L-α-Glycerophosphocholine Contributes to Meat’s Enhancement of Nonheme Iron Absorption1,2

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Abstract
In this research, our aim was to isolate and characterize the substance known as “meat factor,” which is reported to enhance nonheme iron absorption. We used various analytical techniques, and the final step was a human study to measure the effect of a candidate compound on iron absorption. Lean beef was selected for study, as it is known to increase nonheme iron absorption. Cooked ground beef was homogenized and aliquots were taken through a simulated gastric and intestinal digestion. This was followed by purification using fast protein liquid chromatography. The fractions were collected and applied to a Caco-2 cell system designed to measure iron absorption using radioiron. Fractio

Materials and Methods
Preparation of meat fractions. Lean beef was boiled in deionized water for 20 min, homogenized, freeze-dried, ground to a fine powder with a pestle and mortar, sieved through a 450-μm mesh, and stored at −20°C. Freeze-dried material (1 g) was homogenized with 1 mol/L sodium chloride over ice and subjected to a simulated gastric and small intestinal digestion at 37°C in a shaking water bath. The pH was adjusted to 2 using 5 mol/L HCl, pepsin (Sigma P7125) was added, and the mixture was incubated for 1 h. The pH was raised to 7.5, pancreatin-bile (Sigma P1750, Sigma B8381) was added, and the mixture was incubated for 2 h. Aliquots of the digest were transferred to Eppendorf tubes, centrifuged at 1000 × g for 5 min, placed on ice for 30 min, the fat layer was removed, and the supernatant was transferred to a clean tube and stored at −20°C. The meat digest was thawed and centrifuged at 190 × g for 5 min, the supernatant was transferred to a clean tube and 300 μL was injected into a Triton Superdex column (Amersham Biosciences), to separate the components within the meat digest according to molecular size. A 0.2 mol/L phosphate buffer (2.84 g disodium hydrogen phosphate and 5.84 g sodium chloride in 1 L Milli-Q water) at pH 7.4 (using 5 mol/L HCl) with a flow rate of 0.3 mL/min was used. Each fast protein liquid

Introduction
The enhancing effect of meat and fish on iron absorption is well documented but, despite considerable effort, the identification of the “meat factor” remains elusive. Proposed candidates include amino acids (1), peptides (2,3), and, more recently, glycosaminoglycans components (4), in addition to the long established gastric acid–promoting effect of meat (5).

The aim of our research was to separate fractions from cooked beef after it had undergone simulated gastric and intestinal digestion, and to test their effect on iron uptake into Caco-2 cells using 55Fe. The active fractions containing the lower molecular weight components were selected for further investigation, as it was simpler to isolate and identify lower molecular weight compounds. Mass spectrometric techniques were used to measure the mass and identify the compound present in the most active fraction. Finally, the effect of a commercial supply of the compound on iron absorption from a vegetarian meal was tested in human volunteers.
chromatography run lasted 2–3 h, and the eluate from at least 12 runs was pooled and stored at −20°C. The fractions were desalted by adding 10 g Dowex mixed bead ion exchange resin (Sigma CAS 103591–115, batch 11210EA), which had been rinsed for several days in Milli-Q water. The meat fraction and beads were mixed for 48 h at 4°C and the beads were removed by filtration. The desalted fractions were freeze-dried.

In vitro studies on meat digest fractions using Caco-2 cells. Caco-2 cells were maintained in DMEM containing 20% fetal bovine serum, 1% nonessential amino acids, 2 mmol/L L-glutamine; 500 kU/L penicillin, and 500 mg/L streptomycin. Cells from a TC7 subclone (Inserm U505) were used between passages 25–30, seeded at a density of 1 × 10⁶ cell/L in 24-well plates and grown for 21 d in the above medium.

Freeze-dried meat fractions were resuspended in HBBS, pH 6.5, and 0.5 mL/well added to the Caco-2 cell monolayers. Initial studies were carried out using 12 pooled fractions (labeled MF2–MF13) and 5 of these fractions were selected for further fractionation and analysis.

