Chapter 3

Metabolic effects of zinc oxide nanoparticles

Glycemic Control
Chapter 3

Metabolic effects of zinc oxide nanoparticles

3.1. Introduction

Once the intracellular zinc levels increase, several cellular functions are expected to be affected. Effects of ZON on some of these metabolic parameters are presented in this chapter. The parameters investigated were shortlisted based on the results of earlier efficacy studies in diabetic rats (Umrani and Paknikar, 2014). Reduction of non-fasted and fasted blood glucose levels in ZON treated rats suggested the involvement of multiple glucose metabolism pathways including gluconeogenesis and glycolysis. Therefore, Glucose 6 phosphatase (G-6-Pase) and Phosphoenolpyruvate carboxykinase (PEPCK), as well as Glucokinase (GCK) expression were investigated. Improved glucose tolerance observed in the previous study prompted an investigation of Glucose transporter 4 (GLUT4) translocation and glucose uptake after ZON treatment. To study the effect of ZON on insulin sensitivity and signaling, protein kinase B (PKB) and protein tyrosine phosphatase 1B (PTP1B) phosphorylation levels were studied. ZON treatment to diabetic rats (earlier studies) also resulted in a reduction of TG and FFA levels suggesting improved lipid metabolism. Therefore, to study the role of ZON in lipolysis, hormone sensitive lipase (HSL) inactivation was studied. Thus, comprehensive in vitro studies were designed to identify the key mediators of anti-diabetic actions of ZON.

3.2. Materials and methods

3.2.1. Cells and culture conditions

HepG2 (human hepatocarcinoma), L6 (rat myoblasts) and 3T3L1 (mouse pre-adipocytes/fibroblasts) cells were procured from cell repository, National Centre for Cell Sciences, Pune. HepG2 cells were maintained in Eagle's Minimum
Essential Medium; and L6 and 3T3L1 cells in Dulbecco's Modified Eagle's Medium at 37°C under 5% CO₂ atmosphere. All media were supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Tissue culture medium, serum, and antibiotics were procured from HiMedia Chemicals, India.

3.2.2. Differentiation of pre-adipocyte to adipocytes

3T3L1 pre-adipocytes (1 x 10⁵ cells) were allowed to grow in adipocyte expansion medium (DMEM with 10% bovine calf serum and 1% antibiotic-antimycotic) for 48 h. Next, cells were maintained for another 48 h in adipocyte differentiation medium (DMEM with 10% FBS, 1% antibiotic-antimycotic, 1 μM Dexamethasone, 0.5 mM IBMX and 1 μg/mL Insulin) to induce differentiation. Subsequently, spent medium was replaced with adipocyte maintenance medium (DMEM with 10% FBS, 1% antibiotic-antimycotic and 1 μg/mL Insulin) until differentiation (for 7 to 9 days). Dexamethasone and IBMX were procured from Sigma-Aldrich, USA.

As can be seen in Figure 3.1, undifferentiated cells did not show the presence of lipid droplets (Figure 3.1 A), whereas differentiated cells showed the formation of lipid droplets within the cytoplasm (Figure 3.1 B), which was further confirmed by Oil red O staining (Figure 3.1 C). Differentiated 3T3L1 cells (referred to as 3T3L1 adipocytes) were used in subsequent experiments.

![Figure 3.1](image-url)
3.3. **Cytotoxicity assay**

Cytotoxicity of ZON was assessed in L6 and HepG2 cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent (USB chemicals, Affymetrix, USA). For experiments, 4 x 10^4 cells were seeded in each well of a 96 well plate. After 72 h, spent medium was replaced with fresh medium containing different concentrations of ZON (final concentrations 1, 3, 10, 30 and 100 μg/mL in saline). Saline treated cells served as control. After 24 h, the spent medium containing ZON was removed, and 20 μl of MTT reagent (0.2 mg/mL in PBS) was added. After 4 h, spent medium was removed and the purple colored formazan crystals were dissolved in 200 μl DMSO. Absorbance at 570 nm was recorded using microplate reader (Synergy HT, BioTek Instruments Inc., USA). Percent cell viability after treatment was calculated using the formula:

\[
\text{Cell viability (\%)} = \left( \frac{\text{Absorbance}_{\text{test}}}{\text{Absorbance}_{\text{control}}} \right) \times 100
\]

![Graphs of HepG2 and L6 cytotoxicity](image)

