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Effects of turpentine-induced inflammation on the hypoxic stimulation of intestinal Fe^{3+} absorption in mice

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Summary. Chronic subcutaneous turpentine administration (weekly for 6 weeks) induced a mild normocytic anaemia in mice. In-vitro and in-vivo intestinal Fe^{3+} absorption parameters were, however, not significantly altered from values in saline-treated or untreated mice. Normal mice, when exposed to 3 days hypoxia demonstrated a 2–3-fold increase in iron absorption *in vivo*, mainly due to changes in the amount of iron transferred from the mucosa to the plasma and thence to the carcass. A 2–3-fold increase in *V*max was also observed in in-vitro uptake experiments using isolated duodenal fragments. In contrast, turpentine-treated animals, though demonstrating an enhanced in-vitro maximal uptake capacity, failed to elicit an adaptive response *in vivo* following hypoxic exposure. These findings suggest that a circulating (humoral) factor may be responsible for the inhibition in absorption *in vivo* in this turpentine-induced inflammatory model.

Keywords: turpentine-induced inflammation, intestinal absorption, hypoxia, mice

A mild normocytic anaemia, non-progressive in severity, normally occurs in chronic inflammatory conditions and is accompanied by reduced plasma iron and increased plasma iron-binding capacity (Cartwright & Lee 1971), despite raised tissue storage iron levels (Hershko *et al.* 1974, Feldman *et al.* 1981). Although interest in the disturbance in iron metabolism has been focused on the inhibition in reticulo-endothelial release of iron (Fillet *et al.* 1974; Hershko *et al.* 1974), there is also evidence of altered intestinal absorption of iron in animals with inflammatory reactions induced by endotoxin (Cortell & Conrad 1967; El-Shobaki & Rummel 1985) or turpentine (Hahn *et al.* 1946; Hershko *et al.* 1974). The cellular adaptation in the absorptive pathway during inflammatory disorders is, however, not clearly understood. Moreover, the effect of independently regulated enhancers of iron absorption in chronic inflammation had not been investigated.

In this paper, we investigated the effects in mice of an inflammatory reaction (induced by chronic turpentine treatment) on both the 'uptake' and 'transfer' phases of intestinal Fe^{3+} absorption using in-vitro (isolated fragments) and in-vivo (tied-off loop segments) techniques. Further studies were carried out

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in animals exposed to hypoxia, an independent stimulus for iron absorption (Raja *et al.* 1987a).

Materials and methods

Reagents

All reagents were from either Sigma Chemical Co. Ltd (Poole, Dorset), or BDH Chemicals Ltd (Poole). Radio-iron (^{59}Fe, sp. act, 110–740 MBq/mg Fe) was purchased from Amersham International, Amersham, Bucks.

Animals and hypoxia

Male mice, To strain, 6–7 weeks old, were used for all experiments. Mice were exposed to simulated altitude (15–16 000 ft, 4500–4800 m) by placing them in a hypobaric chamber maintained at 53.3 kPa (0.5 atm) for 3 days. The animals received diet and water *ad lib.* during this period.

Turpentine treatment

Inflammation (abscess) was produced by injecting subcutaneously 0.1 ml of turpentine oil into the intrascapular fat pad, at weekly intervals, for 6 weeks. Control mice were similarly injected with an equivalent volume of sterile 0.15 M NaCl. Some mortality (approximately 30%) was encountered in the experimental groups during chronic treatment. Iron uptake studies were performed a week after the last injection.

Iron absorption studies

In vitro. The method described previously (Raja *et al.* 1987a) was used to determine the initial rates of iron uptake by isolated intestinal mucosal fragments. Mice were sacrificed 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 fragments (2–12 mg wet weight). After rinsing in oxygenated buffer (16 mM Hepes, 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 at 37°C in the same buffer containing $^{59}Fe^{3+}$ as a ferric chelate of nitrilotriacetate (NTA; Fe:NTA, 1:2) with ^{57}Co-cyanocobalamin as the extra-cellular fluid marker. The incubation was terminated by blotting the tissue and rinsing in ice-cold buffer. After re-blotting and weighing, the radioactivity was measured in the tissue and in an aliquot of the medium with a twin channel γ-counter (LKB Wallac 1280, Helsinki, Finland). The emissions of ^{57}Co and ^{59}Fe were separately determined by channel ratio analysis. The uptake, after correction for adherent medium, was expressed as pmol/min/mg wet weight.

