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pH-sensitive transport of Fe^{2+} across purified brush-border membrane from mouse intestine

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Recent studies of Fe^{2+} uptake by mouse proximal intestine brush-border membrane vesicles revealed low-affinity, NaCl-sensitive and high-affinity, NaCl-insensitive, components of uptake (Simpson, R.J. and Peters, T.J. (1985) Biochim. Biophys. Acta 814, 381–388). In this study, the former component is demonstrated to show a strong pH dependence with an optimum of pH 6.8–6.9. Studies at pH 6.5, where the low affinity component is inhibited by more than 25-fold compared with pH 7.2, suggest that the pH-sensitive component represents transport across the brush-border membrane followed by intravesicular binding. Cholate extracts of brush-border membrane vesicles contain pH- and NaCl-sensitive Fe^{2+} binding moieties which may be involved in the transfer of Fe^{2+} across the intestinal brush-border membrane and subsequent binding inside the vesicles. Fe^{2+} uptake by brush-border membrane vesicles from the duodenum of hypoxic mice is higher than uptake by vesicles from control-fed animals, suggesting the existence of a regulable brush-border membrane Fe^{2+} carrier

Introduction

Recent studies of Fe^{2+} uptake by mouse proximal intestine brush-border membrane vesicles have identified two distinct uptake processes, one sensitive to extravesicular sugars and polyols, insensitive to extravesicular NaCl and being a rapid, high-affinity process (probably binding), the other, a slower, lower-affinity two-step process sensitive to NaCl but not to mannitol or cellobiose [1]. This provides a coherent explanation for most of the published studies of Fe^{2+} uptake by intestinal brush-border membrane vesicles (see references in Ref. 1).

Knowledge of the precise nature of the two-step, high-capacity uptake is critical to understanding the significance of the work of Cox and O'Donnell [2,3] on a putative brush-border 'receptor' for Fe^{2+}. Previous studies of the osmotic dependence of Fe^{2+} uptake failed to provide a simple explanation of the nature of the uptake process [1]. Until a satisfactory demonstration is made that Fe^{2+} transport occurs across isolated brush-border membrane, further steps to characterise the biochemical mechanisms of such transport are difficult, thus precluding an understanding of the mucosal uptake mechanism for Fe^{2+}.

In this paper, a further investigation of the effect of pH on the Fe^{2+} uptake processes occurring in brush-border membrane vesicles provides evidence that the predominant uptake is indeed transport across the brush-border membrane. Studies of uptake by vesicles isolated from hypoxic and control-fed animals are also presented.

Abbreviation: Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulphonic acid.

Material and Methods

General methods and materials. Brush-border membrane vesicle preparation, enzyme and protein determinations and materials were as described in Refs. 1 and 4. Fe^{2+} uptake was generally performed as described in Ref. 1. In general the vesicle protein concentration in uptake experiments was adjusted to give maximal uptakes of less than 30% of medium $^{59}Fe^{2+}$ (200 μg protein/ml of assay mixture). Assay mixtures with pH in the range 6.5–7.4 were prepared by mixing double strength assay buffers (0.2 M mannitol/0.2 M NaCl/40 mM Hepes) of pH 6.5 and 7.4. $^{59}Fe^{2+}$/ascorbate mixtures were prepared as described in Ref. 1. The pH of control incubations containing all assay components, except for the $^{59}FeCl_3$ in 10 mM HCl (10 mM HCl alone was used), and vesicles (vesicle resuspension buffer [1] was substituted) was determined and taken as the final assay pH for the corresponding uptake experiments.

Fe^{2+} uptake by vesicle preparations and extracts showed quantitative variability from preparation to preparation while qualitative observations were highly reproducible. Figures generally show representative results which were reproduced on at least three occasions.

