Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle

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Mascher H, Tannerstedt J, Brink-Elfegoun T, Ekbloom B, Gustafsson T, Blomstrand E. Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle. Am J Physiol Endocrinol Metab 294: E43–E51, 2008. First published October 30, 2007; doi:10.1152/ajpendo.00504.2007.—The gain in muscle mass as a result of resistance training is dependent on changes in both anabolic and catabolic reactions. A frequency of two to three exercise sessions per week is considered optimal for muscle gain in untrained individuals. Our hypothesis was that a second exercise session would enlarge the anabolic response and/or decrease the catabolic response. Eight male subjects performed resistance exercise on two occasions separated by 2 days. Muscle biopsies were taken from the vastus lateralis before and 15 min, 1 h, and 2 h after exercise. Exercise led to severalfold increases in phosphorylation of mTOR at Ser2448, p70 S6 kinase (p70S6k) at Ser424/Thr421 and Thr389, and ribosomal protein S6, which persisted for up to 2 h of recovery on both occasions. There was a tendency toward a larger effect of the second exercise on p70S6k and S6, but the difference did not reach statistical significance. The mRNA expression of MuRF-1, which increased after exercise, was 30% lower after the second exercise session than after the first one. MAFbx expression was not altered after exercise but downregulated 30% 48 h later, whereas myostatin expression was reduced by 45% after the first exercise and remained low until after the second exercise session. The results indicate that 1) changes in expression of genes involved in protein degradation are attenuated as a response to repetitive resistance training with minor additional increases in enzymes regulating protein synthesis and 2) the two ubiquitin ligases, MuRF-1 and MAFbx, are differently affected by the exercise as well as by repeated exercise.

atrogen-1; protein translation; p70 S6 kinase

REGULAR RESISTANCE EXERCISE is known to increase muscle mass and strength. The increase in muscle mass is influenced by several factors, including type of training, workload, and frequency of training. A single bout of resistance exercise can increase the rate of protein synthesis up to 48 h after the exercise; however, the rate of protein breakdown is also increased, but to a lesser degree, leading to an improved net protein balance (2, 32). The increased rate of protein synthesis is likely to be mediated through activation of a network of signaling pathways involving protein kinase B (PKB/Akt), the mammalian target of rapamycin (mTOR), and p70 S6 kinase (p70S6k) (12, 31). Activation of p70S6k increases the phosphorylation of the ribosomal protein S6 and thereby enhances the translation of 5′TOP mRNAs, i.e., mRNAs, which encode for ribosomal proteins and elongation factors (22). However, varying results have been reported concerning the effect of resistance exercise on this signaling pathway in human subjects. For example, in a previous study (23), resistance exercise led to a partial phosphorylation of p70S6k but no activation of the enzyme, as evaluated from the lack of effect on S6 phosphorylation. The conclusion drawn was that one session of resistance exercise was not sufficient to fully phosphorylate and activate p70S6k. In contrast, after high-intensity resistance exercise, the Thr389 phosphorylation of p70S6k increased, which should activate the enzyme, in parallel with the muscle protein synthetic rate (12). Furthermore, results from experimental animals (17) indicate that repeated isometric contractions can amplify the effect on p70S6k.

A training-induced increase in muscle mass is a result of an increase in the rate of protein synthesis, a decrease in the rate of degradation, or both. Protein degradation involves the ubiquitin-proteasome pathway (27) and is considered to be regulated by the muscle-specific ubiquitin ligases, muscle atrophy F-box (MAFbx) and muscle-specific RING finger-1 (MuRF-1) (5, 16). The link between the anabolic and catabolic reactions is the activation of the Akt-signaling pathway. Activation of this pathway downregulates the expression of the transcription factors MAFbx and MuRF-1 via inhibition of the Foxo family of transcription factors (15, 37). However, varying results concerning the effect of resistance exercise on MAFbx and MuRF-1 expression in human muscle have been reported. A decrease in the mRNA content of both MAFbx and MuRF-1 was found 3 h after resistance exercise (7), suggesting that protein breakdown was reduced, whereas an increase in the mRNA content of MuRF-1 was found in both type I and type II fibers 4–24 h after resistance exercise, suggesting an increased protein breakdown (43).

