Modulation of zinc transporter expressions by additional zinc in C2C12 cells cultured in a high glucose environment and in the presence of insulin or interleukin 6

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Abstract

Zn status has been related to various chronic diseases presenting oxidative stress and inflammation, such as type-2 diabetes. Zn supplementation has been suggested to be a potential coadjuvant in the management of this condition. Zn transporters constitute a key component in the maintenance of Zn homeostasis. Our aim was to evaluate the modulatory effect of additional Zn on the expression of selected Zn transporters (ZnT1, ZnT5, ZnT7, ZIP6, ZIP7, ZIP10, ZIP14), in myoblast (C2C12) cells cultured in normal and high glucose, and in the absence or presence of insulin, and interleukin 6 (IL 6). Main findings in our study were that in high glucose conditions in absence of insulin or IL 6, additional Zn increased ZnT1, ZIP6 and decreased ZnT5 and ZIP7 expressions. However, this situation is modified by insulin, where incremental Zn induced increased expressions of ZnT1, ZnT5, and all ZIP transporters studied. IL 6 also modified gene expressions; additional Zn caused increased expressions of ZnT1, ZnT7, ZIP6, ZIP7, and ZIP14. This study provides preliminary evidence on the expression of selected Zn transporters in C2C12 cells subjected to high glucose and incremental Zn, emphasizing the relevance of inflammatory and high-insulin environments. The implications of these findings deserve further study.

Introduction

Since 1961, Zn has been recognized as an essential micronutrient in human nutrition. Zn deficiency is related to severe anemia, growth retardation, hypogonadism, and skin abnormalities [24]. Since then, Zn deficiency has been recognized as the main public health problem in developing and industrialized countries [12]. Zn plays key structural, catalytic, gene expression and signaling role. Zn is essential for the activity of more than 300 enzymes [25]. In the 1980s, the “Zn finger” motif was identified in the TFIIIA transcription factor of Xenopus [19]. Since then, more than 20 classes of “Zn fingers” have been described and are known to be functional motifs that interact with a variety of proteins, lipids, and nucleic acids [13]. Zn is also crucial for synthesis, handling and secretion of insulin by pancreatic beta-cells and insulin signaling in peripheral tissues [25].

Zn is mobilized at the cellular and organelle level by Zn transporters. Most of these Zn transporters are organ specific and their expression depend, in addition to the tissue, in part to the presence of Zn atoms and also to other factors [11]. Knowledge of the effects of modulators on Zn transporters is very limited. In humans, two major Zn transporter families have been identified: (1) Zrt-, Irt like protein (ZIP) family (Slc39A); and (2) the ZnT family of Zn transporters (Slc30A) [10]. There are 14 ZIP proteins and 10 ZnT proteins that regulate Zn homeostasis [3, 8, 10]. In general, members of the ZIP family are Zn importers and those from the ZnT family, exporters. For instance, the uptake of Zn is regulated by Zn transporters of the ZIP family, located in membranes such as plasma membrane, the endoplasmic reticulum, and Golgi. ZIP family transporters import Zn from the extracellular environment or from organelles to increase the cytosolic Zn concentration. ZnT family transporters mediate the export of Zn from cytosol into organelles or out of the cell. All ZnT transporters are located in membranes of intracellular organelles except ZnT1 that is located in the plasma membrane [10]. Some Zn transporters have attracted great

attention because its role in highly prevalent conditions, such as type 2 diabetes (T2D), has been clarified. Thus, ZnT8 was demonstrated to be crucial for synthesis and secretion of insulin [4].

Skeletal muscle plays a major role in the uptake of glucose stimulated by insulin in the post-prandial state. Under an insulin resistant state, a condition that precedes T2D, there is a decrease in insulin signaling on IRS-1, the PI-3 kinase PI3K, and Akt, resulting in decreased translocation of GLUT4 with impaired glucose transport across the plasma membrane. As mentioned earlier, Zn also plays a role in the insulin signaling cascade. Several groups have identified that Zn is able to increase tyrosine phosphorylation of the IR-β sub-unit of the insulin receptor and enhance glucose transport in absence of insulin through the PI3K signal transduction pathway [5, 20, 26]. These results have shown that Zn might have insulin-mimetic properties on glucose and lipid metabolism as an inhibitor of protein tyrosine phosphatases as protein tyrosine phosphatase 1B (PTP1B) that is a negative regulator of insulin signaling [28]. Participation of Zn in signaling cascades is in its ion form; therefore, selected Zn transporters may play a pivotal role. This aspect has been limitedly explored. In this study, we analyzed the modulatory effect of additional Zn on the expression of ZIP6, ZIP7, ZIP10, ZIP14, ZnT1, ZnT5, and ZnT7, in myoblast (C2C12) cells cultured in normal and high glucose, and in the presence of insulin, and interleukin 6 (IL 6) a proinflammatory cytokine.

Materials And Methods

Materials

DMEM was purchased from Gibco, Thermo Fisher, USA. Fetal bovine serum (FBS), antibiotic/antimycotic mix, TRIzol LS Reagent, and Turbo DNase-free kit were acquired from Invitrogen, USA. Culture bottles and other reagents were purchased from Sigma-Aldrich, Chem Co, Germany. High Capacity cDNA Reverse Transcription Kit; Fast SYBR® Green Master Mix were purchase from Applied Biosystems, USA.

