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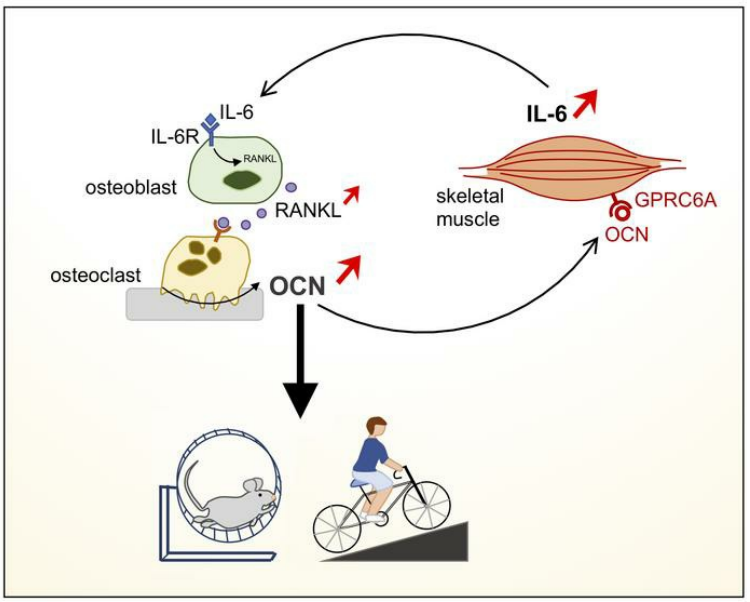
Graphical abstract



Muscle-derived interleukin 6 increases exercise capacity by signaling in osteoblasts

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Given the numerous health benefits of exercise, understanding how exercise capacity is regulated is a question of paramount importance. Circulating interleukin 6 (IL-6) levels surge during exercise and IL-6 favors exercise capacity. However, neither the cellular origin of circulating IL-6 during exercise nor the means by which this cytokine enhances exercise capacity has been formally established yet. Here we show through genetic means that the majority of circulating IL-6 detectable during exercise originates from muscle and that to increase exercise capacity, IL-6 must signal in osteoblasts to favor osteoclast differentiation and the release of bioactive osteocalcin in the general circulation. This explains why mice lacking the IL-6 receptor only in osteoblasts exhibit a deficit in exercise capacity of similar severity to the one seen in mice lacking muscle-derived IL-6 (mIL-6), and why this deficit is correctable by osteocalcin but not by IL-6. Furthermore, in agreement with the notion that IL-6 acts through osteocalcin, we demonstrate that mIL-6 promotes nutrient uptake and catabolism into myofibers during exercise in an osteocalcin-dependent manner. Finally, we show that the crosstalk between osteocalcin and IL-6 is conserved between rodents and humans. This study provides evidence that a muscle-bone-muscle endocrine axis is necessary to increase muscle function during exercise in rodents and humans.

Introduction

The ability to exercise, or exercise capacity, is an evolutionarily conserved physiological function of vital importance because it allows animals living in the wild to escape danger. In addition, exercise provides numerous health benefits to the general population. This explains why there is a growing interest in understanding how this physiological process is regulated (1, 2). Several cytokines have been shown to favor muscle function during exercise and as a result to increase exercise capacity (3). One of them, interleukin 6 (IL-6), sees its circulating levels surge during exercise (4–8). Because *Il6* is expressed by many cell types, this raises the question of the identity of the cell type(s) responsible for its increase in the general circulation during exercise. It has been proposed that once secreted into the general circulation, IL-6 enhances exercise capacity by promoting gluconeogenesis and lipolysis, i.e., the production of the 2 main nutrients for myofibers (3, 7, 9–12). However, this mechanism of action has not been verified in vivo, which leaves open the possibility that IL-6 could regulate exercise capacity through other, yet to be described, means. Those 2 questions are of paramount importance if we want to achieve a comprehensive understanding of how exercise capacity is regulated.

