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The gene expression and activity of calpains and the muscle wasting-associated ubiquitin ligases, atrogin-1 and MuRF1, are not altered in patients with primary hyperparathyroidism

AMY EVENSON, JAMIE MITCHELL, WEI WEI, VITALIY POYLIN, SAREH PARANGI and PER-OLOF HASSELGREN

Section of Endocrine Surgery, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

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Abstract. Hyperparathyroidism (HPT) can be associated with muscle atrophy and weakness. Muscle atrophy is typically caused by increased muscle protein breakdown. The influence of HPT on calpains and the ubiquitin-proteasome pathway, which are important regulators of muscle proteolysis, is not yet known. We examined the expression in skeletal muscle of μ- and m-calpain and the ubiquitin ligases, atrogin-1 and MuRF1, in patients with primary HPT. A biopsy was obtained from the sternohyoid muscle in patients undergoing surgery for primary HPT (n=8) and in normocalcemic control patients undergoing thyroid surgery (n=11). mRNA levels for atrogin-1, MuRF1 and the calcium-regulated proteases, μ- and m-calpain, were determined by real-time PCR. Calpain activity was measured using the calpain-specific substrate, BODIPY-FL-casein, and by zymography. Serum calcium was 11.4±0.46 and 9.5±0.10 mg/dl in HPT and control patients, respectively ($p<0.01$). The corresponding phosphate levels were 2.7±0.2 and 3.6±0.1 mg/dl ($p<0.05$). Parathyroid hormone serum concentration was 286±103 pg/ml (range, 77-946 pg/ml) in patients with HPT and was not measured in control patients. There were no significant differences in mRNA levels for atrogin-1, MuRF1, μ- or m-calpain and in calpain activity between HPT and control patients. The results suggest that the ubiquitin-proteasome and calpain systems are not activated in skeletal muscle in patients with primary HPT, at least not in patients with moderate hypercalcemia.

Introduction

Both primary and secondary hyperparathyroidism (HPT) may be associated with muscle wasting and weakness (1-8). The mechanisms of muscle dysfunction in patients with HPT are not well understood, but impaired energy and protein metabolism with loss of muscle proteins has been implicated as a potential cause (9-11). Several of the hormonal and metabolic abnormalities seen in patients with HPT, including increased parathyroid hormone (PTH) and calcium levels and reduced phosphate levels, could potentially influence muscle protein balance.

Previous reports on the regulation of muscle protein turnover in HPT have been conflicting. Whereas experimental studies in animals provided evidence that PTH may impair energy and protein balance in skeletal muscle (9-11), and calcium may stimulate muscle protein breakdown (12-15), we found previously that protein synthesis and breakdown rates were similar in muscle tissue from patients with HPT and normocalcemic control patients (16). Because, in that study, we measured protein turnover rates in incubated muscle biopsy specimens *in vitro*, it is possible that the potential effects of HPT on muscle protein metabolism were not well reflected by the results. Additional studies, therefore, are needed to test whether patients with HPT have evidence of muscle wasting.

In the present study, we determined the gene expression of the ubiquitin ligases, atrogin-1 and MuRF1, in muscle tissue from patients with primary HPT and normocalcemic control patients. Atrogin-1 and MuRF1 are muscle-specific ubiquitin ligases, which have been shown recently to be substantially upregulated in multiple conditions characterized by muscle wasting (17-20), and their activities are probably rate limiting for ubiquitin-proteasome-dependent muscle proteolysis in those conditions. In fact, changes in mRNA levels for atrogin-1 and MuRF1 have been proposed to be sensitive and specific 'molecular markers' of muscle wasting. In addition, we determined the expression and activity of μ- and m-calpain in muscle from the two groups of patients. This was important because calcium is the most important regulator of calpain activity (21) and the influence of hypercalcemia caused by HPT on muscle calpain expression and activity is not yet known.

