Inhibition of haem-iron absorption in man by calcium

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The inhibiting effect of Ca on non-haem-Fe absorption is well established. Present studies showed that Ca inhibited haem-Fe absorption to the same extent when the same amount of Ca (165 mg Ca as $CaCl_2$) was added to a meal. Attempts were made to examine the mechanism for this inhibition in the present studies. Meat is the only known dietary factor influencing haem-Fe absorption. The present studies were designed to examine whether Ca interfered with the enhancing effect of meat on haem-Fe absorption. We found that the inhibition was the same whether biosynthetically radio-Fe-labelled haemoglobin was given in meals with or without meat. The haem-Fe absorption ratio with:without added Ca was 0·59 (SE 0·07) when Ca was added to a hamburger meal, and 0·52 (SE 0·03) when added to a wheat roll. These values were not significantly different (*t* 0·95; *P* = 0·35). The inhibition of haem-Fe absorption by Ca is, thus, a direct effect on the absorption of haem-Fe and not an indirect counteracting effect of the well-known enhancing effect of meat on haem-Fe absorption. Control studies were conducted to ensure that haem-Fe had not been degraded to non-haem-Fe during preparation of the foods. Since Ca inhibits the absorption of haem- and non-haem-Fe to the same extent, the present results strongly suggest that Ca interferes with the transport of Fe through the mucosal cell, and at a late stage, is common for haem- and non-haem-Fe transport. The observations that Ca strongly interferes with the absorption of both haem- and non-haem-Fe have important nutritional implications.

Iron absorption: Calcium: Man: Haem-iron: Non-haem-iron

Recently we made the unexpected observation that Ca, given as $CaCl_2$, milk or cheese, inhibited the absorption of non-haem-Fe in a dose-related manner (Hallberg *et al.* 1991). In one of these studies we also unexpectedly observed that haem-Fe absorption from hamburgers was also significantly reduced when given together with $CaCl_2$. Since the mechanisms for uptake into the mucosal cells are different for haem- and non-haem-Fe, these results suggested that the inhibition of the absorption of Fe by Ca was not located in the gastrointestinal lumen but rather in some unknown way related to the transport of Fe through the mucosal cells.

It is well known that meat can enhance the absorption of non-haem-Fe (Layrisse *et al.* 1968). It is also well known that considerably more haem-Fe is absorbed from a meal if given together with meat than if given without (Martinez-Torres & Layrisse, 1971; Hallberg *et al.* 1979; Hallberg, 1981). The mechanism behind this enhancing effect of meat on both haem- and non-haem-Fe absorption is still not established. The observation in the previous study (Hallberg *et al.* 1991) that less haem-Fe was absorbed from a hamburger meal when given with Ca may, thus, have two possible explanations. One, that Ca interferes with the enhancing effect of meat on haem-Fe absorption, the other that Ca directly influences the absorption of haem-Fe. These alternatives were considered important to clarify and were, therefore, examined in the present paper by measuring the effect of Ca on

haem-Fe absorption when biosynthetically radio-Fe-labelled haemoglobin was given with and without Ca, either in a meal containing meat (hamburger) or a non-meat meal (wheat rolls).

SUBJECTS AND METHODS

Subjects

Twenty-eight subjects, twelve men and sixteen women, participated in the experiments. All subjects were healthy volunteers aged 24–65 years. Each group included both men and women. Some of the subjects in each group were regular blood donors, which provided a reasonable range of intersubject variation in Fe absorption. Subjects were given written information about the aims and procedures of the study. The project was approved by the ethical committee of the Medical Faculty of the University of Göteborg, Sweden.

Experimental design

Two studies were made to establish whether Ca had a direct inhibiting effect on haem-Fe absorption or if the effect was indirect by counteracting the enhancing effect of meat on haem-Fe absorption. In study 1 biosynthetically ^{55}Fe- or ^{59}Fe-labelled rabbit haemoglobin was mixed into minced meat, and in study 2 the labelled haemoglobin was mixed into the dough of wheat rolls. In each study the same subjects were given meals with and without added Ca when labelling the meals with two different radio-Fe isotopes.

In both studies each meal was served with two wheat rolls, each prepared from 40 g low-extraction (55%) wheat flour to get rolls with a very low content of phytate. The dough also contained yeast, sugar, table salt and water. The non-haem-Fe content of two rolls (one serving) was adjusted to 3·8 mg by adding ferrous sulphate to the dough. The native Fe content of the two rolls was 0·3 mg/80 g flour.