Cholate extracts of brush-border membrane vesicles. Extracts of vesicles were prepared by adding solid sodium cholate or 10% sodium cholate in H_2O to vesicle suspensions (2.5–10 mg vesicle protein/ml). After clarification, the suspensions were centrifuged for 1 h at 165000 × *g* at 4°C. Pellets were resuspended in vesicle resuspension buffer by passage through a 21 gauge needle. Extracts were generally prepared from fresh vesicles. Some experiments were performed on extracts stored at −20°C or extracts prepared from frozen vesicles, however, all results were reproduced with fresh extracts. In general, approx. 70–80% of vesicle protein was released into these extracts. Cholate was removed from the extracts with a 0.5 cm × 15 cm Sephadex G-25 column equilibrated and eluted with 0.1 M mannitol/0.1 M NaCl/20 mM Hepes (pH 7.4).

Experimental animals. Male, To-strain (6–8-week-old) mice were used throughout. Hypoxia was achieved by placing mice in a hypobaric chamber at 0.5 atmosphere for 3 days. These mice consumed approx. 1/3 of the diet eaten by normal mice over 3 days, irrespective of initial body weight (range 20–34 g). Age- and weight-matched control mice were individually fed the mean daily diet consumed by hypoxic mice.

Results and Discussion

Stimulation and inhibition of Fe^{2+} transport rates

The demonstration that Fe^{2+} uptake by brush-border membrane vesicles is transport across the brush-border membrane is difficult, given the contradictory effects of osmotically active solutes on uptake [1]. This is perhaps not surprising given the possibility that Fe^{2+}, if inside the vesicles, might be bound by a variety of functional groups present in proteins and lipids and therefore would not behave as a simple solute. Similar problems might be expected with studies of Fe^{3+} uptake by brush-border membrane vesicles, however the action of membrane permeability perturbents such as detergents or lipid soluble iron ligands can suggest that uptake is indeed transport. If the uptake rate, but not endpoint, is affected by these agents then membrane transport is implicated. Such effects were obtained with the relatively slow Fe^{3+} uptake process operating in mouse brush-border membrane vesicles [4]. Fe^{2+} uptake is much more rapid, hence large stimulation in rate by the detergent cholate cannot be observed (see Fig. 1). More convincing evidence would be obtained by inhibition of the Fe^{2+} transport rate without affecting the endpoint.

Fig. 2 shows the effect of lowering medium pH on Fe^{2+} uptake by mouse proximal intestine brush border membrane vesicles. At pH 6.5 the time course shows two distinct components; a rapid, low capacity component and a long, slow uptake whose endpoint approaches the endpoint obtained at pH 7.2. Addition of low concentrations of 8-hydroxyquinoline stimulates the rate, without greatly affecting the endpoint of the uptake process. These results confirm the presence of two components of uptake; a rapid, low capacity component and a large capacity component, as was proposed from investigations of the effect of NaCl on Fe^{2+} uptake [1]. The rate of the large capacity process was found to be inhibited by 25–56-fold (45 ± 5, mean

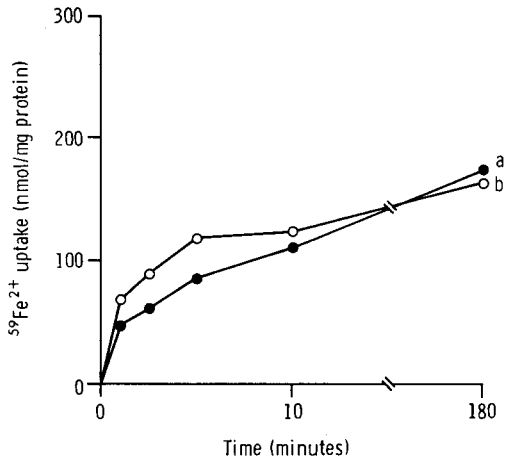

Fig. 1. Effect of cholate on $^{59}Fe^{2+}$ uptake by mouse proximal intestine brush-border membrane: (a) $^{59}Fe^{2+}$ uptake time course for vesicles incubated in 90 μM Fe^{2+}/1.8 mM sodium ascorbate/0.1 M mannitol/0.1 M NaCl/20 mM Hepes, final pH 7.2; (b) as (a) except that 0.01 vol. of 10% cholate was added to the incubation mixture before addition of vesicles. Incubations were performed at 21°C and uptake determined by Millipore filtration, as in Ref. 1. Vesicles were prepared from the proximal one-third mouse small intestine as described previously [1].