Hormones signaling in myofibers can also contribute to the increase in muscle function during exercise. In that regard, we have shown, through its injections in wild-type (WT) mice and the analysis of mice lacking its receptor only in myofibers, that the bone-derived hormone osteocalcin is necessary and sufficient to increase muscle function during exercise in the mouse (13). Osteocalcin also promotes the rise in *Il6* expression in muscle and in circulating IL-6 levels during exercise. On the other hand, in cell culture experiments, IL-6 increased the expression in osteoblasts of *Rankl*, a gene necessary for osteoclast differentiation, a process that is required for the generation of the uncarboxylated and bioactive form of osteocalcin (13–15).

Altogether, the functions of IL-6 during exercise and the relationship between osteocalcin and muscle-derived IL-6 (mIL-6) raise the following questions regarding the mechanism(s) whereby IL-6 promotes exercise capacity: To what extent does muscle contribute to the rise in circulating IL-6 during exercise? Does mIL-6 enhance exercise capacity on its own? If it does, what is its mechanism of action? Can we provide evidence that regulatory events identified in the mouse also take place in humans? The present study was conducted to address these questions.

Results

IL-6 is necessary for the increase in circulating osteocalcin levels observed in response to a training intervention in humans. If the interplay identified in the mouse and taking place during exercise between IL-6 and osteocalcin is of real physiological significance

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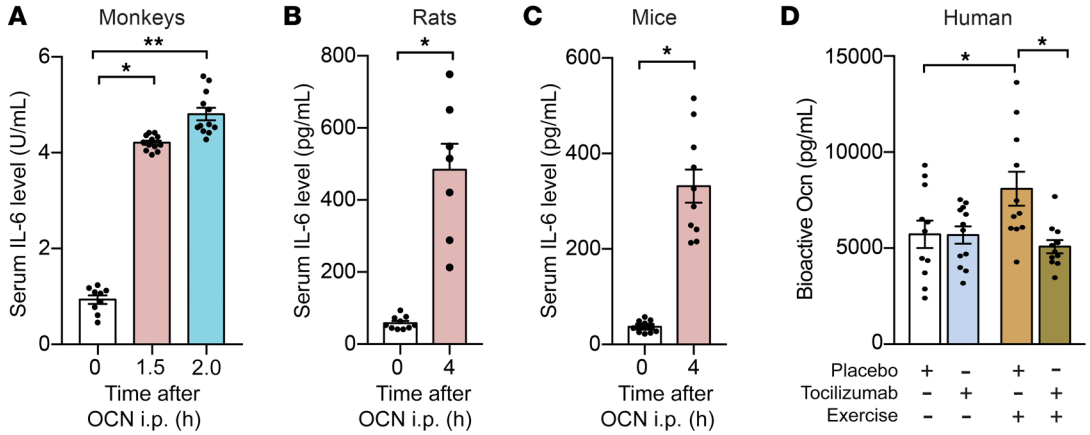

Figure 1. IL-6 is necessary for the increase in circulating osteocalcin levels observed in response to a training intervention in humans. (**A**–**C**) Circulating IL-6 levels in (**A**) rhesus monkeys (14 ± 0.8 years old), (**B**) rats (4 months old), and (**C**) mice (3 months old) treated with vehicle or osteocalcin (Ocn) (13.5 ng/g for monkeys, 30 ng/g for rats, and 30 ng/g for mice). *n* = 12 per treatment. Statistical analyses were conducted using 1-way ANOVA followed by Tukey's post hoc test (**A**) or 2-tailed unpaired *t* test (**B** and **C**). (**D**) Circulating uncarboxylated and bioactive Ocn levels in obese, nondiabetic subjects treated with either placebo or a neutralizing antibody against the IL-6 receptor in combination with or without intensive endurance exercise for 45 minutes, 3 times a week, for 12 consecutive weeks. *n* = 11 per group except no exercise with the tocilizumab group, *n* = 12. Statistical analyses were conducted using 1-way ANOVA followed by the Holm-Sidak post hoc test. All results presented as the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

in determining exercise capacity, it should be observed in other species, including primates (13). In a first test of this contention we found that, as it does in mice, a single injection of recombinant osteocalcin significantly increased circulating IL-6 levels in rats and more importantly from a clinical vantage point, in nonhuman primates (Figure 1, A–C).