**Figure 3.2:** Cytotoxicity assay of ZON in (A) HepG2 and (B) L6 cells. **p<0.01 and ***p<0.001 as analysed by One-way ANOVA followed by Dunnett's multiple comparison test.
Cytotoxicity assay in L6 and HepG2 cells revealed that ZON were safe up to 10 μg/mL concentration. At higher concentration, i.e. 30 μg/mL, a moderate decrease in viability was observed in HepG2 (~10%, Figure 3.2 A) and L6 (~20%, Figure 3.2 B) cells. ZON (100 μg/mL concentration) was found to be toxic to both the cell lines. Based on these results, non-cytotoxic concentrations of ZON (1, 3, 10 μg/mL) were used for further studies.

3.4. Protein phosphorylation studies (PKB, PTP1B, HSL)

The levels of phosphorylated and dephosphorylated forms of proteins were estimated by western blotting. Nitrocellulose membrane (0.45 μm) was procured from Millipore Corporation, USA. Protease inhibitor cocktail; phosphatase inhibitor cocktail 2 and 3; TMB (3,3',5,5'- Tetramethylbenzidine) substrate; anti-PKB, anti-phospho PKB (pSer473), anti-PTP1B, anti-phospho PTP1B (pSer50) and anti-actin antibodies were procured from Sigma-Aldrich, USA. Anti-HSL, anti-phospho HSL (pSer563), and anti-rabbit IgG-HRP antibodies were procured from Thermo Fisher Scientific, USA.

For experiments, HepG2 (5 x 10^6 cells) or L6 (1 x 10^6 cells) or 3T3L1 adipocytes (grown in 25 cm² flasks) were challenged with saline or different concentrations of ZON (1, 3, 10 μg/mL). Insulin (0.1 IU/mL) was used as a positive control. After 24 h, cells were collected by trypsinization and centrifugation (1000 rpm, 10 min). Cells were washed with PBS, resuspended in 500 μl lysis buffer (20 mM HEPES buffer, 1 mM EGTA, 210 mM Mannitol, 70 mM Sucrose) supplemented with protease inhibitor cocktail (1%) and phosphatase inhibitor cocktail 2 and 3 (1% each). The contents were kept on ice for 30 min with intermittent vortexing. The cell lysates so obtained were centrifuged (10000 rpm, 4°C, 10 min) and the supernatants were stored at -20°C until further analysis.

Total protein content was estimated using Bradford reagent (Sigma-Aldrich, USA). Lysates (50 μg protein) were electrophoresed by SDS-PAGE (12% gel).
Protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with BSA (5% in PBST, 3 h) and probed with specific primary antibody. Membranes were incubated overnight at 4°C followed by addition of goat anti-rabbit secondary antibody conjugated to HRP (1:5000 dilution, 2 h). TMB substrate was added to develop the protein bands. Actin was used as endogenous control. For actin immunoblotting, the membrane was stripped and probed using actin antibody. Band intensities were calculated using ImageJ software (National Institute of Health, USA). Protein bands were normalized to actin. The ratio of phosphorylated form to total protein was calculated. Fold change in the treatment group was calculated as compared to saline control.

### 3.4.1. PKB and PTP1B phosphorylation

Western blotting experiments revealed that ZON treatment did not alter the band intensities of total PKB and PTP1B. As expected, phosphorylated PKB and PTP1B (p-PKB and p-PTP1B respectively) band intensities increased in HepG2 (Figure 3.3 A) and L6 (Figure 3.3 B) cells.

(A) HepG2 cells

<table>
<thead>
<tr>
<th>ZON</th>
<th>Saline</th>
<th>1 µg/mL</th>
<th>3 µg/mL</th>
<th>10 µg/mL</th>
<th>Insulin</th>
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<tr>
<td>PKB</td>
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<td>p-PKB S^{473}</td>
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<tr>
<td>PTP1B</td>
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<tr>
<td>p-PTP1B S^{50}</td>
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</tr>
<tr>
<td>Actin</td>
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Figure 3.3: Western blot analysis of PKB and PTP1B phosphorylation in (A) HepG2 and (B) L6 cells.

