POTENTIAL ROLE OF REDOX SENSITIVE SERINE KINASE PATHWAYS IN THE PATHOGENESIS OF INSULIN RESISTANCE IN RHEUMATOID ARTHRITIS

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Abstract

Objectives: Insulin resistance and oxidative stress have been reported in rheumatoid arthritis (RA). However, the molecular basis of insulin resistance in RA is not clearly understood. Recent studies have documented the role of redox sensitive serine kinase pathways in insulin resistance. The aim of the study is to investigate insulin resistance, oxidative stress and lymphocyte redox sensitive - nuclear factor - kB (NF-kB), c-Jun-N-terminal kinase (JNK) and p38 mitogen activated protein kinase (p38MAPK) pathways in RA subjects.

Methods: Twenty newly diagnosed RA patients and 20 healthy volunteers in the age group of 30-50 years were included in this study. Overnight fasting blood was collected from the subjects, red blood cells, lymphocytes and plasma were separated using Ficoll hypaque. Blood oxidative stress parameters, fasting glucose and insulin were estimated. Redox sensitive serine kinase pathways in lymphocytes also studied.

Results: The fasting insulin and HOMA-IR were significantly (p<0.001) higher in RA subjects compared to control subjects. Lymphocyte and erythrocyte reduced glutathione, erythrocyte catalase activity and plasma total antioxidant capacity were significantly (p<0.001) lower in RA subjects. Plasma MDA levels were significantly (p<0.001) higher in RA subjects. Western blotting analysis shows activation of NF-kB pathway in the lymphocytes of RA subjects. There was no significant change in the lymphocyte p38MAPK and JNK pathways between RA and control subjects.

Conclusion: Further studies which explores the role of redox sensitive serine kinase pathways on insulin action in target tissues of insulin will help in the identification of novel therapeutic target for insulin resistance and its complications in RA.

Keywords: Insulin Resistance, Rheumatoid Arthritis, Oxidative stress, Redox Sensitive Kinases

1. Introduction

Insulin resistance is a characteristic feature of type 2 diabetes, obesity, syndrome X and a number of other human disorders. Insulin resistance refers to the decreased ability of circulating insulin to exert its normal biological effects. Insulin regulates glucose homeostasis mainly by increasing the transport of glucose into the skeletal muscle via the tyrosine phosphorylation of insulin receptor and insulin receptor substrate (IRS) proteins. Although genetics may play a role in the pathogenesis of insulin resistance and type 2 diabetes, it has become
increasingly clear that acquired, non-genetic factors represent a critical link in the pathogenesis of insulin resistance\textsuperscript{4,5}. Recent studies have linked the role of oxidative stress in the pathogenesis of insulin resistance\textsuperscript{6,7}. Many recent studies have shown the reactive oxygen species (ROS) induced activation of multiple redox sensitive serine kinase cascades and their role in the pathogenesis of insulin resistance\textsuperscript{8,9}. Such major intracellular redox sensitive serine/threonine kinase pathways include nuclear factor - \text{kB} (NF-\text{kB}), c-Jun-N-terminal kinase (JNK) and p38 mitogen activated protein kinase (p38MAPK)\textsuperscript{7}. NF-\text{kB} pathway comprises family of transcription factors that function as homo or heterodimers in the regulation of the expression of pro-inflammatory, immunomodulatory, and antiapoptotic genes. JNK and p38 MAPK pathways are the member of serine/threonine protein kinase super family of mitogen activated protein kinase (MAPK) pathway. These pathways are ubiquitously expressed and activated by variety of exogenous and endogenous stress signals including ROS, oxidative stress, osmotic stress, proinflammatory cytokines, heat shock and UV irradiation\textsuperscript{10}. The insulin signaling pathway offers number of potential substrates for these activated Ser/Thr kinases including the insulin receptor and the family of IRS proteins\textsuperscript{11}. Increased serine phosphorylation of insulin receptor and IRS-1 are known to impair insulin action\textsuperscript{12,13}. Previous studies have shown an increased activity of these redox sensitive Ser/Thr kinases in patients with type 2 diabetes and animal models of insulin resistance\textsuperscript{14-15}. Thus, it is likely that the oxidative stress induced activation of these redox sensitive serine kinase pathways is the crucial mediators in the progression of insulin resistance. Rheumatoid arthritis (RA) is a debilitating, chronic multisystem disease with an unknown etiology. Numerous studies have reported insulin resistance in RA\textsuperscript{16,17}. Even though insulin resistance in RA is well documented, the molecular basis of insulin resistance in RA subjects is not known. Reports from our laboratory\textsuperscript{18} along with others\textsuperscript{19-21} have reported oxidative stress and impaired redox balance in RA. Many recent studies are demonstrating the activation of multiple redox sensitive serine kinase pathways in RA\textsuperscript{22,23}. However, the components of insulin resistance, oxidative stress and redox sensitive serine kinase pathways are studied individually in RA, their associated role in the pathogenesis of insulin resistance is not investigated together. It is worth investigating the above mentioned events because these studies could yield new insights into the molecular basis of insulin resistance in RA and may help in identifying new pharmaceutical targets for the treatment of insulin resistance and its resultant complications in these subjects. In view of the above, this present study was designed to investigate insulin resistance, oxidative stress and redox sensitive serine kinase pathways (NF-\text{kB}, JNK and p38 MAPK) in lymphocytes of RA patients.

