Chapter 4

Potential Role of Oxidative stress and Redox Sensitive Serine Kinase pathways in the pathogenesis of Insulin Resistance in Rat L6 Myotubes
4.1. Background

A key aspect of mammalian physiology involves the regulation of blood glucose levels. Glucose homeostasis is mainly controlled by the circulating insulin. Insulin facilitates the disposal of blood glucose in the fed state by stimulating its uptake into target tissues, primarily skeletal muscle and fat cells (Kahn, 1996). But it is widely accepted that skeletal muscle, by virtue of its large contribution to body mass, represents the major site of insulin-mediated glucose disposal (DeFronzo et al., 1992). The stimulation of glucose uptake elicited by insulin in both skeletal muscle and fat is achieved by the increased recruitment of the insulin-regulated glucose transporter, GLUT4 to the cell membrane (Shulman, 2000). Reduced insulin sensitivity is a characteristic feature of type 2 diabetes. Even after intense research the molecular mechanism(s) responsible for insulin resistance is not exactly known. One factor that appears to be important in the progression of insulin resistance is oxidative stress. Increased oxidative stress has been recognized to play a role in the pathogenesis of late complications of diabetes, such as renal failure and atherosclerosis (Paolisso et al., 1994). However, the possible involvement of oxidative stress in the pathogenesis and progression of insulin resistance remains controversial (Hansen et al., 1999; Maddux et al., 2001; Rudich et al., 1999).

In the past 20 years, several studies have demonstrated various effects of H$_2$O$_2$ on the insulin signaling machinery (Hansen et al., 1999; Maddux et al., 2001; Rudich et al., 1999). Studies have reported, decreased insulin stimulated glucose transport in 3T3L1 adipocytes and rat L6 myotubes exposed to oxidative stress (Maddux et al., 2001; Rudich et al., 1999). Even though H$_2$O$_2$ induced insulin resistance is reported, it is generally accepted that H$_2$O$_2$ exerts insulinomimetic effects (Goldstein et al., 2005; Kozlovsky,
1997). Studies have proved oxidation-sensitive step(s) with in the insulin signaling machinery (Mahadev et al., 2001). These contradicting results of \( \text{H}_2\text{O}_2 \) on insulin action can be partially explained by the concentration of \( \text{H}_2\text{O}_2 \) used and the time of exposure of cells with \( \text{H}_2\text{O}_2 \). Most of these studies have investigated the effect of \( \text{H}_2\text{O}_2 \) for the short-time (< 30 min) exposure with higher concentrations (millimolar). To the best of our knowledge, the effect of prolonged low grade \( \text{H}_2\text{O}_2 \) concentration on insulin action is not studied. It is worth investigating the effect of prolonged low grade \( \text{H}_2\text{O}_2 \) concentration on insulin action because this mimics the well documented effect of oxidative stress in the pathogenesis and late complications of diabetes mellitus.

Numerous studies have shown that reactive oxygen species (ROS) can function as signaling molecules and activate a number of redox sensitive serine kinase pathways linked to insulin resistance (Evans et al., 2002, Yuan et al., 2001). These pathways include nuclear factor -kB (NF-kB), p38 mitogen activated protein kinase (p38MAPK) and C-Jun-NH\(_2\)- terminal kinase (JNK) pathways (Qiao et al., 1999). Numerous studies have shown activation of these pathways by ROS (Evans et al., 2002; Mercurio et al., 1999) and their increased activity in diabetic tissues (Keran et al., 1997). Even though, the activation of these redox sensitive serine kinase pathways are documented in insulin resistance, the molecular mechanism by which activation of redox sensitive serine kinase pathways leading to insulin resistance is not completely understood.

The role of oxidative stress in the pathogenesis of insulin resistance is mainly supported by several clinical trials that have reported the beneficial role of antioxidants in type 2 diabetic subjects (Jacob et al., 2000; Packer et al., 2000). Many studies have proved the protective effect of antioxidants against the oxidative stress induced insulin resistance (Maddux et al., 2001). Even though the insulin sensitizing/antidiabetic
property of antioxidants is noted, the mechanism by which these antioxidants improve insulin sensitivity is not known. In view of the all above, the present invitro study was aimed to investigate the role of redox sensitive serine kinase pathways and antioxidant treatment on insulin action in rat L6 muscle cells exposed to prolonged low grade oxidative stress.
4.2. Aim and objectives of the in vitro study

Aim

The aim of the in vitro study was to investigate the role of redox sensitive serine kinase pathways (NF-kB, JNK and p38MAPK) in the pathogenesis of insulin resistance in rat L6 myotubes treated with prolonged low grade oxidative stress.

Objectives

a) To study the effect of prolonged low grade oxidative stress and antioxidant treatment on insulin sensitivity in rat L6 myotubes.

b) To study the effect of prolonged low grade oxidative stress and antioxidant treatment on redox balance in rat L6 myotubes.

c) To study the effect of prolonged low grade oxidative stress and antioxidant treatment on proximal insulin signaling (insulin receptor and IRS-1 tyrosine and serine phosphorylation) in rat L6 myotubes.

d) To study the effect of prolonged low grade oxidative stress and antioxidant treatment on redox sensitive serine kinase - NF-kB, p38MAPK and JNK pathways in rat L6 myotubes.
4.3. Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, glucose oxidase, tocopherol acetate (vitamin E), ascorbic acid (vitamin C), α-lipoic acid (LA), 1,1-diphenyl -2-picryl hydrazyl (DPPH), insulin, sodium vanadate, phenyl methyl sulfonil fluoride, aprotinin, leupeptin, okadaic acid and all other chemicals were purchased from Sigma Chemicals (Sigma Chemicals, USA). 2-deoxy-\(^{14}\)C-D glucose was purchased from Amersham Life Sciences (Amersham Life Sciences, UK). Insulin receptor β-subunit, IRS -1, IκBα, P-p38MAPK, p38MAPK, P-JNK1, JNK1 and phospho tyrosine antibodies were purchased from Upstate Biotechnology (Upstate Biotechnology, USA). β-Actin and phospho serine antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, USA). Protein-A agarose slurry was purchased from Bangalore Genei (Bangalore Genei, India).

