Effects of extracellular iron concentration on calcium absorption and relationship between Ca$^{2+}$ and cell apoptosis in Caco-2 cells

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AIM: To determine the method of growing small intestinal epithelial cells in short-term primary culture and to investigate the effect of extracellular iron concentration ([Fe$^{3+}$]) on calcium absorption and the relationship between the rising intracellular calcium concentration ([Ca$^{2+}$]) and cell apoptosis in human intestinal epithelial Caco-2 cells.

METHODS: Primary culture was used for growing small intestinal epithelial cells. [Ca$^{2+}$], was detected by a confocal laser scanning microscope. The changes in [Ca$^{2+}$], were represented by fluorescence intensity (FI). The apoptosis was evaluated by flow cytometry.

RESULTS: Isolation of epithelial cells and preservation of its three-dimensional integrity were achieved using the digestion technique of a mixture of collagenase XI and dispase I. Purification of the epithelial cells was facilitated by using a simple differential sedimentation method. The results showed that proliferation of normal gut epithelium in vitro was initially dependent upon the maintenance of structural integrity of the tissue. If 0.25% trypsin was used for digestion, the cells were severely damaged and very difficult to stick to the Petri dish for growing. The Fe$^{3+}$ chelating agent desferrioxamine (100, 200 and 300 μmol/L) increased the FI of Caco-2 cells from 27.50±13.18 (control, n = 150) to 35.71±13.99 (n = 150, P<0.01), 89.87±43.29 (n = 150, P<0.01) and 104.64±51.07 (n = 150, P<0.01) in a concentration-dependent manner. The positive apoptotic cell number of the Caco-2 cells after being treated with A$^{3+}_{21387}$ increased from 0.32% to 0.69%, 0.90% and 1.10%, indicating that the increase in the positive apoptotic cell number was positively correlated with [Ca$^{2+}$].

CONCLUSION: Ca$^{2+}$ absorbability is increased with the decrease of extracellular iron concentration Fe$^{3+}$ and hindered with the increase of Fe$^{3+}$ consistence out of them. Furthermore, increase of [Ca$^{2+}$], can induce apoptosis in Caco-2 cells.

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Abstract

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INTRODUCTION

Calcium is one of the most important and necessary element which maintains the physiological function. When the absorption of calcium is obstructed, many diseases are induced, such as hypertension, hyperkinesias, colorectal carcinoma, cardiovascular disease and osteoporosis, etc. The iron is one of the most common metals existing in our environment.

In the last decade, a number of studies suggested that the ferric absorption was inhibited by calcium. However, little is known about the effect of iron concentration change on calcium absorption. The purpose of the present study was to investigate the effect of extracellular iron concentration ([Fe$^{3+}$]) on calcium absorption, and to study the effect of rising intracellular calcium concentration ([Ca$^{2+}$]) on the apoptosis of Caco-2 cells.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco. FDA, A$^{3+}_{21387}$ and Fluo-3/AM were purchased from the Laboratory of Molecular Cell Biology of Hebei Normal University. Desferrioxamine (DFO), ferric
ammonium citrate (FAC) and A23187 were dissolved in Ca\(^{2+} /\)Mg\(^{2+}\)-free phosphate-buffered saline containing 0.40 g KCl, 0.06 g KH\(_2\)PO\(_4\), 8 g NaCl, 0.55 g NaHCO\(_3\), and 0.09 g Na\(_2\)HPO\(_4\); 7H\(_2\)O in 1-L liquid.

**Cell preparation**

Caco-2 cells, from cell store room of Shanghai Cell Academic Institution of CAS, were cultured in DMEM supplemented with 10% fetal bovine serum. Culture medium was changed every 2-3 d. The 20-30\(^{th}\) generation of Caco-2 cells cultured for 3 d at 37 \(^\circ\)C was digested by trypsin and the cell concentration was fixed to about 1×10\(^6\)/mL. They were then seeded into six-well plates and continuously cultured. The culture liquid was made up of \(\phi\) (FCS) = 10%, DMEM \(\phi\) (high sugar) = 85%, double antibiotics in which the final concentration of penicillin was 100 IU/mL and that of streptomycin was 100 μg/mL.