Patients and methods

Patients undergoing surgery for primary HPT in the section of endocrine surgery of the department of surgery at the Beth Israel Deaconess Medical Center, Boston, MA, were

Correspondence to: Dr Per-Olof Hasselgren, Department of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, USA
E-mail: phasselg@bidmc.harvard.edu

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included in the study. The diagnosis was based on elevated calcium and parathyroid hormone (PTH) levels in serum. Normocalcemic patients undergoing thyroid surgery served as control. The initial parts of the surgical procedures for HPT and thyroid disease were identical. With patients under general endotracheal anesthesia, a transverse neck incision measuring approximately 4 cm was performed. After development of upper and lower skin flaps, the neck was opened between the strap muscles. A biopsy specimen measuring approximately 1x0.5x0.5 cm was obtained from the medial edge of one of the sternohyoid muscles using atraumatic technique and avoiding cautery. The specimen was immediately removed from the operative field, frozen in liquid nitrogen, and brought to the laboratory for processing. After the muscle biopsy specimen had been removed, small bleeding vessels in the muscle were carefully controlled with ligatures and cautery, whereafter the operation continued in a routine fashion. The muscle biopsy procedure prolonged the operation by no more than 5-10 min. There were no complications from the muscle biopsy procedure in any of the patients.

Serum levels of calcium, phosphate, and PTH were determined as part of the patients' routine care and were measured in the clinical laboratories of the Beth Israel Deaconess Medical Center.

A written consent form was obtained from all patients. The study was approved by the Committee on Clinical Investigations at the Beth Israel Deaconess Medical Center.

Measurement of mRNA levels. mRNA levels for μ- and m-calpain and for atrogin-1 and MuRF1 were determined by real-time PCR as described previously (22). Total RNA was extracted by the acid-guanidinium thiocyanate-phenol-chloroform method using Tri reagent (MRC, Cincinnati, OH). The RNA was treated with DNase and real-time PCR was performed for quantification of human μ- and m-calpain and atrogin-1 and MuRF1 mRNA expression, with amplification of 18S RNA as endogenous control. TaqMan analysis and subsequent calculations were performed with an ABI PRISM 7700 sequence detection system (Perkin-Elmer, Foster City, CA). Analyses were performed in triplicate and the results were normalized to 18S mRNA and expressed as arbitrary units.

Measurement of muscle calpain activity. Muscle calpain activity was determined using two different methods. First, calpain activity was determined by measuring the degradation of the fluorogenic calpain-specific substrate 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-proprionic acid labeled casein (BODIPY-FL) casein as described previously (22,23). Using this technique, the overall net calpain activity was measured in muscle extracts and results were expressed as fluorogenic units (FU). The method does not differentiate between μ- and m-calpain activity and because the endogenous calpain inhibitor, calpastatin, is present in the tissue extract, any changes in calpain activity observed in this assay may reflect changes in calpain activity itself or changes in calpastatin activity. The second method used to measure calpain activity was zymography as described in detail previously (22,24,25). With this technique, the calpains were separated by electrophoresis and the activity of μ- and m-calpain was measured individually and independent of calpastatin by determining the degradation of casein in the gel used for separation of the enzymes.

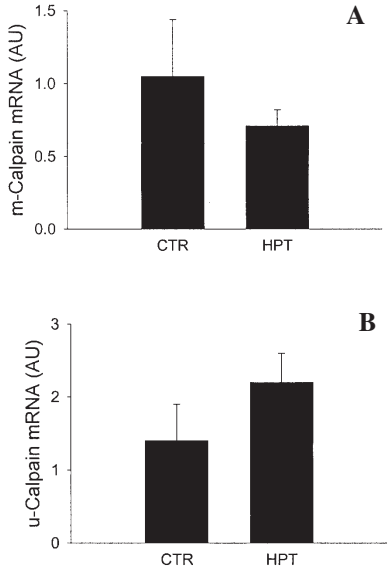

Figure 1. mRNA levels for m-calpain (A) and (B) μ-calpain in muscle from normocalcemic control patients (CTR) and patients with primary HPT. Results were normalized to 18S mRNA levels and expressed as arbitrary units (AU). Results are means ± SEM with n=11 (control) and n=8 (HPT).