Cell culture

Rat L6 myoblasts (American Type Culture Collection) were cultured (37\(^0\)C, 5% CO\(_2\)) in growth medium (DMEM, 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin). Once the myoblasts reached 70-80% confluence, the L6 myoblasts were allowed to differentiate into myotubes in differentiation medium (DMEM, 2% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin) spontaneously (Blair et al., 1999). The differentiation of myoblasts into myotubes was checked by the morphology and fusion of myotubes under the microscope. All the following experiments were carried out in the differentiated myotubes.
Hydrogen peroxide treatment

The rat L6 myotubes were exposed to oxidative stress with H\textsubscript{2}O\textsubscript{2} generating system. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was generated in medium by an enzymatic glucose oxidase/glucose system. To optimize the concentration of glucose oxidase and time of H\textsubscript{2}O\textsubscript{2} treatment, the L6 myotubes were treated with different concentrations of glucose oxidase for different time intervals. Since H\textsubscript{2}O\textsubscript{2} is a cytotoxic agent, H\textsubscript{2}O\textsubscript{2} induced cell toxicity was investigated by cell viability assay for each of the concentrations of glucose oxidase used at different time intervals as follows.

Myotubes were differentiated from myoblasts in 24 well cultured plates as mentioned above and were incubated with DMEM (phenol red free) supplemented with 0.5 % BSA at different concentrations (0, 25, 50, 75 and 100 mU/ml) of glucose oxidase and 5 mmol/l glucose for different time intervals (2, 4, 8, 12, 16 and 24 hrs). Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) induced cytotoxicity was measured at different time intervals using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (Ferrai., 1990). Briefly, myotubes were incubated with 50 μl of MTT solution (4 mg/ml PBS) for 100 μl of DMEM (phenol red free) for 3 hours at 37 °C, 5 % CO\textsubscript{2} for the formation of formazan crystals. After 3 hours the medium was gently removed and the plates were dried on tissue paper. The formazan crystals formed inside the cells were dissolved in 100 μl of DMSO. The H\textsubscript{2}O\textsubscript{2} induced cytotoxicity was determined by measuring the absorbance of formazan crystals at 570 nm. We found, 25 mU/ml (low grade) of glucose oxidase treatment for 12 hours (chronic treatment) is not cytotoxic to myotubes (section 4.4 and Fig. 4.1). So, we have chosen 25 mU/ml glucose oxidase treatment for 12 hours as a prolonged low grade oxidative stress model for our further experiments.
Antioxidant and hydrogen peroxide treatment

Rat L6 myotubes were incubated with vitamin E (5 μM), vitamin C (500 μM), LA (300 μM) and antioxidant mixture (5 μM vitamin E, 500 μM vitamin C and 300 μM LA) in DMEM supplemented with 0.5 % BSA for 18 hours. After antioxidant treatment, myotubes were washed with DMEM supplemented with 0.5 % BSA and incubated in DMEM (phenol red free) supplemented with 0.5 % BSA, 25 mU/ml glucose oxidase and 5 mmol/l glucose for 12 hours. Hydrogen peroxide (H₂O₂) generated by the glucose/glucose oxidase system was estimated from the media collected at different time intervals using Amplex red – hydrogen peroxide/peroxidase assay kit (Molecular Probes, USA) following the manufacture’s protocol. Cytotoxicity of the treatment was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described above. Creatinine Kinase (CK) activity was measured in the culture medium using colorimetric kit (Teco Diagnostics, USA) in 550 Express plus autoanalyser (Bayers Diagnostics, USA) as a measure of muscle damage.

Determination of glucose transport into myotubes

Insulin stimulated glucose uptake by the myotubes was measured as described previously (Blair et al., 1999). Rat L6 myotubes were treated with antioxidants (18 hrs) followed by H₂O₂ (12 hrs) as described above. After H₂O₂ treatment, L6 myotubes were washed with DMEM supplemented with 0.5 % BSA and then incubated with serum free DMEM for 3 hrs. After serum starvation, insulin (100 nmol/l) was added to the serum free medium and further incubated for 30 min. After insulin stimulation, myotubes were washed with HBS (140 mmol/l NaCl, 20 mmol/l Hepes pH 7.4, 5.0 mmol/l KCl, 2.5 mmol/l MgSO₄, 1.0 mmol/l CaCl₂) and incubated with 2 Deoxy –¹⁴ C – D glucose (0.5 μCi/ml) in HBS for 10 min. After 10 min, radioactive medium was aspirated rapidly and
cells were washed with ice-cold isotonic saline (0.9 % NaCl). Cells were lysed in 0.05 M NaOH and radioactivity in the lysate was determined by liquid scintillation counting (Packyard Top Count LSC, USA). Protein content in lysates was estimated by the method of Bradford (Bradford., 1976). Non-specific glucose uptake was determined in the presence of cytochalasin B (50 μmol/l) an inhibitor of facilitative glucose transport and was subtracted from total uptake.

Antioxidant assays

Rat L6 myotubes were treated with antioxidants (18 hrs) followed by H2O2 (12 hrs) as described above and lysed in 0.5 ml of PBS (pH 7.4) by repeated freezing and thawing. Total antioxidant status of cell lysates were quantified by trolox equivalent antioxidant capacity assay (TEAC) using 1, 1-Diphenyl – 2 – Picryl Hydrazyl (DPPH) as described previously (Nikolaidis et al., 2004). Briefly, total antioxidant activities of lysates were determined spectrophotometrically at 517 nm by quantifying the decrease in the absorbance of free radical DPPH after the addition of cell lysates. The antioxidant capacities of samples were measured against a trolox standard and expressed as TEAC. Protein concentration in the cell lysates was estimated by the methods of Bradford (Bradford., 1976). The total antioxidant capacity of the cell lysates were expressed as TEAC (μM/mg protein)

Reduced glutathione concentration in cell lysates was estimated using Ellman’s reagent [5,5’- Dithio bis – 2 – nitrobenzoic acid (DNB)] as described by Beutle et al (Beutle et al., 1963). To 0.5 ml of cell lysates, 1.0 ml of Na2HPO4 (0.3 M) and 2.0 ml of DNB reagent (0.4 % DNB and 1 % sodium citrate) were added. This mixture was incubated at room temperature for 30 min. After 30 min of incubation, the absorbance was measured at 412 nm. A standard graph was drawn using reduced glutathione as
standard and the reduced glutathione concentration in the cell lysates was calculated. Protein concentration in the cell lysates was estimated by the method of Bradford (Bradford., 1976). The reduced glutathione concentration in the cell lysates was expressed as mg glutathione/mg of protein.

**Insulin signaling analysis**

Insulin signaling analysis in rat L6 myotubes was performed as described previously (Keren et al., 1997; Sadd et al., 1992). Rat L6 myotubes were treated with antioxidants (18 hrs) followed by \( \text{H}_2\text{O}_2 \) (12 hrs) as described above and incubated with serum free DMEM for 30 min. After serum starvation, insulin (100 nmol/l) was added to the serum free medium and incubated for 15 min. Myotubes were washed with ice-cold PBS and scraped into ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM sodium vanadate, 1 mM phenyl methyl sulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 0.5 μg/ml okadaic acid). Cell lysates were centrifuged at 12,000g for 15 min at 4°C. Protein content of the supernatant collected was estimated by the method of Bradford (Bradford., 1976). The cell lysates were stored at -70°C for subsequent analysis.

