Altered REDD1, myostatin, and Akt/mTOR/FoxO/MAPK signaling in streptozotocin-induced diabetic muscle atrophy

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Submitted 2 August 2011; accepted in final form 25 October 2011

Hulmi JJ, Silvennoinen M, Lehti M, Kivelä R, Kainulainen H. Altered redd1, myostatin, and Akt/mTOR/FoxO/MAPK signaling in streptozotocin-induced diabetic muscle atrophy. Am J Physiol Endocrinol Metab 302: E307–E315, 2012. First published November 8, 2011; doi:10.1152/ajpendo.00398.2011.—Type 1 diabetes, if poorly controlled, leads to skeletal muscle atrophy, decreasing the quality of life. We aimed to search highly responsive genes in diabetic muscle atrophy in a common diabetes model and to further characterize associated signaling pathways. Mice were killed 1, 3, or 5 wk after streptozotocin or control. Gene expression of calf muscles was analyzed using microarray and protein signaling with Western blotting. We identified translational repressor protein REDD1 (regulated in development and DNA damage responses) that increased sevenfold and was associated with muscle atrophy in diabetes. The diabetes-induced increase in REDD1 was confirmed at the protein level. This result was accompanied by the increased gene expression of DNA damage/repair pathways and decreased expression in ATP production pathways. Concomitantly, increased phosphorylation of AMPK and dephosphorylation of the Akt/mTOR/S6K1/FoxO pathway of proteins were observed together with increased protein ubiquitination. These changes were especially evident during the first 3 wk, along with the strong decrease in muscle mass. Diabetes also induced an increase in myostatin protein and decreased MAPK signaling. These, together with decreased serum insulin and increased serum glucose, remained altered throughout the 5-wk period. In conclusion, diabetic myopathy induced by streptozotocin led to alteration of multiple signaling pathways. Of those, increased REDD1 and myostatin together with decreased Akt/mTOR/FoxO signaling are associated with diabetic muscle atrophy. The increased REDD1 and decreased Akt/mTOR/FoxO signaling followed a similar time course and thus may be explained, in part, by increased expression of genes in DNA damage/repair and possibly also decrease in ATP-production pathways.

MUSCLE ATROPHY in poorly controlled diabetes, also called diabetic myopathy, is a clinical condition in which skeletal muscle size and strength/endurance are reduced (21, 39). Adequate muscle size and strength capacity enable work at a lower relative submaximal level during normal daily activities. In addition to locomotion, skeletal muscles are also important in many other functions such as glucose homeostasis. Adequate size and function of skeletal muscle can attenuate further diabetic complications.

In type 1 diabetes, the major reason for diabetic muscle atrophy is still under debate, but it is a function of protein synthesis/protein breakdown. Animal studies using severe experimental diabetes have studied this phenomenon directly from the muscles without any reflection of the other tissues, such as the splanchnic region. These studies have shown that both the increase in muscle proteolysis and the decrease in muscle protein synthesis contribute to diabetic muscle atrophy (12, 24, 28, 33). However, the signaling pathways possibly linked with muscle loss in diabetic myopathy have not been thoroughly studied throughout the different time points of the muscle atrophy process. Therefore, in the present study, we searched by transcriptional profiling highly responsive genes in streptozotocin (STZ)-induced diabetes that would be associated with muscle atrophy. We identified translational repressor protein REDD1 (regulated in development and DNA damage responses) as a possible mediator in this process; it was robustly increased by diabetes. REDD1 was originally found to be upregulated in response to hypoxia (3, 43), but later it was shown to be induced also after, e.g., DNA damage (9) and energy stress/deficit (31). These pathways may also be activated in type 1 diabetes. Furthermore, REDD1 decreases mammalian target of rapamycin complex 1 (mTORC1) signaling (3). Supporting this, REDD1 overexpression by electroporation of skeletal muscle in vivo has caused a 10% decrease in muscle fiber size (10). On the other hand, insulin itself is an anabolic hormone able to directly activate, e.g., mTOR and mitogen-activated-protein kinase (MAPK) signaling (35, 46). Therefore, the purpose of the present study was to further investigate which of these pathways or possibly others would be associated with muscle atrophy and enhanced expression of REDD1. Furthermore, MAPK signaling was also explored together with myostatin, a negative regulator of muscle size (32, 38). By combining the approaches of muscle transcriptome and protein signaling, we have tried to provide, to date, the most complete picture of diabetic muscle atrophy signaling in a common diabetic animal model with a state of severe lack of insulin.

