Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats

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Submitted 10 November 2008; accepted in final form 27 January 2009

ENDURANCE WEIGHT-BEARING or impact exercise with moderate intensity such as running is known to benefit calcium metabolism by increasing bone formation rate, bone mineral density (BMD), and bone strength and by decreasing urinary calcium excretion, thereby inducing positive calcium balance in trained animals (22, 26). These beneficial effects of endurance running are contributed, in part, by the enhanced intestinal calcium absorption (58). However, little is known regarding the mechanism by which exercise affects the intestinal calcium transport.

In contrast to running, swimming is an unloaded nonimpact exercise that is suitable for several groups of patients, such as cardiovascular, postmenopausal, osteoporotic patients, and patients, who need to prevent high blood pressure or impact that can cause bone and joint injuries (17, 38, 43, 49). Although the effect of swimming on bone metabolism may not be as obvious as seen in running, it appears to increase bone mass, particularly that of the lower extremities in young female swimmers (15). Long-term swimming with moderate intensity is suggested to have a rehabilitative effect on bone in postmenopausal women, since it can increase BMD in estrogen-deficient aging animals (38). Hart et al. (22) also demonstrated a greater bone mass, BMD, and bone strength in ovariectomized swimming rats. However, the effect of endurance swimming on the intestinal calcium absorption has never been reported.

Calcium traverses the intestinal epithelium through the transcellular and paracellular pathways (24). The transcellular calcium transport is a 1,25(OH)2D3-dependent three-step process consisting of 1) calcium entry from the luminal side via the transient receptor potential vanilloid family calcium channels (TRPV) 5 and 6, 2) cytoplasmic calcium translocation in a calbindin-D9k-bound form, and 3) basolateral calcium extrusion via the plasma membrane Ca2+/H+ exchanger (PMCAb) and Na+/Ca2+ exchanger (NCX1; Ref. 24). On the other hand, the paracellular calcium transport is regulated by the tight junction that acts as a size and charge-selective barrier for ion movement (52). It has been shown that some transmembrane tight junction proteins of the claudin family, e.g., claudin-2, -3, and -12, which polymerize to form ion-selective paracellular channels, can regulate the transepithelial calcium transport (8, 18). Interestingly, the expression of claudin-3 was found to be 1,25(OH)2D3 dependent (32). Another transmembrane protein of tight junction, occludin, is also important for the maintenance of epithelial integrity (52). A number of cytoplasmic tight junction proteins, e.g., zonula occludens proteins (ZO)-1, -2, and -3 and cingulin, could also regulate the expression, distribution, and functions of claudins (21, 52). Moreover, the paracellular calcium transport may occur with the solvent drag, which is driven by the paracellular standing sodium gradient created by Na+/K+ ATPase (7). Generally, the transcellular calcium transport is mostly confined to the duodenum, proximal jejunum, and cecum, whereas the paracellular mechanism is found in all segments (16, 29). The expression pattern of those calcium transport-related genes has been, however, intensively studied in the duodenum but not in the distal segments.

Previous investigations (8, 47, 51) demonstrated that the enhanced intestinal calcium absorption in several physiological and pathological conditions, such as exposure to 1,25(OH)2D3,
estrogen supplementation, or chronic metabolic acidosis, was usually correlated with upregulation of genes related to transcellular and/or paracellular calcium transport. Thus the enhanced calcium absorption associated with endurance swimming may also result from the altered expression of these genes. In addition, endurance exercise was known to induce transport of other ions and long-term metabolic changes in the intestinal epithelial cells (3, 19, 33, 53). Thus a genome-wide study using microarray should help indentify other intestinal genes that were affected by endurance swimming.

Therefore, the objectives of the present study were 1) to study mRNA expression of genes related to calcium absorption along the whole length of the intestine, 2) to demonstrate the effect of endurance swimming on the intestinal calcium absorption as well as on the plasma calcium and inorganic phosphate concentrations, 3) to investigate the effects of swimming on the expression of genes related to calcium absorption, and 4) to identify other swimming-altered intestinal genes by using microarray technique.

**MATERIALS AND METHODS**

**Animals.** Female Sprague-Dawley rats, weighing 180–200 g (10-wk-old), were obtained from the National Laboratory Animal Centre (Thailand). They were housed in the laboratory animal husbandry unit for at least 7 days before the experiments under 12:12-h light-dark cycle and were fed regular pellets, containing 1.0% calcium and 0.9% phosphorus (Perfect Companion, Bangkok, Thailand), and distilled water ad libitum. The room had a temperature of 20–25°C, humidity of 50–60%, and average illuminance of 150–200 lux in the daytime. Food intake was recorded daily. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University (Bangkok, Thailand). All animals were cared for in accordance with the principles and guidelines of the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.”

