ARTICULOS DE ACTUALIZACION / REVIEW ARTICLES

Calcium as a nutrient involved in the synthesis and localization of proteins that facilitate iron uptake and efflux in enterocytes

El calcio como nutriente implicado en la síntesis y localización de proteínas que participan en la captación y eflujo del hierro en los enterocitos

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ABSTRACT
Iron deficiency is the most common type of nutritional deficiency in the world, and calcium intake is low in many populations. Developing a supplement that contains both micronutrients would be an excellent method to prevent iron deficiency and increase calcium intake. Human and cellular studies have shown that calcium inhibits iron absorption. Currently, the mechanisms underlying this inhibitory effect have not been elucidated. Because calcium is involved in cell signaling pathways, it may affect the regulation of the expression or localization of proteins involved in iron uptake and efflux in enterocytes. The aim of this review was to describe the processes involved in the absorption of dietary iron and the regulation of the expression and activity of proteins involved in this absorption. Additionally, the potential mechanisms by which calcium affects the expression and localization of these proteins are also discussed.
Key words: iron, uptake, efflux, absorption, calcium, Caco-2.

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INTRODUCTION

Iron is an essential nutrient that is involved in important physiological processes. Different factors affect the nutritional status of this mineral and increase the risk of deficiency, such as low consumption of meat, high consumption of absorption inhibitors, high nutritional requirements, and high iron loss resulting from different pathological conditions (1). According to the World Health Organization, iron deficiency is the most common nutritional deficiency in the world, primarily affecting children, pregnant women, and women of reproductive age (2,3). Conversely, an analysis of calcium intake in men and women from 20 countries showed that depending on the age group, between 10-40% of men and 40-50% of women do not consume their daily calcium requirement(4). Based on these findings, the design and implementation of strategies for nutritional intervention in various populations are necessary to improve the intake of these micronutrients and prevent iron deficiency, especially among vulnerable groups.

A potential solution to this problem is the development of a supplement that contains both minerals. However, there is evidence that suggests that low doses of calcium consumed in combination with food (5,6) can inhibit iron absorption. Two types of dietary iron are traditionally recognized: non-heme (plant foods and salts used for food fortification and supplementation) and heme (found in meat).Calcium is the only component of the diet that has a recognized effect on the absorption of these two types of iron (5,6). Hallberg et al. (6) reported that 40 mg of calcium, in the form of calcium chloride, added to a preparation decreased the uptake of 3.8 mg of non-heme iron by 40% (6). Subsequently, they found that 165 mg of calcium chloride decreased the absorption of the heme iron in burgers and wheat rolls by 41% and 48%, respectively (5). In contrast, Gaitan et al.(7) found that when an iron salt is consumed together with calcium on an empty stomach, this inhibitory effect of calcium only occurs at doses higher than 800 mg. Furthermore, Cook et al. (8) showed that the type of calcium salt used determines the inhibitory effect and that citrate and phosphate salts inhibit iron absorption when ingested on an empty stomach. Based on these findings, it is necessary to determine the precise short-, medium-, and long-term effects of calcium on iron absorption. An interesting experimental model for evaluating these effects is the cell line

Caco-2, which is easy to use and produces results that strongly correlate with the absorption of this mineral in humans (9,10). Furthermore, this model can also be used to investigate the cellular mechanisms involved in nutrient absorption.

The aim of this review was to describe the processes involved in the absorption of dietary iron and the regulation of the expression and activity of the proteins involved in this absorption. Additionally, the potential mechanisms by which calcium affects the expression and localization of these proteins are also discussed.