Myostatin, a member of the transforming growth factor-β superfamily, has recently been identified as an inhibitor of skeletal muscle mass. Inactivating mutations of the myostatin gene in cattle and mice are associated with generalized increase in skeletal muscle. Similarly, disruption of myostatin gene expression is associated with dramatic increases in skeletal muscle mass due to muscle fiber hyperplasia and/or hypertrophy (30, 39). The biological effects of myostatin on muscle mass were initially attributable mainly to inhibition of myoblast proliferation (38), although McFarlane et al. (29) re-
cently reported that myostatin positively influences expression of the ubiquitin-proteasome components and suggested that the myostatin-induced decrease in muscle mass also involves activation of protein degradation within the skeletal muscle fibers.

Resistance exercise training two to three times per week is recommended to induce an increase in muscle mass in relatively untrained individuals (3, 19, 26, 35). Our hypothesis was that a second exercise session within the present time frame should enlarge the effect on enzymes involved in translation initiation and elongation and thus activate these enzymes and/or decrease the catabolic reactions. To test this hypothesis, the subjects performed two sessions of leg press exercise with the same relative workload and duration as in a previous study (23), and the effects on selected markers for anabolic and catabolic pathways were evaluated. The two sessions were separated in time by 2 days, representing resistance exercise training two to three times per week.

MATERIALS AND METHODS

Subjects. Eight healthy male subjects participated in the study after being fully informed orally and in writing of its purpose and the possible risks involved before giving their informed consent. The subjects did not perform endurance or resistance exercise on a regular basis. The mean age (±SD) was 23 (±1) yr, height 181 (±1) cm, weight 75 (±4) kg, body mass index 22.9 (±1.1) kg/m², and maximal oxygen uptake (VO₂ max) 3.91 (±0.16) l/min. The investigation was performed according to the principles outlined in the Declaration of Helsinki. The Ethics Committee of the Karolinska Institute approved the study protocol.

Preparatory tests. Prior to the experiment, the subjects participated in two preparatory tests. The first test was designed to determine their one-repetition maximum (1RM). During the 1RM test, a leg press was performed at a 90–180° knee angle. The load was progressively increased until the subject could not perform more than one single repetition. The subjects reached 1RM within five to six trials. Subjects performed 10 repetitions at 80% of 1RM. The second preparatory exercise test was performed to familiarize the subjects with the intensity and the repetition frequency of the test exercise. During the second preparatory exercise test, the subjects performed the exercise routine scheduled to be performed during the study protocol.

VO₂ max was determined by use of an on-line system (Amis 2001 Automated Metabolic Cart; Innovision, Odense, Denmark) during running on a treadmill. The speed and incline of the treadmill was gradually increased until exhaustion (1).

Diet and exercise control. The subjects were asked to refrain from vigorous physical activity for 2 days before the experiment and during the 2 days between the two resistance exercise sessions. They were also asked to follow a controlled diet 2 days before the first exercise session and during the 2 days between the two exercise sessions. The diet scheme was prepared by a nutritionist and consisted of 57 energy points for 30 min, a catheter was inserted into the antecubital vein, and a resting blood sample was taken. After local anesthesia had been administered to the skin, subcutaneous tissue, and fascia, a resting muscle biopsy was taken from the lateral part of the quadriceps muscle (vastus lateralis) using a Weil-Blakesley conchothome (AB Wisex, Möln达尔, Sweden) as described by Henriksson (20).

The subjects exercised at 100 W for 10 min on a cycle ergometer as a warmup bout. Thereafter, they were seated in the leg press machine and performed resistance exercise at a workload corresponding to ~80% of 1RM. The protocol consisted of four sets of 10 repetitions, with a 5-min rest period between sets. The resistance exercise protocol lasted for a total of 20 min. The same training protocol was used in a previous study (23).

Blood samples were taken before exercise, after the second set during exercise, immediately after exercise, and at 15, 30, 60, 90, and 120 min in the recovery period. After exercise the subjects rested in a supine position, and 15 min, 1 h, and 2 h after exercise, muscle biopsy samples were taken from the vastus lateralis. The first biopsy sample was taken 12–15 cm above the midpatella and the following samples 2–4 cm proximal to the previous biopsy point. All four biopsies on 1 day were taken from the same leg, and each biopsy sample was taken from a separate incision. Four of the subjects had biopsies taken from the right leg on the first exercise session and from the left leg on the second exercise session and vice versa.

After the first resistance session, the subjects had lunch, continued to follow the standardized diet, refrained from vigorous physical activity, and reported again to the laboratory in the morning 2 days later in a fasted state. The same protocol as during the first exercise session was followed during the second one.

All biopsy samples were immediately (within 10 s) frozen in liquid nitrogen and stored at −80°C for later analysis. The choice of time points for biopsy sampling was based on previous data (10, 12, 14, 23) showing early changes in signaling proteins after a single resistance exercise session in human subjects. The mRNA quantification was performed on biopsy samples taken before and 2 h after exercise on the basis of the observation that the mRNA content of myogenic genes did not increase until 2 h after resistance exercise (42).

Western immunoblot analyses. Samples containing 30 µg total protein were separated by SDS-PAGE for 60 (Akt), 90 [GSK-3 and eukaryotic elongation factor 2 (eEF2)], or 120 min (mTOR, p70S6K, and S6) at 200 V using 4–20% gels (Akt, mTOR, p70S6K, and S6), 7.5% (eEF2), or 10% gels (GSK-3) on a Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). All eight samples from one subject were run on the same gel. After electrophoresis, gels were incubated in transfer buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 mM Na₃VO₄, 2 mM DTT, 1% Triton X-100, 20 µg/ml leupeptin, 50 µg/ml aprotonin, 40 µg/ml PMSF) at a dilution of 80 µl/mg of dry-weight muscle. Homogenates were rotated for 60 min at 4°C, centrifuged at 10,000 g for 10 min at 4°C, and stored at −80°C. Protein was determined in aliquots of the supernatant diluted 1:10 in distilled water using a bichinchoninic acid protein assay ( Pierce Bio-technology, Rockford, IL).

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was obtained by subtracting 18S rRNA or GAPDH CT values from their respective primary antibody solutions for 1 h, followed by washing in TBS-T (Akt and p70S6k on Thr389) or TBS-T containing 2.5% nonfat dry milk and incubated with their appropriate secondary antibody diluted in their respective target CT values. The expression of each target was then evaluated by 2−ΔCT (40).

Statistics. Data are presented as means ± SE. Differences between the first and second exercise session for plasma concentrations of glucose and lactate during exercise and recovery were evaluated by comparing the corresponding areas under the time/concentration curves. The concentrations at two consecutive time points were averaged, multiplied by the time span, and summed for the whole exercise or recovery period. The comparison between the areas was then made using Student’s t-test for paired observations. A one-way ANOVA was employed to evaluate changes in mRNA expression over time. A two-factorial (time, exercise session) repeated-measures ANOVA was employed to compare changes in kinase phosphorylation over time between the two exercise sessions. When a significant overall effect was indicated, a Fisher least significant difference post hoc test was performed. Significance was accepted at P < 0.05.

RESULTS

The mean value of the subjects’ maximal strength, 1RM, was 255 ± 20 kg, and the average workload during leg press was 198 ± 16 kg, corresponding to 78 ± 1.7% of 1RM. Five subjects performed the stipulated 4 × 10 repetitions at both exercise sessions. Two subjects performed one to two repetitions less on the second exercise session, and one was unable to perform four sets during his first exercise session; he performed 28 repetitions (2 × 10 followed by 8 repetitions) on the first occasion and the same in the second exercise session.

Plasma concentrations of glucose and lactate. The plasma concentration of glucose did not change throughout the experiment in any of the exercise sessions (data not shown). The plasma concentration of lactate increased significantly during the leg press exercise in both the first and second exercise sessions. The level immediately after exercise was 9.60 ± 1.55 mmol/l, and the second exercise session was 8.69 ± 1.05 mmol/l, corresponding to a significant increase in mTOR phosphorylation at Ser2448 at 15 min, 1 h, and 2 h after exercise on both occasions but only at Ser2481 after the second exercise session (Fig. 3, A and B). However, the ANOVA did not reveal a significant difference
between the first and second exercise sessions. The phosphorylation of p70S6k at Thr421/Ser424 and Thr389 was increased six- to ninefold 15 min after exercise and remained elevated 1 and 2 h into recovery after both exercise sessions (Fig. 3, C and D). No significant difference between the sessions was detected; however, for the Thr389 phosphorylation, six of eight subjects showed a greater phosphorylation 15 min and 1 h after the second resistance session compared with the first session. Phosphorylation of the ribosomal protein S6 was elevated 12- to 20-fold from 15 min to 2 h after the first exercise, statistically significant at 1 h after exercise, and 30- to 40-fold from 15 min to 2 h recovery after the second exercise (Fig. 4A). There was a tendency for greater phosphorylation after the second exercise session ($P < 0.1$). The phosphorylation of eEF2 was reduced by 50–70% 15 min after exercise and remained decreased 1 and 2 h after exercise in both exercise sessions (Fig. 4B).

**mRNA content.** Analyses of mRNA expression were performed on the biopsy samples taken before and 2 h after exercise on both occasions. The mRNA expression of MAFbx (atrogin-1) was reduced by 32% on the morning before the second exercise session and by 40% after the second exercise session compared with before the first exercise session, but no significant change was detected during the exercises (Fig. 5, A)

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**Fig. 2.** Phosphorylation of Akt (A), GSK-3α (B), and GSK-3β (C) in skeletal muscle before and 15 min, 1 h, and 2 h after resistance exercise performed twice and separated in time by 48 h. Representative immunoblots are shown above each graph. Values in the graph are arbitrary units (means ± SE for 8 subjects).
The mRNA expression of MuRF-1 was increased 2 h after both exercise sessions, twofold after the first exercise session and 1.6-fold after the second exercise session. The mRNA expression was 40% higher after the first than after the second exercise session (Fig. 5, C and D). The mRNA expression of myostatin was reduced by 45% 2 h after the first exercise session, and the level remained reduced 48 h later. There was no further decrease 2 h after the second exercise session (Fig. 5 E and F).

DISCUSSION
Resistance training is known to increase both anabolic and catabolic processes, presumably to ensure both quantitative as
well as qualitative changes in the remodeling of skeletal muscle. In the present study we aimed to evaluate whether two training sessions separated by 48 h affected selected pathways in these two counteracting processes differently. The hypothesis of the present study was that a second exercise session following a 48-h recovery period should induce a larger effect on enzymes involved in protein synthesis and/or decrease the catabolic reactions evaluated as changes in mRNA expression of the muscle-specific ubiquitin ligases MAFbx and MuRF-1.

The results indicate that repeated exercise sessions attenuate the increase in gene expression of MuRF-1 and further reduce the expression of MAFbx but have only a minor additional effect on enzymes regulating protein synthesis compared with a single session.

The resistance exercise led to increases in mTOR (Ser2448), p70S6k (Thr389), and S6 phosphorylation 15 min after exercise, which persisted for 2 h into recovery after both the first and the second exercise sessions, with no concurrent increase in Akt phosphorylation. Akt-independent phosphorylation of signaling molecules in the mTOR pathway following resistance exercise has been found previously (9, 14) and might suggest the possibility of another pathway involved in the activation of these molecules in human skeletal muscle. The findings indicate that translation initiation is stimulated after exercise similarly on both occasions, which is further supported by the decrease in eEF2 phosphorylation in the recovery period after exercise. A decrease in eEF2 phosphorylation is considered to activate the enzyme and, hence, also the elongation phase of translation (34, 36). This notion is supported by the recent results reported by Dreyer et al. (12) showing a decrease in eEF2 phosphorylation in the 2-h recovery after resistance exercise that occurred in parallel with an increase in the rate of muscle protein synthesis. There was not the expected enlarged effect after the second exercise session, although there was a tendency to a larger effect on p70S6k and S6, but the difference did not reach statistical significance. There was no remaining effect of the first exercise session on the phosphorylation status of the kinases measured on the morning 48 h later. This may not be expected; however, one study has demonstrated a sustained effect on Akt and p70S6k phosphorylation using a different experimental setup. Cuthbertson et al. (11) reported a 2.5- and 3.5-fold elevated p70S6k and Akt phosphorylation, respectively, 24 h after either dynamic concentric or dynamic eccentric exercise. In the latter study, the subjects were given a mixture of carbohydrates and amino acids immediately after and repeatedly throughout the 24-h recovery period, which may have prolonged the exercise effect. In the present study, the subjects had a meal 2.5 h after exercise and followed a standardized dietary scheme until the evening before the second exercise session.

In a previous study (4, 23), the same type of resistance exercise as in the present one had no effect on mTOR, p70S6k (Thr389), or S6 phosphorylation, suggesting that the amount of training was insufficient to activate translation initiation. There is no obvious explanation for the divergent results. The subject groups in the two studies were similar regarding physical and physiological parameters, except for the maximal muscle strength (1RM), which was 20% lower in the present study, 3.37 compared with 4.22 kg/kg body wt in the study by Karlsson et al. (23). This difference in maximal strength may suggest that the subjects in the present study were less trained in resistance exercise. Coffey et al. (9) have shown a different response to resistance exercise in endurance- and strength-trained subjects; an increase in Thr389 phosphorylation of p70S6k was found only in endurance-trained subjects 3 h after exercise. Although the subjects in the present study were not endurance trained, they did not perform resistance exercise regularly, which may explain, at least in part, why they respond more strongly to a single session of resistance exercise.
There was a marked increase in plasma lactate concentration during exercise, which indicates that the muscle fibers are greatly activated and that there is a considerable contribution from anaerobic energy processes (Fig. 1). The same high levels of plasma lactate were found during leg extension exercise, 10 repetitions at 70% of 1RM (12), and these authors discuss a possible inhibitory effect of acidosis on the rate of protein synthesis; however, a possible influence on the rate of protein synthesis would be the same in the two exercise sessions.

We also hypothesized that a decrease in activation of catabolic reactions would occur following the second resistance training session. In support of this, we found that the mRNA content of MuRF-1, which increased after exercise, was lower after the second than after the first exercise session. Furthermore, the MAFbx expression was reduced by 32% 48 h after the first exercise session and by 40% 2 h later after the second exercise session in relation to the content before the first exercise session. A decrease in mRNA expression of MAFbx has been reported (25) after dynamic lengthening exercise; a 60% decrease was noted already 3 h after exercise, and the effect persisted for up to 24 h after exercise, whereas no significant effect was found after concentric exercise. In another study (8), a tendency to a decrease in mRNA expression

![Fig. 5. Changes in mRNA expression of muscle atrophy F-box (MAFbx; A and B), muscle-specific RING finger-1 (MuRF-1; C and D), and myostatin (E and F) normalized to ribosomal GAPDH or 18S in skeletal muscle before and 2 h after resistance exercise performed twice and separated in time by 48 h. Values in the graph are arbitrary units (means ± SE for 8 subjects). *P < 0.05 and #P < 0.01 vs. before exercise, and +P < 0.05 for comparison, 1st session vs. 2nd session of exercise.](image-url)
of MAFbx was found 3 h after resistance exercise in endurance-trained subjects, but no change was found in strength-trained subjects. In the current study, there was a small and statistically insignificant decrease 2 h after resistance exercise and a 32% reduction of MAFbx expression 2 days after the first exercise session, which is in line with previous data.

Consistent with our hypothesis, the MuRF-1 response to exercise was reduced following the second exercise session. Yet an increase was observed, in contrast to MAFbx expression, where a slight decrease was seen following the resistance exercise sessions. Importantly, no systematic differences due to a general change in muscle mRNA content in response to resistance training could explain this finding. Such effects would have influenced both factors in a similar fashion, and furthermore, no differences in the expression pattern were found with the two housekeeping genes used. Also, the increase in mRNA content of MuRF-1 noted in the present study agrees with the results of Yang et al. (43), who also studied relatively untrained subjects. They found a 1.4-fold increase in MuRF-1 expression in both type I and type II fibers 4 and 24 h after leg extension exercise, with $3 \times 10$ repetitions at 65% of 1RM. In contrast, in a study on highly trained subjects who had participated in regular resistance training for many years, a decrease in MuRF-1 expression was noted 3 h after leg press exercise, with $8 \times 5$ repetitions at approximately 85% of 1RM (7). Those authors suggested that the long-term resistance exercise training may have attenuated protein breakdown and enhanced net protein balance, in line with previous measurements of protein turnover in trained and untrained individuals (32). An interesting observation is that 18-wk training carried out by hemodialysis patients, particularly endurance training, reduced the amount of 14-kDa actin fragment in muscle, which was correlated to the rate of protein degradation (41).

Nonetheless, the present findings indicate that 1) changes in expression of genes involved in protein degradation seem to be attenuated as a response to repetitive resistance training sessions and 2) the two markers for protein degradation are differently affected by the exercise as well as by repeated exercise. The observation that the two ubiquitin ligases, MAFbx and MuRF-1, behave differently is novel and indicates a divergent regulation of the two and, furthermore, that they may have different functions in protein degradation induced by resistance exercise. These factors are assumed to act in a coordinated fashion in response to stimuli that influence the muscle protein balance, and therefore, it is possible only to speculate about the mechanisms behind the changes in expression of MAFbx and MuRF-1. The Akt-Foxo signaling pathway has been considered to be the main regulating pathway for both factors (5, 13, 18, 37). Although the mRNA expression of MAFbx was downregulated and that of MuRF-1 was lower 2 h after the second exercise, no significant increase in Akt phosphorylation was observed at the selected time point. However, there was a tendency for elevated Akt phosphorylation (Fig. 2A) and possibly activation 1 and 2 h after the second session, which could account, at least in part, for the changes in mRNA expression of the ubiquitin ligases. In contrast to Akt, the phosphorylation of mTOR and p70S6k was significantly elevated after exercise (Figs. 2 and 3), suggesting that they were activated, but Sandri et al. (37) concluded that the mTOR/p70S6k pathway is not important in the regulation of MAFbx in atrophic cells. On the basis of our results, it is tempting to suggest that signaling pathways other than the Akt-pathway may play a physiologically significant role in the regulation of these factors in response to resistance training. Examples of possible alternative pathways involved could be the p38 MAP kinase and the NF-κB pathways (6, 28).

Myostatin expression was markedly reduced after the first exercise session (Fig. 5), which is similar to results in a recent study on women (33) but is in contrast to results from others (7, 21) who found no change in myostatin expression in the early recovery period. Twenty-four hours after resistance exercise, mRNA expression of myostatin was reduced by 44% in both male and female subjects (24). A similar reduction was noted 48 h after leg press exercise in old males who had completed a 21-wk resistance training program, although no change in myostatin expression was noted in untrained individuals (21). Interestingly, myostatin was recently (29) reported to inhibit Akt phosphorylation in C2C12 cultured cells, suggesting that reduced levels of myostatin may stimulate the Akt pathway. It is tempting to speculate that the low myostatin expression observed at the start of the second exercise (Fig. 5) may promote an activation of the Akt pathway, although an alternative biological effect of reduced myostatin levels is a release of its inhibition of myoblast proliferation (30, 38).

In summary, the results of the present study indicate that 1) repeated exercise sessions separated in time by 48 h have only a minor additional effect on signaling proteins regulating protein synthesis compared with a single session, 2) repeated exercise sessions attenuate the changes in mRNA expression of genes involved in muscle protein degradation as shown by reduced expression of MURF-1 and MAFbx, 3) the two markers for protein degradation are differently affected by repeated resistance exercise bouts, 4) the expression of myostatin is downregulated after the first exercise session and remains reduced after the second one, and 5) signaling pathways other than Akt may play a physiologically significant role in the regulation of these factors in response to resistance training.

NOTE ADDED IN PROOF

During the review process of this manuscript, data have been published supporting our finding that the two ubiquitin ligases MuRF-1 and MAFbx are differently affected by acute exercise. (Louis E, Raue E, Yang Y, Jemiolo B, Trappe S. Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. J Appl Physiol 103: 1744–1751, 2007).

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