Muscle-specific overexpression of NCOAT<sup>GK</sup>, splice variant of O-GlcNAcase, induces skeletal muscle atrophy

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¹Department of Pharmacology and Toxicology, ²Department of Cell Biology, ³Department of Physiology and Biophysics, ⁴Graduate Biomedical Sciences Program, and ⁵Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Alabama at Birmingham, Birmingham, Alabama

Submitted 7 April 2010; accepted in final form 17 December 2010


Huang P, Ho SR, Wang K, Roessler BC, Zhang F, Hu Y, Bowe DB, Kudlow JE, Paterson AJ. Muscle-specific overexpression of NCOAT<sup>GK</sup>, splice variant of O-GlcNAcase, induces skeletal muscle atrophy. Am J Physiol Cell Physiol 300: C456–C465, 2011. First published December 22, 2010; doi:10.1152/ajpcell.00124.2010.—The protein O-linked β-N-acetylglucosaminyltransferase 1 (O-GlcNAc transferase) is highly expressed in the pancreas (1, 20). The NCOAT gene encodes two splice variants, one with a catalytic domain (O-GlcNAcase) and the other with a catalytic domain and an N-terminal regulatory domain (O-GlcNAcase-inactive splice variant). The overexpression of NCOAT in rats results in the development of severe diabetes (28), and has been linked to the protein posttranslational modification of O-GlcNAc (31). This protein modification (O-GlcNAcylation) involves the addition of O-GlcNAc to Ser and Thr residues and is fueled via the hexosamine biosynthetic pathway (HBP). It has been reported that hyperglycemia and free fatty acids can increase flux through the HBP and induce β-cell apoptosis (13, 39). Additionally, streptozotocin, an inhibitor of O-GlcNAcase and diabeticogenic agent, causes increased O-GlcNAc modification and induces apoptosis in cardiomyocytes (5) and Schwann cells (12). The Bastide group has reported that a number of contractile muscle proteins are O-GlcNAcylated (22) and that variations in O-GlcNAc can regulate muscle protein homeostasis, playing a role in the development of muscular atrophy (9, 10, 24). Our laboratory has shown that O-GlcNAc modification of the proteasome can inhibit function, causing accumulation of short half-life proteins, including some proapoptotic factors (7, 19, 30, 41, 42, 56). Yang et al. (51) have also shown that O-GlcNAcylation of p53 prevents its degradation via the ubiquitin-proteasome system (UPS).

The O-GlcNAc modification is dynamic, and its addition to proteins is catalyzed by O-GlcNAc transferase (OGT) and is removed by O-GlcNAcase (NCOAT) (21, 27, 33). Both proteins are expressed ubiquitously but with varying enzyme activity. Skeletal muscle, brain, and pancreas have higher levels of O-GlcNAcase activity compared with other tissues (14), and OGT has the same tissue distribution; in particular, OGT is highly expressed in the pancreas (1, 20). The NCOAT gene has been linked to type II diabetes in Mexican Pima Indians (15, 29). Furthermore, our laboratory has cloned a splice-variant of the NCOAT gene (NCOAT<sup>GR</sup>) from the brain of the spontaneously diabetic Goto-Kakizaki (GK) rat, lacking exon 8 and disrupting O-GlcNAcase catalytic activity (45). The overexpression of NCOAT<sup>GR</sup> retarded mammary ductal branching (3, 48) and caused premature cataracts in transgenic mice (47), functionally behaving as a dominant-negative NCOAT. However, the effect of NCOAT<sup>GR</sup> on other glucose-sensitive tissues, such as skeletal muscle, has yet to be determined.