RESEARCH DESIGN AND METHODS

Animals. Male NMRI mice (n = 30; Harlan, The Netherlands) were housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM). The mice had free access to tap water and food pellets (R36; Labfor, Stockholm, Sweden). The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the University of Jyväskylä Ethics Committee for Animal Care and Use ( Permit No. LSLH-2005-00875/Ym-23). All efforts were made to minimize suffering.

At the age of 10 wk, the animals were randomly assigned to diabetic and control groups. Experimental type 1 diabetes was induced...
by a peritoneal injection of STZ (Sigma-Aldrich, Lyon, France; 180 mg/kg, 0.1 mol/l sodium citrate buffer, pH 4.5). An equal volume of buffer was injected into the control mice. Diabetes was confirmed by urine glucose that was >200 mg/dl 72 h after the injection of STZ. Two mice died in total (one from the 3-wk group and one from the 5-wk group). This did not alter the total number, as we had reserve diabetic mice to protect against this eventuality.

Muscle sampling. Mice were randomly assigned to six groups \((n = 5\) per group). Groups were named as follows: healthy control mice based on the length of the experiment in weeks \((C1, C3, C5)\) and diabetic mice \((D1, D3, D5)\). Animals were weighed before and after the experiment. The mice were killed by cervical dislocation. Calf muscles (soleus, gastrocnemius, and plantaris) were immediately removed, weighed, and frozen in liquid nitrogen. Analyzed muscle group represents average phenotype of skeletal muscles containing both slower, type I and faster, type II muscle fibers.

mRNA measurements by microarray. Total RNA was extracted from the calf muscle complex with TRIzol Reagent (Invitrogen, Carlsbad, CA) and further purified with RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer’s protocols. The concentration and purity of RNA were determined by spectrophotometry at wavelengths of 260 and 280 nm. The integrity of RNA was tested by agarose gel electrophoresis. For the microarray analysis, an equal amount of RNA from each sample was pooled within each group, resulting in six arrays. They were analyzed with an Affymetrix GeneChip MG U74Av2 (Affymetrix, Santa Clara, CA). The microarray analyses were performed by the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. Arrays were scanned using a GeneArray Scanner G2500A (Agilent, Palo Alto, CA). Image analysis and data processing were performed using Microarray Suite 5.0 (Affymetrix) and GeneSpring 6.1 (Silicon Genetics, Redwood City, CA) software as previously described (29). In brief, all chips were scaled (global scaling) to the target intensity of 50, and the data were subjected to robust normalization. MIAME guidelines were followed during array data generation, preprocessing, and analysis. The complete data set is publicly available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession nos. GSE1659).

Tissue processing for protein analysis. The proximal part of the calf muscle complex was pulverized in liquid nitrogen and then homogenized in ice-cold buffer as earlier with small modifications (18, 19): 20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 100 mM b-glycerophosphate, 1 mM Na3VO4, 2 mM DTT, 1% NP-40, 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor cocktail (P 78443; Pierce, Rockford, IL). Homogenates were rotated for 30 min at 4°C and centrifuged at 10,000 \(g\) for 10 min at 4°C to remove cell debris. Total protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology) in triplicates with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