**Fluo3-AM loading**

The Caco-2 cells were incubated with Fluo3-AM working solution containing 0.03% Pluronic F-127 (the final concentration of Fluo3-AM was 20 μmol/L) at 37 \(^\circ\)C for 40 min. After incubation, the cells were washed thrice at 25 \(^\circ\)C with Ca\(^{2+} /\)Mg\(^{2+}\)-free phosphate-buffered saline to remove extracellular Fluo3-AM.

**Measurement of [Ca\(^{2+}\)]**

After Fluo3-AM loading, the cells were mounted on the small pool of Teflon printed slice, and covered with cover glass. Only the cells with rod shape and visible striations were used for experiments. The fluorescence signals were detected with a confocal laser scanning system (Biorad lasersharp MRA2, Oxfordshire, UK), which was equipped with a Nikon E-600 eclipse microscope. An argon laser was used to excite Fluo3 at 488 nm and emit at 530 nm. \([\text{Ca}^{2+}]\) changes were represented with fluorescence intensity (FI).

**Experimental protocols**

The experiments consisted of three groups: (1) Effect of DFO on intracellular calcium concentration ([Ca\(^{2+}\)]): FI was measured after 100, 200 and 300 μmol/L DFO were added to normal phosphate-buffered saline containing Ca\(^{2+}\) and Mg\(^{2+}\) for 20 min (\(n = 150\)). Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline was used as a control (\(n = 150\)). (2) Effect of FAC on [Ca\(^{2+}\)]: FI was measured after 10, 50 and 100 μmol/L FAC were added to normal phosphate-buffered saline containing Ca\(^{2+}\) and Mg\(^{2+}\) for 20 min (\(n = 150\)). Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline was used as a control (\(n = 150\)). (3) Effect of calcium ionophore A23187 on [Ca\(^{2+}\)]: FI was measured after 0.1, 1.0, and 10 μmol/L A23187 were added to normal phosphate-buffered saline containing Ca\(^{2+}\) and Mg\(^{2+}\) for 20 min (\(n = 150\)). Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline was used as a control (\(n = 150\)).

**FCM examination method**

The Caco-2 cells of the 20-30\(^{th}\) generation were planted to a conflux monolayer, and the course of medication was the same as the previous one. PBS (0.01 mol/L) was added to the cells after they were digested, the cells were then centrifuged and the supernatant fluid was discarded. The cells were fixed with cold ethanol of 70%, and then stored at -4 \(^\circ\)C overnight for flow cytometry (FCM) analysis.

**Data processing**

The cells were dealt with confocal assistant software, and 150 cells were disposed for each sample. The data were analyzed by ANOVA and LSD examination by STAT software, and the examination results were expressed as mean±SD.

The coherence examination of \(\chi^2\)-test was used for the test of apoptotic percentage, and the test method of \(df=1\) and \(f>5\) for two groups was used. The calculating formula was \(\chi^2 = \Sigma (f_i-f_e)^2/f_e\), where practice frequency is denoted with \(f_i\), theoretical frequency is denoted with \(f_e\), and the summation is denoted with \(\Sigma\). The test level of \(\chi^2\)-test was \(\alpha = 0.05\), and \(P<0.05\) was considered statistically significant.

**RESULTS**

**Primary culture of epithelial cells**

The intestinal mucosa suspension after being digested was constituted of recess epithelial cells observed under an inverted microscope. The cell livability was more than 95.7% digested by the mixture of collagenase IX and neutral proteinase I after trypsin blue staining. There were undispersed epithelial cells and elastic fiber in the discarded depositions (Figure 1A). Primary cultured cells of intestinal mucosa were adhered after 24-48 h and then converged into groups of cell colonies after 4-6 d (Figure 1B), and the cells joined into pieces after 10-12 d. They were monolayer and polygon cells and pavement-like (Figure 1C). Cell borderlines were clear and not overlapped. The cytoplasm was abundant and their nuclei were round and olivary, the chromatin in the nuclei was very sparse with 1-2 nucleoli. There were few smooth muscle cells, fibroblasts and glial cells when observed under the microscope. The cells cultured in this experiment were not subcultured.