Densitometric analyses showed that ZON treatment (10 μg/mL) increased PKB phosphorylation by ~1.5 fold, similar to insulin, in HepG2 and L6 cells (Figures 3.4 A and 3.4 B), indicating PKB activation. Further, ZON treatment increased PTP1B phosphorylation by ~1.5 fold in HepG2 and L6 cells (Figure 3.4 C and 3.4 D respectively), indicating PTP1B inactivation.
Figure 3.4: Densitometric analyses showing fold change in phosphorylated PKB/PKB in (A) HepG2 and (B) L6 cells; and fold change in phosphorylated PTP1B/PTP1B in (C) HepG2 and (D) L6 cells. *p<0.05 and **p<0.01 as analysed by One way ANOVA followed by Dunnett’s multiple comparison test.
3.4.2. HSL phosphorylation

ZON treatment to 3T3L1 adipocytes decreased p-HSL band intensity in a dose-dependent manner, indicating HSL inactivation. Intensities of HSL bands were unaltered (Figure 3.5 A). The decrease in p-HSL was to the tune of ~2 fold after ZON treatment (10 µg/mL), significantly different from control (Figure 3.5 B). A minor decrease in p-HSL was observed after insulin treatment, although not statistically significant.

(A)

Figure 3.5: (A) Western blot analysis of HSL phosphorylation in 3T3L1 adipocytes. (B) Fold change in phosphorylated HSL/HSL in 3T3L1 adipocytes. **p<0.01 and ***p<0.001 as analysed by One way ANOVA followed by Dunnett's multiple comparison test.
3.5. GLUT4 translocation assay

GLUT4 translocation was studied by immunofluorocytochemistry. L6 cells (1 x 10^5 cells) were exposed to saline or different concentrations of ZON (1, 3, 10 μg/mL). Cells treated with Insulin (0.1 IU/mL) served as positive control. After 12 h, spent medium was removed, cells were washed twice with PBS and fixed with paraformaldehyde (4%, Sigma-Aldrich, USA) for 15 min. Cells were then permeabilized using Triton-X 100 (0.1% in PBS, Sigma-Aldrich, USA) for 15 min and blocked using BSA (1% BSA in PBS containing 0.3 M glycine and 0.1% Triton-X100) for 30 min. Next, cells were incubated with the anti-GLUT4 primary antibody (1:1000 dilution, 1h, Thermo Fisher Scientific, USA) followed by goat anti-rabbit FITC conjugated secondary antibody (1:100 dilution, 1 h in dark, Thermo Fisher Scientific, USA). Cell nuclei and cytoskeleton were counterstained with Hoechst 33342 (1:5000 dilution) and Rhodamine-Phalloidin (1:1000 dilution) respectively. Images were taken at 400X magnification using fluorescence microscope (Eclipse 80i, Nikon, Japan) under FITC (Green), TRITC (Red) and DAPI (Blue) filters.

Similar experiments were performed in 3T3L1 adipocytes (grown on lysine coated coverslips).

As can be seen from Figure 3.6 and 3.7, bright green fluorescence at the membrane surface in ZON treated cells indicated increased GLUT4 translocation in L6 cells and 3T3L1 adipocytes, respectively. Similarly, GLUT4 translocation was also observed in insulin-treated cells.
Figure 3.6: GLUT4 translocation in L6 cells. The first column shows nuclei stained blue with Hoechst 33342, the second column shows cytoskeleton stained red with Rhodamine-Phalloidin, the third column shows GLUT4 probed with anti-GLUT4 antibody followed by immunostaining with FITC tagged secondary antibody, and the last column shows merged images.
**Figure 3.7:** GLUT4 translocation in 3T3L1 adipocytes. The first column shows nuclei stained blue with Hoechst 33342, the second column shows cytoskeleton stained red with Rhodamine-Phalloidin, the third column shows GLUT4 probed with anti-GLUT4 antibody followed by immunostaining with FITC tagged secondary antibody, and the last column shows merged images.
3.6. Glucose uptake assay

Glucose uptake was studied using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino) -2-Deoxyglucose (2-NBDG, Sigma-Aldrich, USA), a fluorescent analog of glucose. Briefly, L6 cells (2 x 10^4 cells) or 3T3L1 adipocytes (grown on lysine coated coverslips) were exposed to saline or different concentrations of ZON (1, 3, 10 µg/mL) or insulin (0.1 IU/mL) in glucose-free, serum-free medium containing 2- NBDG (40 µM) for 1 h. Cells were then washed with PBS, mounted on slides and visualized under FITC filter of a fluorescence microscope (Eclipse 80i, Nikon, Japan). Images were taken at 400X magnification and fluorescence intensity was calculated using ImageJ software (National Institute of Health, USA).