**Swimming protocol.** The present swimming protocol known to be a nonimpact endurance exercise with moderate intensity was modified from the methods of Elhaı̅meur et al. (14) and the American Physiological Society’s “Resource Book for the Design of Animal Exercise Protocols.” In brief, rats were randomly divided into two groups. The swimming group was assigned to perform endurance swimming for 2 wk, while the age-matched controls remained sedentary in a swimming pool filled with tap water to a depth of 5 cm. Swimming rats were initially trained for a week until they could swim nonstop 1 h/day. Swimming frequency was 5 days/wk. Four rats concurrently swam in a glass swimming pool with a dimension of 0.8 × 0.8 × 0.8 m³. Each rat swam in a space of 0.4 × 0.4 × 0.8 m³ filled with 50 cm of tap water. The water temperature was maintained at 30–32°C. Body weight was recorded before training and then every swimming day for both control and swimming groups. Twelve hours after the last swimming session, animals were killed for determination of calcium flux or gene expression. To confirm that changes in calcium transport observed in the swimming group were due to the 2-wk training, calcium fluxes were determined in the age-matched untrained rats subjected to a single-bout swimming. In some experiments, the transepithelial calcium fluxes were immediately determined after the last swimming session (i.e., without 12-h rest).

**Tissue preparation.** A median laparotomy was performed while the animals were under 50 mg/kg pentobarbitone sodium intraperitoneal (Abbott, North Chicago, IL) anesthesia. Intestinal segments, including duodenum (10 cm), proximal and distal jejenum (10 cm each), ileum (8 cm), cecum (4 cm), and proximal and distal colon (8 cm each), were removed, rinsed in an ice-cold bathing solution, and cut longitudinally along the radix mesenterii to expose the mucosa. The duodenal segment was then mounted in a modified Ussing chamber to measure calcium flux as described previously (28). The tissue was incubated for 20 min in the chamber before the 60-min calcium flux measurement was carried out. As for the quantitative real-time PCR (qRT-PCR) and microarray studies, intestinal epithelial cells were collected by scraping the mucosal surface once with an ice-cold glass slide (10, 28). Gastrocnemius muscle was collected for analysis of citrate synthase activity. Femora were dissected for determination of BMD and bone mineral content (BMC). Arterial blood sample (5 ml) was drawn from the left ventricle. Wet and dry heart weights were also determined.

**Measurement of BMD and BMC.** Femoral BMD and BMC were assessed by dual-energy X-ray absorptiometer (model Lunar PIXImus2; GE Medical Systems, Madison, WI), operated with a software version 2.10. The dual-energy supply was 80/35 kVp at 50 μA.

**Bathing solution.** The bathing solution, continuously gassed with humidified 5% CO₂-95% O₂, contained (in mmol/l) the following: 118 NaCl, 47 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution was maintained at 37°C (pH 7.4) and had an osmolality of 290–292 mosmol/kg H2O as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA). Water used in calcium flux measurement had a resistance >18.3 MΩ cm and a free-ionized calcium concentration <2.5 mmol/l.

**Electrical measurement.** Three electrical parameters, i.e., potential difference (PD), short-circuit current (Iₛ), and transepithelial resistance (TER), were determined as previously described (28). A pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/l KCl) were placed near each surface of the mounted duodenal tissue for measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) and finally to a PowerLab 4/30 operated with a software Chart 5.2.2 for Mac OS X (ADInstruments; Colorado Springs, CO). Another pair of Ag/AgCl electrodes was placed close to the other end of the hemichamber to supply Iₛ, which was also measured by a PowerLab 4/30 connected in series to the EVC-4000 current-generating unit (World Precision Instruments).

**Calcium flux measurement.** Transepithelial calcium fluxes were determined by the modified method of Charoenphandhhu et al. (8). Although we measured calcium fluxes in both small and large intestine, the duodenum was of interest because it is the most efficient site for calcium absorption (16, 29). After a 20-min incubation in the Ussing chamber, the tissue was bathed on both sides with fresh bathing solution. One side was ⁴⁵CaCl₂-containing bathing solution (initial amount of 5 mCi/ml, final specific activity of ~450–500 mCi/mol, Amersham, Buckinghamshire, UK; cold calcium concentration of 1.25 mmol/l). The transepithelial calcium flux (Jₜₚ₋ₗ; C from the hot side (H) to the cold side (C) was calculated by Equations 1 and 2.

\[
J_{H \rightarrow C} = R_{H \rightarrow C}(S_H - A) \quad (1)
\]

\[
S_H = C_H/C_{tot} \quad (2)
\]

where \(R_{H \rightarrow C}\) is the rate of tracer appearance in the cold side (cpm/h); \(S_H\) is the specific activity in the hot side (cpm/mmol); \(A\) is the surface area of the tissue (0.69 cm²); \(C_H\) is the mean radioactivity in the hot side (cpm/ml); and \(C_{tot}\) is the total calcium in the hot side (mmol). Radioactivity of ⁴⁵Ca was analyzed by liquid scintillation spectrophotometry (model Tri-Carb 3100TR; Perkin-Elmer, Boston, MA). Total calcium concentration of the bathing solution was analyzed by atomic absorption spectrophotometry (model SpecTRAA-300; Varian Techron, Springvale, Australia).