In this current study, we report on the effects of overexpressing NCOAT<sup>GR</sup> in the skeletal muscle of transgenic mice utilizing the muscle creatine kinase (MCK) promoter (37). Induced expression resulted in the development of severe muscle atrophy due to myocyte apoptosis, occasionally causing the death of the animal. We demonstrated that proteasome function decreased in the affected cells concomitant with increased cellular O-GlcNAc levels and that some proapoptotic factors, such as p53 and caspase 3, accumulated. Our study provides evidence to link the nutrient status of a muscle cell with the development of muscle atrophy (17, 36), the activation of cell death and muscle loss (12). The Bastide group has reported that a number of contractile muscle proteins are O-GlcNAcylated (22) and that variations in O-GlcNAc can regulate muscle protein homeostasis, playing a role in the development of muscular atrophy (9, 10, 24). Our laboratory has shown that O-GlcNAc modification of the proteasome can inhibit function, causing accumulation of short half-life proteins, including some proapoptotic factors (7, 19, 30, 41, 42, 56). Yang et al. (51) have also shown that O-GlcNAcylation of p53 prevents its degradation via the ubiquitin-proteasome system (UPS).

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with the disruption of homeostasis and atrophy, through the O-GlcNAc modification.

MATERIALS AND METHODS

Mice. To generate the skeletal muscle-specific transgene, the 1.5-kb cDNA fragment containing the MCK promoter was synthesized by PCR using oligonucleotides: 5′-GATGAATTCCTGGCTGGCTGGCTCCTG-3′ and 5′-GACTGAAATTCATGATGGCACCTCCCAGGCGAGAAG-3′, with mouse liver genomic DNA as template, and cloned into BlueScript KS(−) at the EcoRI site. The MCK gene was excised from BlueScript KS(−) with ClaI and ligated into the K14-tetracycline-responsive transcriptional activator (rtTA) transgene (50), replacing the K14 sequence using standard cloning techniques. The 4.1-kb KpnI-KpnI fragment containing the entire transgene cassette was isolated, and the purified DNA was microinjected into one-cell B6xSJL mouse zygotes at a concentration of 2 ng/ml, carried out at the University of Alabama at Birmingham Transgenic Mouse Facility. Founder animals were screened by PCR using oligonucleotides designed to anneal to MCK (5′-CAGTGAGCAAGTCAGCCCTTG-3′) and rtTA (5′-CTTCTTTAGCGACTTGATGCT-3′).

The tetracycline-responsive element (TRE)-enhanced green fluorescent protein (EGFP)-NCOATGK transgene was modified from the TRE-NCOATGK transgene used previously (48) by inserting the EGFP sequence from plasmid pEGFP-C1 (Invitrogen) between the NCOAT untranslated region and NCOATGK. The 5.6-kb ClaI-XhoI fragment containing the entire transgene cassette was purified and microinjected as described above. Founder animals were screened by PCR using oligonucleotides designed to anneal to NCOAT (5′-CCTTGACCTCCCTCTCTCTG-3′) and the SV40 polyadenylation sequence terminating the transgene (5′-GTCCTTGGGGTCTTACCTTTCTC-3′).

MCK-rtTA mice were cross-bred with TRE-EGFP-NCOATGK mice to generate bitransgenic animals, which were genotyped by PCR as above, using tail-snip DNA as previously described (31). Doxycycline (Dox) was administered to mice via a grain-based rodent diet containing Dox (6 g/kg) (catalog no. F4096; Bio-Serv, Frenchtown, NJ) 4 wk after birth. To terminate transgene expression, the Dox diet was replaced with the normal rodent diet. All mice were handled in an accredited university animal resources facility with the approval of the Institutional Animal Care and Use Committee in accordance with institutional animal care policies.

Fig. 1. Schematic of transgenes and characterization of muscle creatine kinase (MCK)-reverse tetracycline-responsive transcriptional activator (rtTA) transgenic mice. A and B: schematics of the transgene constructs for the rtTA driven by the MCK promoter (A) and the tetracycline-responsive element (TRE)-LacZ transgene (B) (18, 25). C: β-Galactosidase activity assays showing β-Gal expression levels of three MCK-rtTA founder transgenic mice and controls on a doxycycline (Dox) diet. RLU, relative light units. D: β-Gal expression in various tissues taken from line 1 transgenic founder, with or without Dox induction. E–G: frozen sections of skeletal muscle from wild-type (WT) mice (control) and bitransgenic (MCK/LacZ) mice with Dox were stained using X-gal (blue), the substrate of β-galactosidase.

