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Food additives such as sodium sulphite, sodium benzoate and curcumin inhibit leptin release in lipopolysaccharide-treated murine adipocytes *in vitro*

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Abstract

Obesity leads to the activation of pro-inflammatory pathways, resulting in a state of low-grade inflammation. Recently, several studies have shown that the exposure to lipopolysaccharide (LPS) could initiate and maintain a chronic state of low-grade inflammation in obese people. As the daily intake of food additives has increased substantially, the aim of the present study was to investigate a potential influence of food additives on the release of leptin, IL-6 and nitrite in the presence of LPS in murine adipocytes. Leptin, IL-6 and nitrite concentrations were analysed in the supernatants of murine 3T3-L1 adipocytes after co-incubation with LPS and the food preservatives, sodium sulphite (SS), sodium benzoate (SB) and the spice and colourant, curcumin, for 24 h. In addition, the kinetics of leptin secretion was analysed. A significant and dose-dependent decrease in leptin was observed after incubating the cells with SB and curcumin for 12 and 24 h, whereas SS decreased leptin concentrations after 24 h of treatment. Moreover, SS increased, while curcumin decreased LPS-stimulated secretion of IL-6, whereas SB had no such effect. None of the compounds that were investigated influenced nitrite production. The food additives SS, SB and curcumin affect the leptin release after co-incubation with LPS from cultured adipocytes in a dose- and time-dependent manner. Decreased leptin release during the consumption of nutrition-derived food additives could decrease the amount of circulating leptin to which the central nervous system is exposed and may therefore contribute to an obesogenic environment.

Key words: Adipocytes: Adipokines: Antioxidants: Leptin

Changes in lifestyle including overnutrition and physical inactivity have led to a rise of excess body weight during the last decades. Severe obesity is the sixth most important risk factor contributing to the overall burden of diseases worldwide due to the main adverse consequences such as CVD, metabolic disorders and several types of cancer[1]. It is well recognised that obesity is associated with a state of chronic inflammation due to different pathogenic mechanisms[2]. Lipid accumulation leads to adipocyte hypertrophy, cellular stress, increased lipolysis and activation of pro-inflammatory pathways, resulting in an increased production and secretion of pro-inflammatory cytokines by adipocytes and macrophages[3]. Moreover, new evidence supports the idea that some key aspects of the mammalian host–gut microbial relationship could play a major role in obesity. LPS is continuously produced within the gut by the death of Gram-negative bacteria and is absorbed into the intestinal capillaries to be transported by lipoproteins leading to a state of metabolic endotoxaemia, which seems to be a potential pathway to initiating and maintaining a state of low-grade inflammation associated with obesity[4–6].

Among the inflammation-related cytokines, adipocytes secrete various adipokines, which play a major role in energy homeostasis by exerting multiple favourable effects on lipid and carbohydrate metabolism[7]. It is recognised that these beneficial effects are removed in states of severe obesity. Leptin, which is produced in proportion to fat stores, plays a crucial role in the regulation of appetite, food intake and energy homeostasis by signalling the information of available energy within the central nervous system[8–10]. Various studies have investigated a potential effect of food composition on circulating leptin levels over the last years[11–13]. However, studies analysing the influence of food preservatives, which are widely used to preserve aliments, are scarce.

The addition of food preservatives such as antioxidants and food colourants prevents the growth of bacteria, fungi and

Abbreviations: LPS, lipopolysaccharide; SS, sodium sulphite; SB, sodium benzoate.

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other micro-organisms. Furthermore, these additives decelerate oxidation of fats preventing rancidity, and inhibit ageing and discolouration of food. The daily intake of food has increased substantially and is responsible for the dramatic rise of the prevalence of obesity inducing metabolic disorders such as insulin resistance, diabetes mellitus, inflammation and blood lipid disorders[1,14]. As a consequence of increased food ingestion, the intake of antioxidant food supplements has increased exponentially.

The aim of the present study was to analyse the effect of the widely used food preservatives sodium sulphite (E221, SS), sodium benzoate (E211, SB) and the spice and food colourant curcumin (E100) on the leptin release of unstimulated and LPS-stimulated adipocytes, in order to investigate a potential contribution of these diet-derived agents to the development of obesity-related metabolic perturbations.