**Total RNA and cDNA preparation.** With the use of TRIzol reagent (Invitrogen, Carlsbad, CA), the total RNA was prepared from intestinal epithelial cells, as previously described (10, 28). The total RNA was treated with RQI DNase (Promega, Madison, WI) and later...
purified with RNeasy Mini kit (Qiagen, Valencia, CA). The purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which fell in the range of 1.8–2.0. Integrity of RNA was analyzed by denaturing agarose gel electrophoresis with the 28S:26S-rRNA band appearing approximately twice as intense as the 18S-rRNA band. The total RNA samples were used for both qRT-PCR and microarray. As for qRT-PCR, 1 μg total RNA was reverse-transcribed with oligo-dT20 primer and iScript kit (Bio-Rad, Hercules, CA) by a thermal cycler (model MyCycler; Bio-Rad). GAPDH, a housekeeping gene, served as a control gene to check the consistency of the reverse transcription (percent coefficient of variation <5%; n = 30).

**qRT-PCR and sequencing.** Primers used in the present study (Supplemental Table S1; supplemental data for this article are available online at the Am J Physiol Endocrinol Metab website.) were designed by Oligo 6 (Molecular Biology Insights, Cascade, CO) and Primer VALIDATOR 1.4 (Narut Software, Bangkok, Thailand) or as previously used by Chaoenphandhu et al. (8). qRT-PCR and melting curve analyses were performed by Bio-Rad MiniOpticon with iQ SYBR Green SuperMix (Bio-Rad). Gene expression levels were first normalized by GAPDH expression. The PCR products were also visualized on a 1.5% agarose gel stained with 1.0 μg/ml ethidium bromide under a ultraviolet transilluminator (Alpha Innotech, San Leandro, CA). After electrophoresis, all PCR products were extracted by the HiYield Gel/PCR DNA extraction kit (Real Biosystems, Foster City, CA). qRT-PCR experiments were performed in triplicate.

**Microarray.** The total RNA samples were obtained from six swimming rats and six age-matched control rats. The Illumina’s high-performance BeadArray technology was used in the present microarray study because of the reported high selectivity and sensitivity in gene expression profiling as a result of the high redundancy of each probe built in the BeadArray (31). As for the protocol, Illumina TotalPrep RNA amplification/in vitro transcription kit (catalog no. I11791; Ambion, Austin, TX) was used to amplify and generate biotinylated cRNA from 500 ng total RNA from duodenal scraping for hybridization with Illumina BeadChip array. After purification, biotinylated duodenal cRNA was quantified with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA); 750 ng of biotinylated cRNA from each duodenal sample were individually hybridized on the RatRef-12 Expression BeadChip (binary manifest file version V1.0_R0_1122119_A; Illumina, San Diego, CA). Each BeadChip constructed with BeadArray technology contains 12 whole-genome gene expression arrays, thus allowing 12 independent samples to be hybridized to a single chip. Each array, which can probe 21,910 genes, consists of 22,523 oligonucleotide probes (50-base gene-specific sequence and 29-base address sequence per probe) selected primarily from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database (release 16; http://www.ncbi.nlm.nih.gov/RefSeq). After incubation at 58°C for 20 h, the BeadChip was washed, blocked, and incubated at room temperature with streptavidin-Cy3-containing solution (Illumina) according to the manufacturer’s instruction. Fluorescent signals were scanned with BeadStation 500GX Genetic Analysis System (Illumina). Image registration and data extraction were automatic with BeadStudio 3.1.1 (Illumina). Complete details of the microarray data can be found in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under Accession No. GSE13499.

**Citrate synthase activity assay.** Gastrocnemius muscles from 2-wk-swimming rats were collected at 12 h posttraining. To determined citrate synthase activity, muscles were homogenized in ice-cold homogenizing buffer (pH 7.4) containing 20 mmol/l HEPES, 0.25 mmol/l sucrose, and 1 mmol/l EDTA, as previously described (48). Immediately before assay, 50 μl homogenate were diluted with 1.2 ml of 100 mmol/l Tris buffer (pH 8.0) containing 3 mmol/l acetyl CoA, and 1 mmol/l 5,5′-dithiobis-2-nitrobenzoic acid (all purchased from Sigma). To determine the basal activity, the diluted homogenate was read for the optical density change at 412 nm every 30 s for 4 min at 25°C by a spectrophotometer (model UV-2550; Shimadzu, Kyoto, Japan). The reaction was started by adding oxaloacetate (Sigma) dissolved in 100 mmol/l Tris buffer and 100 mmol/l K2HPO4 (3:1) to obtain a final concentration of 6.5 mmol/l.