Prior to iron uptake measurement, the cell culture medium was removed, the cells were washed twice with HBBS, and a third aliquot of fresh HBBS was then added to each well. Uptake was initiated by adding 55FeCl₃ and 54FeCl₃ (1:1 ratio, final concentration 10 µmol/L) to each well and the cells were incubated for 2 h at 37°C on a rocking shaker (6 oscillations/min). Control wells contained only FeCl₃. Ascorbic acid (AA)⁵ (final concentration 100 µmol) was added to some wells as a positive control. At the end of the incubation period, cells were washed twice with a rinse solution (140 mmol/L NaCl; 5 mmol/L KCl; 10 mmol/L HEPES) and incubated for 10 min at room temperature with a removal solution (140 mmol/L NaCl; 5 mmol/L KCl; 50 mmol/L sodium hydrosulfite; 1 mmol/L bathophenanthroline disulfonic acid). After 10 min, the removal solution was discarded and the cells were washed again with the rinse solution. The final stage was the solubilization of the cells using 0.5 mol/L NaOH, and the cell samples were subjected to scintillation counting to determine iron uptake.

Liquid chromatography/MS and tandem MS analysis. A Lichrosorb 5-µm Diol 250 × 4.6-mm column (Phenomenex) was used with acetonitrile (solvent A) and unbuffered water (solvent B) solvents were initially used to analyze extracts from the meat digest. The solvent program, used at a flow rate of 1 mL/min, is shown in Table 1. Mass spectra and tandem mass spectra were obtained using a Quattro II triple quadrupole mass spectrometer (Micromass) coupled to a Jasco PU-1585 triple pump HPLC, equipped with an AS-1559 cooled autoinjector, a CO-1560 column oven and UV-1575 UV detector (Jasco) were used to identify the structure as L-α-glycerophosphocholine (L-α)⁶. The accurate mass measurement of the component present in the meat digest fraction with the greatest iron enhancing effect in the Caco-2 cell system was determined by Hall Analytical Laboratories, using a Micromass LCT mass spectrometer.

The final step in the identification procedure was accomplished by comparing the extracted compound with the authentic material. Tandem MS (MS/MS) spectra obtained by product ion analysis of the [M + H]+ ions (m/z 258) at a collision energy of 25 eV and a collision gas (Argon) pressure of 87 mPa were identical and had the same retention time. A comparison of the MS/MS spectra of the meat factor compound and authentic L-α was shown (Fig. 1). Because of the zwitterionic nature of glycerophosphocholine, the HPLC method yielded a poor peak shape; therefore liquid chromatography/MS was used to analyze later samples (6).

In vitro studies using Caco-2 cells. The effect of L-α on iron uptake from vegetarian lasagna that had undergone simulated digestion was measured in Caco-2 cells (American Tissue Culture Collection, passage 40) (7). A quantity of lasagna containing 0.4 g of protein (8 g) was cooked, homogenized in liquid nitrogen, and mixed with 120 mmol/L NaCl (8 mL). The food homogenate solution was adjusted to pH 2 with 5 mol/L HCl, after which radioactive and cold iron in the form of ferric chloride ([55FeCl₃], [54FeCl₃]) was added. Pepsin solution (1 g pepsin in 0.1 mol/L HCl) was then added to the food homogenate and incubated at 37°C for 1 h on a rocking shaker (55 oscillations/min). After 1 h, the pH of the food homogenate was increased to pH 6 using 1 mol/L NaHCO₃, after which a pancratin/bile solution was added (0.1 g bile + 0.6 g pancratin in 50 mL NaHCO₃). The final solution of the food homogenate was increased to 13 mL with 120 mmol/L NaCl, of which 1.5 mL was used in the iron uptake experiments as outlined above.

Human study. A commercial supply of the active compound identified from the in vitro studies (L-α) was obtained from Chemi S.p.A. (50–52% L-α; 47–49% mannitol; white/off-white powder) and used for the human study. A randomized cross-over trial was undertaken in 13 women aged 18–45 y with low iron stores (serum ferritin <30 µg/L) to compare the effect of a commercial supply of the active component in the meat digest (L-α) with that of AA on iron absorption from a Mediterranean vegetarian lasagna meal (Linda McCartney) (Table 2), using the stable isotope erythrocyte incorporation technique (8). Serum ferritin was measured in the screening blood samples at BUPA Hospital, Norwich, UK, using a commercial chemiluminescent immunoassay analyzer (Immullite 2000, Table 1. Solvent program used to analyze extracts from the meat digest

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5 Abbreviations used: AA, ascorbic acid; GAG, glycosaminoglycans; MS/MS, tandem mass spectrometry; L-α, L-α-glycerophosphocholine.

* Values are means ± SEM, n = 3. Asterisks indicated greater than (*) or less than (**) control, P < 0.05.
Diagnostic Products) plus C-reactive protein (Vitros 250 Analyser system) to eliminate falsely high ferritin resulting from infection/inflammation. The magnitude of the iron enhancing effect was measured, with L-a and AA being present in the meal at the same enhancer:iron molar ratio (2:1). The study was approved by the IFR Human Research Governance Committee and the Norwich Local Research Ethics Committee and all volunteers gave written informed consent.