Experimental methods

Cell culture

Murine 3T3-L1 fibroblasts cells were obtained from the American Type Culture Collection (ATCC-CL-173; Manassas, VA, USA) and cultured in 5% CO_2 at 37°C. The cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Karlsruhe, Germany) supplemented with 5 mM-glucose, 10% heat-inactivated bovine serum, 2 mM-L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every 2 d. At 2 d after reaching confluence, the pre-adipocytes were treated with a medium to induce differentiation as described previously[15]. In short, 2 d post-confluence cultured cells were supplemented with 0·5 mM-1-methyl-3-isobutylmethylxanthine + 1·0 mM-dexamethasone + 10 μg/ml insulin + 10% fetal bovine serum for another 2 d. Then the cells were kept in a culture medium with 10 μg/ml insulin + 10% fetal bovine serum for another 2 d. After differentiation, the culture medium was replaced every 2nd day using a culture medium with 10% fetal bovine serum. As determined by light microscopy, over 90% of the cells that were used for the experiments were differentiated. All experiments were performed within 10–14 d post-differentiation.

Experimental procedures

First, the adipocytes were incubated with increasing concentrations of SS, SB and curcumin for 24 h to analyse a possible dose-dependent effect on leptin secretion. Accordingly, the cells were washed twice with PBS and incubated with 1 mM- and 10 mM-SS, 10 mM- and 20 mM-SB, or 10 μM- and 50 μM-curcumin.

After the incubation of cells with food additives, we stimulated the adipocytes with 1 μg/ml LPS (Sigma-Aldrich, Munich, Germany) and incubated for 24 h before the measurement of leptin for the dose–response experiments. To assess the effect of LPS on the secretion of pro-inflammatory cytokines, IL-6 concentrations were measured in the cell culture supernatant. Furthermore, the concentration of the nitrite in the cell culture supernatant was determined, which represents a stable end product of nitrite oxide and thus an estimate of NO synthase activity.

Finally, time–course experiments were conducted using 1 mM-SS, 10 mM-SB and 50 μM-curcumin. We incubated the cells for 6, 12 and 24 h with and without LPS, and measured the leptin levels in the supernatant. All the experiments were replicated three times in triplicates.

Cell viability

To measure the possible effects of the food additives or LPS on the viability of 3T3-L1 adipocytes during the experiments, we used the lactate dehydrogenase release assay. Lactate dehydrogenase was measured in the cell culture supernatant by using an autoanalyser (ABX-Cobas Mira; Roche Diagnostic, Mannheim, Germany) at the beginning and at the end of all incubations according to the manufacturer's instructions. Lactate dehydrogenase is an ubiquitous, intracellular-located enzyme, which is detectable in the cell culture supernatant only after cell lysis due to cytotoxicity.

Analysis of leptin secretion

Leptin concentrations were measured in the cell culture supernatant using an ELISA kit for mouse leptin from Research and Diagnostic Systems (Quantikine® M Murine; R&D Systems, Minneapolis, MN, USA; inter-assay CV% <5; intra-assay CV% $<3{\cdot}8$).

Analysis of IL-6 secretion

IL-6 concentrations were determined in the cell culture supernatant using an ELISA kit for mouse IL-6 (Quantikine® Mouse IL-6; R&D Systems; inter-assay CV% $<7{\cdot}6$; intra-assay CV% $<3{\cdot}5$).

Analysis of nitrite

Nitrite concentrations in cellular supernatants were determined photometrically using the Griess reaction. Thereby, nitrite in the samples was quantitatively converted to a diazonium salt, which was then coupled with *N*(1-naphthyl) ethylenediamine dihydrochloride, forming an azo dye that was read at 540 nm in a spectrophotometer.

Statistical analysis

Data are expressed as means and standard deviations unless otherwise indicated. Normality of data was assessed using the Shapiro–Wilk test. Differences between groups were analysed by one-way ANOVA. *Post hoc* adjustment for multiple comparisons was applied according to the method of Bonferroni.