**Arterial blood gas analysis and blood chemistry.** Blood gas analysis was performed with an automated equipment (model Ultra C; Nova Biomedical, Waltham, MA) to obtain plasma pH, partial pressure of O2, partial pressure of CO2, and HCO3− concentration. Total plasma calcium and inorganic phosphate were determined by modified o-creolphosphate complexone and phosphomolybdate-based kits, respectively, by using Dimension Rxi analyzer (Dade Behring, Marburg, Germany). Free-ionized calcium was measured by ion-selective electrode (model Stat Profile CCX; Nova Biomedical) under an anaerobic environment. Plasma lactate concentration was analyzed by the lactate oxidase technique with Stat Profile CCX system (Nova Biomedical).

**Data analysis.** Body weight, heart weight, blood chemistry, muscle enzyme activity, and calcium flux are expressed as means ± SE. Unless otherwise specified, comparisons between two groups were performed by Student’s t-test, while multiple comparisons were performed by one-way ANOVA with Dunnett’s posttest. The level of significance for all statistical tests was P < 0.05. Data were analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA). Differential gene expression of microarray and qRT-PCR was considered significant when there was a twofold or greater difference in expression between the control and swimming groups (9). Microarray data were analyzed by BeadStudio Gene Expression Module 3.2.3 and Genome Viewer 3.1.7 (Illumina). False discovery checks were performed with the “compute false discovery rate” function of the BeadStudio. The Illumina’s Cubic-Spline normalization algorithm was applied to adjust sample signals to minimize the effects of variation arising from nonbiological factors. Comparison between two groups of samples (control vs. swimming) was done with the t-test differential expression algorithm (detection P value < 0.05). Gene Ontology (GO) analysis for characterizing the biological properties of gene products was performed with AmiGO (http://amigo.geneontology.org) according to the GO Consortium’s instruction at the GO website (http://www.geneontology.org). The results of qRT-PCR were compared using 2−ΔΔCt method (34) and expressed as log2 means ± SE. All graphics were obtained using GraphPad Prism 5.

**RESULTS**

**Cardiac hypertrophy and the enhanced citrate synthase activity confirmed successful exercise training.** After 2 wk of swimming, the wet heart weight, dry heart weight, wet heart weight per body weight, and dry heart weight per body weight were significantly increased (Fig. 1, A and D). Swimming also increased the citrate synthase activity in gastrocnemius muscle by ∼40% (Fig. 1E) but did not change plasma lactate concentration (Table 1). Swimming rats manifested normal plasma concentrations of calcium, phosphate, and HCO3− as well as normal plasma pH and arterial blood gas (Table 1). Arterial blood gas and blood chemistry determined immediately after the last training session (Table 1) and 12 h posttraining (data not shown) were not different. Our results indicated that the present training was a nonimpact endurance exercise with moderate intensity (36). However, a 2-wk period may be too short to demonstrate the beneficial effect of swimming on bone, since femoral BMD and BMC of swimming rats were not changed (Supplemental Fig. S1).
Endurance swimming stimulated the transepithelial calcium transport in the duodenum, proximal jejunum, and cecum. In the Ussing chamber study, the duodenum of swimming rats that had a 12-h rest before the calcium flux determination showed an increase in the transepithelial calcium transport by ~39% (Fig. 2A). However, when calcium flux was measured immediately after swimming, there was no change in the transepithelial calcium transport (Fig. 2A). In addition, endurance swimming did not affect the electrical parameters, namely potential difference, short-circuit current, and TER, in the duodenal epithelium (Table 2). Besides duodenum, endurance swimming (with 12-h rest) significantly increased the mucosa-to-serosa calcium flux in the proximal jejunum and cecum by ~120 and ~32%, respectively, whereas it decreased that in the proximal colon by ~34% (Fig. 2B). Single-bout swimming in untrained rats changed neither calcium flux (Fig. 2A) nor electrical parameters (Table 2).

Epithelial cells of all intestinal segments expressed essential genes required for calcium absorption. Since endurance swimming significantly altered the intestinal calcium absorption, we further investigated the expression of the genes associated with calcium transport in the entire length of the intestine. Before the differential expression study in swimming rats, the normal profiles of calcium transporter genes were demonstrated. The results showed that all intestinal segments, i.e., duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon (n = 10 each), strongly expressed the genes involved in the transcellular calcium transport, i.e., TRPV5, TRPV6, calbindin-D9k, PMCA1b, and NCX1 (Fig. 3A), as well as those possibly involved in the paracellular ion transport, i.e., Na⁺-K⁺-ATPase (β1-subunit), ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12 (Fig. 3B). A classical