Each volunteer was allocated to 1 of 2 groups (A or B) and completed 2 separate iron absorption studies (phase 1 and 2), separated by a period of at least 18 d. During each phase, they consumed 4 test meals (Table 1): 2 were extrinsically labeled with 10 mg $^{57}$Fe and 2 with 2 mg $^{58}$Fe plus 8 mg natural abundance iron, with or without 46 mg L-a or 100 mg AA. We estimated that we needed to provide a minimum of 18 mg $^{57}$Fe to ensure there was measurable enrichment in the red blood cells of the most abundant stable isotope label used in our study. Therefore, each person consumed 2 similar meals (one at midday and 1 in the evening), providing a total of 20 mg iron. Each 320 g portion of lasagna provided 1350 kJ, 19.8 mg iron, 63% compared with 54% with lasagna alone ($P = 0.045$). Under control conditions, cells took up $0.10 \text{ fmol Fe}$, which was less than that when lasagna alone ($69 \pm 11 \text{ fmol Fe}$, $P = 0.045$) or lasagna + L-a ($86 \pm 19 \text{ fmol Fe}$, $P = 0.009$) was included.

Thirteen volunteers completed the human iron absorption study but the results from 1 person were not included because her C-reactive protein was above the normal range. There was a significant negative correlation (Pearson’s product-moment correlation) between plasma ferritin concentration and % iron fractions (1–17) either inhibited or had no effect on iron uptake, whereas the lower molecular weight fractions stimulated iron uptake. Samples 24–29 generated from MF11 resulted in iron uptake that was $156 \pm 34\%$ of the control ($P = 0.01$, Fig. 2B).

The active compound in the meat fraction with the greatest enhancing effect on $^{55}$Fe uptake into Caco-2 cells had a mass of 257.22 and, from mass spectra (Fig. 1), it was identified as 2-$\{[25S]-2,3$-dihydroxypropoxy$\}$-oxido-phosphoryl$\}$-oxethyl-trimethyl-azanium glycerophosphocholine ($C_{17}H_{20}$ NO$_5$P), also referred to as L-a-glycerophosphorylcholine, L-a-glycerophosphorylcholine, L-a-glycerophosphorylcholine, L-a-glycerophosphorylcholine, or 2-$\{[2,3$-dihydroxy-propoxy$\}$-hydroxy-phosphoryloxyl$\}$-ethyl trimethyl-amonium betaine (L-a). When tested in the in vitro system, L-a showed a dose dependent effect on iron uptake compared with control (no L-a present) and reached a plateau (Fig. 3). L-a increased iron uptake from vegetarian lasagna by 63% compared with 54% with lasagna alone ($P = 0.010$). Under control conditions, cells took up $32 \pm 0.10 \text{ fmol Fe}$, which was less than that when lasagna alone ($69 \pm 11 \text{ fmol Fe}$, $P = 0.045$) or lasagna + L-a ($86 \pm 19 \text{ fmol Fe}$, $P = 0.009$) was included.

### Results

In the initial Caco-2 cell screen (Fig. 2A), we identified 2 meat fractions that stimulated iron uptake (MF4 and MF11) and 3 fractions that inhibited iron uptake (MF3, MF6, and MF8). When each of these 5 samples were fractionated to provide 5 or 6 subfractions (29 fractions in total), the higher molecular weight subfractions (1–17) either inhibited or had no effect on iron uptake, whereas the lower molecular weight fractions stimulated iron uptake. Samples 24–29 generated from MF11 resulted in iron uptake that was $156 \pm 34\%$ of the control ($P = 0.01$, Fig. 2B).

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Thirteen volunteers completed the human iron absorption study but the results from 1 person were not included because her C-reactive protein was above the normal range. There was a significant negative correlation (Pearson’s product-moment correlation) between plasma ferritin concentration and % iron
absorption from both sets of test meals (Phase 1; \(^{57}\)Fe; \(r = -0.82, P = 0.001; ^{56}\)Fe; \(r = -0.87, P = 0.0002\) and Phase 2; \(^{57}\)Fe \(r = -0.63, P = 0.027\); \(^{56}\)Fe \(r = -0.63, P = 0.028\)), confirming the established relationship between efficiency of absorption and body iron stores. Iron absorption from the 2 vegetarian lasagna meals was 3.4 ± 2.7% (control 1) and 3.5 ± 2.9% (control 2) in phase 1 and 2, respectively, which is equivalent to 0.34 mg and 0.35 mg isotopic iron, respectively. Absorption increased with phase 1 and 2, respectively, which is equivalent to 0.34 mg and 0.35 mg, respectively. Absorption increased with phase 1 and 2, respectively, which is equivalent to 0.34 mg and 0.35 mg, respectively. Absorption increased with phase 1 and 2, respectively, which is equivalent to 0.34 mg and 0.35 mg, respectively.

