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Endurance Training Improves Leg Proton Release and Decreases Potassium Release During High-Intensity Exercise in Normoxia and Hypobaric Hypoxia

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ABSTRACT

Aim: To assess the impact of endurance training on skeletal muscle release of H^+ and K^+.

Methods: Nine participants performed one-legged knee extension endurance training at moderate and high intensities (70%–85% of W_{peak}), three to four sessions·week^{-1} for 6 weeks. Post-training, the trained and untrained (control) leg performed two-legged knee extension at low, moderate, and high intensities (40%, 62%, and 83% of W_{peak}) in normoxia and hypoxia (~4000 m). The legs were exercised simultaneously to ensure identical arterial inflow concentrations of ions and metabolites, and identical power output was controlled by visual feedback. Leg blood flow was measured (ultrasound Doppler), and acid–base variables, lactate- and K^+ concentrations were assessed in arterial and femoral venous blood to study K^+ and H^+ release. Ion transporter abundances were assessed in muscle biopsies.

Results: Lactate-dependent H^+ release was similar in hypoxia to normoxia ($p = 0.168$) and was lower in the trained than the control leg at low–moderate intensities ($p = 0.060$–0.006) but similar during high-intensity exercise. Lactate-independent and total H^+ releases were higher in hypoxia ($p < 0.05$) and increased more with power output in the trained leg (leg-by-power output interactions: $p = 0.02$). K^+ release was similar at low intensity but lower in the trained leg during high-intensity exercise in normoxia ($p = 0.024$) and hypoxia ($p = 0.007$). The trained leg had higher abundances of Na^+/H^+ exchanger 1 ($p = 0.047$) and Na^+/K^+ pump subunit α ($p = 0.036$).

Conclusion: Moderate- to high-intensity endurance training increases lactate-independent H^+ release and reduces K^+ release during high-intensity exercise, coinciding with increased Na^+/H^+ exchanger 1 and Na^+/K^+ pump subunit α muscle abundances.

1 | Introduction

Repeated muscle contraction causes fatigue, that is, reduces the ability to produce force and power output. Skeletal muscle fatiguability may originate from a reduced motor drive, insufficient oxygen (O_2) and substrate availability, reactive O_2 and nitrogen species production, and intracellular metabolic and ionic perturbations [1, 2]. During exercise, regulating transmembrane ion gradients is essential to maintain sarcolemmal excitability and intracellular pH [2, 3]. Contraction-induced intracellular accumulation of H^+ (reduced pH) is counteracted by intracellular buffer systems, facilitated diffusion via the lactate/H^+ cotransporters (monocarboxylate transporters [MCT] 1 and 4), in exchange for Na^+ by the Na^+/H^+ exchanger

1 (NHE1), and diffusion of undissociated lactic acid [3]. The Na^+/K^+ pump, the Na^+-K^+-$2Cl^-$ cotransporter (NKCC), and the K^+ inward rectifier channel 2.1 play pivotal roles in preventing interstitial K^+ accumulation and preserving sarcolemmal excitability during repetitive skeletal muscle activation [2, 4].

During low- and moderate-intensity exercise, skeletal muscle releases modest amounts of H^+ and K^+ to the venous blood [5–10]. However, at high-intensity endurance and sprint-like exercise, muscle acidosis and the interstitial concentrations and the release rates of H^+ and K^+ increase dramatically [5–7], reflecting insufficient K^+ re-uptake, and H^+ buffering and efflux capacity. A few studies have investigated the impact of training on H^+ dynamics during exercise [5, 6, 11, 12] and the implication of training-induced elevations of H^+ transporters on performance [13–15]. Increased abundance of MCT and NHE1 has been reported following 7–8 weeks of interval training at supramaximal intensities together with maintained [11] or increased [6] leg H^+ release during high-intensity one-legged knee extension (1L-KE) exercise. These findings coincided with reduced intracellular-to-interstitial H^+ and lactate gradients after training, entailing an increased or maintained muscle H^+ efflux at a lower driving force across the sarcolemma.

On the contrary, 7 weeks of intensified training with reduced training volume did not alter MCT and NHE1 abundances nor the H^+ release during high-intensity cycling [5]. The impact of training on the abundance of the Na^+/K^+ pump and its relationship with K^+ dynamics also remains controversial. For instance, when well-trained cyclists replaced their regular training with speed-endurance- and high-intensity interval training, the Na^+/K^+ pump abundance, the interstitial $[K^+]$, and the leg K^+ release were unaltered despite improved performance [5]. Conversely, 7 weeks of high-intensity interval training of one leg reduced interstitial $[K^+]$ during steady-state submaximal and incremental exercise to exhaustion in the trained compared with the control leg, while leg K^+ release was unaltered [7].

A reason for the discrepancies might be that muscle release rates of H^+ and K^+ vary on the arterial inflow concentrations of ions and metabolites that change during repeated trials when a trained and an untrained leg are compared on two trials on the same day or two different days separated by a training intervention. Herein, we examined the impact of 6 weeks of one-legged (1L-KE) moderate- to high-intensity endurance training on H^+ and K^+ release rates by exercising the trained leg and the untrained control leg simultaneously during two-legged knee extension (2L-KE) at three different power outputs in normoxia and acute hypobaric hypoxia (4000 m): at low (40% of the normoxic peak power output), moderate (62%), and high intensities (83%). Leg blood flow was measured (ultrasound Doppler), and acid–base variables, lactate- and K^+ concentrations were assessed in arterial and femoral venous blood from both legs to study leg K^+ and H^+ release (lactate-dependent and lactate-independent). This experimental approach allows the study of ion shifts while the trained and untrained legs are perfused by having similar physicochemical characteristics, thus avoiding an order effect and the influence of day-to-day/trial-to-trial variations in arterial inflow concentrations of ions and metabolites. Furthermore, we assessed the impact of training on ion transporter abundances in muscle biopsies from vastus lateralis. We hypothesized that the trained leg would have a higher H^+ release and lower K^+ release than the control leg during high-intensity exercise, especially in hypoxia.

2 | Materials and Methods

2.1 | Subjects and Ethics Approval

Nine recreationally active men (mean ± SD: age: 27.5 ± 5.2 years; height: 1.79 ± 0.07 m; weight: 79.0 ± 10.6 kg; $\dot{V}O_{2max}$: 55.5 ± 9.5 mL kg^{-1} min^{-1}) were recruited for participation and completed the study. The Norwegian School of Sport Sciences Ethics Committee approved this study (03-020517). Oral and written informed consents were obtained from all participants before the start of this investigation, which was carried out following the Declaration of Helsinki, except for pretrial study registration in a publicly available database. Data from other aspects of this study are published elsewhere [16].

2.2 | Experimental Design

After 6 weeks of 1L-KE endurance training of one of the legs, both legs were exercised simultaneously during 2L-KE at three different power outputs in normoxia and hypobaric hypoxia corresponding to 4000 m above sea level: at 40 ± 3%, 62 ± 4%, and 83 ± 4% of the normoxic peak power output ($\dot{W}_{peak}$). Submaximal and near-maximal power outputs were chosen to ensure identical absolute power output produced by the trained and the nontrained leg, although there was a substantial difference in training status. This was ensured using a real-time feedback system displaying the balance in power output between legs so that the subject could adjust the contribution of each leg to the total power output during 2L-KE. LBF was measured, and blood samples were drawn simultaneously from catheters indwelling the femoral veins of both legs and from a femoral artery.

Before and after the training intervention, pulmonary $\dot{V}O_{2max}$ and $\dot{W}_{peak}$ during cycling, 1L-KE (both legs) and 2L-KE in normoxia were measured and described in detail in a previous publication [16]. A muscle biopsy from each thigh was sampled before and after training in a rested state. The subjects were asked to refrain from physical activity the day before testing and sampling of muscle biopsies.

