

## The capacity of foodstuffs to induce innate immune activation of human monocytes *in vitro* is dependent on food content of stimulants of Toll-like receptors 2 and 4

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### Abstract

The ingestion of fatty meals is associated with a transient, low-grade systemic inflammatory response in human subjects, involving the activation of circulating monocytes and the secretion of pro-inflammatory cytokines. However, it is not yet clear how different foodstuffs may promote inflammatory signalling. In a screen of forty filter-sterilised soluble extracts from common foodstuffs, seven were found to induce the secretion of TNF- $\alpha$  and IL-6 from human monocytes *in vitro*. To investigate what may differentiate inflammatory from non-inflammatory food extracts, stimulants of Toll-like receptor (TLR) 2 and TLR4 were quantified using human embryonic kidney-293 cells transfected with each TLR, and calibrated with defined bacterial lipopeptide (BLP) and lipopolysaccharide (LPS) standards. These assays revealed that while most foods contained undetectable levels of TLR2 or TLR4 stimulants, all TNF- $\alpha$ -inducing foods contained stimulants of either TLR2 (up to 1100 ng BLP-equivalent/g) or TLR4 (up to 2700 ng LPS-equivalent/g) in both the soluble and insoluble fractions. TLR stimulants were present mainly in meat products and processed foods, but were minimal or undetectable in fresh fruit and vegetables. The capacity of food extracts to induce TNF- $\alpha$  secretion in monocytes correlated with the content of both TLR2 ( $r$  0.837) and TLR4 stimulants ( $r$  0.748), and was completely abolished by specific inhibition of TLR2 and TLR4. LPS and BLP were found to be highly resistant to typical cooking times and temperatures, low pH and protease treatment. In conclusion, apparently unspoiled foodstuffs can contain large quantities of stimulants of TLR2 and TLR4, both of which may regulate their capacity to stimulate inflammatory signalling.

**Key words:** Inflammation: Endotoxins: Food: Toll-like receptors

Dietary factors have long been understood to play a critical role in the development of diseases such as atherosclerosis and insulin resistance<sup>(1)</sup>. As more recent evidence has indicated that chronic inflammatory processes also underpin the development of these diseases, potential mechanisms linking nutrition and inflammatory signalling are being sought. Recently, it has been shown that the ingestion of fatty meals is associated with the transient activation of circulating monocytes and increases in circulating inflammatory markers, such as TNF- $\alpha$  and IL-6<sup>(2–4)</sup>. We and others showed that these responses may be due, at least in part, to the induction of mild postprandial endotoxaemia, which was found to occur after a fatty meal in human subjects and in animal models<sup>(5–10)</sup>. To date, it has been widely considered that the source of this circulating endotoxin is the resident intestinal microflora. However, in light of the recent finding that chylomicrons are the likely vehicle for endotoxin translocation in response to a fatty meal<sup>(10)</sup>, we sought a re-evaluation of this notion.

Specifically, as the small intestine is likely to be the primary site of endotoxin absorption after a fatty meal, yet only low levels of endogenous endotoxin are present in the small intestine due to the very limited microflora present in this region of the gut<sup>(11)</sup>, alternative potential sources of post-prandial endotoxin were sought.

We therefore aimed to determine whether common foodstuffs may contain appreciable quantities of endotoxin or other similar agents that may be capable of eliciting innate immune activation of human monocytes. In particular, we chose to quantify the levels of stimulants of Toll-like receptor (TLR) 2 and TLR4 in food extracts, as these receptors have been shown to play key roles in murine models of atherosclerosis<sup>(12–14)</sup> and insulin resistance<sup>(15–19)</sup>. Moreover, experimental administration of the ligands of TLR2 and TLR4, namely bacterial lipopeptides (BLP) and lipopolysaccharides (LPS), to animal models of these diseases has been shown to result in a marked increase in both atherosclerosis and insulin resistance<sup>(6,12,20–23)</sup>.

**Abbreviations:** BLP, bacterial lipopeptide; HEK, human embryonic kidney; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular patterns; TLR, Toll-like receptor.

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Forty extracts were therefore prepared from twenty-seven foodstuffs common to the Western diet, and the capacity of each to induce the secretion of IL-6 and TNF- $\alpha$  from human monocytes was measured and compared with the abundance of stimulants of TLR2 or TLR4 in each foodstuff, as quantified using a novel TLR transfectant-based bioassay<sup>(24)</sup>. We furthermore aimed to establish whether the biological activities of such stimulants may be sensitive to commonly used cooking regimens, or to low pH and protease environments similar to those that may be encountered in the stomach before entry to the small intestine, and whether they may reflect endogenous TLR stimulants or microbial food contaminants.

## Materials and methods

### Preparation of food extracts

Fresh foods from four major categories (fruit and vegetables, dairy, meat and processed foods) were purchased from local supermarkets or retail food outlets and were taken directly to the laboratory for processing on the same day. All foods showed no obvious signs of spoilage or degradation, as assessed by lack of 'off-odours', unusual colouration or visible blemishes associated with plant diseases, and were within advertised 'use-by' dates. Fruit and vegetables were peeled and chopped before processing in the uncooked form. Minced meats were also uncooked before assay, although precooked processed foods (including those purchased from fast-food outlets) were assayed in the cooked form as they would be bought or consumed. In each case, 25 g of fresh produce were homogenised in 250 ml PBS using a domestic blender (full power for 1 min) which was thoroughly cleaned and rinsed between the sample processing. A 1 ml aliquot of each homogenate was then heat sterilised (100°C for 10 min) to represent the insoluble pathogen-associated molecular patterns (PAMP), such as LPS and lipopeptide, present in each foodstuff (termed heat-killed food extract), and these were applied to cells in subsequent experiments as a suspension of insoluble particles. A second aliquot of each food suspension was then clarified by centrifugation (13 000 g for 5 min), and the resulting supernatant was filter sterilised (0.22  $\mu$ m; Acrodisc, San Francisco, CA, USA) to represent the soluble PAMP present in each sample (termed sterile-filtered food extract). Sterile-filtered food extract and heat-killed food extract samples were stored at -20°C before batch assay for BLP and LPS contents.