Cell culture

The cell line C2C12 (ATCC® CRL-1772) was used. It was cultured with low or high glucose concentration and with normal or high Zn concentration, and in the absence or presence of insulin or Interleukin 6 (IL 6). The intracellular Zn concentration and the expression of metallothionein-2 (MT2) and selected Zn transporters (ZnT1, ZnT5, ZnT7, ZIP6, ZIP7, ZIP10, and ZIP14) were determined. C2C12 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine se-rum (FBS) and 10 IU/mL penicillin and streptomycin. Basal glucose and Zn content were 25 mM and 5 μM, respectively (control). Cells were maintained at standard temperature and CO2.

C2C12 cells were incubated for 24 hours with: Zn (ZnSO4) 10 or 100 μM and/or glucose 10 or 30 mM, in addition, the cells were challenged with insulin 1 nM or IL 6 5 nM for 24 h. The different treatments were: Gluc 10/Zn 10= glucose 10 mM, Zn 10 μM; Gluc 10/Zn 100= glucose 10 mM, Zn 100 μM; Gluc 30/Zn 10= glucose 30 mM, Zn 10 μM; Gluc 30/Zn 100= glucose 30 mM, Zn 100 μM; Gluc 10/Zn 10/Ins= glucose 10 mM, Zn 10 μM, insulin 1 nM; Gluc 10/Zn 100/Ins= glucose 10 mM, Zn 100 μM, insulin 1nM;

Gluc 30/Zn 10/Ins= glucose 30 mM, Zn 10 μM, insulin 1 nM; Gluc 30/Zn 100/Ins= glucose 30 mM, Zn 100 μM, insulin 1 nM; Gluc 10/Zn 10/IL6= glucose 10 mM, Zn 10 μM, interleukin 6 5 nM; Gluc 10/Zn 100/IL6= glucose 10 mM, Zn 100 μM, interleukin 6 5 nM; Gluc 30/Zn 10/IL6= glucose 30 mM, Zn 10 μM, interleukin 6 5 nM; Gluc 30/Zn 100/IL6= glucose 30 mM, Zn 100 μM, interleukin 6 5 nM.

The intracellular Zn concentration and mRNA relative abundance of ZnT1, ZnT5, ZnT7, ZIP6, ZIP7, ZIP10, ZIP14, and MT2 were determined by qRT-PCR. The experiments were made in triplicate and repeated at least three times.

Intracellular Zn concentration in C2C12 cells

For total Zn quantification, C2C12 cells were separated into two aliquots. One of them was lysed with nitric acid (65%) (SupraPure, Merck, Chemical Co., Darmstadt, Germany) overnight at 60 ºC. This sample was used to measure the total Zn concentration, using an atomic absorption spectrophotometer (Perkin-Elmer 2280). A 3-point standard curve was used, with Zn standard solution (CertiPur 1.19806, Merck, Chemical Co., Darmstadt, Germany). Total metal content was expressed as μg metal/μg protein per sample. The second aliquot was used to determine total protein concentration [16].

qRT-PCR of Zn transporter genes

RNA from C2C12 cells was extracted using Trizol reagent according to product protocol (Invitrogen). Extracted total RNA was treated with RNase-Free DNase Set (Qiagen) according to product instructions. Total RNA concentration was measured by absorption at 260 nm. RNA purity and concentration were checked by determining the OD ratio at 260/280 nm using a Biowave II Spectrophotometer. Reverse transcription of RNA (1.5 μg) was done using an Affinity Scrip cDNA Synthesis Kit (Stratagene). Real Time PCR was performed using Brilliant II SYBR Green QPCR Master Mix (Stratagene) on a Step One, Applied Biosystems (USA). Beta-2-microglobulin (B2M) was used as housekeeping gene.

The primers used were: (1) B2M: CCGCCTCACATTGAAATCCA and CTGCAGGCGTATGTATCAGT (NM_009735.3); (2) ZnT1: CACGACTACCCATT-GCTCAAGGA and CGTCAACGTCTCGAAGCTCTTTCA (NM_009579.3); (3) ZnT5: TGCTCTTTGACTGCTCGGCTTT and CTCTATTCGGCCATACCCATAGGA (NM_022-885.2); (4) ZnT7: TGCAAAGAACTCCTCCCTCGTTG and TCCTTGTAACTG TTGCACCCTCTG (NC_000069.7); (5) ZIP6: ACTGCCGGCTTGTTCATGTATGTC and ACTGCCGGCTTGTTCATGTATGTC (NC_000084.6); (6) ZIP7: TCTGGCCATTGG TGCTTCCTTT and AGACTGGACCAGGATGGCAAAA (NC_000083.6); (7) MT2A: TCGGAAGCCTCTTTGCAGAT and CGCCTGCAAATGCAAACAATG (NC_000074.6); (8) Zip10: ATAGCCTGGATGGTGATCATGGGT and TATGGATGTGCTGATGCCT CCAGT (NC_0000 67.6); (9) ZIP14: TCCATTGGAACGCTGCTCTC and AAACAC CATGCAGACTTGGAG (NM_001135152.1);

The products of each set of primers were confirmed using agarose gel electrophoresis. The number of mRNA copies of target and housekeeping genes were calculated according to the standard curve method. PCR amplification efficiency of each primer pair was calculated from the slope of the standard curve.