$\pm$ S.E., $n = 6$) by lowering the pH from 7.2 to 6.5. The large capacity component demonstrates properties consistent with it being a transport process across the brush border membrane.

Fig. 2 plus the results in Ref. 1 suggest at least 3 distinct Fe^{2+} binding sites may be implicated in brush-border membrane vesicles; (1), a high-affinity, low-capacity binding site, relatively pH- and NaCl-insensitive and possibly on the outside surface of the vesicles, (2) a lower-affinity, highly pH-sensitive transporter which presumably binds Fe^{2+}, (3) intravesicular site(s), less sensitive to reduction of extravesicular pH from 7.2 to 6.5 and presumably with large binding capacity for Fe and a higher affinity for Fe^{2+} than the transporter.

Fig. 3 shows the pH dependence of initial ($t = 1$ min) Fe^{2+} uptake by vesicles at 90 μM Fe^{2+} in the pH range 7.2 to 6.5. This dependence is similar to that reported for rabbit duodenal vesicles by Cox and O'Donnell [2] and shows an optimum at about pH 6.8–6.9. Those workers, however, did not consider a possible multi-component nature of uptake. Fig. 3 also shows the pH dependence of initial uptake with the presence of NaCl and at low Fe^{2+} concentrations. These show no optimum in the pH

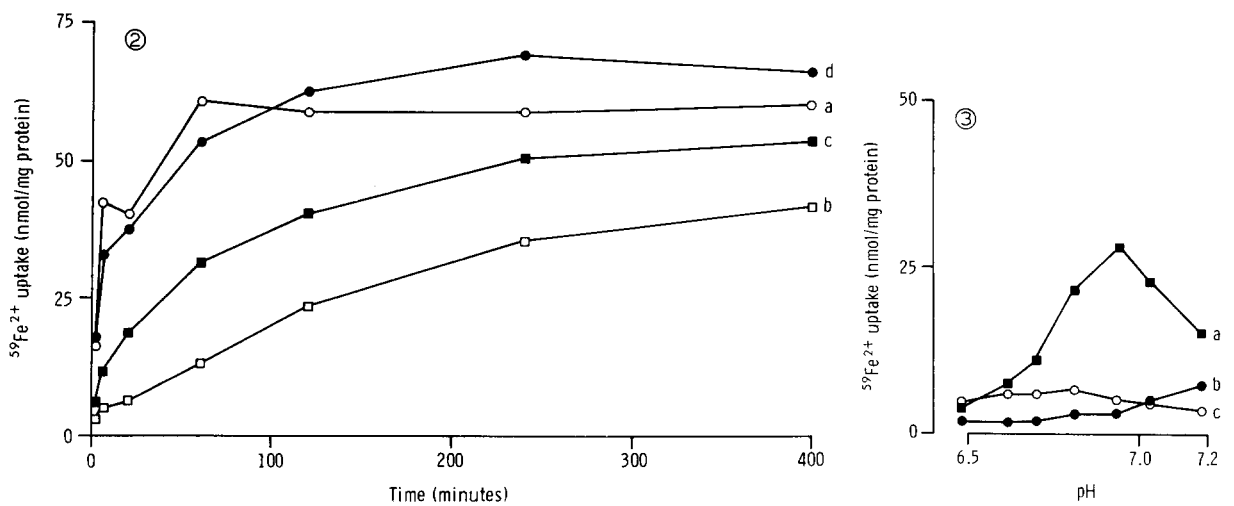

Fig. 2. Effect of lowering pH and addition of 8-hydroxyquinoline on Fe^{2+} uptake by brush-border membrane vesicles: (a) vesicle uptake at pH 7.2, conditions were as Fig. 1a; (b) vesicle uptake at pH 6.5, other conditions were as Fig. 1a; (c) as (b) except that 1.8 μM (final) 8-hydroxyquinoline (hemisulphate salt) was added to the incubation mixture; (d), as (c) except that the final 8-hydroxyquinoline concentration was 9 μM.