The cell keratin was the characteristic antigen ingredient of epithelial tissue. The antibody of cell keratin marked with biotin was used in this experiment to show qualitatively the rat intestinal epithelial cells. The immunocytochemistry result showed that the intestinal epithelial cells were positive brown (Figure 1D), and that the negative group was not stained (Figure 1E).

**Effect of DFO on [Ca\(^{2+}\)]**

Fe\(^{3+}\) was chelated by adding different concentrations of DFO into the culture liquid. DFO (100, 200 and 300 μmol/L) increased [Ca\(^{2+}\)] of Caco-2 cells in a concentration-dependent manner. The FI value of Caco-2 cells was 35.71±13.99 (\(n = 150\), \(P<0.01\)), respectively. The FI value of control was 27.50±13.18 (control, \(n = 150\), \(P<0.01\), respectively). The calculating formula was \(\Sigma (f_i-f_e)^2/f_e\), where practice frequency is denoted with \(f_i\), theoretical frequency is denoted with \(f_e\), and the summation is denoted with \(\Sigma\). The test level of \(\chi^2\)-test was \(\alpha = 0.05\), and \(P<0.05\) was considered statistically significant.
Effect of ferric ammonium citrate (FAC) on [Ca\(^{2+}\)]

Fe\(^{3+}\) was increased by adding different concentrations of Fe\(^{3+}\) donor FAC into the culture liquid. The FI value of Caco-2 cells was examined, the result showed that FAC (10, 50 and 100 \(\mu\)mol/L) decreased the FI from 44.43±14.14 (control, \(n = 150\)) to 185.85±33.77 (\(n = 150\), \(P<0.01\)), 122.73±58.47 (\(n = 150\), \(P<0.01\)), and 53.29±19.82 (\(n = 150\), \(P<0.01\)), respectively. There was a significant difference among the treatments (\(P<0.01\), Figure 2B). It showed that the transportation of Ca\(^{2+}\) into the cells could be induced by the slight increase of [Fe\(^{3+}\)], but the transportation of Ca\(^{2+}\) into the cells was hindered with the continuous increase of [Fe\(^{3+}\)] in the cells.

Effect of calcium ionophore A23187 on [Ca\(^{2+}\)]

The cell livability was examined with FDA, and its final concentration was 10 mg/L. FDA could be decomposed and take on green fluorescence when it enters the live cells, but it cannot be decomposed when it enters the dead cells and take on fluorescence. The live nature of cells was observed by confocal laser scanning microscope, and the cell livability was more than 90%.

The FI of blank control without A23187 and Fluo-3/AM was very feeble (25.47±6.48, \(n = 150\)), and that of negative control without A23187 was relatively feeble (40.45±13.95, \(n = 150\)). The FI of negative control was obviously stronger than that of blank control (\(P<0.01\)). Calcium ionophore A23187 (0.1, 1.0 and 10 \(\mu\)mol/L) increased the FI of Caco-2 cells from 40.45±13.95 (control, \(n = 150\)) to 45.19±21.95 (\(n = 150\), \(P<0.01\)), 89.87±43.29 (\(n = 150\), \(P<0.01\)) and 104.64±51.07 (\(n = 150\), \(P<0.01\)) in a concentration-dependent manner. There was a significant difference among the treatments (\(P<0.01\), Figure 2C).

