Effects of Zinc on Glucose-6-Phosphate Dehydrogenase in Buffalo Rat Liver Cells

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"Effects of Zinc on G6PDH Activity"

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Effects of Zinc on Glucose-6-phosphate Dehydrogenase in Buffalo Rat Liver Cells

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Abstract

In recent years, interest has surfaced in the use of insulin-mimicking agents for diabetic therapies. Zinc has been shown to have insulin-like effects *in vivo* and *in vitro* but has never been studied for its ability to mimic insulin in the regulation of carbohydrate and fatty acid metabolism. Therefore, we studied the effects of zinc on glucose-6-phosphate dehydrogenase (G6PDH), an enzyme stimulated by insulin that is imperative in both carbohydrate and fatty acid metabolism. Because G6PDH is highly regulated in liver cells, a stable rat liver cell line, Buffalo Rat Liver (BRL), was used. Endogenous G6PDH activity and G6PDH promoter activity were both significantly stimulated at 1 μM [Zn²⁺]. Two antioxidants, trolox C and catalase, eliminated the zinc-induced increase of endogenous G6PDH activity and G6PDH promoter activity respectively. Further studies were performed to investigate whether zinc effects are produced due to the generation of reactive oxygen species. Upon addition of trolox C and zinc, BRL cell death was decreased compared to the zinc alone treatment. A compound known to eliminate glutathione, BSO, had no effect on BRL cell viability. These results infer that the mechanism of zinc action does involve the generation of reactive oxygen species (ROS) but that zinc does not produce ROS through the depletion of glutathione. Zinc has also been shown here to be toxic only at very high concentrations, a phenomenon seen with vanadate and selenate, two well-studied insulin mimetics. The results presented here demonstrate that zinc regulates G6PDH similarly to insulin and generates enthusiasm for zinc as a possible insulin mimic.

Introduction

Glucose-6-phosphate dehydrogenase (G6PDH—EC 1.1.1.49) is the rate-limiting enzyme in the pentose phosphate pathway and a key contributor to carbohydrate and fatty acid metabolism. In the fed state, as much as 60% of the glucose present is directed through the pentose phosphate pathway. This pathway, which is regulated at the G6PDH reaction, supplies the body with both NADPH, the major reducing equivalent and an essential participant in lipogenesis, and ribose-5-phosphate, the sugar necessary for nucleic acid synthesis. Although G6PDH is considered a "housekeeping" enzyme, it is highly regulated in liver cells. Insulin, vanadate and selenate (insulin mimetics), and reactive oxygen species (ROS) have all been shown to regulate G6PDH. In the presence of insulin, liver G6PDH expression is increased through a cascade of events. This
cascade is initiated by insulin binding to the insulin receptor and mediated by tyrosyl phosphorylation of signal proteins in the phosphatidylinositol-3 (PI-3) kinase pathway (Journal of Biological Chemistry in press). Vanadate and selenate, two postulated insulin mimetics, have also been shown to induce the expression of G6PDH in rat hepatocytes (Stapleton et al., 1995). Furthermore, our lab and others have shown that the insulin receptor and some signal proteins in the insulin pathway (insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3 (PI-3) kinase, and mitogen activated protein kinase (MAPK)) are activated by the presence of vanadate and/or selenate (Stapleton et al., 1996; Heffetz & Zick, 1989; Heffetz et al., 1990; unpublished results).

Recently, data showing that G6PDH expression may also be regulated by the oxidative state of the cell has been published. Diquat and ethanol, two compounds known to enhance oxidative stress, have been shown to increase G6PDH activity at the transcriptional level (Cramer et al., 1995; Kleitzen et al., 1994). Because G6PDH is the primary source of NADPH, the major reducing equivalent responsible for maintaining the oxidative state of cells, it is not surprising that G6PDH levels have been shown to increase due to the presence of oxidative stressors. Figure 1 shows the role that NADPH produced from G6PDH plays in reducing reactive oxygen species.

Understanding the regulation of G6PDH by insulin compared to insulin mimetics and ROS is important for possible diabetic therapies involving insulin mimetics.

Zinc, a postulated insulin mimic, is an essential trace element (like selenium) that enters the cells through a high affinity, saturable pathway (Taylor & Simmons, 1994). It is a cofactor for over 200 enzymes, some of which play a role in maintenance of cell functions including transport processes and subcellular organelle integrity (Vallee & Auld, 1990). The concentration of zinc in
the blood is approximately 15 μM, however, only about 0.2 nM of that is not bound to albumin (Taylor & Simmons, 1994).