Melting curve analysis was constructed to verify the presence of gene-specific amplification and for absence of primer dimers.

Statistical Analysis

The results are expressed as median and interquartile range. Gene expression results are expressed as fold change related to Beta-2-microglobulin (B2M) used as housekeeping gene. Statistical analyses were performed with Graph-Pad Prism 6.0 software (San Diego, CA, USA). The data were analyzed depending on the normality of the results. Comparisons were conducted according to One-way ANOVA (Tukey´s post-hoc test) or Kruskal-Wallis test (Dunn´s post-hoc test) as corresponds. Statistical significance was considered when $p< 0.05$.

Results

Total Zn concentration and MT2 relative expression

Total Zn content in C2C12 cells exposed to different stimuli is shown in Figure 1A. In general, increasing Zn from 10 μM to 100 μM but not increasing glucose in the media was accompanied with greater intracellular Zn concentrations. Regarding MT2 expressions (Figure 1B), although there was a general agreement with intracellular Zn concentration results, increased expression values in 100 μM in the three conditions studied, were of higher magnitude compared than observed in intracellular Zn content changes.

Relative expression of Zn transporters (ZnT1, ZnT5 and ZnT7) and ZIP transporters (ZIP 6, ZIP 7, ZIP 10 and ZIP 14) in a normal and a high glucose environment. Effects of additional Zn.

The relative expression of Zn transporters ZnT1, ZnT5 and ZnT7 is shown in Figure 2A. Under normal Zn conditions (10 μM), glucose increase from 10 mM to 30 mM was accompanied by a significant decrease of ZnT7 but not ZnT1 or ZnT5 expressions. Additional Zn had a similar effect on ZnT1 and ZnT7 expressions, that is, decreased expressions under normal glucose conditions (10 mM) and increasing expressions (in the case of ZnT7 a not significant trend) in high glucose conditions (30 mM). ZnT5 expressions had the opposite pattern.

In Figure 2B are shown the results of the relative expressions of ZIP 6, ZIP 7, ZIP 10 and ZIP 14 transporters. Under normal Zn conditions (10 μM), glucose increase from 10 mM to 30 mM was accompanied by a reduced expression of ZIP6, and ZIP14 and an increased expression of ZIP 10, while ZIP 7 was unaffected. Increasing Zn from 10 μM to 100 μM had varied effects on the expression of the ZIP transporters studied, although they were dependent of glucose content of the media. Thus, additional Zn showed reduced expressions of all ZIP transporters in the 10 mM glucose (in the case of ZIP10 a not significant trend). In high glucose conditions (30 mM), additional Zn increased ZIP6 and decreased ZIP7 expressions, while ZIP 10 and ZIP14 were unaffected.

Relative expression of Zn transporters (ZnT1, ZnT5 and ZnT7) and ZIP transporters (ZIP 6, ZIP 7, ZIP 10 and ZIP 14) in a normal and a high glucose environment. Effects of additional Zn in the presence of insulin.

The results of the relative expression of the ZnT (Figure 3A) and ZIP (Figure 3B) transporters studied under similar conditions as described above but adding insulin to the media is displayed in these Figures. These experimental conditions were mainly aimed to observe, in the cell culture model, the effects resembling what it is observed in vivo in a high glucose and insulin resistance situation. It is noteworthy that in high glucose conditions increasing Zn from 10 μM to 100 μM had a marked increasing effect on ZnT1, ZnT5, ZIP6, ZIP7, ZIP10 and ZIP14 expressions. Moreover, in the case of ZnT1 and ZIP10 relative expression reached values well above 20 fold in contrast with the observations in the absence of insulin (see Figures 2A and 2B) where the highest expressions were below 10 fold.

Relative expression of Zn transporters (ZnT1, ZnT5 and ZnT7) and ZIP transporters (ZIP 6, ZIP 7, ZIP 10 and ZIP 14) in a normal and a high glucose environment. Effects of additional Zn in the presence of interleukin 6.

In Figure 4 are shown the results of the relative expression of the ZnT and ZIP transporters studied under similar conditions as described in Figures 2A and 2B but adding a proinflammatory agent (interleukin 6). In this experiment, conditions used were mainly aimed to observe, in the cell culture model, the effects resembling what it is observed in vivo in a high glucose and systemic inflammation situation. Under these conditions additional Zn induced increased expression of ZnT1 and ZnT7 but not ZnT5 (Figure 4A). In terms of the response of ZIP transporters, additional Zn stimulated ZIP6, ZIP7, ZIP14 but not ZIP10 expressions (Figure 4B).