These results led us to further expand our analysis and to measure circulating levels of bioactive osteocalcin in obese, nondiabetic human subjects that had either rested or had undergone a 12-week-long aerobic training period (8). This 12-week training period consisted of endurance exercise sessions (45 minutes of biking) 3 times a week. We used for that purpose a novel and sensitive ELISA that specifically measures circulating uncarboxylated and bioactive human osteocalcin (13, 14). This endurance exercise training protocol resulted in a 60% increase in circulating osteocalcin levels in these individuals compared with resting individuals. Of note, this increase was sustained for at least 2 days after the cessation of exercise. To the best of our knowledge, this represents the first evidence that performing endurance exercise 3 times a week for a relatively long period of time (3 months) is enough to induce a significant increase in circulating osteocalcin levels in humans (Figure 1D).

In the third experiment, we measured circulating osteocalcin levels in cohorts of exercising obese nondiabetic subjects that received either placebo or an anti–IL-6 receptor (anti–IL-6R) antibody (tocilizumab) during the training period (8). We observed that in individuals that had received the anti–IL-6R antibody, the increase in circulating osteocalcin levels triggered by exercise had virtually disappeared (Figure 1D). Albeit of a correlative nature, these latter data are an indication that IL-6 may regulate circulating osteocalcin levels in humans as well during exercise.

mIL-6 is needed for maximal exercise capacity. In view of the conservation of regulation between mice and humans presented above, we asked to what extent does muscle contribute to the increase in circulating IL-6 levels observed during exercise and does mIL-6 actually regulate exercise capacity?

For that purpose we crossed *Il6*$^{fl/fl}$ mice with *Hsa-MerCreMer* mice that express the *Cre* recombinase in an inducible manner specifically in myofibers (Figure 2A and refs. 16, 17). *Cre* expression was induced in 5-week-old mice by intraperitoneal (i.p.) injections of tamoxifen (10 mg/mL) 4 days in a row followed by feeding with tamoxifen-containing chow diet (1 mg/20 g of body weight) for 2 to 4 weeks before analysis (18). This resulted in a highly efficient deletion of *Il6* in oxidative (soleus), glycolytic (extensor digitalis or EDL), and mixed-fiber muscle (gastrocnemius). In contrast, no deletion of *Il6* could be detected in the heart or any other tissues tested (Figure 2, B and C). *Il6*$_{Hsa}^{-/-}$ mice were obtained at the expected Mendelian ratio, had a normal life span, and appeared overtly normal.

The contribution of muscle to circulating IL-6 levels during exercise was assessed by measuring its levels in male and female *Il6*$_{Hsa}^{-/-}$ and control mice before and after exercise (running on a treadmill at a constant speed until exhaustion or for 50 minutes) (13). We observed that although circulating IL-6 levels increased robustly in control mice after exercise, they did not in *Il6*$_{Hsa}^{-/-}$ mice (Figure 2D and Supplemental Figure 1, A and B). These results indicate that the vast majority of the IL-6 molecules present in general circulation during exercise originate from muscle.

