THE EFFECT OF INSULIN ON EXPRESSION OF GENES AND BIOCHEMICAL PATHWAYS IN HUMAN SKELETAL MUSCLE

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INTRODUCTION

Diabetes is one of the metabolic diseases which have reached to the proportions of epidemic. The number of individuals suffering from diabetes is around 150 million worldwide which will double in next 20 years. According to the WHO data for diabetes, there are 346 million people affected with diabetes worldwide, and by the end of year 2004 around. 3.4 million diabetic individual died, this number of deaths according to WHO projections will be double in between 2005 and 2030 [1]. Diabetes is classified into various types but the two major forms are Type 1 (Autoimmune disease caused by destruction of insulin producing beta cells of pancreas) and Type 2 diabetes (caused by impairment in production of insulin by beta cells). In this paper we will discuss mainly about Insulin action on the gene expression. Various in-vivo studies have shown that skeletal muscles are the major site for the insulin dependent glucose disposal and we also know that in type 2 diabetes insulin resistance is the main metabolic feature [2]. The primary defect in the development of type 2 diabetes is not known but the impairment in insulin action in skeletal muscles have been recognized to be an early defect in the pathogenesis of type 2 diabetes [3]

MECHANISM OF INSULIN SIGNALING

Whole body glucose homeostasis is governed by its uptake by insulin sensitive and insulin insensitive tissues. Insulin secretion is tightly regulated to maintain the blood glucose level in physiological limits. An adequate amount of insulin storage depends on transcriptional and translational regulation of insulin biosynthesis. Various important studies performed in euglycaemic first-degree relatives of Type 2 diabetic patients, developed skeletal muscle insulin resistance as a nearly defect in the pathogenesis of Type 2 diabetes [4]. Therefore, impaired insulin action on the glucose metabolism in skeletal muscle tissues should not be underestimated.

There are two interlocking pathways which play an important role in allowing insulin to regulate gene expression. These are:
- Ras-MAPK pathway (Mitogen activated protein kinase, Ras- GTP binding protein)
- Phosphatidylinositol-3-Kinase Pathway (PI3-Kinase).

To understand better about the mechanism of insulin action in skeletal muscles, Hyperinsulinemic-euglycaemic-clamp technique is used to obtain the skeletal muscle biopsies under basal and insulin stimulated condition in insulin sensitive individuals. In this study, it is to demonstrate that insulin is responsible for regulating gene expression for a large number of transcription factors, metabolic pathway and bio-cellular function. This comprehensive assessment of the differential expression in response to insulin can be assessed using cDNA microarray technology.

The figure below shows that insulin signalling pathway activates the glucose transport and metabolism, protein synthesis and gene expression. It shows that after activation of the insulin receptor there is increased phosphorylation of members of the IRS family, which results in the activation of various signalling pathways like PI 3-kinase dependent and independent signalling and AMPK activation. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism.

**Fig. 1. Outline of pathways regulating glucose transport and GLUT4 translocation to the plasma membrane in skeletal muscle**\(^5\).
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MAPK PATHWAY:

Mitogen Activated Protein Kinase is a signalling cascade that transmits the extracellular signals into the intracellular targets via network of stimulated proteins acting together thus controlling large number of major cellular events like embryogenesis, cell differentiation and proliferation and apoptosis. In mammals there at least 4 distinct groups of MAPKs, these are Extra Cellular Signal Related Kinases (ERK), Jun Amino Terminal Kinases (JNK1/2/3), p38 proteins (p38 α, β, γ and δ) and ERK5. These groups are activated by respective MAP2K like MEK1/2 for ERK1/2, MAP3K for p38, MAP4K for JNKs and MEK5 for ERK5 [6].