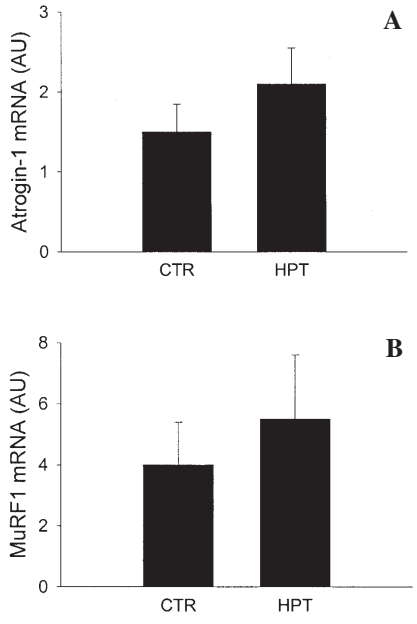

Figure 2. mRNA levels for (A) atrogin-1 and (B) MuRF1 in muscle from normocalcemic control patients (CTR) and patients with primary HPT. Results are means ± SEM with n=11 (control) and n=8 (HPT).

Statistics. Results are presented as means ± SEM. Student's t-test was used for statistical analysis.

Results

Eight consecutive patients with primary HPT and 11 consecutive normocalcemic control patients were included in the

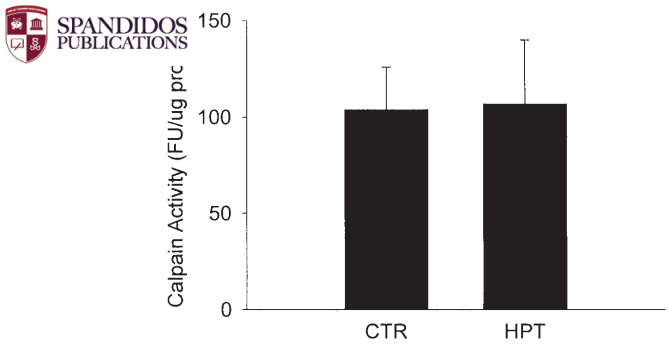

Figure 3. Calpain activity in muscle tissue from normocalcemic control patients (CTR, n=11) and patients with HPT (n=8). Calpain activity was determined by measuring the degradation of the fluorogenic calpain-specific substrate BODIPY-FL casein and results were expressed as fluorogenic units (FU)/μg protein.

study. Age and sex distribution in the two groups of patients as well as serum calcium, phosphate, and parathyroid hormone (PTH) levels are shown in Table I. Patients with HPT were older than the control patients and there were more male patients in the HPT group than in the control group. The uneven distribution of age and gender between the two groups of patients reflects the fact that consecutive patients were included in the study. Six of the patients with HPT had a parathyroid adenoma and 2 patients had multiglandular hyperplasia. Among the control patients, 3 had a multinodular goiter, 4 had a follicular adenoma, 2 had papillary cancer, and 2 had Graves' disease that had been treated with antithyroid drugs and μ-blockers before surgery and who were biochemically and clinically euthyroid at the time of surgery.

Two of the patients with primary HPT were symptomatic: 1 patient had polyuria and polydypsia and 1 patient presented with hypercalcemic crisis (serum calcium of 14.8 mg/dl and affected mental status). Among the 6 patients with asymptomatic HPT, the indication for parathyroidectomy was increased 24 h urinary calcium excretion in 1 patient, osteoporosis in 1 patient, and significant hypercalcemia (>11.5 mg/dl) and/or patient's desire in the remaining 4 patients. None of the patients with HPT reported muscle weakness or other muscle symptoms.