**Western blotting analysis of insulin receptor content**

Cell lysate (100 μg of protein) was resolved by 8.0 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows. The 30 % acrylamide and bis-acrylamide mixture was prepared by dissolving 29 gms of acrylamide and 1 gm of bis-acrylamide in milliQ water (Millipore, USA). The 8.0 % separating gel was prepared by mixing 7.5 ml of 30 % acrylamide mixture, 6.5 ml of lower Tris buffer (1.5 M Tris-HCl, pH 8.8), 12.5 ml of milliQ water, 0.1 ml of 10 % SDS, 0.2 ml of 10% ammonium
per sulfate (APS) and 20 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED). The 2.0 % stacking gel was prepared by mixing 0.6 ml of 30 % acrylamide mixture, 0.930 ml of upper Tris buffer (0.5M Tris-HCl, pH 6.8), 2.1 ml of milliQ water, 20 µl of 10 % SDS, 50 µl of 10 % APS and 10 µl of TEMED. This gel is mounted in the electrophoresis apparatus (Bio-Rad MINI – vertical system, USA) and the electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 250 mM glycine and 0.1 % SDS) was poured to the top and bottom of the reservoirs. Cell lysates (100 µg of protein) were boiled with Laemmli (Laemmli., 1970) sample buffer (50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol, 2 % SDS, 0.1 % bromophenol blue and 10 % glycerol) for 2 min and proteins were separated at 100 voltage until the bromophenol blue reaches the bottom of the separating gel.

After separation, the proteins were transferred (wet-transfer) from the acrylamide gel to the nitrocellulose membrane by the method of Towbin et al (Towbin et al., 1979) as follows. Six pieces of Whatmann (3MM) paper and one piece of nitrocellulose membrane (Sigma Chemicals) were submerged in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037 % SDS and 20 % methanol, pH 8.3). The transfer cassette was fixed by keeping the nitrocellulose membrane towards the anode and the SDS-polyacrylamide gel towards the cathode. The nitrocellulose membrane and SDS-polyacrylamide gel were kept in between the Whatmann papers (3 in each side) and the air bubbles were removed using a glass pipette as roller. After assembling, the cassette was submerged in transfer buffer in the transfer unit (Biotech Laboratories, India). The proteins were transferred to the nitrocellulose membrane by applying current (1 mA/cm²) for the period of 1.0 - 1.5 hours.
After transferring the proteins, the nitrocellulose membrane was stained with Ponceau S stain (2 % Ponceau S, 30 % TCA and 30 % sulfoalicylic acid) and the molecular weight markers [Broad range (10-250 KDa), Bangalore Genei, India] were marked. The non-specific sites of the nitrocellulose membrane were blocked with blocking buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20 (TBS-T) containing 5 % bovine serum albumin (BSA)] for 2 hours at room temperature. After 2 hrs, the membrane was washed with TBS-T for 10 min at room temperature. Washing was repeated for three times and the membrane was incubated with anti insulin receptor-β subunit antibody (1: 1000 dilutions in blocking buffer) for overnight at 4°C. The membrane was washed with TBS-T for three times (10 min each) and incubated with horseradish peroxidase (HRP) coupled secondary antibody (1:10,000 dilutions in blocking buffer) for 2 hours at room temperature. The membrane was washed with TBS-T for three times (10 min each) and the protein bands were visualized on X-films by the enhanced chemiluminescence method using Amersham ECL – kit (Amersham Life Sciences, Buckingham, UK) following manufacture's instructions. The bands were scanned with a densitometer (Bio-Rad, Model GS-710, USA) and the band densities were quantified by Quantity 1 software (Bio-Rad, USA). Further, the membrane was stripped of bound antibodies by incubating the membrane at 55°C for 30 minutes in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 nM β-mercaptoethanol and 2% SDS) and then reprobed with antibody specific to β-actin (1:500 dilutions in blocking buffer). The band density of insulin receptor was normalized with the band density of internal control, β-actin.
Immunoprecipitation analysis of insulin receptor tyrosine and serine phosphorylation

Cell lysate (500 µg of protein) was incubated with anti insulin receptor - β subunit antibody (30 µg) for overnight at 4°C. The immune complex was captured by adding 50 µl of protein A-agarose beads for 2 hours at 4°C. Immune complex was pelleted at 12,000g for 15 min at 4°C and washed three times with cell lysis buffer. The immune complex was suspended in Laemmli sample buffer (Laemmli., 1970) and boiled for 2 min. Protein A agarose was removed from the denatured proteins by centrifugation at 12,000g for 15 min at 4°C. The supernatant was resolved on 8.0 % SDS-PAGE and further electrotransferred to nitrocellulose membrane as described earlier. The nitrocellulose membrane was blocked for 2 hours with blocking buffer and washed with TBS-T as described earlier. Proteins were immunoblotted with the antibody specific for phosphorylated tyrosine (1:1000 dilutions in blocking buffer) for overnight at 4°C and washed with TBS-T for three times. The membrane was further incubated with HRP-coupled secondary antibody (1:10,000 dilutions in blocking buffer) for 2 hours at room temperature and washed three times with TBS-T. Protein band detection and quantification was performed using ECL-kit as mentioned earlier. Further, the immunoblot was stripped of bound antibodies and then reprobed with an antibody specific to phosphorylated serine (1:1000 dilutions in blocking buffer). The membrane was further stripped of bound antibodies and then reprobed with antibody specific to insulin receptor- β subunit antibody (1:1000 dilutions in blocking buffer). The band densities of insulin receptor tyrosine and serine phosphorylation were normalized with the band density of immunoprecipitated insulin receptor protein.
Western blotting analysis of insulin receptor substrate-1 content

Cell lysate (100 µg of protein) was resolved by 8.0 % SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibody specific to IRS-1 as described above. Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to β-actin. Protein band detection and quantification was performed using ECL-kit as mentioned above. The band density of IRS-1 was normalized with the internal control β-actin.

