly affected by dark chocolate consumption. These correlative findings suggest a direct linear inhibitory effect of cocoa flavanols on stress reactivity of pro- but not anti-inflammatory intracellular but not extracellular measures. Notably, application of different cocoa flavanol concentrations would have been necessary to prove this speculation. Given the proposed role of the pro-inflammatory stress response in mediating the link between psychosocial stress and increased CVD risk (Steptoe and Kivimaki, 2012, 2013), our findings may add to the current knowledge of mechanisms underlying beneficial effects of dark chocolate on cardiovascular health.

Our findings are generally in line with *in vitro* studies reporting anti-inflammatory effects of cocoa flavanols (Mao et al., 2000a; Mao et al., 2000c; Mao et al., 2002) including inhibitory effects of cocoa flavanols on pro-inflammatory cytokine expression in mitogen- and glucose-stimulated human immune cells (Mao et al., 2000a; Mao et al., 2000c; Wu et al., 2009). However, flavanol effects on inflammatory measures upon stimulation by psychological stress have not yet been investigated, either in *in vitro* stress simulations or in animal or human studies. Given our findings, the previously reported anti-inflammatory cocoa flavanol effects seem to be extendable to psychosocial stress or more precisely, to de novo synthesis of inflammatory responses to stress. However, it should be acknowledged that the

temporal relation between stress-induced NF- κ B activation and related mRNA level increases on the one hand and the resulting de novo protein synthesis and ultimate release into circulation on the other hand is hitherto unknown in humans and needs further scrutiny.

We propose the following mechanism to explain our inhibitory dark chocolate effects on inflammatory stress reactivity: Based on in vitro studies demonstrating that flavanols including epicatechin are capable of inhibiting NF- κ B at different levels in the activation pathway (Fraga and Oteiza, 2011) dark chocolate consumption seems to directly inhibit NF- κ B-BA. More precisely, flavanols have been shown to inhibit NF- κ B activation by preventing both (1) degradation of the NF- κ B inhibitor I κ B α (and thus transition from inactive to active NF- κ B) through anti-oxidative actions and (2) binding of NF- κ B to DNA through direct interaction with active NF- κ B (Fraga and Oteiza, 2011; Mackenzie et al., 2004). We speculate that these mechanisms result in the observed attenuated increase in NF- κ B-BA in reaction to stress in the dark-chocolate-group. Considering that NF- κ B-BA subsequently promotes gene expression of IL-1 β and IL-6 (Pahl, 1999) probably also in reaction to activation by stress (Kuebler et al., 2015), we interpret that inhibitory flavanol effects on NF- κ B-BA stress reactivity are likely to mediate the observed lower stress reactivity of IL-1 β and IL-6 mRNA expression in our study.

In contrast to the intracellular inflammatory measures under study, we could not find a corresponding inhibiting flavanol effect on plasma cytokines. We offer three possible, although admittedly speculative biological mechanisms that might underly the missing associations between mRNA and plasma cytokine levels. First, the observed cocoa-effects on stress induced changes in the intracellular measures under study might have been too weak to manifest in corresponding changes in plasma cytokine levels. Second, cocoa flavanols might not affect stress-induced increases in plasma cytokines that do not result from de novo synthesis. This corresponds with our previous study - having demonstrated for the first time in

humans - that stress-induced increases in pro-inflammatory plasma cytokines, within 120 min post stress, are unlikely to derive from de novo synthesis based on stress-induced NF- κ B activation (Kuebler et al., 2015). Third, given our previous in-vitro study findings in human whole blood samples, where we found a substantial increase in plasma cytokine levels following a 6 h incubation period with a supra-physiological stimulus (Wirtz et al., 2004a; Wirtz et al., 2004b, c; Wirtz et al., 2004d; Wirtz et al., 2003), and given our limited post-stress interval of 120 min, we cannot rule out that the observed cocoa-effects on stress-induced changes in the intracellular measures under study may eventually result in corresponding plasma cytokine effects later than 120 min post stress. Notably, in the absence of any trend in this direction in our plasma data, the latter explanation might be considered speculative and possibly unjustified. Future larger scale studies are needed to clarify the biological mechanisms underlying the missing associations between mRNA and plasma cytokine levels and to rule out the probability of type II error; i.e., to falsely not detect true buffering effects of acute dark chocolate intake on stress reactivity of pro-inflammatory plasma cytokines.

4.2. Effects of chocolate consumption prior to stress induction

In terms of inflammatory activity prior to stress induction, we found that dark chocolate intake related to a marginally significant increase in mRNA levels of the anti-inflammatory cytokine IL-10 as compared to placebo-chocolate intake. This finding corroborates evidence from previous in vitro experiments with human immune cells indicating that anti-inflammatory actions of dark chocolate are partly due to its stimulatory effects on mRNA expression of anti-inflammatory cytokines (Mao et al., 2000a; Mao et al., 2000b). Interestingly, we did not observe an association between plasma epicatechin and IL-10 mRNA-levels suggesting a non-linear relationship between cocoa flavanols and IL-10

mRNA expression in circulating blood cells. With respect to potential underlying mechanisms, we speculate that extracellular signal-regulated kinase (ERK) or the transcription factors signal transducer and activator transcription (STAT)-6 and -3 might be involved (Ramiro-Puig and Castell, 2009; Saraiva and O'Garra, 2010). In contrast to IL-10 mRNA, none of the other inflammatory measures were affected by dark chocolate consumption prior to stress contradicting the only hitherto published study reporting an inhibitory effect of cocoa flavanols on the activation of NF- κ B (Vazquez-Agell et al., 2013). Several methodological reasons may contribute to the apparent inconsistency, including post-consumption monitoring period (2hvs.6h), measurement of NF- κ B activity (binding-activity-to-DNA vs. phosphorylation of p65), gender ratio (men/women: 45/0vs.9/9), and cocoa product (dark chocolate vs. cocoa powder with water).