2.3 | Exercise Training

The subjects conducted supervised 1L-KE training of one of the legs (counterbalanced between right and left legs) three to four times per week over 6 weeks, as described previously [16]. Briefly, the sessions were (1) 35–65 min at 70% of $\dot{W}_{peak}$ (progressed 70%–82% of pretraining $\dot{W}_{peak}$), (2) 10 min warm-up,

20 min "all-out" at the highest possible power output (progressed 78%–91% of pretraining $\dot{W}_{peak}$) and a cooldown of 5 min, and (3) 10 min warm-up, and four to six repetitions of 5 min at 85% of $\dot{W}_{peak}$ (progressed 85%–94% of pretraining $\dot{W}_{peak}$), which was interspersed with recovery periods of 2 min and followed by a 5-min cooldown.

2.4 | Catheterization and Preparation for the Main Experiment

To enable two subjects per day, the subjects were scheduled to report to the laboratory either at 8:00 or 14:00. Catheters were placed percutaneously under local anesthesia (2% lidocaine) using the Seldinger technique. A 20-gauge catheter (Arrow ref. #ES-04150; Teleflex Medical, Wayne, PA, USA) was placed into the right femoral artery, 2–5 cm below the inguinal ligament and advanced 8 cm in the proximal direction. Another two 20-gauge catheters were placed in the femoral veins, 2 cm below the inguinal ligament and advanced 8 cm in the proximal direction. All catheters were sutured to the skin.

2.5 | Blood Sampling and Analytic Procedures

Blood was sampled using heparinized syringes (safePICO; Radiometer, Denmark). Venous blood samples were taken simultaneously from both legs immediately after the arterial sample and quickly analyzed for hemoglobin concentration ([Hb]), hemoglobin O_2 saturation (SO_2), the tension of O_2 (PO_2) and CO_2 (PCO_2), pH, plasma potassium concentration ($[K^+]$), and plasma lactate concentration ($[La^-]$) using ABL90 FLEX (Radiometer, Denmark).

2.6 | Femoral Arterial Blood Flow

LBF was measured in the femoral artery of both legs using ultrasound Doppler (Vivid E95; GE Vingmed Ultrasound AS, Horten, Norway) equipped with a linear transducer (9L-D; GE Vingmed Ultrasound AS) operating with an image frequency of 10 MHz and a Doppler frequency of 3.7–4.0 MHz. Blood velocity was measured in the common femoral artery distal to the inguinal ligament but proximal to its bifurcation into superficial and profound femoral branches. The insonation angle was minimized and always below 60°. A low-velocity filter was applied to reject noise created by turbulence at the vessel wall. The arterial diameter was determined during systole (mean of three heart cycles) from B-mode images with the transducer parallel to the vascular walls. Blood velocity was measured continuously and averaged over ~45 s for each leg. LBF was measured ~1.0–1.5 min earlier in the left leg compared with the right leg, but with no difference between the mean of training and control legs (counterbalancing).

2.7 | Calculations

Plasma bicarbonate concentration ($[HCO_3^-]$) and base excess ([BE]; mmol L^{-1}) in oxygenated whole blood ($SO_2 = 1.0$), as a quantitative measurement of the nonrespiratory (i.e., metabolic) component of the acid–base balance, was calculated using the Henderson–Hasselbalch equation and the Zander equation, respectively (Equations 1–4) [17, 18]. It was employed pH-variable Z and pK′, and the [BE] was adjusted to fully oxygenated conditions:

$$[BE] = Z \times \left(\left(\left[HCO_3^- \right] - 24.26 \right) + (9.5 \times 1.63 \times [Hb]) \times (pH - 7.4) \right) - 0.2 \times [Hb] \times \left(1 - SO_2\right) \quad (1)$$

$$Z = 1 - \text{hematocrit} \times \left(0.43 + 0.28 \times (pH - 7.4) + 0.082 \times (pH - 7.4)^2\right) \quad (2)$$

$$\left[HCO_3^- \right] = 0.0304 \times PCO_2 \times 10^{pH - pK'} \quad (3)$$

$$pK' = 6.125 - \log_{10} \times \left(1 + 10^{pH - 8.7}\right) \quad (4)$$

Venous–arterial concentration differences (v-a-diff) of $[K^+]$, [La] and [BE] were computed as the difference between femoral arterial and femoral venous concentrations for each leg. The leg K^+ release was calculated by multiplying the v-a-diff in $[K^+]$ by the plasma flow. Plasma flow was calculated as LBF × (1-Hct/100). The leg H^+ release (mmol min^{-1}) was calculated by multiplying the LBF by the v-a-diff in [BE]. The lactate-dependent leg H^+ release was calculated by multiplying the plasma flow by the v-a-diff in $[La^-]$, considering the 1:1 relationship between La and H^+ muscle transmembrane co-transport by MCT1 and MCT4 [19]. The lactate-independent H^+ release was then the difference between the total leg H^+ release and the lactate-dependent H^+ release.

2.8 | Hypobaric Hypoxia

Hypobaric hypoxia was produced using a hypobaric chamber (Norwegian Universal Technology, Norway). The background conditions were strictly regulated to maintain stable chamber pressure, temperature, and fractions of O_2 and CO_2. Gas fractions were regulated using O_2 supply (AGA, Norway) and CO_2 scrubbers (Sofnolime; Molecular Products Ltd., UK). The hypoxic experiment was carried out with a barometric pressure of 622 ± 3 mbar matching the pressure at 4000 m above sea level. The normoxic experiment was carried out at 200 m above sea level (986 ± 6 mbar) inside the hypobaric chamber.

2.9 | Knee Extension Ergometer

Knee extension exercises were performed on an electromagnetically braked ergometer that isolates leg muscle contractions to quadriceps femoris during knee extensions [20], as described in detail previously [16]. Adding a steel bar to the pedal arm on either one or both sides of the flywheel, both 1L-KE and 2L-KE can be performed. Strain gauges were incorporated in the steel bars, and the angles of the pedal arms were continuously monitored. The work performed on the flywheel was calculated and recorded by custom-made software. The kicking frequency (kept at 60 rpm) and the work expressed as the balance between the legs were displayed in real-time on a monitor in front of the subjects to ensure equal involvement of both legs.

2.10 | Exercise Protocol and Timing of Measurements

The normoxic experiment was conducted before the hypoxic experiment and separated by 1 h of supine rest. First, a baseline measurement was conducted after 10 min rest seated in the KE ergometer. After that, three 8-min bouts of 2 L-KE exercise at low (47 ± 7 W; i.e., 23.5 W by each leg), moderate (73 ± 10 W; i.e., 36.5 W by each leg), and high intensities (97 ± 13 W; i.e., 48.5 W by each leg) were conducted interspersed by 5-min passive rest. The three power outputs equaled 40 ± 3%, 62 ± 4%, and 83 ± 4% of normoxic 2 L-KE $\dot{W}_{peak}$, respectively. Post hoc analysis showed equal involvement of legs; the trained leg accounted for 50.3 ± 0.8%, 50.6 ± 0.8%, and 50.8 ± 1.3% of the power output at low-, moderate-, and high-intensity exercise in normoxia, respectively. In hypoxia, despite employing an identical electromagnetic braking force on the flywheel, post hoc analysis from the strain gauges in the steel bars identified 5.2 ± 2.2% lower power output than in normoxia. Therefore, the low-, moderate-, and high-intensity exercises were carried out at 44 ± 7 W, 69 ± 10 W, and 92 ± 13 W, respectively, and the trained leg accounted for 50.3 ± 1.2%, 50.4 ± 1.1%, and 50.5 ± 1.6% of the power output. The slightly lower power output in hypoxia than in normoxia was likely caused by lower air resistance to the rotating flywheel due to the lower barometric pressure. Since these differences were small (5%) and had no implication for the between-leg differences, the statistical analyses have not incorporated any correction when comparing exercise responses during normoxia and hypoxia. During each bout, the subjects used the first 1–2 min to fine-tune equal involvements of legs. After 3 min, B-mode imaging of the left femoral artery began, followed by Doppler measurements at 4 min on the left leg and approximately from 5 min on the right leg. Two blood samples from each vessel were drawn during the Doppler measurements (~4.5 and ~5.5 min), and the duplicates were averaged. At the end of each exercise bout, the subjects were asked to rate the perceived intensity for each leg separately using the CR-10 scale [21].