Zinc has been shown to have insulin-like affects at both the organismal and cellular level. At the organismal level, ZnCl₂ administered orally to diabetic rats reduced blood glucose levels as much as 50% after 2 hours of treatment (Shisheva et al., 1992). At the cellular level, several studies have shown that the presence of zinc stimulates glucose transport and inhibits lipolysis in rat adipocytes (Ezaki, 1989; May and Contoreggi, 1982). Zinc addition to adipocytes also increased glucose oxidation by glycolysis and the pentose phosphate pathway (May & Contoreggi, 1982; Shisheva et al., 1992) and stimulated lipogenesis (Coulston & Dandona, 1980). Like insulin, zinc has been shown to be an antagonist of glucagon (cAMP) with regards to glycolysis regulation (Brand & Kleineke, 1996).

Zinc has also been shown to increase oxidative stress in the cell. Deibel et al. postulated that zinc plays an oxidative stressor role in the neurodegeneration hypothesis for Alzheimer's disease (Deibel et al., 1996). Furthermore, metallothionein (MT), a known scavenger of free radicals, has also been shown to protect mice against zinc toxicity in vivo (Kelly et al., 1996). May and Contoreggi postulate that zinc mimics insulin, in part, through the generation of H₂O₂, a reactive oxygen species, because insulin-like effects of zinc were inhibited upon catalase addition (May and Contoreggi, 1982). However, the action of zinc due to the production of H₂O₂ was not confirmed in a later study (Ezaki, 1989).
Oxidative stress on the cell is produced through the generation of free radicals and other reactive oxygen species. A free radical is defined as any species that has one or more unpaired electrons (Halliwell & Gutteridge, 1984). Oxygen free radicals include hydroxyl radical (OH\(^{•}\)), superoxide (O\(_2^{•}\)), alkoxy (RO\(^{•}\)), and peroxyl (ROO\(^{•}\)) radicals (Pietrangelo, 1996). Although H\(_2\)O\(_2\) does not have one or more unpaired electrons, it causes similar effects as the oxygen free radicals and is easily converted to the very toxic OH\(^{•}\) radical as shown in Equation 1. Collectively, oxygen free radicals and hydrogen peroxide are referred to as reactive oxygen species (ROS). The oxidation of biological molecules, membranes, and tissue by ROS is associated with such pathological events as cancer, aging, and diabetes mellitus (Paolisso & Giugliano, 1996). Furthermore, ROS can cause DNA damage, activate procarcinogens, damage proteins, cause lipid oxidation, and alter the cellular antioxidant system (Bagchi et al., 1997; Paolisso & Giugliano, 1996).

It is not surprising that zinc has been shown to generate oxidative stress because transition metals, which frequently have unpaired electrons, are excellent catalysts for the generation of oxidative stress. Two primary mechanisms through which metals induce oxidative stress are 1) single e\(^{-}\) withdrawal and 2) depletion of glutathione. Through the transfer of single electrons, metals can cause the formation of ROS by catalyzing the reactions shown in Equation 1.

\[
\text{Equation 1: } \text{O}_2 \xrightarrow{e^{-}} \text{O}_2^{-} \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{OH}^{•}
\]

Iron and copper have extensively been studied and shown to produce highly reactive hydroxyl radicals by the mechanism outlined in Equation 2 (Deibel et al., 1996; Freeman & Crapo, 1982).

\[
\text{Equation 2: } \text{M}^{(n+1)+} + \text{H}_2\text{O}_2 \rightarrow \text{M}^{(n+1)+} + \text{OH}^{•} + \text{OH}^{-}
\]

Co\(^{3+}\) and V\(^{3+}\) are also capable of oxidizing various compounds by single-electron withdrawal. However, other metals can create oxidative stress through depleting glutathione. Glutathione has
a high affinity for metals and is a substrate in the reduction of $\text{H}_2\text{O}_2$ by GSH peroxidase as shown in Figure 1. Reduced levels of glutathione increase the susceptibility of cells to damage by $\text{H}_2\text{O}_2$ because the natural defense mechanism against ROS (Figure 1) is compromised (Paolisso & Giugliano, 1996). Cd$^{2+}$, Hg$^{2+}$, Ni$^{2+}$, and Pb$^{2+}$ increase oxidative stress through the depletion of glutathione (GSH) (Stohs & Bagchi, 1995).

![Role of G6PDH in the natural defense mechanism against reactive oxygen species](image)

Zinc, because it exists solely in the divalent state, is unlikely to participate in Equation 1 to produce ROS (May and Contoreggi, 1982). However, zinc has been shown to be a potent inhibitor of glutathione reductase (GSSG reductase) in liver homogenates (Mize et al., 1962) and may generate oxidative stress by inhibiting the reduction of GSSG to GSH, thereby depleting glutathione levels. However, the mechanism by which zinc causes oxidative stress is still unclear.