Discussion

Iron is one of the most abundant micronutrients. Excessive iron also has cell toxicity and induces cell damnification. So there are rigid regulating mechanisms in the body to keep the balance of iron metabolism. Duodenum and jejunum are the main place of iron absorption and the hinge position in regulating the balance of iron metabolism. It is known that iron in the enteric cavity is carried into the small intestine epithelia by divalent metal transporter 1 in its membrane with Fe\(^{2+}\) formation. It is commonly considered that calcium inhibits iron absorption\(^{[1-5]}\). Iron absorption was hindered by adding CaCl\(_2\) and this function was in a dose-dependent
manner by separating rat gastrointestinal loop and observing the iron absorption after the rat was supplied with different doses of calcium (CaCl$_2$)\textsuperscript{[6-10]} . At present, the mechanism of reciprocity between calcium and iron has not yet been elucidated. Although the absorption methods of heme iron and nonheme iron were different, their absorption was also hindered by calcium. Hallberg \textit{et al}\textsuperscript{[6]} thought that the same transport carrier was used by calcium and iron, and there existed competition and inhibition between them in the transport process from mucous membrane cells to blood plasma. This track of nonheme iron was the same to heme iron. There were only few reports on the calcium absorption under the effect of iron. Calcium absorption was not influenced by the increase of Fe$^{3+}$ concentration, but it was increased by iron only in the instance of the ratio of 20 to 1 of iron to calcium under the effect of different Fe$^{3+}$ in the brush border vesicle\textsuperscript{[11-16]} . In the present study, we demonstrated that the Fe$^{3+}$ chelating agent DFO increased the Ca$^{2+}$ concentration of Caco-2 cells in a concentration-dependent manner and the FAC decreased the Ca$^{2+}$ concentration of Caco-2 cells, suggesting that calcium absorption is influenced by [Fe$^{3+}$]. But the idiographic
mechanism of calcium absorption increased by a low dose of Fe²⁺ and inhibited by a high dose of Fe²⁺ remains to be further established.

In the present study, calcium ionophore A23187 increased the Ca²⁺ concentration of Caco-2 cells in a concentration-dependent manner. Ca²⁺ being the important second messenger in cells is the signal of subsistence and death, and almost all physiological activities are regulated by Ca²⁺, for instance, flop of heart, secretion of hormone and transfer and reserve of information in cerebrum. The foundation at the start of life and the process of cells developing into special type cells are touched and controlled by Ca²⁺, and cell physiological activity is regulated by Ca²⁺, and then finally cell apoptosis is ongoing under the function of Ca²⁺. Intracellular calcium concentration is commonly between 0.1 and 10 mmol/L, while extracellular calcium concentration is about 0.1 μmol/L. It is the concentration difference that becomes the base of physiological function of Ca²⁺. The concentration of dissociating Ca²⁺ in cell cytoplasts is the pivotal factor to regulate various responses, and there are different kinds of calcium-regulating mechanism to keep its balance. Some studies showed that the transitory increase of Ca²⁺ concentration in cells could be reduced by ATP which can stimulate cells. Biological function can be regulated by mobilization of Ca²⁺ in cells including temporary reaction of muscle constriction, nerve conduction and cell secretion, etc., and permanent reaction of cell differentiation and proliferation. All in all, cell function is regulated by Ca²⁺, and it could pass the stimulating signals of excitomotors onto the enzyme reaction system and functional protein.

Excessive [Ca²⁺], is harmful to cells. Many damaging factors such as insufficient oxygen, toxin, oxidative stress, defective blood perfusion, blood poisoning, ionizing radiation and enteritis can induce the rising of intracellular calcium, which results in cell apoptosis. A23187 is a moving ionic carrier which carries bivalent positive ions of Ca²⁺, Mg²⁺, etc. into cells and two H⁺‘s are carried out of cells at the same time. Ca²⁺ can step into cell cytoplasts immediately if A23187 is added into live cell culture liquid. Therefore, A23187 is widely used to increase the extranuclear dissociated Ca²⁺ concentration in cells. The extranuclear Ca²⁺ concentration can be immediately increased by calcium carrier of A23187 when touched with cells, and then the endogeneity endoprotease, which can make DNA rupture among the nucleosomes into segments between 180 and 200 bp or its diploid segments taking on ladder atlas in the gel electrophoresis, is aroused and cell apoptosis is increased. Inhibition of oxidative phosphorylation process in mitochondria, decrease of mitochondrial membrane electric potential and ATP content in tissues, activation of phosphatase and proteinase and irreversible cell damage have been induced by calcium overload.

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