2.11 | Skeletal Muscle Biopsy

Muscle biopsies (~100–200 mg) were collected from the mid-portion of *vastus lateralis* after local anesthesia, using the Bergström technique with manual suction. The tissue was immediately dissected free from visible fat and connective tissue, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

2.12 | Protein Immunoblot

For western blotting analyses, ~60 mg of muscle tissue was homogenized in 1 mL T-PER (Tissue Protein Extraction Reagent, 78510; Thermo Fischer Scientific, Waltham, MA, USA) and 20 μL Halt Protease & Phosphatase Inhibitor Cocktail (78440; Thermo Fischer Scientific). The tissue lysate was extracted, aliquoted, and stored at −80°C until further analyses. The protein concentration was measured using a commercial kit (BioRad DC Protein Assay, 5000116; Bio-Rad Laboratories, Hercules, CA, USA) and a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). In total, 20 μg of protein was separated by 4%–12% gradient Bis-Tris gels (Invitrogen, Thermo Fisher Scientific) for ~45 min at 200 V in cold buffer (NuPage MES SDS Running Buffer; Invitrogen, Thermo Fisher Scientific). Proteins were subsequently transferred onto a PVDF membrane (Bio-Rad Laboratories) at 30 V for 90 min in cold buffer (NuPage Transfer Buffer; Invitrogen, Thermo Fisher Scientific). Membranes were blocked at room temperature for 2 h in a 5% fat-free skimmed milk (Merck, Darmstadt, Germany) and 0.1% TBS-t solution (TBS: Bio-Rad Laboratories; Tween-20: VWR). Thereafter, the membranes were divided in pieces according to predefined molecular weight using molecular weight ladders (Protein Ladder 310005; GeneON, Ludwigshafen am Rhein, Germany) and incubated overnight (4°C) with primary antibodies against NHE1 (1:300 dilution; sc-136239; Santa Cruz Biotechnology, Dallas, TX, USA), NKCC (1:200; T9-s; developed by Lytle, C./Forbush III, B., obtained from DSHB), Na^+/K^+ pump subunit α (binds to all isoforms $\alpha_{1\text{-}3}$; 1:300; a5-s; developed by Fambrough, D.M., obtained from DSHB), and Na^+/K^+ pump subunit β1 (1:5000; MA3-930; Thermo Fisher Scientific). An anti-mouse IgG (1:15 000; 31430; Thermo Fisher Scientific) secondary antibody was applied for 1 h at room temperature followed by visualization using an HRP detection system (Super Signal West Dura Extended Duration Substrate; Thermo Fisher Scientific). An antibody against the loading control GAPDH (1:3000; ab9484; Abcam, Cambridge, Great Britain) was applied as a secondary probe after using Restore Western Blot Stripping Buffer (21059; Thermo Fisher Scientific). All antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBS-t solution. Between steps, membranes were washed in 0.1% TBS-t and TBS solutions. Chemiluminescence was detected using the ChemiDoc MP system with band intensities quantified using Image Lab 5.1 software (Bio-Rad Laboratories). Pre- and post-samples of both legs were loaded on the same gel in duplicates using a counterbalanced order, and mean values were used for statistical analysis. In addition, a human control sample (a pool of all biopsies in the present project) was loaded in duplicate on each gel, and the average intensity of this sample was used for normalization to allow for semi-quantitative comparisons across gels/subjects [22]. The coefficient of variation of the duplicates on each gel was 13.1%, 14.0%, 10.1%, 8.8%, and 19.3% for NHE1, NKCC, Na^+/K^+ pump subunit α, Na^+/K^+ pump subunit β1, and GAPDH, respectively.

2.13 | Statistical Analyses

Data in text and tables are presented as mean ± standard deviation (SD) and in graphs as mean ± standard error of the mean (SEM). A three-way repeated measures ANOVA with the following three within-subject factors were used for the evaluation of blood flow, venous blood gas measurements, and ion release from the legs: leg (two levels: trained leg and control leg), altitude (two levels: normoxia and hypoxia) and power output (three levels: low-, moderate-, and high-intensity), and the degrees of freedom were adjusted with the Geisser–Greenhouse correction if sphericity was violated. The power output, leg, and altitude contrasts were assessed in addition to their interactions, followed by specific pairwise comparisons with the Fisher least significant difference post hoc test when

appropriate. Arterial blood gas measurements were evaluated by two-way repeated measures ANOVA with altitude and leg as within-subject factors. Between-leg differences in muscle protein abundance were analyzed with a paired-sample *t*-test. The alpha-level was set to <0.05. GraphPad Prism 9 (v.9.2.0; GraphPad Software, CA, USA) was used for statistical analysis.

3 | Results

3.1 | Performance and Exercise Intensity

After the training period, the trained leg achieved 15 ± 8% (mean ± SD) higher $\dot{W}_{peak}$ than the control leg during 1L-KE incremental exercise in normoxia ($p < 0.001$; Figure 1A). During 2L-KE exercise with catheters in normoxia, the low- to high-intensity power outputs represented on the average 8 ± 5%-points lower relative intensities for the trained than the control leg when standardized to one-legged $\dot{W}_{peak}$. The exercise was perceived as more strenuous (CR10 scale) with the control leg ($p = 0.013$, leg contrast), especially during high-intensity exercise ($p = 0.016$, power output by leg interaction). The exercises were also perceived as more strenuous in hypoxia ($p = 0.027$, altitude contrast; Figure 1B).

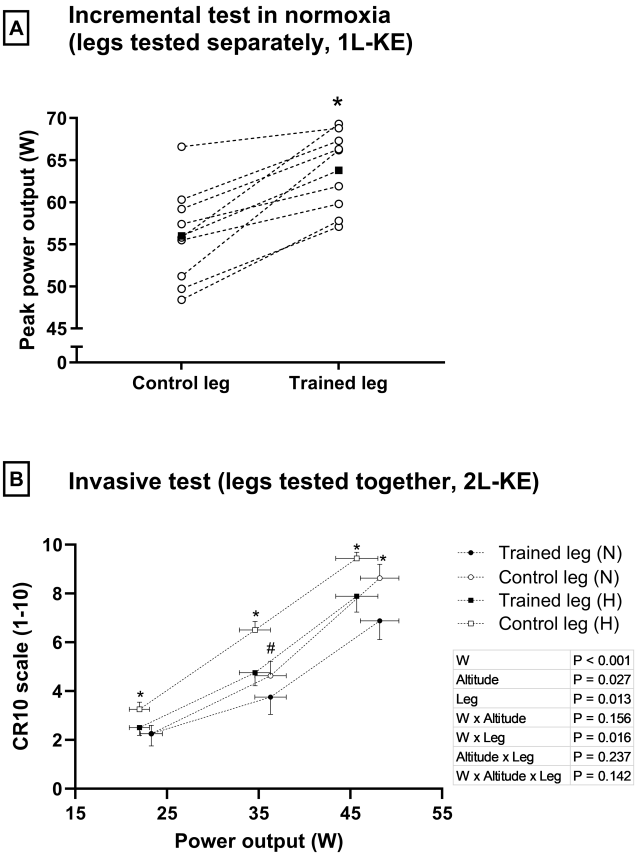

FIGURE 1 | Peak power output during incremental one-legged knee extension (1L-KE) exercise. Legs were tested separately after the training period, and pretraining data have been presented before [16] (A). The perceived intensity during two-legged knee extension (2L-KE) exercise, which was rated for each leg separately using the CR-10 scale (B). The *p*-values from the three-way ANOVA main effects (power output, altitude, and leg) and their interactions are embedded in the graph. The data are means, and error bars denote SEM. *Significant ($p < 0.05$) and #trend toward ($0.05 \leq p \leq 0.10$) difference between legs (post hoc comparisons).

3.2 | Plasma pH, PCO_2, $[HCO_3^-]$ and $[La^-]$, and Blood [BE]

The arterial pH was significantly higher in hypoxia than in normoxia ($p = 0.001$, altitude contrast; Figure 2A) and declined slightly by power output only in normoxia ($p = 0.041$, altitude by power output interaction). The femoral venous pH declined by power output ($p < 0.001$, power output contrast) in a similar fashion in normoxia and hypoxia ($p = 0.143$, altitude by power output interaction). There was a significant power output by leg interaction ($p = 0.013$), with a lower femoral venous pH in the control leg at low- and moderate-intensity (all $p < 0.05$) but not at the high-intensity power output in normoxia and hypoxia. The arterial PCO_2 decreased by power output ($p < 0.001$, power output contrast) and was lower in hypoxia than in normoxia ($p < 0.001$, altitude contrast). The femoral venous PCO_2 tended to be lower in the trained than in the control leg ($p = 0.071$, leg contrast), and the legs tended to change differently by power output ($p = 0.058$, leg by power output interaction; Figure 2B) with a slightly lower value in the trained leg at low- and moderate-intensity exercise ($p = 0.013$–0.093) but with no difference at high-intensity exercise ($p > 0.124$). The arterial and femoral venous $[HCO_3^-]$ was lower in hypoxia than in normoxia ($p = 0.002$ and $p < 0.001$, altitude contrasts for the artery and veins, respectively; Figure 2C), but the femoral vein $[HCO_3^-]$ was not different between legs ($p = 0.917$, leg contrast). The elevated pH and reduced PCO_2 and $[HCO_3^-]$ in hypoxia than in normoxia were associated with elevated pulmonary ventilation ($p < 0.001$, altitude contrast; data not presented).

The arterial [BE] was slightly positive at rest in normoxia (0.7 ± 1.0 mmol L^{-1}) and hypoxia (0.3 ± 1.2 mmol L^{-1}; comparison vs. normoxia: $p = 0.067$) and reduced similarly by power output in hypoxia and normoxia ($p < 0.001$, power output contrast; $p = 0.392$, altitude contrast; $p = 0.198$, altitude by power output interaction; Figure 3A,D), leading to a base deficit of ~5 mmol L^{-1} during high-intensity exercise. Likewise, the femoral vein [BE] decreased similarly by power output in normoxia and hypoxia ($p = 0.109$, altitude by power output interaction), leading to a base deficit of ~8 mmol L^{-1} during high-intensity exercise. The femoral vein [BE] reduced more from low to high intensities for the trained leg than for the control leg (changes in grand mean: $\Delta -7.2 \pm 1.7$ vs. $\Delta -6.9 \pm 1.6$ mmol L^{-1}, respectively; $p = 0.008$, leg by power output interaction).

The arterial and femoral venous $[La^-]$ tended to be higher in hypoxia than normoxia ($p = 0.089$ and $p = 0.093$, respectively, altitude contrasts; Figure 3B,E). The femoral venous $[La^-]$ was higher in the control than in the trained leg ($p = 0.041$, leg contrast). It was a significant power output by leg interaction ($p = 0.044$), and pairwise post hoc analysis revealed significant between-leg differences at all power outputs (all $p < 0.04$) except during high-intensity exercise in hypoxia ($p = 0.831$).

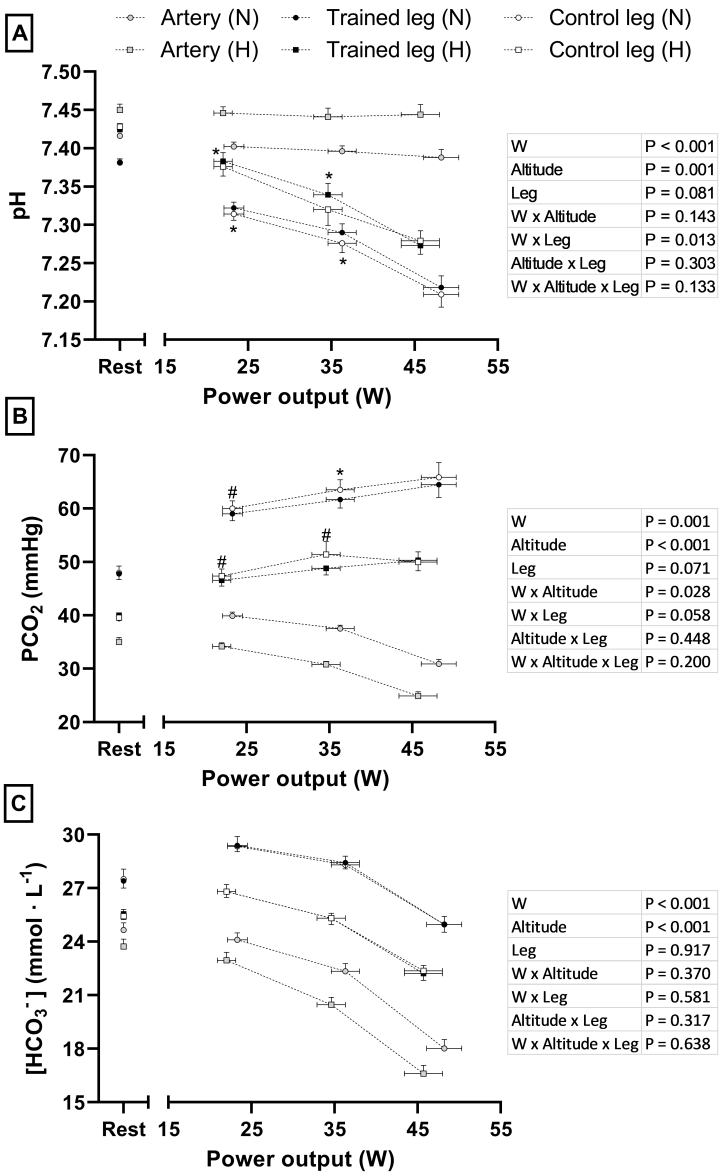

FIGURE 2 | Arterial and femoral venous pH (A), tension of CO_2 (PCO_2) (B), and plasma bicarbonate concentration ($[HCO_3^-]$) (C) in normoxia (N) and hypoxia (H) for the trained leg and the control leg as a function of the power output (W). The *p*-values from the three-way ANOVA main effects (power output, altitude, and leg) and their interactions are embedded in the graphs (only venous samples). The data are means, and error bars denote SEM. *Significant ($p < 0.05$) and #trend toward ($0.05 \leq p \leq 0.10$) difference between legs (post hoc comparisons).

3.3 | Leg Blood Flow

Leg blood flow was higher in hypoxia than in normoxia ($p = 0.017$, altitude contrast) and was slightly higher in the trained leg than in the control leg ($p = 0.044$, leg contrast; Figure 3C,F) with post hoc analysis revealing a significant between-leg difference at the moderate exercise intensity in normoxia ($p = 0.015$) and the low exercise intensity in hypoxia ($p = 0.049$).