Endurance swimming stimulated the transepithelial calcium transport in the duodenum, proximal jejunum, and cecum. In the Ussing chamber study, the duodenum of swimming rats that had a 12-h rest before the calcium flux determination showed an increase in the transepithelial calcium transport by

Table 1. Arterial blood gas and blood chemistry after 2 wk of swimming

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age-matched control (n = 9)</th>
<th>2 wk swimming (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma calcium, mmol/l</td>
<td>2.68±0.07</td>
<td>2.77±0.04</td>
</tr>
<tr>
<td>Ionized calcium, mmol/l</td>
<td>1.17±0.06</td>
<td>1.21±0.03</td>
</tr>
<tr>
<td>Inorganic phosphate, mmol/l</td>
<td>2.79±0.08</td>
<td>3.07±0.07</td>
</tr>
<tr>
<td>Plasma pH</td>
<td>7.39±0.05</td>
<td>7.33±0.02</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td>35.43±2.66</td>
<td>34.93±1.55</td>
</tr>
<tr>
<td>Partial pressure O₂, kPa</td>
<td>9.66±0.43</td>
<td>8.76±0.43</td>
</tr>
<tr>
<td>Partial pressure CO₂, kPa</td>
<td>2.73±0.39</td>
<td>2.80±0.26</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>3.23±0.65</td>
<td>1.87±0.35</td>
</tr>
</tbody>
</table>

Values are means ± SE. All parameters were immediately determined after the last swimming session.

Fig. 2. A: duodenal calcium transport in the control, 2-wk swimming, and single-bout swimming rats. All rats were age matched. Calcium fluxes were measured immediately after swimming (Im.) or 12 h postswimming. B: mucosa-to-serosa calcium fluxes determined at 12 h postswimming in the proximal (PJ) and distal jejunum (DJ), ileum (I), cecum (CE), and proximal (PC) and distal colon (DC). Since the intestinal tissue was bathed on both sides with physiological bathing solution containing 1.25 mmol/l calcium, the observed calcium fluxes represented the active calcium transport. *P < 0.05, **P < 0.01, compared with the control group. Numbers in parentheses represent the number of tissues.
Interestingly, expressions of some calcium transporter genes, pressed along the whole length of the intestine (Fig. 3) or 12 h postswimming.

basolateral side. Electrical parameters were measured immediately after swim-

nuclear receptor for 1,25(OH)2D3, i.e., VDR, was also expressed along the whole length of the intestine (Fig. 3A).

Endurance swimming differentially changed the expression of calcium transporter genes in the small and large intestine. Expression of calcium transporter genes in swimming rats was demonstrated by qRT-PCR. Since 2 wk of swimming did not change GAPDH expression (Fig. 4A), mRNA expression levels were first normalized by GAPDH expression. As seen in Fig. 4, B and G, the small intestine (n = 10) responded to endurance swimming by upregulating VDR and the transcellular genes, i.e., TRPV5, TRPV6, calbindin-D9k, PMCA1b, and NCX1, whereas the large intestine (n = 10) showed no response or responded by decreasing those transcripts. Regarding the paracellular genes, the expression of Na+–K+–ATPase (β1-subunit) and claudin-12 was modestly changed after swimming (Fig. 5, A and I). The expression of ZO-1, ZO-2, ZO-3, cingulin, and occludin was markedly upregulated in the duodenum but was downregulated in the colon (Fig. 5, B–F). Interestingly, clau-
din-2 was upregulated in most intestinal segments, while clau-
din-3 was upregulated in the duodenum, ileum, and cecum but was downregulated in the colon. The present data also indicated that the proximal parts of the small intestine, which are generally the efficient sites for calcium absorption (16, 29), responded well to endurance swimming by increasing the expression levels of calcium transporter genes.

Microarray study revealed transcriptome responses of the duodenal epithelial cells after 2 wk of endurance swimming. Besides calcium absorption, endurance exercise has been reported to affect absorption of other electrolytes, nutrients, and water as well as intestinal permeability, presumably at the genomic level (3, 19, 33, 53). Therefore, the genome-wide microarray study was performed in duodenal cells from control (n = 6) and swimming rats (n = 6) to demonstrate transcriptome responses after swimming. Among the 21,910 genes, microarray analysis detected a total of 490 genes being upregulated by more than twofold (459 known genes and 31 unknown genes) and 211 genes downregulated by lower than −2-fold (188 known genes and 23 unknown genes) in the duodenal epithelial cells of swimming rats (Fig. 6, A and B; Supplemental Tables S2 and S3). Analysis of the distribution of fold changes demonstrated that ~60% of the swimming-altered transcripts were increased in the range of two- to fourfold (267 known genes and 20 unknown genes) or were decreased in the range of −2- to −4-fold (108 known genes and 12 unknown genes). To verify the microarray results, 13 known genes with sequences available in the NCBI database were selected to be tested by qRT-PCR according to their biological functions as defined in GO categories, i.e., one to three genes per each category (Supplemental Table S1). As shown in Fig. 6C, the qRT-PCR results of all transcripts agreed with the microarray results in response to swimming.