In study 1 two hamburgers were served together with the two wheat rolls and 150 ml water. The two hamburgers in each meal contained together (g) minced meat 85, onion 5, boiled potatoes 8, egg 5, salt and pepper. Radio-Fe-labelled rabbit haemoglobin (^{55}Fe or ^{59}Fe) was mixed into the meat batter before cooking. Each serving contained 1 mg added haem-Fe and 0·5 mg native haem-Fe. The cooking was done at low temperature to avoid a conversion of haem-Fe into non-haem-Fe (Schricker & Miller, 1983; Martinez-Torres *et al.* 1987). As shown below, special studies were done to ensure that the present cooking procedure did not degrade the haem-Fe to non-haem-Fe.

In study 2, the ^{55}Fe- or ^{59}Fe-labelled rabbit haemoglobin was added to the water when making the dough. The two wheat rolls contained together 3 mg haem-Fe. They were served with butter and 150 ml water.

The haemoglobin-labelled hamburgers served with two plain rolls (study 1) or the haemoglobin-labelled wheat rolls (study 2) were given to each subject with or without 165 mg Ca as CaCl$_2$ (**a** and **b** respectively) on alternate mornings after an overnight fast on four consecutive days in the order **abba** or **baab**. The **a** and **b** rolls were labelled with the two different radio-Fe isotopes, ^{55}Fe and ^{59}Fe. A blood sample was drawn 2 weeks after serving the last meal to determine the content of ^{55}Fe and ^{59}Fe. The total retention of ^{59}Fe was measured by whole-body counting at the same time, and the total retention of ^{55}Fe was calculated from the ^{55}Fe:^{59}Fe value in erythrocytes. An oral reference dose (see p. 535) was then given to the fasting subject as well as a second dose on the following morning. The absorption of the reference dose was then measured by whole-body counting 2 weeks later (for the method of expression of results, see p. 535).

To examine if haem-Fe had been converted into non-haem-Fe by the baking and cooking procedures, two kinds of control experiments were made. The usual method to measure

whether the haem-Fe content in a meal had decreased during cooking is to determine the difference between the contents of total Fe and non-haem-Fe in the foods, before and after cooking (Schricker & Miller, 1983; Martinez-Torres et al. 1987).

Considering the importance of the present findings of an inhibition of the absorption of haem-Fe by Ca, we also conducted an experiment (study 3) where exactly the same hamburger meal as in study 1 was served to ten healthy volunteers. The haem-Fe in the meal was labelled as before with the ^{55}Fe-labelled rabbit haemoglobin. The non-haem-Fe in the same meal was also labelled with a trace amount of inorganic Fe using ^{59}Fe-labelled $FeCl_3$ in 0·1 M-HCl. Two servings were made of these doubly labelled hamburgers. After 2 weeks the Fe absorption of a ^{59}Fe-labelled reference dose was measured (see below).

Labelled haemoglobin was prepared by intravenous administration of radio-Fe into rabbits. Details of the procedure were previously described (Hallberg, 1980). In study 1 and study 2 each meal contained 37 kBq ^{55}Fe or ^{59}Fe. In study 3, where only two meals were served, each meal contained 75 kBq ^{55}Fe and 75 kBq ^{59}Fe.

Oral reference doses

A solution of 10 ml 0·01 M-HCl containing 3 mg Fe as $FeSO_4$ and 30 mg ascorbic acid labelled with ^{59}Fe was used as a reference in all studies. The 10 ml vials containing the Fe solution were rinsed twice with water, and this was also consumed. Each subject received two reference doses on two consecutive mornings after an overnight fast. No food or drink was allowed for 3 h after the reference dose. Each subject received a total of 36 kBq ^{59}Fe.

Expression of results of Fe absorption measurements

The mean of the individual absorption ratios, Fe absorption from meals with or without added $CaCl_2$, is an expression of the difference in bioavailability of haem between the two meals and an expression of the effect of the added Ca. These mean ratios are considered to be the most accurate basis for comparisons between different experiments (Magnusson et al. 1981).

The absorption of haem-Fe is much less influenced by the Fe status than non-haem-Fe absorption. This is the reason why absorption values in the present paper have not been adjusted to a 40% reference dose absorption as we usually do with non-haem-Fe absorption values.