Fig. 3. Dependence of initial uptake of Fe^{2+} by brush-border membrane vesicles on pH: (a) incubation conditions other than pH were as in Fig. 1a, incubation time was 1 min; (b) as (a) except that 1.8 M NaCl was added; (c) as (a) except that the Fe^{2+} concentration was 9 μM and the sodium ascorbate 180 μM.

range studied and indicate that the high-affinity, NaCl-independent component of uptake is relatively pH independent. Correction of total uptake for this component would therefore lead to the very pronounced inhibition of the 'transport' component found when the pH was lowered from 7.2 to 6.5 (Fig. 2 above).

On the basis of these results it may be proposed that an Fe^{2+} transporter with a characteristic pH dependence may be present in the mouse (and presumably rabbit) proximal intestine brush-border membrane. The above interpretations were further tested by studies with detergent extracts of brush-border membrane which may contain iron binding components but should not demonstrate a transport component of uptake.

Fe^{2+} uptake by cholate extractable constituents of brush-border membrane vesicles

Fig. 4A suggests that cholate extracts of brush-border membrane vesicles contain Fe^{2+} binding components which are retained by 0.22 μm Millipore filters after dilution of the cholate in the assay mixture. The time course for uptake by these extracts was found to be characteristically more rapid (uptake terminated by 20 min, usually within 10 min) than by intact vesicles.

The ability of extracts to bind large amounts of Fe^{2+} and be retained by Millipore filters has so far made attempts to reconstitute Fe^{2+} transport activity in phospholipid liposomes unconvincing since extracts alone demonstrate very high apparent Fe^{2+} uptake. Resuspended pellets from the extraction procedure demonstrated approx. 5–10-fold lower apparent Fe^{2+} uptake per mg protein than the extracts when assayed by Millipore filtration.

Fig. 4A also shows that the apparent uptake by vesicle extracts depends on the final cholate concentration in the assay. High cholate concentrations lead to reduced uptake although this appears to be an end-point rather than a rate effect. The reduced uptake at higher cholate concentrations may be due to a reduction in retention of Fe^{2+}-binding components by the filters.

Uptake by extracts was found to be NaCl- and pH-sensitive (Fig. 4B), however, the dependence of uptake on pH and NaCl was variable from extract to extract. Inhibition by 1.8 M NaCl in the range