For the comparison of mean values within the groups during the time–course experiment, one-way ANOVA for repeated measures was used. In the case of significant differences, Bonferroni's *post hoc* tests were performed. The

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Greenhouse–Geisser correction was applied when indicated by Mauchly's test for sphericity. P values $\leq 0{\cdot}05$ were considered statistically significant.

All analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

Results

Effect of lipopolysaccharide and toxicity tests

At 10 d after the initiation of differentiation of murine 3T3-L1 fibroblasts into adipocytes, the cells secreted a significant amount of leptin into the cell culture supernatant. In all the experiments, we used untreated cells as a negative control and LPS (1 μg/ml)-stimulated cells as a positive control. The treatment of adipocytes with LPS for 24 h led to a significant diminution of leptin concentrations by approximately 30 % and a significant increase in IL-6 secretion by nearly 800 % compared with control cells in the absence of LPS. By the measurements of lactate dehydrogenase in the cell culture supernatants at the beginning and at the end of all incubation procedures, we did not observe any influence of LPS or the food additives on cell viability (data not shown).

First, we analysed the release of leptin, IL-6 and nitrite after the treatment of unstimulated and LPS-stimulated adipocytes with SS, SB and curcumin at different concentrations for 24 h. The incubation of cells with food additives in the absence of LPS did not affect leptin levels in the supernatants of adipocyte cultures (data not shown).

The antioxidant, sodium sulphite, reduces leptin release after co-incubation with lipopolysaccharide

Co-incubation of cells with LPS and 1 mM-SS induced a stronger decrease in leptin concentrations by 30 % compared with the LPS-stimulated control in the absence of this food additive ($P \leq 0{\cdot}001$; Fig. 1(a)). Increasing the SS concentration to 10 mM did not further decrease leptin concentrations significantly. However, a significant increase of 57 % of IL-6 was found by augmenting the concentration of SS ($P < 0{\cdot}01$; Fig. 1(b)). With regard to nitrite formation, we found no effect after treatment with SS (Fig. 1(c)).

Sodium benzoate decreases leptin release after co-incubation with lipopolysaccharide

Co-treatment of LPS-stimulated adipocytes with 10 mM-SB decreased leptin levels by 49 % ($P < 0{\cdot}001$; Fig. 2(a)), which was even more pronounced by increasing the concentration of SB to 20 mM (−70 %; $P < 0{\cdot}001$; Fig. 2(a)). No significant effects could be detected on IL-6 and nitrite concentrations after the incubation of LPS-stimulated cells with SB (Fig. 2(b) and (c)).