3.4 | Lactate-Dependent and Lactate-Independent H^+ Release

The lactate-dependent H^+ release increased by power output ($p < 0.001$, power output contrast; Figure 4A,D), and the increase was similar in normoxia and hypoxia ($p = 0.168$, altitude contrast; $p = 0.656$, altitude by power output interaction). There was no main effect of leg ($p = 0.131$, leg contrast), and both legs changed their lactate-dependent H^+ release similarly by power output ($p = 0.129$, leg by power output interaction) and by altitude ($p = 0.163$, leg by altitude interaction). Post hoc analysis revealed a significantly higher lactate-dependent H^+ release in the control leg at the low ($p = 0.006$) and moderate ($p = 0.013$) exercise intensity in normoxia and the moderate exercise intensity in hypoxia ($p = 0.046$).

The lactate-independent H^+ release was higher in hypoxia than in normoxia ($p = 0.034$, altitude contrast; Figure 4B,E) and increased by power output ($p < 0.001$, power output contrast) to a similar extent in normoxia and hypoxia ($p = 0.488$, altitude by power output interaction). There was no main effect of leg

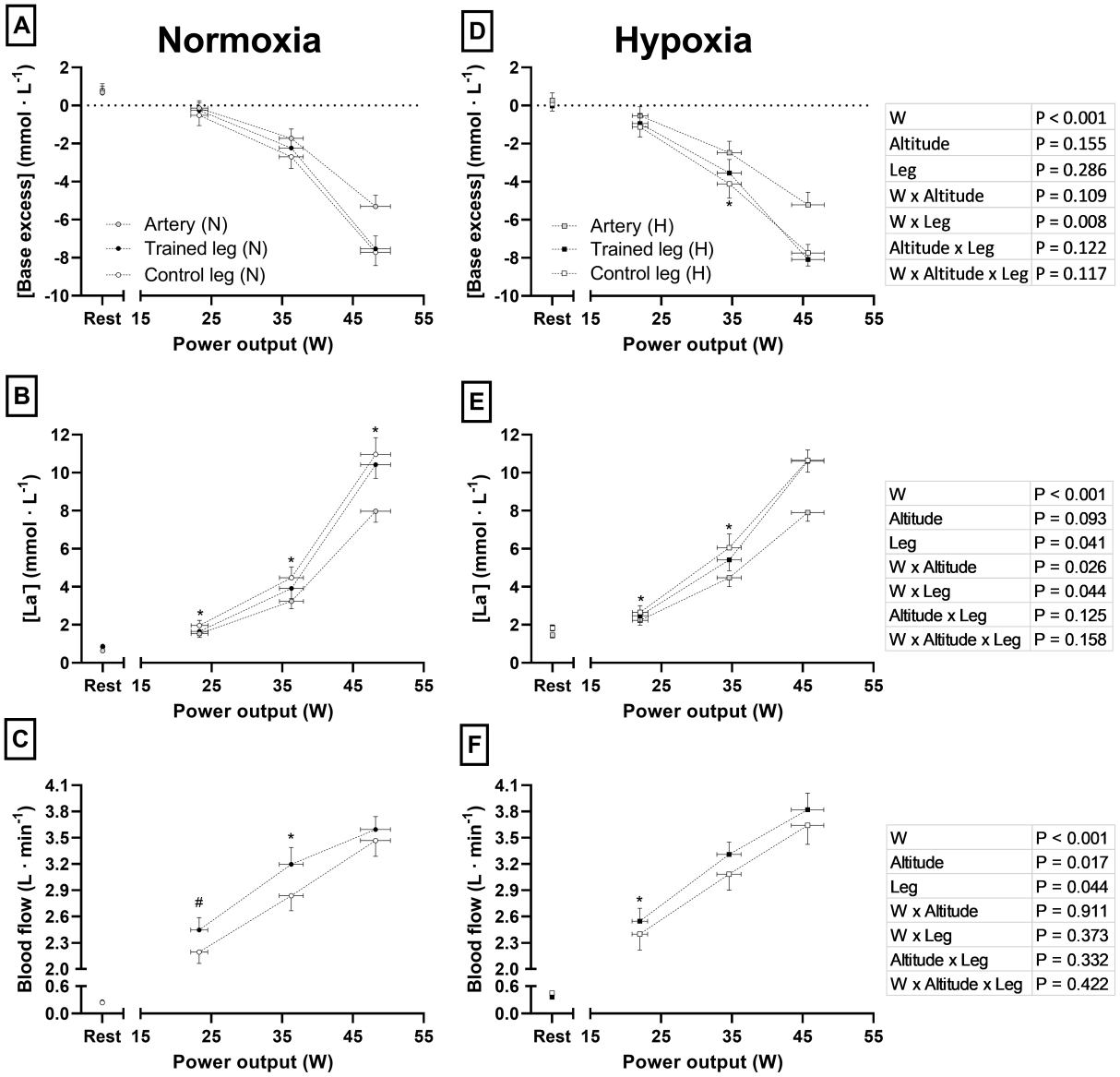

FIGURE 3 | Arterial and femoral venous base excess (A, D) and plasma lactate concentration ($[La^-]$) (B, E) in normoxia (N) and hypoxia (H) for the trained leg and the control leg as a function of the power output (W). In (C) and (F), the leg blood flow is presented. The *p*-values from the three-way ANOVA main effects (power output, altitude, and leg) and their interactions are embedded in the graphs (only venous samples and leg blood flow). The data are means, and error bars denote SEM. *Significant ($p < 0.05$) and #trend toward ($0.05 \leq p \leq 0.10$) difference between legs (post hoc comparisons).

($p = 0.527$, leg contrast), but the trained leg increased its lactate-independent H^+ release more from low to high intensities than the control leg ($p = 0.013$, leg by power output interaction; normoxia: $\Delta 3.7 \pm 2.0$ vs. $\Delta 3.1 \pm 2.4$ mmol min^{-1}, respectively; hypoxia: $\Delta 5.2 \pm 3.4$ vs. $\Delta 3.3 \pm 1.9$ mmol min^{-1}, respectively). When data from both legs were consolidated to further test the impact of altitude, the lactate-independent H^+ release was higher at low (mean difference ± SD: 0.60 ± 1.00 mmol min^{-1}; $p = 0.022$) and moderate intensities (1.55 ± 1.25 mmol min^{-1}; $p < 0.0001$) in hypoxia than normoxia and tended to be higher at high intensity (1.48 ± 3.00 mmol min^{-1}; $p = 0.058$).

The total leg H^+ release (lactate-dependent + lactate-independent) was higher in hypoxia than in normoxia ($p = 0.047$, altitude contrast; Figure 4C,F) and increased by power output ($p < 0.001$, power output contrast) to a similar extent in normoxia and hypoxia ($p = 0.432$, altitude by power output interaction). There was no main effect of leg ($p = 0.652$, leg contrast), but the trained leg increased its H^+ release more from low to the high intensity than the control leg ($p = 0.020$, leg by power output interaction; normoxia: $\Delta 7.9 \pm 3.2$ vs. $\Delta 7.6 \pm 3.6$ mmol min^{-1}, respectively; hypoxia: $\Delta 10.2 \pm 5.2$ vs. $\Delta 7.8 \pm 3.3$ mmol min^{-1}, respectively) and there was a tendency to that this between-leg difference in delta value was more pronounced in hypoxia than in normoxia ($p = 0.096$, power output by altitude by leg interaction). When data from both legs were consolidated to further test the impact of altitude, the total H^+ release was higher at low (0.70 ± 1.23 mmol min^{-1}; $p = 0.027$) and moderate intensities (2.22 ± 1.80 mmol min^{-1}; $p < 0.0001$) in hypoxia than normoxia and tended to be higher at high intensity (1.96 ± 4.33 mmol min^{-1}; $p = 0.079$).