GO analyses of the genes changed by >10-fold further showed that there were a total of 61 annotated genes in the upregulated set and 22 annotated genes in the downregulated set (Fig. 6D). The upregulated genes were predominantly involved in the molecular function (19 genes, 26.76% of >10-fold upregulated genes), cellular metabolic process (10 genes, 14.08%), cellular component (9 genes, 12.68%), regulation of biological process (7 genes, 9.86%), and transport (4 genes, 5.63%). Within the downregulated group, the majority of genes was involved in the molecular function (6 genes, 20.00% of less than −10-fold downregulated genes), cell communication (4 genes, 13.33%), and regulation of biological process (4 genes, 13.33%). The gene names in each category are presented in Supplemental Table S4.

Further analysis of all upregulated and downregulated genes in the transport category (GO identifier 0006810) demonstrated that endurance swimming changed the expression of several genes involved in the transport of ions, such as Ca2+, Na+, K+, Cl–, and Zn2+, and nutrients, such as glucose and amino acids (Fig. 6, E and F). The majority of the swimming-altered transporter genes were related to K+ transport (Supplemental Tables S5 and S6), i.e., 11 upregulated transcripts (32.35% of upregulated transcripts in the transport category) and 4 downregulated transcripts (36.36%). Endurance swimming also increased the expression of some voltage-gated calcium channels, e.g., Cacna1h, Cacng8, and Cacnb3. In addition, a number of the swimming-altered transcripts (Supplemental Tables S2 and S3) were involved in water and electrolyte homeostasis, such as cholinergic receptor (Chrm2), kallikrein-kinin (Klk7 and Kng1), neuropeptide Y (Npy, Npy1r, and Npy2r), aquaporin (Aqp1 and Aqp2), renin (Ren1), arginine vasopressin (Avp), and galanin receptor (Galr3), as well as epithelial permeability and integrity, such as claudin-8 (Cldn8) and epimorphin (Epm1; Refs. 1, 2, 12, 23, 27, 35, 50).

DISCUSSION

How the intestinal calcium absorption was affected by exercise has so far received little attention, although it is the sole pathway to provide calcium for bone mineralization (24). In the present study, we demonstrated, for the first time, that swimming stimulated the intestinal calcium transport in female rats and altered the expression of several genes related to the intestinal calcium absorption. The complete expression profiles of calcium transporter genes along the entire length of the intestine as well as the transcriptome responses of the duodenal epithelial cells to swimming were elucidated. Our swimming protocol was considered an endurance nonimpact exercise with moderate intensity from its ability to induce cardiac hypertro-

phy and enhance muscle enzyme activity without elevation of plasma lactate concentration (36).
Swimming-enhanced calcium absorption has been anticipated, since endurance exercise is widely known to increase bone formation and elevate the plasma level of 1,25(OH)2D3, a classical stimulatory hormone for intestinal calcium transport (22, 57). However, previous studies of physical activity and intestinal calcium absorption usually used impact exercise, such as running, as a model. By using a flat-bed treadmill exercise, Yeh et al. (58) found that the exercise-trained female rats had a higher rate of duodenal calcium absorption than the sedentary controls. In well-trained male athletes orally administered with stable strontium as a marker of calcium absorption, Zittermann et al. (61) demonstrated increases in the fractional calcium absorption and serum 1,25(OH)2D3 level after a short-term moderate exercise bout. Another study (60) in male athletes with a minimum of 8 h/wk of endurance sport activities confirmed a 4% increase in the fractional strontium absorption compared with the age-matched sedentary controls. In contrast, immobilization by sciatic denervation in female rats led to a decrease in the duodenal calcium absorption, especially the passive components (58). A report (42) in paraplegic rats.

Fig. 3. Expression of nuclear vitamin D receptor (A; VDR) and genes related to the transcellular calcium transport, i.e., transient receptor potential vanilloid family calcium channels 5 and 6 (TRPV5 and TRPV6), calcium-binding protein calbindin-D28k (CaBP), plasma membrane Ca2+-ATPase (PMCA1b), and Na+/K+-ATPase (NKA), zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12, in the intestinal epithelial cells of normal rats. Seven intestinal segments, i.e., duodenum (D), proximal and distal jejunum, ileum, cecum, and proximal and distal colon (PJ, DJ, I, CE, PC, and DC, respectively), were used in this study (n = 10 per intestinal segment). Data were obtained from quantitative real-time PCR (qRT-PCR). GAPDH is a housekeeping gene used for normalization. Results are expressed as log means ± SE. *P < 0.05, cecum vs. duodenum (Mann-Whitney test).
showed increases in fecal calcium and phosphorus contents, presumably as a result of a decrease in the intestinal absorption of these elements. The present study demonstrated that swimming could also enhance the intestinal calcium absorption, similar to that seen during impact exercise. Although the stimulatory effect of swimming on calcium absorption was clearly observed at 2 wk, no change in BMD and BMC was observed in 2-wk-swimming rats (Supplemental Fig. S1). Since Hart et al. (22) were able to demonstrate positive bone response after 12 wk of swimming, it was likely that a detectable change in bone may require a longer than the 2-wk exercise training period.