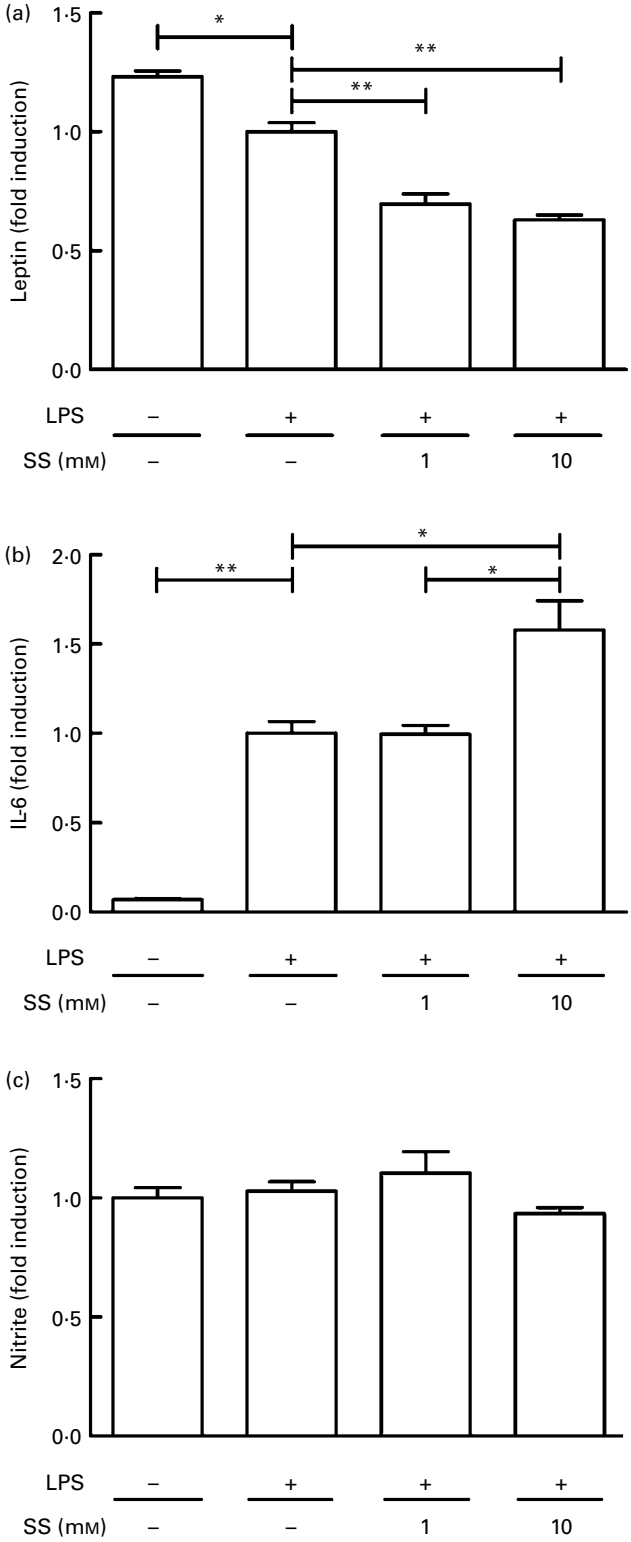

Fig. 1. Influence of sodium sulphite (SS) in lipopolysaccharide (LPS; 1 μg/ml)-treated 3T3-L1 cells on (a) leptin secretion, (b) IL-6 release and (c) NO formation after 24 h of treatment. The control conditions in the absence or presence of 1 μg/ml LPS are shown. Fold induction was related to the control group with LPS in the absence of SS. All experiments were performed in triplicates. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different as assessed by one-way ANOVA with Bonferroni's adjustment: * $P \leq 0{\cdot}01$ and ** $P \leq 0{\cdot}001$.

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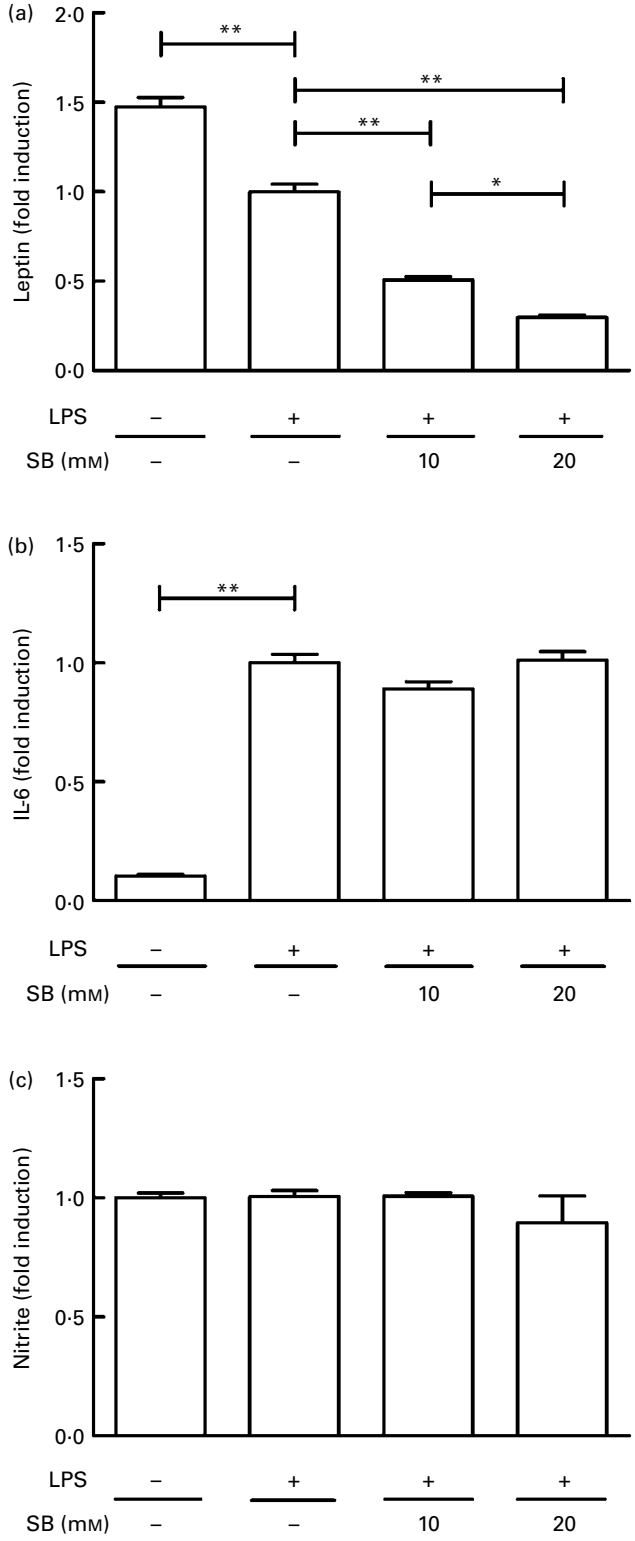