TERMINATION OF INSULIN SIGNALLING AND NEGATIVE REGULATION:

Enzymes that are important in the attenuation of PtdIns(3,4,5)P₃ signalling are phosphatase and tensin homologue found on chromosome 10 (PTEN, a 3’ phosphatase) and the family of SRC homology 2 containing inositol 5’-phosphatase (SHIP, a 5’ phosphatase) proteins, which include two gene products SHIP1 and SHIP2[7]. These phosphatases help in degrading PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂ or PtdIns(3,4)P₂ respectively. Internalization of the insulin-insulin receptor complex into endosomes and the degradation of insulin by insulin degrading enzyme also results in termination of Insulin signalling [8]. (IDE) (Bevan 2001).

MATERIALS & METHODS:

Skeletal muscle biopsies were taken from 20 insulin sensitive or diabetic individuals, before and after insulin infusion in each case. RNA was then extracted from these individuals before and after hyperinsulinemic-euglycaemic-clamp technique and used in a microarray experiment.

HYPERINSULINEMIC-EUGLYCAEMIC-CLAMP TECHNIQUE:

It is one of the gold standards in measuring the Insulin resistance. With this technique we calculate the amount of glucose required to compensate the increased levels of insulin without causing hypoglycaemia. It is a 2 hour long procedure where insulin is infused at a rate of 10-120 mU per m² per minute through a peripheral vein. In order to compensate for this insulin infusion, 20% glucose is infused to maintain blood glucose levels between 5 and 5.5 mmol/l which is monitored after every 5 – 10 minutes. During the last 30 minutes of the glucose infusion, the insulin sensitivity is determined. If high levels of glucose (7.5 mg/min or higher) are required, the patient is insulin-
sensitive whereas very low levels (4.0 mg/min or lower) indicate that the body is resistant to insulin action. Levels between 4.0 and 7.5 mg/min are suggestive of "impaired glucose tolerance," which is an early sign of insulin resistance.

**MICROARRAY ANALYSIS:**

The RNA was labelled and hybridised to Affymetrix Human Genome U95A Arrays (this contains 12626 genes, almost about half of the human genome). The amount of binding of the labelled RNA to the chip reflects the activity of that gene in a sample. Binding is measured with a laser that excited the labelled RNA. Average expression values were then calculated for each gene across each group, so that we can work out how much each gene changes when insulin is added. A list of genes whose expression changed significantly (p<0.05, tested with empirical Bayes analysis) was created, then this was filtered for fold change of at least twofolds. Thus we have a list of genes whose expression changes under test conditions.

The next thing to do is compare groups to each other. Firstly, we looked at genes that changed more than 2 folds up or down in normal muscle when exposed to insulin then performed pathway enrichment. Briefly, a cellular process such as proliferation will contain hundreds of genes. If a total of, for eg., 5% of genes change across a whole microarray experiment, we might expect that by chance 5% of genes in any biological grouping of genes will change. Any deviation from this might indicate that this process is specifically impacted in a disease state. A total of 132 genes met our threshold values and these were fed into the pathway analysis tool to look for over-representation of biological processes. In Table 1, you’ll see the top 5 most impacted pathways. MAPK and insulin signalling are at the top of the list. Pathways are ranked by P value, and the number of genes that one would have expected to change is shown, along with the observed value. Next, we compared diabetic muscle, with and without insulin. This time only 32 genes met the threshold. This is consistent with loss of function if you compare to normal muscle response. In Table 2, the top pathway is still MAPK, but insulin signalling has disappeared from the list. As above, there are so few genes involved in the other pathways listed that these aren’t really worth pursuing.