Discussion

Zn status has been related to various chronic diseases, especially those presenting oxidative stress and inflammation, such as T2D. Zn supplementation has been suggested to be a potential coadjuvant in the management of this condition as result of its roles in oxidative stress, inflammation and apoptosis [25]. Furthermore, zinc has shown to be crucial for insulin synthesis and secretion at the pancreatic β cell [4] and has also shown to have insulin-mimetic properties [28]. Several studies have shown an inverse relationship between Zn availability and cellular oxidative stress. Oteiza et al., [22] showed the increase of reactive oxygen species (ROS) production in 3T3 cells under low Zn concentrations. Ho et al., [6] demonstrated that ROS levels raised in Zn deficient rat C6 glioma cells; increased ROS production was related to an oxidative DNA damage in these cells. In addition, studies in lung fibroblasts, liver cells, neuroblastoma cells and prostate epithelial cells showed increased levels of ROS and oxidative damage when grown in low Zn environments [7, 31, 32].

Inflammation is another major risk factor for chronic diseases and Zn has been pointed to be essential for the normal function of the immune system in both innate and adaptive immunity response. Zn deficiency results in reduced antibody production and cell-mediated immune response impairment. Also,

Zn deficiency contributes to a chronic inflammatory state. These conditions are of particular interest in diseases such as T2D and cardiovascular diseases [30]. Zn regulates two major transcription factors that modulate inflammation: nuclear factor kappa-B (NF-κB) and hypoxia-inducible factor-1 alpha (HIF 1α), controlling the central inflammatory cascade [14]. Our group has recently published results on the anti-apoptotic effects of additional zinc by modifying the expression of pro and antiapoptotic genes in cultured myoblasts [1].

In order to make the effects of zinc in distinct tissues and cells possible, this element needs to be mobilized to the cell/organelle target. This is accomplished by Zn transporters. Although in general terms ZnT transporters are mainly exporters from cytoplasm to organelles or to the extracellular space, and ZIP transporters are mainly importers to the cytoplasm, there are relevant particularities related to tissues and specific conditions [10]. In the context of T2D, ZnT8 has been the center of attention because its crucial role in the pancreatic beta cell has been demonstrated. Furthermore, its deletion causes major alterations in insulin synthesis and secretion [29]. This Zn transporter is almost exclusively expressed in these cells [11].

In glucose management, in addition to pancreas and liver, skeletal muscle is highly relevant because it represents the largest mass of glucose utilization tissue and at the same time the major mass of body Zn. Knowledge on conditions present in insulin resistance and T2D, such as hyperglycemia, hyperinsulinemia, and inflammation, and how they relate to Zn is very limited. In order to explore such relationships, we used a myoblast cell model assessing the effect of additional Zn on selected Zn transporters cultured in normal and high glucose and in the absence or presence of insulin and a proinflammatory stimulus (IL 6).

The main findings in our study were that in high glucose conditions in absence of insulin or IL 6, additional Zn increased ZnT1, ZIP6 and decreased ZnT5 and ZIP7 expressions. However, this situation is marked modified in the presence of insulin, where incremental Zn induced increased expressions of ZnT1, ZnT5, and all four ZIP transporters studied. Presence of IL 6 in the media also modified gene expressions. Thus, additional Zn caused increased expressions of ZnT1, ZnT7, ZIP6, ZIP7, and ZIP14. We consider these preliminary observations interesting, although we agree that implications are far from being fully understood as result of the limited available information. On the other hand, these observations can serve as point of departure for further studies.

Information in the literature related to the issue under study is very limited. For instance, studies in skeletal muscle cell line C2C12 have shown that glucose metabolism is enhanced by ZIP7 via Akt phosphorylation, ZIP7 activates insulin receptor signaling by its binding to PTP1B [21]. Huang et al., [9] studied ZnT7 KO mice. They reported that a combination of decreased insulin secretion and increased insulin resistance may be responsible for the glucose intolerance observed in ZnT7 KO mice. The activity of the insulin signaling pathway was down-regulated in myocytes isolated from the femoral muscle of Znt7 KO mice. Besides the studies on ZnT7 and ZIP7 in muscle cells, knowledge on potential roles of other Zn transporters in the glucose/insulin homeostasis, is scarce. For instance, Paskavitz et al., [23]

studied the changes in Zn transporter expressions as differentiation of C2C12 cell progresses. In terms of studies on insulin and the ZIP transporters studied here, there is an absence of reports in muscle cells. There are a few studies however, in ZIP6 and ZIP7 in pancreatic β cells [15] and ZIP14 in pancreatic [18], pre-adipocytes [17] and hepatic cells [2]. Regarding ZnT transporters in muscle cells no information was found with respect to ZnT1 and ZnT5. There are some evidence demonstrating that ZnT7 deficiency had adverse effects on fatty acid metabolism and insulin action in subcutaneous, but not epididimal fat in mice [27].

Conclusions

This study provides preliminary evidence on the expression of selected ZnT and ZIP transporters in C2C12 cells subjected to high glucose and incremental Zn, emphasizing the relevance of inflammatory and high-insulin environments. The implications of these findings in T2D using Zn supplements deserve further study.

Declarations

Author Contributions:

MR, MAG and MAO conception and design of research. MAG performed experiments. MAG and MAO analyzed data. MAG, MR and MAO interpreted results of experiments. MAG and MAO prepared figures. MAG drafted manuscript. MAG, MR, and MAO edited and revised manuscript. MAO and MR approved final version of the manuscript.