Fig. 2. Influence of sodium benzoate (SB) in lipopolysaccharide (LPS; 1 μg/ml)-treated 3T3-L1 cells on (a) leptin secretion, (b) IL-6 release and (c) NO formation after 24 h of treatment. The control conditions in the absence or presence of 1 μg/ml LPS are shown. Fold induction was related to the control group with LPS in the absence of SB. All experiments were performed in triplicates. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different as assessed by one-way ANOVA with Bonferroni's adjustment: * $P \leq 0{\cdot}01$ and ** $P \leq 0{\cdot}001$.

The food colourant and antioxidant curcumin decreases leptin secretion after co-incubation with lipopolysaccharide

A significant decrease in leptin release by 18 % was detected after the incubation of the cells with 10 μM-curcumin ($P < 0{\cdot}01$; Fig. 3(a)), which was further suppressed with 50 μM by 87 % ($P < 0{\cdot}001$; Fig. 3(a)). In contrast to SS and SB, co-treatment of LPS-stimulated adipocytes with 50 μM-curcumin significantly decreased IL-6 concentrations by 57 % in the supernatant of the cells (Fig. 3(b)). Again, no effect of curcumin was detected on the formation of nitrite (Fig. 3(c)).

Antioxidants influence leptin secretion over time

Next, we investigated the effect of food additives on leptin secretion into the cell culture supernatant as a function of time (Fig. 4). In unstimulated cells, leptin concentrations rose from 49·6 (SD 28·3) pg/ml after 6 h to 185·7 (SD 55·1) pg/ml after 12 h, and to 854·4 (SD 203) pg/ml after 24 h of treatment (all $P < 0{\cdot}001$ *v.* 6 h). Incubation of the cells with LPS diminished leptin levels significantly after 12 and 24 h of treatment to 115·5 (SD 40·9) pg/ml and 529·2 (SD 138·9) pg/ml, respectively (all $P < 0{\cdot}01$ when compared with unstimulated cells). After 6 h of treatment, no significant changes in leptin levels could be detected on treatment with LPS alone or in combination with the food additives. Co-treatment of LPS-stimulated cells with SS further diminished leptin levels slightly but significantly in relationship with treated cells with LPS after 24 h of treatment ($P < 0{\cdot}05$; Fig. 4(a)). In contrast, co-treatment of cells with SB or curcumin resulted in a substantial suppression of leptin release into the supernatant after 12 and 24 h compared with LPS-stimulated adipocytes (Fig. 4(b) and (c)).