O-GlcNAc INDUCES MUSCLE ATROPHY
**β-Galactosidase activity assays and β-Gal staining.** Confirmation of rtTA transgene expression in muscle was accomplished by breeding the MCK-rtTA founder lines with a tetracycline-responsive LacZ transgenic mouse line (tetOLacZ) obtained from Dr. J. Segre (18, 25). Bitransgenic mice were administered Dox-water, drinking water containing 2 mg/ml Dox-HCl (RPI, Mt. Prospect, IL) sweetened with 1 ml Sweet’N Low per 100 ml water, for 3 days. The mice were then killed and the muscle tissues were collected and either homogenized under a fluorescent microscope.

**Northern blot analysis.** Total RNA was prepared from the muscle using the guanidine isothiocyanate extraction method and separated on denaturing formaldehyde gels, as described previously (34). Northern blots were hybridized with 32P-labeled NCOAT cDNA probes (34).

**Immunohistochemistry.** Paraffin sections were prepared from muscle tissue dissected from adult mice, fixed in PBS-buffered formalin, and embedded in paraffin. Sections were cut at 5-μm thickness and stained with hematoxylin and eosin for histopathological evaluation. Immunohistochemistry was conducted following the protocol outlined by Vector Laboratories (Burlingame, CA). Muscle paraffin sections were deparaffinized and subjected to antigen examination. A M.O.M kit (Vector) was employed when using mouse monoclonal antibodies, anti-O-GlcNAc (RL2) (1:250 dilution) and anti-p53 (1:100 dilution; Santa Cruz).

**Western blot analysis.** Muscle samples from bitransgenic mice that had been treated with or without Dox administration, or from other control mice, were homogenized in ice-cold homogenization buffer, as described previously (32). Protein content was determined using the Bio-Rad assay. Aliquots containing equal amounts of protein were prepared and separated on 10% SDS-PAGE. The mouse monoclonal antibodies against RL2, p53 (Santa Cruz), Bax (Santa Cruz), active caspase 3 (Cell Signaling), GFP (Sigma), and β-actin (Sigma), and polyclonal antibodies against caspase 9 and p21WAF1 (NeoMarkers) were used in the analysis.

**Proteasome activity assays (fluorogenic peptide substrate assays).** Proteasome activity assays were conducted following the method described previously (56). Skeletal muscle harvested from the hind legs of Dox-treated transgenic mice was homogenized in proteasome lysis solution (56), and aliquots (20 μl) of these muscle extracts containing 30 μg proteins were incubated at 37°C for 90 min with 2 μl of the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-4-methylcoumary-7-amide (suc-LLVY-AMC) (100 μM). The reaction was stopped by addition of 900 μl SDS (1%). The activity was determined as the increase in fluorescence of the products.

**Fig. 2.** Schematic of the TRE-enhanced green fluorescent protein (EGFP)-NCOATGK transgene and characterization of transgene expression. A: transgene construct consisting of the TRE upstream of the EGFP in frame with the NCOATGK gene. UTR, untranslated region. B and C: tissue sections of skeletal muscle from control or Dox-treated bitransgenic (MCK/EGFP-NCOATGK) mice were prepared in Technovit 8100, and EGFP expression was observed under a fluorescent microscope. D: Northern blot analysis of total cellular RNA (15 μg) from skeletal muscle of bitransgenic mice fed Dox for 12 h, 24 h (1d), and 48 h (2d), hybridized with a 32P-labeled NCOAT fragment, identifying the 3.5-kb transcript representing the chimera GFP-NCOATGK mRNA. E: Western blot analysis of 40 μg protein from homogenized muscle extracts of wild-type mice, bitransgenic mice with Dox (+), or Dox-treated mice followed by withdrawal from Dox (±). The 130-kDa EGFP-NCOATGK protein was detected in muscle of Dox-treated bitransgenic mice, identified using antibody to GFP. F: Western blot analysis of 40 μg tissue lysates from mouse tissues as indicated, probing with antibodies to GFP and actin. C, control; T, transgene.
from hydrolysis of the peptide. The fluorescence was measured using a Barnstead/Turner fluorometer equipped with 360-nm excitation and 450-nm emission filters. As a control, an aliquot of each lysate was also preincubated with the proteasome inhibitor MG132 (10 μM) for 30 min before addition of the proteasome peptide substrate.