Iron absorption: Intestinal absorption is the term used to refer to the passage of nutrients from the intestinal lumen to the blood stream. Iron is primarily absorbed by duodenal enterocytes (11). In humans, the mechanisms for the intestinal absorption of the two types of dietary iron are different. Because of the duodenal pH, non-heme iron is in its ferric oxidation state (Fe+3) (12)and is reduced to its ferrous oxidation state (Fe+2) by the DcytB (Duodenal cytochromeB) protein, which is located in the apical membrane of enterocytes (13). Subsequently, this iron is transported into the cytosol through the DMT1 (Divalent MetalTransporter 1) apical transporter(14). In contrast, heme iron is released from the food matrix and is taken up by the HCP1 (Heme Carrier Protein 1) apical protein (15).It has been suggested that HCP1 functions as a membrane receptor that mediates endocytosis (16) or as a heme iron transporter (17). Thus, the protoporphyrin ring enters a cytoplasmic vesicle where the iron is released by the enzyme heme-oxygenase (HO-1) (18) and enters the cytosol through a currently unknown mechanism that is potentially mediated by DMT1(17). After this release, the two types of dietary iron become indistinguishable to the cell, forming a cytoplasmic iron pool that is stored in the protein ferritin or transported from the cytosol to the blood stream. The efflux of iron, which occurs at the basolateral membrane of the enterocyte, is mediated by ferroportin (Fpn), the only currently identified iron exporter protein (19). After efflux, Fe+2 must be oxidized to Fe+3 by the protein hephaestin, which is required for its incorporation into transferrin, which is the protein that transports this mineral in the blood to the tissues (20).

In addition to the classic dietary forms of iron described, recent findings indicate that a type of organic iron associated with animal and plant ferritin can be taken up by clathrin-dependent endocytosis(21,22); however, the receptor that regulates this mechanism has not yet been identified. AnFe+3 absorption pathway, involving theβ3-Integrin-Mobilferrin-Pathway (IMP), has also been proposed(23).Currently, however, there is not enough evidence that supports this pathway as a major route of iron uptake. The proposed iron absorption mechanisms are shown in figure 1.

Regulation of molecules involved in iron absorption:

Certain conditions of the enterocyte regulate the expression of proteins involved in iron absorption, such as intracellular iron status and hypoxia (24). Furthermore, at the systemic level, individual iron nutritional status, hypoxia, and inflam-

FIGURE 1

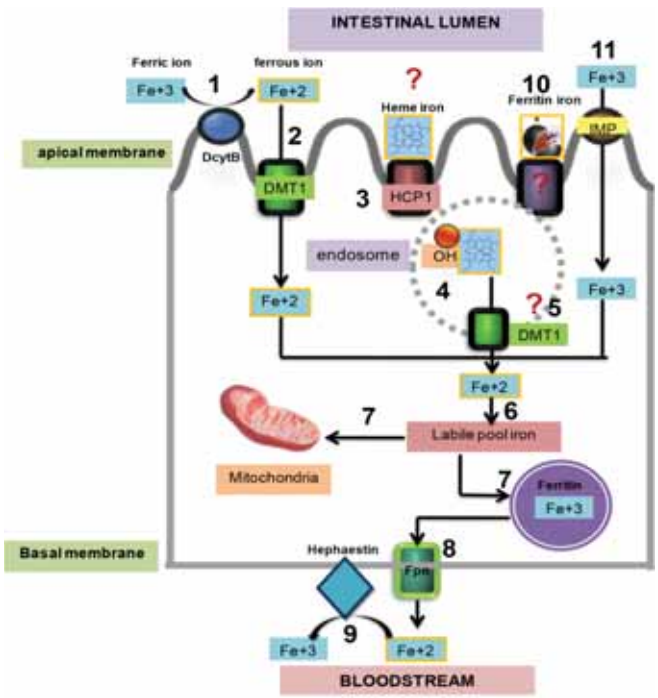

Figure 1. Iron absorption mechanisms in the enterocyte.1. Reduction of Fe+3 to Fe+2 by DcytB.2. Apical uptake of Fe+2by DMT1 3. Apical heme iron uptake by HCP1 through a currently unknown mechanism 4. Heme degradation by the enzyme HO. 5. Vesicular release of iron, which is potentially mediated by DMT1.6. Labile iron pool formation. 7. Distribution of iron to mitochondria or cytoplasmic ferritin based on the needs of the cell. 8. Efflux of iron through the basolateral membrane by Fpn. 9. Re-oxidation of Fe+2 through hephaestin prior to binding to transferrin for blood transport. 10 and 11.Alternative mechanisms of iron uptake from ferritin and Fe+3.

mation determine the amount of iron absorption through the hepatic secretion of the polypeptide hepcidin (Hpc) (25,26). This polypeptide forms a receptor-ligand complex with Fpn, favoring its ubiquitination and subsequent degradation and thus decreasing iron efflux from the enterocyte (27). For the purposes of this review, the local mechanisms of iron regulation will be emphasized considering that the Caco-2 model is most useful for these types of studies.