Discussion

In the present study, the food preservatives SS and SB as well as the food colourant, curcumin, diminished leptin production in the cell culture supernatants of LPS-treated murine adipocytes in a dose- and time-dependent fashion. All tested compounds possessed antioxidant and/or radical-scavenging properties, which could play a role in interfering with the signal transduction cascades that modulate leptin production. The function of leptin was originally perceived as a signal that prevented obesity, since leptin-deficient ob/ob mice and leptin-resistant db/db mice develop hyperphagia and, consequently, severe obesity[8,16]. Supplementation of leptin to genetically deficient ob/ob mice increases their metabolic rate, body temperature and general activity, and decreases food intake, body weight and adiposity[8,17–19]. Basal or fasting leptin levels are strongly correlated with adipose tissue mass, percentage of body fat or BMI in both healthy adults and those with type 2 diabetes mellitus[12,20,21–23]. Despite these strong correlations, people with similar degrees of adiposity have circulating leptin concentrations that vary considerably, due to the influence of several factors on leptin metabolism such as insulin, glucocorticoids and

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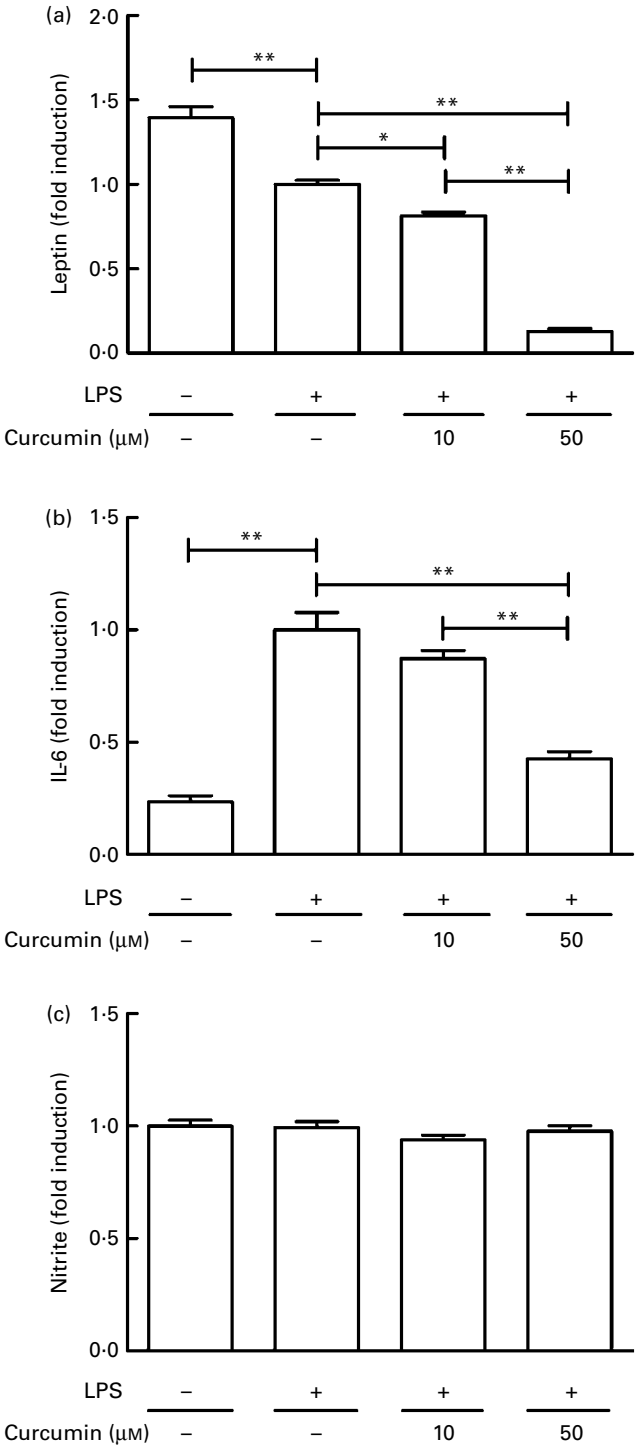

Fig. 3. Influence of curcumin in lipopolysaccharide (LPS; 1 μg/ml)-treated 3T3-L1 cells on (a) leptin secretion, (b) IL-6 release and (c) NO formation after 24 h of treatment. The control conditions in the absence or presence of 1 μg/ml LPS are shown. Fold induction was related to the control group with LPS in the absence of curcumin. All experiments were performed in triplicates. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different as assessed by one-way ANOVA with Bonferroni's adjustment: * $P \leq 0{\cdot}01$ and ** $P \leq 0{\cdot}001$.