Fluorescence microscopy images showed increased green fluorescence in ZON as well as insulin-treated L6 cells as compared to saline control (Figure 3.8 A), indicating increased glucose uptake. Fluorescence intensity analysis showed statistically significant increase in 2-NBDG uptake after ZON treatment as compared to saline control (Figure 3.8 B). Similar results were obtained in 3T3L1 adipocytes (Figure 3.9 A), however, statistical significance was not achieved (Figure 3.9 B).
Figure 3.8: (A) Glucose uptake assay in L6 cells (B) Fluorescence intensity analysis. *p<0.05 and **p<0.01 as analysed by One way ANOVA followed by Dunnett's multiple comparison test.
3.7. Gene expression studies (G-6-Pase, PEPCK, GCK)

3.7.1. RNA isolation

RNA isolation reagents (Tri-reagent, chloroform, isopropanol) were procured from Sigma-Aldrich, USA. HepG2 cells (3 x 10^6) were seeded in 25 cm² tissue culture flasks and incubated at 37°C and 5% CO₂ atmosphere. After 72 h, spent medium was removed and cells were incubated in glucose-free, serum-free medium for 3 h. Cells were then exposed to saline or different concentrations of ZON (1, 3, 10 μg/mL) or insulin (0.1 IU/mL). After 18 h, RNA isolation was performed as described below.

Tri-reagent (1 mL) was added to flasks, and cell suspensions were transferred to 1.5 mL microcentrifuge tubes. Chloroform (0.2 mL) was added and tubes were shaken vigorously for 15 s. After 10 min, tubes were centrifuged (12000 g, 15 min, 4°C) to obtain three phases. The top most layer (aqueous phase) containing RNA was transferred to fresh 1.5 mL microcentrifuge tubes. Isopropanol (0.5 mL) was added; after 5-10 min tubes were centrifuged (12000 g, 10 min, 4°C) to obtain RNA pellet. The pellets were washed with ethanol (75%, 1 mL) and centrifuged (12000 g, 5 min, 4°C). Further, pellets were air dried for 5-10 min and resuspended in TE buffer (40 μl, pH 7.5). DNase treatment was given according to manufacturer's instructions to remove any
contaminating DNA. RNA absorbance was recorded using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA).

3.7.2. cDNA synthesis

ReadyScript cDNA synthesis mix (Sigma-Aldrich, USA) was used for cDNA synthesis, according to manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was reverse transcribed to cDNA for 30 min at 42°C in a 20 μl reaction volume. cDNA was stored at -20°C until further analysis.

3.7.3. Quantitative Real-Time PCR

Gene expression was studied using real-time PCR. Primers for PEPCK (forward- 5'-ATT CTG GGT ATA ACC AAC CC-3', reverse 5'- GTT GAT GGC CCT TAA ATG AC-3'), G-6-Pase (forward- 5'-ACT GTG CAT ACA TGT TCA TC -3', reverse 5'-TGA ATG TTT TGA CCT AGT GC-3'), GCK (forward- 5' AAG CAG CAT TCA GCA CAC -3', reverse 5'- AAT CTT AGG TTG GGC ATG GA - 3') and GAPDH (forward 5'- ACA GTT GCC ATG TAG ACC-3', reverse 5'-TTT TTG GTT GAG CAC AGG-3') were procured from Sigma-Aldrich, USA. Real-time PCR was performed using LightCycler® FastStart DNA Master PLUS SYBR Green I kit (Roche Holding AG, Switzerland). The PCR protocol included a preincubation step (at 95°C for 5 min) followed by 50 cycles of amplification (denaturation at 95°C for 10 s, annealing at primer specific temperature for 10 s, and extension at 72°C for 45 s) and the final step of cooling (at 37°C for 5 min) using LightCycler 2.0 instrument (Roche Holding AG, Switzerland). Melting curve analysis was performed to check the purity of the PCR products. Fold change in expression of G-6-Pase and PEPCK was calculated by the 2^ΔΔCt method. GAPDH was used as endogenous control.

ZON treatment to HepG2 cells resulted in significant reduction of G-6-Pase (Figure 3.10 A) and PEPCK (Figure 3.10 B) expression, similar to insulin,
suggesting inhibition of gluconeogenesis. No significant difference in GCK expression was observed after ZON treatment (Figure 3.10 C), indicating no effects on glycolysis.

