Antioxidants preserve redox balance and inhibit c-Jun-N-terminal kinase pathway while improving insulin signaling in fat-fed rats: evidence for the role of oxidative stress on IRS-1 serine phosphorylation and insulin resistance

R Vinayagamoorthi, Zachariah Bobby and M G Sridhar
Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605 006, India
(Correspondence should be addressed to Z Bobby; Email: zacbobby@yahoo.com)

Abstract
The oxidative stress-sensitive c-Jun-N-terminal kinase (JNK) pathway is known to be activated in diabetic condition and is involved in the progression of insulin resistance. However, the effect of antioxidants on JNK pathway and insulin resistance has not been investigated. The present study was aimed to investigate the effect of antioxidants on redox balance, insulin sensitivity, and JNK pathway in high-fat-fed rats. Male Wistar rats were divided into four groups: the control group – received a rodent chow; control + antioxidant group – fed with rodent chow supplemented with 0.2% (w/w) vitamin E, 0.3% (w/w) vitamin C, and 0.5% (w/w) α-lipoic acid; high-fat group – received high-fat diet; and high fat + antioxidant group – fed with high-fat diet supplemented with above antioxidants. Fat feeding to rats for 9 weeks significantly increased IRS-1 serine phosphorylation, reduced insulin–stimulated IRS-1 tyrosine phosphorylation and insulin sensitivity. High-fat diet also impaired redox balance and activated the redox-sensitive serine kinase – JNK pathway. Antioxidant supplementation along with high-fat diet preserved the free radical defense system, inhibited the activation of JNK pathway, and improved insulin signaling and insulin sensitivity. The present study shows for the first time that antioxidants inhibit JNK pathway and IRS-1 serine phosphorylation while improving insulin sensitivity in fat-fed rats. These findings implicate the beneficial effect of antioxidants in obesity-/dyslipidemia-induced insulin resistance in humans.


Introduction
The incidence of type 2 diabetes is rapidly increasing in all parts of the world. It has been estimated that over 200 million people will be afflicted with the disease by the end of this decade. The pathogenesis of type 2 diabetes is not understood in any great detail, but it has become increasingly clear that acquired and non-genetic factors represent a critical link in the pathogenesis of type 2 diabetes (Saltiel & Kahn 2001, Leng et al. 1999). Insulin resistance and several defects in insulin signaling have been reported in rats fed with high-fat diet. The high-fat diet-fed rodent is a model of research interest because it might provide insights into the mechanisms underlying insulin resistance in obese individuals with impaired glucose tolerance. Even though insulin resistance in obesity has been demonstrated, the molecular basis for the obesity and/or high-fat-induced insulin resistance is not clear.

Recently, we (Vinayagamoorthi et al. 2006) along with others (Rudich et al. 1997, 1999, Maddux et al. 2001) reported the role of oxidative stress in the pathogenesis of insulin resistance. A decline in reduced glutathione, vitamin C, vitamin E, and lipoic acid is noted in patients with diabetes (Shigeta et al. 1961, Maxwell et al. 1997, Opara et al. 1999). In vitro studies and studies in animal insulin resistance models have shown that antioxidants, especially α-lipoic acid, vitamin E, and vitamin C improve insulin sensitivity (Evans & Goldfine 2000, Jacob et al. 2000). When rat L6 muscle cells and mouse 3T3L1 adipocytes were exposed to oxidative stress, insulin-stimulated glucose uptake was inhibited (Rudich et al. 1999, Maddux et al. 2001). Antioxidant treatment to these cell lines protects against the oxidative stress-induced insulin resistance (Maddux et al. 2001, Evans et al. 2002). However, the outcomes of several clinical studies have yielded disappointing results when these antioxidants were used independently (Yusuf et al. 2000). Studies have shown that when these antioxidants were used in combination with high-fat diet, insulin resistance was improved. This study was aimed to investigate the effect of antioxidants on redox balance, insulin sensitivity, and JNK pathway in high-fat-fed rats.
their insulin-sensitizing/antidiabetic action was enhanced through their interaction and effective regeneration of endogenous antioxidants (Evans et al. 2002). Although the insulin-sensitizing property of antioxidants is identified, the potential mechanism(s) by which antioxidants improve insulin sensitivity in insulin resistance is not clearly known.