Immunoprecipitation analysis of IRS-1 tyrosine and serine phosphorylation

Cell lysate (500 µg of protein) was incubated overnight at 4°C with IRS-1 antibody. The immune complex was captured by adding 50 µl of protein A-agarose beads for 2 hours at 4°C. Immune complex was pelleted at 12,000g for 15 min at 4°C and washed three times with homogenization buffer. The immune complex was suspended in Laemmli sample buffer (Laemmli., 1970) and boiled for 2 min. Protein A-agarose was removed from the denatured proteins by centrifugation at 12,000g for 15 min 4°C. The supernatant was resolved on 8.0 % SDS-PAGE and further electrotransferred to nitrocellulose membrane. Proteins were immunoblotted with the antibody specific for phosphorylated tyrosine and the immunoblot was stripped off bound antibodies and then reprobed with an antibody specific to phosphorylated serine. Further, the membrane was deprobed and then reprobed with IRS-1 antibody used for immunoprecipitation. Protein band detection and quantification were performed as mentioned earlier. The band densities of IRS-1 tyrosine and serine phosphorylation were normalized with the band density of immunoprecipitated IRS-1 protein.
Western blotting analysis of NF-kB pathway

Cell lysate (100 µg of protein) was resolved by 12 % SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibody specific to IkBα as mentioned above. Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to β-actin. Protein band detection and quantification were performed as described above. The band density of IkBα was normalized with the internal control β-actin.

Western blotting analysis of JNK pathway

Cell lysate (100 µg of protein) was resolved by 12 % SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibody specific to phosphorylated JNK1 as mentioned above. Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to JNK1. Protein band detection and quantification were performed as described above. The band density of phosphorylated JNK1 was normalized with the band density of JNK1 protein.

Western blotting analysis of p38MAPK pathway

Cell lysate (100 µg of protein) was resolved by 12 % SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibody specific to phosphorylated p38MAPK as mentioned above. Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to p38MAPK. Protein band detection and quantification were performed as described above. The band density of phosphorylated p38MAPK was normalized with the band density of p38MAPK protein.
Statistical analysis

Data are expressed as mean ± SEM. Differences between means were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s post-test. A ‘p’ value less than 0.05 was considered as statistically significant.
4.4. Results

Effect of glucose oxidase concentration (source of \( \text{H}_2\text{O}_2 \)) and incubation time on rat L6 muscle cell viability:

To optimize the glucose oxidase concentration and incubation time, we incubated the rat L6 muscle cells with different concentrations (0, 25, 50, 75 and 100 mU/ml) of glucose oxidase and 5 mmol/l glucose for different time (2, 4, 8, 12, 16 and 24 hrs). The \( \text{H}_2\text{O}_2 \) induced cytotoxicity was measured by MTT assay. Fig 4.1. shows the cytotoxic effect of \( \text{H}_2\text{O}_2 \) generated by different concentrations of glucose oxidase at different time intervals. Since our objective of the study was to investigate the effect of prolonged low grade oxidative stress on insulin action, from the results (Fig. 4.1) we have chosen 25 mU/ml glucose oxidase treatment for 12 hours as a prolonged low grade oxidative stress model for our further experiments.

Effect of 25 mU/ml of glucose oxidase and 5 mmol/L of glucose on \( \text{H}_2\text{O}_2 \) concentration in the medium:

Estimation of \( \text{H}_2\text{O}_2 \) generated by the glucose oxidase (25 mU/ml) and glucose (5 mmol/L) system in the media at different time intervals was carried out using Amplex red – hydrogen peroxide/peroxidase assay kit. The \( \text{H}_2\text{O}_2 \) concentration in the medium was achieved at a steady state of 52 ± 12 \( \mu \text{M/L} \) after 30 min and this concentration was maintained throughout the 12 hour experimental period (Fig. 4.2).

Effect of \( \text{H}_2\text{O}_2 \) and antioxidants on insulin stimulated glucose uptake in rat L6 myotubes:

The effect of \( \text{H}_2\text{O}_2 \) and antioxidants on glucose transport into rat L6 myotubes is shown in Fig. 4.3. When myotubes were stimulated with insulin (100 nM for 30 min), there was a significant increase (2 – 2.5 fold, \( p < 0.001 \)) in glucose transport into the cells.
compared to basal levels. Treatment of cells with 25 mU/ml of glucose oxidase in the presence of 5 mM glucose for 12 hours maintained a H₂O₂ concentration of 52 ± 12 μM/L in the medium. When cells were incubated at this concentration of H₂O₂, there was no significant effect on basal glucose transport, whereas the insulin stimulated glucose uptake was completely abolished (p < 0.001).

Pretreatment of L6 cells with vitamin E (5 μM), LA (300 μM) and antioxidant mixture (5 μM vitamin E, 500 μM vitamin C and 300 μM LA) for 18 hours did not have significant effect on glucose transport in cells not treated with H₂O₂. However, these antioxidants completely restored (p < 0.001) the insulin stimulated glucose transport in cells exposed to H₂O₂. Vitamin C (500 μM) treatment did not have significant effect on the insulin stimulated glucose transport in cells treated with and without H₂O₂.

These data clearly demonstrate prolonged low grade H₂O₂ treatment causes insulin resistance in rat L6 myotubes and pretreatment with antioxidants (vitamin E, LA and antioxidant mixture) prevents this H₂O₂ induced insulin resistance.

Effect of H₂O₂ and antioxidants on redox balance in rat L6 myotubes:

H₂O₂ treatment to rat L6 myotubes significantly (p < 0.01) decreased the reduced glutathione levels [Fig. 4.4 (A)] and total antioxidant capacity [Fig. 4.4 (B)]. These results indicate H₂O₂ treatment impaired intracellular redox balance in L6 cells. Pretreatment of L6 cells with vitamin E, LA and antioxidant mix preserved the intracellular redox balance. Whereas vitamin C pretreatment was not effective against the H₂O₂ induced oxidative stress in L6 myotubes.

Effect of H₂O₂ and antioxidants on rat L6 myotube toxicity:

To determine whether H₂O₂ and antioxidants treatment cause toxicity to L6 myotubes, we performed cell viability (MTT) assay [Fig. 4.5 (A)] and measured the
release of muscle cytosolic marker enzyme, creatine kinase activity in the medium [Fig. 4.5 (B)]. MTT assay and creatine kinase activity did not show significant difference between the groups. Thus, under the experimental conditions of the present study, the effect antioxidants observed are due to the protection against the oxidative stress and not due to protection of the cells from cell death.

Effect of H₂O₂ and antioxidants on proximal insulin signaling in rat L6 myotubes:

Treatment of rat L6 myotubes with H₂O₂ and antioxidants did not have significant effect on insulin receptor content [Fig. 4.6 (A)], insulin stimulated insulin receptor tyrosine phosphorylation [Fig. 4.6 (B)] and basal insulin receptor and insulin stimulated insulin receptor serine phosphorylation [Fig. 4.6 (C)]. These results indicate that prolonged low grade oxidative stress did not have any effect at the level of insulin receptor.