![Fig. 1. A: Body weight. B: mass of calf muscle complex. C: serum glucose. D: serum insulin. A*: filled bar, before; open bar, 1, 3, or 5 wk after STZ or control injection in mice. B, C, and D: filled bar, control mice; open bar, diabetic mice. D1, D3, and D5 are diabetes 1, 3, or 5 wk after STZ, respectively. *P < 0.05 difference from control; #significant decrease from D1 to D3.](http://ajpendo.physiology.org/)
Western immunoblot analyses. Aliquots of muscle lysate were solubilized in Laemmli sample buffer and heated at 95°C to denature proteins. Samples containing 30 μg of total protein were separated by SDS-PAGE for 90 min at 200 V using 4–20% gradient or 10% gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at a 300-mA constant current for 3 h on ice at 4°C. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 2 h and then incubated overnight at 4°C with primary antibodies. Membranes were then washed in TBS-T and incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Jackson ImmunoResearch Europe) for 1 h followed by washing in TBS-T. All the antibodies were diluted in TBS-T containing 2.5% nonfat dry milk or BSA [hypoxia-inducible factor 1α (HIF-1α)]. Proteins were visualized by ECL (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology) and quantified (band intensity × volume) using a ChemiDoc XRS in combination with Quantity One software (v. 4.6.3; Bio-Rad Laboratories). For ubiquitin immunoblots, membranes were blocked overnight and incubated with a horseradish peroxidase-conjugated anti-ubiquitin antibody for 1 h at room temperature. Automatic band detection of the Bio-Rad software was used to analyze intensities of all the visible bands. The sum of all the bands from the range of ~20 to 250 kDa was taken into analysis to avoid the size area where free ubiquitin is (~8 kDa).

Uniformity of protein loading was confirmed by staining the membrane always with Porcine S and by reprobing the membrane with an antibody against GAPDH (Abcam, Cambridge, MA). Our earlier studies and preliminary experiments confirmed a proportional linear relation between protein loaded and the strongest band in Porcine S at ~42 kDa in quantification between 5 and 60 μg of total protein loaded (19). A less well proportional and linear relationship was found between GAPDH, α-actin (1:20,000, Sigma), or staining of myosin heavy chain left in the gel after blotting. Therefore, the results are presented as relative to Porcine S at ~42 kDa. The mean results remained unchanged when presented as unrelative or as relative to GAPDH (not shown).

Antibodies. Antibodies recognized phosphorylated Akt at Ser473, rpS6 at Ser240/244, p38 MAPK at Thr180/Tyr182, ERK1/2 MAPK at Thr202/Tyr204, S6K1 at Thr389, FoxO1 at Thr24, mTOR at Ser2448, rpS6 at Ser240/244, p38 MAPK at Thr180/Tyr182, and eEF2 at Thr56 (Cell Signaling Technology). Myostatin (detecting myostatin at size 42 kDa in quantification between 5 and 60 μg of total protein loaded) (19). A less well proportional and linear relationship was found between myostatin, α-actin (1:20,000, Sigma), or staining of myosin heavy chain left in the gel after blotting. Therefore, the results are presented as relative to myostatin at ~42 kDa. The mean results remained unchanged when presented as unrelative or as relative to myostatin (not shown).

Serum insulin and glucose. Serum glucose was analyzed with a HemoCue B-Glucose analyzer (HemoCue, Angelholm, Sweden). Serum insulin was analyzed with a Merckodia Insulin ELISA Kit according to manufacturer’s protocol (Merckodia, Uppsala, Sweden).

Statistical methods. Differences between groups were evaluated by unpaired t-test or two-way analysis of variance where appropriate. When the Shapiro-Wilk test revealed that Western blot data were not normally distributed, all those values were log-transformed. SSPS v. 13.0 for Windows was used for statistical analyses (SPSS, Chicago, IL). The level of significance was set at P ≤ 0.05. Statistical analysis of the microarray data was performed as previously described (29). In brief, a one-sided Wilcoxon’s signed rank test was used to determine which genes were expressed above the background (P = 0.04) and to determine significant changes in expression between treatment groups (increased at P ≤ 0.0025 and decreased at P ≥ 0.9975) and when the log2 (D vs. C) was over 0.3 or less than −0.3. Calculation of the magnitude of the change in expression was based on differences between corresponding probe pair intensities across the two arrays and one-step Tukey’s biweight estimate statistics. Data are expressed as means ± SD except where designated.

RESULTS

Body weight, muscle mass, and serum glucose and insulin levels. Diabetes led to muscle atrophy. The decrease of calf muscle complex mass was prominent already in D1 (P < 0.05), and the mass decreased slightly further to D3; but at 5-wk muscle it had stabilized to the level of D3 (Fig. 1A). A similar temporal trend was seen in the whole body (Fig. 1B) and the MQF muscle weights (not shown). Serum glucose concentrations were significantly increased in D3 compared with C3 and at 5 wk compared with D1 (P < 0.05) (Fig. 1C).

