ABSTRACT: In this study we investigate the effect of a single session of high-intensity contractions on expression of pleiotropic genes and, in particular, those genes associated with metabolism in soleus muscle from electrically stimulated (ES) and contralateral (CL) limbs. The right limbs of male Wistar rats were submitted to contractions by 200-ms trains of electrical stimulation at 100-Hz frequency with pulses of 0.1 ms (voltage 24 $\pm$ 3 V) delivered each second for 1 hour. Soleus muscles were isolated 1 hour after contraction, and gene expression was analyzed by a macroarray technique (Atlas Toxicology 1.2 Array; Clontech Laboratories). Electrical stimulation increased expression in 92 genes (16% of the genes present in the membrane). Sixty-six genes were upregulated in both ES and CL soleus muscles, and expression of 26 genes was upregulated in the ES muscle only. The most altered genes were those related to stress response and metabolism. Electrical stimulation also raised expression of transcription factors, translation and posttranslational modification of proteins, ribosomal proteins, and intracellular transducers/effectors/modulators. The results indicate that a single session of electrical stimulation upregulated expression of genes related to metabolism and oxidative stress in soleus muscle from both ES and CL limbs. These findings may indicate an association with tissue hypertrophy and metabolic adaptations induced by physical exercise training not only in the ES but also in the CL non-stimulated muscle, suggesting a cross-education phenomenon.

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CHANGES OF GENE EXPRESSION IN ELECTRICALLY STIMULATED AND CONTRALATERAL RAT SOLEUS MUSCLES

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Regular exercise training promotes several morphological and physiological changes in the human musculoskeletal system. This mechanism is blunted as soon as the stimulus that promoted the changes ceases.[1] According to the overload principle, strength gains are not reached unless the training intensity exceeds a certain threshold. In addition, strength gain as well as neuromuscular adaptations exhibit high specificity, which means that functional and structural adaptations are restricted to the muscle subjected to exercise stress. However, it has been reported that long-term unilateral training may affect the homologous muscle in the contralateral limb. This phenomenon, known as cross-education, was first described more than a century ago.[2] Cross-education may help prevent strength loss in an injured limb if the opposite contralateral (CL) limb is trained. Kannus et al.[3] observed that a 3-week period of training in quadriceps muscle leads to an increase of 14% in the strength of the CL quadriceps muscle in humans. This observation supports the proposition

Abbreviations: CAT, catalase; CL, contralateral; CS, citrate synthase; CT, cycle threshold; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; DTT, dithiothreitol; dTTP, deoxythymidine triphosphate; ERK, extracellular signal–related kinase; ES, electrically stimulated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; HSP, heat shock protein; JNK, Jun kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; SOD, superoxide dismutase

Key words: cross-education; gene expression; skeletal muscle; metabolism; macroarray; electrical; stimulation; protein synthesis; exercise; soleus muscle

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that keeping the uninjured limb active may benefit the injured one. For athletes, this may speed-up the return to competition and, in the case of elderly and injured patients, it would reduce the period of convalescence in bed and attendant risk of secondary complications.

As a consequence of muscle contraction that occurs during exercise, mechanical and biochemical mechanisms trigger coordinated signals that modulate expression of key genes responsible for cell structure and function.[4–6] These changes lead to adaptations usually observed in trained subjects. There is evidence of significant hypertrophy (10%) in the untrained CL muscle[7] and increased force output after a training program in the CL unstimulated muscle.[2] However, there are few data regarding molecular changes in the CL limb after electrical stimulation–induced muscle contraction.

The aim of this study was to examine changes induced by a single session of electrical stimulation on expression of genes (mRNA) associated with muscle hypertrophy and metabolism in the electrically stimulated (ES) and CL soleus muscles from the stimulated group, as compared with control non-stimulated rats, by using macroarray and real-time polymerase chain reaction (PCR) techniques. Several studies have addressed soleus muscle function and metabolism. Thus, there is reason to investigate the effect of ES on molecular mechanisms in soleus muscle. The soleus muscle was also chosen due to the well-established association between selected genes and oxidative capacity.