Chemical measurements

Samples of rolls and hamburgers were freeze-dried and ground to a powder in a porcelain mortar. Weighed amounts of this powder were analysed for total Fe (Björn-Rasmussen et al. 1976), Ca (Clegg et al. 1981) and phytic acid-P, using the Association of Official Analytical Chemists' method (Harland & Oberleas, 1986). In rolls with a low content of phytate, 6 ml of the original HCl extract was used instead of 1 ml (original method) and the amount of NaOH–EDTA was increased proportionally. This modification markedly increased the lower limit of detection of phytate in the extract. Non-haem-Fe was determined according to Hallgren (1953).

Fe absorption measurements

Relative absorption of ^{55}Fe and ^{59}Fe was calculated from analyses of blood samples. Absolute absorption of the two tracers was calculated from whole-body counting of ^{59}Fe and the relative absorption of the two tracers. Analyses of ^{55}Fe and ^{59}Fe in blood were made by a modification of the method described by Eakins & Brown (1966), using a liquid-scintillation spectrometer (Tri-Carb, Packard Instruments, Texas, USA). All procedures and methods of calculation have been described previously (Björn-Rasmussen et al. 1974; Hallberg, 1980).

Table 1. *Effect of calcium as calcium chloride (165 mg Ca) on haem-Fe absorption from a meat-containing meal (hamburger) in human subjects**

| Subject | Haem-Fe absorption (%) | | Fe absorption (%) (reference dose) | Absorption ratio (with:without added Ca) |
	Without added Ca	With added Ca		
M†	21·2	12·9	36·0	0·61
F	40·4	9·7	82·1	0·24
F	15·4	9·8	36·6	0·63
F†	38·7	20·4	86·0	0·53
M	17·4	15·6	27·3	0·89
M†	16·8	12·3	45·2	0·73
F	9·8	6·5	34·8	0·66
M	21·8	9·3	12·9	0·43
Mean	22·7	12·1	45·1	0·59
SEM	3·9	1·5	9·1	0·07

M, male; F, female.
* For details of procedures, see pp. 534–535.
† Regular blood donor.

Statistical methods

All statistical analyses were made using a Statview II computer program (Abacus Concepts, Inc., Berkeley, CA, USA). For statistical comparisons the means with their standard errors of the individual absorption ratios in each experiment were used. The possible statistical significance of the difference between the mean absorption and 1 was examined by an unpaired, two-sided t test.

RESULTS

The addition of 165 mg Ca as $CaCl_2$ to the hamburgers in study 1 containing biosynthetically ^{55}Fe- or ^{59}Fe-labelled haemoglobin reduced the haem-Fe absorption by 41%. The absorption ratio with:without Ca was 0·59 (SE 0·07) which was highly significantly different from 1 (t 5·86; $P < 0.001$; Table 1).

In study 2, where 165 mg Ca was added to wheat rolls containing biosynthetically ^{55}Fe- or ^{59}Fe-labelled haemoglobin, haem-Fe absorption was reduced by 48%. The absorption ratio with:without Ca was 0·52 (SE 0·03), which was highly significantly different from 1 (t 13·1; $P < 0.001$; Table 2).

The inhibition of haem-Fe absorption by Ca in studies 1 and 2 was not statistically different. The t value in an unpaired two-sided test was 0·945 ($P = 0.35$).

In study 3, the absorption values of haem-Fe and non-haem-Fe from hamburgers identical to those served in study 1 were compared. The mean absorption of haem-Fe was 22·1 (SE 1·77)% and of non-haem-Fe 9·8 (SE 2·03)% (n 10). The mean value for the individual absorption ratios (haem-Fe:non-haem-Fe) was 3·71 (SE 1·01). This was significantly different from 1, t 2·7; $P = 0.0122$. Since these ratios were skewly distributed the same analysis was also made after log transformation. The logarithms of the ratios were also significantly different from 0 (t 4·17; $P = 0.0012$). The mean reference dose absorption in study 3 was 40·0 (SE 6·87)%. The reason for the skewness of the ratios is that the absorption of non-haem-Fe was strongly related to the absorption from the reference dose (r^2 0·771), whereas the corresponding relationship between absorption of haem-Fe and absorption of reference doses was much lower and not significant (r^2 0·042).