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Declarations.

Ethics Approval and Consent to Participate. This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest.

Manuel Ruz and Miguel Arredondo received stipends from the research project FONDECYT 1120323. The rest of authors declare that they have no conflicts of interest

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Figures

Figure 1

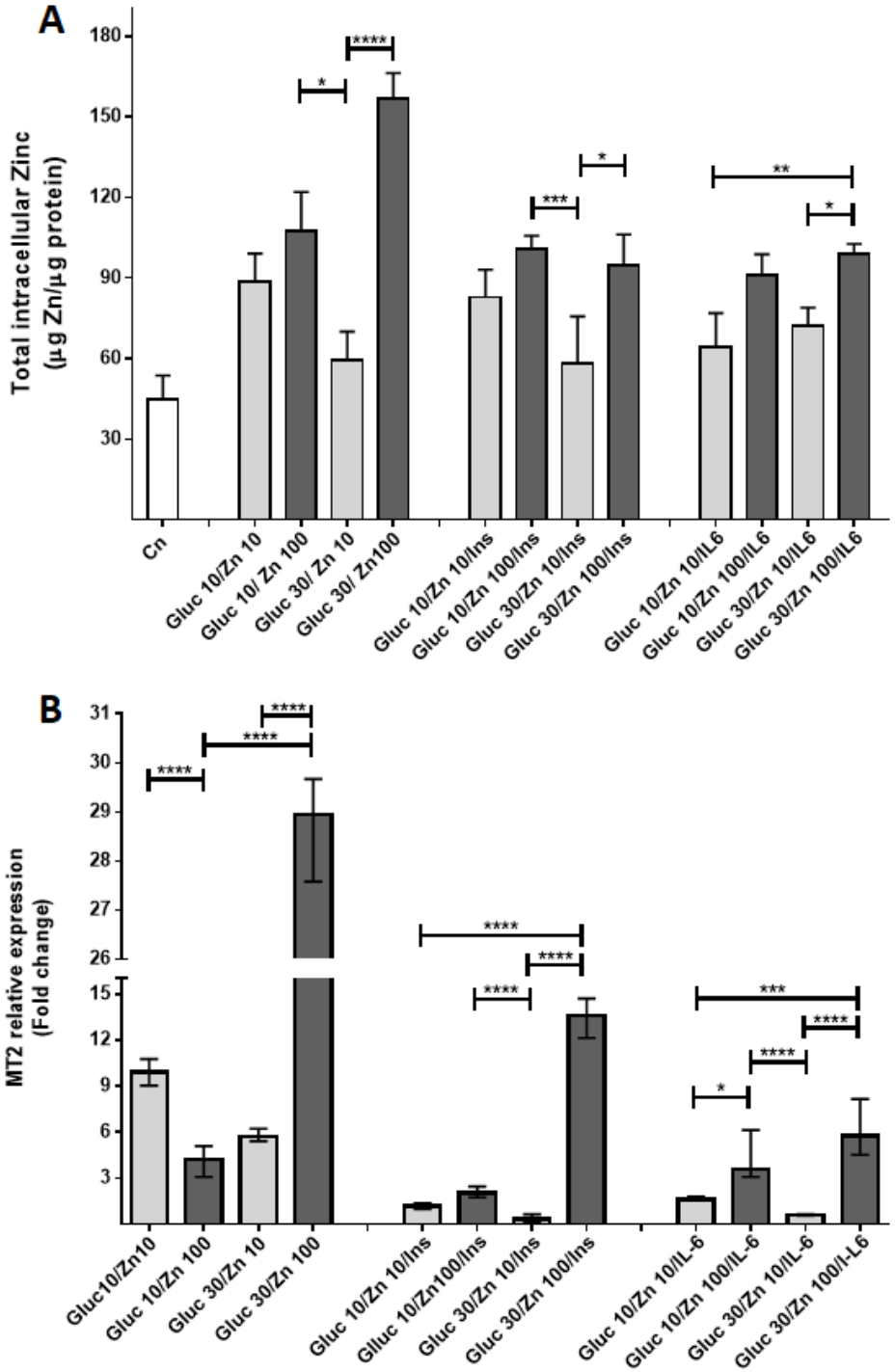

Figure 1

Total Zn concentration (Panel A) and Metallothionein 2 (MT2) (Panel B) relative expression. C2C12 cells cultured for 24 hours with different stimulus: Cn= control; Gluc 10/Zn 10= glucose 10 mM, Zn 10 µM; Gluc 10/Zn 100= glucose 10 mM, Zn 100 µM; Gluc 30/Zn 10= glucose 30 mM, Zn 10 µM; Gluc 30/Zn 100= glucose 30 mM, Zn 100 µM; Gluc 10/Zn 10/Ins= glucose 10 mM, Zn 10 µM, insulin 1 nM; Gluc 10/Zn 100/Ins= glucose 10 mM, Zn 100 µM, insulin 1nM; Gluc 30/Zn 10/Ins= glucose 30 mM, Zn 10 µM,