### Challenge of human monocytes

Venous blood was collected by venipuncture from consenting healthy human subjects (age 22–35 years) according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Leicester College of Medicine Research Ethics Committee. Exclusion criteria

included self-reporting of a previous diagnosis of any chronic inflammatory disease (such as arthritis or inflammatory bowel disease), infection within the previous 4 weeks or use of any medication other than oral contraceptives within the last week. Written informed consent was obtained from all the subjects. Peripheral blood mononuclear cells were prepared by density gradient centrifugation at 800 g for 25 min using Histopaque-1077 (Sigma, St Louis, MO, USA). Recovered cells were washed twice in PBS, re-suspended in Roswell Park Memorial Institute–10% fetal calf serum (Sigma) and plated in ninety-six-well plates at  $4 \times 10^5$  cells per well. Monocytes were prepared from peripheral blood mononuclear cells by plastic adherence (1 h at 37°C), followed by gentle washing to remove non-adherent cells. Remaining monocytes, which were of approximately 85–90% purity as measured by forward and side scatter distribution and CD14-positive staining as measured by flow cytometry, were then challenged by adding a 1:20 dilution of each sterile-filtered food extract in tissue culture medium. After incubation at 37°C for 4 h, the supernatants were removed for the assay of TNF- $\alpha$  content by L929 cell bioassay as described previously<sup>(25)</sup>, or IL-6 levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA) after 18 h.

### Toll-like receptor transfection reporter assays and quantification of Toll-like receptor stimulants

For transfection assays, human embryonic kidney (HEK)-293 cells were plated in ninety-six-well plates at  $10^4$  cells per well and transfected after 24 h using GeneJuice (Novagen, Madison, WI, USA) according to manufacturer's instructions. The amounts of construct per well were 30 ng of human TLR2, TLR4 (co-expressing MD-2) or TLR5 (InvivoGen, San Diego, CA, USA), 30 ng of pCD14 (kind gift of Professor Christopher Gregory, University of Edinburgh) and 10 ng of NF- $\kappa$ B-sensitive luciferase reporter construct (pELAM). Cells were grown for 3 d post-transfection before 18 h challenge. Promoter expression was calculated as fold induction relative to cells cultured in medium alone (SD). Endogenous expression of TLR in HEK-293 cells was ruled out by RT-PCR (data not shown). *Escherichia coli* R1 (NCTC 13114) LPS was prepared as described previously and was repurified by phenol–water extraction to remove TLR2-stimulating lipopeptide contaminants<sup>(25)</sup>. Synthetic BLP Pam<sub>3</sub>CSK<sub>4</sub> and flagellin of *Salmonella typhimurium* were purchased from InvivoGen. LPS, Pam<sub>3</sub>CSK<sub>4</sub> or flagellin were unable to activate HEK-293 cells transfected with CD14 alone, or with non-corresponding TLR (data not shown). Standard curves were prepared using 10-fold dilutions of BLP and LPS from 100 to 0.1 ng/ml, or from 2000 to 20 ng/ml for flagellin, in duplicate. Log-transformed PAMP concentrations were then plotted against fold induction of NF- $\kappa$ B reporter to generate a standard curve. This was used to estimate the concentrations of TLR2, TLR4 and TLR5 stimulants present in each food sample relative to standard

Pam<sub>3</sub>CSK<sub>4</sub>, LPS or flagellin as described previously<sup>(24)</sup>. The quantity of TLR stimulants present in each extract is therefore presented as a relative biological activity with respect to Pam<sub>3</sub>CSK<sub>4</sub>, LPS or flagellin. For example, results presented as 200 ng BLP-equivalent/g mean that each gram of food contains TLR2 stimulants with a capacity to stimulate TLR2 signalling equal to that of 200 ng Pam<sub>3</sub>CSK<sub>4</sub>. Food extracts were measured at 1:10 dilution in Dulbecco's modified Eagle's medium–10% fetal calf serum in duplicate. If signals exceeded the range of the standard curve, further dilutions were prepared and re-assayed. As transfected cells were sensitive to a minimum of 0.1 ng LPS or Pam<sub>3</sub>CSK<sub>4</sub> per ml, the minimum concentration of foodborne PAMP detectable by the assay was 10 ng PAMP per g food.