The synthesis of certain proteins involved in iron absorption in the enterocyte is determined by the cytosolic IRPs (Iron Regulatory Proteins) IRP1 and IRP2 (28). These proteins bind to mRNA sequences known as IREs (Iron Responsive Elements). The IRP binding site occurs in the 3'- or 5'-UTR (Untranslated Regions) of mRNAs that encode proteins involved in the uptake, storage, and efflux of iron from the enterocyte. The formation of IRP/IRE complexes in the 5'-UTR region of mRNA inhibits mRNA translation, while binding in the 3'-UTR region prevents mRNA degradation by ribonucleases, promoting translation (27,29). Currently, twelve IRE-containing mRNA have been described, seven of which contain an IRE in the 5'-UTR region, and among these are mRNAs that encode ferritin (heavy chain and light chain) and Fpn. The other five identified mRNAs contain an IRE in the 3'-UTR region, among these is an mRNA that encodes DMT1 (29,30). As previously mentioned, cellular iron concentration regulates the different mechanisms of IRP1 and IRP2 action. In states of cellular iron repletion, IRP1 acquires an iron atom to form an iron-sulfur cluster [4Fe-4S], giving it the function of cytosolic aconitase but with a low affinity for IRE (31).In a state of iron deficiency, IRP1 contains only 3 iron atoms [3Fe-4S] in the active site, dramatically increasing its affinity for IRE. IRP2 is structurally similar to isoform 1 but lacks the Fe-S cluster. In a state of cellular iron repletion, a cysteine-rich IRP2 domain is oxidized, promoting its ubiquitination and subsequent proteasomal degradation. In contrast, this protein is stabilized in a state of cellular iron deficiency (28). Despite these structural differences between IRP1 and 2, these proteins interact similarly with mRNA. An efficient IRP/IRE interaction ensures the proper expression of the target genes of these proteins. Studies in genetically modified mice in which the expression of IRP1 and IRP2 is suppressed resulted in poor DMT1 expression, Fpn overexpression in the duodenum, and increased mortality compared with mice that normally express these IRPs (30). These findings emphasize the importance of these proteins in not only iron metabolism but in maintaining biological homeostasis.

When the cell is exposed to low levels of iron, there is a decrease in the intracellular iron pool. This decrease in iron activates the IRPs, increasing their affinity for mRNA IRE. DMT1 mRNA, with an IRE at the 3' end, is recognized by regulatory proteins, stabilizing the RNA and promoting message translation, thus increasing the synthesis of this protein and ultimately favoring the uptake of iron (31). In contrast, the binding of IRPs in the 5'-UTR region of Fpn mRNA inhibits its translation (31), thereby reducing iron transport from the enterocyte through the basement membrane into the blood vessels. Ferritin formation is also altered by an IRP/IRE complex in the 5'-UTR region, which negatively affects the synthesis of this protein (32). Altogether, these regulatory mechanisms tend to increase the availability of iron to the cell. Conversely, when the cell is sufficiently exposed to iron, the pool of intracellular iron increases, and the IRPs are inhibited, decreasing their interactions with IREs, which increases the expression of cellular ferritin and Fpn and decreases DMT1 synthesis; therefore, the efflux of iron into the blood stream is favored to prevent mineral accumulation within the cell. The local regulatory mechanisms of the proteins involved in iron absorption mediated by IRP/IRE are summarized in figure 2.

In contrast to DMT1, Fpn and ferritin, the regulation of HCP1 synthesis is not determined by the intracellular iron status. Other conditions of the cellular environment may regulate the expression of this protein, such as hypoxia(15), heme oxygenase (HO-1) activity (33), and potentially the concentration of the minerals that cause the activation ofHO-1 (34). The availability of intracellular iron, however, does change the cellular localization of HCP1.For example, in deficient cells, HCP1 is translocated from cytoplasmic vesicles to the apical membrane (15).