Elevated levels of fatty acids are known to cause the production of reactive oxygen species (ROS) and oxidative stress (Carlsson et al. 1999, Rao & Reddy 2001). Many studies have shown that the ROS induces activation of multiple stress-sensitive serine kinase cascades and their role in the pathogenesis of insulin resistance (Adler et al. 1999, Qiao et al. 1999). One such major intracellular serine kinase target for oxidative stress is C-Jun-N-terminal kinase (JNK). The JNK pathway is the member of serine/threonine protein kinase superfamily of mitogen-activated protein kinase pathway. The JNK pathway is activated by variety of exogenous and endogenous stress signals including oxidative stress, osmotic stress, proinflammatory cytokines, heat shock, and u.v. irradiation (Tibbles & Woodgett 1999). The activation of JNK pathway is known to interfere with insulin action (Agirre et al. 2000). It has been reported that JNK pathway is activated in various tissues under diabetic condition (Purves et al. 2001, Hirosumi et al. 2002, Qiao et al. 1999). Thus, it is likely that the oxidative stress-induced activation of JNK pathway is a crucial mediator of the progression in insulin resistance. Even though oxidative stress, the activation of JNK pathway and impaired insulin signaling is reported in high-fat feeding, their associated role in the pathogenesis of insulin resistance and effect of antioxidants are not studied in detail. It is worth investigating the interrelationship between the above-mentioned biochemical events because these studies could yield new insights into the molecular basis of obesity-/dyslipidemia-induced insulin resistance as well as help to identify new pharmaceutical targets for the treatment of type 2 diabetes. In view of the above, the present study was designed to investigate the effect of antioxidants on redox balance, insulin action, and JNK pathway in high-fat-fed rats which closely mimic the obesity-/dyslipidemia-induced insulin resistance in humans (Buettner et al. 2007).

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, glucose oxidase, 1,1-diphenyl-2-picryl hydrazyl (DPPH), insulin (human recombinant), sodium vandate, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin, okadaic acid, and all other chemicals were purchased from Sigma Chemicals. 2-Deoxy-14C-D-glucose was purchased from Boehringer Mannheim. Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = fasting insulin (µU/ml) x fasting glucose (mM)/22.5 (Pickavance & Wilding 2007).

Analysis of plasma parameters

After 9 weeks of fat feeding, plasma glucose, triglycerides, and total cholesterol were estimated in overnight fasting samples using standard reagent kits in 550 Express plus autoanalyzer (Ciba Corning Diagnostics, MA, USA). Fasting plasma insulin was estimated using rat insulin ELISA kit purchased from Boehringer Mannheim. Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = fasting insulin (µU/ml) x fasting glucose (mM)/22.5 (Pickavance & Wilding 2007).
**Cell culture**

Rat L6 myoblasts (American Type Culture Collection, Manassas, VA, USA) were cultured (37 °C, 5% CO₂) in growth medium (DMEM, 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin). L6 myoblasts were allowed to differentiate into myotubes in differentiation medium (DMEM, 2% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin) as described previously (Vinayagamoorthi et al. 2006).

**Hydrogen peroxide treatment to rat L6 myotubes**

Rat L6 myotubes were incubated with antioxidant mixture (5 µM vitamin E, 300 µM lipoic acid, and 500 µM vitamin C) in DMEM supplemented with 0.5% BSA for 18 h. 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 mM glucose, and 5 mM glucose for 12 h. H₂O₂ generated by this system was estimated from the media collected at different time intervals using Amplex red – hydrogen peroxide/peroxidase assay kit ( Molecular Probes, NJ, USA). Creatine kinase (CK) activity was measured in the culture medium using colorimetric kit (Teco Diagnostics, USA) in 550 Express plus autoanalyzer (Ciba Corning Diagnostics). H₂O₂-induced cytotoxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (Ferrai et al. 1999).

