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Research Article

In vitro studies of iron absorption and activity of glutathione peroxidase in intestinal mucosa of the chicken

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ABSTRACT

We examined the absorption of iron, the activity of selenoprotein glutathione peroxidase (GSH·Px) and cellular compartmentalization of metal in the chicken duodenum and ileum. The method of accumulating mucosa preparation (AMP) was used. It was shown that the intestinal iron accumulation is dose-dependent process, which has two components: transcellular and paracellular. The realization of these pathways is region-specific and depends on exposed iron levels. Slightly elevated iron status of intestinal mucosa does not influence activity of GSH·Px. At the same time the results indicate that the activity of glutathione peroxidase can be altered by iron overload. Immunohistochemistry revealed that stainable iron could be co-localized to the endolysosomal compartment. How the activity of enzyme can be affected by oxidative stress and competitive interactions of iron with selenium are discussed.

Keywords: Glutathione peroxidase; Iron absorption; Intestinal mucosa; Chicken.

1. INTRODUCTION

Iron serves numerous functions in the body relating to the metabolism of oxygen. Ferrous iron can react with oxygen to form superoxide and also can homolytically cleave hydrogen peroxide yielding hydroxyl radicals and hydroxyl ions. These ions are particularly aggressive and elicit toxic effects, which are mainly related to oxidative stress [1]. Moreover, iron is deeply linked to cell death pathways through reactive oxygen species (ROS) production [2]. Therefore most of free iron is safely stored in a non-redox-active form in ferritins. Iron overload is strongly associated with the intensification of free radical oxidation [3]. Glutathione is a main detoxifier of ROS in the intestine. Glutathione peroxidase provides detoxification of peroxides by using reduced glutathione, and is one of the most important antioxidant enzymes [4]. GSH·Px is a selenoprotein, and selenium availability regulates glutathione peroxidase enzyme activity [5]. An excess of certain minerals in the body can antagonize other minerals and cause depletion [6].

Since animals lack mechanisms for iron elimination, iron uptake is strictly regulated. The non-heme iron is ultimately taken up from the gut lumen by divalent metal transporter 1 (DMT1) situated on the microvillus membrane, before joining the labile iron pool in the cytoplasm and transferred to the bloodstream by ferroportin 1 [7]. The mechanism and regulation of intestinal iron absorption are incompletely understood in spite of their pivotal role in the maintenance of body iron homeostasis [8].

Iron metabolism involves iron trafficking along specific cellular compartments, including endosomes and lysosomes [9]. These organelles take center stage in cellular iron accumulation and are involved as a control hub for aging and longevity [10]. The study described here was undertaken to investigate the influence of exposure to iron in concentrations occurring in contaminated food and feed on accumulation and compartmentalization of iron in enterocytes and the activity of GSH·Px in the intestinal mucosa.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

New-hatched Lohmann brown cockerels were obtained from the Latvian poultry company BALTICOVO. All of the experimental procedures were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia, authorisation reference number 13, from December 22, 2008). The chickens were housed in cage units with free access to food and water. Animals received standard full-feed diet. For the *in vitro* study 30 days old chickens were divided into 3 groups (5 in each group): 1 - "Buffer", 2 - "+ Fe 0.512 mM as iron sulfate", 3 - "+ Fe 2.56 mM as iron sulfate". Chickens were sacrificed by decapitation, in accordance with recommendations for the euthanasia of experimental animals of the European Convention [11].

2.2. Determination of iron absorption and GSH-Px activity

The content of iron in chick intestinal mucosa was estimated by atomic absorption spectrophotometry [12], the activity of glutathione peroxydase (GSH·Px) - by a modified Pinto-Bartley method [13].

The intestine was isolated and washed with 10 ml of cooled physiological solution (154 mM NaCl). Then it was placed on ice-cold glass plate. Duodenum and ileum were cut on segments (5 cm) and used for intestinal preparations. Iron binding by the intestinal wall was studied by means of AMP method as developed by Ugolev et al. [14] for investigation of the first stages of transport processes. An everted intestinal segment of birds belonging to groups 1, 2 and 3, mounted on a glass rod, was submerged in 7 ml Tris-buffer containing different concentration of iron (0.512 mM and 2.56 mM). An everted intestinal segment of birds belonging to groups 1, 2 and 3, mounted on a glass rod, was submerged in 7 ml Tris-buffer containing different concentration of iron (0.512 mM and 2.56 mM).

Intestinal AMP were incubated for 30 min at 41ºC. Tris-buffer without iron supplement was used as a control. Buffer composition (mM) was: 4 Tris hydrochloride, 145 sodium chloride, 4 potassium chloride, 20 fructose, pH 7.4. The amount of accumulated iron was calculated as the difference between the iron contents in the mucosa before and after incubation.

2.3. Histological examination

For histological examination, 1-cm segments of intestinal samples from animals of the 2-nd and 3-rd group (duodenum was taken 0.5 cm distal to the ampulla of Vater and ileum - 10 cm proximal to the ileocecal junction) were isolated and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue was cut into 4-µm-thick sections and stained with haematoxylin-eosin and the periodic acid-Schiff (PAS) reagent. Duodenal sections were colored with Perls' Prussian blue stain for iron detection. Late endosomes and lysosomes in the enterocytes were highlighted by immunohistochemistry using an anti-CD68 and anti-TRPV1 antibodies.

2.4. Statistical analysis

All statistics were performed using the program SPSS. Means and standard deviations and significance values were calculated. The results were assessed statistically by *t* tests. Statistical significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

Iron exercised a diversified action: after 30 min incubation in a medium containing 0.512 mM of iron its concentration in the duodenal mucosa amounted to 9.72 ppm, and the iron accumulation was increased by 57.3% (Table 1). At the same time

in the ileal mucosa exposed to lower level of iron only nearly 10% of the metal was accumulated. The obtained data indicated 5.8-fold ability of the duodenum, compared with the ileum, to transfer iron into the mucosa. After applying of 5-times higher iron concentration in the incubation medium the tissue level of this metal increased more than two times and the metal accumulation in the duodenal mucosa was increased by 133.8% vs. 330.7% in the ileal mucosa. Dramatic effects of higher iron exposure on accumulation of this metal in the ileal mucosa with levels more than 30-fold higher than observed for lower levels of iron exposure may be related to greater (paracellular) leakiness of the epithelial barrier in the ileum. It is known, that transcellular active transport of iron across the gut epithelium occurs mainly in the duodenum and jejunum [15]. We conclude that similar to calcium absorption, passive, paracellular absorption of iron predominates in the ileum when dietary iron levels are high [16].

Table 1. Iron accumulation in intestinal mucosa of chickens.

Experimental conditions	Concentration of Fe in intestinal mucosa, ppm		Accumulation of Fe in intestinal mucosa, ppm	
	Duodenum	Ileum	Duodenum	Ileum
1. Buffer	6.18 ± 0.88	3.02 ± 0.30	-	-
2. +Fe (0.512 mM)	9.72 ± 0.65[a]	3.36 ± 0.29	3.54 (+57.3%)	0.32 (+9.9%)
3. + Fe (2.56 mM)	14.40 ± 1.50[a,b]	14.00 ± 1.52[a,b]	8.22 (+133.8%)	10.98 (+330.7%)

[a]Statistically different from the 1st group (P<0,05); [b]Statistically different from the 2nd group (P<0,05)

As revealed by our studies, the activity of GSH·Px in the 2nd group either remains unaffected (in the duodenal mucosa), or decreases insignificantly (in the ileal mucosa), but in the 3rd group both in the duodenum and ileum a statistically significant decreasing trend in GSH·Px activity was observed with increasing iron accumulation in intestinal mucosa (Table 2).

Table 2. Activity of GSH·Px in intestinal mucosa of chickens

Experimental conditions	Activity of GSH·Px µmol GSH/min/g	
	Duodenum	Ileum
1. Buffer	2.37 ± 0.24	1.62 ± 0.25
2. +Fe (0.512 mM)	2.36 ± 0.80	1.46 ± 0.63
3. + Fe (2.56 mM)	1.44 ± 0.38 [a,b].	0.93 ± 0.31 [a]

[a] Statistically different from the 1st group (P<0.05)
[b]Statistically different from the 2nd group (P<0.05)

Stainable iron was found in the small intestinal enterocytes of the chickens in the 3rd group. As shown in Fig. 1-A, iron deposits appeared as a narrow string of punctae in the subapical area all along the brush border. Little or no diffuse staining of the enterocyte cytosol was detected. TRPV1 immunoreactivity was localized in the subapical compartment of the villous enterocytes, having a punctuate appearance (Fig. 1-B). The pattern of CD68 immunoreactivity was quite similar to selective cytoplasmic expression of TRPV1 (Fig. 1-C). It should be emphasized, that both CD68 and TRPV1-positive material and iron deposits within enterocytes were consistently localized to the same area in the vicinity of the brush border. It is well known that the endosomal-localized DMT1 is responsible for mobilizing iron out of endosomes [17]. It was shown that members of the transient receptor potential (TRP) superfamily could function as intracellular cation release channels whose localization is commonly assigned to late endosomes and lysosomes [18]. The obtained results also indicated that TRPV1 is localized to the late endosomes and lysosomes, where TRPV1 may function to transfer the endosomal free Fe^{2+} into the cytoplasm in the transferrin cycle in parallel to DMT1.