The extent to which mIL-6 contributes to exercise capacity during an endurance exercise was determined by measuring the ability of *Il6*$_{Hsa}^{-/-}$ and control mice to perform endurance running. Starting at 3 months of age, male and female *Il6*$_{Hsa}^{-/-}$ mice exhibited a significant reduction in the time and distance they run on a treadmill apparatus when compared with control littermates (Figure 2E and Supplemental Figure 1C). The defect in exercise capacity observed in *Il6*$_{Hsa}^{-/-}$ mice was specific to endurance exercise because grip strength was not affected by the inactivation of mIL-6 (Supplemental Figure 1, D and E). As inferred by these results, treating WT mice with an antibody against IL-6 significantly decreased their exercise capacity (Figure 2F). The severity of the deficit in exercise capacity observed in *Il6*$_{Hsa}^{-/-}$ mice was an incentive to explore its cellular and molecular bases.

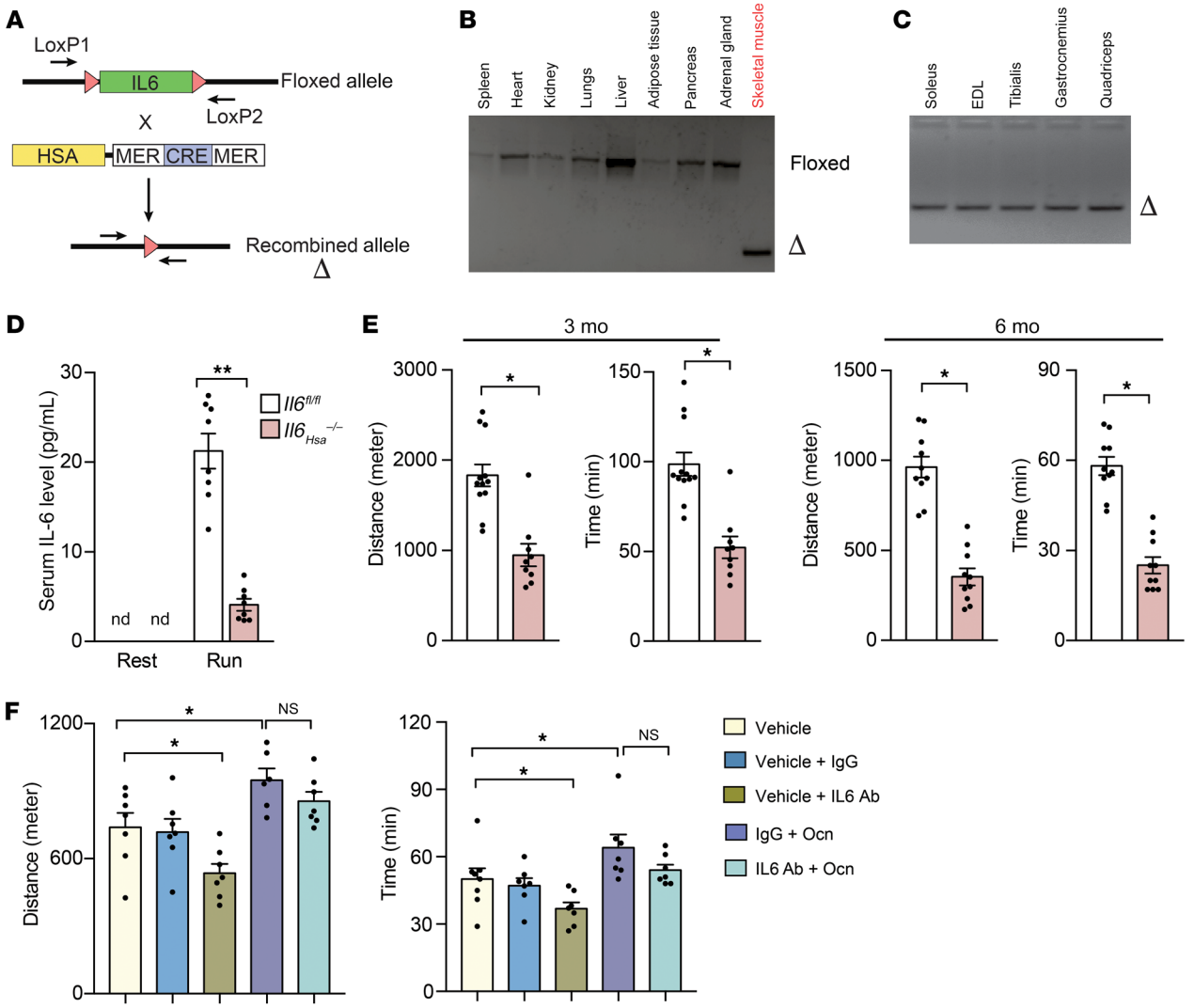

Figure 2. Muscle-derived IL-6 is needed for maximal exercise capacity. (**A**) The promoter of the human α-skeletal actin (*HSA*) gene drives expression of the *MerCreMer* (*MCM*) gene, which harbors a mutated estrogen receptor (Mer) ligand-binding domain on each end of *Cre* recombinase. After crossing with $Il6^{fl/fl}$ mice and treatment with tamoxifen, a Cre-mediated recombination event results in the deletion of the *Il6* gene. (**B**) Detection of *Il6* deletion by PCR on genomic DNA isolated from various tissues of $Il6_{Hsa}^{-/-}$ mice. (**C**) Detection of *Il6* deletion by PCR on genomic DNA isolated from various skeletal muscles of $Il6_{Hsa}^{-/-}$ mice. (**D**) Circulating IL-6 levels in 3-month-old male $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice at rest and after exercise. $n = 8$ per group. (**E**) Performance during an endurance run of 3- and 6-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ female mice. (**F**) Performance during an endurance test (running on a treadmill at 30 cm/s until exhaustion) of 8-month-old mice treated with vehicle or osteocalcin (Ocn, 500 ng/g) and an antibody against IL-6 or a control IgG, $n = 7$ per group. Results presented as the mean ± SEM. Data were analyzed with 2-tailed unpaired *t* test (**D** and **E**) or 1-way ANOVA followed by Tukey's post hoc test (**F**) $^*P < 0.05$; $^{**}P < 0.01$.