**Determination of glucose transport into rat L6 myotubes**

Rat L6 myotubes were treated with antioxidants (18 h) followed by H₂O₂ (12 h) as described above. After glucose/glucose oxidase treatment, L6 myotubes were washed with DMEM supplemented with 0.5% BSA and then incubated with serum-free DMEM for 3 h. After serum starvation, insulin (100 nM) was added to the serum-free medium and further incubated for 30 min. Myotubes were washed with HBS (140 mM NaCl, 20 mM HEPES pH 7.4, 5 mM KCl, 2.5 mM MgSO₄, and 1 mM CaCl₂) and glucose uptake was measured as described previously (Blair et al. 1999). Briefly, after insulin stimulation, myotubes were incubated with 2-deoxy-[¹⁴C]-d-glucose (0.5 µCi/ml) in HBS for 10 min. Radioactive medium was aspirated rapidly and the cells were washed with ice-cold isotonic saline (0-9% NaCl). The cells were lysed in 0.05 M NaOH, and radioactivity was determined by liquid scintillation counting (Packyard Top Count LSC, GMI Inc., MN, USA). Protein content of lysates was estimated by the method of Bradford (1976). Nonspecific glucose uptake was determined in the presence of cytochalasin B (50 µM) an inhibitor of facilitative glucose transport and was subtracted from total uptake.

**Analysis of oxidative stress parameters and antioxidant status in fat-fed rats and rat L6 myotubes**

At the end of 9-week experimental period, plasma and skeletal muscle malondialdehyde (MDA) were estimated by TBARS method (Yagi 1984), and erythrocyte-reduced glutathione content was determined using Ellman’s reagent (5,5′-dithio-bis-2-nitrobenzoic acid) as described by Beutle et al. (1963). Hemoglobin content of blood was estimated by cyanmethemoglobin method of Drabkin & Austin (1932) and the catalase activity in erythrocytes was estimated by the method of Aebi (1984).

Rat L6 myotubes were treated with LA (18 h) followed by H₂O₂ (12 h) as described previously and lysed in 0.5 ml PBS by repeated freezing and thawing. Total antioxidant status of cell lysates was quantified by trolox equivalent antioxidant capacity assay using DPPH as described previously (Vinayagamoorthi et al. 2006). Reduced glutathione concentration in cell lysates was estimated as described by Beutle et al. (1963).

**Insulin stimulation**

At the end of 9-week experimental period, the animals were killed with and without insulin stimulation. Insulin stimulation was performed as described earlier by i.p. injection of 15 units of insulin/kg body weight (Youngren et al. 2001). After an interval of 30 min for the maximum effect of insulin to occur, the animals were killed and the hind limb muscle was removed. Muscle homogenates were prepared in homogenization buffer (50 mM Tris–HCl (pH 7-4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium vandate, 1 mM phenyl methyl sulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, and 0.5 µg/ml okadaic acid) as described previously (Saad et al. 1992).

Rat L6 myotubes were treated with antioxidants (18 h) followed by H₂O₂ (12 h) as described previously and incubated with serum-free DMEM for 30 min. After serum starvation, insulin (100 nM) was added to the serum-free medium and incubated for 15 min. Myotubes were washed with ice-cold PBS and scraped into ice-cold homogenization buffer. Muscle homogenates and cell lysates were centrifuged at 12 000 g for 15 min at 4 °C. Protein content of the supernatant collected was estimated by the method of Bradford (1976).

**Western blotting analysis of JNK pathway**

Muscle homogenates and rat L6 cell lysates (100 µg protein) were resolved by 12% SDS-PAGE, electrotransferred onto nitrocellulose membrane, and immunoblotted with antibody specific to phosphorylated JNK₁. Furthermore, the membrane was stripped of bound antibodies and then reprobed with antibody specific to JNK₁. Protein bands were visualized by enhanced chemiluminescence method using Amersham.
ECL kit (Amersham Life Sciences). Bands were scanned using a densitometer (Bio-Rad, Model GS-710) and quantified by Quantity 1 software (Bio-Rad). The band density of phosphorylated JNK1 was normalized with the total JNK1.