In recent years, interest has surfaced in the use of insulin-mimicking agents for diabetic therapies. It is, therefore, important to assess if postulated insulin mimetic agents control carbohydrate and
fatty acid metabolism similarly to insulin. One way to do this is to observe the effect insulin-mimetics have on G6PDH activity. Zinc, an agent that has been shown to have many insulin-like effects, has never been studied for its ability to stimulate the expression of G6PDH or any other enzymes in fatty acid or carbohydrate metabolism. Thus, it was my goal to study the effect of zinc on G6PDH activity in Buffalo Rat Liver (BRL) cells. Furthermore, since G6PDH is regulated by ROS, it is important to assess if zinc effects on G6PDH are caused by the generation of ROS. In order to do this, 1) the effects on endogenous G6PDH activity were tested with treatments of zinc and an agent that affects ROS production, 2) the effects on the expression of the G6PDH promoter were tested with treatments of zinc and an agent that affects ROS, and 3) the effects on cell viability were examined with treatments of zinc and agents that affect ROS production.

**Experimental Procedures**

**Materials**

ZnCl₂ was used as a source of [Zn²⁺]. Buthionine sulfoximine (BSO) and trolox C were purchased from Aldrich Chemical Company, Milwaukee, WI. DMEM-F12 media was obtained from Life Technologies. The luciferase assay kit was obtained from the Promega Corporation.

**Cell Culture**

Buffalo Rat Liver (BRL) cells, a stable rat liver cell line, were used to study the effects of zinc on glucose-6-phosphate dehydrogenase (G6PDH). The BRL cells were grown in DMEM-F12 media with 5% serum at 37°C under a 5% CO₂ atmosphere. When the cell culture flasks or dishes were
75-85% confluent, the media was replaced with serum-free DMEM media for 24 hours. Various treatments were then added according to each study. In the gene expression study, BRL cells transfected with the luciferase reporter gene driven by the 935 bp promoter region of G6PDH were used to investigate the effect of zinc on the transcriptional activity of G6PDH. Figure 2 displays the plasmid used to create the stably-transfected cells and Figure 3 shows the G6PDH promoter region with various regulatory elements. The exogenous DNA in the host cell was ensured by selective pressure using a co-transfected plasmid which coded for a phosphotransferase capable of enzymatically deactivating G418, a neomycin analog. The stably-transfected BRL cells were created by Mr. Jason Leduc.

**Figure 2**

pGL2—Basic Vector with G6PDH Promoter Insert. Plasmid construct containing the 935 bp promoter region of G6PDH linked to the luciferase reporter gene. Basal transcription of the downstream luciferase reporter gene is driven by the G6PDH promoter.
Sequence of the 935 bp of rat G6PDH DNA that contains the promoter region. This promoter region is the G6PDH sequence that drives luciferase transcription in the plasmid construct shown in Fig. 3. A metal response element (MRE) is postulated to occur at 729 to 735 bp. Other regulatory elements are also noted.

G6PDH Assay

The G6PDH activity assay was performed according to Glaser & Brown, 1955. After 48 hours of zinc treatment, the media was aspirated and 1 mL of 0.1 M potassium phosphate buffer was added to each flask. The cells were then scraped off the flask, homogenized, and centrifuged to remove the membranes. The G6PDH activity of the lysates (supernatant) was measured spectrophotometrically at 340 nm by following the reduction of NADP to NADPH in the presence of glucose-6-phosphate (G6P). In a one mL assay, the final concentrations of reactants...
were 86.3 mM triethanolamine buffer (pH 7.5), 5 mM MgCl₂, 1.2 mM G6P, and 0.37 mM NADP. A 100μL aliquot of cytoplasmic sample was added to the solution of buffer, MgCl₂, and NADP in a cuvette. The reaction was started with the addition of G6P and is linear for approximately 5 minutes. Because the activity of the enzyme is measured as the slope of the linear line associated with the formation of NADPH, it is important that the samples are assayed shortly after G6P is added. A 200μL aliquot was also assayed and should produce a rate double that of the 100μL sample because the rate is proportional to the amount of enzyme added.

LDH Assay

The lactate dehydrogenase (LDH) assay was performed according to Wroblewski & LaDue, 1955. The assay for LDH, an intracellular enzyme released into the extracellular media when cell membranes are compromised, was used as a measure of the point at which zinc began to compromise the cell membranes. LDH catalyzes the reduction of pyruvate to lactate with the subsequent oxidation of NADH to NAD⁺. After 48 hours of zinc treatment, a sample of media was obtained in order to measure the amount of LDH released by the cells. The assay measures the linear, LDH-catalyzed oxidation of NADH in the presence of pyruvate spectrophotometrically at 340 nm. The final concentrations of reagents in a one mL assay were 83 mM potassium phosphate buffer, 0.67 mM pyruvate, and 0.23 mM NADH. A media sample was added to the buffer and pyruvate in a cuvette, followed by the addition of NADH which initiates the reaction. Media samples of 100μL and 200μL were assayed.
Lowry method for protein determination