In vivo. In-situ tied-off duodenal segments (Raja *et al.* 1987b) were used to determine intestinal iron absorption. All experiments were performed under maximal uptake conditions ($Fe^{3+} = 250$ μmol/l), and for an incu-

Table 1. Haematological indices in mice following chronic turpentine treatment

Group	*n*	Erythrocyte count ($\times 10^{12}$/l)	Haemoglobin (g/100 ml)	Haematocrit (%)	Mean corpuscular volume (fl)
Saline-treated controls	5	7.6±0.1	14.5±0.3	38.8±0.9	51.8±0.4
Turpentine-treated	7	7.0±0.1**	12.7±0.3**	34.5±0.7**	50.1±0.6*

Results: mean±s.e.m. for (*n*) animals.
Statistical analysis by Student's *t*-test: * $P > 0.05$; ** $P < 0.01$, as compared with the control group.

bation period of 10 min. The activity of ^{59}Fe present in the duodenal segment is referred to as 'mucosal retention', whilst the activity in the carcass reflects the 'mucosal transfer'. The sum of the 'mucosal retention' and 'transfer' represents the 'total mucosal uptake'. The percentage ^{59}Fe transferred to the carcass is:

$$\frac{\text{Mucosal transfer}}{\text{Total mucosal uptake}} \times 100.$$

Haematology

Heparinized syringes were used for the collection of blood samples via cardiac puncture. A previously calibrated model ZF Coulter Counter (Coulter Electronics, Luton, Beds) was used for measuring the haematological indices. The haematocrit and MCV values were directly read off on the MCV/Haematocrit accessory connected to the Coulter Counter. Haemoglobin concentrations were recorded using a haemoglobinometer (Coulter Electronics, Luton, Beds).

Statistical analysis

Results are expressed as mean ± s.e.m. The significance of differences between experimental groups was tested by an unpaired Student's *t*-test.

Results

The haematological indices were found not to be significantly altered from control values following a single s.c. injection of turpentine (haematocrit/haemoglobin values: normal controls ($n=10$), 48.7 ± 1.4%/15.9 ± 0.4 g/100 ml; 48 h post-injection (3), 46.8 ± 1.3%/16.1 ± 0.2 g/100 ml; 1 week post-injection (3), 48.1 ± 1.7%/15.8 ± 0.3 g/100 ml). In contrast, chronic turpentine treatment (weekly injections for 6 weeks) resulted in the induction of a mild, normocytic anaemia, as reflected by the small, significant reductions in red cell mass, haematocrit and haemoglobin, without any changes in the MCV (Table 1). No distinct changes were however seen in either the 'mucosal retention' or 'mucosal transfer' of iron in the turpentine-treated mice as compared to the saline-treated controls (Table 2). Moreover, the values in both groups were very similar to those obtained in normal untreated mice.

Following hypoxic exposure, control mice demonstrated a threefold increase in net iron absorption, due to changes in both the 'mucosal retention' and 'mucosal transfer': the latter, however, showed the more prominent changes (Table 2). In contrast, turpentine-treated mice exposed to 3 days hypoxia,

Table 2. $^{59}Fe^{3+}$ Absorption from in-situ tied-off duodenal segments

Group	*n*	$^{59}Fe^{3+}$ Absorption (pmol/mg/10 min)			
		Mucosal retention	Mucosal transfer	Total mucosal uptake	Mucosal transfer (%)
Normal controls	8	25.2 ± 2.3	29.1 ± 4.1	54.1 ± 4.4	52.3 ± 4.2
Saline-treated	6	26.8 ± 5.2	20.9 ± 3.2	47.7 ± 7.0	44.8 ± 6.1
Turpentine-treated	7	24.6 ± 2.7	30.6 ± 6.4	55.1 ± 6.4	52.2 ± 5.1
Controls + 3 days hypoxia	4	39.5 ± 6.5**	113 ± 23.2****	152 ± 30****	73.5 ± 1.4***
Turpentine-treated + 3 days hypoxia	3	34.8 ± 2.6*	23.2 ± 8++	57.9 ± 6.3+	38.2 ± 9.0++

Results: mean ± s.e.m. for (*n*) animals.

Statistical analysis by Student's *t*-test: * $P<0.05$ as compared to turpentine-treated mice; ** $P<0.05$; *** $P<0.01$; **** $P<0.001$ as compared to normal controls. + $P<0.05$; ++ $P<0.03$ as compared to values in control mice exposed to hypoxia.

Table 3. Kinetic parameters for in-vitro Fe^{3+} uptake in turpentine-treated animals

Group	*n*	Kinetic parameters Km (μmol/)	Vmax (pmol/mg/min)
Normal controls	10	162±22	11.3±1.5
Saline-treated	9	109±23	13.0±0.9
Turpentine-treated	7	116±16	13.1±0.6
Controls + 3 days hypoxia	6	106±25	27.2±2.8*
Turpentine-treated + 3 days hypoxia	4	133±45	24.0±0.1**

Results: mean±s.e.m. for (*n*) animals.