METHODS

Reagents. Sodium pentobarbital was obtained from Cristalia (Itapira, SP, Brazil); Trizol, random primers, deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), deoxyadenosine triphosphate (dATP), $MgCl_2$, DNAse buffer, DNAse, and Platinum SYBR Green qPCR SuperMix-UDG were purchased from Invitrogen (Carlsbad, California). Sodium dodecylsulfate (SDS) was obtained from Merck (Darmstadt, Germany). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalase, superoxide dismutase 2 (SOD2), heat shock protein 70 (HSP70), HSP90, and mitogen-activated protein kinase 1 (MEK) were from IDT (Coralville, Iowa). Express hybridization solution and termination mix were obtained from Clontech Laboratories (Mountain View, California). Reverse transcriptase *Revertaid* M-MuLV was from MBI Fermentas (Burlington, Ontario, Canada). ^{33}P-labeled ATP was purchased from GE Healthcare (Waukesha, Wisconsin).

Animals. Twenty male adult (9 weeks of age, about 200 g) albino rats (Wistar strain) from the Institute of Biomedical Sciences of the University of São Paulo were divided in two groups: (a) control ($n = 10$) and (b) electrically stimulated ($n = 10$). In the electrically stimulated group, we examined the stimulated (ES) and the contralateral non-stimulated (CL) soleus muscles.

The rats were housed 5 per cage at 20°–23°C and a 12:12-hour light–dark cycle and had ad libitum access to rat chow (Nuvilab CR1; Nuvital Nutrientes, Curitiba, Paraná) and water. The experimental protocol used was approved by the ethics committee of the Institute of Biomedical Sciences of the São Paulo University.

Electrical Stimulation. Rats were anesthetized with sodium pentobarbital (75 mg/kg body weight) and had their right sciatic nerve exposed through a lateral section on the thigh where a platinum electrode was connected. The rats were then fastened on an acrylic platform with a metallic bar crossing the right knee to fix the limb. Another metallic bar was fixed at the Achilles tendon, connecting the hindfoot to a force transducer (Myograph F-2000; Narco Bio-Systems, Austin, Texas) that indicated the generated tension by using a polygraph (Narco Bio-Systems). The contralateral limb was fastened to the platform by adhesive tape. Rats were kept under external warming to maintain core temperature during the entire procedure.

The animals were subjected to an intense electrical stimulation protocol as described elsewhere.[8,9] Briefly, the stimulus consisted of 200-ms trains delivered each second for 1 hour. The pulses in the train were delivered at a frequency of 100 Hz with a duration of 0.1 ms. The voltage was 24 ± 3 V. In order to reach maximum force output, the muscle rest length and the stimulation voltage were adjusted at the beginning of each experiment. Soleus muscles from ES and CL limbs were removed 1 hour after electrical stimulation. Control rats were subjected to the same conditions as the experimental group, including surgery and fixation to the platform, but with no electrical stimulation. The tissues were extracted, immediately frozen in liquid nitrogen, and kept at −70°C for the assays. Rats were killed by cervical dislocation.

Analysis of Gene Expression by Macroarray. ***Total RNA Extraction.*** Total RNA was obtained from 100 mg of soleus muscle using 1 ml of Trizol reagent. After 5-minute incubation at room temperature, 200 μl of chloroform was added to the tubes and centrifuged at 12,000*g*. The aqueous phase was transferred to another tube. RNA was pelleted by centrifugation (12,000*g*) with cold ethanol, and then dried in air. RNA pellets were eluted in RNase-free water and treated with DNase I. The RNA preparation was then stored at −70°C until the time of the experiment. RNA was quantified by measuring absorbance at 260 nm. The purity of the RNA preparation was assessed by the 260/280-nm ratio on a 1% agarose gel stained with ethidium bromide at 5 mg/ml. These samples were used for macroarray and real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis.