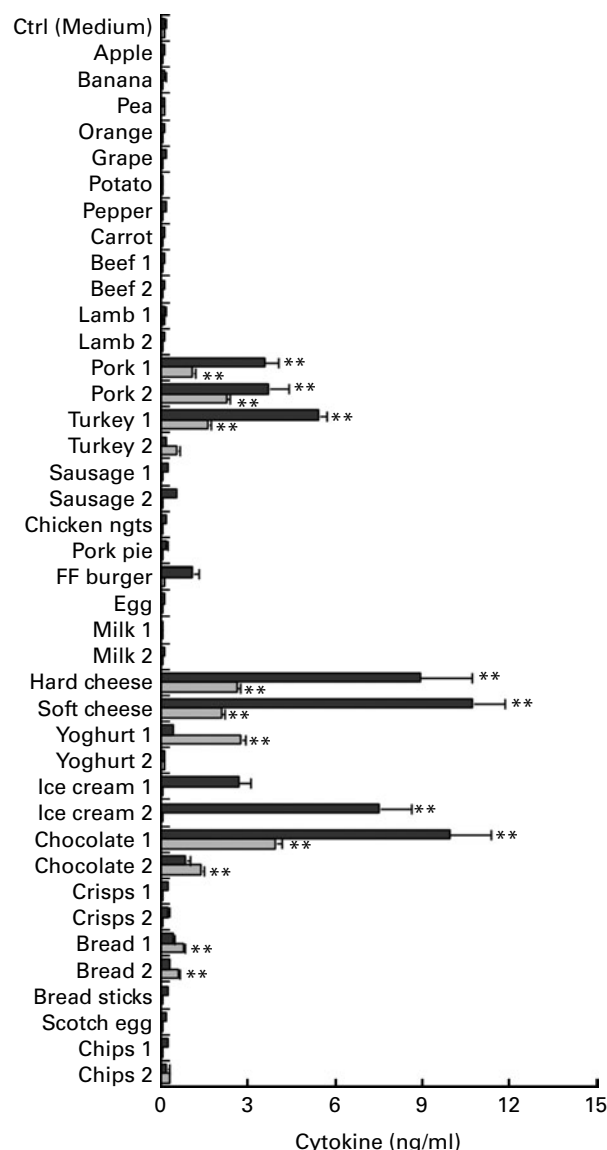
### Pathogen-associated molecular pattern treatments

To determine whether the biological activities of LPS, lipopeptide or flagellin may be altered by typical cooking times and temperatures, solutions of *E. coli* LPS, Pam<sub>3</sub>CSK<sub>4</sub> or *S. typhimurium* flagellin (100 ng/ml) were prepared in normal saline. Samples were then maintained at 100°C for 1–120 min, before cooling, diluting 1:10 in Dulbecco's modified Eagle's medium–10% fetal calf serum and applying to HEK-293 cells transfected with TLR2, TLR4/MD2 or TLR5 for measurement of the capacity to induce TLR signalling as described earlier. Alternatively, aliquots of each PAMP were adjusted to pH 1.0 for 2 or 3 h by the addition of HCl. Samples were then neutralised by the addition of NaOH solution and applied to TLR-transfected HEK-293 cells as described earlier. As a negative control, parallel samples were supplemented with an equal molarity of NaCl to account for increased salinity of samples due to acid–base neutralisation. In separate experiments, LPS, lipopeptide or flagellin preparations were treated with proteinase-K at 37°C for 1 h at neutral pH, then heated at 80°C for 10 min to inactivate the enzyme before addition to transfected HEK-293 cells. Control samples were also heat treated for 10 min. In some experiments, 10 µg/ml polymyxin-B (Sigma) were added to the samples for 10 min before assay to determine if TLR4 stimulants were of LPS origin.

In other experiments, monocytes were pretreated with 25 µg/ml oxidised palmitoyl arachidonyl phosphocholine (Sigma) prepared by dry film air oxidation in sterile glass tubes for 72 h and re-suspension in culture medium as described previously<sup>(26)</sup>, before addition of food extracts or 100 ng Pam<sub>3</sub>CSK<sub>4</sub> or LPS per ml. Oxidised palmitoyl arachidonyl phosphocholine was used at 25 µg/ml as this has been shown previously to result in specific inhibition of TLR2 and TLR4 signalling, but not in signalling initiated via other TLR or cytokine receptors<sup>(26,27)</sup>.

### Statistics

Results were compared by ANOVA using Tukey's or Dunnett's post-test. Differences were considered to be significant at  $P < 0.05$ .



**Fig. 1.** Induction of monocyte cytokine secretion by sterile food extracts. Human primary monocytes were cultured with filter-sterilised food extracts diluted 1:20 in tissue culture medium. Secretion of TNF- $\alpha$  (■) and IL-6 (□) was measured at 4 and 18 h, respectively, and is shown as means with their standard errors. ngts, Processed nuggets; FF, purchased from a 'fast-food' outlet. \*\* Mean values were significantly different when compared with medium alone ( $P < 0.001$  by ANOVA with Dunnett's test).

## Results

### Stimulation of human monocytes by soluble extracts of common foodstuffs

The soluble extracts of twenty-seven commonly available foodstuffs were prepared by filter sterilising a homogenate of each foodstuff to exclude intact bacterial cells. Each food product was processed on the same day of purchase, showed no signs of spoilage and was within the advertised 'use-by' date. A 1:20 dilution of each sterile-filtered extract was then prepared in the tissue culture medium and applied to human monocytes for 4 h. Although the extracts of most foods did not stimulate TNF- $\alpha$  secretion, the