mIL-6 favors exercise capacity in part through osteocalcin. Although trying to unravel how mIL-6 enhances exercise capacity, we observed that circulating osteocalcin (encoded by the *Ocn* gene) levels did not increase in $Il6_{Hsa}^{-/-}$ mice after exercise, as they did in control mice (Figure 3A). Moreover, $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ mice exhibited a deficit in exercise capacity as severe as the one observed in $Il6_{Hsa}^{-/-}$ mice, whereas single heterozygous mice did not, and that circulating osteocalcin did not increase in $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ mice during an endurance exercise. This genetic epistasis experiment indicated that osteocalcin mediates mIL-6 regulation of adaptation to exercise in the mouse, as it appears to do in humans (Figure 3, B and C).

If the aforementioned hypothesis is accurate, then one would expect that osteocalcin would correct, at least in part, the deficit in exercise capacity observed in mice lacking mIL-6. In a positive control experiment, we observed that an injection of IL-6 before exercise increased circulating IL-6 levels, corrected as it should the deficit in exercise capacity observed in the $Il6_{Hsa}^{-/-}$ mice, and restored the surge of circulating osteocalcin levels during endurance exercise (Figure 3, D–F). This IL-6 injection also corrected the deficit in exercise capacity seen in $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ mice (Figure 3G). More importantly for our purpose, an injection of osteocalcin corrected the majority of the deficit in exercise capacity seen in $Il6_{Hsa}^{-/-}$ mice during endurance exercise. Of note, circulating Il-6 levels increased following these osteocalcin injections, suggesting that osteocalcin can release IL-6 from organs other than muscle (Figure 3, H and I). These data support the notion that osteocalcin is a mediator of mIL-6's ability to enhance exercise capacity without excluding the possibility that other mediators of this action exist.