**Insulin signaling analysis**

Muscle homogenates (250 mg protein) and rat L6 cell lysates (500 mg protein) were incubated overnight at 4 °C with insulin receptor and IRS-1 antibodies. The immune complex was captured by adding 50 ml protein A-agarose beads for 2 h at 4 °C. The immune complex was pelleted at 12 000 g for 15 min at 4 °C and washed thrice with homogenization buffer. The immune complex was suspended in Laemmli (1970) sample buffer and boiled for 5 min. Protein A agarose was removed from the denatured proteins by centrifugation at 12 000 g for 15 min at 4 °C. The supernatant was resolved by 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 of bound antibodies and then reprobed with an antibody specific to phosphorylated serine. Protein band detection and quantification were performed as mentioned previously. The band densities of tyrosine and serine phosphorylation were normalized with the band densities of immunoprecipitated insulin receptor and IRS-1 respectively.

**RT-PCR analysis of antioxidant enzymes in male Wistar rats**

At the end of 9-week experimental period, RT-PCR analysis was performed in skeletal muscle of male Wistar rats as described previously (Piro et al. 2002, Bhor et al. 2004). Briefly, total RNA was extracted from rat skeletal muscle using Trizol reagent (Invitrogen). The cDNA was synthesized from total RNA (2 μg) using cDNA synthesis kit (Roche Molecular Biochemicals) following the manufacturer’s protocol. The PCR was performed using 2 μl cDNA in a total volume of 20 μl reaction. Amplification of all the genes of interest was done with the forward and reverse gene-specific primers (Bhor et al. 2004) at a concentration of 0.6 μM in PCR mixture (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris–HCl (pH 9.0), 200 μM each dNTP, 2.5U Taq polymerase). Reverse transcription and gene amplification reactions were carried out in a gradient thermal cycler (Eppendorff, Gradient Mastercycler, Germany). The electrophoresis analysis of PCR products was performed in 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. The gels were photographed using a gel-documentation system (Bio-Rad, Gel Doc EQ) and were analyzed in Quantity 1 software (Bio-Rad).

**Statistical analysis**

Data are expressed as mean ± S.E.M. Analysis of difference between the means of groups was done by one-way ANOVA followed by Tukey’s post-test. A P value <0.05 was considered statistically significant.

**Figure 1** Antioxidants improve insulin sensitivity in fat-fed rats and H₂O₂-treated rat L6 myotubes: male Wistar rats were fed with specific diet for 9 weeks (filled square, control (n = 9); filled diamond control antioxidants (n = 10); filled triangle high fat diet (HFD; n = 8); X HFD + antioxidants (AO; n = 8)). At the end of 9 weeks of treatment, (a) i.p. glucose tolerance test and (b) i.p. insulin tolerance test were performed as described in Materials and Methods. Data are expressed as means with their standard errors indicated by vertical bars. Area under curve (AUC) for the glucose tolerance test of control, control antioxidants, HFD, and HFD + antioxidants was 15 087 ± 894, 14 543 ± 702, 22 333 ± 1453*, and 16 108 ± 769 respectively. The AUC for the insulin tolerance test of control, control + antioxidants, HFD, and HFD + antioxidants was 4352 ± 239, 4416 ± 227, 5816 ± 263*, and 4442 ± 232 respectively. *P<0.01 compared with control and antioxidant-treated groups. (c) Rat L6 myotubes were pre-treated with antioxidants for 18 h and then exposed to H₂O₂ for 12 h. 2-Deoxy-14C-D-glucose uptake was measured in myotubes after insulin stimulation as described in the Materials and Methods. Data represent mean ± S.E.M. of three independent experiments done in triplicate. *P<0.001 in comparison with basal glucose uptake.
Results

**Antioxidants improve insulin sensitivity in fat-fed rats and H\textsubscript{2}O\textsubscript{2}-treated L6 myotubes**

Glucose and insulin tolerance tests (Fig. 1(a) and (b)) in male Wistar rats showed that fat feeding for 9 weeks increases the area under curve significantly (*P*<0.01) compared with control and antioxidant-supplemented groups. The fasting insulin and HOMA-IR (Table 1) were significantly (*P*<0.001) increased in fat-fed group compared with other groups. Increased area under curve and HOMA-IR in fat-fed group show impaired glucose tolerance and insulin resistance. Antioxidant supplementation along with high-fat diet improves the glucose tolerance and insulin sensitivity in fat-fed rats.