Table 2. *Effect of calcium as calcium chloride (165 mg Ca) on haem-Fe absorption from a non-meat meal (wheat rolls) in human subjects**

| Subject | Haem-Fe absorption (%) | | Fe absorption (%) (reference dose) | Absorption ratio (with:without added Ca) |
	Without added Ca	With added Ca		
M†	29·4	19·7	58·7	0·67
M†	24·3	15·4	22·7	0·63
M†	18·0	10·9	46·7	0·60
F	9·7	3·4	39·9	0·35
F†	41·6	23·9	35·6	0·57
F	17·7	9·5	22·2	0·54
F	17·3	9·6	53·5	0·56
F	19·2	7·7	35·0	0·40
M	11·5	4·6	12·0	0·40
M	14·6	7·1	31·2	0·49
Mean	20·3	11·2	35·8	0·52
SEM	2·3	1·7	4·5	0·03

M, male; F, female.

* For details of procedures, see pp. 534–535.

† Regular blood donor.

The contents of total Fe and non-haem-Fe were determined in the dough and the bread, and in the hamburgers before and after cooking. The studies were done in triplicate. All foods, cooked and uncooked, were freeze-dried at the same time. There was no increase in the content of non-haem-Fe in the hamburgers after cooking that might have suggested a degradation of haem-Fe. In the bread containing haem-Fe there was a slight increase in the content of non-haem-Fe after baking (26 mg/kg dry weight before baking and 28 mg/kg dry weight after baking). The total Fe content was 57 mg/kg dry weight and the calculated haem-Fe content thus decreased from 31 to 29 mg or 6%.

DISCUSSION

A main new finding in the present paper was that Ca inhibited haem-Fe absorption whether given with or without meat. This implies that the inhibition by Ca is a direct effect on the absorption of haem-Fe. The possibility that the inhibiting effect of Ca on haem-Fe absorption from hamburger meals might be due to a counteraction of the well-known enhancing effect of meat on haem-Fe absorption could, thus, be excluded.

The inhibiting effect of Ca on haem-Fe absorption was of the same magnitude as the inhibiting effect on the absorption of non-haem-Fe observed in a previous paper (Hallberg et al. 1991). The individual non-haem-Fe absorption ratios in the previous paper when wheat rolls were served with and without 165 mg Ca (mean value 0·54 (SE 0·07)) were not different from the individual haem-Fe absorption ratios in the present study (mean value 0·52 (SE 0·03)) when wheat rolls containing labelled haemoglobin were given with and without the same amount of Ca (Fig. 1). An unpaired, two-sided t test showed that the difference between the two means was not statistically significant (t 0·48, $P = 0·64$).

The inhibiting effect of Ca on haem-Fe absorption reported in a previous paper (Hallberg et al. 1991) was slightly lower (mean absorption ratio 0·76 (SE 0·07)) than in the present studies. The difference, however, from present results (0·59 (SE 0·07)) was not
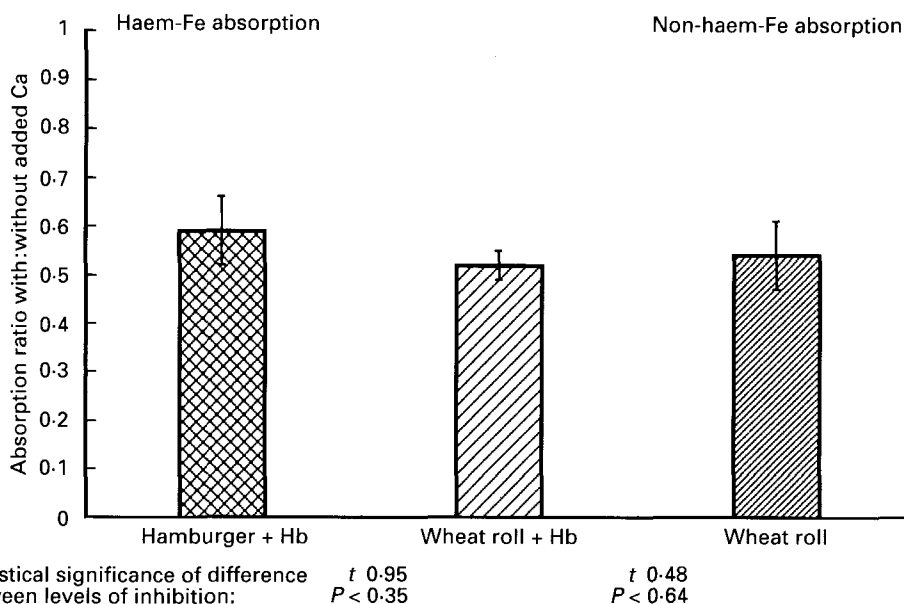
Fig. 1. Effect of calcium on inhibition of haem- and non-haem-Fe absorption in human subjects. Inhibition by Ca (165 mg as calcium chloride) of haem-Fe absorption from a hamburger meal and a wheat roll (present study) was similar to that of haem-Fe absorption from wheat rolls and non-haem-Fe absorption in a previous study (Hallberg *et al*. 1991). For detail of procedures, see pp. 534–535.