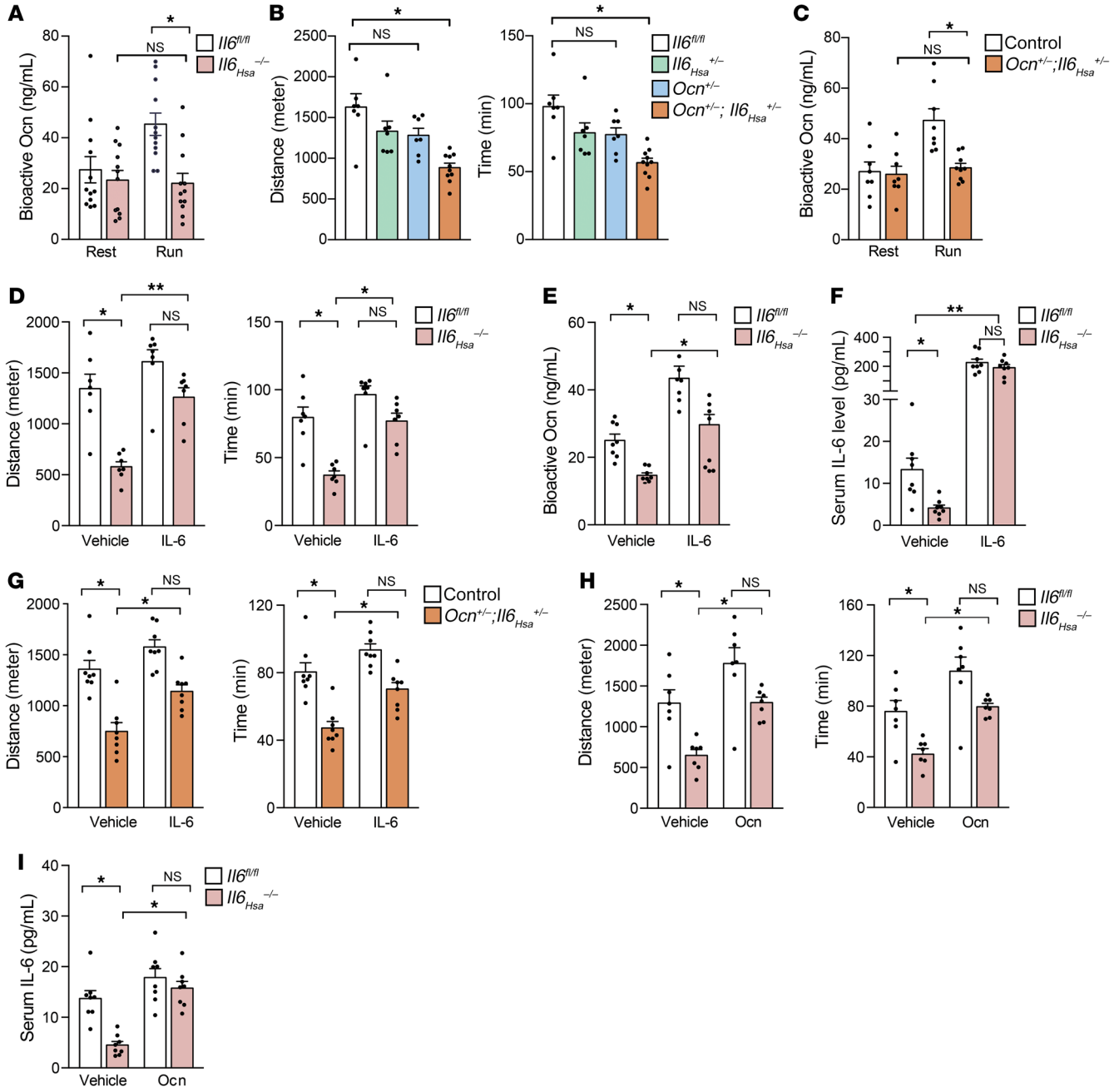

Figure 3. mIL-6 favors exercise capacity in part through osteocalcin. (**A**) Circulating bioactive osteocalcin (Ocn) levels in 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice at rest and after exercise, $n = 12$. (**B**) Performance during an endurance run of 3-month-old $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ and control ($Il6_{Hsa}^{+/-}$, $Ocn^{+/-}$, and WT) mice, $n = 8$–12. (**C**) Circulating Ocn levels in 3-month-old control ($Il6^{fl/fl}$, $Ocn^{+/-}$, and WT mice) and $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ mice at rest and after exercise, $n = 8$. (**D**) Performance during an endurance run of 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice after an i.p. injection of IL-6 (3 ng/g), $n = 8$. (**E**) Circulating Ocn levels during an endurance run in 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice after an i.p. injection of IL-6 (3 ng/g), $n = 8$. (**F**) Circulating IL-6 levels during an endurance run in 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice after an i.p. injection of IL-6 (3 ng/g), $n = 8$. (**G**) Performance during an endurance run of 3-month-old control ($Il6^{fl/fl}$, $Ocn^{+/-}$, and WT mice) and $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ mice after an i.p. injection of IL-6 (3 ng/g), $n = 8$. (**H**) Performance during an endurance run of 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice after an i.p. Ocn injection (120 ng/g), $n = 7$. (**I**) Circulating IL-6 levels during an endurance run in 3-month-old $Il6^{fl/fl}$ and $Il6_{Hsa}^{-/-}$ mice after an i.p. injection of Ocn (120 ng/g), $n = 8$. These results are representative of 3 independent experiments. Data were analyzed by 1-way ANOVA followed by Tukey's post hoc test. Results presented as the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

mIL-6 contributes to the maintenance of muscle mass in an osteocalcin-independent manner. In the course of this analysis we observed a second muscle phenotype in $Il6_{Hsa}^{-/-}$ mice. Indeed, and even though myostatin (*Mstn*) expression was not affected (Supplemental Figure 1E), $Il6_{Hsa}^{-/-}$ mice exhibited a significant decrease in the weight of oxidative muscles, e.g., the soleus, the most mobilized muscle type during an endurance exercise, in 3-month-old $Il6_{Hsa}^{-/-}$ mice compared with control littermates (Figure 4A). One experimental line of evidence indicates, however, that mIL-6 regulates muscle function independently of its regulation of muscle