Synthesis of cDNA Probes. A pool of total RNA from three soleus muscles of each group (control, ES, and CL) was prepared using 10 μg of RNA from each muscle, as described earlier. The array experiment was performed three times with three different pools of RNA. The RNA was pooled to reduce the individual variability.[10]

cDNA probes were synthesized using a pure total RNA labeling system according to the manufacturer's recommendations (Atlas Kit; Clontech Laboratories, Mountain View, California). Briefly, 15 μg of total RNA pool from soleus muscles of each group and 2 μl of primer mix (a mixture of primers relative to the genes spotted in the macroarray membrane) were heated at 70°C for 5 minutes in a thermal cycler (Techne-Genius, Oxford, UK). The temperature was decreased to 50°C, and 13.5 μl of the mix of the following reagents were added: 4 μl of 5× reaction buffer; 0.5 μl of 100 mM dithiothreitol (DTT); 2 μl of 10× dNTP mix (dCTP, dGTP, dTTP, dATP); 5 μl of (α-^{33}P)ATP (at 10 μCi/μl); and 2 μl of reverse transcriptase. The mixture was incubated for 25 minutes at 50°C, which was stopped by adding 2 μl of Termination Mix. The ^{33}P-labeled probe was purified from unincorporated nucleotides by passing the reaction mixture through a push column (NucleoSpin Extraction Spin Column; Clontech).

Macroarray Membrane Hybridization. The Atlas Toxicology 1.2 Array (Clontech) was used to analyze the effect of electrical stimulation on gene expression. The list of genes in the macroarray membranes is available on the Clontech website (http://www.clontech.com/support/tools.asp?product_tool_id=157578&tool_id=157579). The membrane was prehybridized for 30 minutes at 68°C in Express Hyb containing 50 μg of freshly denaturated salmon sperm DNA. Subsequently, the membrane was hybridized during 18 hours at 68°C with ^{33}P-labeled denaturated probe (2×10^6 cpm/ml). The membrane was then washed four times at 68°C with 1 × SSC and 0.1% SDS, followed by washing in 1 × SSC, 1% SDS, and then exposed to phosphor screen for 48 hours and scanned on an imaging system (Storm 840; Molecular Dynamics, Sunnyvale, California).

Analysis of Macroarray Results. Changes in gene expression from IP and CL muscles were analyzed by comparison with results of expression observed in soleus muscle from control rats using Array-Pro Analyzer, version 4 software (Media Cybernetics, Silver Spring, Maryland). The results were presented as the mean of normalizations performed using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is present in the membrane. Triplicate hybridizations using separate sets of nylon membranes were performed for all conditions. Only signals that differed from control by at least 1.8-fold in the three independent experiments were considered to be significantly regulated. A similar procedure was used in our previous studies.[11–13]

Real-Time RT-PCR Analysis. Real-time RT-PCR, using specific primers, was performed to confirm the differential expression of the mRNAs detected in the macroarray analysis. Total RNA was extracted from soleus muscles as described previously. Total RNA (2 μg) was reverse transcribed to cDNA using reverse transcriptase *Revertaid* M-MuLV.

Expression of SOD2 (MnSOD), catalase, heat shock protein HSP70, HSP90, and MAP kinase kinase 1 (MEK1) was determined by real-time PCR (Rotor Gene-3000; Corbett Research, Mortlake, Australia) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The sequences of the primers were designed using information from the GenBank public database of the National Center for Biotechnology Information. These sequences and the thermal cycler protocol are described in Table 1.

The relative quantitation value of each target gene was analyzed using a comparative cycle threshold (CT) method.[14,15] The following formula was used to calculate the relative amount of transcript in the sample and normalized to endogenous reference (GAPDH): $2^{-\Delta\Delta CT}$, where ΔCT is the difference in CT between the gene of interest

Table 1. Standardized conditions for real-time RT-PCR analysis.