**Statistical analysis.** Experiments involving transgenic mice were done in replicate, using three mice for each condition involving Western blot and histochemical analyses, and as many as 30 mice were used in the body and tissue weight measurements. Error bars on graphs correspond to mean values ± SE. Assays for proteasome activity and densitometry measurements of O-GlcNAc levels were analyzed using paired t-tests, while statistical significance on the weight data was determined by a parametric, unpaired t-test, assuming equal variance and normal distribution as determined with GraphPad Prism software.

**RESULTS**

**Characterization of inducible muscle-specific EGFP-NCOATGK bitransgenic mice.** From our previous studies, overexpression of the O-GlcNAcase splice variant NCOATGK caused developmental defects in mammary and lens tissues of transgenic mice (47, 48), along with significant increases in cellular O-GlcNAc levels. The role of O-GlcNAcylation in muscle development (10) and the importance of this tissue in the regulation of glucose metabolism and insulin resistance (4, 11) are well established. The following studies were designed to determine the role of O-GlcNAcase, the enzyme that removes the O-GlcNAc modification, in skeletal muscle. The tetracycline-inducible system was used in which the MCK promoter drove expression of the reverse Tet-activator protein, rtTA, in skeletal muscle (Fig. 1A). Three MCK-rtTA transgenic founder mice were identified by PCR, as described in MATERIALS AND METHODS, and each was bred with the TRE-LacZ transgenic line (Fig. 1B) to determine expression efficiency of the rtTA protein. As determined by the β-galactosidase assay, founder line 1 was clearly the greatest expresser (Fig. 1C) and was the transgenic line used in the subsequent experiments. An examination of other tissues in the Dox-treated bitransgenic mice showed undetectable LacZ expression in uterus and kidney, with barely detectable activity in the heart and skeletal muscle from the untreated animals (Fig. 1D). Immunohistochemistry β-Gal staining of paraffin-embedded muscle sections confirmed LacZ expression in the Dox-treated bitransgenic animals (Fig. 1, E–G). These studies demonstrated the tight transcriptional control of the tetracycline system and the target specificity of the MCK promoter, that virtually all transgene expression occurs solely in skeletal muscle and only when induced by Dox.

![Figure 3](http://ajpcell.physiology.org/)

**Fig. 3.** Phenotype of MCK-EGFP-NCOATGK mice. **A:** male littermates on the Dox diet and a comparison of body size of wild-type control and bitransgenic mice at 6 wk of age. **B:** representative pictures of hind leg of male control and bitransgenic mice. **C:** weights of the hind legs of male mice, 2 wk following Dox treatment. **D:** percent body weight change of 25-wk-old male mice consisting of gene negative controls and bitransgenic littermates with or without Dox. Tg, transgenic; nTg, nontransgenic. **E:** weights of tissues taken from mice in **D** at euthanasia. Statistics were derived from 12 male mice for study in **C** and ~30 male mice for studies in **D** and **E**, using a nonparametric Student’s t-test assuming unequal variance. Standard error bars are shown with the P values where appropriate.
The TRE-EGFP-NCOAT\textsuperscript{GK} transgenic mouse line was generated by a similar method (48), with the addition of an EGFP cDNA sequence as depicted in Fig. 2A. This transgenic line was bred with the MCK-rtTA transgenic mouse to generate bitransgenic litters identified by PCR genotyping, as described in MATERIALS AND METHODS. The EGFP-NCOAT\textsuperscript{GK} transgene was activated by administering a Dox diet for 2 wk, starting 4 wk after birth. Skeletal muscle tissues were dissected from a mixture of wild-type, single transgenic, and bitransgenic mice with or without Dox and embedded in Technovit 8100, and examined under a fluorescent microscope. Expression of EGFP-NCOAT\textsuperscript{GK} fusion protein was clearly apparent in the tissues (Fig. 2B and C). The activation of transgene transcription was confirmed by Northern blotting, and was both time dependent (Fig. 2D), and dose dependent (data not shown) upon Dox treatment (drinking water).