Hydrogen peroxide (H₂O₂) and antioxidants treatment did not have significant effect on IRS-1 content [Fig. 4.7 (A)]. However, treatment of L6 myotubes with H₂O₂ significantly reduced (p < 0.01) the insulin stimulated IRS-1 tyrosine phosphorylation compared to cells not treated with H₂O₂ [Fig. 4.7 (B)]. In addition, H₂O₂ treatment significantly increased (p < 0.01) the basal serine phosphorylation of IRS-1 compared to cells not treated with H₂O₂ [Fig. 4.7 (C)]. Pretreatment of L6 cells with vitamin E, LA and antioxidant mix inhibited the H₂O₂ induced IRS-1 serine phosphorylation and improved the insulin stimulated IRS-1 tyrosine phosphorylation. Vitamin C pretreatment did not have significant effect on H₂O₂ induced IRS-1 serine phosphorylation and the decreased insulin stimulated tyrosine phosphorylation. These data clearly demonstrate H₂O₂ induced insulin resistance is associated with increased IRS-1 serine
phosphorylation. Antioxidant supplementation inhibits the H₂O₂ induced IRS-1 serine phosphorylation and improved insulin stimulated IRS-1 tyrosine phosphorylation.

**Effect of H₂O₂ and antioxidants on redox sensitive serine kinase pathways:**

H₂O₂ treatment to L6 myotubes significantly decreased (p < 0.01) the IkBα content [Fig. 4.8 (A)] and increased (p < 0.01) the JNK₁ phosphorylation [Fig. 4.8(B)] compared to cells not treated with H₂O₂. These results suggest the activation of redox sensitive NF-kB and JNK pathways by H₂O₂. Pretreatment of L6 cells with vitamin E, LA and antioxidant mix inhibited the H₂O₂ activation of induced NF-kB and JNK pathways. Vitamin C pretreatment failed to inhibit the H₂O₂ induced activation of NF-kB and JNK pathways. H₂O₂ and antioxidant pretreatment did not have significant effect on p38MAPK activation [Fig. 4.8 (C)].

In summary, our results demonstrate treatment of rat L6 myotubes with prolonged low grade oxidative stress (H₂O₂) decreased the insulin stimulated IRS-1 tyrosine phosphorylation and caused insulin resistance (decreased insulin stimulated glucose uptake). This oxidative stress induced insulin resistance in L6 myotubes is associated with impaired intracellular redox balance, activation of redox sensitive NF-kB and JNK pathways and increased IRS-1 serine phosphorylation. Antioxidant pretreatment restored the redox balance, inhibited the activation of redox sensitive NF-kB and JNK pathways and retained the insulin action in L6 myotubes exposed to oxidative stress.
Figure 4.1. Effect of the concentration of glucose oxidase and incubation time on rat L6 muscle cell viability: Rat L6 myotubes were incubated with different concentrations of glucose oxidase and 5 mM of glucose to generate H₂O₂. The cell viability MTT assay was carried out at different time intervals as described in the methods. The H₂O₂ induced cytotoxicity was compared with the cells not treated with H₂O₂. Data represent mean ± SEM of three independent experiments done in triplicate. *p<0.01 in comparison to cells not treated with H₂O₂. The arrow indicates the prolonged low grade oxidative stress (25 mU/ml for 12 hrs) which is not cytotoxic chosen for the further experiments. GO = glucose oxidase.
Figure 4.2. Concentration of H₂O₂ generated in the medium during the course of incubation: Rat L6 muscle cells were treated with 25mU/ml of glucose oxidase and 5 mM glucose for 12 hours. The H₂O₂ generated in the medium by the system was estimated at different time intervals during this 12 hours. Data represent mean ± SEM of three independent experiments done in triplicate. GO = glucose oxidase, Glc = glucose.
Effect of H$_2$O$_2$ and antioxidants on insulin stimulated glucose uptake in rat L6 myotubes: Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H$_2$O$_2$ for 12 hours. 2- deoxy-¹⁴C-glucose uptake was measured in myotubes after insulin stimulation as described in the methods. Data represent mean ± SEM of three independent experiments done in triplicate. *p < 0.001 in comparison to basal glucose uptake, †p < 0.001 in comparison to insulin stimulated glucose uptake in cells not treated with H$_2$O$_2$ of respective group. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA = lipoic acid pretreatment, Mix = Pretreatment with mixture of antioxidant.
Figure 4.4 (A). Effect of H$_2$O$_2$ and antioxidants on reduced glutathione levels in rat L6 myotubes.

Figure 4.4 (B). Effect of H$_2$O$_2$ and antioxidants on total antioxidant capacity in rat L6 myotubes.

Figure 4.4. Effect of H$_2$O$_2$ and antioxidants on redox balance in rat L6 myotubes:

Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H$_2$O$_2$ for 12 hours. Reduced glutathione [Fig. 4.4 (A)] and total antioxidant capacity [Fig. 4.4 (B)] was estimated in the cell lysates as described in the methods. Data represent mean ± SEM of three independent experiments done in triplicate. *p < 0.01 compared to cells not treated with H$_2$O$_2$. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA = lipoic acid pretreatment, Mix = Pretreatment with mixture of antioxidant. TEAC = Trolax equivalent antioxidant capacity.
Figure 4.5 (A). Effect of H₂O₂ and antioxidants on rat L6 myotubes viability.

Figure 4.5 (B). Creatine kinase released by rat L6 myotubes after H₂O₂ and antioxidant treatment.

Figure 4.5. Effect of H₂O₂ and antioxidants on the viability of rat L6 myotubes: Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H₂O₂ for 12 hours. The cell viability assay [Fig. 4.5 (A)] and creatine kinase released by myotubes in the medium [Fig 4.5 (B)] were estimated as described in the methods. Data represent mean ± SEM of three independent experiments done in triplicate. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA = lipoic acid pretreatment, Mix = Pretreatment with mixture of antioxidant.
Figure 4.6 (A). Effect of H$_2$O$_2$ and antioxidants on insulin receptor content in rat L6 myotubes.

Figure 4.6 (B). Effect of H$_2$O$_2$ and antioxidants on insulin receptor tyrosine phosphorylation in rat L6 myotubes.
Figure 4.6 (C). Effect of H₂O₂ and antioxidants on insulin receptor serine phosphorylation in rat L6 myotubes.

Figure 4.6. Effect of H₂O₂ and antioxidants on insulin receptor in rat L6 myotubes:

Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H₂O₂ for 12 hours. Insulin receptor content, insulin receptor tyrosine and serine phosphorylation were studied in cell lysates by immunoprecipitation and western blotting analysis as described in methods. Fig. 4.6 (A); Effect of H₂O₂ and antioxidants on insulin receptor content. Fig. 4.6 (B); Effect of H₂O₂ and antioxidants on insulin receptor tyrosine phosphorylation. Fig. 4.6 (C); Effect of H₂O₂ and antioxidants on insulin receptor serine phosphorylation. A representative immunoblot of three independent experiments is shown. Results shown are mean ± SE of three experiments. *p < 0.001 compared to basal. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA = lipoic acid pretreatment, Mix = Pretreatment with mixture of antioxidant.
Figure 4.7 (A). Effect of H$_2$O$_2$ and antioxidants on IRS-1 content in rat L6 myotubes.