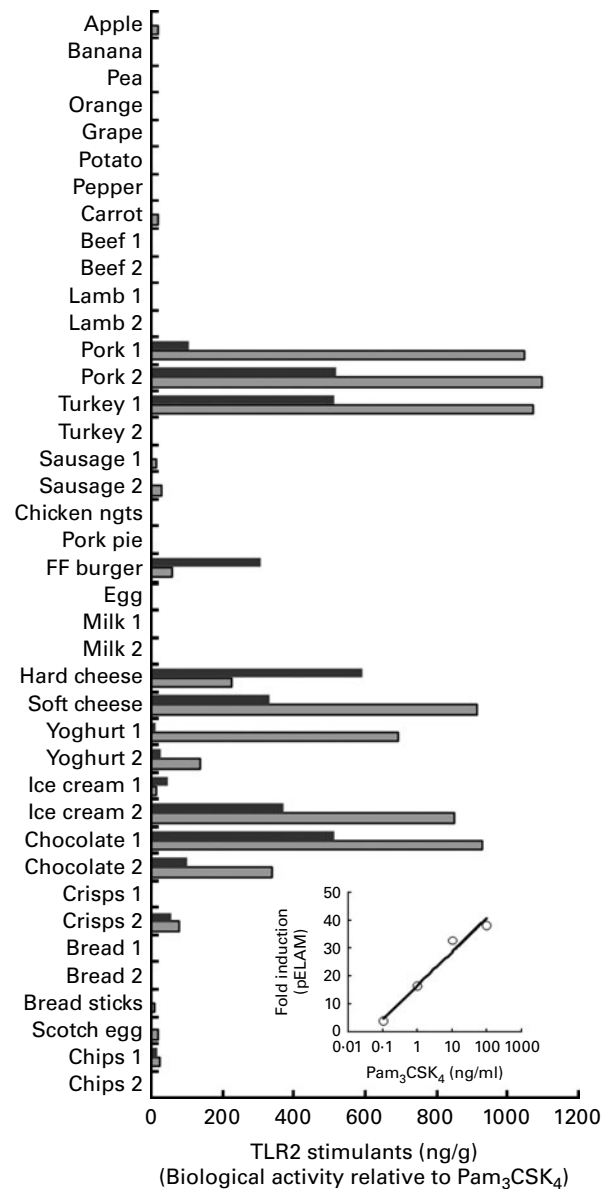
extracts of three minced meats, two cheeses, one ice cream and one chocolate product induced significant secretion of TNF- $\alpha$  relative to cells cultured in the medium alone ( $P < 0.001$ , Fig. 1). Similar results were obtained using the monocytes of four different subjects, and also by using the measurement of monocyte secretion of IL-6 (Fig. 1). Murine J774 macrophages also secreted TNF- $\alpha$  in response to treatment with the same extracts that promoted TNF- $\alpha$  secretion from human monocytes, confirming the biological activity of these samples (data not shown).

#### Quantification of stimulants of Toll-like receptor 2, Toll-like receptor 4 and Toll-like receptor 5 in food extracts

In order to investigate what factors may be responsible for the ability of some food extracts to promote cytokine secretion by monocytes, while related foods did not, we next quantified the abundance of stimulants of TLR2 and TLR4 in both the soluble (sterile-filtered food extract) and insoluble (heat-killed food extract) fractions of each food extract. These fractions were examined separately as it was considered that PAMP that remain attached to bacteria are unlikely to translocate from the small intestine into the blood. Specifically, it has been shown that while intact bacteria are efficiently excluded from the circulation by intestinal epithelial cell tight junctions, a small fraction of labelled soluble molecules, particularly those with molecular weights  $< 60$  kDa, can translocate from the lumen of the small intestine into the circulation via non-specific uptake mechanisms during the absorptive phase, remaining antigenically intact or biologically active after transport<sup>(28–30)</sup>. However, since it remains possible that PAMP that are contained within bacteria may become solubilised to a more readily absorbed form during the digestive process, PAMP were also quantified in the insoluble fraction of each foodstuff.

In terms of TLR2 stimulants, it was found that most foods did not contain either detectable soluble or insoluble stimulants of TLR2 (Fig. 2). However, three minced meats (two pork and one turkey), one cooked fast-food outlet burger, two cheeses, two ice creams and two chocolate products all contained detectable TLR2 stimulants, with levels ranging from 55 to 588 ng/g in the soluble fraction, and from 80 to 1096 ng/g in the insoluble fraction, as measured in terms of their biological activities relative to the synthetic BLP Pam<sub>3</sub>CSK<sub>4</sub>.

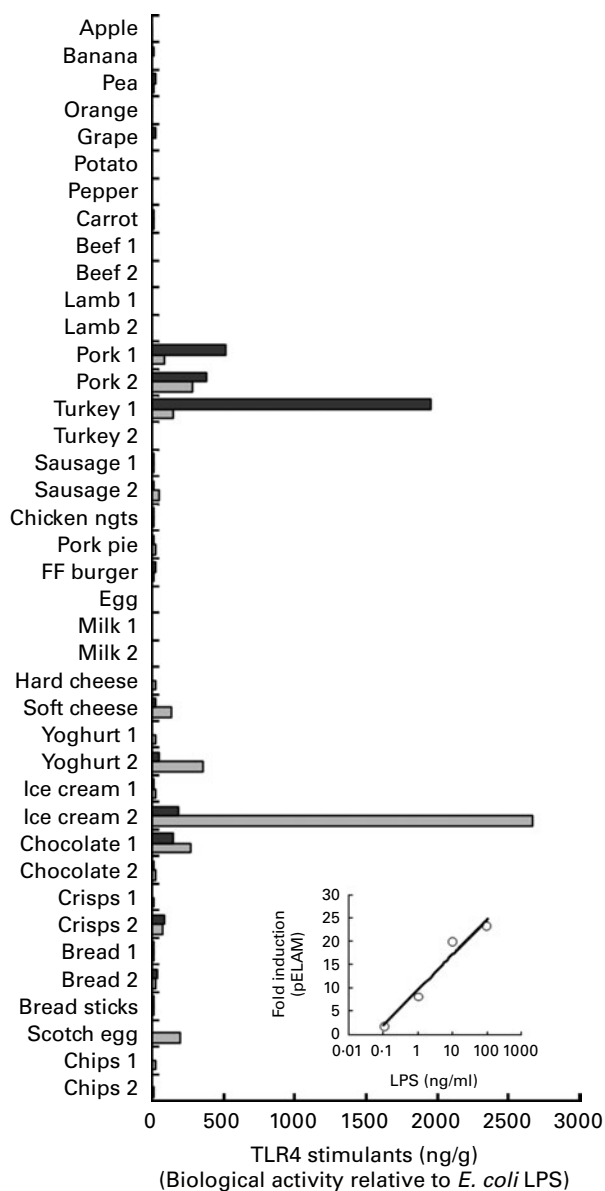
Next, because the stimulus amoebocyte lysate (LAL) assay is readily confounded by common food constituents such as  $\beta$ -glucans<sup>(31)</sup>, and generates a false-positive reaction to TLR4 antagonist-type LPS<sup>(32,33)</sup>, we found that it could not be used to detect TLR4 stimulants in this type of study. To circumvent these problems, expression of the native human receptor for hexa-acyl LPS, TLR4/MD2, was instead used to detect TLR4 stimulants in each of the food samples. Examination of the abundance of TLR4 stimulants using this technique revealed that most food