Addition of 25 mU/ml glucose oxidase, 5 mM glucose for 12 h resulted in a H\textsubscript{2}O\textsubscript{2} concentration in the medium that achieved a steady state of 40±10 μM after 30 min. When cells were incubated at this concentration of glucose oxidase, basal uptake was not affected, whereas insulin-stimulated glucose uptake was completely abolished (Fig. 1(c)). Pretreatment with antioxidants had no effect on basal glucose transport, but restored insulin-stimulated glucose uptake in cells exposed to oxidative stress.

**Effect of high-fat diet and antioxidants on plasma lipids, body weight, and epididymal fat weight**

As shown in Table 1, fat feeding for 9 weeks in male Wistar rats significantly increases the fasting plasma triglycerides (*P*<0.001), total cholesterol (*P*<0.01), body weight, and epididymal fat weight (*P*<0.001) compared with control group. This reveals induction of insulin resistance by high-fat feeding in rats closely mimics the obesity-/dyslipidemia-induced insulin resistance in humans. Antioxidant supplementation along with high fat did not have any significant effect on the above parameters.

**Antioxidants inhibit IRS-1 serine phosphorylation and improve insulin-stimulated IRS-1 tyrosine phosphorylation in fat-fed rats and H\textsubscript{2}O\textsubscript{2}-treated rat L6 myotubes**

Fat feeding to rats and H\textsubscript{2}O\textsubscript{2} treatment to L6 myotubes did not have significant effect on insulin receptor tyrosine and serine phosphorylation (data not shown). However, feeding rats with high-fat diet and H\textsubscript{2}O\textsubscript{2} treatment to L6 myotubes significantly (*P*<0.01) reduced the insulin-stimulated tyrosine phosphorylation of IRS-1 compared with control and antioxidant-treated groups (Fig. 2(a) and (c)). In addition, above treatments significantly (*P*<0.01) increased the basal serine phosphorylation of IRS-1 compared with other groups (Fig. 2(b) and (d)). Antioxidant treatment inhibited the high-fat diet and H\textsubscript{2}O\textsubscript{2}-induced IRS-1 serine phosphorylation and improved the insulin-stimulated IRS-1 tyrosine phosphorylation.

**Antioxidants restore redox balance and inhibit redox-sensitive JNK pathway in fat-fed rats and H\textsubscript{2}O\textsubscript{2}-treated rat L6 myotubes**

Fat feeding to rats for 9 weeks significantly (*P*<0.001) increased the plasma and skeletal muscle TBARS (Table 1) and expression of skeletal muscle antioxidant enzymes (Fig. 4(a)) compared with control and antioxidant groups. High-fat feeding significantly (*P*<0.001) increased the erythrocyte catalase activity and decreased the reduced glutathione concentration (Table 1) compared with control and antioxidant groups. High-fat diet also activated the redox-sensitive JNK pathway in skeletal muscle (Fig. 3(a)). Antioxidant supplementation preserved the redox balance in fat-fed rats and inhibited the activation of JNK pathway.