It was noted that the enhanced calcium absorption was not observed immediately after exercise. This phenomenon could be explained by the fact that exercise normally suppressed the absorptive functions of the intestine by a number of mechanisms, e.g., modulation of autonomic outflow and reduction in mucosal blood supply (3), and such effects appeared to persist even in the removed tissue. On the other hand, an increase in calcium absorption at 12 h postswimming in 2-wk-trained rats and the absence of such a finding in single-bout control rats (n = 10). Gene expression was first normalized by GAPDH. Results are expressed as log₂ means ± SE. Twofold upregulation or downregulation are indicated by dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column.
with the highest rate of calcium absorption (30). Moreover, the distal jejunum and ileum also expressed all transcellular genes, even though they predominantly transport calcium via the paracellular passive mechanism (16, 29). Regarding the paracellular genes, all intestinal segments strongly expressed Na\(^+/\)H\(^+\)-K\(^+/\)H\(^+\)-ATPase and the relevant tight junction genes. It has been shown that Na\(^+/\)H\(^+\)-K\(^+/\)H\(^+\)-ATPases lining the paracellular membranes were essential for generation of solvent drag, which was a driving force for the paracellular ion transport, especially in the proximal small intestine (7). Claudin-2 and -12 created cation-selective tight junctional pores for the paracellular calcium movement (18). Expression of these paracellular genes was found to increase in conditions known to enhance the paracellular calcium transport, such as chronic metabolic acidosis (8, 10). Thus the present expression profiling suggested that both small and large intestine expressed important genes required for the transcellular and paracellular calcium transport.

Up until now, nothing has been known regarding changes in the expression of the calcium transporter genes in the intestine of exercise-trained rats. Studies (46) in immobilized rats

![Fig. 6. A–I: fold changes in the expression levels of the paracellular genes, i.e., \(\beta1\)-subunit of Na\(^+/\)K\(^+/\)ATPase (NKA), ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12, in the epithelial cells collected from the duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon of swimming (n = 10) vs. age-matched control rats (n = 10). Gene expression was first normalized by GAPDH. Results are expressed as log2 means ± SE. Twofold upregulation or downregulation are indicated by dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column.](http://ajpendo.physiology.org/)

Fig. 5. A–I: fold changes in the expression levels of the paracellular genes, i.e., \(\beta1\)-subunit of Na\(^+/\)K\(^+/\)ATPase (NKA), ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12, in the epithelial cells collected from the duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon of swimming (n = 10) vs. age-matched control rats (n = 10). Gene expression was first normalized by GAPDH. Results are expressed as log2 means ± SE. Twofold upregulation or downregulation are indicated by dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column.
showed decreases in the mRNA expression of TRPV5, TRPV6, calbindin-D_28k, but not PMCA_{1b} in the rat duodenum, with no change in the colon. Herein, we provided evidence that swimming, in contrast to immobilization, upregulated several intestinal genes for the transcellular calcium transport in the proximal small intestine and cecum, the important sites for active calcium absorption. Of interest was the ~210-fold upregulation of NCX1 mRNA in the duodenum, which probably contributed substantially to the observed swimming-stimulated transcellular calcium transport, despite the fact that NCX1 transport capacity is ~20% of PMCA_{1b} (55). It was possible that PMCA_{1b} expression was near maximum under normal condi-

A

B

E

F

Upregulated transcripts > 2-fold (34)

Downregulated transcripts < -2-fold (11)
transcellular calcium flux and L-type Cav activity (40) as well as the microarray data. Because of a strong correlation between Relative contributions of Cav and parvalbumin to the total calcium absorption remain to be investigated. It was speculated that their roles may become more significant when TRPV6 calcium absorption in the colonic segment (16).

Such changes should not have much impact on the overall calcium absorption because of the relatively low fractional calcium absorption in the colonic segment (16).

Of interest was also the swimming-induced upregulation of the voltage-gated calcium channel (Ca$_v$) subunits as shown in the microarray data. Because of a strong correlation between transcellular calcium flux and L-type Cav activity (40) as well as inhibition of the intestinal calcium absorption by the L-type Ca$_v$ blocker verapamil (45), the altered Ca$_v$ expression, in addition to TRPV5 and TRPV6, could partially explain the swimming-enhanced duodenal calcium transport. Upregulation of parvalbumin by approximately sixfold (Supplemental Table S2) may also help facilitate the cytoplasmic calcium translocation in the duodenal cells, similar to calbindin-D$_{9k}$ (6). Relative contributions of Ca$_v$ and parvalbumin to the total calcium absorption remain to be investigated. It was speculated that their roles may become more significant when TRPV6 and/or calbindin-D$_{9k}$ are absent or saturated as in the TRPV6/ calbindin-D$_{9k}$ double knockout mice in which active calcium transport occurred at a normal rate (5).