**Fig. 2.** Quantification of Toll-like receptor (TLR) 2 stimulants in food extracts. Filter-sterilised food extracts (representing soluble foodborne TLR stimulants) or heat-killed food suspensions (representing insoluble foodborne TLR stimulants) were diluted 1:20 in tissue culture medium and applied to human embryonic kidney-293 cells transfected with NF- $\kappa$ B reporter, CD14 and TLR2. Reporter activity was measured at 18 h and converted to lipopeptide equivalents using standard curves on the same plate using Pam<sub>3</sub>CSK<sub>4</sub> as standard. A typical standard curve for measurement of biological activity relative to Pam<sub>3</sub>CSK<sub>4</sub> is also shown (inset).  $r^2$  0.9688 (293-TLR2). ■, Soluble; □, insoluble.

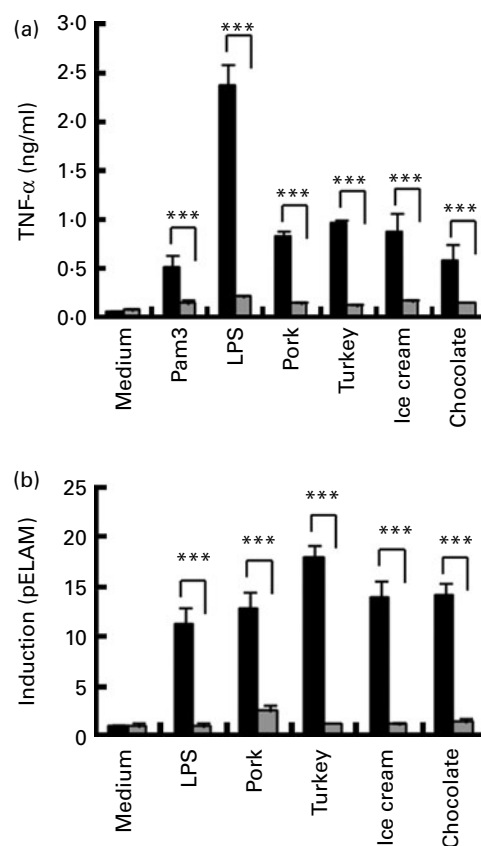
extracts examined contained little or no detectable TLR4 agonist-type molecules. However, the same three minced meats which contained TLR2 stimulants also contained abundant TLR4 stimulants, while one ice cream, one yoghurt and one chocolate product also contained elevated endotoxin concentrations. Levels of TLR4 stimulants in these products ranged from 50 to 1959 ng/g in the soluble fraction, and from 89 to 2667 ng/g in the insoluble fractions, relative to *E. coli* LPS (Fig. 3).





**Fig. 3.** Quantification of Toll-like receptor (TLR) 4 stimulants in food extracts. Filter-sterilised food extracts (representing soluble foodborne TLR stimulants) or heat-killed food suspensions (representing insoluble foodborne TLR stimulants) were diluted 1:20 in tissue culture medium and applied to human embryonic kidney-293 cells transfected with NF- $\kappa$ B reporter, CD14, TLR4 and MD2. Reporter activity was measured at 18 h and converted to LPS-equivalents using standard curves on the same plate using *Escherichia coli* R1 LPS as standard. A typical standard curve for measurement of biological activity relative to LPS is also shown (inset).  $r^2$  0.9609 (293-TLR4/MD2). ■, Soluble; □, insoluble; ngts, processed nuggets; FF, purchased from a 'fast-food' outlet.

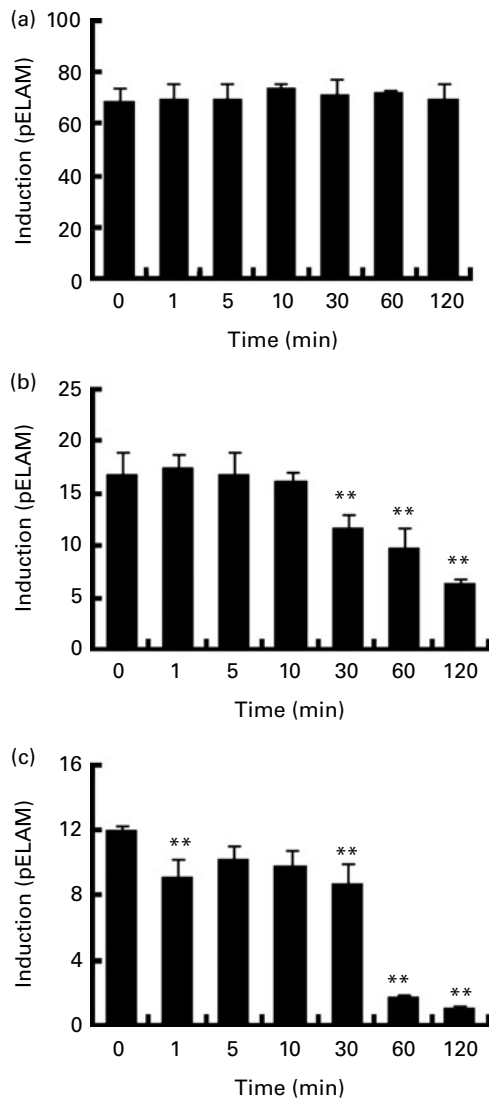
Measurement of TLR5 stimulants in each soluble extract revealed that only three samples contained flagellin levels above the limit of detection in this assay (200 ng/g). Specifically, one yoghurt, one ice cream and one chocolate product contained soluble flagellin levels ranging from 240 to 376 ng/g, in terms of comparison to *S. typhimurium* flagellin standard. Notably, none of the food extracts induced NF- $\kappa$ B activation in HEK-293 cells transfected with CD14 and reporter alone, i.e. without TLR2 or TLR4 transfection, indicating that the food extracts did not



