INTRODUCTION

Skeletal muscle tissue is the major amino acids reservoir in the body and plays an essential role in the regulation of nitrogen balance and glycemic control. Muscle growth, maintenance and regeneration is controlled by not fully elucidated cooperation between signals mediated by hormones, peptide growth factors and muscle-specific transcription factors. Sequential expression of MyoD and myogenin is required for the formation of myofibers and muscle-specific gene expression during muscle development and regeneration (1, 2). Among the factors that negatively regulate growth and regeneration of muscle tissue is myostatin, the physiological role of which is to prevent the muscle tissue overgrowth in different stages of organism development. The activation of myostatin expression is thought to play a role in muscle wasting (3) and in catabolic conditions induced in vitro (4).

Type 1 and type 2 diabetes are characterized by a loss of insulin action in skeletal muscle, that leads to...
changes in glucose and lipid metabolism, gene expression and protein phosphorylation (5). Recent studies performed on animal models and humans have been done to assess diabetes-associated alterations in gene expression, however the full set of specific modifications is not identified. Our previous observations showed that STZ-diabetes alters intracellular signaling of insulin in skeletal muscle and can contribute to disruption of anabolic signals in skeletal muscle and further impairment of protein synthesis (6, 7).

In the present study we focused our interest on potential changes in the expression of genes involved in protein metabolism and myogenic differentiation markers in skeletal muscle of streptozotocin (STZ)-diabetic mice, a model of insulin-dependent diabetes mellitus (IDDM). Transcriptional profiling using microarrays, with their capacity for simultaneous monitoring thousands of genes, provides a powerful tool for understanding the critical transcriptional changes that occur in diabetic skeletal muscle.

MATERIAL AND METHODS

Animals

These experiments were carried out in accordance with the opinion of Local Ethic Committee (No 30/2007), as well as the specific national laws on the protection of animals were followed. Mice used for the experiments were selected for body weight reduction and originated from an outbred stock obtained with cross-breeding of four inbred strains (A/St, BALB/c, BN/a, C57B1/6JN) (8). Mice (male, 12-week-old, n = 6/group) were supplied by Faculty of Animal Sciences, Warsaw University of Life Sciences and housed under controlled environmental conditions (temperature 22°C, 12-hour-darkness period starting at 18.00). Mice were given free access to standard laboratory chow diet and water before the beginning of the experiment. Diabetes was induced by single intravenous injection of streptozotocin (STZ, 50 mg/kg b. w., dissolved in 0.01 mol/l citrate buffer, pH 4.5) at 09.00-10.00 h, to overnight-fasted mice. Control group received an equal volume of vehicle. All animals were returned to ad libitum feeding conditions 6 hours after injection. Onset of diabetes in STZ-treated mice was verified by glycosuria using diagnostic sticks 72 hours after STZ injection.

Sampling

Fourteen days after STZ injection animals were anesthetized with ketamine (20 mg/kg b.w.) and xylazine (10 mg/kg b.w.). Gastrocnemius muscles were dissected and blood was drawn from the heart for further analyses. Muscle samples were frozen in liquid nitrogen and stored at -80°C until analysis. At the end of the experiment all STZ-treated mice had high plasma glucose as compared to the controls (17.1 ± 2.9 mmol/l and 6.1 ± 1.6 mmol/l, respectively).