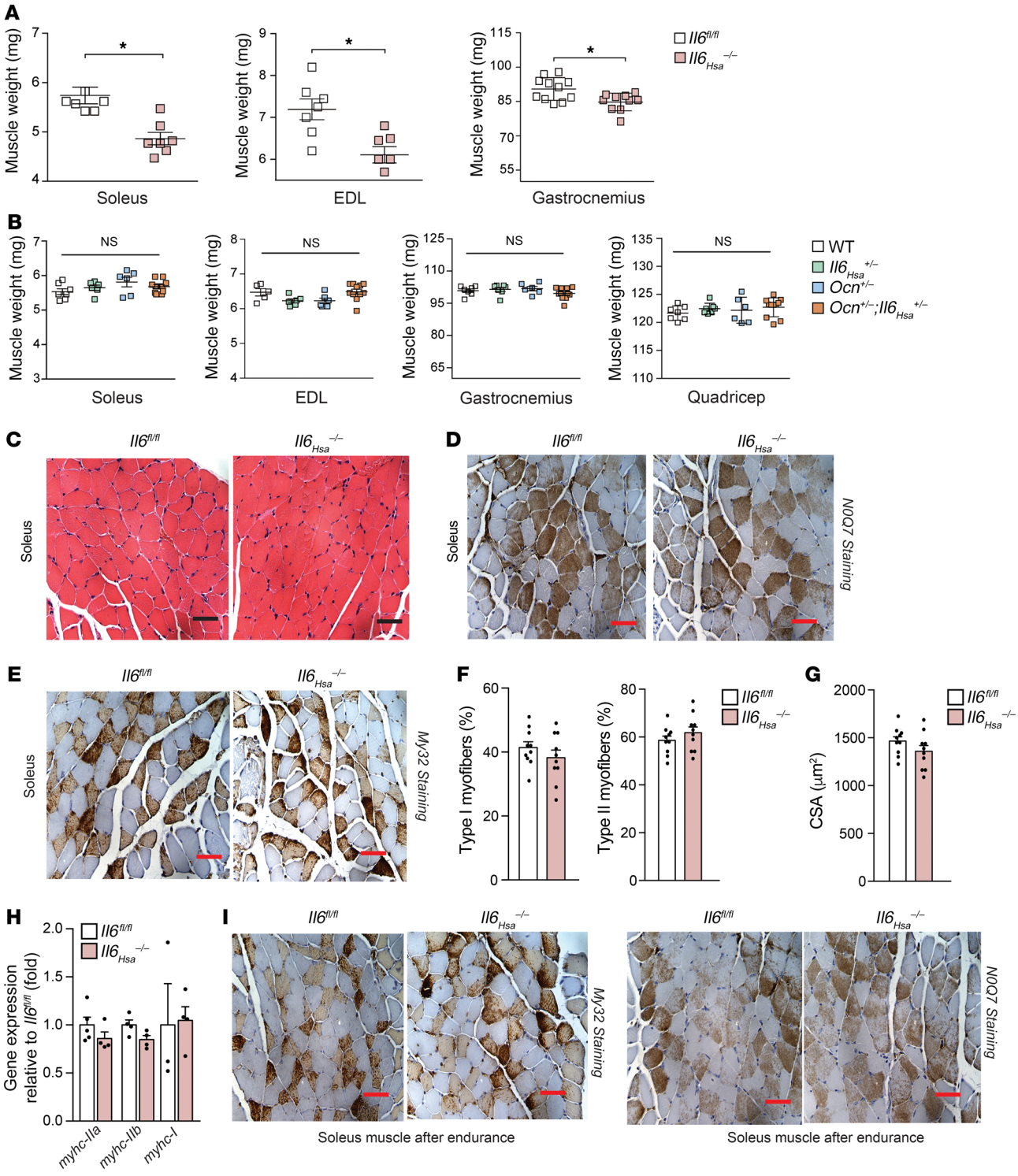

Figure 4. mIL-6 contributes to the maintenance of muscle mass in an osteocalcin-independent manner. (**A**) Weight of hind limb muscles of 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates, n = 6–11. (**B**) Weight of hind limb muscles in 3-month-old $Ocn^{+/-}$ $Il6_{Hsa}^{+/-}$ compound mutant mice and control littermates (control group includes WT, $Ocn^{+/-}$, and $Il6_{Hsa}^{+/-}$ mice), n = 5–7. (**C**) Representative H&E staining of soleus muscle fibers in 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates. Scale bars: 100 μm. (**D** and **E**) Representative histology with (**D**) N0Q7 (MHC I slow twitch fibers) and (**E**) MY32 (MHC II fast twitch fibers) staining of soleus muscle of 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates. Scale bars: 100 μm. (**F**) Distribution of type 1 and type II myofibers in 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates. Scale bars: 100 μm. (**G**) Measurement of cross-sectional area (CSA) of muscle fibers in 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates. (**H**) Expression of myosin heavy chain genes in gastrocnemius muscle measured by qRT-PCR. (**I**) Representative histology with N0Q7 and MY32 staining of soleus muscle after endurance exercise in 3-month-old female $Il6_{Hsa}^{-/-}$ and control littermates. Scale bars: 100 μm. These results are representative of 3 independent experiments. Data shown in **A**, **F**, and **G** were analyzed by 2-tailed unpaired t test and data in **B** and **H** by 1-way ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SEM. *$P < 0.05$.