GenBank accession no.	Gene	Sense primer	Antisense primer	Annealing temperature (°C)
M17701	GAPDH	5′GGTGCTGAGTATGTCGTGGAG3′	5′ATATTTCTCGTGGTTCACACCC3′	56
X54793	HSP60	5′ATGGGTGCCTATGCTCCTGAGC3′	5′ATGGGTGCCTATGCTCCTGAGC3′	58
X54793	HSP70	5′GAGATCATCGCCAACGACCAG3′	5′CTCGCCCTTGTAGTTCACCTGC3′	60
S45392	HSP90	5′ACCAGGTAGGCAGAGTAGAAGC3′	5′GACTACGGGACCTGTTCTAAGC3′	57
X56600	SOD2	5′GACCTGCCTTACGACTATG3′	5′TACTTCTCCTCGGTGACG3′	55
M11670	Catalase	5′ATTGCCGTCCGATTCTCC3′	5′CCAGTTACCATCTTCAGTGTAG3′	55
M64300	MEK1	5′AGTGAGGAGACGGCATTCACCC3′	5′CTCGTTCGCTTTGGTATGCCC3′	58

Sequences of the primers and the temperatures and number of cycles used are shown for each gene studied. RT-PCR, reverse transcription–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; MEK1, mitogen-activated protein kinase kinase 1; SDHA, succinate dehydrogenase complex.

and the housekeeping, and ΔΔCT for the sample = mean ΔCT of the sample − mean ΔCT of the control sample (used as calibrator).

GAPDH was used as the housekeeping gene based on information described by others[15] and reported in our previous studies.[8,10] The decision to use GAPDH was also based on the use of the GeNorm program. This program determines the most stable reference genes from a set of tested genes in a given cDNA sample panel. It allows calculation of a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of reference genes. GeNorm allows calculation of gene expression stability measured (M) for a reference gene as the average pairwise variation (V) for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability.

Statistical Analysis. The differences between control and CL or ES muscles were assessed by nonpaired Student's *t*-test. Comparison between CL and ES muscle was performed by paired Student's *t*-test. Significance was set at $P < 0.05$. Results are presented as mean ± SEM. Analyses were performed using GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, California, www.graphpad.com).

RESULTS

Increased expression of 92 genes (16% of the genes present in the membrane) was observed in the ES soleus muscle as compared with non-stimulated controls (Table 2). Expression of 66 genes was upregulated in both ES and CL muscles, whereas expression of 26 genes was upregulated in the ES muscle only (Table 2). The intensity of increase in gene expression of the CL soleus muscle was clearly lower than that for ES (Table 2).

The most altered genes were those related to stress response (13 genes) and metabolism (19 genes). Electrical stimulation also increased expression of genes related to transcription factors (8 genes), cell cycle (4 genes), translation and post-translational modification (5 genes), protein modification enzymes (2 genes), ribosomal proteins (5 genes), growth factors and chemokines (2 genes), intracellular transducers/effectors/modulators (5 genes), amino acid metabolism (1 gene), metalloproteinases (2 genes), protein turnover (3 genes), calcium-binding proteins (2 genes), DNA synthesis/recombination/repair (4 genes), and protease inhibitors (3 genes). Expression of the HSP90 gene showed the most pronounced increases, by 73.8- and 13.4-fold in the ES and CL muscles, respectively.

Expression of a number of genes related to stress response, metabolism, and protein synthesis was increased by at least tenfold: Hsp70/Hsp90-organizing protein; T-complex protein 1; HSP60; suppression of tumorigenicity 13; HSP90, thioredoxin 2; superoxide dismutase 1 and 2; cytochrome P450; hydroxysteroid 11β dehydrogenase 2; enoyl coenzyme A hydratase; acetyl-coenzyme A dehydrogenase; peroxiredoxin 1; protein disulfide isomerase–related protein; nucleophosmin 1; protein disulfide isomerase; defender against cell death 1; and ribosomal proteins S3, S19, L6, and L13A (Table 2).