Microarray analysis

Total RNA from gastrocnemius muscles samples was isolated using Total RNA kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water and RNA quantity was measured using NanoDrop (NanoDrop Technologies, USA). The samples with adequate amount of RNA were treated with DNase I to eliminate DNA contamination. Subsequently the samples were purified using RNeasy MiniElute Cleanup Kit (Qiagen, Germany). The samples were again analyzed using BioAnalyzer (Agilent, USA) to measure final RNA quality and integrity. Total RNA (10 µg) was reverse-transcribed using SuperScript Plus Indirect cDNA Labelling kit (Invitrogen, USA) according to the manufacturer's protocol. Single strand cDNA was labeled with Alexa 555 or Alexa 647 dyes (Invitrogen, USA). Efficiency of dyes incorporation was measured using NanoDrop (NanoDrop Technologies, USA). Afterwards the samples were randomly paired (one sample from diabetes mouse and one from control mouse) in one tube and hybridized. Before hybridization microarray slides Operon Mouse V4.0.1, containing 35852 oligonucleotide probes representing 25000 genes and around 38000 transcripts (Operon, Germany) were pre-hybridized according to the manufacturer's protocol. Hybridization was performed using automatic hybridization station HybArray12 (PerkinElmer, USA). Slides were fixed in hybridization chambers and after O-ring conditioning probes were added. Hybridization of slides was performed using 18 hours of hybridization protocol provided by manufacturer of microarrays. After hybridization slides were automatically and manually washed.

Acquisition and analysis of hybridization intensities were performed using microarray scanner ScanArray HT and ScanExpress software (PerkinElmer, USA). Mean spot intensity values were automatically normalized (Lowess method) by ScanExpress software and used for further analyses. Selection of differently expressed genes has been developed based on microarray data and aimed the selection of the most differently expressed genes in
the groups compared, based on some group comparison measures. This approach is typically used for dimensionality reduction/feature selection in the class prediction studies (9), where it is attempted to build a classification model able to predict class membership of samples, based on their gene expression profiles. This approach is also used to observe the set of arbitrarily defined size of the most differently expressed genes across the groups of samples compared. This method assumes some measure (statistical or heuristic) to express difference in expression among classes, and ranks genes by the value of the measure computed individually for each gene. Different measures of difference in expression were reported in literature, while recent comprehensive study devoted to robustness of microarray technology (10) recommend fold-difference to be used for ranking differently expressed genes. Since various measures express difference among classes in a specific way, there is no single generally accepted method of gene selection (11).

In this work we decided to select the most differently expressed genes in the groups compared, based on some group comparison measures. The methods of ranking genes used will be explained with the following notation. Let \( x_i, y_i, i=1,2,...,n \), represent data related to the \( n \) samples tested in a microarray experiment, where \( x_i = [x_{i,1}, x_{i,2},..., x_{i,d}] \in \mathbb{R}^d \) denotes the vector of expressions of \( d \) genes (transcripts) measured for the sample \( i \), and \( y_i \in \{c_1, c_2\} \) denotes membership associated with the sample \( i \). In this study as elements of vectors \( x_i \) was used mean pixel intensity for a spot as measured by the chip scanner.

Prior to the actual gene ranking stage, data were preprocessed in order to ensure equal mean intensity of each sample. Technically, each of the vectors \( x_i, i=1,2,...,n \), was multiplied by a rescaling factor defined as \( \text{avg}(x_i)/\text{avg}(x) \), where

\[
\text{avg}(x) = \frac{1}{d} \sum_{i=1}^{d} x_{i,j}
\]

thus rescaling its intensity to the intensity of sample 1.

The following methods of ranking of genes were used:

- Wilcoxon statistics,
- Fold change.

Gene selection based on the Wilcoxon statistics (9) requires that for each fixed gene \( j, j=1,...,d \), nonparametric rank test is performed comparing two groups of samples \( \{x_{i,j} | y_i = c_1\} \) against \( \{x_{i,j} | y_i = c_2\} \). This gives a \( p \)-value whose small value (e.g. below 0.05 threshold) indicates that expression of gene \( j \) for the groups \( c_1 \) and \( c_2 \) should be considered different. Sorting the list of genes by increasing \( p \)-value places the most differently expressed genes on top of this list.

Selection of differently expressed genes using the fold difference measure requires that for each gene \( j, j=1,...,d \), the ratio of mean expressions of this gene for class \( c_1 \) and \( c_2 \) as \( \mu_1 \) and \( \mu_2 \), respectively, then a convenient expression of the fold difference is given by the term \( |\log \mu_1 - \log \mu_2| \), which produces high values if either of the means exceeds the other. Sorting the list of genes by decreasing value of this measure gives the most differently expressed genes on top of the list.