The samples were assayed for protein concentration using the Lowry method (Lowry et al., 1951). The principle behind the Lowry method is a comparison between the amount of color development from known pure proteins and that of experimental samples. Color develops due to the formation of a purple protein-copper complex when the peptide nitrogen atoms combine with Cu$^{2+}$. Color also appears due to the reduction of the phosphomolybdate-phosphotungstate reagent (Folin-Ciocalten reagent) with tyrosine and tryptophan residues. In this procedure, 50 mL of 2% Na$_2$CO$_3$ in 0.1 N NaOH, 0.5 mL of 2% sodium potassium tartrate, and 0.5 mL of 1% CuSO$_4$ were mixed together. Two mL of this solution was added to an appropriate amount of sample in a test tube. The amount of sample varies because the concentration range of the assay is 5-100 µg of protein. This mixture of solution and sample was mixed well and allowed to stand at room temperature for ten minutes. After which, 0.2 mL of 1N Folin-Ciocalten “phenol reagent” was added to the test tube and color was allowed to develop for 30 minutes. Next, the samples were placed in cuvettes and the absorbance was read at 500 nm on the spectrophotometer. Along with the experimental samples, a “standard protein” of known concentration was assayed and used to construct a calibration curve using a best fit of the line through the standard protein absorbance values. In this procedure, bovine serum albumin (BSA) was used as the standard protein. The amount of protein in the experimental samples was determined by comparing the absorbance of the experimental sample to the best line fit of the BSA standard. The concentration of the protein in the sample is important to calculate the specific activity (rate/concentration) of the enzyme. This specific activity can then be compared to the specific activity of other samples.
Luciferase Assay

The luciferase assay is based on the measurement of light emitted from the oxidation of luciferin catalyzed by luciferase. Promega’s Luciferase Assay system was used for this assay. The luciferase gene acts as a reporter gene for the G6PDH promoter located upstream. After 6 hours of zinc treatment on the stably-transfected cells, the media was aspirated and the cells washed with phosphate-buffered saline (PBS). Next, 1X lysis buffer from the assay kit was added to the flask and the cells were scraped off the bottom. After 15 minutes of allowing the cell material to lyse, the solution was placed in an eppendorf tube and the debris was collected by centrifugation at 14,000 rpm. The lysate (supernatent) was then assayed for luciferase activity quantified by a scintillation counter. Experimental samples were added to 100μL of luciferin (substrate) to commence the reaction. Due to the speed of the reaction, it was important that the samples and luciferin were in solution together for less than one minute before quantifying.

Fluorescence Viability Assay

The fluorescence viability assay was performed according to Yazdanbakhsh et al., 1987 with some modifications. After treatments, the media was aspirated and washed with phosphate-buffered saline (PBS). Ethidium bromide (10μg/mL) was added to cell dishes and the number of dead cells were scored using fluorescence microscopy. Fluorescein diacetate was not used to stain the living cells due to problems of diffraction associated with the plastic culture dishes. Trolox C (6-hydro-2,5,7,8-tetramethylchromane-2-carbonic acid) (Strubelt et al., 1996) and buthionine sulfoximine (BSO) were agents used to alter the oxidative state of the cell. Trolox C was dissolved in distilled water by first adjusting the pH to 12 and then readjusting the pH to 7.4 (Wu et al., 1996).
Statistical Analysis

The luciferase, LDH, and G6PDH activities are presented as fold increases from the no addition control. Activity per unit of protein was used as the specific activity of each sample. Fluorescent viability study results are shown as percentage of viable cells per flask. Results are reported as means +/- SEM. A one-tailed students t-test was used to indicate significance (p<0.05).

Results

Effects of zinc on endogenous G6PDH activity. BRL cells were treated with varying concentrations of zinc for 48 hours and assayed for endogenous G6PDH activity. Protein concentrations were measured to determine the specific activity of G6PDH. A concentration-dependent effect of zinc on G6PDH activity is shown in Figure 4. Maximal zinc stimulation of G6PDH occurred at 1 μM. The fold increase in G6PDH activity at this concentration matched the fold increase in G6PDH activity from the insulin treatment. The increases in G6PDH activity at 0.5, 1, and 5 μM [Zn^{2+}] were significant compared to the no addition control. This finding is an important first step in investigating whether zinc stimulates carbohydrate and fatty acid metabolism similarly to insulin.
Effect of zinc on endogenous G6PDH activity in Buffalo Rat Liver cells. ZnCl₂ was used as the source of [Zn²⁺]. Ins=insulin treatment (300ng/ml). The cells were treated with zinc concentrations for 48 hours and incubated in DMEM media. An insulin treatment was used to compare the effects of zinc to insulin. [Zn²⁺] significantly increased, equivalent to insulin, the endogenous G6PDH activity. Maximal stimulation occurred at 1 μM [Zn²⁺]. The results are displayed as fold increase from the no addition control (NA).