Figure 4.7 (B). Effect of H$_2$O$_2$ and antioxidants on IRS-1 tyrosine phosphorylation in rat L6 myotubes.
Figure 4.7 (C). Effect of H$_2$O$_2$ and antioxidants on IRS-1 serine phosphorylation in rat L6 myotubes.

Figure 4.7. Effect of H$_2$O$_2$ and antioxidants on IRS-1 in rat L6 myotubes: Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H$_2$O$_2$ for 12 hours. IRS-1 content, IRS-1 tyrosine and serine phosphorylation were studied in cell lysates by immunoprecipitation and western blotting analysis as described in methods.

Fig. 4.7 (A); Effect of H$_2$O$_2$ and antioxidants on IRS-1 content. Fig. 4.7 (B); Effect of H$_2$O$_2$ and antioxidants on IRS-1 tyrosine phosphorylation. Fig. 4.7 (C); Effect of H$_2$O$_2$ and antioxidants on IRS-1 serine phosphorylation. A representative immunoblot of three independent experiments is shown. Results shown are mean ± SE of three experiments.

*P < 0.001 compared to basal. *P<0.01 compared to insulin stimulated H$_2$O$_2$ untreated cells. *p<0.001 compared to H$_2$O$_2$ untreated cells. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA= lipoic acid pretreatment, Mix = Pretreatment with mixture of antioxidant.
Figure 4.8 (A). Effect of H$_2$O$_2$ and antioxidants on NF-κB pathway in rat L6 myotubes.

Figure 4.8 (B). Effect of H$_2$O$_2$ and antioxidants on JNK pathway in rat L6 myotubes.
Figure 4.8 (C). Effect of H$_2$O$_2$ and antioxidants on p38MAPK pathway in rat L6 myotubes.

Figure 4.8. Effect of H$_2$O$_2$ and antioxidants on redox sensitive serine kinase pathways in rat L6 myotubes: Rat L6 myotubes were pretreated with antioxidants for 18 hours and then exposed to H$_2$O$_2$ for 12 hours. NF-kB, JNK and p38MAPK pathways were studied in cell lysates by western blotting analysis as described in methods. Fig. 4.8 (A); Effect of H$_2$O$_2$ and antioxidants on NF-kB pathway. Fig. 4.7 (B); Effect of H$_2$O$_2$ and antioxidants on JNK pathway. Fig. 4.7 (C); Effect of H$_2$O$_2$ and antioxidants on p38MAPK pathway. A representative immunoblot of three independent experiments is shown. Results shown are mean ± SE of three experiments. *p<0.001 compared to H$_2$O$_2$ untreated cells. Vit E = vitamin E pretreatment, Vit C = vitamin C pretreatment, LA= lipoic acid pretreatment, MIX = Pretreatment with mixture of antioxidant.
4.5. Discussion

In the present invitro study, we have investigated the effect of prolonged low grade oxidative stress and antioxidant treatment on insulin action, redox balance and redox sensitive serine kinase pathways in cultured rat L6 myotubes. Treatment of L6 myotubes with \( \text{H}_2\text{O}_2 \) decreased the insulin stimulated IRS-1 tyrosine phosphorylation and glucose uptake. \( \text{H}_2\text{O}_2 \) treatment impaired the redox balance, activated the redox sensitive serine kinase NF-\( \text{kB} \) and JNK pathways and increased IRS-1 serine phosphorylation. Antioxidant treatment restored insulin action may be by inhibiting the NF-\( \text{kB} \) and JNK pathways through preserving intracellular redox balance in \( \text{H}_2\text{O}_2 \) treated cells. Even though, previous reports (Hansen et al., 1999; Maddux et al., 2001; Rudich et al., 1999) are in support of our findings that oxidative stress induced inhibition of insulin stimulated glucose uptake and insulin signaling, evidence have also been provided that \( \text{H}_2\text{O}_2 \) has an insulinomimetic effect (Goldstein et al., 2005; Kozlovsky., 1997). These discrepancies can be partly explained by the differences in experimental design and in concentration of reagents used. The insulin like action of \( \text{H}_2\text{O}_2 \) is reported in cells which were exposed to \( \text{H}_2\text{O}_2 \) for a shorter duration (< 60 min) at millimolar concentrations (Rudich et al., 1997).

For decades, the insulin like action of \( \text{H}_2\text{O}_2 \) was attributed with involvement of an oxidation step in the action of insulin (Mahadev et al., 2001). But only recently, the potential molecular mechanism responsible for ROS induced insulin action has been suggested. These studies have hypothesized that insulin binding to its receptor triggers \( \text{H}_2\text{O}_2 \) generation by activating the enzyme NADPH oxidase (Nox) (Goldstein et al., 2005; Lambeth et al., 2004; Mahadev et al., 2004). This insulin stimulated production of \( \text{H}_2\text{O}_2 \) modifies the catalytic thiol residues of protein tyrosine phosphatases (PTPs) (Heffetz et al., 1990). Oxidative modifications of PTPs inactivate these enzymes and retain the
tyrosine phosphorylated form of insulin receptor and IRS-1 (Montfort et al. results in prolonged insulin action in target cells. The above said insulin observed only when cells were exposed to acute and higher concentrations the present study shows inhibition of insulin action by prolonged low grade stress.

The mechanisms by which H2O2 and other mediators of oxidative stress cause insulin resistance are not known. The present study shows, oxidative stress did not have significant effect on basal glucose transport, however it completely abolished the insulin stimulated glucose uptake. Several reports have claimed decreased insulin stimulated PI3K activity and GLUT4 translocation in cells exposed to oxidative stress (Blair et al., 1999; Rudich et al., 1999). These results clearly demonstrate an oxidative stress induced defect in proximal insulin signaling pathway. However, the exact component of insulin signaling machinery affected by oxidative stress is not clearly identified. Our result shows, decreased insulin stimulated IRS-1 tyrosine phosphorylation and increased basal IRS-1 serine phosphorylation in cells exposed to H2O2. Studies have reported increased IRS-1 serine phosphorylation as a potential molecular mechanism for insulin resistance (Keren et al., 1997; Sykiotis et al., 2001). A major negative regulatory role to insulin action is attributed to agents that enhance serine phosphorylation of IRS-1. In cultured muscle and fat cells, okadaic acid, an inhibitor of serine phosphatases, increased IRS-1 serine phosphorylation and inhibited its insulin stimulated tyrosine phosphorylation (Tanti et al., 1994; Roth et al., 1994). Similarly, tumor necrosis factor α (TNF-α), diminishes insulin induced tyrosine phosphorylation of IRS-1 while it induces serine phosphorylation of IRS-1 (Hotamisligil et al., 1996; Kanety et al., 1996). In the present study, we identified the prolonged low grade oxidative stress induced increased IRS-1
serine phosphorylation, decreased insulin stimulated tyrosine phosphoryl

cultured myotubes. Now the question to be answered is how increa
phosphorylation leads to impaired insulin signaling.