H\textsubscript{2}O\textsubscript{2} treatment to L6 cells significantly (*P*<0.01) decreased the total antioxidant capacity (Fig. 4(b)), decreased the reduced glutathione levels (Fig. 4(c)), and activated the redox-sensitive serine kinase JNK pathway (Fig. 3(b)). Pretreatment with antioxidant restored the redox balance and inhibited the oxidative stress-induced activation of JNK.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=9)</th>
<th>Control + AO (n=10)</th>
<th>HFD (n=8)</th>
<th>HFD + AO (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Glucose (mg/dl)</td>
<td>84.5±15.3</td>
<td>84.9±12.9</td>
<td>81.6±7.3</td>
<td>90.25±11.5</td>
</tr>
<tr>
<td>2 Insulin (μU/ml)</td>
<td>21.03±2.57</td>
<td>22.32±3.18</td>
<td>43.23±4.49†</td>
<td>25.43±3.97†</td>
</tr>
<tr>
<td>3 HOMA-IR</td>
<td>4.2±0.6</td>
<td>4.6±1.05</td>
<td>8.6±1.2†</td>
<td>5.6±1.3</td>
</tr>
<tr>
<td>4 Total cholesterol (mg/dl)</td>
<td>6.52±8.1</td>
<td>6.52±8</td>
<td>8.3±13.2†</td>
<td>8.52±12.3†</td>
</tr>
<tr>
<td>5 Triglycerides (mg/dl)</td>
<td>693.7±7.1</td>
<td>68.8±6.5</td>
<td>111.1±12.9†</td>
<td>107.5±12.0†</td>
</tr>
<tr>
<td>6 Body weight (g)</td>
<td>272±9.8</td>
<td>282.5±8.8</td>
<td>33.0±12.4†</td>
<td>324.0±287†</td>
</tr>
<tr>
<td>7 Epididymal fat weight (g)</td>
<td>1.31±0.23</td>
<td>1.24±0.27</td>
<td>3.01±0.32†</td>
<td>3.18±0.54†</td>
</tr>
<tr>
<td>8 Plasma TBARS (micromols/l)</td>
<td>2.88±0.48</td>
<td>2.83±0.44</td>
<td>4.10±0.31†</td>
<td>3.01±0.60†</td>
</tr>
<tr>
<td>9 Muscle TBARS (micromols/mg protein)</td>
<td>11.86±5.3</td>
<td>11.52±6.5</td>
<td>21.15±6.1†</td>
<td>10.86±5.4</td>
</tr>
<tr>
<td>10 Erythrocyte-reduced glutathione (mg/g Hb)</td>
<td>4.74±0.45</td>
<td>4.76±0.78</td>
<td>2.88±0.39†</td>
<td>4.36±0.67†</td>
</tr>
<tr>
<td>11 Erythrocyte catalase (K/ml)</td>
<td>8.34±1.46</td>
<td>6.16±0.1</td>
<td>13.51±2.28†</td>
<td>7.03±2.16†</td>
</tr>
</tbody>
</table>

*P*<0.05 compared with control, †*P*<0.001 compared with control. HFD, high-fat diet, AO, antioxidants.

Table 1 Effect of high-fat diet and antioxidants on biochemical and oxidative stress parameters. Male Wistar rats were fed with specific diet for 9 weeks. After 9 weeks of treatment, blood parameters were estimated in overnight fasting sample. Results shown are mean±S.E.M.

www.endocrinology-journals.org
pathway. To determine whether H$_2$O$_2$ treatment causes cell toxicity, we performed the cytotoxicity (MTT) assay and measured the release of skeletal muscle cytosolic marker enzyme creatine kinase (CK) in culture medium after incubation with glucose oxidase. The MTT assay and CK activity in the culture medium did not show any significant difference between the groups (data not shown). Thus, under the experimental conditions of the present study, the effect of antioxidants is due to the protection against the oxidative stress and not due to simply protecting the cells from cell death.

Discussion

In the present study, we have investigated the effect of antioxidants on insulin sensitivity, proximal insulin signaling, redox balance, and JNK pathway in fat-fed rats and cultured rat L6 myotubes. Fat feeding to rats for 9 weeks impaired insulin sensitivity, redox balance, and insulin-stimulated IRS-1 tyrosine phosphorylation. High-fat diet significantly increased the IRS-1 serine phosphorylation and activated JNK pathway. When we exposed rat L6 myotubes to oxidative stress using a H$_2$O$_2$ generating system, the insulin-stimulated glucose transport and IRS-1 tyrosine phosphorylation were decreased. Oxidative stress also increased the IRS-1 serine phosphorylation, impaired intracellular redox balance, and activated the redox-sensitive serine kinase – JNK pathway. Antioxidant treatment restored redox balance, inhibited JNK activation and IRS-1 serine phosphorylation, preserved insulin-stimulated IRS-1 tyrosine phosphorylation, and improved insulin sensitivity in both fat-fed rats and H$_2$O$_2$-treated rat L6 myotubes.