* Indicates significance (p<0.05); n=5

**Effect and mechanism of zinc on G6PDH expression using a reporter construct.** Cells stably-transfected with a plasmid (Figure 2) containing 935 bp of the G6PDH promoter region driving the luciferase reporter gene were used to investigate the effect of zinc on the recently isolated G6PDH promoter region (Figure 3) (Rank et al., 1994). Because luciferase is not found in mammals, any luciferase activity must be from the induction of the gene located on the plasmid. If zinc stimulates the expression of G6PDH transcriptionally by acting on the G6PDH promoter, then luciferase activity will be present. The stably-transfected BRL cells were treated with 1 μM
[Zn$^{2+}$] for 6 hours. Figure 5 shows that 1 μM [Zn$^{2+}$] treatment resulted in more than a two-fold increase in luciferase activity which matches the increase in endogenous G6PDH activity associated with this treatment. In order to determine whether the induction of G6PDH by zinc could be due to the generation of reactive oxygen species, cells were treated with a catalase + zinc treatment. Catalase does not enter the cells but eliminates extracellular H$_2$O$_2$ by catalyzing Equation 3.

\begin{equation}
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\end{equation}

Because intracellular and extracellular H$_2$O$_2$ concentrations are at equilibrium, it is assumed that catalase decreases to concentration of H$_2$O$_2$ inside the cell by causing H$_2$O$_2$ efflux. The zinc + catalase treatment returned the luciferase activity to the no addition control value. This suggests that H$_2$O$_2$ is important in the zinc-induced increase in G6PDH promoter activity.

![Figure 5](image)

**Figure 5**
Effect of zinc on luciferase activity after 6 hours. ZnCl$_2$ was used as the source of [Zn$^{2+}$]. Luciferase transcription was driven by the G6PDH promoter region in the plasmid construct. 1 μM [Zn$^{2+}$] treatment resulted in a 2.0-fold increase in luciferase activity. This suggest that zinc acts transcriptionally to promote an increase in G6PDH activity. Catalase (130 U/ml), an antioxidant, restored the luciferase activity to the no addition value suggesting that the mechanism of zinc action may be due to the generation of ROS. The results are displayed as fold increase from the no addition control (NA).

* Indicates significance (p<0.05), n=4
Mechanism of zinc action on endogenous G6PDH expression. In order to further assess the possible mechanism of zinc action at the concentration that induced maximal G6PDH activity, endogenous G6PDH activity was measured with a treatment of 1 μM $[\text{Zn}^{2+}]$ and trolox C. Cells were treated with zinc and/or trolox C for 48 hours. Figure 6 shows that trolox C effectively eliminated the increase in G6PDH activity seen with 1 μM $[\text{Zn}^{2+}]$. Trolox C alone created a slight significant increase in G6PDH which is unexplained. Reactive oxygen species appear to play a role in the zinc-induced increase in endogenous G6PDH activity and G6PDH promoter activity.

![Effect of Zn and trolox C on endogenous G6PDH activity](image)

**Figure 6**
Effect of zinc and trolox C on endogenous G6PDH activity. ZnCl$_2$ was used as the source of $[\text{Zn}^{2+}]$. Cells were treated for 48 hours and then assayed for G6PDH activity. Trolox C effectively reduced the zinc-induced increase in G6PDH activity. Therefore, it is evident that ROS play a role in the induction of G6PDH activity by zinc. * indicates significance (p< 0.05) compared to the no addition control (NA). ** indicates significance compared to the 1 μM $[\text{Zn}^{2+}]$ treatment; n=3
Assessment of zinc toxicity. The decrease in endogenous G6PDH activity (Figure 4) at zinc concentrations of 5 μM and 10 μM occurred because the cells were compromised as zinc began to exert toxic effects. To assess toxic zinc effects, the media of the cells treated with zinc was measured for LDH activity, a compound that is released by alive cells into the media due to compromised membranes. Figure 7 shows the LDH activity at appropriate zinc concentrations. At 5 μM zinc, the LDH activity increased significantly suggesting that zinc begins to cause cell membranes to be compromised at this concentration. The decrease in LDH activity at 10 μM is indicative of cell death. A lack of increased LDH activity at 1 μM [Zn$^{2+}$], the concentration that induced maximal G6PDH activity, suggests that this concentration does not significantly compromise the cell membranes.
Effect of Zn on LDH activity in Buffalo Rat Liver cells