Six genes that had their expression increased by electrical stimulation as indicated by macroarray were chosen for further analysis by real-time RT-PCR. A marked increase of mRNA levels of SOD2 (7.8- and 5.9-fold), catalase (8.5- and 6.2-fold), HSP70 (6.1- and 3.8-fold), HSP60 (3.2- and 2.2-fold), and MEK1 (6.8- and 4.8-fold) was observed in the ES and CL soleus muscles, respectively, as

Table 2. Modifications of gene expression in stimulated and contralateral soleus muscle isolated 1 hour after electrical stimulation.

GenBank accession no.	Gene name	ES/Ct	CL/Ct
Transcription factors and related proteins			
X63369	*Zinc finger protein 36*	3.9	2.2
X63594	*Nuclear factor of kappa light-chain gene enhancer in B-cells inhibitor, alpha*	3.4	
D10862	*Inhibitor of DNA binding 1, helix–loop–helix protein (splice variation)*	4.1	2.3
Z12020	*Mdm2, transformed 3T3 cell double minute 2*	2.8	
AF090306	*Retinoblastoma binding protein 7*	4.7	2.5
Z17223	*Mesenchyme homeo box 2*	5.1	2.1
U17013	*POU domain class 2, transcription factor 1*	5.4	2.2
X17163	*V-jun sarcoma virus 17 oncogene homolog (avian)*	5.3	1.9
Cell cycle			
M18416	*Early growth response 1*	3.7	2.2
D14013	*Cyclin C*	2.9	
X70871	*Cyclin G1*	5.7	2.0
D31838	*Wee1 tyrosine kinase*	2.9	
Intercellular adhesion			
S80439	*OB-cadherin 1*	3.8	2.0
Stress response			
M86389	*Heat shock 27-kDa protein 1*	4.9	
X15705	*Testis-specific heat shock protein–related gene hst70*	3.3	
Y00054	*Heat shock protein 8*	4.7	2.8
X15187	*Tumor rejection antigen (gp96) 1*	5.6	3.0
M69246	*Serine (or cysteine) proteinase inhibitor, clade H, member 1*	4.9	2.4
Y15068	*Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)*	**20**	**11.0**
U83843	*T-complex protein 1 eta subunit (TCP1-eta); CCT-eta (CCTH; CCT7); HIV-1 NEF-interacting protein (rat homolog of human)*	**20.2**	**11.0**
D90345	*T-complex 1*	**33.7**	**12.3**
X54793	*Heat shock protein 60 (liver)*	**16.7**	5.6
Z27118	*Heat shock 70-kDa protein 1*[A]	5.9	3.1
X82021	*Suppression of tumorigenicity 13 (colon carcinoma) HSP70-interacting protein*	**11.0**	5.6
S45392	*Heat shock 90-kDa protein beta (HSP90-beta); HSP84; HSPCB*	**73.8**	**13.4**
S78556	*Mitochondrial stress-70 protein precursor (MTHSP70); 75-kDa glucose-regulated protein (GRP75); peptide-binding protein 74 (PBP74); mortalin; HSPA9*	6.5	2.0
Voltage-dependent Ionic Channels			
M21730	*Annexin 5*	2.7	
Metabolism			
M22413	*Carbonic anhydrase 3*	8.5	
M31788	*Phosphoglycerate kinase 1*	5.7	
U73525	*Thioredoxin 2*	**19.5**	6.4
Y00404	*Superoxide dismutase 1*	**23.9**	5.5
M11670	*Catalase*	**14.0**	8.0
X56600	*Superoxide dismutase 2*	**31.8**	**14.3**
D12770	*Solute carrier family 25 (mitochondrial adenine nucleotide translocator) member 4*	7.4	
U09540	*Cytochrome P450, subfamily 1B, polypeptide 1*	4.1	
M21208	*Cytochrome P450, subfamily 17*	**13.6**	6.9
L03294	*Lipoprotein lipase*	7.7	2.9
J04171	*Glutamate oxaloacetate transaminase 1*	6.7	
M18467	*Glutamate oxaloacetate transaminase 2*	6.6	3.0
J05405	*Heme oxygenase 2*	6.2	3.5
D30647	*Acyl-coenzyme A dehydrogenase, very long chain*	4.5	
U22424	*Hydroxysteroid 11beta dehydrogenase 2*	**17.1**	7.7
M23995	*Aldehyde dehydrogenase family 1, subfamily A4*	8.6	4.7
X15958	*Enoyl coenzyme A hydratase, short chain 1*	**24.7**	9.4
J05029	*Acetyl-coenzyme A dehydrogenase, long chain*	**15.7**	7.0
D30035	*Peroxiredoxin 1*	**20.6**	6.1
X12367	*Glutathione peroxidase 1*	6.2	
Translation/post-translational modification/protein folding			
M86870	*Protein disulfide isomerase–related protein (calcium-binding protein, intestinal-related)*	**12.1**	6.9
L24804	*Progesterone receptor–associated protein*	4.2	
M75715	*Eukaryotic translation termination factor 1*	2.0	
J03969	*Nucleophosmin 1*	**19.0**	6.3