2. Materials and methods

2.1. Subjects: A total of 20 newly diagnosed rheumatoid arthritis patients (12 females and 8 males) in the age group of 30-50 years were included in
the study. Patients with rheumatoid arthritis overlapping with other connective tissue disorders such as systemic lupus erythematosus, polymyositis and osteoarthritis were excluded from the study. In addition subjects with any acute infections or with co-existing systemic diseases such as coronary artery disease, hypertension or chronic renal failure were excluded. As control, 20 age matched healthy volunteers (11 females and 9 males) were recruited. None of the subjects were receiving any form of drugs. The experimental procedures were approved by the Institute Human Ethics Committee and informed consent was obtained from all the participants.

2.2. Blood Collection and Lymphocyte separation: After 12 hours over night fasting blood sample (8 mL) was collected from the subjects in heparinizied bottles. Lymphocytes were isolated from the blood as described previously. Briefly, 8 ml of heparinized blood was added slowly along the sides of the tube containing 8ml of Ficoll hypaque (Histopaque 1077, Sigma, USA) and centrifuged at 400 g for 20min. The lymphocytes (white interphase layer), plasma (upper layer) and red blood cells (bottom layer) were carefully separated. The lymphocytes were washed with PBS (pH 7.4) three times. Lymphocyte preparation was divided into two parts for the estimation of reduced glutathione and western blotting analysis of NF-kB, p38 MAPK and JNK pathway.

2.3. Analysis of plasma biochemical parameters: Plasma glucose was estimated in fasting samples using standard reagent kit adapted to the 550 Express Plus clinical chemistry analyzer (Bayers Diagnostics, USA). Fasting plasma insulin was estimated using insulin ELISA kit (United Biotech Inc, USA). From the fasting glucose and insulin values the homeostatic model assessment-insulin resistant (HOMA-IR) was calculated using the following formula:

\[ \text{HOMA-IR} = \frac{\text{fasting insulin (µU/ml)} \times \text{fasting glucose (mM/L)}}{22.5}. \]

2.4. Analysis of oxidative stress parameters: Plasma malondialdehyde (MDA) was estimated by thiobarbituric acid reactive substances (TBARS) methods. The MDA values were calculated using the molar extinction coefficient for the MDA-TBA complex of 1.56X10^6 at 560 nm and are expressed as µM/L. The plasma total antioxidant capacity was quantified by trolox equivalent antioxidant capacity assay (TECA) using 1,1-Diphenyl-2-Picryl Hydrazyl (DDPH) as described previously. Erythrocyte and lymphocyte reduced glutathione concentration was determined spectrophotometrically using Eliman’s reagent. Erythrocyte catalase activity was estimated by the method of Aebi and the hemoglobin content of blood was estimated by cyanmethemoglobin method of Drabkin and Austin.