statistically significant when applying an unpaired, two-tailed t test (t 1·73; $P = 0·10$). A possible reason for the lower inhibition by Ca in the previous study (0·76 v. 0·59) might be related to the fact that a frozen batch of biosynthetically [55]Fe-labelled haemoglobin more than 2 years old was used in the previous study to label the haem-Fe in the hamburger given without Ca (Hallberg *et al*. 1991). This presumption is supported by the observed low mean absorption of haem-Fe of 14·6 % from these hamburger meals given without Ca in that study. This value is lower than is usually observed from haem-Fe given together with meat (Hallberg *et al*. 1979) and should be compared with the present absorption of 22·7 %. In this perspective it is, thus, possible that the haemoglobin used in the previous study might have been partly denatured during the long storage, and that the inhibitory effect of Ca on haem-Fe absorption might have been underestimated.

In the interpretation of the present results it is important to be able to exclude that any appreciable fraction of the haem-Fe had been converted into non-haem-Fe during the cooking, since that would invalidate our conclusion that Ca had an effect on the absorption of haem-Fe. The possibility of a degradation, however, was strongly contradicted by the observations that there was no increase in the non-haem-Fe content after cooking or baking in the hamburger or the bread. An even stronger support for the conclusion that haem-Fe was not even functionally degraded during cooking was obtained in study 3, in which both haem- and non-haem-Fe absorption were measured from an identical hamburger as served in study 1. The mean absorption of haem-Fe was about the same in study 3 as in study 1, and about the same as that observed by us and others from similar meals, containing both cooked and uncooked haem-Fe. Moreover, the absorption of the haem-Fe tracer was not related to the absorption from the reference dose, which was in sharp contrast to the absorption of the non-haem-Fe tracer from the same meals. It has previously been shown that this difference in pattern of absorption of haem- and non-

haem-Fe in relation to Fe status is typical for the two types of Fe (Hallberg *et al.* 1979; Bezwoda *et al.* 1983). There are, thus, different independent pieces of evidence strongly supporting the conclusion that haem-Fe had not been degraded to non-haem-Fe during cooking of the hamburgers and that only negligible amounts of the haem-Fe in bread might have been degraded. The observed effect of Ca in the present two studies (study 1 and study 2) must, thus, be considered as true inhibitory effects of Ca on haem-Fe absorption.

The mechanisms of absorption of haem- and non-haem-Fe are different. Haem-Fe is absorbed into the mucosal cells via a special receptor (Gräsbäck *et al.* 1979) and in the form of an Fe–porphyrin complex that is split within the mucosal cells (Weintraub *et al.* 1968; Raffin *et al.* 1974). The balance of evidence indicates, however, that the final transport stages of Fe from the mucosal cells to the plasma are common for haem- and non-haem-Fe (Hallberg & Sölvell, 1967). The present results thus strongly support the conclusion that there is a competitive inhibition between Ca and Fe in some of these final stages of intracellular transport in the intestinal mucosal cells. Further studies are needed to clarify the nature of such a stage and to investigate the possibility that there may be similar interactions between Ca and other minerals.

The practical implication of the present results is that the inhibition of Fe absorption by Ca is valid for both haem- and non-haem-Fe in the diet. In the large groups of subjects with high Fe requirements (children, teenagers and women at child-bearing age) attempts should be made to keep the Ca content low in most of the main meals providing most of the haem- and non-haem-Fe. This can be done, for example, by reducing the intake of dairy products with these meals and by covering most of the Ca requirements in breakfast meals and in the in-between meals. Both Fe and Ca are nutritionally essential. Present interactions between Ca and Fe must be considered in dietary recommendations, in the composition of single meals, and in the design of daily menus in order to satisfy the requirements for both nutrients in a feasible way.

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