Figure 3.10: Fold change in G-6-Pase (A), PEPCK (B) and GCK (C) gene expression after treatment with ZON or insulin. **p<0.01 and ***p<0.001 as analysed by One way ANOVA followed by Dunnett's multiple comparison test.
3.8. Discussion

Earlier efficacy studies (Umrani and Paknikar, 2014) demonstrated anti-diabetic effects of ZON, where increased serum insulin levels were observed in treated rats. This observation raised an important question as to whether the observed biochemical effects of ZON are secondary to increased insulin levels or are they effects of ZON per se? To answer this question, mechanism of action studies were carried out in vitro. As mentioned in the introduction section, the putative mediators of ZON effects were shortlisted based on previous observations in diabetic rats (Umrani and Paknikar, 2014).

One of the primary abnormalities in diabetes is impaired insulin signaling due to increased protein tyrosine phosphatase 1B (PTP1B) activity. PTP1B is a protein phosphatase which negatively modulates insulin signaling by dephosphorylating insulin receptors and its substrates (Obanda and Cefalu, 2013). It is known that PTP1B itself is inactivated by phosphorylation at serine 50 residue, thereby impairing its ability to dephosphorylate insulin receptors and substrates (Bakke and Haj, 2015). In the present work, ZON treatment to HepG2 and L6 cells increased phosphorylated (pSer50) PTP1B levels signifying its inactivation. Interestingly, other researchers have also reported inhibition of PTP1B enzyme activity by zinc ions (Wilson et al., 2012; Bellomo et al., 2014).

Another important intermediate in insulin signaling is PKB. Physiologically, insulin or insulin-like growth factors (IGF-1) activate PI 3-kinase, which in turn activates PKB by phosphorylation at serine 473 residue. ZON treatment to HepG2 and L6 cells, in our study, increased levels of phosphorylated (pSer473) PKB resulting in its activation, suggesting improved insulin signaling in the liver as well as muscle. These results are in agreement with another study where di(hinokitiolato)zinc complex (25 µM) treatment to 3T3L1 adipocytes increased phosphorylation of PKB and GSK3β (Naito et al., 2011). In the present study, we have obtained similar results at a lower concentration of ZON (~12.27 µM
zinc). In view of these results, we propose that ZON treatment can enhance insulin signaling by virtue of its effects on PTP1B inactivation and PKB activation.

Several reports suggest that PKB mediates many of the downstream events of PI 3-kinase activation, including GLUT4 translocation, glucose uptake, glycogen synthesis, protein synthesis, preadipose cell differentiation and regulation of gene expression (Lai et al., 2012; Olson, 2012). Amongst these downstream events, a key metabolic effect is GLUT4 translocation. GLUT4 is a glucose transport protein expressed in insulin-responsive tissues viz., muscles and adipose tissue. Physiologically insulin-mediated GLUT4 translocation (from vesicles to cell membrane) enhances glucose uptake in these tissues thereby regulating blood glucose levels. In the present study, ZON treatment to L6 cells and 3T3L1 adipocytes resulted in increased GLUT4 translocation to the membrane surface, analogous to insulin. GLUT4 translocation consequently increases glucose uptake, which was assessed by 2-NBDG assay in the present study. Our results showed that treatment with ZON increased glucose uptake in L6 cells (~3 fold) as well as 3T3L1 (~1.3 fold) adipocytes. These results are in agreement with the study reported by Naito et al. (2011) where di(hinokitiolato)zinc complex caused GLUT4 translocation and enhanced glucose uptake in adipocytes. Interestingly, GLUT4 translocation is also mediated by insulin-regulated aminopeptidase (IRAP) which is a zinc-dependent metalloprotein (Morgan et al., 2011). Effects of ZON on IRAP association with GLUT4 vesicle can be investigated in future.