Studies on calcium and its effect on iron absorption in a cellular model

Studies in cell models have shown that calcium has an inhibitory effect on the absorption of non-heme iron. In 2010, Thompson et al. (35) analyzed the dose-response effect of increasing calcium concentrations (in the form of calcium chloride; 0-2.5 mM) on iron (in the form of ferric ammonium citrate) absorption (30μM) in Caco-2 cells. The calcium:iron (Ca:Fe) molar ratio in this study was not determined because the number of iron atoms in ferric ammonium citrate is not standard, and the author did not report the type of iron salt used. In this study, the cells cultured for two hours in the presence of 1.25 and 2.5 mM calcium chloride demonstrated a significant reduction in iron uptake (determined by cell ferritin formation) compared with the cells cultured in the absence of calcium (ANOVA $p<0.05$). This inhibitory effect may result from the use of citrate, which has been proposed to be an iron inhibitor in humans (8). Moreover, it is also important to consider that ferric ammonium citrate is capable of binding iron-generating complexes that may affect its absorption. Conversely, in a review on this subject, Lönnerdal (36) reported that there is a decrease in basolateral iron transport in Caco-2 cells incubated in the presence of 1 μM Fe (FeSO4) and 100 μM calcium chloride compared with cells cultured in the absence of calcium; the culture times were 1.5 and 4 hours (the Ca:Fe molar ratios were 0:1 and 100:1, respectively). Moreover, Gaitan et al. (37) evaluated, the effect of calcium (in the form of calcium chloride) on the uptake, efflux, and net absorption of a 2 μM iron solution (FeSO4) in Caco-2 cells; the calcium was administered at Ca:Fe molar ratios of 50:1 - 1000:1. In this study, calcium did not affect the net absorption of iron; however, iron uptake by the cells was significantly higher than uptake by the control group at molar ratios of 500:1 and 1000:1(ANOVA $p<0.009$), while the efflux of iron decreased significantly at a molar ratio of 1000:1 (ANOVA $p<0.002$). The overall analysis of these previous studies suggests that calcium negatively affects iron absorption and that this effect is due to decreased efflux, which is consistent with the human studies by Hallberg et al. (5,6).

Currently, there are no widely accepted mechanisms explaining the effect of calcium on iron absorption. Initially, a competitive mechanism was proposed to explain this effect. However, this mechanism is losing support because the proteins involved in calcium uptake by the enterocyte are different from those involved in iron uptake (38). Furthermore, Gunshin et al. (14) reported that calcium is not a substrate of DMT1, and Shawki and Mackenzie (39) also suggested a lack of competition between these two minerals for this transporter. It has been suggested that calcium modifies the

expression and localization of proteins involved in iron uptake and transport from the enterocyte into the blood stream, and this proposed mechanism would explain the inhibitory effect. Based on this proposal, two studies have evaluated the effect of calcium on the expression/localization of DMT1 and Fpn (35,36) in a cellular model.

In the first study, Thompson et al. (35) assessed the subcellular fractions of DMT1 and Fpn in Caco-2 cells. The