Table 1. Changes in REDDI/p53/p63 pathway of DNA damage response [7] and in hypoxia markers

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Other DNA damage markers

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Gene expression is expressed in relation to control at the corresponding time point. D1, D3, and D5 are diabetes 1, 3, or 5 wk after STZ, respectively. mRNAs having changes considered significant are shown. Statistically significant changes are expressed by typeface: italic, upregulated; boldface, downregulated. A one-sided Wilcoxon’s signed rank test was used to determine which genes were expressed above the background (P < 0.04) and to determine significant changes in expression between treatment groups (increased at P ≤ 0.0025 and decreased at P ≥ 0.9975) and when the log2 (D vs. C) was over 0.3 or less than −0.3. The level of significance was set at P ≤ 0.05. Statistical analysis of the microarray data was performed as previously described (29). In brief, a one-sided Wilcoxon’s signed rank test was used to determine which genes were expressed above the background (P = 0.04) and to determine significant changes in expression between treatment groups (increased at P ≤ 0.0025 and decreased at P ≥ 0.9975) and when the log2 (D vs. C) was over 0.3 or less than −0.3.
In accordance with the results from mRNA profiling, a search for highly responsive genes that might be associated with muscle atrophy after transcriptional profiling revealed that, in particular, the translational repressor REDD1 mRNA was robustly increased after experimental type 1 diabetes: about seven- to eightfold in D1 and D3, but only twofold in D5 compared with controls (Table 1). Similarly, gene expression of DNA damage markers in the p53/REDD1 pathway (9) showed a robust increase in D1 and D3 tending to almost all decreasing close to control levels in D5 (Table 1). Also, other, less well characterized DNA-damage markers showing a change are listed in Table 1. The expression of hypoxia-responsive genes were either slightly increased or close to the control levels in diabetic muscle (Table 1).

Conversely decreased gene expression of ATP production pathways in D1–D5 (Table 2 and Table 3) was observed. No difference in glucose or insulin among D1, D3, or D5 was observed.

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Protein expression and signaling. REDD1 protein was significantly increased at all time points (D1, D3, D5) in diabetic mice compared with respective controls, but especially in D1 (Fig. 2A), thus following REDD1 mRNA in microarray data. Upstream of REDD1, HIF-1α remained unchanged (Fig. 2B) similarly to the mRNA levels in microarray data (Table 1). Also upstream of REDD1, p36 protein increased at D1 (Fig. 2C) similarly to microarray data (Table 1), and the phosphorylation of AMPK increased at D1–D3 (Fig. 2D). Myostatin protein increased in diabetic muscles at all time points compared with controls (Fig. 2E).

Akt/mTOR signaling. The phosphorylations (p-) of Akt, S6K1, and rpS6 were decreased in D1 and D3 but not in D5 compared with controls (Fig. 3). Total Akt did not change, whereas rpS6 at D3 showed a slight but significant decrease and total S6K1 decreased at D1, D3, and D5. p-mTOR and total mTOR did not change due to D (Fig. 3D). In accordance with its upstream regulator Akt, p-FoxO1 decreased at D1–D3, and, similar to p-rpS6, increased at D5 (Fig. 4A). Dephosphorylation of eEF2 was observed in D1 and D3 (Fig. 4A).

MAPK signaling. p38 was dephosphorylated in D1–D5 compared with controls, revealing significance in D3 and in D5 (Fig. 4B). p-ERK1/2 tended to decrease in D1–D5 (P = 0.06; Fig. 4C). No significant changes in total p38 were observed (Fig. 4B), but total ERK1/2 was higher than control in D1–D5 (Fig. 4C). Therefore, the decrease in p-ERK1/2, when calculated in relation to total ERK1/2, turned out to be significant in D1–D5.
Ubiquitinated proteins and ubiquitin ligases. Total ubiquitination of proteins increased 1.8 ± 0.3-fold in D1 and 1.6 ± 0.2-fold in D3 (P < 0.05), but only a trend existed in D5 (1.3 ± 0.2-fold, P = 0.09). Our microarray data contained only one ubiquitin ligase, Nedd4, and that also increased 1.5-fold in D1–D3 and only 1.2-fold in D5. The result of Nedd4 was verified, and mRNA levels of three other ubiquitin ligases (MAFbx, Mdm2, and MuRF1) were measured with real-time