Our data showed that chickens of the 3rd group had lower GSH·Px activity in the intestinal mucosa than did animals in the 1st and 2nd group. It is likely that in the 3rd group iron reaches damaging levels, exceeding the homeostatic capacity of the

enterocytes. Decreased GSH·Px activity has been reported in tissues where oxidative stress occurs in several pathological animal models [19]. It is known, that an excess of iron in tissues can induce hydroxyl radical formation. This effect was likely promoted by the recycling of chelated, inactive Fe^{3+} to the active Fe^{2+} state by the Fenton reaction in the mitochondria [20]. Fe^{2+} is extremely toxic because it can rapidly react with hydrogen peroxide and molecular oxygen to produce reactive oxygen species. Proteins are oxidatively damaged by the combined action of free radicals and the trace metal ions such as Fe^{2+} and Cu^{2+} [21]. In our experiments oxidative damage to GSH·Px may also affect its activity. The seeming paradoxical dissociation between considerable iron accumulation in the ileal mucosa in the 3rd group and only moderate down-regulation of GSH·Px activity comparable to that in the duodenal mucosa can be explained by the preferential use of paracellular route of iron transport under these circumstances.

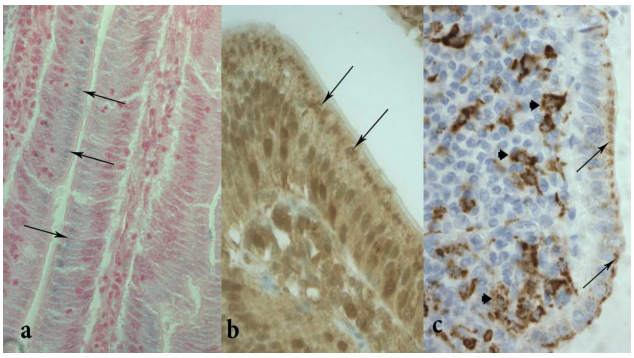

Figure 1. A - Iron histochemistry with Perls' staining of chicken intestinal mucosa from a third group. Iron deposits in the subapical compartment of villous enterocytes, x40. B - TRPV1-positive punctae in the subapical compartment of villous enterocytes (arrows) x40. C - CD68 expression in the enterocytes with the subapical pattern (arrows). Likewise, high levels of CD68 expression are associated with macrophages (arrowheads), x40.

According to the reports, supplementary iron reduces selenium bioavailability [22]. Therefore, reduction of the activity of selenoprotein GSH·Px during the iron overload may be related at least in part to the competitive iron interactions with selenium, thus reducing its bioavailability.

Understanding of the ways and control of transition metal uptake and translocation is very important particularly because some of them can be highly toxic when accumulate in the cells. Our animal model of iron overload has demonstrated the accumulation of selective iron subapical deposits colocalized with endolysosomal markers. Labile iron can readily generate ROS, and sequestration in the endolysosomal apical system may represent one of many protective mechanisms that exist within the absorptive epithelial cell. These data seems to support the theory that at least half of the iron transported across the villous enterocytes uses a vesicular pathway and that a significant portion of the vesicular pathway involves the endolysosomal system, which is located en route towards the basolateral membrane [23, 24].

Low iron levels may have a link with cognitive health later on in life. For example, the patients with anemia had a higher risk of developing dementia compared with those who were not anemic [25]. Eating foods high in iron can help prevent dementia. On the other hand, our results showed, that the iron supplementation can cause side effects and, consequently, compromise the life expectancy mainly for elderly populations, because age-related iron overload is a known contributor to multiple degenerative diseases, including cancer, liver fibrosis and heart attack [26-31].

4. CONCLUSION

In conclusion, after iron treatment (0.512 mM in the incubation medium) GSH-Px activity remains unchanged despite the accumulation of metal in the intestinal mucosa. However pathological accumulation of the iron within the intestinal mucosa (2.56 mM in the incubation medium) elicits toxic effects, reducing the activity of GSH-Px, which are mainly related to oxidative stress. The endolysosomal compartment plays an important role in cellular iron homeostasis in the iron-overloaded state.

AUTHORS' CONTRIBUTIONS

JM: Study design and interpretation of the protocol and guidance; acquisition of the data; obtained funding; drafting of the manuscript; critical revision of the manuscript for important intellectual content;

AG and NB: Study concept and design; analysis and interpretation of the data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical expertise; study supervision. GK, SV, GS: analysis and interpretation of the data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; administrative, technical and material support. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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