**Immunoblotting analysis**

Gastrocnemius muscles were homogenized in ice-cold extraction buffer, as described previously (6) and centrifuged at 10000g for 30 min at 4°C. Protein content in resulting supernatants was measured by Bradford reaction.

For immunoblotting analysis of MyoD, myogenin and myostatin, aliquots of muscle extracts corresponding to 50 µg of total protein were subjected to SDS-PAGE under reducing conditions. Electrotransfer of separated proteins to nylon membrane (Millipore Corp., Bedford, MA, USA) was conducted for 1 h at 100 V. Membranes were then saturated in TBS buffer (20 mM Tris-base, 500 mM NaCl, pH 7.5) supplemented with 5% nonfat powdered milk for 1 h and subsequently probed with appropriate primary antibody: anti-MyoD (12), anti-myogenin (13), and anti-myostatin antibody which allow to assess an active form of this protein (14), for 16 h at 4°C. After extensive washing in TBS containing 0.05% Tween-20, membranes were exposed to the appropriate second antibody conjugated with horseradish peroxidase. Membranes were also reprobed with anti-actin antibody (15) to ensure that all lanes contained equal amounts of total protein (loading control). The blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer’s instructions. Different exposure times were used to ensure that bands were not saturated. The data were expressed in arbitrary units presenting the changes related to control values ± S.E.M. The results were statistically evaluated using ANOVA, with actin data included as a covariate (general linear model). Statistical significance was set at \( P<0.05 \). The analyses were performed using SPSS 12.0PL for Windows (SPSS Inc. & SPSS Poland).

**RESULTS**

The microarray analysis revealed alterations in the expression of 84 gene transcripts in the gastrocnemius
muscle of STZ-diabetic mice. This alterations resulted in a fold change of >1.6 (increase or decrease), and 28 of these transcripts were upregulated, while 56 were declined. The identified gene transcripts were classified by biological process ontology terms using The Panther Classification System database (www.pantherdb.org). Among categories for biological process involvement of altered genes were: cell cycle, cell proliferation and differentiation, amino acid metabolism, protein metabolism and modification, lipid, fatty acid and steroid metabolism, nucleoside, nucleotide and nucleic acid metabolism, signal transduction, immunity and defense, homeostasis and transport (Fig. 1).

With regard to protein metabolism and modification in the gastrocnemius muscle of STZ-diabetic mice there was a marked downregulation in gene transcripts for: general transcription factor II A1, Gtf2a1 (-1.88 fold of value obtained in the control group, \(P=0.016309\)), TATA box binding protein, Tbp (-2.17 fold of value obtained in the control group, \(P=0.037373\)), eukaryotic translation initiation factor 4E nuclear import factor 1, Eif4enif1 (-1.61 fold of value obtained in the control group, \(P=0.037373\)), and eukaryotic translation elongation factor I ß2, Eef1b2 (-1.95 fold of value obtained in the control group, \(P=0.010406\)).