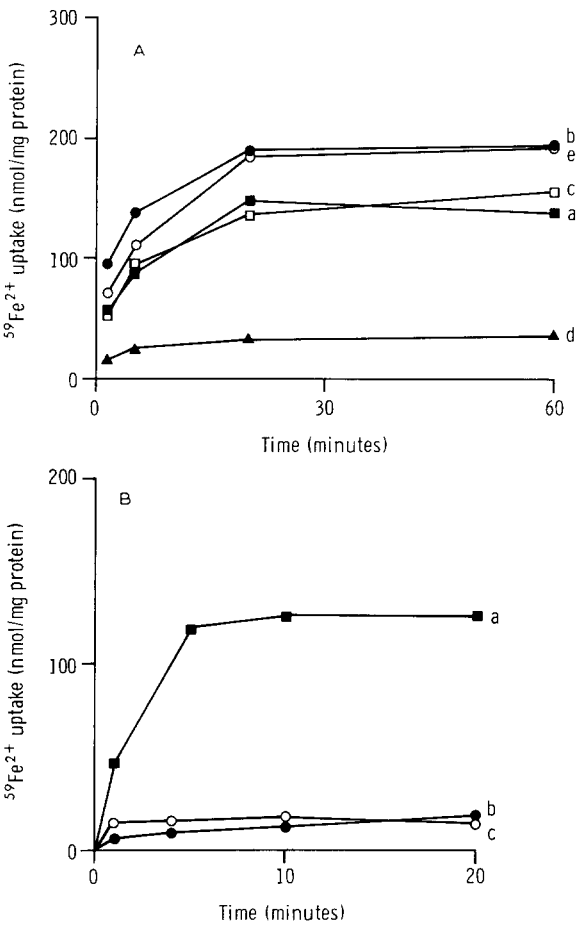

Fig. 4. Uptake of Fe^{2+} by cholate extracts of mouse proximal intestine brush-border membrane vesicles. (A) Extracts were prepared by addition of 10% (w/v) cholate to vesicle preparations (see Materials and Methods) and centrifugation at 165000 $\times g$ for 1 h. The clear extract was passed down a Sephadex G-25 column and the excluded fraction collected. This cloudy, approx. 5-fold diluted extract was assayed for Fe^{2+} uptake as for vesicles in Fig. 1a in the presence of final cholate concentrations (w/v) of: (a) 0%; (b), 0.18%; (c), 0.35%; (d) 0.7%. In (e), original extract prior to gel filtration was also assayed for Fe^{2+} uptake with a final cholate concentration of 0.18%. Results for both extracts are expressed relative to assayed protein. (B) Extract was prepared by addition of 10% cholate (w/v) and centrifugation at 165000 $\times g$ for 1 h. Fe^{2+} uptake was assayed: (a) as in Fig. 1a (final cholate concentration, 0.24%); (b) as (a) except the pH was 6.5; (c), as (b) except 2.4 μM 8-hydroxyquinoline was added.

3–100-fold has been obtained. Uptake on reducing pH from 7.2 to 6.5 decreased 2.2–13-fold. It was also found that uptake at low pH is not stimulated by 8-hydroxyquinoline (Fig. 4B). These findings

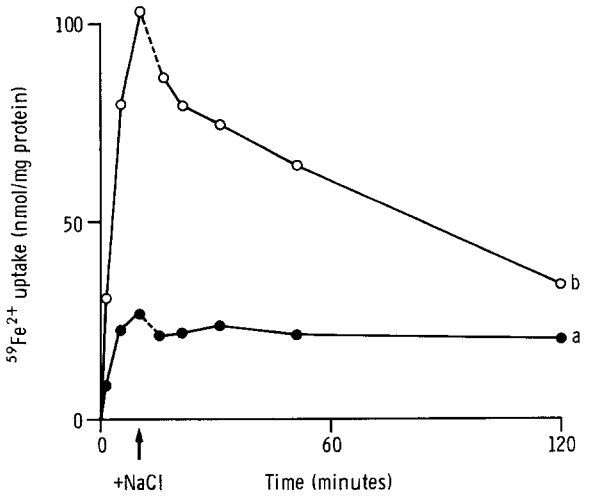

Fig. 5. Effect of added NaCl on $^{59}Fe^{2+}$ taken up by (a) brush-border membrane vesicles and (b) cholate extract. Incubation media were as in Fig. 1a. At 11 min, solid NaCl was added to a final concentration of 1.8 M. This dissolved within 30 s. The extract was prepared as described in Fig. 4A from the same vesicles preparation as was used for (a).

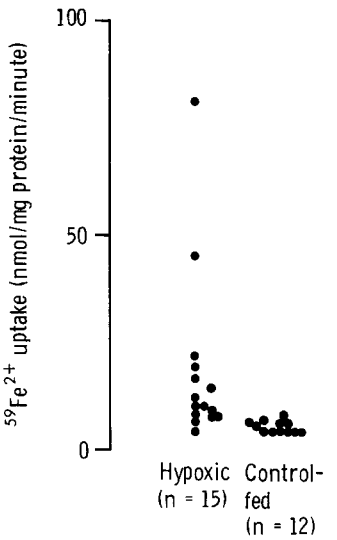

Fig. 6. Initial uptake by duodenal brush-border membrane vesicles prepared from hypoxic and control-fed mice. Uptake values are for 1 min incubation as in Fig. 1a with the number of preparations between parentheses.

are consistent with the proposal that extracts contain mixtures of Fe^{2+} binding components with different pH and NaCl sensitivities.