Fig. 2. Effect of STZ-induced diabetes on protein level of REDD1 and its known/suggested upstream regulators (A), HIF-1α (B), p63 (C), and phosphorylation (p-) of AMPKα at Thr172 (D). E: myostatin. All results are normalized to Ponceau S. Filled bars, control mice; open bar, diabetic mice. Results are means ± SE; n = 5 in each group. *P < 0.05 difference from control at each time point except in the case of p-AMPK, which shows significance at weeks 1–3 grouped in diabetes vs. control. F: representative blots.

Fig. 3. Effect of STZ-induced diabetes on Akt/mTOR signaling. Filled bars, phosphorylated, open bars total protein levels in diabetic mice. Result is shown as normalized to control in each time point: D1/C1, D3/C3, and D5/C5. A: p-Akt at Ser473. B: p-S6K1 at Thr389. C: p-rpS6 at Ser240/244. D: p-mTOR at Ser2448. E and F: representative blots. Results are means ± SE; n = 5 in each group. All results are normalized to Ponceau S. *P < 0.05 difference from control at each time point.
PCR. Diabetes increased the levels of transcripts of all these ubiquitin ligases in D1 and D3 ($P < 0.05$) but only slightly or not at all in D5 (unpublished results), supporting the findings mentioned above.

No change was observed in GAPDH or Ponceau S (see representative blot images in Figs. 2F and 3F, respectively). No change was observed in the control group from C1 to C5 in any proteins.

**DISCUSSION**

The present data show that, in the skeletal muscle of STZ-induced diabetic mice, during rapid muscle atrophy, dramatically increased REDD1 expression coincides with the upregulated expression of DNA damage/repair pathway genes, increased phosphorylation of AMPK, and decreased Akt/mTOR/S6K1 signaling. Furthermore, decreased FoxO phosphorylation and increased protein ubiquitination in muscle were observed in diabetic mice. Practically, all these changes disappeared at 5 wk, even though serum insulin levels and insulin function, as indicated by increased blood glucose, remained low throughout the experiment. Myostatin protein was increased and MAPK signaling decreased in diabetes, but they did not match the time course of muscle atrophy.

The overexpression of REDD1 specifically in muscle has been shown to cause a 10% decrease in muscle fiber size (10) and in cells to decrease mTORC1 signaling (3), an important pathway regulating muscle protein synthesis and myofiber size (2, 23). Moreover, in vitro, REDD1 induction decreases cell size, whereas the opposite is true with REDD1 repression (44). Possible candidates for observed increased REDD1 in STZ-induced diabetes could be, at least, DNA damage (9), energy stress (31), and hypoxia (3, 43). DNA damage was not directly measured, but DNA damage marker p63 protein was increased in early diabetes. This occurred together with increases in, e.g., GADD45, Perp, and p21 mRNAs in the rather well-known pathway shown to mediate DNA damage and increase REDD1 expression, perhaps especially by DNA alkylation (9). Diabetic hyperglycemia can be associated with increased production of reactive oxygen species and that, in turn, with DNA damage (6, 7, 14). Thus, oxidative stress and STZ-induced direct DNA alkylation (9, 45) may together have activated the DNA damage/repair pathways in the present study. The importance of oxidative stress in diabetic muscle atrophy was demonstrated earlier; restoring oxidative imbalance with antioxidant treatment could attenuate most of the muscle loss in STZ-induced diabetes (1).