Western blot analysis, using an anti-GFP antibody, determined the expression of the EGFP-NCOAT\textsuperscript{GK} protein in skeletal muscle (Fig. 2F). Only the Dox-treated bitransgenic animals expressed the protein, confirming the tight control of the tetracycline-inducible system functioning at the level of protein expression. Withdrawal of the Dox diet for 2 wk showed a significant reduction in EGFP-NCOAT\textsuperscript{GK} levels, demonstrating the temporal expression of the transgene under the control of Dox induction.

Overexpression of EGFP-NCOAT\textsuperscript{GK} causes skeletal muscle atrophy in mouse. We next examined the effect of overexpressed EGFP-NCOAT\textsuperscript{GK} on the mouse phenotype using \textsuperscript{30} mice, consisting of a mixture of bitransgenic males with or without Dox, and wild-type male mice. All mice appeared normal in terms of body mass and behavior before supplementation of their diet with Dox. Between 1 and 2 wk of Dox treatment, bitransgenic mice experienced progressive weight loss compared with their littermate controls, with significant changes in their posture and gait (Fig. 3A). The loss of body weight generally occurred after 10 days and became highly significant after 14 days (Fig. 3D). This weight loss can be attributed to the dramatic loss of skeletal muscle mass, shown here in the hind legs (Fig. 3B) and also in the front legs of these mice (not shown). Interestingly, our previous studies have shown that the male mice were affected more than female mice, resulting in \textsuperscript{80} male mortality between 2 and 4 wk of Dox treatment (data not shown). The sex-sensitive effect of EGFP-NCOAT\textsuperscript{GK} expression in muscle is not currently known, and further study is needed to investigate this phenomenon. Although the exact cause of death is unclear, examina-

Fig. 4. Histology of bitransgenic muscle. A–E: representative cross sections of muscle tissues from 25-wk-old (A and B) or 6-wk-old (C–E) mice treated with Dox for 2 wk. Following 2 wk of treatment, Dox was replaced with the regular diet for half the mice, which were allowed to recover for 2 more weeks (E). Sections were stained with hematoxylin and eosin. Magnification, ×7.5 or ×100. F: measurement of myofiber area from 6 groups of mice treated as above, counting 400 fibers from each group, principally from gastrocnemius. Areas were calculated using ImageJ software to obtain mean values ± SE. A Student’s \textit{t}-test was used to establish significant differences (*\textit{P} < 0.01).
tion of the principle tissues appeared normal and, excluding the gastrocnemius calf muscle, the transgenic tissue weights did not differ significantly from the control animals (Fig. 3E). This was not surprising since the transgene is not measurably expressed in these tissues (Fig. 2F). Withdrawal of Dox before the affected animals reached the critical end point could prevent the death of male bi-transgenic mice, advocating a regulatory role for O-GlcNAcylation in the development of muscle atrophy.