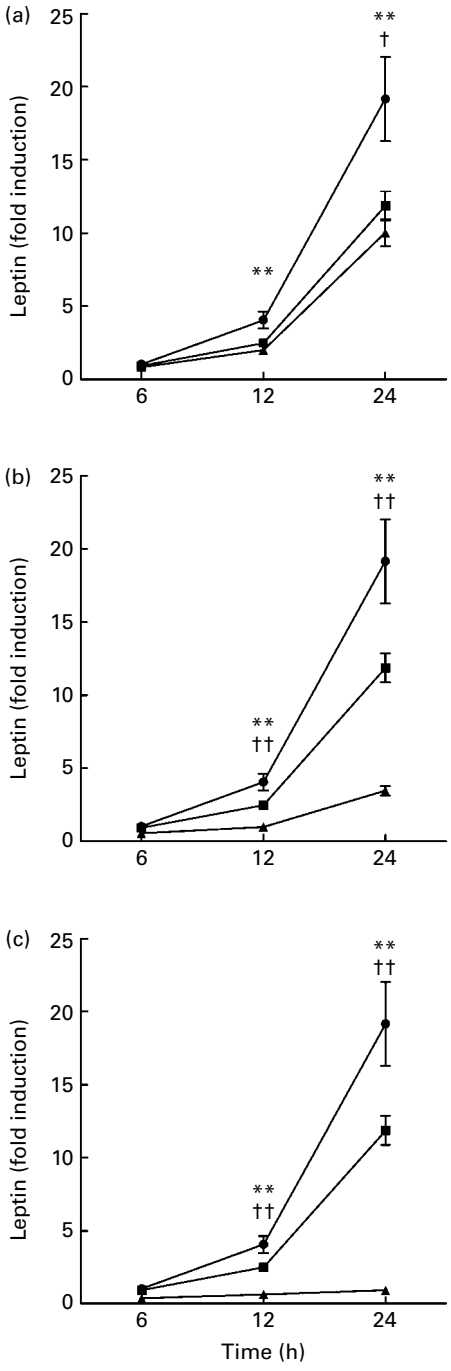

Fig. 4. Time course of leptin production in unstimulated and lipopolysaccharide (LPS; 1 μg/ml)-stimulated 3T3-L1 cells, and in cultures co-incubated with LPS (1 μg/ml) and (a) 1 mM-sodium sulphite (–▲–), control group – LPS (–●–), control group + LPS (–■–); (b) 10 mM-sodium benzoate (–▲–), control group – LPS (–●–), control group + LPS (–■–) or (c) 50 μM-curcumin (–▲–), control group (–●–), control group + LPS (–■–). Experiments (*n* 3) were performed in triplicates. Values are means, with their standard errors represented by vertical bars. ** Mean values were significantly different from those of leptin levels at 6 h to levels at 12 and 24 h in the presence of LPS and food additives within the condition as determined by one-way repeated-measures ANOVA, Bonferroni's method applied ($P < 0{\cdot}001$). Mean values were significantly different of leptin concentration after incubation with LPS in the presence of food preservatives when compared with the control in the presence of LPS alone as determined by one-way ANOVA with Bonferroni's adjustment: † $P < 0{\cdot}05$ and †† $P < 0{\cdot}01$.

catecholamines, suggesting that leptin metabolism is regulated by a complex network[24].

Based on the current data, different mechanisms induce and maintain a chronic state of inflammation in obese people[2]. Among others, it could be shown that in obese states the amount of Gram-negative bacteria differs compared with that in lean individuals. Moreover, due to the increased decay of Gram-negative bacteria, the release of LPS and its absorption in the gut capillaries may induce metabolic endotoxaemia and support chronic inflammation[4–6].