Reversibility of Fe^{2+} uptake by extracts

We reported previously [1] that NaCl inhibits Fe^{2+} uptake by intact vesicles when present throughout the assay but does not displace Fe^{2+} which has already been taken up (see Fig. 5a). This effect suggests that uptake by vesicles is a two-step process, i.e., transport followed by binding. Fig. 5b demonstrates that Fe^{2+} uptake by extracts is reversible and bound Fe^{2+} can be largely displaced by addition of high NaCl concentrations. This finding is again consistent with our interpretation that uptake by extracts is a simple binding process.

We believe that the contrast between the effects of NaCl, 8-hydroxyquinoline, cholate and incubation time on Fe^{2+} uptake by intact vesicles and their effects on Fe^{2+} uptake by extracts provides strong evidence that vesicle Fe^{2+} uptake is predominantly a transport process.

Adaptive regulation of Fe^{2+} uptake by brush-border membrane vesicles

A significant change in total mucosal Fe^{2+} uptake in vivo by mouse duodenum has been observed when hypoxic animals are compared with control-fed animals [6]. If vesicles are prepared from the duodenum of control-fed animals (Fig. 6), initial Fe^{2+} uptake is found to be significantly reduced when compared with hypoxic animals ($P < 0.0005$, Wilcoxon's rank sum test for nonnormally distributed data [7]). These results confirm that the major Fe^{2+} uptake component operating in isolated brush border membrane vesicles is subject to adaptive changes in parallel with in vivo changes in flux of Fe^{2+} across the brush-border membrane. Cox and O'Donnell [5] have demonstrated that uptake of Fe^{2+} by rabbit brush-border membrane vesicles is enhanced when vesicles are isolated from Fe-deficient rabbits. Previously we reported no significant increase in uptake by vesicles prepared from hypoxic compared with normal mice [1]. This finding may relate to the observation that total mucosal Fe^{2+} uptake in vivo is not enhanced by hypoxia when compared with normally fed animals [6]. Increased Fe^{2+} absorption in hypoxia is due to enhanced transfer of Fe^{2+} from the intestinal mucosa to the animal [6]. Enhancement of Fe^{3+} uptake by brush-border membrane vesicles in hypoxia has been previously reported [8].

Conclusions

(1) Fe^{2+} uptake by mouse proximal intestine brush-border membrane vesicles consists of a rapid, high-affinity, pH- and NaCl-insensitive, low-capacity binding and a slower, lower-affinity, highly pH- and NaCl-sensitive transport across the brush-border membrane to high-capacity binding site(s) within the vesicles.

(2) Fe^{2+} transport by vesicles is subject to adaptive regulation in response to physiological stimuli.

(3) Cholate extracts of brush-border membrane vesicles contain pH and NaCl-sensitive Fe^{2+} binding moieties.

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References

1 Simpson, R.J. and Peters, T.J. (1985) Biochim. Biophys. Acta 814, 381–388
2 Cox, T.M. and O'Donnell, M.W. (1981) Biochem. J. 194, 753–759
3 O'Donnell, M.W. and Cox, T.M. (1982) Biochem. J. 202, 107–115
4 Simpson, R.J. and Peters, T.J. (1984) Biochim. Biophys. Acta 772, 220–226
5 Cox, T.M. and O'Donnell, M.W. (1980) Biochem. Int. 1, 446–454
6 Simpson, R.J., Raja, K.B. and Peters, T.J. (1986) Biochem. Soc. Trans., in the press
7 Wetherill, G.B. (1967) Elementary Statistical Methods, Methuen and Co. Ltd., London
8 Simpson, R.J., Raja, K.B. and Peters, T.J. (1985) Biochim. Biophys. Acta 814, 8–12