2.5. Western blotting analysis of redox sensitive serine kinase pathways in lymphocytes: Lymphocyte lysates were prepared in the lysis buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM sodium vandate, 1mM phenyl methyl sulfonyl fluoride, 1 mM aprotinin, 1mM leupeptin ans 0.5 µg/ml okadaic acid) as described previously. Cell lysates were centrifuged at 12,000g for 15 minutes at
4°C. Protein content of the supernatant was estimated by the method of Bradford. To study the NF-kB pathway, cell lysates (100 µg of protein) were resolved by 12% SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibody specific to IkBα (Upstate Biotechnology, USA). Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to β-actin (Santa Cruz Biotechnology, USA). For the analysis of p38 MAPK and JNK pathways, cell lysates (100 µg of protein) were resolved by 12% SDS-PAGE, electrotransferred to nitrocellulose membrane and immunoblotted with antibodies specific to phosphorylated p38 MAPK and JNK1 (Upstate Biotechnology, USA). Further, the membrane was stripped of bound antibodies and then reprobed with antibodies specific to p38 MAPK and JNK1 (Upstate Biotechnology, USA). Protein bands were visualized by the enhanced chemiluminescence method with Amersham ECL – kit (Amersham Life Sciences, Buckingham, UK). The bands were scanned with a densitometer (Bio-Rad, Model GS-710, USA) and was quantified by Quantity 1 software (Bio-Rad, USA).

2.6. Statistical Analysis: Results were shown as mean ± SD. Statistical significance of difference between the groups was evaluated using student’s t-test. A ‘p’ value less than 0.05 was considered statistically significant.

2. Results

The fasting plasma insulin levels and HOMA-IR were significantly (p<0.001) higher in RA subtests compared to control subjects. Lymphocyte and erythrocyte reduced glutathione, erythrocyte catalase activity and plasma total antioxidant capacity were significantly (p<0.001) lower in RA subjects compared to control subjects. Plasma MDA levels were significantly (p<0.001) higher in RA subjects compared to control subjects. Western blotting analysis shows a significant (p<0.01) decrease in IkB levels in lymphocytes of RA subjects compared to control subjects. Decreased IkB levels in lymphocytes of RA subjects indicate the activation of NF-kB pathway in the lymphocytes of RA subjects. There was no significant change in the lymphocyte p38MAPK and JNK pathways between RA and control subjects.

4. Discussion

In the present study, we have investigated HOMA-IR, plasma MDA and total antioxidant activity, erythrocyte and lymphocyte reduced glutathione, erythrocyte catalase activity and redox sensitive serine kinase pathways (NF-kB, p38MAPK and JNK) in RA subjects. Our results show, insulin resistance, oxidative stress and activation of lymphocyte NF-kB pathway in RA subjects compared to controls. In agreement with previous studies, our results have shown insulin resistance and oxidative stress in RA. Insulin resistance seems to be the main metabolic abnormality which alters glucose metabolism, decreases the sensitivity of peripheral tissues to insulin in patients with RA. Several studies have demonstrated in RA, insulin resistance and oxidative stress are the strong predictors of
cardiovascular disease. Thus the identification of molecular basis of insulin resistance in RA seems to have clinical relevance. Numerous studies have shown that oxidative stress can cause insulin resistance. Recently, we along with others reported the role of oxidative stress in the pathogenesis of insulin resistance. We demonstrated reduction in insulin stimulated glucose uptake in rat L6 muscle cell lines when exposed to oxidative stress and its reversal on vitamin E treatment. In patients with diabetes oxidative stress and reduced antioxidant machinery are documented. Through vitro and animal models of insulin resistance, it has been found that antioxidants, especially α-lipoic acid, vitamin E and vitamin C improve insulin sensitivity. Reactive oxygen species (ROS) can function as signaling molecules and activate a number of redox sensitive Ser/Thr kinase cascades linked to insulin resistance. Recent studies have documented increased IRS-1 serine phosphorylation as a potential molecular mechanism for insulin resistance. IRS-1 contains more than 30 serine residues providing a great potential site for phosphorylation by Ser/Thr kinases. In patients with type 2 diabetes and animal models of insulin resistance IRS-1 serine phosphorylation was found to be increased. 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. 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. Indentation of specific stimulus and serine kinase pathways which impair insulin action is an intense area of research. Recent studies are documenting oxidative stress induced activation of NF-kB, p38 MAPK and JNK pathways and their role in insulin resistance. We found an oxidative stress induced activation of these redox sensitive kinase pathways, increased IRS-1 serine phosphorylation and insulin resistance in vitro (rat L6 muscle cell line) and animal models (high fat fed rats) of insulin resistance. Evidence from cellular models and transgenic animals demonstrated the role of these pathways in the pathogenesis of insulin resistance. Support for the importance of these pathways in insulin resistance is provided by the results of gene knockout experiments in mice. Suppression/inhibition of redox sensitive serine kinase pathways improves insulin sensitivity in animal models of insulin resistance.