Histology of skeletal muscle of EGFP-NCOAT<sup>GR</sup>-expressing mice. To further study the effects of NCOAT<sup>GR</sup> expression in muscle, hematoxylin and eosin-stained paraffin sections of the midcalf region, including the medial and lateral gastrocnemius, soleus, and extensor digitorum longus muscles, were examined. The total cross-sectional areas are depicted for a control and transgenic mouse (Fig. 4, A and B), showing the large difference in muscle mass with comparable bone size. Myofiber degeneration and cell loss were accompanied by the disassembly of myofiber structure (Fig. 4D). A necrotic focus invaded by phagocytic cells can be seen in the center of the image. Also apparent was a greater presence of dead myofibers with reduced structure and an increased number of dying myofibers with accumulations of nuclei attempting to reverse the cell death process. The myofiber cross-sectional area of bitransgenic mice after 2 wk of Dox induction was reduced by more than twofold compared with those of control mice (Fig. 4F). To reverse the atrophy of skeletal muscle in bitransgenic mice, we replaced the Dox diet with normal rodent food and analyzed the muscle tissue after 2 wk. Thepause in expression of NCOAT<sup>GR</sup> almost restored the myofibers to their pre-Dox state (Fig. 4E). A reduction in accumulation of nuclei in the center of myofibers indicated the successful rescue from atrophy. These data so far demonstrate that overexpression of EGFP-NCOAT<sup>GR</sup> protein in skeletal muscle alone can cause a dramatic decrease in muscle mass, particularly in male mice, and that this tissue loss appears to be part of the apoptotic process.

EGFP-NCOAT<sup>GR</sup> expression causes O-GlcNAc-dependent inhibition of proteasome activity. It has been reported that O-GlcNAc modification of the Rpt2 subunit of the proteasome complex inhibits ATPase activity and proteasome function in cell culture (56) and in transgenic mice (3, 47). We therefore examined proteasome function in skeletal muscle as a function of protein O-GlcNAc levels. Western blot analysis of muscle tissues extracted from Dox-treated bitransgenic mice showed a general increase in O-GlcNAcylated proteins as determined by immunoblotting with anti-O-GlcNAc antibody (RL2) (Fig. 5, A and B). The increase in O-GlcNAc is likely a result of NCOAT<sup>GR</sup>, which is catalytically inactive, blocking the endogenous O-GlcNAcase from removing the O-GlcNAc modi-

![Image][1]

**Fig. 5. Inhibition of proteasome with excess O-linked β-N-acetylglucosamine (O-GlcNAc) modification.** A: Western blot analysis of protein extracts (40 μg) from muscle of wild-type and bitransgenic mice on Dox, using anti-O-GlcNAc antibody, RL2. The extracts from bitransgenic mice 2 wk following Dox withdrawal are also shown (±). B: densitometry for the O-GlcNAc blot shown in A, showing SE error bars for 3 separate experiments. C and D: muscle paraffin sections of wild-type (C) and bitransgenic mice on Dox (D) were subjected to immunostaining using anti-O-GlcNAc antibody. E: the proteasome LLVY peptidase activity assays performed on muscle extracts, from multiple mice, giving mean values ± SE. A Student’s t-test was used to establish significant differences (*P < 0.01).
fication from cellular proteins (48). Withdrawal of Dox from the bitransgenic mice returned levels of O-GlcNAc to almost that of control mice (Fig. 5B). Immunostaining muscle tissue sections with RL2 showed moderate O-GlcNAc staining in the EGFP-NCOATGK-expressing tissues (Fig. 5, C and D), confirming the results seen in the Western blot.

The proteasome peptidase activity was determined in skeletal muscle protein extracts from control and Dox-treated bitransgenic mice in the chymotrypsin-like LLVY peptide activity assay, as described in MATERIALS AND METHODS (Fig. 5E). Increased O-GlcNAc modification in the EGFP-NCOATGK-expressing mice corresponded to a 30% decrease in proteasome activity, demonstrating that NCOATGK-mediated impairment of proteasome activity occurred either directly through Rpt2 O-GlcNAcylation or indirectly through some intermediate interacting protein. Since reduced proteasome activity is known to increase the levels of proapoptotic factors such as p53 and BAX, an O-GlcNAc-mediated blockade of the UPS provides a link between O-GlcNAcylation and cell death-dependent muscle atrophy.

