Biochimica et Biophysica Acta, 984 (1989) 262–266
Elsevier

BBAMEM 74552

Membrane potential dependence of Fe(III) uptake by mouse duodenum

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(Received 15 March 1989)
(Revised manuscript received 5 June 1989)

Key words: Hypoxia; Iron absorption; Valinomycin; Nigericin; Glucose uptake; (Mouse duodenum)

Intestinal iron uptake by mouse duodenal fragments is inhibited in the absence of oxygen and glucose from the incubation medium and by a variety of metabolic inhibitors. The mechanism of energy coupling to iron uptake is, however, unclear. In vitro experiments using duodenal fragments showed Fe^{3+} uptake to be markedly inhibited, in a reversible fashion, by the replacement of incubation medium Na^+ by K^+. Addition of phloridzin to the medium failed to affect iron uptake, suggesting that the above effect was not a consequence of reduced glucose uptake. Substitution of Na^+ by Rb^+ also potently reduced duodenal iron uptake. Replacement of medium NaCl by either mannitol or choline chloride had no significant effect on Fe^{3+} uptake, thus excluding the possibility of the Fe^{3+} uptake process being Na^+-dependent. Similar observations were made with duodenal fragments from animals with enhanced Fe^{3+} absorption, due to chronic hypoxia. Valinomycin (1–5 μM) increased the uptake of both glucose and Fe^{3+}. Higher concentrations (22.5 μM) of the ionophore were inhibitory. In vivo studies (tied-off segments) using Rb^+-containing medium confirmed the inhibitory effects of univalent cations on Fe^{3+} absorption. Enhanced absorption of Fe^{3+} was also demonstrable in vivo, with low concentrations of valinomycin and nigericin added to the luminal medium. These observations suggest that the Fe^{3+} uptake process may be dependent on the brush-border membrane potential.

Introduction

Iron homeostasis is maintained primarily by controlling intestinal iron absorption. The absorptive mechanism entails at least two kinetically distinct steps: (i) 'uptake' of iron from the lumen into the mucosa and (ii) following intracellular processing, the 'transfer' of some/all of the iron from the mucosa to the plasma [1]. Our previous studies have shown the 'uptake' phase to involve at least two pathways [2]: Pathway one is facilitated by free fatty acids [3], whilst pathway two, which is quantitatively more important, requires metabolically active cells to function [2]. The latter pathway is sensitive to metabolic inhibitors [2] and exhibits saturation-kinetics (carrier-mediated) and adaptive responses to changes in iron requirements [4]. The coupling between enterocyte iron uptake and the expenditure of metabolic energy is, however, unclear. There are at least two possible means by which the two processes may be interlinked. Firstly, ATP may be hydrolysed during the carrier-mediated transport of the metal, analogous to the Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase systems operating at many cell membranes. Alternatively, iron transport may be coupled to ionic gradients either directly (co-transport of other ions) or indirectly through electrogenic ion-transport. In this study, we set out to investigate the latter possibilty, through manipulation of ionic gradients and thus, the electric potentials across the duodenal mucosa.

Materials and Methods

Reagents. All chemicals and biochemicals were from either BDH Chemicals (Poole, Dorset) or Sigma Chemical Co Ltd. (Poole). Radiochemicals were from Amersham International (Amersham, Bucks).

Animals. Male mice, To strain, 6–8 weeks old were used throughout. Hypoxia was initiated by placing mice in a hypobaric chamber maintained at 53.3 kPa (0.5 atm) for 3 days.

In vitro uptake studies. A previously described in vitro technique [4] was used for determining initial rates of iron uptake by intestinal mucosal fragments. Mice

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were killed by cervical dislocation and pieces of duodenal tissue removed from the first 5 cm proximal to the pylorus. The tissue was cut longitudinally and sectioned into tissue fragments (2–10 mg wet weight). After rinsing in oxygenated buffer (16 mM Hepes-Tris, 125 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 10 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4), the fragments were incubated in the same buffer containing $^{59}Fe^{3+}$-nitrilotriacetate (NTA) with ^{57}Co-cyanocobalamin as the extracellular fluid marker. Parallel incubations were performed with tissue fragments from the same animal, using media in which ionic manipulations had been introduced. Such a protocol minimises the likelihood of differences arising due to the variability in uptake rates which may occur between different groups of mice. Ionophores were dissolved in ethanol before use. Control media contained corresponding amounts of ethanol (0.1% final concentration). Inclusion of ethanol at these concentrations has been found not to affect the duodenal Fe^{3+} uptake rate. Fragment uptake was corrected for non-mucosal (serosal) entry of $^{59}Fe^{3+}$ by performing similar incubations with right side out tied-off duodenal segments [4].