There were no differences in mRNA levels for μ-calpain, m-calpain, atrogin-1, or MuRF1 in muscles from HPT and control patients (Figs. 1 and 2). There was a significant positive correlation between serum calcium levels and muscle MuRF1 mRNA concentrations among patients with HPT (r^2=0.919, p<0.001). There were no statistically significant correlations between serum calcium and mRNA levels for μ- and m-calpain or atrogin-1. Calpain activity, determined by the BODIPY-FL assay (22,23), was almost identical in the two groups of patients (Fig. 3). Zymography also did not reveal any differences in calpain activity between the two groups of patients (not shown).

Discussion

In the present study, we examined whether the expression of certain genes that are commonly upregulated in muscle wasting conditions is increased in muscle from patients with HPT. The rationale for the study were the facts that calcium is an important regulator of muscle wasting and that patients with HPT may have evidence of muscle atrophy and weakness. The present results suggest, however, that the expression and activity of calpains, as well as the ubiquitin-ligases, atrogin-1 and MuRF1, are not altered in the skeletal muscle of patients with HPT.

Table I. Age and sex distribution, PTH, calcium and phosphate levels in normocalcemic control patients and in patients with primary HPT.

	Control (n=11)	HPT (n=8)
Age (years)	35±3 (27-63)	49±3[a] (33-65)
Male:Female	1:10	4:4
PTH (pg/ml)	N.D.	286±103 (77-946)
Calcium (mg/dl)	9.5±0.1	11.4±0.5[a] (10.4-14.8)
Phosphate (mg/dl)	3.6±0.1	2.7±0.2[a] (2.3-3.2)

Results are means ±SEM. Figures in parentheses are ranges. N.D., not determined. [a]p<0.05 vs control.

Primary HPT is associated with increased serum levels of PTH and calcium and reduced phosphate levels. Previous studies suggest that each of these changes may cause negative protein and energy balance in skeletal muscle and result in muscle wasting. For example, Garber (9) reported that treatment of incubated rat epitrochlearis muscles with intact bovine parathyroid hormone or a synthetic 1-34 fragment of the hormone stimulated protein breakdown and inhibited protein synthesis in a concentration-dependent manner. Interestingly, when muscles from uremic rats were examined, basal protein breakdown rates were higher than in muscles from rats with normal kidney function but protein breakdown rates were not further increased by PTH, suggesting that these muscles had become resistant to the hormone. In other experiments, treatment of rats *in vivo* with PTH resulted in increased calcium uptake and reduced energy levels in skeletal muscle (10,11), similar to the situation in muscle-wasting conditions (26-28). Because, in one study (10), the effects of PTH on muscle energy metabolism were prevented by verapamil, the authors suggested that the effects of PTH were mediated by the increased calcium uptake in skeletal muscle.

A number of previous reports support a role of increased calcium concentrations in the regulation of muscle proteolysis. For example, when incubated rat muscles were treated with calcium or the calcium ionophore, A23187, protein breakdown rates were increased (13,14,26). In other studies, muscle calcium levels were elevated in conditions characterized by muscle wasting, and blocking the increase in calcium levels prevented the increase in muscle protein breakdown (27,28). Calcium is the most important regulator of calpain activity (21)

and in recent experiments, we found evidence that calcium also regulates muscle proteasome activity (29). Interestingly, in the present study, there was a significant positive correlation between serum calcium and muscle MuRF1 mRNA levels. More studies are needed to test whether calcium actually regulates the expression of MuRF1.

In addition to elevated PTH and calcium levels, there is evidence that reduced phosphate concentrations may also regulate muscle protein and energy metabolism. For example, Brautbar *et al* (30) reported that phosphate depletion resulted in impaired energy metabolism in skeletal muscle and other studies provided evidence that muscle phosphate levels were reduced during PTH administration in rats (31).