Table 2. Continued.

GenBank accession no.	Gene name	ES/Ct	CL/Ct
X02918	*Protein disulfide isomerase (prolyl 4-hydroxylase, beta polypeptide)*	**22.8**	9.5
Protein modification enzymes			
U94340	*ADP-ribosyltransferase 1*	5.5	4.1
Y13336	*Defender against cell death 1*	**37.3**	**18.1**
Ribosomal proteins			
X51536	*Ribosomal protein S3*	**15.8**	9.4
X51707	*Ribosomal protein S19*	**16.3**	3.3
X58465	*Ribosomal protein S5*	2.6	
X87107	*Ribosomal protein L6*	**24.0**	8.3
X68282	*Ribosomal protein L13A*	**47.6**	7.6
RNA processing, turnover, and transport			
U22893	*Cold shock domain protein A*	5.4	
D17711	*Heterogenous nuclear ribonuclopotein K*	3.9	*1.9*
D84418	*High-mobility group box 2*	4.1	2.3
Receptors			
U59809	*Insulin-like growth factor 2 receptor*	2.2	
M25804	*Nuclear receptor subfamily 1, group D, member 1*	4.5	2.7
L06482	*Retinoid X receptor alpha*	3.2	
U84402	*Smoothened*	6.1	3.2
Growth factors, cytokines, and chemokines			
M32167	*Vascular endothelial growth factor*	6.8	2.9
U06436	*Chemokine (C—C motif) ligand 5*	4.0	
Intracellular transducers/effectors/modulators			
M64301	*Mitogen-activated protein kinase 6*	4.8	2.7
D14592	*Mitogen-activated protein kinase kinase 2*	5.0	*1.9*
M64300	*Mitogen-activated protein kinase kinase 1*	5.8	2.8
L27112	*Stress-activated protein kinase alpha II*	3.4	*1.9*
L27129	*Mitogen-activated protein kinase 8*	5.5	3.0
Amino acids metabolism			
D17615	*Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*	3.7	
Calcium-binding proteins			
L18889	*Calnexin*	4.5	2.4
X53363	*Calreticulina*	7.1	2.3
Protein turnover			
X62671	*Finkel–Biskis–Reilly murine sarcoma virus ubiquitously expressed*	5.9	2.0
D63673	*Peroxisomal biogenesis factor 6*	4.1	2.0
AF095740	*Neural precursor cell expressed, developmentally downregulated gene 8*	5.2	1.9
Metalloproteinases			
U46034	*Matrix metalloproteinase 11 (stromelysin 3)*	3.9	1.8
D87336	*Bleomycin hydrolase (BLM hydrolase, BLMH, BMH)*	4.9	2.2
Cysteine proteases			
D63378	*Glucose-regulated protein, 58 kDa*	5.8	2.5
Protease inhibitors			
L31884	*Tissue inhibitor of metalloproteinase 2*	5.6	2.2
J02635	*Alpha-2-macroglobulin*	8.4	4.1
D00753	*Serine protease inhibitor*	3.0	
Intermediate filament proteins			
X62952	*Vimentin*	5.7	
DNA synthesis, recombination, and repair			
Y00047	*Proliferating cell nuclear antigen*	4.3	2.3
J03250	*DNA topoisomerase I*	3.5	1.9
D00144495	*Apurinic/apyrimidinic endonuclease 1*	3.5	
AJ006070	*Recombination activation protein 1*	7.2	3.5
Non-classified proteins			
L28818	*Involucrin gene*	6.0	
M17698	*Thymosin, beta 10*	6.8	2.3
D50093	*Prion protein*	9.3	3.9