Statistical analysis by Student's *t*-test: * $P<0.001$ as compared to normal controls; ** $P<0.001$ as compared to turpentine-treated mice.

though demonstrating an elevation in haemoglobin levels (16.7±0.5(4) g/100 ml, $P<0.001$, as compared to values in turpentine-treated non-hypoxic group, 12.7 ±0.3(7) g/100 ml), failed to elicit an adaptive response to iron absorption.

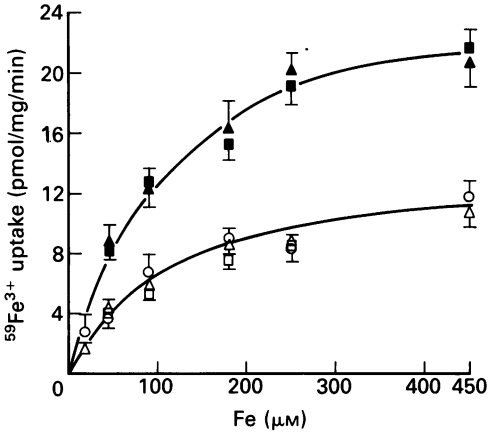

Fig. 1. $^{59}Fe^{3+}$ uptake by mouse duodenum at various medium iron concentrations. Results shown as mean±s.e.m. for 3–9 animals. □, Normal controls; ○, saline-treated controls; △, turpentine-treated with (closed symbols) or without (open symbols) 3 days hypoxia.

Effects of turpentine treatment on in-vitro Fe^{3+} uptake

Mucosal Fe^{3+} uptakes performed *in vitro* using isolated duodenal fragments, demonstrated saturation kinetics over the medium iron concentration range 18–450 μM, with an excellent fit to Michaelis–Menten type curves in both groups of mice (Fig. 1). From such concentration–velocity studies, the apparent kinetic constants, Km (affinity constant) and Vmax (maximal uptake capacity), for Fe^{3+} influx were calculated with the direct linear plot of Eisenthal and Cornish-Bowden (1974). The kinetic parameters in the turpentine-treated mice did not differ significantly from values in either saline-treated or normal animals (Table 3). Furthermore, an adaptive response of similar magnitude to that in control mice (i.e. a 2–3-fold increase in Vmax) was found when the experimental group was exposed to 3 days hypoxia (Table 3).

Discussion

As a single injection of turpentine failed to perturb the haemoglobin/haematocrit levels in mice, a protocol involving weekly injections for 6 weeks was used in the study. The

inflammatory response induced by such treatment resulted in the development of a mild, normocytic anaemia. The degree of anaemia is comparable to the values reported previously (Gutnisky & Van Dyke 1963).

Fe^{3+} absorption studies performed *in vivo* demonstrated no changes in either the 'mucosal retention' or 'mucosal transfer' (or, thus, in the 'total mucosal uptake') following turpentine treatment. Moreover, the values in the experimental group were very comparable to those seen in untreated controls. These findings contrast with previous observations (Cortell & Conrad 1967; Hershko *et al.* 1974; El-Shobaki & Rummel 1985) which showed decreased intestinal iron absorption in animals with simulated inflammatory disorders; the discrepancy may be attributable to differences in either the species used (mice as compared to rats), the experimental methods, the duration of endotoxin/turpentine treatment (chronic as compared to acute (single-dose) treatment) or possibly the iron status of the animals. Our study, however, demonstrated no significant perturbation in haematological indices in mice within 48 h of a single turpentine injection.

Control mice, when exposed to 3 days hypoxia, demonstrated a threefold increase in total iron absorption mainly attributable to variation in the transfer of ^{59}Fe from the mucosa via the plasma to the carcass. Experiments performed *in vitro* using isolated duodenal fragments from such animals also exhibited a 2–3-fold increase in *V*max, the maximal uptake capacity, without any changes in *K*m. In contrast, iron absorption values determined *in vivo* remained unchanged when turpentine-treated mice were exposed to 3 days hypoxia. Elevation of the haemoglobin levels in this group may have been due to (i) bone marrow responding normally in this inflammatory model (Gutnisky & Van Dyke 1963), with iron possibly being donated by tissues other than the intestine, and/or (ii) haemo-concentration following decompression. However, failure of the in-vitro experiments to confirm the abolition of the in-vivo adaptive response in turpentine-treated hypoxic animals suggests that a humoral factor may be responsible for the in-vivo inhibition in absorption in this inflammatory model.

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