**Fig. 4.** Effect of inhibition of Toll-like receptor (TLR) 2 and TLR4 on food extract-induced inflammatory signalling. (a) Primary human monocytes were incubated with filter-sterilised food extracts previously established to contain lipopolysaccharide (LPS) and bacterial lipopeptide (BLP) in the presence or absence of 25  $\mu$ g/ml oxidised palmitoyl arachidonyl phosphocholine (OxPAPC), a specific inhibitor of signalling via TLR2 and TLR4, but not via other TLR. TNF- $\alpha$  secretion was measured at 4 h. (b) Capacity of LPS-containing food extracts to stimulate TLR4 signalling in transfected human embryonic kidney-293 cells was measured in the presence or absence of polymyxin-B (PMB), a specific inhibitor of bioactivity. Means and standard deviations are shown. Mean values were significantly different: \*\*\* $P$  < 0.001 (compared by ANOVA with Tukey's test). (a) ■, -OxPAPC; □, +OxPAPC. (b) ■, -PMB; □, +PMB.

possess inherent capacity to stimulate NF- $\kappa$ B signalling in these cells in the absence of TLR2 or TLR4 (data not shown).

To assess the reproducibility of these findings, three food extracts which were originally found to contain both TLR2 and TLR4 stimulants were subjected to repeat assays on three further occasions. These subsequent assays revealed very similar patterns of TLR stimulants in each foodstuff, with inter-assay CV for concentrations of TLR2 stimulants approximately 27% and TLR4 stimulants approximately 20% over three freeze-thaw cycles. This level of variation is typical for cell-based bioassays in which the useful dynamic range of the assay is spread over several orders of magnitude (i.e. from 10 to 10 000 ng/g in this case). Food extracts which were negative for TLR stimulants in the first screen were also negative in subsequent assays, indicating that the results are not due to spontaneous contamination arising during sample processing or measurement (data not shown).



**Fig. 5.** Thermal stability of lipopeptide, lipopolysaccharide (LPS) and flagellin. 100 ng/ml Pam<sub>3</sub>CSK<sub>4</sub>, *Escherichia coli* LPS or flagellin in PBS were heated at 100°C for 0–120 min. Samples were then cooled and diluted 1:10 in medium, and the induction of NF- $\kappa$ B-sensitive reporter (pELAM) was measured in human embryonic kidney-293 cells transfected with Toll-like receptor (TLR) 2 (a), TLR4/MD-2 (b) or TLR5 (c). Means and standard deviations are shown. Mean values significantly different when compared with untreated pathogen-associated molecular patterns: \*\* $P < 0.01$  ( $t = 0$ , compared by ANOVA with Dunnett's test).

#### Capacity of food extracts to stimulate TNF- $\alpha$ secretion is dependent on Toll-like receptor 2 and Toll-like receptor 4

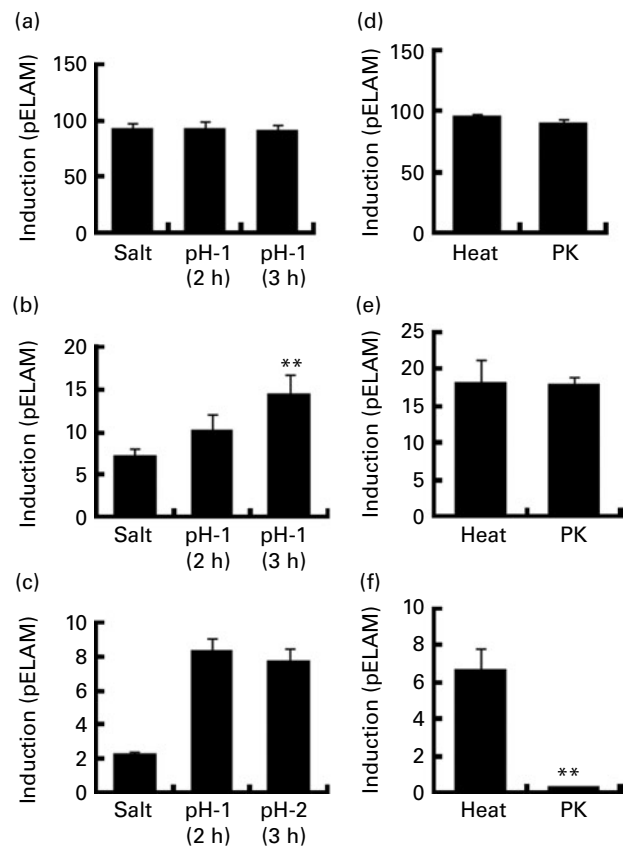
Monocyte TNF- $\alpha$  secretion was found to correlate with the content of both TLR2 and TLR4 stimulants ( $r$  0.837 and 0.745, respectively, each  $P < 0.0001$ ) when food products with detectable PAMP levels were compared. To determine if TLR2 and TLR4 stimulants are required for the induction of TNF- $\alpha$  secretion, human monocytes were treated with each foodstuff in the presence or absence of oxidised palmitoyl arachidonyl phosphocholine, a compound that we and others have shown specifically inhibits signalling via TLR2 and TLR4, but not via other TLR or cytokine receptors<sup>(26,27)</sup>. Combined blockade of TLR2 and TLR4

with oxidised palmitoyl arachidonyl phosphocholine completely abrogated TNF- $\alpha$  secretion in response to each stimulant-containing foodstuff (Fig. 4(a)).