We also investigated other candidates upstream of REDD1 and mTORC1. AMP-activated protein kinase (AMPK) is a well-known sensor of cellular energy status (16). Increases in the AMP/ATP ratio can lead to activation of AMPK marked by increased phosphorylation at Thr172 (17). In the present study, diabetes induced a dramatic decrease in the expression of genes related to ATP production, which, although not directly measured in the present study, may lead to a decreased amount of muscle ATP (11) and, thus, also an increase in AMP. This microarray observation was indeed associated with increased phosphorylation of AMPK at D1–D3 but not at week 5. Interestingly, in vitro AMPK activation induces REDD1 (44). Activating AMPK in C2C12 myoblast cultures with AICAR was shown to decrease myotube size (52) and has been associated with decreased mTORC1 signaling and protein synthesis (51). However, the importance of this pathway in the muscle loss of diabetic mice is not known, warranting more in vivo evidence. Interestingly, in response to energy stress/energy deficit, a decrease in cell size is less pronounced and the dephosphorylations of S6K1 and rpS6 do not seem to occur when REDD1 is deleted (44). McGhee et al. (31) recently showed that, ~3 days following diabetes-inducing alloxan to rats, REDD1 protein was increased in skeletal muscle when the rats fasted overnight. In the present experiment, mice were not food deprived, but it could be speculated that the decrease in muscle ATP production pathways and increase in phosphorylated AMPK induced by the lack of insulin may have allowed...
the large, possibly mainly DNA damage induced the REDD1 response to occur.

Hypoxia could also be responsible for the observed REDD1 induction, since hypoxia increases REDD1 levels through HIF-1α protein (43). Capillary density in skeletal muscles was significantly decreased in the diabetic animals in our previous study (25). However, the present data do not support the role of hypoxia in REDD1 induction in diabetic muscle, at least not through HIF-1α. We also have to acknowledge the possibility of ER stress in inducing REDD1, as ATF4 and eIF2α (not shown) were slightly (~1.3-fold) elevated in the microarray data 1 wk after STZ. This cascade has also been shown to induce REDD1 (49). Insulin itself can also affect REDD1. Low insulin cannot, however, directly cause the observed REDD1 response, as insulin per se increases REDD1 in skeletal muscle (13). On the other hand, at least in adipocytes, REDD1 protein is decreased with hypophysiological concentrations of insulin (37). Therefore, in STZ-induced experimental diabetes, the possible effects of insulin are probably masked by the stronger effects of the activation of DNA damage/repair pathways and possibly also energy stress or decreased ATP production capacity, which both induce rather than suppress REDD1 in muscle.

rpS6 and S6K1 showed decreased phosphorylation at D1–D3, coinciding with the robust decrease in muscle size. Kimball et al. (24) showed that decreased muscle protein synthesis in alloxan-induced diabetic rats was associated with decreased mTORC1 signaling. In addition to pharmacologically induced diabetes, pancreatectomized rats also show decreased mTORC1 signaling and protein synthesis, as well as muscle and myofiber atrophy (15). These and our results combined indicate that mTORC1 inactivation and, thus, decreased protein synthesis signaling (34) in different diabetic rodents may contribute to loss in muscle mass.

In the present study, FoxO1 and Akt were dephosphorylated, but again only at D1–D3. The Akt phosphorylation site Ser473 investigated in the present study can be considered an important and much used marker of Akt activation, occurring through mTORC2 (42). The phosphorylation of FoxO’s by activated Akt results in their nuclear export (4). In contrast, the observed decrease in p-FoxO, probably through decreased upstream Akt activation in diabetes, would thus increase nuclear localization of FoxO, perhaps increasing the transcription of ubiquitin ligases. Indeed, increased mRNA levels of ubiquitin ligases were observed in D1–D3 and to a smaller extent in D5. Moreover, total protein ubiquitination, estimated by an antibody against ubiquitin, showed an increase at D1 and D3 and only a trend toward an increase at D5. Increased ubiquitinated proteins in our study and the same phenomenon observed in the earlier study indicate increased muscle protein degradation soon after STZ injection (28).

On the basis of our interpretations of the present and other data sets, we have proposed possible signaling mechanisms associated with, and possibly contributing to, muscle atrophy in STZ-induced diabetes in Fig. 5. It is likely that the effects reported are both indirect and direct effects of the lack of insulin. Insulin is an anabolic hormone activating, e.g., mTORC1 signaling (35, 46). Downstream of insulin, p-AS160 at Thr642, a marker of insulin-Akt signaling (36), was decreased ~20–30% in diabetic mice (P < 0.05, not shown) without a similar clear time course as muscle atrophy, REDD1, and mTORC1 signaling. Accordingly, blood insulin also remained low and its function based on high blood glucose during weeks 1–5. Yet, our cross-sectional data suggest that muscle size seemed to decrease mainly during the first 1–3 wk, especially in week 1. These results argue against insulin deprivation directly being the sole factor in the muscle atrophy in STZ-mouse model. Possible changes in several other hormones than insulin, such as growth hormone, insulin-like growth factor I, and cortisol, can also partially contribute to the observed muscle atrophy in STZ-induced diabetes (41, 47).