Figure 7
Effect of zinc on LDH activity in Buffalo Rat Liver cells. ZnCl₂ was used as the source of [Zn²⁺]. Ins=insulin treatment (300 ng/ml). LDH, an enzyme released by compromised membranes, is a common indicator of toxicity. BRL cells were treated for 48 hours with varying concentrations of zinc or insulin and the media was assayed for LDH activity. Cells were significantly compromised at 5 μM [Zn²⁺]. At 10 μM [Zn²⁺], cell death was indicated. Neither insulin nor the optimal concentration of zinc for maximal G6PDH activity showed a significant increase in LDH activity compared to the no addition control. The results are displayed as fold increase from the no addition control (NA). * Indicates significance (p<0.05); n=3 for 0.5, 5, 10 μM [Zn²⁺], n=4 for all other treatments.

Mechanism of zinc action. In order to further investigate the potential role of ROS in the mechanism of zinc action, flasks were treated with zinc and agents that alter the oxidative state of the cell. Cell viability was then quantified. The results using trolox C are shown in Figure 8 and the results using BSO are shown in Figure 9. Trolox C is a water-soluble analog of Vitamin E and a known antioxidant that protects against lipid peroxidation (Chen & Tappel, 1996) and ROS in rat hepatocytes (Wu et al., 1991). It enters the cell and thus can sequester intracellular oxygen.
radicals. Cells were treated with zinc and 2mM trolox C. After 24 hours of treatment, the media was aspirated and percent viability scored. The percent of dead cells was quantified using fluorescence microscopy to visualize ethidium bromide chelated to the DNA of the dead cells. Trolox C had a slight but significant protective effect on the cell viability of BRL cells treated with zinc at and below 90 μM. At very high zinc concentrations, trolox C had no effect possibly due to an overabundance of ROS. This data suggest that zinc, in part, could produce toxic effects due to the generation of free radicals (ROS). Although trolox C did not have a significant effect on cell viability at 1 μM [Zn²⁺], very little cell death at this concentration made it difficult to assess the effect of trolox C on cell viability at this concentration. However, the effects caused by zinc at higher concentrations appear to involve the generation of ROS and, therefore, ROS must also be formed at lower zinc concentrations but may not induce cell death.
Effect of zinc and trolox C on Buffalo Rat Liver cell viability. ZnCl₂ was used as the source of [Zn²⁺]. BRL cell viability was measured using fluorescence microscopy after treatments of zinc and trolox C (2mM) for 24 h. Ethidium bromide was used to stain the DNA of the dead cells. The significant, slightly protective effect of trolox C suggest that the mechanism of zinc action in part involves the generation of ROS. However, at high concentrations of zinc, no effect of trolox C could be seen. Results are shown as percent viable cells. * indicates significance (p< 0.05) compared to the same [Zn²⁺] treatment without trolox C, n=4

Zinc and BSO were added to BRL cells to investigate if the mechanism of zinc action involves the generation of ROS by depletion of glutathione. BSO is a synthetic amino acid that depletes glutathione by irreversibly inhibiting gamma-glutamylcysteine synthetase. Inhibition of this enzyme eliminates a critical step in glutathione biosynthesis (Keogh et al., 1994; Bironaite & Ollinger, 1997) and thus inhibits the body’s natural defense mechanism against ROS (Figure 1). The BRL cells were pre-treated with BSO for 24 h and then treated with zinc. After approximately 24 h of zinc treatment, the cells were scored for percent viability. Figure 9 shows
that zinc's toxic effects were not significantly altered due to the elimination of GSH by BSO.

BSO alone at higher concentrations did show a decreased cell viability in a concentration dependent manner. A slight, non significant decrease in cell viability can be seen with treatment of both zinc and BSO.

![Figure 9](image)

**Figure 9**
Effect of zinc and BSO on BRL cell viability. ZnCl₂ was used as the source of [Zn²⁺]. BRL cell viability was measured using fluorescence microscopy after treatments of zinc and BSO (2mM unless otherwise noted) for 24 h. Ethidium bromide was used to stain the DNA of the dead cells. Results are shown as percent viable cells. * indicates significance (p< 0.05) compared to the same [Zn²⁺] treatment without BSO, n=4
Discussion

It is important to assess whether possible insulin mimetics regulate carbohydrate and fatty acid metabolism as insulin does. One way to do this is to study G6PDH, the rate-limiting enzyme in the pentose phosphate pathway which is important in both carbohydrate and fatty acid metabolism. Insulin increases G6PDH activity through a phosphorylation cascade of events that includes the insulin receptor, IRS-1, PI-3 kinase, and S6K (JBC in press). Vanadate and selenate, two transition metals and postulated insulin mimetics, have also been shown recently to stimulate G6PDH activity (Stapleton, et al. 1995). Zinc, also a transition metal, has been shown in several studies to have insulin-like effects in vivo and in vitro (Shisheva et al., 1992; Ezaki, 1989; May & Contoreggi, 1982; Coulston & Dandona, 1980) but its effects on G6PDH activity have never been studied until now. The results in this study demonstrate that zinc does mimic insulin by increasing G6PDH activity. This is a key step into investigating the insulin-like effects of zinc on carbohydrate and fatty acid metabolism. The mechanism by which zinc exerts this effect is still unclear, but the generation of ROS is suggested.