Glucose uptake by fragments was performed by the *in vitro* technique described above, except that the incubation medium contained 10 mM 3-*O*-methyl-D-[^{3}H]glucose and ^{51}Cr-EDTA as the ECF marker. After incubation, tissue samples were counted for gamma radiation, before being oxidised in a Tri-Carb sample oxidiser, Model B306 (Canberra Packard, Pangbourne, Berks). The resulting material was counted for the tritium label in 'Monophase S' (Canberra Packard) scintillation fluid, and the glucose uptakes (corrected for the extracellular fluid (ECF) space) expressed as nmol/mg tissue per min.

In vivo iron uptake. In situ tied-off duodenal segments were used to determine intestinal iron absorption [5]. A small volume (50–100 μl) of physiological medium containing $^{59}Fe^{3+}$ as Fe^{3+} : NTA (1 : 2) was instilled into the duodenal segment of the anaesthetised mouse. The segment was prewashed with 0.6 ml of 0.15 M NaCl (at 37 °C) before the addition of $^{59}Fe^{3+}$. Following a 10 min incubation, the segment was removed, flushed with ice cold NaCl (0.15 M), weighed and counted in a gamma counter. The carcass was counted similarly for 5 min in a high resolution bulk sample counter [6]. After weighing the duodenal segment, the activity of ^{59}Fe present in either the tissue (mucosal retention) or carcass (mucosal transfer) was expressed as pmol/mg wet weight intestine. All experiments were performed under maximal uptake (V_{max}) conditions (i.e. Fe^{3+} concentration, 250 μmol/l).

Intracellular Na^+ and K^+ measurements. Isolated duodenal tissue (4–5 cm) was cut longitudinally, blotted and incubated in the appropriate incubation medium for 10 min. Thereafter, it was removed, blotted thoroughly and the mucosa scraped off on an iced plate. The weighed tissue was suspended in 20 mM nitric acid, and homogenised in a small Dounce homogeniser (Kontes Glass Co. Ltd., NJ, U.S.A.). After brief centrifugation (1800 rpm × 5 min) the supernatant was separated and analysed for the Na^+ and K^+ content with a Corning 430 Flame Photometer.

Results

Importance of glucose and oxygen for in vitro Fe^{3+} uptake

In addition to the effect of metabolic inhibitors [2], the dependence of the initial Fe^{3+} uptake process on the metabolic energy was demonstrated by the reduction in the mucosal flux of ^{59}Fe when fragments were incubated in either glucose-free medium or medium aerated with nitrogen (Table I).

Effect of univalent cations on Fe^{3+} uptake

Table II shows the effect of replacing medium Na^+ with equimolar amounts of other cations on duodenal Fe^{3+} uptake. Incubation of tissue fragments in a medium in which Na^+ had been almost totally replaced by K^+ (final Na^+ concentration = 10 mM) was accompanied by changes in the tissue cation concentration (tissue Na^+: control medium 0.09 ± 0.01 μmol/mg tissue, low Na^+/high K^+ medium 0.06 ± 0.01 (2) μmol/mg; tissue K^+: control medium 0.07 ± 0.003 (4) μmol/mg, low Na^+/high K^+ medium 0.13 ± 0.01 (2) μmol/mg) and resulted in a marked reduction in the mucosal Fe^{3+} uptake rate. In contrast, addition of phloridzin (a specific hexose transport inhibitor; [7]) or the equimolar replacement of medium Na^+, K^+ and Cl^- with mannitol did not have any significant effects upon the duodenal Fe^{3+} uptake rates.

Substitution of medium NaCl by choline chloride also failed to alter the Fe^{2+} uptake rate. Similar observations were made using fragments from hypoxic (3 d at 0.5 atm) animals (Table III), a suitable model of enhanced iron absorption [4]. These data thus indicate firstly, that the reduced iron uptake is not a conse-

TABLE I

Effect of glucose and oxygen on in vitro Fe^{3+} uptake

Result: mean uptake ± S.E. for 6–8 determinations. The uptake values have been corrected for non-mucosal entry of ^{59}Fe. * $P < 0.01$, ** $P < 0.001$ as compared to values in normal oxygenated medium.