Table 1. Differences in level of gene transcripts involved in protein metabolism in STZ-diabetic mice in comparison to control mice. The altered gene transcripts and fold changes were determined as described in Material and Methods. The results are expressed as the mean ± S.D. The mean fold changes represent average of 6 pairwise comparisons among individual animals (\(P<0.05\)). Ctrl - control group, STZ - STZ-diabetes. \(P\)-value was measured using Wilcoxon statistics.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene ID</th>
<th>Gene name, symbol</th>
<th>Ontology</th>
<th>Ctrl Mean ± S.D.</th>
<th>STZ Mean ± S.D.</th>
<th>Mean fold change</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>83602</td>
<td>General transcription factor II A1, Gtf2a1</td>
<td>Basal transcription factor, transcription cofactor, nucleic acid binding, general mRNA transcription activities</td>
<td>2054.17±1363.90</td>
<td>1086.33±746.82</td>
<td>-1.88</td>
<td>0.016309</td>
</tr>
<tr>
<td>2.</td>
<td>21374</td>
<td>TATA box binding protein, Tbp</td>
<td>Nucleic acid binding</td>
<td>3381.50±2433.51</td>
<td>1553.50±820.98</td>
<td>-2.17</td>
<td>0.037373</td>
</tr>
<tr>
<td>3.</td>
<td>74203</td>
<td>Eukaryotic translation initiation factor 4E nuclear import factor 1, Eif4enif1</td>
<td>Translation initiation factor, protein biosynthesis</td>
<td>1078.00±412.57</td>
<td>671.17±180.16</td>
<td>-1.61</td>
<td>0.037373</td>
</tr>
<tr>
<td>4.</td>
<td>55949</td>
<td>Eukaryotic translation elongation factor Iß2, Eef1b2</td>
<td>Translation elongation factor, protein biosynthesis</td>
<td>1127.50±655.76</td>
<td>576.66±254.67</td>
<td>-1.95</td>
<td>0.010406</td>
</tr>
<tr>
<td>5.</td>
<td>66177</td>
<td>Ubiquitin-like 5, Ub5</td>
<td>Proteolysis</td>
<td>1400.83±1046.53</td>
<td>835.83±583.27</td>
<td>-1.67</td>
<td>0.024975</td>
</tr>
<tr>
<td>6.</td>
<td>108086</td>
<td>Ubiquitin conjugating enzyme 7 interacting protein 1, Ubc7ip1</td>
<td>Proteolysis</td>
<td>460.83±234.58</td>
<td>273.33±132.60</td>
<td>-1.68</td>
<td>0.016309</td>
</tr>
</tbody>
</table>
1.67, $P=0.024975$) and ubiquitin conjugating enzyme 7 interacting protein 1 (-1.68, $P=0.016309$) were also downregulated in streptozotocin-diabetic mice (Table 1).

As was shown in Fig. 2a, MyoD protein level was slightly but significantly increased in skeletal muscle of diabetic mice (to 106% of value obtained in the control group, $P<0.05$). Protein level of myogenin in muscle of STZ-diabetic mice was markedly decreased (to 25% of value obtained in the control group, $P<0.05$) (Fig. 2b). The expression of the active form of myostatin (26 kDa) in muscle of diabetic mice was elevated by approx. 70% ($P<0.05$) when compared to the control (Fig. 2c). The protein level of actin was similar in skeletal muscle of control and diabetic mice (Fig. 2 insert).

DISCUSSION

The rates of protein synthesis and breakdown in each cell must be balanced precisely, since even a small decrease in synthesis or a small acceleration of degradation, if sustained, can result in a marked loss of mass in the organism (16). It is particularly important in regard to the skeletal muscle, which accounts for nearly half of all protein in the body (17). The aim of the present study was to gain more insight into the molecular mechanisms contributing to the effects of diabetes on protein metabolism and modification in skeletal muscle. A better understanding of these changes could help to develop strategies for intervention in muscle wasting associated with diabetes. We studied skeletal muscle gene expression profile in animal model of insulin-independent diabetes mellitus and compared it with the non-diabetic state. Presented results are the first report concerning diabetes-associated alterations in genetic mechanisms controlling protein metabolism in skeletal muscle of mice selected for body weight reduction. A relationship between the activity of GH-IGF-I axis and the insulin sensitivity has been intensively studied. Thus it was interesting to explore whether the attenuation of growth-promoting mechanisms (i.e. reduced GH-IGF-I axis activity, manifested by the body weight reduction), could interfere with potential diabetes-evoked modifications of expression of genes involved in muscle protein turnover. Using microarray analysis we found that STZ-diabetes altered the expression of 84 gene transcripts in the gastrocnemius muscle, which may suggest that the transcriptome appears to be very stable or that diabetes is not reflected by great changes in the gene expression profile in skeletal muscle. The effect of STZ-induced type 1 diabetes on skeletal muscle gene expression has been studied previously (18) and cDNA microarray approach revealed that of 16,392 individual gene sequences 120 were differentially expressed. In other report, microarray analysis of 19,000 genes in skeletal muscle of insulin resistant patients did not display significant changes when compared to insulin sensitive subjects (19).