FIGURE 2

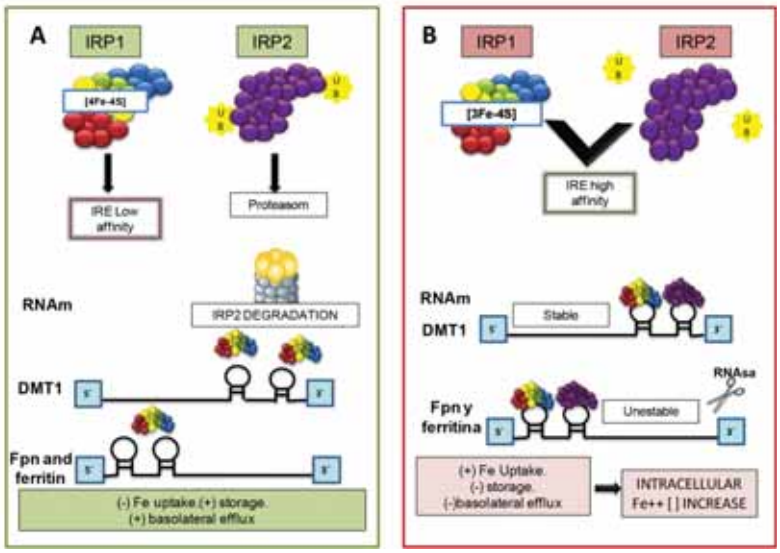

Figure 2. Local regulation of proteins involved in iron absorption through the IRP/IRE system. A. Cellular regulation of proteinin a state of iron repletion. IRP1 is configured in an iron-sulfur [4Fe-4S] cluster that reduces its affinity for IRE. Thus, IRP1/IRE weak interactions prevent DMT1 mRNA stabilization and the destabilization of Fpn and ferritin mRNAs. The result is a decrease in the expression of DMT1 and increases inthe expression of cellular Fpn and ferritin, which tend to reduce the availability of free iron within the enterocyte. IRP2 is ubiquitinated and degraded by the proteasome/ubiquitin system. The result is a decrease in iron uptake and increases in iron storage and basolateral iron transport. B. Cell regulation in a state of iron depletion. IRP1 is configured in an iron-sulfur [3Fe-4S] cluster, dramatically increasing its affinity for IRE. IRP2 is not ubiquitinated and can bind to the IRE. IRPs/IRE strong interactions favor the stabilization of DMT1 mRNA and the destabilization of Fpn and ferritin mRNA. The result is an increase in DMT1 expression and decreases in the expression of cellular Fpn and ferritin, which tend to increase the availability of free iron within the enterocyte.

FIGURE 3

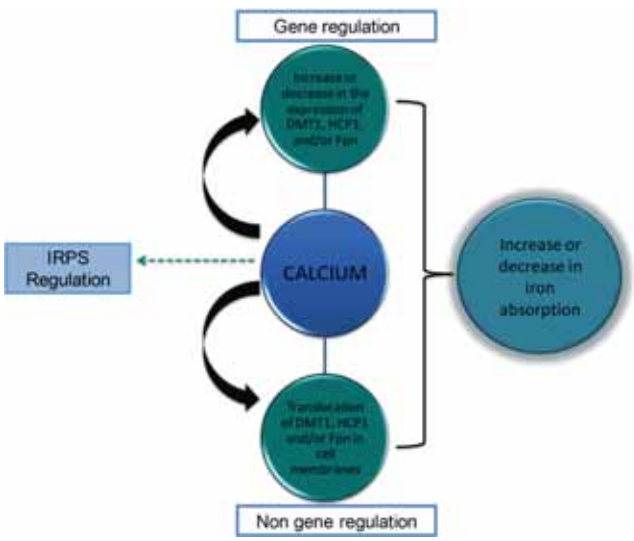

Figure 3. Potential calcium pathways regulating the expression and localization of proteins involved in iron absorption. A. Gene regulatory pathway in which calcium can increase or decrease the expression of DMT1, HCP1, and Fpn. B. Non-gene regulatory pathway involving the translocation of DMT1, HCP1, and Fpn in cell membranes. C. Alternate regulatory pathway explaining the effect of calcium on modulating the expression of IRPs.

cells were incubated with 30 µM ferric ammonium citrate, 2.5 mM calcium chloride, or 30 µM ferric ammonium citrate + 2.5 mM calcium chloride for 4 hours. After sedimentation by centrifugation, the fractions used to determine the expression and localization of the proteins of interest were obtained. Although there were no differences in protein expression between the treatments, in the cells treated with iron, calcium or both nutrients, the translocation of DMT1 to the cell membrane was significantly decreased compared with the cells cultured in the absence of iron and calcium (ANOVA $p<0.05$). The results obtained in the presence of iron are not surprising because DMT1 is down regulated in the presence of iron (30); however, the modulator effect observed in the cells cultured in the presence of calcium alone is striking. In contrast, the amount of membrane-associated Fpn did not appear to differ between the groups. Furthermore, in the second study, Lönnerdal (36) determined the DMT1 and Fpn mRNA expression levels and the amount of membrane-associated Fpn. The author reported that there were no significant differences in the DMT1 mRNA expression between the cells incubated in the absence or presence of calcium for 1.5 h ($p=0.06$). However, the cells cultured for 4 hours with 100µM calcium demonstrated a significant increase in DMT1 expression (p-value not reported), which may explain the findings obtained by Gaitan et al. (37), who observed that calcium significantly increases iron uptake in Caco-2 cells. Regarding Fpn mRNA expression, Lönnerdal (36) found no significant differences between the cells incubated with and without calcium for 1.5 or 4 hours. In contrast, the membrane-associated Fpn was lower in the cells incubated with 100 µM calcium for 1.5 h (p-value not reported) and was higher in the cells cultured under the same conditions for 4 h. It is possible that the decrease in Fpn in the basolateral membrane after the 1.5 h incubation found by Lönnerdal explains the effects of calcium on lowering the iron efflux reported by Gaitan al. (37). To corroborate these findings, however, it is necessary to unify the experimental conditions of the two studies in terms of incubation times and Ca:Fe molar ratios. It should be taken into consideration that the discrepancy between the results obtained by Thompson et al. (28) and Lönnerdal (36)on the effect of calcium on the cellular localization of DMT1 and Fpn may be due to the different iron and calcium salts used.