Next, as it has been suggested that alternative ligands beyond LPS may also be capable of stimulating TLR4-dependent signalling (such as SFA and heat shock proteins<sup>(34,35)</sup>), we examined the capacity of polymyxin-B to inhibit the TLR4 stimulation induced by sterile-filtered extracts. Polymyxin-B, which is a cationic antibiotic that specifically binds LPS and sequesters it from the receptors of the innate immune system, blocked TLR4 signalling from each of the products examined, suggesting that the TLR4 stimulants in the foodstuffs examined are endotoxins and not other types of molecule (Fig. 4(b)).

#### Heat stability of lipopeptide, lipopolysaccharide and flagellin

To determine whether foodborne lipopeptides, LPS or flagellins may be destroyed by typical cooking temperatures



**Fig. 6.** Effects of low pH and protease treatment on biological activities of lipopeptide, lipopolysaccharide (LPS) and flagellin. 100 ng/ml Pam<sub>3</sub>CSK<sub>4</sub> (a), *Escherichia coli* LPS (b) or flagellin (c) were adjusted to pH 1.0 with HCl for 2 or 3 h, then neutralised with NaOH, or treated with equivalent molarity of NaCl at 37°C. Capacity of each pathogen-associated molecular patterns (PAMP) to signal via Toll-like receptor (TLR) 2, TLR4 or TLR5 was then measured in TLR-transfected human embryonic kidney-293 cells. Alternatively, 100 ng/ml Pam<sub>3</sub>CSK<sub>4</sub> (d), *E. coli* LPS (e) or flagellin (f) were treated with proteinase-K (PK) at 37°C for 1 h before heat treatment at 80°C for 10 min to neutralise enzyme. Means and standard deviations are shown. Mean values were significantly different: \*\*  $P < 0.01$  v. PAMP treated with salt only ((a)–(c)) or heat only ((d)–(f)), compared by ANOVA with Dunnett's test.

and times, aliquots of Pam<sub>3</sub>CSK<sub>4</sub>, *E. coli* LPS and flagellin boiled in saline at 100°C for up to 2 h were tested for their remaining capacity to stimulate signalling via their respective TLR. The biological activity of Pam<sub>3</sub>CSK<sub>4</sub> was not measurably reduced by heating for up to 2 h (Fig. 5(a)). LPS retained its biological activity up to about 10 min, but further heating led to a modest but significant reduction in biological activity after 30 min (Fig. 5(b)). By contrast, the biological activity of flagellin was almost completely abolished by 1 h (Fig. 5(c)). As the internal temperature of cooking foodstuffs rarely exceeds 100°C due to evaporation of moisture from the food surfaces under most circumstances<sup>(36)</sup>, these results suggest that typical cooking times and temperatures are not likely to greatly reduce the biological activity of contaminating LPS or lipopeptides in food products.

#### *Resistance of lipopeptide, lipopolysaccharide and flagellin to low pH and protease treatment*

As ingested PAMP must pass through the stomach before entry to the small intestine, we next tested whether lipopeptide, LPS or flagellin may be resistant to low pH or protease treatment. Low pH (followed by neutralisation) did not affect the capacity of BLP to stimulate TLR2, while the activity of LPS was increased by low pH treatment, presumably due to release of lipid-A<sup>(37)</sup>. Likewise, low pH increased the biological activity of flagellin, probably due to increased monomerisation, as expected<sup>(38)</sup>. However, while the biological activities of LPS and lipopeptide were unaffected by protease treatment, proteinase-K abolished the bioactivity of flagellin (Fig. 6).

#### Discussion

A wealth of evidence now exists to suggest that the induction of inflammatory signalling via stimulation of innate immune receptors plays a key role in the development of both atherosclerosis and insulin resistance<sup>(12–19)</sup>. However, it is not yet clear how nutritional factors may regulate innate immune function or inflammatory signalling<sup>(2–4)</sup>. One possibility, supported by several recent studies, is that fat-induced translocation of small quantities of bacterial endotoxin from the gut into the circulation could play a role in this process<sup>(5–10)</sup>. To date, it has been assumed that this endotoxin is likely to derive from the endogenous host microflora. However, as the small intestine has recently been identified as the likely site of diet-induced LPS translocation<sup>(10)</sup>, and since the small intestine contains only very low levels of endogenous bacteria (and hence LPS)<sup>(11)</sup>, we tested the hypothesis that common foodstuffs may also contain stimulants of TLR2 or TLR4, since these receptors have been identified as key innate immune mediators involved in metabolic diseases<sup>(12–19)</sup>.

The measurement of concentrations of TLR stimulants in food products presented several difficulties. First, as

TLR stimulants can be derived from any type of micro-organism, they show an inherently large antigenic and molecular diversity, which precludes the use of traditional ELISA or MS techniques. Next, we found that the most widely used assay for the detection of endotoxins in foodstuffs<sup>(39–42)</sup>, namely the LAL assay, was not suitable for this type of study for several reasons. First, it is well established that the limulus assay generates a positive reaction to  $\beta$ -glucans which can be common in foodstuffs, thereby potentially generating false-positive results<sup>(31)</sup>. Next, work from our own laboratory and those of other workers has shown that several forms of non-enterobacterial lipid-A, which can often be antagonists of TLR4 and LPS signalling in human cells, stimulate a positive reaction in the limulus assay<sup>(32,33)</sup>. Notably, many environmental and foodborne organisms possess a non-enterobacterial lipid-A structure which does not stimulate human TLR4/MD-2<sup>(37)</sup>. Finally, as the LAL assay is insensitive to lipopeptides and flagellins, it cannot be used to quantify these PAMP<sup>(43)</sup>. To circumvent these problems, and in order to retain relevance to human PAMP receptor specificity, we quantified foodborne concentrations of TLR stimulants using a recently developed bioassay employing TLR-deficient cells transfected with human TLR2, TLR4/MD-2 or TLR5<sup>(24)</sup>.