In our model, we found no direct effect on leptin secretion after incubation with antioxidants in the absence of LPS, at low levels of IL-6. However, at high levels of IL-6 due to LPS co-incubation, leptin secretion was significantly less than in the absence of antioxidants. These results suggest that a state of inflammation may be a prerequisite for the observed effect on leptin secretion. In the past 30 years, SS has become one of the leading food preservatives in the food sector throughout the world. The measured concentrations of SS in food vary between 0·8 mM, i.e. in dried potatoes, and 1·6 mM, i.e. in wine and dried fruits[25]. In 1983, the Joint Expert Committee on Food Additives of the FAO of the WHO established an acceptable daily intake level of 0·7 mg/kg body weight. A constant fraction of sulphite agents that enter the body via ingestion is metabolised in the liver. However, a finite amount will pass through the organ and enter the systemic circulation. Approximately, 10 % of the ingested dose is excreted unchanged in the urine[26,27].

Another widely used preservative is SB, which is known for its hydroxyl-radical scavenging, bacteriostatic and fungistatic properties under acidic conditions[28]. The Joint Expert Committee on Food Additives of the FAO/WHO established an acceptable daily intake level of 5 mg/kg body weight. However, recent studies have suggested that SB is linked to allergic reactions and, moreover, to hyperactivity in children[29–31]. The possible effects of SS and SB on specific metabolic pathways are scarce, albeit SB is a well-documented hydroxyl-radical scavenger and was recently found to suppress Th1-type immune responses *in vitro* [32,33].

Another compound with a strong antioxidant capacity is curcumin, the major component of turmeric (*Curcuma longa*). *In vitro* and animal studies have shown that curcumin exerts various beneficial properties such as anti-inflammatory, anti-neoplastic, anti-cancer and anti-ischaemic effects[34–36]. Recently published studies have elucidated the effect of curcumin on metabolisms of obesity and insulin resistance in various animal models. Taken together, these studies demonstrate decreased leptin levels after curcumin consumption in animal models[37–39]. In our study, we were able to confirm and to extend these results using a cell culture system of differentiated adipocytes, where we could show a significant decrease in leptin levels already after 12 h of exposure to curcumin. After an incubation time of 24 h, curcumin also decreased LPS-induced IL-6 levels in the supernatant, which is consistent with the reported anti-inflammatory properties of curcumin[40]. However, another possible explanation for the strong effects of curcumin on leptin release could be the induction of apoptosis, as described by various studies[41,42]. For SS and SB, mechanisms of apoptosis do not explain the obtained diminution of leptin levels, as IL-6 and nitrite were not affected during the whole experiments.

As we did not observe any effect of the antioxidants on leptin secretion in the absence of LPS, we assumed that the activation of the inducible NO synthase could provide a possible explanation for the affected leptin secretion in LPS-stimulated cells only. Although LPS is well known to activate inducible NO synthase in macrophages and dendritic cells of the skin, thereby elevating concentrations of nitrite, which in turn may affect leptin secretion[43–45], in our model, we did not observe any effect on nitrite release after treatment with LPS alone or in combination with the food additives. These results are in line with previously published studies, which show that only a combination of LPS, TNF-α and interferon-γ affects inducible NO synthase activity in murine 3T3-L1 cells, suggesting that the induction of this enzyme is mediated by a complex network of interactions involving inflammatory cytokines and LPS at the level of the adipocytes[46,47].

Taken together, our data suggest a possible effect of the food additives SS, SB and curcumin on the release of leptin from adipocytes in a state of chronic inflammation, which is associated with obesity. Decreased leptin release during the consumption of nutrition-derived food additives would decrease the overall amount of circulating leptin to which the central nervous system is exposed and could thereby influence food intake and contribute to an obesogenic effect[9].

In the murine 3T3-L1 cell culture system, we could analyse the sole effect of the investigated compounds avoiding interfering factors, which affect leptin metabolism, i.e. circulating insulin, glucocorticoids or catecholamines. From the data obtained in the present *in vitro* study, however, it is unclear how food additives interfere in a complex system such as the human organism with regard to leptin metabolism. Therefore, it is unclear to what extent any conclusion from the present *in vitro* study can be extrapolated to the *in vivo* situation, and clearly more studies are needed to investigate the potential contribution of diet-derived agents in a complex organism and a possible influence on the development of obesity.

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