Previous studies in high-fat-fed rats demonstrated severe insulin resistance and defects in proximal insulin signaling pathway (Saad et al. 1992). Hansen et al. (1998) reported a
decreased insulin-stimulated IRS-1 tyrosine phosphorylation in fat-fed rats. In the present study, fat feeding for 9 weeks significantly increased the IRS-1 serine phosphorylation and decreased the insulin-stimulated IRS-1 tyrosine phosphorylation. Numerous studies have documented increased IRS-1 serine phosphorylation as a potential molecular mechanism for insulin resistance (Le Roith & Zick 2001, Sykiotis & Papavassiou 2001, Anna et al. 2005).

**Figure 3** Antioxidants inhibit JNK pathway in fat-fed rats and H2O2-treated rat L6 myotubes. Western blotting analysis of JNK pathway was performed in (a) male Wistar rat skeletal muscle homogenates and (b) rat L6 myotube lysates as described in Materials and Methods. A representative immunoblot of three independent experiments is shown. Results shown are mean ± s.e.m. of three experiments. C = control, C + A = control + antioxidant, H = HFD, H + A = HFD + antioxidant, and AO = antioxidant pre-treatment. *P < 0.001 compared with control and antioxidant groups.

**Figure 4** Antioxidants preserve the redox balance in fat-fed rats and H2O2-treated rat L6 myotubes. (a) RT-PCR analysis of antioxidant enzymes in skeletal muscle mRNA of male Wistar rats. A representative gel picture of four independent experiments is shown. Results shown are mean ± s.e.m. of four experiments. C = control, C + A = control + antioxidant, H = HFD, H + A = HFD + antioxidant. *P < 0.001 compared with control and antioxidant groups. (b) Total antioxidant status in cell lysates of rat L6 myotubes. (c) Reduced glutathione levels in cell lysates of rat L6 myotubes. AO = antioxidant pre-treatment. Data represent mean ± s.e.m. of three independent experiments done in triplicate. *P < 0.01 compared with control and antioxidant-treated cells. GPX, glutathione peroxidase; GSH, glutathione; TEAC, Trolox equivalent antioxidant capacity.
patients with type 2 diabetes and animal models of insulin resistance, IRS-1 serine phosphorylation was found to be increased (Qiao et al. 1999). IRS-1 contains a total of 232 serine/threonine amino acid residues that provide a potential site for phosphorylation by Ser/Thr kinases (Werner et al. 2004). Serine phosphorylation of IRS-1 impairs its interaction with the juxtamembrane domain of insulin receptor and thus renders IRS-1 as a poorer substrate for insulin receptor kinase (Paz et al. 1997). Numerous agents that induce insulin resistance, such as TNF-α, okadaic acid, platelet-derived growth factor, and angiotensin II, increase IRS-1 phospho-serine content (Sykiotis & Papavassiliou 2001). Basal levels of IRS-1 serine phosphorylation are increased in cells under various conditions, leading to observable shifts in electrophoretic mobility (Tanti et al. 1994, Mothe & Obberghen 1996). The magnitudes of the shifts demonstrate that multiple sites are phosphorylated. Numerous studies have shown that serine phosphorylation of IRS-1 (in mouse) at ser302, ser407, ser612, ser636, ser731, and ser789 inhibits its interaction with insulin receptor (Mothe & Obberghen 1996). Out of all this, the inhibitory effect of IRS-1 ser407 phosphorylation on insulin action is extensively studied (Tanti et al. 1994, Mothe & Obberghen 1996, Paz et al. 1997, Sykiotis & Papavassiliou 2001). However, there are reports for the requirement of clusters of serine phosphorylation to disturb IRS-1 function (Werner 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 impaired insulin action in fat-fed rats and cultured rat L6 myotubes. However, additional studies are clearly required to identify the oxidative stress induced specific phosphorylated serine residues and their role in insulin action.