Medium	Uptake (pmol/mg per min)
Normal medium (aerated with 95% O_2/5% CO_2)	5.7 ± 0.5
Glucose-free medium	3.4 ± 0.3 *
Normal medium (aerated with 95% N_2/5% CO_2)	2.4 ± 0.4 **

TABLE II

Effect of cations on mucosal $^{59}Fe^{3+}$ uptake by duodenal fragments from normal mice

Results: mean ± S.E. for 6–10 determinations. Duodenal fragments were obtained from normal mice. These fragments were divided into two groups, one group incubated in control medium and the other in experimental medium. The tissue samples were pre-incubated in the appropriate medium for 5 min, followed by a further 5 min in similar medium containing ^{59}Fe and ^{57}Co. The uptake values for the duodenal fragments have been corrected for non-mucosal entry. Medium Fe^{3+} 250 μmol/l, NTA 500 μmol/l. Statistical analysis by Student's '*t*'-test: * $P < 0.01$, ** $P < 0.001$.

Medium	Uptake (pmol/mg per min)		Inhibition (%)
	control	experimental	
Low Na^+/high K^+	5.0±0.4	1.2±0.2 **	76
Phloridzin (0.5 mM)	3.9±0.6	4.4±1.0	0
Mannitol	3.1±0.4	3.1±0.7	0
Choline chloride (NaCl-free)	3.8±0.4	3.6±0.7	5
Replacing Na^+			
Li^+	3.1±0.4	1.4±0.4 *	55
Cs^+	3.8±0.4	1.6±0.3 **	58
Rb^+	3.2±0.4	0.2±0.3 *	94

quence of reduced glucose uptake and secondly, that iron uptake is not a Na^+-dependent process. K^+, on the other hand, appears to have marked inhibitory effects.

The data in Fig. 1 shows that fragments preincubated in normal physiological medium had a reduced uptake rate when transferred to a medium containing a low Na^+/high K^+ concentration. In addition, fragments which had been preincubated in the low Na^+/high K^+ medium had normal Fe^{3+} uptake rates when transferred to normal medium, suggesting that the inhibition is reversible.

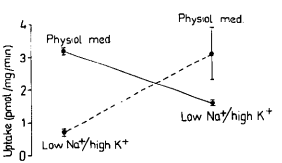

Fig. 1. Duodenal fragments, isolated from control animals were incubated in either physiological or low Na^+/high K^+ media. After 5 min, $^{59}Fe^{3+}$ uptakes were determined in half the fragments in each medium. The remaining fragments were transferred to either low Na^+/high K^+ or physiological medium, respectively, and uptakes determined after a further 5 min. Results are means ± S.E. for 6–10 determinations. Medium Fe^{3+} 90 μM. Molar ratio Fe^{3+}/NTA, 1:2.

Marked inhibition in uptake rate (> 50%) was also observed when medium Na^+ was replaced by other monovalent cations (Tables II and III). Rb^+ was found to have the most potent effect: A 50% reduction in the Fe^{3+} uptake rate (I_{50} value) was observed at approx. 30 mM Rb^+. The inhibitory effects of Rb^+ on Fe^{3+} uptake was confirmed in vivo with in situ tied-off duodenal segments injected with control or Rb^+-containing (125 mM) medium (total mucosal uptake: control medium 61.6 ± 3.6 (3) pmol/mg per 10 min; +Rb medium 41.9 ± 4.9 (5), $P < 0.05$).

Effect of ionophores on Fe^{3+} uptake

Electric potentials exist across cellular membranes, due to ionic gradients between the intra- and extra-cellular environments (inside being more negative).

TABLE III

Effect of cations on mucosal $^{59}Fe^{3+}$ uptake by duodenal fragments from 3d hypoxic mice

Results: mean ± S.E. for 3–8 determinations. Duodenal fragments were pre-incubated in either control or experimental medium for 5 min followed by a further 5 min in similar medium containing ^{59}Fe and ^{57}Co. The uptake values have been corrected for the non-mucosal entry. Medium Fe^{3+} 250 μM, NTA 500 μM. Statistical analysis by Student's '*t*'-test: * $P < 0.001$.