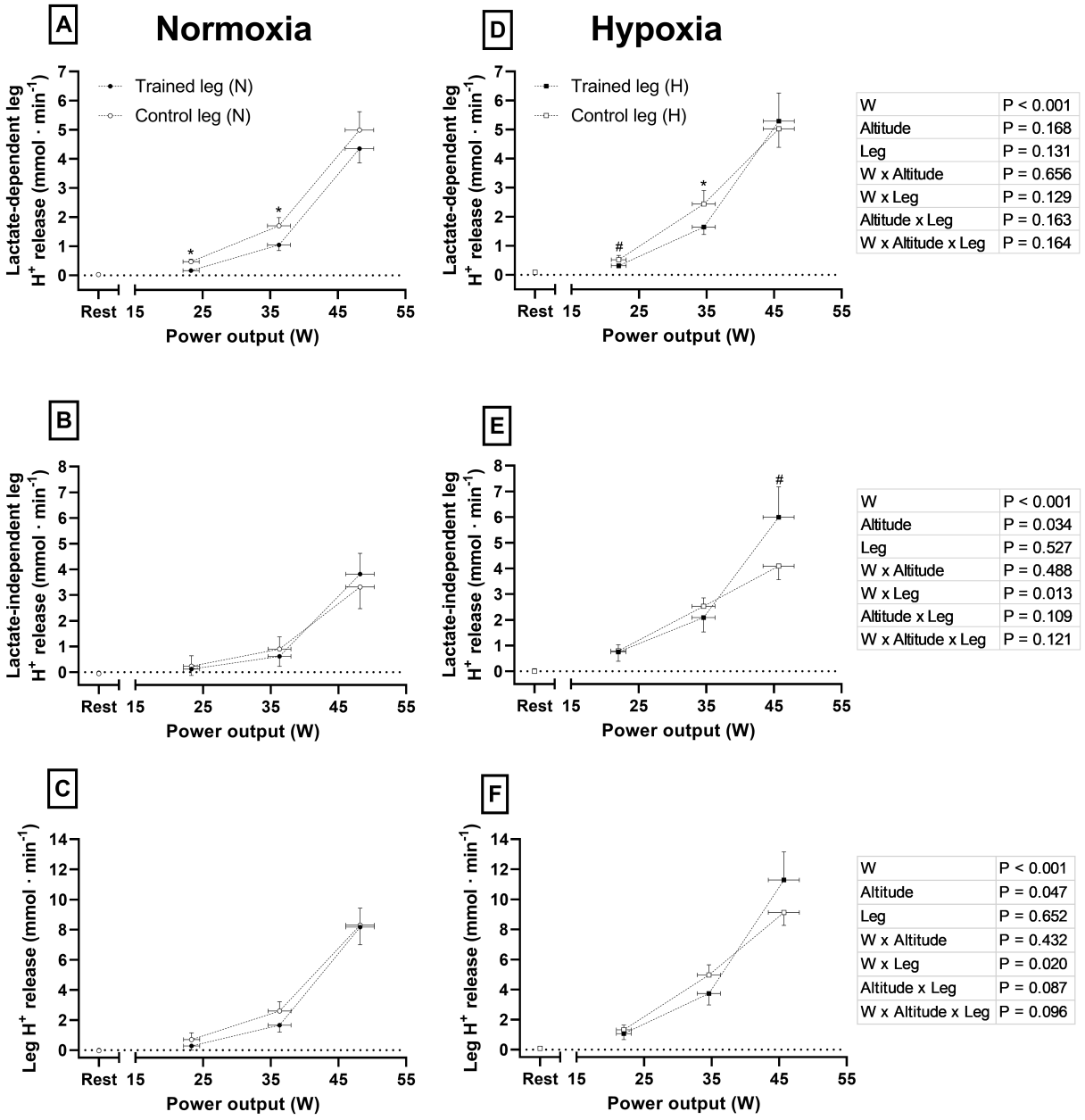

FIGURE 4 | Lactate-dependent H^+ release (A, D), the lactate-independent H^+ release (B, E), and the total leg H^+ release (C, F) for the trained leg and the control leg in normoxia (N) and hypoxia (H) as a function of the power output (W). The *p*-values from the three-way ANOVA main effects (power output, altitude, and leg) and their interactions are embedded in the graphs. The data are means, and error bars denote SEM. *Significant ($p < 0.05$) and #trend toward ($0.05 \leq p \leq 0.10$) difference between legs (post hoc comparisons).

3.5 | Leg K^+ Release

The arterial and femoral venous [K^+] were lower in hypoxia than in normoxia ($p = 0.001$ and $p = 0.038$, altitude contrasts for the artery and veins, respectively; Figure 5A,C) and increased by power output ($p < 0.001$, power output contrasts for artery and veins). This increase by power output was largest in hypoxia ($p = 0.025$ and $p = 0.006$, power output by altitude interactions for artery and veins, respectively). The femoral venous [K^+] was not different between legs at low–moderate exercise intensities, but increased more by power output in the control leg ($p = 0.002$, leg by power output interaction), leading to a significantly higher concentration than in the trained leg during high-intensity exercise in normoxia ($p = 0.014$) and hypoxia ($p = 0.003$).

The leg K^+ release increased by power output ($p < 0.001$, power output contrast) and was similar in normoxia and hypoxia ($p = 0.160$, altitude contrast; $p = 0.278$, power output by altitude interaction). It was a tendency to a main effect of leg ($p = 0.054$, leg contrast) and a significant leg-by-power output interaction ($p = 0.004$), with pairwise post hoc tests revealing no significant between-leg differences at low–moderate exercise intensities ($p > 0.078$) but a significantly lower K^+ release in the trained leg than in the control leg at the highest exercise intensity in normoxia ($p = 0.024$) and hypoxia ($p = 0.007$).