How can calcium modify the expression or localization of DMT1, HCP1, and Fpn?

Calcium is a nutrient with very important biological functions; therefore, the cytoplasmic concentration of this mineral is highly regulated. At baseline, cells have a calcium concentration of approximately 100 mM and are activated when the concentration increases to approximately1000mM. This concentration increase is associated with the role of calcium as a second messenger in cell signaling pathways (40). Changes in the cytoplasmic calcium concentration are transient and are primarily the result of the opening of channels located in the endoplasmic reticulum, allowing the efflux of this mineral from this organelle to the cytoplasm, which is driven by the concentration gradient generated by the ATPases located in the reticular membrane. The opening of these channels is mediated by the ligands and receptors associated with signaling pathways related to phosphatidylinositol (PI)(40–42),cyclic ADP-ribose, and sphingosine-1-phosphate (40,41).

An increase in the calcium concentration results in the activation of two calcium-dependent proteins, Ca2+-calmodulin-dependent kinase II (CAMDKII) and protein kinase C (PKC), which are involved in cell signaling cascades that modulate gene expression (43) or regulate the activity of different proteins through their phosphorylation, thus modulating their activity and subcellular localization among other proteins. Additionally, the phosphorylation of certain proteins results in the regulation of other cell signaling cascades.

These above-mentioned mechanisms have been frequently studied in nerve, muscle, immune, and liver cells (38,39). However, there is evidence that the phosphorylated inositol receptor is present in rat enterocytes (44), suggesting that these mechanisms of calcium-dependent cellular signaling also play an important role in regulating the functions of the enterocyte, the absorption of nutrients being the most important of these functions.

Furthermore, enterocytes specialized in nutrient absorption uptake calcium through two mechanisms, a vitamin D-independent paracellular mechanism and a vitamin D-dependent transcellular mechanism, in which the uptake is accomplished by TRPV 5 and 6 channels (38) located in the apical membrane. The transcellular mechanism primarily occurs in the duodenum, where the absorption of dietary iron also takes place. Therefore, it is conceivable that the combined intake of calcium and iron would promote the uptake of both minerals and transiently increase their cytoplasmic concentrations. In the case of calcium, these changes could activate the cell signaling pathways dependent on this nutrient (45). Considering the numerous functions of calcium, it may also be assumed that these changes would modulate gene expression as well as the function and localization of proteins involved in the uptake and transport of iron in the enterocyte, such as DMT1, HCP1, and Fpn, by increasing or decreasing expression or by affecting protein localization changes. The potential effects of calcium on gene expression and the localization of proteins involved in iron absorption are summarized in figure 3.

CONCLUSION

Because of a lack of evidence definitively supporting the effect of calcium on iron absorption and the regulation of genes and the localization of proteins involved in this process, it is necessary to conduct studies in cellular models to address these issues and find answers to the currently unresolved questions. The findings of this research, combined with the results of certain recent studies elude to the potential use of a supplement that contains both micronutrients, which could be part of comprehensive nutrition programs that seek to prevent, control, or treat deficiencies in high-risk individuals or groups.

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RESUMEN

La deficiencia de hierro es la deficiencia nutricional más común en el mundo y la ingesta de calcio es baja en la mayoría de poblaciones. El desarrollo de un suplemento que contenga ambos micronutrientes sería una excelente estrategia para prevenir la deficiencia de hierro y aumentar la ingesta de calcio. Estudios en humanos y en células han demostrado que el calcio inhibe la absorción del hierro. Actualmente los

mecanismos que explican este efecto inhibitorio no están claramente dilucidados. Dado que el calcio está implicado en la señalización celular, podría afectar la regulación de la expresión y localización de proteínas implicadas en la captación y eflujo de hierro en los enterocitos. El objetivo de esta revisión es describir los procesos implicados en la absorción del hierro dietario y la regulación de la expresión y actividad de las proteínas implicadas en esta absorción. Además, se discutirán los posibles mecanismos por los cuales el calcio afecta la expresión y localización de esas proteínas

Palabras clave: hierro, captación, eflujo, absorción, calcio, Caco-2.

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