Medium	Uptake (pmol/mg per min)		Inhibition (%)
	control	experimental	
Low Na^+/high K^+	11.0±0.9	1.4±0.4 *	87
Phloridzin (0.5 mM)	12.2±1.8	11.1±0.6	9
Mannitol	15.9±1.3	14.9±0.3	6
Choline chloride	10.9±1.3	10.1±0.8	7
Replacing Na^+			
Li^+	11.0±0.9	2.2±0.5 *	80
Cs^+	11.0±0.9	2.6±0.3 *	76
Rb^+	11.0±0.9	0.2±0.3 *	98

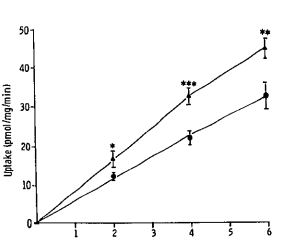

Fig. 2. Time course for $^{59}Fe^{3+}$ uptake by mouse duodenal fragments in the presence (▲) and absence (●) of 5 μM valinomycin. Fe^{3+} = 250 μM, molar ratio Fe^{3+}/NTA, 1:2. Results are means ± S.E. for 4–7 determinations at each time point. Statistical analysis by Student's '*t*'-test: * $P > 0.05$, ** $P < 0.05$, *** $P < 0.02$ as compared to control values at that specific time point.

TABLE IV

$^{59}Fe^{3+}$ absorption from in vivo tied-off duodenal segments

Results: mean ± S.E. for (n) animals. Segments were prewashed with 0.15 M NaCl (at 37°C) prior to $^{59}Fe^{3+}$ incubation. Fe^{3+} 250 μM, molar ratio Fe/NTA, 1:2. * $P < 0.02$, ** $P < 0.01$ as compared to control values.

Medium	n	$^{59}Fe^{3+}$ absorption (pmol/mg per 10 min)		
		mucosal retention	mucosal transfer	total mucosal uptake
Normal	4	28.5 ± 1.7	20.5 ± 1.5	49.0 ± 2.1
+ 5 μM valinomycin	4	39.9 ± 2.3 **	24.6 ± 1.8	64.4 ± 2.7 **
+ 1.5 μM nigericin	3	42.1 ± 3.9 *	31.4 ± 2.9 *	73.6 ± 6.2 **

Changes in membrane potential may thus result from alterations in the ionic composition of the external milieu [8–11]. In order to evaluate the role of the transmembrane potential difference in the uptake of Fe^{3+}, experiments were performed in the presence of valinomycin, which hyperpolarises membranes by specifically increasing the K^+ permeability [12]. Fragments incubated in normal physiological medium containing valinomycin (5 μM) did exhibit enhanced $^{59}Fe^{3+}$ uptake rates, as compared to control values (Fig. 2). Stimulation was even evident, though to a lesser extent, at lower valinomycin (1 μM) concentrations (incubation time 4 min, Fe^{3+} uptake: control 29.9 ± 1.9 (5) pmol/mg; + valinomycin 37.8 ± 4.2 (6), $P < 0.2$). In experiments in which a higher valinomycin concentration (22.5 μM) was used and the fragments preincubated (5 min) in this medium, a reduction in the corrected mucosal uptake was seen (control 3.4 ± 0.1 (3) pmol/mg per min; + valinomycin 2.2 ± 0.1 (3), $P < 0.05$).

Nigericin, another ionophore which increases K^+ permeability, though to a lower extent than valinomycin [13], was also found to significantly stimulate the duodenal Fe^{3+} uptake rate (Fe^{3+} = 250 μM: control medium 7.1 ± 0.4 (7) pmol/mg per min; + 1.4 μM nigericin 8.7 ± 0.5 (9) pmol/mg per min, $P < 0.05$).

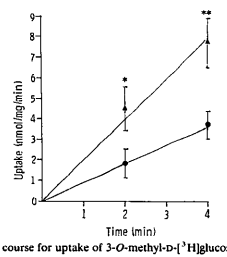

Fig. 3. Time course for uptake of 3-*O*-methyl-D-[^{3}H]glucose by mouse duodenum, in the presence (▲) and absence (●) of 5 μM valinomycin. Results are means ± S.E. for three or four determinations at each time point. Statistical analysis by Student's '*t*'-test: * $P > 0.05$, ** $P < 0.05$.