**QUALITY ASSESSMENT, MANAGEMENT AND STATISTICAL ANALYSIS OF MICROARRAY DATA:**

As we have 20 individuals in the control group, we get an average value for each gene from the 20 samples before insulin is added and the same 20 samples after. We have used Affymetrix Human Genome U95A Arrays. Data from the Gene Expression Omnibus (GDS3715, http://www.ncbi.nlm.nih.gov/geo/) was uploaded to the Chipster analysis
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package (http://chipster.csc.fi/). A Student-t-test is used to work out if any significant change and also used an arbitrary threshold (we used 2 fold up or down) to decide if any gene changes in expression. Thus we end up with a list of genes whose expression changes when insulin is added. We also group the gene changes by function, to see if some groups of genes are more affected than others. As said earlier insulin has many effects: like glucose uptake, protein synthesis and growth, so we could predict that these pathways would be impacted. On the next page there are the two tables, one showing the groups (or biological processes) that change in normal muscle, and those that change in diabetic muscle. Here we see a reduced response in diabetes, with a loss of insulin signalling and a reduction in MAPK activity.

RESULT:

Table 1. Normal Muscle exposed to insulin:\[^9\]:

<table>
<thead>
<tr>
<th>P value</th>
<th>Expected Count</th>
<th>Actual Count</th>
<th>Pathway Size</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.2e-05</td>
<td>2.85</td>
<td>11</td>
<td>219</td>
<td>MAPK signaling pathway</td>
</tr>
<tr>
<td>0.018282</td>
<td>1.55</td>
<td>11</td>
<td>119</td>
<td>Insulin signaling pathway</td>
</tr>
<tr>
<td>0.038261</td>
<td>0.31</td>
<td>2</td>
<td>24</td>
<td>Cysteine and methionine metabolism</td>
</tr>
<tr>
<td>0.038877</td>
<td>0.76</td>
<td>3</td>
<td>58</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>0.038877</td>
<td>0.76</td>
<td>3</td>
<td>58</td>
<td>Glioma</td>
</tr>
</tbody>
</table>

Table 2. Diabetic Muscle exposed to insulin:\[^9\]:

<table>
<thead>
<tr>
<th>P value</th>
<th>Expected Count</th>
<th>Actual Count</th>
<th>Pathway Size</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001304</td>
<td>0.89</td>
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<td>219</td>
<td>MAPK signaling pathway</td>
</tr>
<tr>
<td>0.001475</td>
<td>0.24</td>
<td>3</td>
<td>58</td>
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<tr>
<td>0.012136</td>
<td>0.17</td>
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<td>42</td>
<td>mTORsignaling pathway</td>
</tr>
<tr>
<td>0.012136</td>
<td>0.17</td>
<td>2</td>
<td>42</td>
<td>Bladder cancer</td>
</tr>
<tr>
<td>0.015677</td>
<td>0.2</td>
<td>2</td>
<td>48</td>
<td>Endometrial cancer</td>
</tr>
</tbody>
</table>

DISCUSSION

The table 1 shows that when normal skeletal muscle is exposed to the insulin, it activates the MAPK signalling pathway, it can also be interpreted that protein synthesis and the genes involved in the cancer are also affected. In table 2, when a diabetic muscle is exposed to insulin, it dampens the MAPK signalling pathway, with no effect on colorectal cancer gene and insulin signalling is no longer detected.
We have studied insulin’s effects on gene expression in human skeletal muscle using Affymetrix microarray technology. This study involved muscle biopsies taken before and during euglycaemic-hyperinsulinemic clamp studies in a group of healthy insulin-sensitive subjects. Insulin also has a great capability in regulating gene expression. This effect of insulin on multiple genes, constitute the basis for systematic and orchestrated regulation of various cellular functions.

After so much of research, still the insulin dependent and insulin independent signalling pathway to glucose transport mapping remains incomplete. Drugs that can target insulin-independent pathways in the regulation of GLUT4 and glucose transport are likely to bypass defects in insulin signalling thus can improve glucose homeostasis in Type 2 diabetic patients. Studies involving exercise training has shown considerable enhancing of insulin sensitivity by increasing post-receptor insulin signalling. However there are still many gaps in understanding that why exercise training mechanism enhances the whole body glucose uptake. Future studies should resolve this question as it can improve the insulin signalling defects in skeletal muscles.

REFERENCES: