Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability

M. H. Stanley Chan, Andrew L. Carey, Matthew J. Watt, and Mark A. Febbraio

Skeletal Muscle Research Laboratory, School of Medical Sciences, RMIT University, Bundoora, Victoria 3083, Australia

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Chan, M. H. Stanley, Andrew L. Carey, Matthew J. Watt, and Mark A. Febbraio. Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability. Am J Physiol Regul Integr Comp Physiol 287: R322–R327, 2004.—To determine the expression and induction of cytokines in human skeletal muscle during concentric contractions, eight males performed 60 min of bicycle exercise, with either a normal (Con) or reduced (Lo Gly) preexercise intramuscular glycogen content. Muscle biopsy samples were obtained before and after exercise and analyzed for glycogen and the mRNA expression of 13 cytokines. Resting muscle glycogen was higher (P < 0.05) in Con compared with Lo Gly and was reduced (P < 0.05) to 102 ± 32 vs. 17 ± 5 mmol U glycosyl/kg dry mass for Con and Lo Gly, respectively. We detected mRNA levels in human skeletal muscle for five cytokines, namely interleukin (IL)-1β, IL-6, IL-8, IL-15, and tumor necrosis factor-α. However, muscle contraction increased (P < 0.05) the mRNA expression of IL-6 and IL-8 alone. In addition, the fold change for both IL-8 and IL-6 was markedly higher (P < 0.05) in Lo Gly compared with Con. Given these results, we analyzed venous blood samples, obtained before and during exercise, for IL-6 and IL-8. Plasma IL-6 was not different at rest, and although the circulating concentration of this cytokine increased (P < 0.05) it increased to a greater extent (P < 0.05) throughout exercise in Lo Gly. In contrast, plasma IL-8 was not affected by exercise or treatment. These data demonstrate that cytokines are not ubiquitously expressed in skeletal muscle and that only IL-6 and IL-8 mRNA are increased during contraction of this mode and duration. Furthermore, the mRNA abundance of IL-6 and IL-8 appears to be influenced by glycogen availability in the contracting muscle.

Interleukins; muscle contraction; real-time polymerase chain reaction

Cytokines are pleiotropic proteins produced by virtually every nucleated cell in the body (28). However, in most tissues, including skeletal muscle, the constitutive expression of these cytokines is either minimal or absent (13). Rather, the expression of cytokines is induced by a myriad of physiological, pathological, or chemical stimuli. Recently, Frost et al. (13) demonstrated that LPS induced the gene expression of a number of cytokines, namely interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-12, IL-1α, IL-1Ra, and TNF-β in mouse skeletal muscle. In addition, it was also demonstrated that 3 h of running exercise, which would result in some tissue damage, induced the mRNA expression of IL-1β, IL-6, IL-8, IL-10, and TNF-α in human skeletal muscle (20). These results were not surprising, because LPS is a potent stimulus for cytokine production in a variety of cell types including blood mononuclear cells, whereas tissue damage will result in neutrophil and macrophage infiltration into skeletal muscle (19), which are known to produce a variety cytokines (1). Work from our group has focused on the effect of “non-damaging” muscle contraction on the induction of cytokines within skeletal muscle. It has been demonstrated that muscle contraction, in the absence of markers of muscle damage, rapidly increases IL-6 mRNA expression in skeletal muscle biopsy samples (23, 25–27), whereas the intramuscular nuclear transcriptional activity of IL-6 is rapidly increased with the onset of such exercise (17). Of note, however, is the observation that unlike LPS, this type of muscle contraction does not increase IL-6 mRNA (30) or protein (22, 24) expression in blood mononuclear cells. This has lead us to hypothesize that the contraction-induced IL-6 expression in skeletal muscle is not a consequence of trauma or damage, but rather is a specific biochemical phenomenon to allow the muscle to release this cytokine to mobilize substrate from fuel depots within the body to facilitate energy metabolism (9). To this end, IL-6 protein is released from skeletal muscle during prolonged knee extensor or bicycle exercise (25–27) and its release is further elevated when intramuscular glycogen stores are low (25) or attenuated when glucose availability is increased (11). In addition, it appears that IL-6 acts in a “hormonelike” manner to increase lipolysis and fat oxidation (31).

In contrast to the observations of contraction-induced skeletal muscle release of IL-6 and the data obtained after 3 h of treadmill running (20), concentric muscle contractions do not increase TNF-α mRNA expression in contracting skeletal muscle (26). In addition, chronic muscular activity downregulates TNF-α gene expression in the skeletal muscles of elderly humans (14). The failure for such muscular contraction to induce TNF-α gene expression in skeletal muscle is not surprising given the marked increase in glucose uptake during exercise and the well-described negative effect of TNF-α on glucose disposal (for review, see Ref. 9). Whether muscle contractions per se increase the expression of other cytokines within skeletal muscle or whether carbohydrate availability mediates such expression is not known and this was the aim of the present study. We chose to study humans during short-term bicycle exercise to minimize any chance of muscle damage inducing a local inflammatory response. We hypothesized that such exercise would not result in the ubiquitous induction of cytokine mRNA expression. Rather, we hypothesized that IL-6 would be the only cytokine markedly increasing its mRNA expression in response to contraction or glycogen availability because of its specific “endocrine-like” role that has been identified.
METHODS

Subjects and Experimental Protocol

Eight active, but not specifically trained, males (24 ± 2 yr, 77 ± 3 kg, and 181 ± 2 cm) participated in the study, which was approved by the Royal Melbourne Institute of Technology Human Ethics Committee. Written informed consent was obtained from all human subjects. Each visited the laboratory and performed an incremental exercise test on a cycle ergometer (Lode, Excalibur, Groningen, The Netherlands) until they reached volitional exhaustion. Peak pulmonary oxygen uptake (V\textsubscript{O}\textsubscript{2peak}) averaged 49.0 ± 3.2 mL·kg\textsuperscript{-1}·min\textsuperscript{-1}. Expired pulmonary gases (Quark b 2, COSMED, Rome, Italy) were collected and analyzed online to compute V\textsubscript{O}\textsubscript{2peak}.

The subjects subsequently visited the laboratory for two experimental trials separated by at least 1 wk and conducted in random order. During these trials they attended the laboratory between 1600 and 1700 to perform glycogen-depleting exercise and returned the following morning at ~0800 to perform the experimental trial. The glycogen-depleting exercise involved two exercise bouts consisting of continuous cycling at 70% V\textsubscript{O}\textsubscript{2peak} for 20 min followed by 20 min intermittent exercise. The intermittent exercise consisted of 2 min cycling at 90% V\textsubscript{O}\textsubscript{2peak} followed by 2 min at 50% V\textsubscript{O}\textsubscript{2peak}. Subjects then performed ~20 min arm-cramping exercise (~50 W) to deplete the upper arms of glycogen. After resting for 5 min, the aforementioned cycling protocol was repeated until subjects reached volitional exhaustion. Five all-out sprints were then performed for 30 s with a 2-min recovery period. The duration of the glycogen-depleting exercise was ~2.5 h (average of 92 ± 3 min cycling exercise). Water intake was permitted throughout all trials, and no adverse events were reported by subjects. Subjects were provided with an overnight food parcel. On one occasion, the 6.2 MJ diet consisted of 79% carbohydrate, 3% fat, and 18% protein (denoted herein as Con); whereas, on the other visit, the 6.4 MJ diet consisted of 4% carbohydrate, 59% fat, and 37% protein (denoted herein as Lo Gly). Subjects were instructed to consume all food before 2200 and were permitted to consume water thereafter.

Subjects arrived at the laboratory the following morning after an overnight fast. They voided, lay supine on a bed, and a Teflon catheter was inserted into an antecubital vein for blood sampling. One leg was prepared for subsequent needle biopsy by making two incisions through the skin and fascia of the vastus lateralis under local anesthesia. Immediately before exercise, a muscle sample was obtained by needle biopsy. The leg was bandaged and the subject moved to the cycle ergometer and commenced cycling at ~70% V\textsubscript{O}\textsubscript{2peak}. Venous blood samples were obtained at 20-min intervals during exercise. In addition, expired pulmonary gases (Quark b 2, COSMED) were collected and analyzed online at 20-min intervals during exercise. A second muscle sample was obtained from the second incision immediately after the cessation of exercise (at 60 min) with the subject on the cycle ergometer. Muscle samples were obtained from the contralateral leg in the subsequent trial.

Muscle Tissue Analyses

Muscle glycogen. A portion of muscle (~10 mg) was freeze-dried, disected free of any blood and connective tissue, powdered, and analyzed for glycogen as previously described (11).

Measurement of mRNA expression. A portion of muscle (~30 mg) was extracted for total RNA using a modification of the acid guanidium thiocyanate-phenol chloroform extraction method of Chomczynski and Sacchi (6) described elsewhere (8). The total RNA was quantified two to three times before 1 ng of each total RNA sample was reverse transcribed in a 10 µl reaction containing 1X TaqMan RT buffer, 5.5 mM MnCl\textsubscript{2}, 500 mM each 2’-deoxynucleoside 5’-triphosphate, 2.5 mM random hexamers, 0.4 U/µl RNase inhibitor, 1.25 U/µl Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) and made up to volume with H\textsubscript{2}O (0.05% DEPC treated).

For basal expression, we were able to detect the expression of interleukin (IL)-β, IL-6, IL-8, IL-15, and tumor necrosis factor (TNF)α. Of these only IL-6 and IL-8 mRNA increased with contraction, and the increase was exacerbated in the presence of low intramuscular glycogen in both of these cytokines.

Control samples were also analyzed where all the above reagents are added to RNA samples except the Multiscribe Reverse Transcriptase. The reverse transcription reactions were performed using a GeneAmp PCR system 2400 (Perkin, Elmer, Wellesley, MA) with conditions at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. EDTA (2 ml 0.5 M; pH 8.0) was added to each sample and stored at ~20°C until further analysis.

Real-time PCR was employed to quantify human IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, the p35 and p40 subunits of IL-12 (IL-12p35, IL-12 p40), IL-15, interferon (IFN)-γ, and TNF-α. All cytokines except IL-6 were analyzed using the Tagman Human Cytokine Gene Expression Plate (Applied Biosystems, Foster City, CA). Human probe and primers for IL-6 were designed (Primer Express version 1.0 Applied Biosystems) from the human gene sequence accessed from GenBank/EMBL as previously described (23). For all genes, a TaqMan probe was labeled with the fluorescent tags FAM (6-carboxyfluorescein) at the 5′ end and TAMRA (6-carboxy-tetramethylrhodamine) at the 3′ end and ribosomal 18S mRNA was also amplified as our reference gene. We quantitated gene expression using a multiplex comparative critical threshold (C\textsubscript{T}) method (Bio-Rad i Cycler IQ, Hercules, CA).

PCR reactions were carried out in duplicate in 50 µl reactions of TaqMan universal PCR master mix (1X), 50 nM TaqMan 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, and probes and primers at specific concentrations. Twenty nanograms of cdNA was amplified using the following conditions: 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Of note, in previous experiments where we have followed these procedures, we compared our gene expression data obtained from real-time PCR methodology with that obtained using a Northern blot and have demonstrated comparable results (25). For each sample, 18S CT values were subtracted from the gene of interest CT values to derive a ΔCT value. The resting value for each subject during each trial was then subtracted from the exercise samples for each subject to derive a ΔΔCT value. The expression of the genes of interest relative to the resting samples was then evaluated using the expression 2\textsuperscript{−ΔΔCT}. The coefficient of variation, determined on the CT value of the 18S samples, was ~1%.

Blood Analyses

Blood for plasma glucose analysis was collected into a tube containing fluoride EDTA, mixed, and spun in a centrifuge at 2200 g for 8 min at 0°C. The plasma was later analyzed for glucose using an

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<th>Cytokine</th>
<th>Basal Expression</th>
<th>Concentric Contraction Inducible?</th>
<th>Influenced by Glycogen Content?</th>
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automated glucose/lactate analyzer (YSI 2300 STAT PLUS, Yellow Springs, OH). Blood samples for plasma IL-6 and IL-8 concentration were drawn into glass tubes containing EDTA. The tubes were spun immediately at 2,200 g for 15 min at 4°C and the plasma was stored at −80°C until analyses were performed. ELISA kits (R&D systems, Minneapolis, MN), were used to quantify plasma IL-6 and IL-8, which were quantified using chemiluminescence. Plasma creatine kinase (CK) was determined by automated enzyme reactions using the International Federation of Clinical Chemistry recommended method (automated analyses for AU5000, Olympus, Tokyo, Japan).

**Histological Determination of Muscle Fiber Cross Sections**

In the present study we did not freeze clamp muscle in isopentane for histological determination. To provide further evidence that bicycle exercise does not induce muscle damage, we collected muscle samples prepared for histochemical determination from a separate cohort of subjects who exercised for a similar duration and intensity on a bicycle ergometer. It is standard practice in our laboratory to perform the glycogen depletion protocol before experimentation to minimize any differences in preexercise glycogen content between subjects. Consequently, the subjects in this cohort also performed the same glycogen depletion protocol before being provided with the identical diet that was given to subjects in Con. Muscle samples obtained before and after exercise were rapidly frozen in isopentane, subsequently sectioned frozen in a cryostat (10-μm sections) and stained with hematoxylin and eosin.

**Statistical Analysis**

Analysis of the IL-6 and IL-8 mRNA measures revealed that the data were not normally distributed. To ensure homogeneity of the

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**Fig. 1.** Contraction-induced increase in interleukin (IL)-6 (top) and IL-8 (bottom) mRNA during exercise with normal (Con) or low (Lo Gly) preexercise intramuscular glycogen content. Values are expressed as a fold change from rest with rest equal to 1 arbitrary unit (means ± SE). *Difference (P < 0.05) compared with Con; n = 8.

**Fig. 2.** Muscle glycogen (top) and plasma glucose (bottom) concentrations during exercise with normal (●) or low (■) preexercise intramuscular glycogen. *Difference (P < 0.05) compared with Con. Values are expressed as mean ± SE; n = 8.

**Fig. 3.** Plasma IL-6 (top) and IL-8 (bottom) concentrations during exercise with normal (●) or low (■) preexercise intramuscular glycogen content. *Difference (P < 0.05) compared with Con. Values are expressed as mean ± SE; n = 8.
data, data were log transformed before statistical analysis. All other parameters were normally distributed. A two-way (trial × time) ANOVA with repeated measures on the time factor was used to compute the statistics (Statistica, Tulsa, OK), with significance accepted with a P value of <0.05. If analyses revealed a significant interaction, a Newman Keuls post hoc test was used to locate specific differences. Data are presented as means ± SE.

RESULTS

Workload and Pulmonary Measures

Subjects cycled at an average of 174 ± 12 W during the experimental trials. Although average \( V_{\text{O2}} \) did not differ when comparing trials (34.7 ± 2.2 vs. 34.2 ± 2.2 ml·kg\(^{-1}\)·min\(^{-1}\) for Con and Lo Gly, respectively), respiratory exchange ratio was higher (\( P < 0.05 \)) in Con (0.90 ± 0.01) compared with Lo Gly (0.81 ± 0.01).

mRNA Abundance of Cytokines

The pattern of cytokine gene expression is presented in Table 1. Of the 13 cytokines analyzed, 5 were detected in the muscle biopsy samples. These were IL-1β, IL-6, IL-8, IL-15, and TNF-α. Only IL-6 and IL-8 were induced (\( P < 0.05 \)) by contraction. In addition, the contraction-induced expression of these cytokines was augmented (\( P < 0.05 \)) during Lo Gly (Fig. 1).

Muscle Glycogen and Plasma Glucose

Resting muscle glycogen was higher (\( P < 0.05 \)) in Con compared with Lo Gly (375 ± 35 vs. 163 ± 27 mmol glycosyl units/kg dry mass for Con and Lo Gly, respectively) and was reduced (\( P < 0.05 \)) to 102 ± 32 vs. 17 ± 5 mmol glycosyl units/kg dry mass for Con and Lo Gly, respectively (Fig. 2). Plasma glucose was not different when comparing trials at rest, and whereas plasma glucose concentration was maintained in Con, it fell (\( P < 0.05 \)) in Lo Gly such that the values at 40 and 60 min were lower (\( P < 0.05 \)) in Lo Gly compared with Con (Fig. 2).

Plasma Cytokines and CK

Plasma IL-6 was not different at rest when comparing trials. Plasma IL-6 increased (\( P < 0.05 \)) after 40 min in both trials, but the increase was greater at 40 and 60 min in Lo Gly such that the concentrations at these times were higher (\( P < 0.05 \)) compared with Con (Fig. 3). In contrast, IL-8 was not different at rest or at any point during exercise when comparing Con with Lo Gly, and exercise per se did not increase plasma IL-8 in either trial (Fig. 3). Plasma CK was not different at rest when comparing trials and was not increased when comparing values at 60 min with those at rest (Table 2).

Histological Determination of Muscle Fiber Cross Sections

We were unable to detect any differences in the general histological architecture of the muscle cross sections before and after exercise. Pre- and postexercise cross sections from a single subject are displayed in Fig. 4.

DISCUSSION

The results from this study demonstrate that cytokines are not ubiquitously expressed in skeletal muscle and the mRNA abundance of IL-6 and IL-8 alone appear to be increased by the type of exercise used in this study. Because the mode of exercise used in the present study results in an absence of observable markers of muscle tissue damage or inflammation and because the contraction-induced increase in IL-8 and IL-6 was much higher when glycogen availability was reduced, our data suggest that the mRNA abundance for these two cytokines is influenced by metabolic processes and unlikely to be related to inflammation secondary to muscle damage.

In the present study we only measured the mRNA abundance and not the rate of gene transcription. Therefore, our measures may reflect an increase in gene transcription and/or increases in mRNA stability or, indeed, a combination of both. Although we have no evidence regarding the contraction-induced rate of transcription for IL-8, Keller et al. (17) demonstrated that the rate of IL-6 gene transcription closely matches the mRNA abundance during exercise with high and low preexercise glycogen levels. In this respect we are confident, at least for our IL-6 measures, that our results reflect the rate of gene transcription. It is well known that skeletal muscle not only contains myocytes, but also smooth muscle cells, fibroblasts, endothelial cells, and macrophages, and these cells...
are also known to produce IL-6 (5, 7, 18, 21). Because we only obtained muscle biopsy samples, it was possible that the elevations seen in IL-6 and IL-8 mRNA expression may have been due to increased mRNA expression in cells other than skeletal myocytes. We have not examined mRNA localization of IL-8 in muscle cells; however, using in situ hybridization, we recently demonstrated that the mRNA abundance of IL-6 increases within the skeletal muscle cells after contraction, and the quantification of mRNA abundance using this method matches the rate seen using RT-PCR in muscle biopsy homogenates (15).

The results from the present study differ somewhat with those that have stimulated muscle with LPS (13) or muscle contraction with a large eccentric component (20). In particular, whereas these two stimuli resulted in a marked increase in the mRNA abundance of TNF-α and IL-1β, we saw no such effect, although both TNF-α and IL-1β were expressed in our tissue samples. Our results are consistent with previous observations in humans during knee extensor exercise (26), which has also been shown to result in minimal muscle damage (27).

It is well known that TNF-α is expressed in response to damage and inflammation (3). In addition, Fielding et al. (12) demonstrated that ultrastructural damage to skeletal muscle is associated with neutrophil infiltration and IL-1β accumulation. In the previous study reported by Nieman et al. (20), the exercise-induced increase in TNF-α and IL-1β was not affected by carbohydrate availability, providing further evidence that the observed increases were probably due to inflammatory and not metabolic processes.