Accumulation of proapoptotic factors and activation of caspases in skeletal muscle of bitransgens. The abnormal activation and accumulation of proapoptotic factors p53 and BAX have been associated with the inhibition and cell death of myocytes grown in high glucose concentrations (16). Moreover, the UPS is the major mechanism responsible for the degradation and removal of these proapoptotic factors (7, 30). To investigate whether impaired proteasome function, through NCOATGK-mediated hyper-O-GlcNAc modification, leads to the accumulation of proapoptotic factors and the activation of the caspase-apoptotic pathway, we determined the expression of these proteins in skeletal muscle from EGFP-NCOATGK-expressing bitransgenic mice. Paraffin sections of muscle tissue dissected from control and Dox-treated bitransgenic mice were stained for p53 (Fig. 6). Significant p53 accumulation was apparent in the tissues from EGFP-NCOATGK-expressing mice compared with controls (Fig. 6, B and E), and this observation was compatible with the observed decrease in proteasome activity (Fig. 5E). The predominant localization of p53 to the nucleus was characteristic of the onset of apoptosis (Fig. 6F). The withdrawal of Dox from the bitransgenic mice halted expression of the dominant-negative O-GlcNCase, causing a decrease in cell O-GlcNAc and reduction in p53 levels (Fig. 6H). Western blot analysis confirmed the accumulation of p53 protein in skeletal muscle (Fig. 7A), and consistent with the earlier results, withdrawal of Dox caused a reduction in p53 protein. These data so far support our hypothesis that EGFP-NCOATGK overexpression causes p53-dependent cell death, and one possible mechanism may be through the hyper-O-GlcNAc-mediated proteasome blockade.

The proapoptotic factors BAX and p21 are downstream genes of the p53 gene. As seen with p53, both BAX and p21 were undetectable in control animals and, like p53, accumulated in the Dox-treated bitransgens (Fig. 7, B and C). Withdrawal of Dox diminished the levels of these proteins. The accumulation of p53 and BAX proteins stimulates the release of cytochrome c from mitochondria, promoting the formation of the “apoptosome,” which cleaves and activates procaspases. The procaspase form of caspase 9 (p46) was cleaved to the activated form (p34) in Dox-treated bitransgenic mice (Fig. 7D). Consequently, the downstream caspase 3 was also processed to its active form (p19) (Fig. 7E). Neither caspase 9 nor caspase 3 was detectable in muscle from the control animals. In summary, the overexpression of NCOATGK in skeletal muscle tissues resulted in the accumulation of proapoptotic factors that normally are cleared by the UPS, and increased caspase apop-
totic markers. The removal of NCOAT\textsuperscript{GK} expression restores O-GlcNAc and proapoptotic proteins to normal levels, rescuing cells from death.

**DISCUSSION**

The O-GlcNcase gene has been linked to type II diabetes (15, 29). We have cloned a spliced variant of this gene from the diabetic GK rat, abbreviated NCOAT\textsuperscript{GK}, which has all the properties of NCOAT (OGT-binding site and histone acetyltransferase domain) minus the O-GlcNcase activity (3, 45, 48). We also reported that the overexpression of NCOAT\textsuperscript{GK} caused disrupted lens fiber cell differentiation and cataracts in mice (47). In the current study, we explored the effect of NCOAT\textsuperscript{GK} on mouse skeletal muscle to demonstrate that disruption of O-GlcNcase activity induced muscle atrophy in transgenic mice.