Myostatin protein was upregulated at all time points in diabetes. This is in accord with a recent study that showed an increase in myostatin protein in murine gastrocnemius muscle 2 wk after STZ injection (50). Myostatin is an important regulator of muscle size. Deletion (32) and muscle-specific downregulation (48) of myostatin increase muscle mass in a dose-dependent manner. On the contrary, selective overexpression of muscle myostatin protein of only ~1.4- to 1.6-fold is sufficient to induce an ~20% decrease in muscle size (38). In theory, the initial 1.5-fold overexpression found at D1 may have contributed in part to the observed 25% muscle atrophy in
muscle mass and cross-sectional area, of which the latter was described previously (significant decrease in muscle fiber cross-sectional area in D, \( P < 0.05 \), D = 2.469 \pm 228 \mu m^2, C = 3.524 \pm 456 \mu m^2) (25).

**Study limitations.** STZ is thought to selectively destroy pancreatic \( \beta \)-cells (5). The atrophic effect of STZ on skeletal muscle seems to be comparable with that of mice with a mutation in the insulin gene (26). In STZ animals, the diabetic phenotype can be mostly prevented with artificial insulin expression in muscle, including decreases in body weight (8, 40) and skeletal muscle mass (30). Moreover, many but not all of the other effects of STZ-induced diabetes are normalized with insulin implant (8). It is, however, acknowledged that, in vitro, STZ also has direct effects on muscle myoblasts (22), a model representing a rather small amount of myogenic cells such as satellite cells in vivo skeletal muscle. Furthermore, one study suggests that decreased fiber size observed 48 h after an STZ dose considered high in rats (120 mg/kg) is not prevented by insulin treatment (22). Our data suggest that some of the detrimental effects of hyperglycemia/hypoinsulinemia together with STZ per se were recovered after \( \sim 2–5 \) wk. Similarly, in rat kidneys, STZ-induced DNA damage seemed to be recovered in \( \sim 3 \) wk (27). The results shown are not explained by the different survival rates of animals in different groups, because only one mouse died in both the 3-wk and 5-wk groups. The relationship between food intake and body weight in STZ-injected rodents seems to be complex (20) and was not measured in the present study. However, it is possible that food intake decreased during the first week after STZ injection when the decrease in muscle mass was accelerated and then later stabilized or increased (20), as was the case with muscle mass and body weight. However, the results of an earlier study suggest that increased protein ubiquitination and protein degradation 3 days after STZ compared with controls were not due to changes in feeding (28). Future studies should investigate mechanistic links between the proteins identified in the current study and muscle atrophy in various diabetic models and also with insulin replacement. Those models, combined with the results of the present study, may provide further understanding of signaling and gene expression changes occurring in diabetics.

The present results provide evidence that diabetic myopathy in an STZ model is associated with the alteration of multiple signaling pathways in which REDD1 may be an integral, previously unrecognized, important factor. mTOR/FoxO and REDD1-associated signaling pathways may provide therapeutic targets to attenuate severe diabetic muscle wasting. Further studies are needed to confirm this suggestion.

**ACKNOWLEDGMENTS**

We especially thank Aila Ollikainen and Simon Walker from the Department of Biological of Physical Activity for help in the laboratory and with the language, respectively.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

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30. Rodgers BD, Strack AM, Dallman MF, Hwa L, Nicoll CS. Corticoste
34. Takasu N, Komiyi A, Asawa T, Nagasawa Y, Yamada T. Strep
36. Unterman TG, Tentel JJ, Oehler DT, Lacson RG, Hogert JF. Effects of glucocorticoids on circulating levels and hepatic expression of insulin-like growth factor (IGF)-binding proteins and IGF-I in the adrenalec