Although previous studies suggest that elevated PTH and calcium levels and reduced phosphate concentrations may impair muscle protein and energy metabolism, those studies were performed in rat muscles. To our knowledge, only one previous study examined protein balance in muscle from human patients with HPT (16) and, in that study, we found that muscle protein synthesis and breakdown rates were not different in patients with HPT and normocalcemic control patients. However, because protein turnover rates were measured in incubated muscle specimens *in vitro*, it may be argued that changes in muscle protein balance may not have been retained *in vitro* and that the results may not have accurately reflected the situation in patients with HPT. In the present study, we expanded our previous observations by determining the expression of atrogin-1 and MuRF1 in muscle from patients with HPT and control patients. Changes in mRNA levels for these ubiquitin ligases are sensitive and specific 'molecular markers' of muscle wasting (17-19), and unchanged mRNA levels for atrogin-1 and MuRF1, as found here, argue against the possibility that the patients had a significant catabolic response in muscle. Because increased calcium levels regulate calpain activity (21), we also determined mRNA levels for μ- and m-calpain and determined their enzymatic activities in muscles from control and HPT patients. Both gene expression and the activity of μ- and m-calpain were unaltered in patients with HPT, lending further support to the conclusion that there was no evidence of HPT-induced muscle wasting among our patients. Thus, the results support our previous report of unaltered protein turnover rates in muscle from patients with HPT (16).

Although we interpret our present and previous (16) results as indicating that patients with primary HPT do not have evidence of muscle wasting, this interpretation needs to be made with caution because there are several other potential explanations for why no differences between the two groups of patients were observed. First, age and sex distribution were not equal between the control and HPT patients, mainly reflecting the fact that consecutive HPT and control patients rather than age- and sex-matched patients, were enrolled in the study. Second, none of the patients reported muscle weakness or other muscle symptoms, although that does not rule out the possibility that some patients had muscle weakness since patients not infrequently report improvement after parathyroidectomy, even when symptoms were not obvious before surgery (32,33). Third, biopsies were obtained from the sternohyoid muscles in the present study because this muscle is easily accessible during the surgical procedure and obtaining the biopsy did not significantly prolong the procedure or impose any significant risk to the patient. Because muscle weakness in patients with HPT most commonly affects the lower extremities (1), it is possible that muscle wasting-associated genes are upregulated in lower extremity muscles, even in the absence of changes in the sternohyoid muscle. Fourth, despite elevated serum calcium levels, it is not known if calcium concentrations were increased in muscle tissue in our patients as has been reported in certain muscle wasting conditions (27,28). Fifth, with the exception of one patient who presented with hypercalcemic crisis, the degree of hypercalcemia was moderate in our patients, and it is possible that more pronounced hypercalcemia would give rise to increased expression in muscle of calpains and ubiquitin ligases. Sixth, unchanged mRNA levels for the ubiquitin ligases do not rule out the possibility that the protein expression and activity of the enzymes were altered. Finally, unchanged expression of the ubiquitin ligases and calpains does not rule out changes of other proteolytic systems. Thus, although the ubiquitin-proteasome and calpain systems are particularly important in muscle wasting, other mechanisms, including increased activity of some of the lysosomal enzymes, may also be important in the development of muscle wasting (15).

Many patients diagnosed with HPT are asymptomatic, reflecting the fact that the diagnosis is frequently made after increased calcium levels have been detected on a chemistry panel. Among symptomatic patients, kidney stones, osteoporosis, peptic ulcer disease and vague mental symptoms, such as fatigue and poor memory, are the most common clinical manifestations, but the disease may be associated with muscle weakness as well (1-4). Indeed, a relatively large number of studies over the last 10-20 years have confirmed muscle weakness as being a common symptom (up to 30-35%) in patients with primary HPT (1-4,34-36). In a recent study of patients with symptomatic HPT, respiratory muscle dysfunction was prevalent (8). In several of those reports, objective evidence (measurement of muscle force or respiratory function) of improved muscle strength after parathyroidectomy was provided even in patients who had not experienced subjective symptoms of muscle weakness. This is important because the lack of subjective symptoms of muscle weakness among the patients with HPT in the present study does not rule out the possibility that at least some of the patients had impaired muscle strength. Regardless of whether this was the case or not, the present results, with observations in a previous study from our laboratory (16), collectively suggest that patients with primary HPT do not have evidence at the molecular level of muscle wasting.

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