GAG, which are present in relatively high amounts in meat, had enhanced effect on nonheme iron absorption has remained elusive. Candidate compounds include amino acids and peptides, particularly those containing cysteine (2), and, more recently, carbohydrate fractions (4). Huh et al. (4) undertook a series of in vitro experiments in which they observed that oligosaccharides, originating from glycosaminoglycans (GAG) isolated from fish, increased iron uptake by Caco-2 cells by 50%, and suggested that GAG may be the active component of the meat factor. However, when tested in a human study, purified sulfated and unsulfated GAG, which are present in relatively high amounts in meat, had no effect on iron absorption (13). The results of the human study were challenged by Jin and Glahn (14), who contended that the purified GAG compounds were not the active components identified in their in vitro studies, and reported that they did not have the same effect when tested in their in vitro system [F. Jin, R. G. Glahn, unpublished data (14)]. It should, however, be noted that there was 100-fold difference in the doses of GAG used: the weight ratio of GAG:Fe in the Caco-2 in vitro model was ~8000:1 compared with ~80:1 in the human in vivo study. It was further suggested that the meat factor may be the combined effect of peptides and other meat constituents (15). Meat protein is digested rapidly by pepsin in the stomach and generates a large quantity of iron-binding low molecular weight peptides (16).

The results of our in vitro experiments demonstrated that 1 of the meat digest fractions significantly enhanced nonheme iron uptake into Caco-2 cells, using a direct measure with radio-labeled iron rather than the indirect ferritin expression method used in parallel with the radio-iron method by Huh et al. (4). The active component of meat digest was L-α-glycerophosphocholine, a hydrolytic product of phosphotidylcholine (lecithin), which occurs in mammalian tissues and fluids (17). Phosphotidylcholine is one of the major phospholipids in cells and is composed of glycerophosphocholine and hydrocarbon chains attached via acyl, alkyl, or alkenyl linkages. It is the most abundant phospholipid in muscle (18), and foods containing the highest concentration of phospholipids include eggs and muscle and organ meats (19). It is also available commercially as a naturally occurring phospholipid precursor and metabolite derived from soy lecithin, which means it could be used to enhance nonheme iron absorption from vegetarian meals.

We investigated the effect of commercially available L-α on iron absorption from a Mediterranean vegetarian lasagna meal. The dose of L-α administered in the iron absorption study (46 mg) was selected because it was equivalent to the amount found in a medium portion of meat (19), thereby ensuring that the iron absorption test was relevant to the human diet. The dose of ascorbic acid added to the test meals was chosen such that the molar ratio to iron was the same as L-α, namely 2:1. Ascorbic acid is a potent enhancer of nonheme iron absorption and its effect is dependent on the iron molar ratio (20). The mechanism of action is chemical binding, which prevents the iron from either being precipitated as insoluble ferric hydroxide with the increase in pH as the chyme leaves the stomach and enters the duodenum, or being bound to other dietary substances (e.g., phytate and tannins) that prevent it being taken up by Divalent Metal Transporter-1 in the epithelial cells. Divalent metal ions are able to bind to lecithin and phosphatidylycholine, with size being an important determinant, suggesting that steric characteristics influence the phospholipid-metal interaction (21). It is possible that this is the mechanism whereby lecithins play a role in enhancing iron absorption.

The data from our human study suggest that phospholipids from beef may have an enhancing effect on nonheme iron absorption. Our results lend support to the argument that the meat factor effect stems from the binding of iron with a mixture of peptides from digested proteins and other constituents of meat, such as phospholipids. Iron deficiency anemia is still a major worldwide problem and any strategies that increase iron bioavailability would be of great benefit. L-α is water soluble, it caused no detectable sensory changes in the food to which it was added, and it appears to be relatively stable, although this would need confirmation with further testing in different food matrices. Adding L-α to food would be one way of enhancing the absorption of nonheme iron from the diet.

**FIGURE 4** % Iron absorption in women from vegetarian lasagna alone or with ascorbic acid or L-α. Values are means ± SEM. Asterisks indicate different from control. *\(P < 0.05\), **\(P = 0.01\).
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Literature Cited