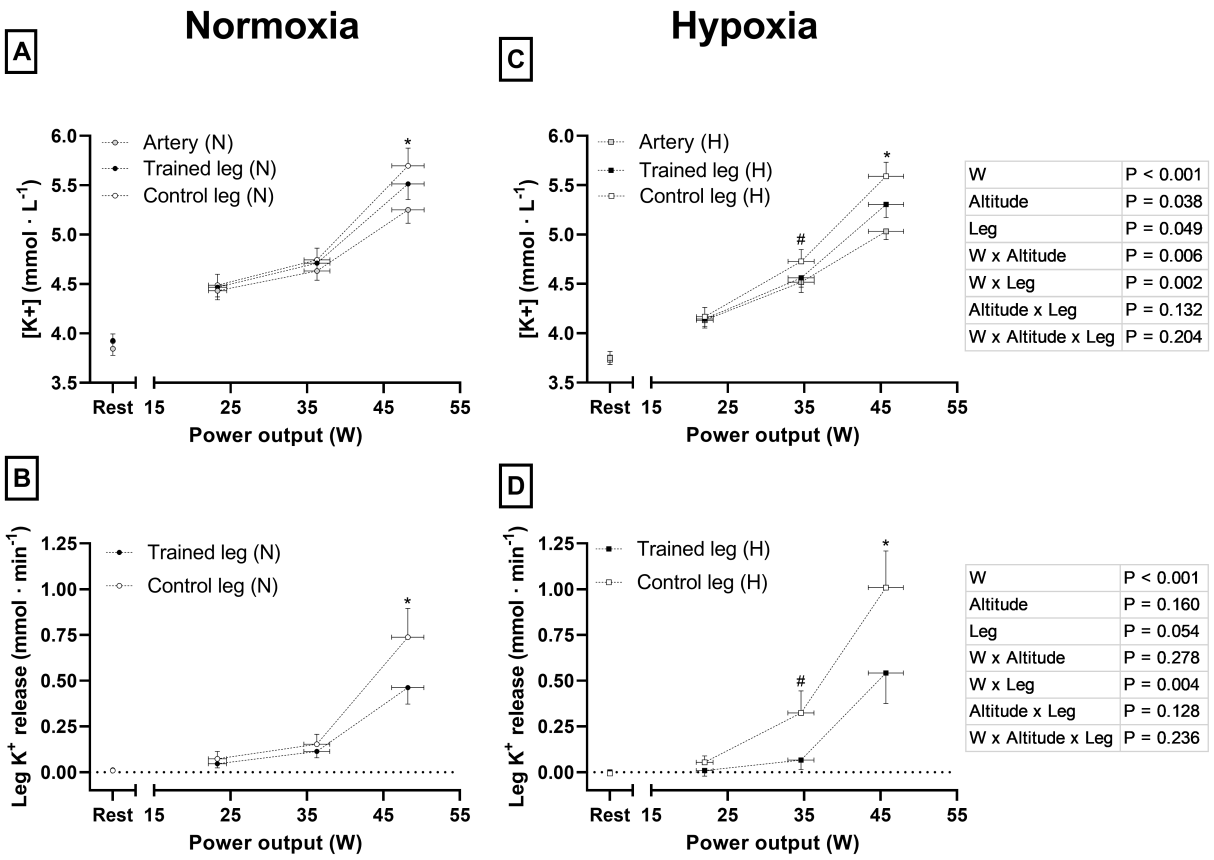

FIGURE 5 | Arterial and femoral venous plasma K^+ concentration ($[K^+]$) (A, C) and the leg K^+ release (B, D) for the trained leg and the control leg in normoxia (N) and hypoxia (H) as a function of the power output (W). The *p*-values from the three-way ANOVA main effects (power output, altitude, and leg) and their interactions are embedded in the graphs. The data are means, and error bars denote SEM. *Significant ($p < 0.05$) and #trend toward ($0.05 \leq p \leq 0.10$) difference between legs (post hoc comparisons).

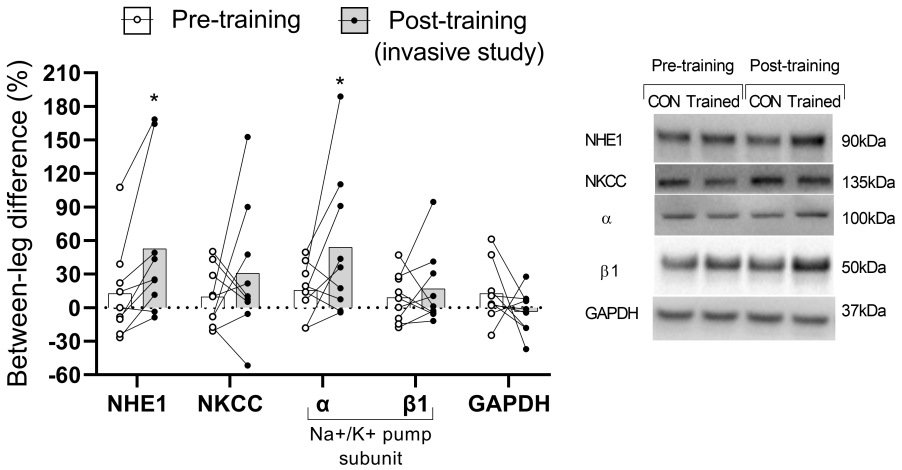

FIGURE 6 | Individual and mean between-leg differences in the Na^+/H^+ exchanger 1 (NHE1), the Na^+-K^+-$2Cl^-$ cotransporter (NKCC), and the Na^+/K^+ pump subunit α and β1 along with the loading control GAPDH before and after the training period. Representative blots for all proteins are shown on the right with their observed molecular weight. *Significant difference between legs at time-point ($p < 0.05$).

3.6 | Ion Transporter Abundance

Representative blots for all proteins are shown in Figure 6. No between-leg differences were identified pretraining ($p > 0.085$). Post-training, the trained leg achieved a higher abundance of NHE1 ($53 \pm 67\%$; $p = 0.047$) and Na^+/K^+ pump subunit α ($54 \pm 65\%$; $p = 0.036$) than the control leg while Na^+/K^+ pump subunit β1 ($17 \pm 34\%$; $p = 0.174$) and NKCC ($31 \pm 60\%$; $p = 0.161$) were similar.

4 | Discussion

The major findings of the present study were that moderate- to high-intensity endurance training improved skeletal muscle lactate-independent H^+ release during high-intensity exercise in hypoxia, coinciding with a higher abundance of NHE1. We also show that the total leg H^+ release was higher in hypoxia than in normoxia due to higher lactate-independent H^+ release, while the lactate-dependent H^+ release was similar. Lastly, training reduced the leg K^+ release during high-intensity exercise irrespective of altitude, which coincided with upregulated muscle Na^+/K^+ pump subunit α abundance and a higher peak power output during incremental 1L-KE exercise.

The experimental design used in the present study, with the simultaneous exercise of one trained and one control leg, offers several advantages over a pre- to post-test design or separate tests for a trained and an untrained control leg. An order effect and day-to-day variations in metabolism and performance are avoided, and the arterial inflow concentrations of substrates, ions, and metabolites are identical for both legs at all power outputs. Thus, any between-leg difference in the uptake or release of a substance should reflect local metabolism and/or altered blood flow regulation.

4.1 | Effects of Training on H^+ Release

The lactate-dependent and lactate-independent leg H^+ releases were relatively small at the low- to moderate-intensity power outputs and increased dramatically at the highest power output, as observed by others [5–7]. The lactate-dependent H^+ release was slightly lower in the trained leg than in the control leg at the low–moderate power outputs, likely due to the lower relative exercise intensity, higher capillary-to-fiber ratio and abundance of mitochondrial proteins (previously published data) [16], and slightly higher blood flow that may have attenuated the reliance on anaerobic energy production in the trained leg, but this difference vanished at the highest power output. These findings are similar to previous studies where moderate- and high-intensity endurance training have reduced muscle lactate release at moderate exercise intensity but increased the release during maximal two-legged cycling [23] and 1L-KE exercise [6]. This is caused by a lower intramuscular lactate accumulation at fixed submaximal power outputs after training and the tight relationship between the intramuscular lactate concentration and leg lactate release [24]. High-intensity training is also shown to upregulate glycolytic enzymes such as phosphofructokinase and pyruvate dehydrogenase [25] and the abundance of MCT [6] that may enhance the maximal production and release rates of lactate/H^+ during maximal exercise. This is further supported by increased lactate/H^+ peak release rates in sarcolemmal giant vesicles obtained from muscle homogenates of rat [26] and human [11] muscles after training. Thus, training-induced improvements in lactate-dependent H^+ efflux capacity are likely to be seen at maximal exercise intensities (i.e., in situations with high intramuscular acidosis and lactate accumulation), and it may be that the highest power output utilized in the present study was slightly too low for its detection (83% of $\dot{W}_{peak}$), especially since the relative intensity was lower for the trained than the control leg.

Importantly, the lactate-independent and the total leg H^+ releases were similar between legs at low–moderate intensities but increased more by power output for the trained than the control leg (significant power output by leg interactions: both $p < 0.02$), supporting that training enhances lactate-independent H^+ efflux capacity and improves pH regulation during high-intensity exercise. Moderate- and high-intensity training has previously been shown to reduce intracellular lactate- and H^+ accumulation at fixed power outputs [6, 12, 24], causing lower intracellular-to-interstitial lactate and H^+ gradients [6, 12]. Thus, a similar or larger H^+ release from the trained leg at a lower driving force across the sarcolemma than the control leg entails improved H^+ efflux capacity and potentially less fatigue development during high-intensity exercise [27]. This is supported by the lower perceived exertion for the trained leg during 2 L-KE, and the higher time to exhaustion (Δ108 s) [16] and peak power output (Δ8 W) during incremental 1L-KE exercise for the trained leg than the control leg. However, the potentially lower intracellular-to-interstitial lactate and H^+ gradients cannot be confirmed for the subjects in the present study since acute muscle biopsies were not taken directly after exercise. Nevertheless, the post-training muscle biopsies revealed a larger abundance of NHE1 in the trained leg than in the control leg, which likely contributed to the improved lactate-independent H^+ release at the highest power output in hypoxia. Increased NHE1 expression was found after 6 weeks of blood flow-restricted interval training, but did not improve the lactate-independent H^+ release compared with the control leg [12]. Similarly, 7–8 weeks of 1L-KE training at supramaximal intensities (~150% of 1L-KE $\dot{V}O_{2max}$) increased MCT and NHE1 abundances but resulted in both unchanged [11] and increased [6] lactate-dependent and lactate-independent H^+ releases during intense, exhaustive exercise. Despite these discrepancies, all the above studies have either found an increased H^+ release or a maintained H^+ release in combination with reduced intracellular-to-interstitial lactate and H^+ gradients during high- to maximal intensity exercise [6, 11, 12], thus, strongly pointing toward improved H^+ efflux capacity after training spanning moderate to supramaximal intensities.

4.2 | Effects of Training on K^+ Release

The trained leg displayed lower femoral venous [K^+] and leg K^+ release than the control leg during high-intensity exercise in normoxia and hypoxia, with no significant difference at low–moderate intensities. Unaltered leg K^+ v-a-diff or release rates after vs. before training at low–moderate exercise intensities [7, 28, 29] and at exhaustion during maximal exercise (i.e., at a higher power output after training) [7, 30] are frequent findings. This stresses that the same absolute power output at a high or near-maximal exercise intensity is most appropriate to facilitate the detection of training-induced improvements in K^+ handling [29], as in the present study (83% of 2L-KE $\dot{W}_{peak}$). The lower K^+ release at high-intensity exercise may result from a higher abundance of Na^+/K^+ pump subunit α in the trained leg (+54%) that elevated the capacity for K^+ re-uptake in the contracting cells. Theoretically, lower muscle K^+ efflux during repolarization can also induce a lower K^+ release from exercising muscles [2]. For instance, K^+ efflux is higher in fast-twitch than slow-twitch fibers in rats under electrical stimulation [31]. However, no difference in fiber type distribution existed between legs [16], and the

between-leg difference in relative exercise intensity was small, indicating a similar recruitment pattern. The ATP-sensitive K^+ channel (K_{ATP}) opens and causes K^+ efflux at low intracellular pH and ATP [32], and intensified training reduces K_{ATP} abundance [5]. Therefore, it may be that a lower abundance of K_{ATP} and/or less opening of K_{ATP} due to lower reliance on anaerobic energy turnover (i.e., lower glycolytic flux and H^+ accumulation) contributed to the lower K^+ release in the trained leg during high-intensity exercise. Despite this, increased K^+ re-uptake by the contacting cells is the most probable explanation for the reduced K^+ release from the trained leg. This may have counteracted the rundown of the muscle membrane potential, supported sarcolemmal excitability, and contributed to improved performance and lower perceived exertion during exercise with the trained leg. Previous training interventions (endurance-, speed-endurance-, and high-intensity interval training) have largely found increased abundance of one or more of the Na^+/K^+ pump subunits (subunit α1, subunit α2 and β1, or total complex) [7, 13–15, 29, 33], but reports of an unchanged abundance after training also exist [5]. Also, whether increased Na^+/K^+ pump abundance translates into improved K^+ homeostasis remains controversial. Lower rates of K^+ accumulation in arm venous blood during intense exercise have been observed after training that has coincided with improved short- [14, 15] and long-duration [14] performance. Conversely, high-intensity interval training of one leg did not alter leg K^+ release during submaximal steady-state exercise or incremental exercise to exhaustion compared with the control leg. In the latter study, however, the trained leg's interstitial $[K^+]$ was reduced, as measured by microdialysis, which indicates improved K^+ handling [7]. In another study, when well-trained cyclists replaced their regular training with speed-endurance- and high-intensity interval training, the interstitial $[K^+]$ and the leg K^+ release were unaltered despite improved performance [5]. An explanation for the discrepancies with no improvement in K^+ homeostasis in the latter study may be that no changes in muscle K^+ transmembrane proteins were observed, potentially explained by their initial high training status [5].

4.3 | Effects of Hypoxia on Acid–Base Balance and H^+ Release

As expected, the arterial and venous pH increased, and PCO_2 and $[HCO_3^-]$ decreased in acute hypoxia versus normoxia due to increased ventilatory drive and blood CO_2 removal [34]. Conversely, the nonrespiratory component of the acid–base balance (i.e., [BE]) was unaffected by hypoxia and indicated unchanged acid–base balance at rest ($[BE] \sim 0.3–0.7\,mmol\,L^{-1}$) but with progressing metabolic acidosis leading to a base deficit of ~5 and ~8 $mmol\,L^{-1}$ on the arterial and venous side, respectively, during high-intensity exercise. Interestingly, we observed a higher H^+ release in hypoxia than in normoxia, owing to increased lactate-independent- but not lactate-dependent H^+ release. Few previous studies have examined the influence of hypoxia on muscle H^+ release [34–36]. At submaximal and near-maximal power outputs during incremental cycling (100–300 W), total leg H^+ release was unaltered compared with sea level when acutely breathing a hypoxic gas mixture of 12.6% O_2, equaling 4100 m [36]. However, in another study, breathing 10% O_2 was shown to increase leg H^+ release at the same absolute submaximal power outputs (80–240 W) as at sea level, but the H^+ release reached the same levels at exhaustion (~240 W in hypoxia vs. ~340 W at sea level) [34]. Therefore, our observation of a slightly increased H^+ release during submaximal exercise in hypoxia is likely an effect of increased relative exercise intensity increasing the glycolytic rate and intracellular metabolic perturbations rather than a higher H^+ efflux capacity in hypoxia per se.

5 | Conclusions

The present study shows that 6 weeks of moderate-intensity continuous endurance training combined with high-intensity interval training increases leg lactate-independent H^+ release and reduces leg K^+ release during high-intensity exercise that coincides with higher NHE1 and Na^+/K^+ pump subunit α abundances.

5.1 | Perspective

Intracellular proton accumulation (reduced muscle pH) and interstitial accumulation and release of potassium to the venous blood are modest during low–moderate intensities but accelerate during high-intensity exercise. Herein, training was shown to efficiently improve leg lactate-independent H^+ release and reduce leg K^+ release during high-intensity exercise. These improvements in regulating transmembrane ion gradients may support sarcolemmal excitability and intracellular pH regulation and delay perceived muscle fatigue during intense exercise.

Author Contributions

Conception and design of the experiment: Ø.S. and J.H. Data collection: Ø.S., C.C., J.A.C., and J.H. Analysis of data: Ø.S. Interpretation of data: Ø.S., C.C., J.A.C., and J.H. Writing the first draft: Ø.S. Revising the manuscript: Ø.S., C.C., J.A.C., and J.H. All authors have read and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper. There are no financial conflicts of interest to disclose. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Data Availability Statement

The data supporting these findings are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.

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