The results from these assays showed that several commonly consumed foods can contain large quantities of both TLR2 and TLR4 stimulants, reaching up to 1.1  $\mu$ g BLP-equivalent or 2.7  $\mu$ g LPS-equivalent per gram of food. Although to our knowledge the measurement of TLR2 or TLR5 stimulants in foodstuffs has not been attempted previously, the present findings are supported by earlier demonstrations of high levels of endotoxin in some foodstuffs using the LAL assay. For example, Jay *et al.*<sup>(41)</sup> showed that freshly purchased beef mince can contain up to 7.4  $\mu$ g LPS/g, and Gehring *et al.*<sup>(40)</sup> showed that milk can contain between 1 and 100 ng LPS/ml. However, it should be noted that the LAL-based assays used in these previous studies are likely to have significantly overestimated the genuine TLR4-stimulating potential of foodborne endotoxins, for the reasons outlined earlier<sup>(31–33)</sup>.

It has been proposed that in addition to microbial molecules, several molecules of eukaryotic origin, such as SFA or heat-shock proteins, may also stimulate TLR2 or TLR4 signalling<sup>(34,35)</sup>. However, several lines of evidence suggest that the TLR2 and TLR4 stimulants detected in each foodstuff reflect molecules derived from microbial sources, rather than endogenous food-derived TLR-stimulating molecules. For example, in several cases, very similar foodstuffs (most notably the minced meats) contained abundant TLR2 and TLR4 stimulants, while others of the same food type did not. Polymyxin-B also efficiently inhibited the TLR4 signalling of each positive food extract, suggesting that LPS, and not endogenous food molecules, is the agent responsible for TLR4 signalling in these extracts. Notably, while all of the foodstuffs examined here contained SFA, not all the extracts stimulated TLR

signalling, lending further support to our recent demonstration that SFA do not stimulate TLR2 or TLR4 signalling<sup>(43)</sup>. These findings therefore suggest that apparently unspoiled foodstuffs may nevertheless contain at some point in their preparation or processing a sufficient microbial load to release TLR2 and TLR4 stimulants into their growth environment. This notion is supported by many previous studies showing that certain commonly consumed foodstuffs can contain a high bacterial load before cooking, such as fresh beef mince which has often been shown to contain approximately  $10^5$ – $10^7$  colony forming units/g<sup>(41)</sup>. Notably, however, the purpose of the present study was not to examine the microbial quality of each foodstuff, since PAMP biological activity is retained independently of bacterial viability or cooking. Further studies are therefore warranted to establish which types of foodborne micro-organism may represent the dominant contributors to PAMP contaminants in each type of food product.

Another key question that remains to be addressed in future studies is whether the levels of TLR2 and TLR4 stimulants that we have identified in these foods are of physiological relevance. Previous studies in mice suggest that approximately 0.2% of orally ingested radiolabelled LPS can be absorbed into the circulation when dietary fat is present to facilitate absorption<sup>(10)</sup>, and such LPS was shown to retain its biological activity after translocation from the gut into the circulation<sup>(44)</sup>. Remarkably, oral gavage of mice with as little as 39  $\mu$ g of LPS results in systemic cytokine release<sup>(45)</sup>, while higher doses of oral LPS reactivated both ovalbumin- and collagen-induced arthritis in mice<sup>(44,46)</sup>. If human subjects also absorb 0.2% of ingested LPS, these findings suggest that a meal containing 100  $\mu$ g LPS could lead to the absorption of 200 ng LPS. By way of comparison, a bolus injection of 140 ng LPS results in marked systemic inflammation, including IL-6 and TNF- $\alpha$  releases, in healthy human subjects<sup>(47,48)</sup>. Notably, however, recent studies have established that the amount of LPS absorbed from the intestine may be regulated not only by the presence of dietary fat, but potentially also by other dietary components. For example, while the ingestion of cream induced postprandial endotoxaemia and circulating inflammatory markers in human subjects, ingestion of isoenergetic orange juice or glucose drinks did not<sup>(8)</sup>. In contrast to a high-fat high-carbohydrate meal, which was shown to increase endotoxin levels and circulating mononuclear cell expression of TLR2 and TLR4, a meal rich in fibre and fruit did not<sup>(7)</sup>. The consumption of orange juice also appeared to reduce the postprandial increases in circulating endotoxin and inflammatory markers induced by a high-fat meal<sup>(9)</sup>.

In terms of potential for absorption of dietary lipopeptides, it is interesting to note that BLP has very similar physico-chemical properties to LPS and could therefore also translocate via similar pathways. Indeed, oral administration of synthetic lipopeptides was shown to result in systemic immune responses in mice<sup>(49)</sup>. Thus, it is tempting

to speculate that the occasional ingestion of meals high in LPS and/or BLP could promote transient, mild, systemic inflammatory episodes that predispose subjects to the development of atherosclerosis and insulin resistance. If future studies establish this to be the case, the potential health benefit of modifying food preparation protocols to minimise potential contamination with these agents may merit further investigation.

In conclusion, the present findings indicate that pro-inflammatory stimulants of TLR2 and TLR4 can be present at levels of potential biological significance in many foodstuffs common to the Western diet. Further studies are warranted to establish whether these contaminants are of pathological relevance in the context of common chronic inflammatory diseases, such as atherosclerosis, insulin resistance and arthritis.

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