Keren et al (Karen et al., 1997) showed that increased serine phosphorylation of
IRS-1 inhibits its interaction with the jexxtamembrane region of insulin receptor, turning
them to poorer substrate for activated insulin receptor kinase activity and fail to undergo
appropriate tyrosine phosphorylation. Studies have also shown increased IRS-1 serine
phosphorylation facilitates the ubiquitin mediated degradation of IRS-1 and thereby
causes insulin resistance (Pederson et al., 2001). But the results of present study are not in
favor of the later concept of serine phosphorylation mediated degradation of IRS-1
because our data did not show any significant difference in the IRS-1 levels (Fig. 4.7)
between the cells treated with and without H2O2. Most relevant to our results, patients
with type 2 diabetes and animal models of insulin resistance have near normal amounts of
the IRS-1 protein and in these subjects and animals only functional deficiencies in IRS-1
have been identified (Vikram et al., 1999). Therefore in the present study, the oxidative
stress induced increased IRS-1 serine phosphorylation could have mediated functional
defects in IRS-1 and impaired insulin signaling.

IRS-1 contains 232 serine and threonine residues making up, nearly 19% of its
1231 aminoacid moieties, providing great potential for multisite phosphorylation. Basal
levels of IRS-1 serine phosphorylation are increased in cells under various conditions,
leading to observable shifts in electrophoretic mobility (Mothe et al., 1996; Tanti et al.,
1994). The magnitudes of the shifts demonstrate that multiple sites are phosphorylated.
However, not all IRS-1 serine phosphorylation is inhibitory to insulin action. It has been
shown that phosphorylation of serine residues adjacent to the phospho tyrosine binding
(PTB) domain of IRS-1 (ser^{266}, ser^{325} and ser^{368} in mouse) protects the IRS-1 from the action of tyrosine phosphatases and maintains the tyrosine-phosphorylated form (Evans et al., 2002; Weigert et al., 2005). Numerous studies have shown that serine phosphorylation of IRS-1 (in mouse) at ser^{302}, ser^{307}, ser^{612}, ser^{636}, ser^{731} and ser^{789} inhibits its interaction with insulin receptor (Isabelle et al., 1996). Out of all this, inhibitory effect of IRS-1 ser^{307} phosphorylation on insulin action is extensively studied (Keren et al., 1997; Sykiotis et al., 2001; Mothe et al., 1996; Tanti et al., 1994). However, there are studies which report, for the requirement of clusters of serine phosphorylation for the disturbance of IRS-1 function (Eric et al., 2004). To this end, it is evident that different stimuli may phosphorylate different serine residues of IRS-1 and inhibit insulin action. Thus the specific signals and the phosphorylated serine residues which inhibit IRS-1 function need to be identified. In the present study we identified the oxidative stress induced increased global serine phosphorylation of IRS-1 and an associated impaired insulin action in cultured rat L6 myotubes. However, further studies are required to identify the oxidative stress induced phosphorylation of specific serine residues and their role in insulin action.

Recent studies have proved that ROS can act as signaling molecules and activate number of redox sensitive serine kinase pathways (Evans et al., 2003). Elevated multiple serine kinase cascades have been recognized in insulin resistance (Qiao et al., 1999). There are number of potential targets for these kinases in the insulin signaling pathway, including insulin receptor and IRS-1. Identification of such redox sensitive serine kinase pathways and their role in the pathogenesis of insulin resistance is an intense area of research. While a number of redox sensitive serine kinase pathways have been recognized, the role of NF-kB, p38MAPK and JNK pathways are well established in the
insulin resistance (Evans et al., 2002). However the effect of prolonged low grade oxidative stress on these pathways and the precise role of redox sensitive serine kinase pathways on insulin signaling are not studied together. Our results show the activation of NF-κB and JNK pathway and increased IRS-1 serine phosphorylation in L6 myotubes exposed to oxidative stress. Previous studies have shown that IRS-1 contains more than 30 serine residues which provide potential sites for these activated kinases (Kera et al., 1997). NF-κB pathway is the most extensively studied intracellular pathway as a target of ROS and oxidative stress (Mercurio et al., 1999). Many factors implicated in the development of insulin resistance such as TNF-α, free fatty acids (Boden., 1994; Shulman., 2000) and serine phosphatase inhibitors (Jullien et al., 1993; Tanti et al., 1994) are able to activate the inhibitory kappa kinase (IKK) complex and NF-κB pathway. Interestingly, insulin-sensitizing drugs such as thiazolidinediones inhibit NF-κB activity (Ghanim et al., 2001). Adiponectin, a cytokine secreted by adipose cells whose plasma levels are negatively correlated with insulin resistance (Maeda et al., 1996), inhibits IKK activity in cells (Quchi et al., 2000). Moreover, diet-induced insulin resistance is ameliorated in IKK deficient mice (Yan et al., 2001). However, molecular mechanisms by which activation NF-κB pathway may specifically interact with insulin signaling pathways are not well understood. Our result show oxidative stress induced activation of NF-κB pathway, increased IRS-1 serine phosphorylation and insulin resistance. The possible explanation for the NF-κB pathway induced insulin resistance in the present study could be oxidative stress induced activation of IKK. IKK is a redox sensitive serine kinase that plays central role in NF-κB pathway (Zhan et al., 2002). Recent study by Zhan et al (Zhan et al., 2002) in cell lines demonstrated IRS-1 as a substrate for activated IKK. They demonstrated the binding of IKK with IRS-1 and its phosphorylation.
Numerous studies have shown serine phosphorylation of IRS-1 at Ser\textsuperscript{307} as a potential inhibitory site for insulin action (Keren et al., 1997; Sykiotis et al., 2001; Mothe et al., 1996; Tanti et al., 1994).