Figure 4 shows that zinc effectively stimulates G6PDH activity to the same extent that insulin does. Furthermore, it is suggested that this stimulation is due to a zinc effect on the G6PDH promoter as demonstrated by the results shown in Figure 5. The finding that zinc increases G6PDH activity demonstrates that zinc does regulate one aspect of carbohydrate and fatty acid metabolism like insulin and the insulin mimetics, vanadate and selenate. However, the mechanism through which zinc exerts its action needs to be further investigated.
One possible explanation for the zinc-induced increase in G6PDH activity is that zinc increases oxidative stress on the cell. Several studies have shown that increased G6PDH activity is seen when cells are treated with compounds known to produce ROS (Cramer et al., 1995; Kleitzen et al., 1994) probably because a major product of the reaction catalyzed by G6PDH is NADPH, the major reducing compound in the body. Moreover, several studies have shown ROS to be insulin-mimetics similar to vanadate and selenate. For example, H$_2$O$_2$, a common ROS, increases glucose oxidation to CO$_2$, glucose uptake, lipogenesis, and glycogenesis (Czech et al., 1974; Lawrence & Larner, 1978; May & de Haen, 1979a). Furthermore, hydrogen peroxide has been shown to stimulate tyrosine phosphorylation of the insulin receptor, and several signal proteins important in the insulin signal pathway including IRS-1 and PI3K (Heffetz & Zick, 1989; Heffetz et al., 1990; May & de Haen, 1979a). In fact, several studies have suggested that H$_2$O$_2$ acts as a second messenger for insulin because insulin caused an increase in intracellular H$_2$O$_2$ production (May & de Haen, 1979a; May & de Haen, 1979b). A recent study on the insulin-like effect of H$_2$O$_2$ on enzymes in carbohydrate metabolism showed that H$_2$O$_2$ acts as an insulin mimetic in the regulation of phosphoenolpyruvate carboxykinase, an important carbohydrate metabolism enzyme (Sutherland et al., 1997). Therefore, zinc, and possibly vanadate and selenate, may exert insulin-like effects mediated by H$_2$O$_2$ or another ROS. The data in Figure 5 that shows a decrease in zinc-induced G6PDH promoter activity due to catalase supports this notion. Also, zinc-induced endogenous G6PDH activity was eliminated upon addition of an antioxidant, trolox C, which again suggests that zinc’s insulin-like effects with regards to G6PDH regulation were caused by an increase in ROS.
Our lab has recently demonstrated that cadmium, a nonessential and extremely toxic metal that is in the same periodic family as zinc, increases the expression of G6PDH in a concentration-dependent manner in Buffalo Rat Liver (BRL) cells. Furthermore, we have shown that this concentration-dependent increase in G6PDH activity is inhibited by addition of catalase, an antioxidant that eliminates H$_2$O$_2$. These results suggest that cadmium causes an increase in G6PDH activity through the generation of ROS which is consistent with the zinc data presented here. Although zinc and cadmium are both IIB transition metals, they differ in biological significance because zinc is essential while the body recognizes cadmium as a toxic substance. The results of the LDH leakage study demonstrate the non-toxic effects of zinc (Figure 7). Unlike cadmium, zinc did not show an increase in LDH leakage at the concentration that produced maximal G6PDH activity. Furthermore, concentrations ten-fold larger than the concentration that produced maximal G6PDH activity were needed to cause cell death with zinc. This non-toxic effect of zinc is more similar to vanadate and selenate than cadmium; however, the effect of zinc on G6PDH appears to be similar to cadmium.