Two weeks of overexpression of the EGFP-NCOAT\textsuperscript{GK} chimera protein in bitransgenic mice produced dramatic symptoms, including moderate body weight loss, which was mostly attributed to severe muscle mass reduction, and impaired mobility and gait, with 70–80% morbidity in male mice. We are not sure of the cause of lethality for the bitransgenic male mice since the gross pathology and mass of the organs appeared normal. One explanation could be the lack of food intake caused by an inability to chew due to muscle loss, although the addition of moistened food was only partially helpful. Additional studies are needed to help explain the sexual dimorphism regarding O-GlcNAcylation-induced atrophy. Interestingly, a few male bitransgenic mice survived the challenge of overexpression of EGFP-NCOAT\textsuperscript{GK}, regaining their health with time. The withdrawal of Dox from the bitransgenic mouse diet slows the expression of EGFP-NCOAT\textsuperscript{GK} in muscle, reverses the symptoms of muscle loss, and is able to prevent the lethality experienced in the male bitransgenic mice, evidence that overexpression of NCOAT\textsuperscript{GK} was the major cause of this muscle atrophy. Reducing NCOAT\textsuperscript{GK} expression and restoring the function of wild-type O-GlcNcase likely saves the animal from muscle loss before a critical end point is reached.

The pathology of the affected bitransgenic skeletal muscle may not resemble true cachexia of cancer patients, and the accelerated symptoms seen in the mice may have other contributing factors associated with downregulating the O-GlcNcase enzyme. The abnormal presence of higher-than-normal O-GlcNacylated proteins may itself be toxic, although this is unlikely since increases in glucose and glucosamine above physiological levels lead to increased O-GlcNAc and yet in some systems appear to be protective, as reported by Chatham’s group (6, 57) and others (49, 54). Also, from our previous studies (3, 47), and in this work, the levels of O-GlcNAcylated protein are not extraordinarily elevated in the general cellular proteome. However, there are clearly multiple factors driving myofibers into apoptosis, and one approach in deciphering their action will be a proteomic study of O-GlcNAc-modified proteins.

From the studies of Fiordaliso et al. (16), the abnormal activation and accumulation of proapoptotic factors p53 and BAX were associated with hyperglycemia-induced myocyte death. As we observed in the tetracycline-inducible, MCK-rTA/TRE-EGFP-NCOAT\textsuperscript{GK} bitransgenic mice, overexpression of EGFP-NCOAT\textsuperscript{GK} in skeletal muscle also resulted in the accumulation of p53, BAX, and p21, with the activation of caspase 9, 3, and 21, as shown in Fig. 7.

**Fig. 7. Accumulation of proapoptotic factors and activation of caspases in muscle of bitransgenic mice.** Western blots of protein extracts (40 μg) of wild-type (control) and bitransgenic mice with Dox treatment (+) or 2 wk following Dox withdrawal (±). Blots were probed with antibodies to p53 (A), BAX (B), p21 (C), caspase 9 (D), activated caspase 3 (E), and β-actin (F).
caspase 9 and caspase 3, suggesting that apoptosis may contribute to muscle atrophy through O-GlcNAcylation. Tumor suppressor p53 and its downstream genes, p21 and BAX, are normally degraded by the proteasome complex. Zhang et al. and others showed that increasing cellular O-GlcNAcylation reduced proteasome proteolytic function (47, 55, 56). Indeed, overexpression of NCOATGK in skeletal muscle of the bitransgenic mice also caused an elevation of cellular protein-O-GlcNAc modification as well as impairment of proteasome proteolytic activity. These results support our hypothesis that O-GlcNAcylation-mediated proteasome blockade, caused by the inhibition of O-GlcNACase, induces muscle atrophy in mouse skeletal muscle. Although proteasome inhibition by O-GlcNAc may serve as a major cause of the accumulation of proapoptotic factors and the activation of the caspase cascade, it is also possible that NCOATGK and O-GlcNAc have a pleiotropic effect on muscle cells, such as regulating transcription (52, 53), affecting protein-protein interaction between p53 and its negative regulator mdm2 (26), the direct regulation of p53 itself (51), and contractile protein function (8–10, 22, 24, 26). Therefore, O-GlcNAc overexpression of NCOATGK in skeletal muscle of the bitransgenic mouse tissues.

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ACKNOWLEDGMENTS

We thank Dr. Trenton R. Schoeb for assistance with the pathological analysis of the transgenic mouse tissues.

GRANTS

This work was supported by funding from National Institutes of Health Grants CA095021 and DK043652.

DISCLOSURES

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