insulin 1 nM; Gluc 30/Zn 100/Ins= glucose 30 mM, Zn 100 µM, insulin 1 nM; Gluc 10/Zn 10/IL6= glucose 10 mM, Zn 10 µM, interleukin 6 5 nM; Gluc 10/Zn 100/IL6= glucose 10 mM, Zn 100 µM, interleukin 6 5 nM; Gluc 30/Zn 10/IL6= glucose 30 mM, Zn 10 µM, interleukin 6 5 nM; Gluc 30/Zn 100/IL6= glucose 30 mM, Zn 100 µM, interleukin 6 5 nM. Results are presented as Median ± Interquartile range. MT2 results are expressed as fold change related to Beta-2-microglobulin (B2M) used as housekeeping gene. Statistical comparisons: *p< 0.05; **p< 0.01; ***< 0.001; ****< 0.0001. Comparisons not shown are not significant p>0.05.

Figure 2

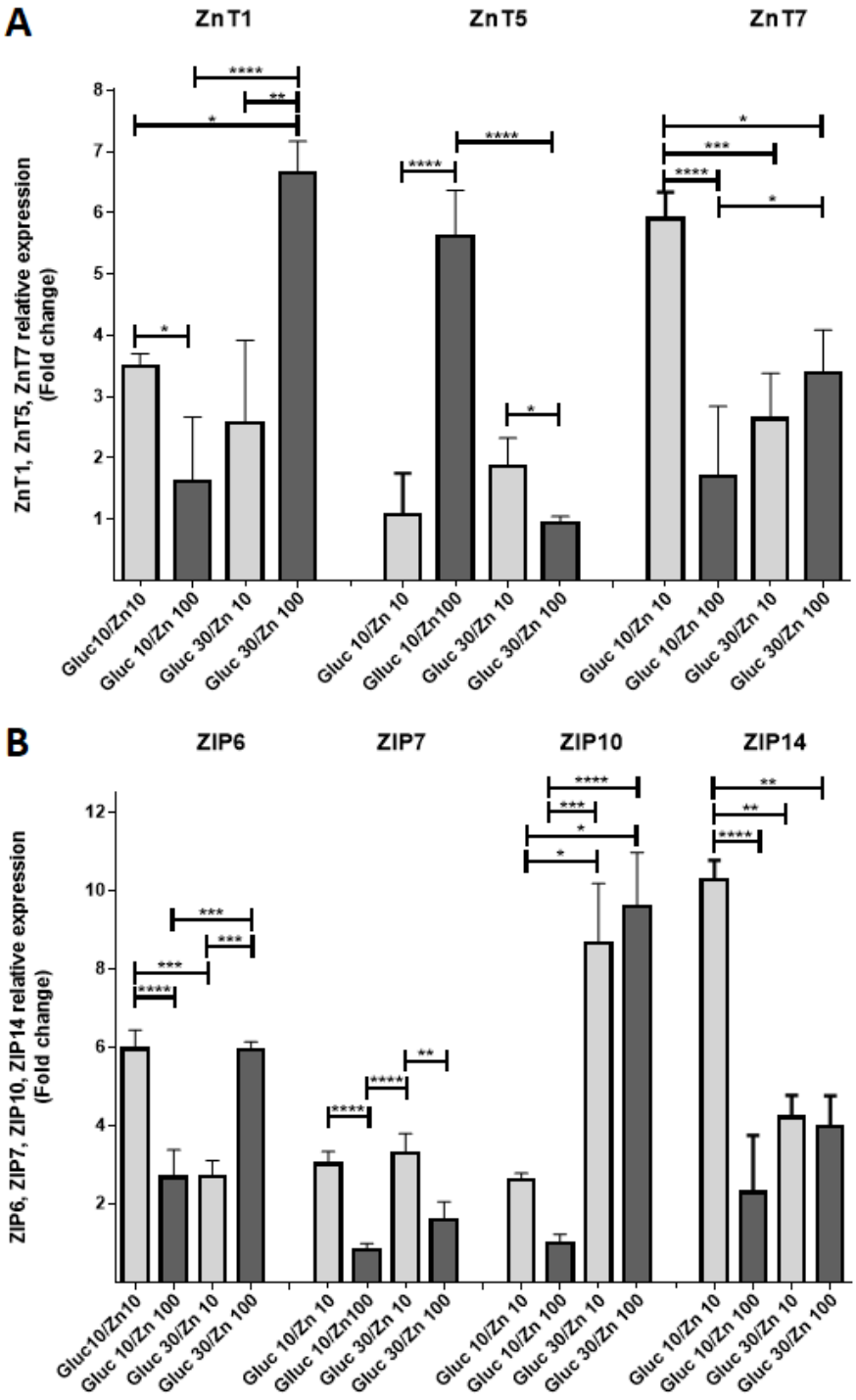

Figure 2

Relative expression of ZnT1, ZnT5, and ZnT7 (Panel A) and ZIP6, ZIP7, ZIP10 and ZIP14 (Panel B). Effects of additional Zn. C2C12 cells cultured for 24 hours with different stimulus: Gluc 10/Zn 10= glucose 10 mM, Zn 10 µM; Gluc 10/Zn 100= glucose 10 mM, Zn 100 µM; Gluc 30/Zn 10= glucose 30 mM, Zn 10 µM; Gluc 30/Zn 100= glucose 30 mM, Zn 100 µM. Results are presented as Median ± Interquartile range. Results are expressed as fold change related to Beta-2-microglobulin (B2M) used as housekeeping

gene. Statistical comparisons: *p< 0.05; **p< 0.01; ***< 0.001; ****< 0.0001. Comparisons not shown are not significant p>0.05.