If zinc is an insulin-mimetic in similar ways to vanadate and selenate or if insulin-like effects of zinc are mediated by hydrogen peroxide, zinc should cause phosphorylation of the insulin receptor and other insulin signal pathway proteins. Hydrogen peroxide, vanadate and selenate have all been shown to increase phosphorylation of the insulin receptor and insulin signal cascade proteins (Stapleton et al., 1996; Heffetz & Zick, 1989; Heffetz et al., 1990; unpublished results). A recent study however found that zinc does not stimulate the autophosphorylation or kinase activity of the insulin receptor in rat adipocytes (Ezaki, 1989). But, the phosphorylation of these proteins was quantified after only forty minutes of metal treatment. In a more recent study using selenate, it
was found that increased phosphorylation of insulin signal proteins was not seen until at least one hour and was not maximal until at least three hours in rat hepatocytes and adipocytes (Stapleton et al., 1996). Also, the study done by Ezaki concluded that cadmium does not stimulate phosphorylation of proteins involved in the insulin signal cascade (Ezaki, 1989); however, data from our lab has shown that cadmium does in fact stimulate phosphorylation of insulin signal cascade proteins. This stimulation of phosphorylation by cadmium was not seen with incubations less than one hour and was maximal with incubations much longer than one hour (unpublished results). Therefore, it is apparent that metals take longer than other activators to cause increased phosphorylation of insulin pathway signal proteins possibly due to the time needed to create ROS which act as second messengers. And so, the lack of autophosphorylation or kinase activity of the insulin receptor with zinc treatments suggested by Ezaki may be caused by an incubation time that was too short as was the case with cadmium.

Studies on the mechanism of zinc action were further done using BSO and trolox C to affect the production of ROS. An increase in cell viability with trolox C addition was not seen until 5 \( \mu \text{M} \) \( [\text{Zn}^{2+}] \) and this was slight. Large effects of trolox C were not seen until very high concentrations of zinc suggesting that zinc toxicity involves the formation of ROS. Vanadate and selenate have also been shown to become toxic only at very high concentrations possibly through the generation of ROS (unpublished results). This is consistent with other similarities between zinc and vanadate and selenate. An incomplete restoration to cell viability levels by trolox C suggests that zinc toxicity may involve other mechanisms besides the generation of ROS. This is supported by a study of various metals that concluded that metals are nonspecific toxicants and are involved in a number of mechanisms that affect cell viability (Strubelt et al., 1996). Although trolox C did not
cause an increase in cell viability at 1 μM [Zn$^{2+}$], it is probable that slight amounts of ROS are
created at 1 μM [Zn$^{2+}$] but these ROS formed are not enough to kill the cells.

A study on cell viability with zinc and BSO treatments was also performed to investigate the
mechanism of zinc action. BSO, an inhibitor of the body's natural antioxidant defense mechanism
by depleting glutathione (GSH), did not have an effect on zinc toxicity. Mize and Langdon found
that zinc inhibits hepatic glutathione reductase which would deplete GSH (Mize & Langdon,
1962) and leave the cell more susceptible to oxidative stress. Treatment of BSO, which
eliminates GSH, should have a potentiating effect when combined with zinc if zinc causes
oxidative stress through the depletion of GSH. However, the results displayed in Figure 9 do not
show a potentiating effect of GSH on zinc toxicity. BSO also did not affect the toxicity of
cadmium in intestinal epithelial cells (Keogh et al., 1994). Keogh et al. suggested that cadmium
toxicity may involve the binding to specific high-affinity thiol groups and not glutathione (Keogh
et al., 1994). The zinc data presented here also suggests that zinc toxicity is not mediated through
binding of GSH although more specific studies on GSH content with zinc treatments should be
done to confirm or dispute this.

It is important to note that the fluorescence cell viability assay distinguishes between cell death
and cell survival and therefore it is difficult to assess the effects of trolox C and BSO at non-toxic
zinc treatments. However, zinc has been shown to cause cell death due, in part to ROS and so
zinc would also cause ROS at lower concentrations. The fact that these lower concentrations of
zinc are non-toxic yet still increase G6PDH activity suggest that G6PDH may act as a sensor to
even small amounts of ROS. The increase in G6PDH activity can eliminate the ROS by using
NADPH to reduce these compounds. This appears to be the case with vanadate and selenate (Berg et al., 1995) and, based on the results shown here, zinc also appears to elicit such a response. The non-toxic effect of zinc at 1 μM is confirmed by the LDH activity. Based on the LDH study, cell death did not begin to occur until 10 μM [Zn$^{2+}$] but membranes began to become compromised at 5 μM [Zn$^{2+}$]. Although the cell viability studies did not begin to show significant cell death until 30 μM [Zn$^{2+}$] while the LDH graph suggested cell death at 10 μM [Zn$^{2+}$], it is important to note that cells were treated for 48 hours in the LDH study and only 24 hours in the cell viability study. This time difference can be used to explain the difference in toxicity levels of zinc between the LDH and cell viability studies.

These results generate enthusiasm for zinc as a possible insulin-mimetic and suggest a role of ROS in the action of zinc. It also appears that G6PDH acts as a sensor of non-toxic ROS. Furthermore, zinc has, for the first time, been shown to increase activity of an important enzyme if carbohydrate and fatty acid metabolism similarly to insulin. Further studies need to be done to confirm the that zinc acts at the transcriptional level and to investigate where on the G6PDH promoter the zinc action could be mediated. Also, further investigation into the mechanism of zinc action is needed.
Cited References


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