Results are shown as electrically stimulated (ES) and control (Ct) or contralateral (CL) and control ratios. Total RNA was isolated, retrotranscribed, ^{33}P-labeled, and hybridized to the cDNA array membrane presenting 1176 transcripts of known genes. The signals were then analyzed with Pro-Analysis Array-Pro Analyzer software, version 4 (Media Cybernetics, Silver Spring, Maryland) and expressed as IP and Ct or CL and Ct ratios. Only signals that differed from the cells isolated in the pre-diet period by at least twofold were considered significant. See Methods for details of the calculation. Bold entries indicate expression of genes that were increased by at least tenfold.

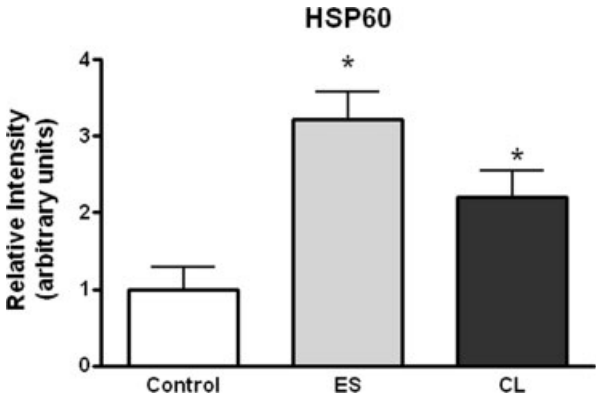

FIGURE 1. mRNA levels of heat shock protein 60 (HSP60) in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control.

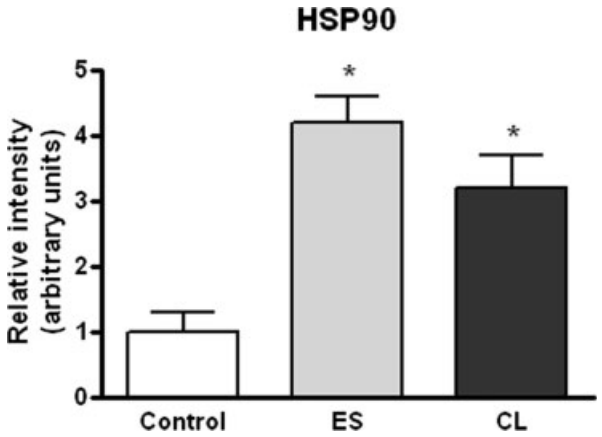

FIGURE 3. mRNA levels of HSP90 in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control.

compared with non-stimulated (control) muscles (Fig. 1). Although the magnitude was not the same, the direction of changes induced by electrical stimulation was consistent in both macroarray and real-time RT-PCR analysis. Expression of HSP70 (Figure 2), MEK1 (Figure 6), catalase (Figure 4), and SOD2 (Figure 5), was differentially modulated in ES and CL soleus muscle.