Figure 3

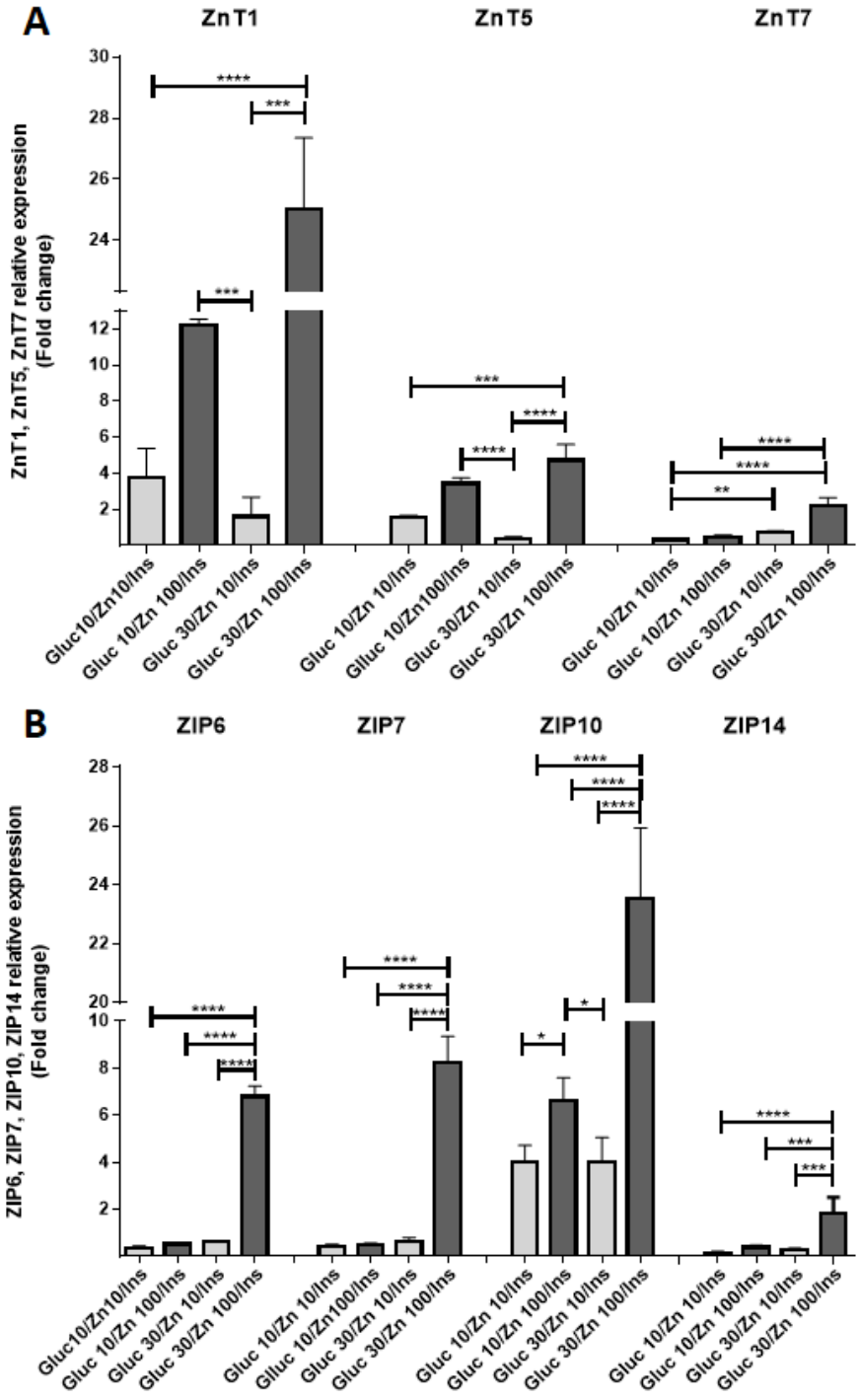

Figure 3

Relative expression of ZnT1, ZnT5, and ZnT7 (Panel A) and ZIP6, ZIP7, ZIP10 and ZIP14 (Panel B). Effects of additional Zn in the presence of Insulin. C2C12 cells cultured for 24 hours with different stimulus: Gluc 10/Zn 10/Ins= glucose 10 mM, Zn 10 µM, insulin 1 nM; Gluc 10/Zn 100/Ins= glucose 10 mM, Zn 100 µM, insulin 1nM; Gluc 30/Zn 10/Ins= glucose 30 mM, Zn 10 µM, insulin 1 nM; Gluc 30/Zn 100/Ins= glucose 30 mM, Zn 100 µM, insulin 1 nM. Results are presented as Median ± Interquartile range. Results are expressed as fold change related to Beta-2-microglobulin (B2M) used as housekeeping gene. Statistical comparisons: *p< 0.05; **p< 0.01; ***< 0.001; ****< 0.0001. Comparisons not shown are not significant p>0.05.