Although our data cannot categorically rule out the presence of some muscle damage, the evidence strongly argues against such an occurrence. First, as discussed, we saw no evidence that either TNF-α or IL-1β were increased by contraction, and these cytokines are often, if not always, elevated when muscle is damaged (12, 13, 20). Second, whereas our resting plasma CK levels were in the high range of normal, there was no indication that CK rose during 60 min of exercise. This is in contrast with previous studies where subjects ran (32) or performed eccentric cycling (29) for 60 min. In these studies, where exercise involved an eccentric component, an increase in CK was observed after 60 min. Third, we recently demonstrated that this mode of exercise results in intramyocellular increases in both IL-6 mRNA and protein but does not increase IL-6 mRNA in the area surrounding the fibers (15). Finally, whereas our histological data are from a separate cohort of subjects, these subjects nonetheless underwent a similar exercise regimen, including exercise the previous day. The histology did not provide any evidence of ultrastructural damage. Our data suggest, therefore, that during short-term (60 min) continuous exercise there is no observable muscle damage.

Our observations that IL-6 increased with noncontracting skeletal muscle and that the increase was augmented when intramuscular glycogen levels were low are entirely consistent with previous studies that have examined this relationship (17, 25). Although in the present study we did not sample leg IL-6 release, because of the fact that leg IL-6 release can account for much of the plasma IL-6 accumulation (27), our plasma IL-6 data suggest that, like previous studies (25), circulating IL-6 appears to be related to glycogen availability. Recently, however, using in situ hybridization and immunohistochemical techniques, we identified that IL-6 is produced almost exclusively by type 2 muscle fibers (15). Given that type 1 fibers are recruited during prolonged contraction, we showed that IL-6 was inversely related to glycogen content within muscle fibers (15). Although these recent results seem somewhat contradictory to the present and previous (17, 25) findings, there is a viable explanation for the apparent anomaly. In the present and previous (17, 25) studies, muscle glycogen levels were analyzed from mixed fiber muscle biopsies and patterns of glycogen depletion within specific fiber types were not obtained. It is likely, however, that as type 1 fibers became depleted of muscle glycogen, type 2 fibers were subsequently recruited, producing and then releasing IL-6.

A key finding from this study was that like IL-6, IL-8 mRNA is induced by concentric muscle contraction and that its induction is markedly exacerbated by low intramuscular glycogen stores. It must be noted, however, that whereas IL-6 increased ~150-fold in Lo Gly, the increase in IL-8 was ~40-fold (Fig. 1). In addition, whereas IL-6 increased in the circulation in both trials, but to a greater extent in Lo Gly, no such increase in IL-8 was observed. Hence, there appear to be differences in the biological profile of contraction-induced increases in these two cytokines. IL-8 has been shown to increase in skeletal muscle, but is blunted by carbohydrate ingestion throughout exercise (20). However, the biological significance of this increase was not discussed in this previous paper (20) and little is known regarding the significance of the increase in skeletal muscle IL-8 mRNA. IL-8 was identified little over a decade ago (2) and is classed as one of 40 chemotactic peptides ranging from 8 to 14 kDa. In this respect, the biological action and size of IL-8 are quite different compared with IL-6. In recent years, biological roles have been identified for muscle-derived IL-6, both as an intramyocellular signal for heat shock proteins (10) and as an endocrine-like molecule to be released from the muscle to induce lipolysis (31). In the current study, we saw no evidence that IL-8 was increased into the circulation, so in this respect, it is unlikely to have significant circulatory actions. Whereas it is possible that IL-8 has some biological role to play in the etiology of muscle metabolism, it is also possible that its induction is simply a consequence of a disruption to myocellular homeostasis. In most cell types IL-8 is markedly induced via the MAPK pathways. In particular, p38 appears to be a potent upstream signaling molecule for IL-8 in monocytes, neutrophils, and smooth muscle cells (for review, see Ref. 16). We recently demonstrated that during muscle contraction, the nuclear abundance of p38 MAPK is phosphorylated, a process that is augmented when intramuscular glycogen availability is reduced (Chan MHS, McGee SL, Watt MJ, Hargreaves M, and Febbraio MA, unpublished observations).

In summary, our data demonstrate that cytokines are not ubiquitous in skeletal muscle and that IL-8, like IL-6, is influenced by the glycogen availability within the contracting muscle. The biological role of IL-8 during exercise is yet to be elucidated.

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