Besides the transcellular genes, swimming also altered the expression of paracellular genes in several intestinal segments (Fig. 5). Expression of claudin-2, -3, and -12, which could form cation-selective paracellular pores (18, 52), was increased in many segments of the small intestine, suggesting an enhanced paracellular calcium transport in these parts. The absence of changes in TER after swimming further evinced that claudins probably create calcium-selective pores without formation of nonselective paracellular pores or widening of the tight junction that usually leads to the alteration in the trans-epithelial potential difference and a reduction in TER (20).

Upregulation of other tight junction genes, e.g., ZOs, cingulin, and occludin, which could regulate the ion-selective functions of claudins (21, 52), was predominantly observed in the duodenum, whereas the colon manifested downregulation of these genes. Since Na$^+$-K$^+$-ATPase expression was slightly changed (Fig. 5), it was postulated that swimming did not alter the solvent drag-induced calcium transport in the small intestine.

The mechanism of swimming-altered gene expression is currently unknown, but it might be mediated by 1,25(OH)$_2$D$_3$, as the plasma 1,25(OH)$_2$D$_3$ concentration is typically elevated after endurance exercise (57, 58, 60). In contrast, immobilization was associated with suppressed 1,25(OH)$_2$D$_3$ synthesis (46).

Expression of several genes, such as TRPV6, calbindin-D$_{9k}$, PMCA$_{1b}$, and claudin-3, is also 1,25(OH)$_2$D$_3$ dependent (32, 51). In the present study, the expression patterns of those genes in swimming rats were consistent with that of VDR.

In addition to calcium absorption, microarray analysis revealed that swimming altered a number of intestinal functions, including cell communication, metabolism, and transport of several ions and nutrients, e.g., Na$^+$, K$^+$, Cl$^-$, and glucose. It is not known as to why the majority of upregulated transporters was related to K$^+$ transport. However, previous investigation suggested that the voltage-gated K$^+$ channels regulated membrane potential, which was, in turn, important for the Na$^+$-dependent absorption of glucose and amino acids in the small intestine (37). Although the physiological functions of most swimming-altered genes have never been investigated, a number of genes are possibly involved in water and electrolyte homeostasis, such as cholinergic receptor, kallikrein-kinin, neuropeptide Y, aquaporin, renin, vasopressin, and galanin receptor (2, 12, 23, 25, 35, 50). These genes could partially explain changes in water and electrolyte metabolism during exercise training (3). Upregulation of claudin-8, known to act as a paracellular cation barrier to Na$^+$, K$^+$, H$^+$, and NH$_4^+$, as well as a regulator of epithelial integrity (1), may be a part of the compensatory change to prevent allergen leakage and intestinal hyperpermeability during exercise, as previously reported in humans and dogs (13, 33). Furthermore, the epithelial integrity may be upheld by upregulating genes of local factors which could stimulate growth and differentiation of the intestinal epithelial cells, such as epimorphin and insulin (Supplemental Table S2; Refs. 27, 39). Similar to the effects of exercise on biotransformation in hepatocytes (59), swimming increased transcripts of certain intestinal genes, e.g., UDP-glucuronosyltransferase (Udpgt2), gluthione S-transferase (Gsto2), and paraoxonase (Pon1), which could neutralize xenobiotics and protect the body from toxic substances (11, 44, 56).

In conclusion, we demonstrated that swimming significantly stimulated calcium absorption in the duodenum, proximal jejunum, and cecum of female rats, presumably by upregulating crucial genes for transcellular and paracellular calcium transport, such as TRPV6, calbindin-D$_{9k}$, PMCA$_{1b}$, NCX1, and claudin-2. Swimming also altered the expression of genes pertaining to transport of ions and nutrients as well as water homeostasis, although further investigation is required to determine their physiological significances. Our findings concerning the swimming-enhanced calcium absorption thus help elucidate the physiological adaptation of calcium metabolism during nonimpact exercise, which should benefit cardiovascular, postmenopausal, and osteoporotic patients. The microarray data also provide direction for further investigations in the regulation of intestinal permeability and electrolyte homeostasis during exercise.

ACKNOWLEDGMENTS

We thank Kanogwun Thongchote for excellent technical assistance.

GRANTS

This research was supported by grants from the Faculty of Science, Mahidol University, the Commission on Higher Education, and the Thailand Research Fund (RSA5180001 to N. Charoenphandhu and RTA5080008 to N. Krishnamra).

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