Increased intake of fat and its derivatives are reported to cause oxidative stress (Bakker et al. 2000). Elevated fats can cause oxidative stress due to increased β-oxidation (Otczalk & Schonfeld 1993, Carisson et al. 1999) and mitochondrial respiration (Rao & Reddy 2001). In the process of mitochondrial respiration, molecular oxygen is essential for the production of ATP. During the course of normal oxidative phosphorylation between 0-4 and 4-0% of the total oxygen consumed is converted into the free radical superoxide anion (O$_2^-$) (Chance et al. 1979, Boveris 1984). Subsequently, O$_2^-$ can be converted into other ROS. However, the endogenous antioxidant system that exists within the cells neutralize these ROS and maintains redox balance. Oxidative stress sets in when endogenous antioxidant network fails to provide a compensatory response to restore cellular redox balance. In agreement with previous studies (Rudich et al. 1997, 1999, Maddux et al. 2001), our results from cultured rat L6 myotubes demonstrate oxidative stress can cause insulin resistance. Recently we reported the reduction in insulin-stimulated glucose uptake in rat L6 muscle cell lines when exposed to oxidative stress and its reversal on vitamin E treatment (Vinayagamoorthi et al. 2006). ROS can function as signaling molecules and activate a number of redox-sensitive Ser/Thr kinase cascades linked to insulin resistance (Evans et al. 2002). Our results show that fat feeding to rats impairs redox balance and activates the redox-sensitive serine kinase – JNK pathway. 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. Suppression of JNK pathway improves insulin sensitivity in db/db mice and sucrose-fed rats (Nakatani et al. 2004). Several studies have shown inhibitors of JNK pathway improve insulin signaling and insulin sensitivity (Kaneto 2005, Liu & Rondinone 2005). Agirre et al. (2000) showed in CHO cells, that anisomycin a strong activator of JNK, stimulated the binding activity of JNK to IRS-1, and inhibited the insulin-stimulated tyrosine phosphorylation of IRS-1.

Our results from rat L6 myotubes demonstrate that oxidative stress-induced insulin resistance is associated with the activation of JNK pathway and increased IRS-1 serine phosphorylation. Antioxidant pre-treatment to rat L6 myotubes preserved redox balance, inhibited JNK pathway and IRS-1 serine phosphorylation, retained insulin-stimulated IRS-1 tyrosine phosphorylation, and restored insulin sensitivity. In this regard, here we propose (Fig. 5) that fat feeding to rats increases ROS formation and causes redox imbalance. This results in the activation of redox-sensitive JNK cascade. Oxidative stress-induced activation of JNK in turn phosphorylates IRS-1 and increases phosphor-serine content of IRS-1. Increased IRS-1 serine phosphorylation inhibits its interaction

![Figure 5](https://www.endocrinology-journals.org)

---

**Figure 5** Proposed mechanism for insulin resistance and beneficial effect of antioxidants in high-fat-fed rats.
with insulin receptor and decreases insulin-stimulated IRS-1 tyrosine phosphorylation. This results in insulin resistance in fat-fed rats. The insulin-sensitizing property of antioxidants in high-fat-induced insulin resistance could be related to their ability to preserve the intracellular redox balance and thereby preventing the activation of redox-sensitive JNK pathway and IRS-1 serine phosphorylation. In conclusion, the present study helps us to understand the molecular basis of high-fat-induced insulin resistance and insulin-sensitizing property of antioxidants. More detailed understanding of the role of oxidative stress and antioxidants on redox-sensitive Ser/Thr kinase cascades in humans and animal models of insulin resistance might open novel therapeutic targets for the treatment of type 2 diabetes and its complications.

Acknowledgements

We are grateful to Indian Council of Medical Research (ICMR) for providing senior research fellowship to R.V. The authors do not have conflict of interest in this work.

References


www.endocrinology-journals.org


LeRoith D & Zick Y 2001 Recent advances in our understanding of insulin action and insulin resistance. Diabetes Care 24 588–597.


Received in final form 3 February 2008 Accepted 20 February 2008 Made available online as an Accepted Preprint 20 February 2008