In the present study we identified insulin resistance, oxidative stress and activation of lymphocyte NF-kB pathway in RA subjects. Even though, numerous studies have reported the activation of these (NF-kB, p38 MAPK and JNK) pathways and their diverse effect in various tissues of RA patients, our results shows for the first time the activation of redox sensitive NF-kB pathway and insulin resistance in RA subjects. However the role of other two redox sensitive serine kinase pathways (p38 MAPK and JNK) are well established in the pathogenesis of insulin resistance, in the present study
we did not find activation of these pathways in lymphocytes of RA subjects. Studies have revealed the role of increased TNF-α level in the pathogenesis of insulin resistance in RA\textsuperscript{46}. It has been proved that TNF-α increases the serine phosphorylation of IRS-1 and cause insulin resistance\textsuperscript{47,48}. Activation of NF- B pathway stimulates the transcription of target genes involved in diverse function. One such important target for NF- B pathway is TNF-α expression\textsuperscript{49}. In the present study the insulin resistance caused by the activation of redox sensitive NF- B pathway is due to the activation of inhibitory kappa kinase (IKK) or via the TNF-α expression is not clear. One of the major limitations of the present study is elucidation of the redox sensitive serine kinase pathways in lymphocytes. As such lymphocytes are not the primary target tissue for insulin action, further studies are clearly warranted in potent insulin sensitive tissues like skeletal muscle, adipose tissue and liver to firmly establish the role of redox sensitive serine kinase in the pathogenesis of insulin resistance in RA. In conclusion, future studies in RA subjects which reveal the role of redox sensitive serine kinase pathways on insulin action in the target tissues will open novel therapeutic targets for the treatment of insulin resistant and its resultant complications in RA.

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![Fig.1(A). Western blotting analysis of NF-kB pathway.](image)
**Fig. 1(B).** Western blotting analysis of p38MAPK pathway

**Fig. 1(C).** Western blotting analysis of JNK pathway.
Fig.1. Western blotting analysis of NF-kB, p38MAPK and JNK pathways in lymphocytes.

Lymphocyte lysate (100µg of protein) was resolved by 12% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Analysis of NF-kB, p38MAPK and JNK pathways was performed using specific antibodies as described in methods. A representative immunoblot of five independent experiments is shown. Fig.1(A); Western blotting analysis of NF-kB pathway. Fig.1(B); Western blotting analysis of p38MAPK pathway. Fig.1(C); Western blotting analysis of JNK pathway. *p<0.01 compared to control.

Table.1. Biochemical and oxidative stress parameters in healthy control and rheumatoid arthritis (RA) subjects. *p<0.001. TAEC = trolax antioxidant equivalent capacity

| S.No | Parameter                        | Control (n=20) | RA (n=20) |
|------|---------------------------------|----------------|------------|
| 1    | Age (years)                     | 41.7 ± 9.2     | 42.5 ± 8.3 |
| 2    | BMI (kg/m²)                     | 21.5 ± 2.0     | 22.2 ± 2.6 |
| 3    | Glucose (mg/dl)                 | 83.45 ± 13.1   | 91.0 ± 16.5 |
| 4    | Insulin (µU/ml)                 | 12.9 ± 5.7     | 67.5 ± 26.3* |
| 5    | HOMA-IR                         | 2.61 ± 1.13    | 15.0 ± 8.5* |
| 6    | MDA (µM/L)                      | 2.53 ± 0.67    | 4.2 ± 1.9* |
| 7    | Lymphocyte GSH (mg/mg protein)  | 4.4 ± 0.7      | 2.1 ± 1.2* |
| 8    | Erythrocyte GSH (mg/gHb)        | 6.3 ± 1.5      | 3.3 ± 1.2* |
| 9    | Erythrocyte Catalase (K/ml)     | 20.17 ± 5.6    | 8.5 ± 2.4* |
| 10   | Plasma Total Antioxidant (TAEC) capacity (µmol/ml) | 123.4 ± 31.6 | 73.4 ± 30* |