In the present study, we also identified the activation of JNK pathway by H\textsubscript{2}O\textsubscript{2} in rat L6 myotubes. Previous studies in Chinese hamster ovary (CHO) cells showed H\textsubscript{2}O\textsubscript{2} induced activation of JNK pathway and its role in apoptosis (Yuasa et al., 1998). Our results demonstrate for the first time H\textsubscript{2}O\textsubscript{2} induced activation of JNK pathway and its role in the pathogenesis of insulin resistance. Evidence from cellular models and transgenic animals demonstrated the role of JNK in the pathogenesis of insulin resistance. Support for the importance of JNK pathway in insulin resistance is provided by the results of gene knockout experiments in mice. It was shown that insulin resistance is substantially decreased in mice homozygous for a targeted mutation in the JNK gene (Liu et al., 2005). Suppression of JNK pathway improves insulin sensitivity in db/db mice and sucrose fed rats (Yoshihisa et al., 2004). Studies have shown that inhibitors of JNK pathway improves insulin signaling and insulin sensitivity (Kaneto, 2005). Aguirre et al (Aguirre et al., 2005) showed in CHO cells, anisomycin a strong activator of JNK, stimulates the binding activity of JNK to IRS-1 and inhibits the insulin-stimulated tyrosine phosphorylation of IRS-1. In a series of invitro and invivo experiments Aguirre et al (Aguirre et al., 2002) showed that JNK can phosphorylate IRS-1 at Ser\textsuperscript{612}, Ser\textsuperscript{632}, Ser\textsuperscript{652}, and Ser\textsuperscript{731} and inhibits insulin action. Thus, it is likely that oxidative stress induced activated JNK pathway is a crucial mediator of the progression of insulin resistance found in type 2 diabetes.

Previous studies in rat L6 myotubes have shown that H\textsubscript{2}O\textsubscript{2} mediated inhibition of insulin stimulated glucose transport was accompanied by the activation of p38MAPK.
Treatment with LA and specific inhibitors of p38MAPK restored the insulin action in H$_2$O$_2$ treated cells (Maddux et al., 2001; Obata et al., 2000). These results demonstrate the role of oxidative stress induced p38MAPK activation and its role in insulin resistance. However, our results did not show activation of p38MAPK by prolonged low grade oxidative stress. Even though H$_2$O$_2$ mediated activation of p38MAPK is reported, in agreement with our results p38MAPK activity is not significantly elevated in diabetic tissues from humans and animal models (Blair et al., 1999). In contrast, several studies have reported that activation of p38MAPK pathway enhances insulin sensitivity in muscle (Moriguchi et al., 1996; Waskiewicz et al., 1995). There are reports which support the insulin induced activation of p38MAPK in 3T3-L1 adipocytes and L6 myotubes (Taha et al., 1997; Tsakiridis et al., 1996). Studies have also shown that inhibition of p38MAPK pathway by selective inhibitors impairs insulin signaling and glucose transport in cell lines (Sweeney et al., 1999). These results support the beneficial role of p38MAPK activation on insulin action. The contradicting results of various studies could be due to the difference in the experimental design. Blair et al (Blair et al., 1999) showed that acute exposure (<30 minutes) of cells with H$_2$O$_2$ activates p38MAPK whereas chronic treatment did not have significant effect on p38MAPK pathway. Since in the present study we have investigated p38MAPK pathway after chronic treatment (12hrs) with H$_2$O$_2$, no significant effect was observed on the pathway. However, further studies are warranted to firmly establish the role of p38MAPK on insulin action.

Through invitro studies and animal models of diabetes, it has been found that antioxidants improve insulin sensitivity (Evans et al., 2003). Several clinical trials have also demonstrated that treatment with vitamin E, vitamin C, LA and glutathione improves
insulin sensitivity in insulin resistant individuals and patients with type 2 diabetes (Jacob et al., 2000). The beneficial effect of LA has been quantitated by euglycemic-hyperinsulinemic clamp (Evans et al., 2002). Maddux et al (Maddux et al., 2002) reported the protection against the oxidative stress induced insulin resistance in rat L6 muscle cells by micromolar concentrations of LA. It has been shown that vitamin E treatment improves free radical defense and insulin sensitivity in fructose induced insulin resistant rats (Packer et al., 2000). Oral administration of vitamin C has been shown to improve the endothelial dysfunction in patients with type 2 diabetes (Lugo et al., 2000). Several studies have shown that over expression of antioxidant enzymes in cell lines improves insulin sensitivity (Evans et al., 2000). Even though, the beneficial effect of antioxidants in insulin resistance is well documented, the molecular mechanism of action these antioxidants are not known. Results from the present study show that H2O2 treatment of L6 myotubes inhibit insulin stimulated glucose uptake. Pretreatment with antioxidants (vitamin E, LA and mixture of antioxidants) restored the insulin stimulated glucose transport in L6 cells treated with H2O2. Antioxidant treatment also preserved the intracellular redox balance in L6 cells treated with H2O2. To the best our knowledge, for the first time our result showed the role of antioxidants in preserving the intracellular redox balance and prevention of the activation of redox sensitive NF-kB and JNK pathways and IRS-1 serine phosphorylation while improving insulin sensitivity. Although the beneficial effect of vitamin C in type 2 diabetes is reported, in the present study, under the experimental conditions vitamin C was not protective against oxidative stress and oxidative stress induced insulin resistance. In agreement with our results, previous studies by Maddux et al (Maddux et al., 2001) demonstrated that vitamin C (300 μM) treatment was not protective against the oxidative stress induced insulin resistance in L6
myotubes. Abundis et al (Abundis et al., 2001) showed that oral administration of vitamin C to obese diabetic subjects improved the vascular function, but there was no beneficial effect on insulin sensitivity. Hui et al (Hui et al., 2006) demonstrated poor uptake of vitamin C by skeletal muscle compared to vascular and endothelial cells. In the present study, a possible explanation for the inability of vitamin C to protect against oxidative stress induced insulin resistance could be due to its poor entry in to L6 myotubes.

Taken together, our results demonstrate activation of redox sensitive serine kinase pathways by oxidative stress. These activated serine kinases might phosphorylate the IRS-1 and increase their phospho-serine content. Increased serine phosphorylation of IRS-1 decreases the extent of its insulin stimulated tyrosine phosphorylation and leads to insulin resistance. The protective mechanism of antioxidants on oxidative stress induced insulin resistance is associated with their ability to preserve the intracellular redox balance and prevention of the activation of redox sensitive serine kinase pathways and IRS-1 serine phosphorylation.
4.6. Conclusion

The present invitro study shows strong evidence for the role of oxidative stress in the pathogenesis of insulin resistance. Our study demonstrates the role of redox sensitive serine kinase pathways on insulin signaling and insulin resistance. Our results also reveal the molecular basis of insulin sensitizing property of antioxidants. Even though we identified the activation of redox sensitive serine kinase pathways (NF-kB and JNK) and increased IRS-1 serine phosphorylation, further studies are required to identify the specific phosphorylated serine residues and involvement of other serine kinase pathways. Moreover, additional studies which explore the effect of antioxidants and specific inhibitors of serine kinases on insulin signaling in animal models of insulin resistance and insulin resistant subjects will open novel therapeutic targets to delay/prevent the insulin resistance and its complications.