PKB activation leads to several downstream effects including altered gene expression of glucose metabolism pathways (Manning and Cantley, 2007). Gluconeogenesis is a process of generation of glucose from non-carbohydrate precursors (viz., lactate, pyruvate, glycerol, etc.). In the diabetic state, G-6-Pase and PEPCK, the rate limiting enzymes of gluconeogenesis pathway, are overexpressed resulting in increased hepatic glucose output and contributing to
fasting hyperglycemia. Insulin is known to inhibit gluconeogenesis pathway by suppressing G-6-Pase and PEPCK activity and expression (Kim et al., 2013; Guan and Chen, 2014). Therefore, it was worthwhile to study, if treatment with ZON can modulate G-6-Pase and PEPCK expression. Our results showed that ZON treatment significantly decreased expression of G-6-Pase and PEPCK, similar to insulin, suggesting decreased gluconeogenesis. Several reports suggest a role of metal ions in inhibition of gluconeogenesis. As early as 1964, Rutman et al. (1964) reported the inhibitory effect of Zn\(^{2+}\), Cu\(^{2+}\), and Cd\(^{2+}\) on glucose production from amino acids and tricarboxylic acid cycle intermediates. Cameron et al. (2012) observed that the insulin-like effects of clioquinol on G-6-Pase and PEPCK expression were zinc dependent. Despite evidence of the role of zinc ions in gluconeogenesis, reports on G-6-Pase and PEPCK expression by zinc compounds are not available. To the best of our knowledge, we report for the first-time inhibition of gluconeogenesis by zinc oxide nanoparticles. Interestingly, a recent report by Kim et al. (2016) has shown the involvement of a corepressor SMILE in the inhibition of gluconeogenesis in insulin signaling. Though the molecular details of the protein are not yet available, it would be interesting to study if zinc has any regulatory effect on this corepressor.

In the diabetic state, glucose utilization is also compromised. Insulin regulates hepatic glucose homeostasis by inducing glucokinase gene expression to activate glycolysis (Guan and Chen, 2014). However, in our study, no significant effects of ZON were observed with respect to GCK expression. These results, suggest that either zinc does not regulate glycolysis or the effects are not mediated through GCK gene expression. A detailed investigation on this aspect may be carried out. Nevertheless, our results suggest that ZON treatment enhances insulin signaling, increases glucose uptake and decreases gluconeogenesis, thus exerting multiple beneficial effects on glucose metabolism.
Diabetes is also a state of altered lipid metabolism, where excessive free fatty acids interfere with insulin signaling and contribute to insulin resistance (Stinkens et al., 2015). Hormone-sensitive lipase (HSL) is considered as a key regulatory enzyme of lipolysis contributing to free fatty acid release. Literature reports suggest that HSL gets activated by phosphorylation at serine 563 residue (Moon et al., 2012), whereas its dephosphorylation (and therefore inactivation) results in inhibition of lipolysis. Physiologically, insulin is known to inhibit lipolysis (Sears and Perry, 2015). In diabetes (an insulin resistant state), insulin’s regulation over lipolysis is lost. Nakayama et al. (2008) reported HSL inhibitory activity of zinc allixin complex, where they could observe decreased FFA release and increased glucose uptake in adipocytes. Recently, researchers have reported that dietary zinc supplementation decreases HSL enzyme activity and mRNA levels in weaned piglets (Zhang et al., 2014). In agreement with these reports, we have observed decreased levels of phosphorylated HSL after ZON treatment, suggesting its inactivation and suppression of lipolysis. These results indicate that ZON plays a beneficial role in lipid metabolism too. Recently, Maxel et al. (2015) reported an interesting correlation between zinc influx through ZIP14 and PPARγ activation, which further improves insulin sensitivity and glucose metabolism in adipocytes. This study opens a new avenue for a detailed investigation on the effects of ZON on PPARγ activation and subsequent effects on glucose metabolism in adipocytes.

3.9. Salient findings

The salient findings of the studies on metabolic effects of ZON are listed below (summarized in Figure 3.11):

- ZON treatment increases insulin sensitivity through PTP1B inactivation.
- Treatment with ZON results in insulin mimetic effects viz., PKB activation, GLUT4 translocation and enhanced glucose uptake.
- G-6-Pase and PEPCK repression by ZON suggests reduced gluconeogenesis and hepatic glucose output.

- HSL inactivation suggests beneficial effects of ZON on lipid metabolism.

**Figure 3.11**: Proposed mechanism for anti-diabetic activity of ZON

Besides the direct effects of ZON on glucose and lipid metabolism, there could be other effects on cellular defence mechanism. For example, zinc is a component of SOD (an endogenous anti-oxidant enzyme) and is known to alleviate oxidative stress. Considering this possibility, effects of ZON on the cellular anti-oxidant status were investigated (See Chapter 4).