In the present study a significant downregulation of gene transcripts for important regulators of protein transcription and translation was observed (Table 1). The general transcription factor II A1 (TFIIA1) plays a role in the regulation of transcription by RNA polymerase II through stabilizing the interaction between general transcription factor TFIIID and DNA and thus determines the transcriptional efficiency (20). The TATA box binding protein (TBP) is a subunit required by all three of the RNA polymerases
in the process of gene expression (21). Eukaryotic translation initiation factor 4E nuclear import factor 1, a nucleocytoplasmic shuttling protein containing an eIF4E-binding site, mediates the nuclear import of eIF4E, the least abundant of the initiation factors considered to be the limiting factor for cap-dependent translation initiation (22). In view of the important role of these proteins in regulation of translation machinery, diabetes-induced downregulation of these genes can limit the process of protein synthesis.

A reduced circulating level of insulin and supressed activation of PI-3K/PKB pathway observed in catabolic conditions associated with insulin resistance accelerates muscle proteolysis through an increased expression and activity of the ubiquitin-proteasome proteolytic pathway (23). The ubiquitin-proteasome pathway in muscle is activated in several states associated with muscle wasting such as: uremia, sepsis, fasting, diabetes, cancer cachexia and glucocorticoid treatment (18). Suprisingly, we found downregulation of gene transcripts for ubiquitin-like 5 and ubiquitin conjugating enzyme 7 interacting protein 1 in skeletal muscle of streptozotocin-diabetic mice (Table 1). The sequence homology and structure prediction algorithms indicate that the ubiquitin-like 5 protein has a structure similar to ubiquitin (24). It has been shown that protein with identical amino acid sequence contributes to development of obesity and type 2 diabetes in the Israeli sand rat (25). Although the role of ubiquitin-like 5 protein has not yet been fully elucidated, its widespread expression in all tissue types suggests that this protein has an important role (26). Ubiquitin conjugating enzyme 7 interacting protein 1 acts as an E3 ubiquitin ligase (TRIAD3), which accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes and transfers it to substrates promoting their degradation by the proteasome. Although its precise role in muscle is unclear, it belongs to the family of E3 ubiquitin ligases among which are atrogin-1/MAFbx and MuRF-1, well known from contributing to muscle atrophy (18, 27, 28). Previous reports using microarray showed that STZ-diabetes in rats strongly induced genes involved in protein degradation, including polyubiquitins, Ub fusion proteins, the Ub ligases atrogin-1/MAFbx and MuRF-1, multiple but not all subunits of the 20S proteasome and its 19S regulator (18, 29). However, recent study has demonstrated that the reduction in muscle mass during STZ-induced diabetes in mice is not due to an increased expression of MuRF1 and atrogin-1 (30, 31). The discrepancy between our and other results regarding the expression of the ubiquitin system may be probably a reflection of different susceptibility to STZ-induced diabetes (32). Severity of STZ-induced diabetes and following muscle mass reduction and the extent of protein degradation reflected by the activity of proteolytic systems can vary among different mouse strains. To our knowledge, the present study is the first report analyzing the pattern of transcriptional changes in muscle of STZ-diabetes in mice selected for body weight reduction.

In the simpliest hypothetic model of muscle wasting associated with insulin resistance, the general acceleration of proteolysis results from increased expression of all or many elements of the ubiquitin-proteasome system in muscle. Our results suggest these alterations are not general features of changes in skeletal muscle during catabolic conditions. Protein degradation plays a role in maintaining normal physiological homeostasis and adapting to new homeostatic states, since the ubiquitin-proteasome pathway selectively eliminates abnormally folded or damaged proteins that have arisen by missense or nonsense mutations, biosynthetic errors or damaged by free radicals (16, 17). Downregulation of genes that control the activity of ubiquitin-proteasome pathway can be one of the mechanisms of diabetic complications during untreated hyperglycemia. On the other hand, the marked downregulation of gene expression observed under hyperglycemic conditions may be result of altered transcriptional regulatory mechanisms and modifications of mRNA stability (33).