4.3. Strengths and limitations of the study

Our study has several strengths including the randomized-placebo controlled experimental design with its unique placebo chocolate for a best possible match to the experimental dark chocolate, and the use of a well-validated standardized acute psychosocial stress protocol. Moreover, we enrolled participants with a broad age range and matched on age. However, our study has also its limitations. First, the generalizability of our findings might be limited to healthy men, acute psychosocial stress, and the cytokines IL-1 β , IL-6, and IL-10. It remains to be elucidated whether the anti-inflammatory effects of dark chocolate intake also apply to women, cardiovascular patients, chronic psychosocial stress exposure, and other cytokines. Second, because of the relatively small sample size our data should be interpreted with caution until confirmed in larger studies. Third, the clinical relevance of the observed intracellular anti-inflammatory effects of acute dark chocolate consumption regarding cardiovascular health remains to be elucidated. Fourth, potential effects of regular chocolate

consumption over a longer period of time on intracellular inflammatory activity and stress reactivity remain unclear and should be addressed in future studies. Fifth, we cannot rule out the possibility that changes in immune cell subtypes due to stress or other (non-) immunogenic factors may have influenced our cytokine mRNA results. However, given the kinetics of stress-induced leukocyte subset redistribution, which seems to disappear within minutes after stress cessation (e.g. Buske-Kirschbaum et al., 2007), and given non-significant group differences in leukocyte and neutrophil counts in whole blood over time (data not shown), we consider it unlikely that changes in leukocyte subpopulations over time significantly confounded the observed group differences in mRNA levels.

4.4. Conclusion

In sum, our findings indicate that acute flavanol-rich dark chocolate exerts anti-inflammatory effects both by increasing mRNA expression of the anti-inflammatory cytokine IL-10 and by attenuating stress reactivity of the pro-inflammatory transcription factor NF- κ B-BA and of the pro-inflammatory cytokines IL-1 β and IL-6 at mRNA-levels. Future studies are needed to replicate our findings in other samples and settings, and to investigate their clinical relevance.

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Figure legends

Figure 1. A to D. NF- κ B binding-activity (NF- κ B-BA; A), IL-1 β mRNA (B), IL-6 mRNA (C), and IL-10 mRNA (D) reactivity to psychosocial stress (Trier Social Stress Test, TSST) in the dark-chocolate-group and in the placebo-chocolate-group. Values are means $\pm$ SEM; asterisks indicate significant group differences (*, $p < .05$).

Figures 1. A to D.

A

B

C **D**

Table 1. Participant characteristics by study group

	Dark-chocolate (n=31)	Placebo-chocolate (n=34)	P-value
Age (years)	34.5±1.6 (21-50)	36.8±1.5 (20-50)	.30
Body-mass-index (kg/m ²)	25.0±0.8 (19.7-39.8)	25.2±0.7 (18.7-36.0)	.84
Mean-arterial-blood pressure (mmHg)	89.6±1.8 (72.2-117.3)	91.3±1.6 (75.8-112.3)	.48
Epicatechin before stress and after chocolate intake (ng/ml)	40.5±2.9 (16.7-77.2)	<5	<.001
Epicatechin 120min post stress (ng/ml)	16.7±1.1 (8.8±30.6)	<5	<.001
Epinephrine-baseline (pg/ml)	26.6±3.6 (3.0-90.4)	19.9±2.1 (3.0-54.6)	.33
Epinephrine-stress- change (pg/ml)	28.7±4.9 (-13.0-104.5)	40.5±6.0 (5.3-157.5)	.035
Cortisol-baseline (pg/ml)	10.1±1.5 (2.3-33.0)	9.9±1.2 (2.2-32.2)	.90
Cortisol-stress-change (nmol/l)	9.1±1.2 (-2.3-21.8)	14.9±1.4 (0.1-35.3)	.004

Values are given as means±SEM (range)

Table 2. Cytokine plasma levels assessed at five time points

	Before chocolate consumption	Immediately before stress	60 min after stress	90 min after stress	120 min after stress
Interleukin-1 β (pg/ml)					
Dark chocolate (n=30)	0.05 $\pm$ 0.007	0.06 $\pm$ 0.007	0.06 $\pm$ 0.008	0.06 $\pm$ 0.007	0.07 $\pm$ 0.009
Placebo chocolate (n=33)	0.06 $\pm$ 0.007	0.06 $\pm$ 0.006	0.06 $\pm$ 0.006	0.08 $\pm$ 0.012	0.07 $\pm$ 0.008
Interleukin-6 (pg/ml)					
Dark chocolate (n=30)	0.30 $\pm$ 0.07	0.70 $\pm$ 0.13	1.13 $\pm$ 0.19	1.14 $\pm$ 0.27	1.48 $\pm$ 0.26
Placebo chocolate (n=34)	0.23 $\pm$ 0.04	0.89 $\pm$ 0.18	1.54 $\pm$ 0.29	1.89 $\pm$ 0.40	1.61 $\pm$ 0.29
Interleukin-10 (pg/ml)					
Dark chocolate (n=30)	0.19 $\pm$ 0.22	0.22 $\pm$ 0.39	0.34 $\pm$ 0.09	0.26 $\pm$ 0.05	0.21 $\pm$ 0.04
Placebo chocolate (n=34)	0.16 $\pm$ 0.01	0.28 $\pm$ 0.07	0.36 $\pm$ 0.09	0.31 $\pm$ 0.09	0.29 $\pm$ 0.09

Note. Values are given as means $\pm$ SEM.