Figure 4

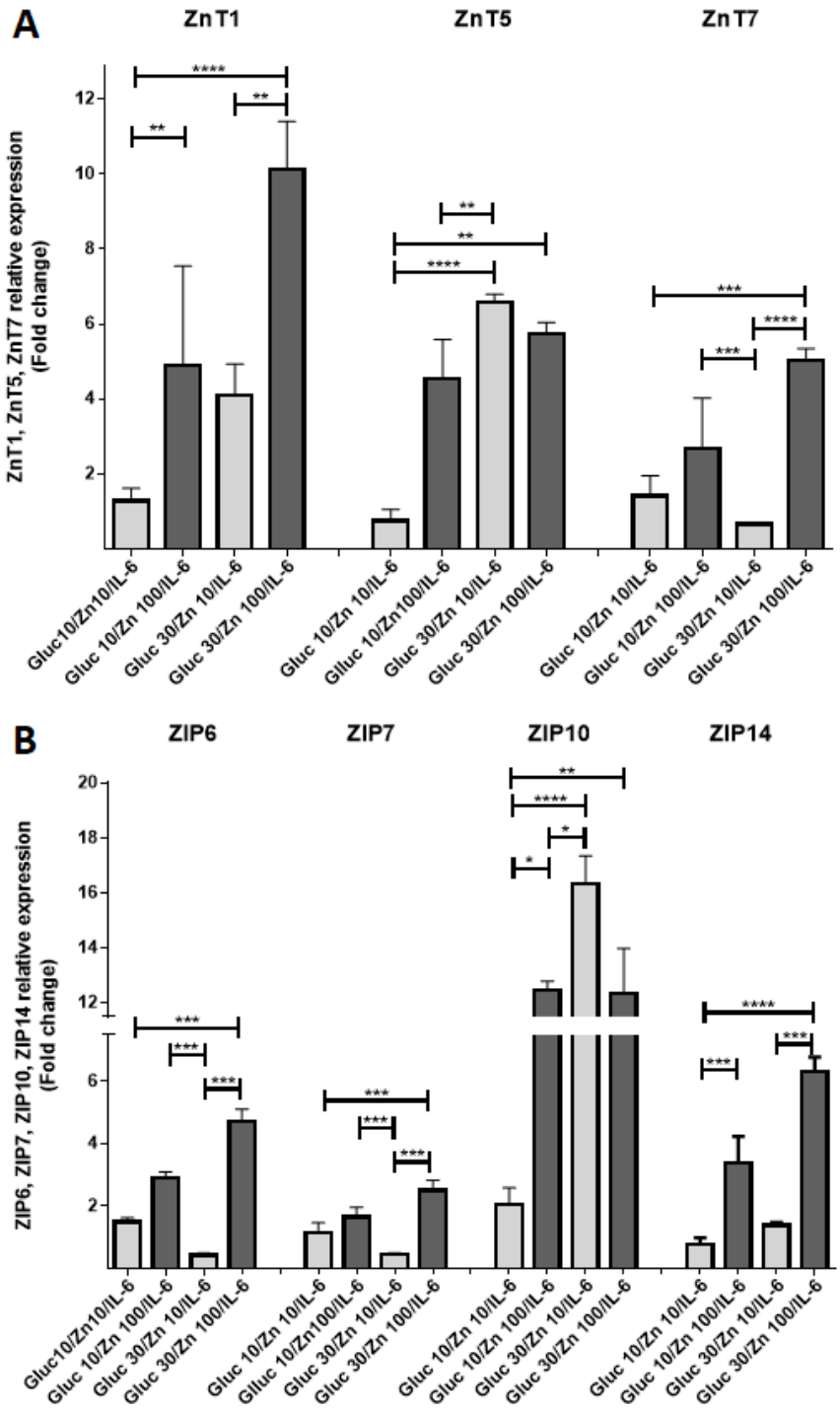

Figure 4

Relative expression of ZnT1, ZnT5, and ZnT7 (Panel A) and ZIP6, ZIP7, ZIP10 and ZIP14 (Panel B). Effects of additional Zn in the presence of Interleukin 6. C2C12 cells cultured for 24 hours with different stimulus: Gluc 10/Zn 10/IL6= glucose 10 mM, Zn 10 µM, interleukin 6 5 nM; Gluc 10/Zn 100/IL6= glucose 10 mM, Zn 100 µM, interleukin 6 5 nM; Gluc 30/Zn 10/IL6= glucose 30 mM, Zn 10 µM, interleukin 6 5 nM; Gluc 30/Zn 100/IL6= glucose 30 mM, Zn 100 µM, interleukin 6 5 nM. Results are presented as

Median ± Interquartile range. Results are expressed as fold change related to Beta-2-microglobulin (B2M) used as housekeeping gene. Statistical comparisons: *p< 0.05; **p< 0.01; ***< 0.001; ****< 0.0001. Comparisons not shown are not significant p>0.05.