Since the microarray used in the present study did not include probes for some gene transcripts for transcriptional factors involved in muscle regeneration process, we decided to examine their expression at the protein level. According to the present results, STZ-diabetes inhibits myogenin expression, however it exerts slight, but significant stimulatory effect on MyoD level (Fig. 2a, b). The observed decline in myogenin level is in agreement with crucial role of myogenin in the progression of normal fusion, particularly at the onset of fusion. Elimination of myogenin results in affected development of muscle and inhibits myotube formation in vitro and in vivo (34, 35). A recent microarray gene profiling study has revealed a set of 140 muscle-expressed genes, the expression of which was downregulated in embryonic tongue muscle of mice lacking myogenin (36). However, in a previous study muscle atrophy in STZ-treated rats was associated with 50% reduction in the levels of MyoD and myogenin (37). Recently, Lehti and coworkers (38) have demonstrated in STZ-diabetic mice decreased mRNA level of MyoD but that of myogenin was below the detection limit. With regard to observed diabetes-induced modification in MyoD expression, one could ask a question whether such a minimal difference between control and diabetic
mice has a biological significance. A potential explanation could be the activation of satellite cells induced by an increased systemic level of proinflammatory cytokines accompanying diabetes. Nevertheless, a biological significance of diabetes-associated changes in myogenic regulatory factors expression requires further investigations.

Finally, we emphasize that STZ-diabetes enhances the expression of myostatin (Fig. 2c). It is important to note that the previous data concerning the expression of this protein during diabetes are not consistent. In gastrocnemius muscle of hyperglycemic STZ-diabetic rats myostatin mRNA levels were comparable to controls (30). On the contrary, in NMRI mice STZ-induced diabetes decreased myostatin mRNA (38). This apparent discrepancy between our and other results in the literature may be caused by the differences in the regulation of myostatin expression at the level of mRNA and the protein. It may also result from the different experimental procedure, the specificity of the antibody used or the way of data analysis. It has been shown that myostatin inhibits muscle differentiation through the downregulation of myogenic regulatory factors expression (39, 40). Hence, it is possible that diabetes-induced increase in myostatin expression exerts an inhibitory effect on myogenin and impairs the growth and regeneration of skeletal muscle.

The immunoblotting analysis was performed independently of the microarray, since the microarray employed for the analysis did not contain the oligonucleotide probes for the MyoD and myostatin gene transcripts. According to this analysis, STZ-diabetes had no effect on the expression of gene transcript for myogenin (not shown). On the contrary, the immunoblotting revealed that STZ-diabetes inhibits myogenin expression pointing to the post-transcriptional and post-translational regulation.

Alterations in the expression of the muscle specific regulatory factors can result from the modifying effect of hyperglycemia-associated enhanced production of reactive oxygen species. According to Aragno and coworkers (37), oxidative stress may play a role in diabetes-related impaired muscle repair, since vitamin E administration to STZ-diabetic rats reverses oxidative imbalance and restores the content of myogenic regulatory factors MyoD and myogenin. Indeed, in vitro H2O2 inhibits myogenesis at the level of muscle-specific protein expression (41).

In conclusion, 1) STZ-diabetes attenuates expression of gene transcripts involved in the process of transcription and translation, which may affect skeletal muscle protein biosynthesis and lead to nitrogen imbalance, 2) impaired expression of gene transcripts involved in the regulation and activity of the ubiquitin-proteasome pathway may contribute to attenuation of mechanisms eliminating damaged proteins in STZ-diabetes, 3) changes in the expression of key myogenic factors, manifested by a decrease in myogenin level and enhancement of myostatin expression may be one of the mechanisms limiting skeletal muscle growth and regeneration associated with diabetes.

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