Absorption studies performed in vivo, confirmed the enhancement in uptake rates, when low concentrations of valinomycin (5 μM) or nigericin (1.5 μm) were included in the incubation medium (Table IV).

Effect of valinomycin on in vitro glucose uptake

The importance of Na^+ in the transmembrane transport of sugar(s) across the small intestine is well documented [14,15]. More recent studies on intestinal cells have, however, proposed that the membrane potential, in addition to the Na^+-gradient is, an important component of the thermodynamic driving force for glucose transport [11,16]. The stimulation in glucose uptake in the presence of valinomycin (5 μM) in the medium (Fig. 3) confirms this proposal. Detectable changes in the uptake rates were also evident at low concentrations (1 μM) of the ionophore (control 1.4 ± 0.2 (3) nmol/mg per min; + valinomycin 2.4 ± 0.4 (3) nmol/mg per min, $0.05 < P < 0.10$). Higher concentrations of ionophore were inhibitory (control 1.6 ± 0.2 (4) nmol/mg per min; + 22.5 μM valinomycin 0.5 ± 0.2 (4) nmol/mg per min, $P < 0.01$).

Discussion

Our previous studies on ^{59}Fe uptake from Fe-NTA have shown that iron is specifically transferred from the chelate complex at the cell surface, whilst the intact chelate is excluded from cell entry [4]. This saturable (carrier-mediated) process is greatly affected by the presence of metabolic inhibitors or the absence of oxygen/glucose in the medium. The mechanism by which the metabolic energy is coupled to the uptake process, however, remains unclear. The transmembrane potential, which depends on the ionic environment [8] has been shown to affect the epithelial transport of many Na^+-coupled solutes [17]. Alanine transport in hepatocytes has also been reported to be sensitive to the membrane potential gradient [18,19]. This study was therefore performed to determine whether the electrogenic uptake of Fe^{3+} is influenced by the ionic (electric) gradient across the cell membrane; This was investigated by manipulating changes in the ionic composition of the bathing medium. Substitution of medium NaCl with either mannitol or choline chloride, had no distinct effects on Fe^{3+} uptake, suggesting that the uptake process is not Na^+-dependent. Furthermore, the failure of phloridzin to affect uptake, suggests that iron-transport is not dependent on the Na^+-dependent glucose carrier. However, the almost complete replacement of medium Na^+ by K^+, a manoeuvre associated with membrane depolarisation [20,21] resulted in a marked, but reversible, inhibition in Fe^{3+} uptake. Other cations in the

same group as K^+ also had inhibitory effects, but to a variable degree. The similarity in the degree of inhibition by K^+ and Rb^+ may be ascribed to the fact that the ionic radii of the two ions are very similar (K^+ 0.133 nm, Rb^+ 0.148 nm [22]).

Further evidence for the possible involvement of the membrane potential in the Fe^{3+} uptake process was provided by experiments performed with specific ionophores. Valinomycin (a neutral ionophore with an equilibrium selectivity for K^+ over Na^+ of about 10000 : 1 [23]) resulted, at low concentrations (1–5 μM), in an increase in the Fe^{3+} uptake rate. Nigericin, a related compound, also had a similar stimulatory effect on the Fe^{3+} uptake rate. Analogous changes in uptake of glucose, a membrane-potential sensitive process [11], were also observed on the inclusion of valinomycin (1–5 μM) in the medium. These observations suggest that the stimulatory effect of valinomycin on Fe uptake is due to membrane hyperpolarisation. The inhibition in uptake at the higher concentration of valinomycin (22.5 μM) may be attributable to the secondary effects of the ionophore on cellular energy metabolism viz. uncoupling of mitochondria [24]. This effect also indicates that the stimulation in iron uptake by low concentrations of valinomycin, is not due to valinomycin-catalysed iron transport.

The inhibitory effects of univalent cations and the transient stimulation in Fe^{3+} uptake seen with K^+ ionophores, suggests that the brush-border membrane potential is an important driving force for the active uptake of $^{59}Fe^{3+}$ by mouse duodenum. A recent study [21] demonstrating the electrogenic uptake of non-transferrin bound iron by the liver to be dependent on the transmembrane potential difference, further supports these findings.

Acknowledgments

We are very grateful to Professor R. Hider for helpful discussions, to Dr. C. Edmonds and Mr. C. Willis for their help and use of the Flame Photometer and to Ms. Rosamund Greensted for secretarial assistance.

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