DISCUSSION

The major finding observed in this study was an overall induction of gene expression in the ES soleus muscle when compared with non-stimulated controls. A similar effect was observed in the CL muscle, but of a lower magnitude. The macroarray analysis revealed an increased expression of 92 genes after electrical stimulation, from which 66 genes were upregulated in both ES and CL muscles. So, the CL leg muscles also respond to muscle contractions. This effect has also been described by other investigators who used the terms contralateral effect or cross-education.[16] Improvement of contralateral muscle force after a training program has been associated with neural adaptations at central (cortical) and peripheral levels.[17,18] However, the full mechanism involved in the cross-education phenomenon remains poorly understood.[2,16,18]

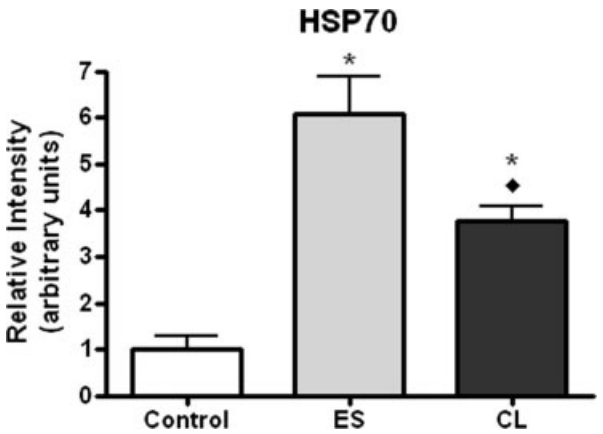

FIGURE 2. mRNA levels of HSP70 in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control; ◆$P < 0.05$ compared with values from electrically stimulated muscle.

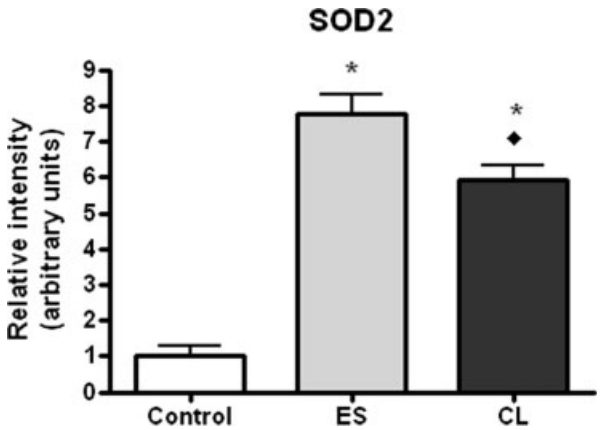

FIGURE 4. mRNA levels of Mn-superoxide dismutase (SOD2) in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control; ◆$P < 0.05$ compared with values from electrically stimulated muscle.

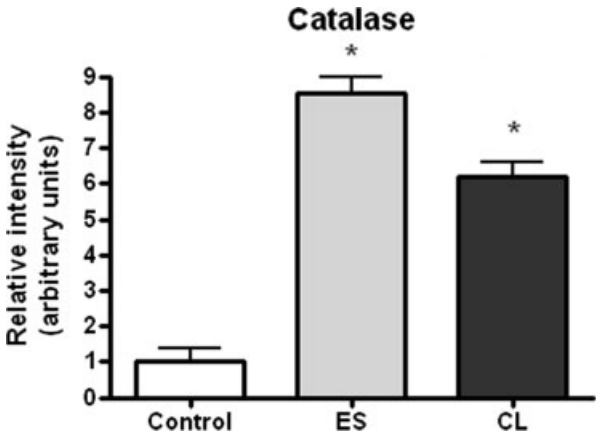

FIGURE 5. mRNA levels of catalase in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control.

Expression of several genes related to stress response, protein synthesis, and metabolism was increased by at least tenfold due to muscle contraction (Table 2). This fact indicates that a single session of electrical stimulation is enough to increase the expression of genes involved with hypertrophy and metabolic adaptations. One report showed a marked increase in maximal activity (63%) and expression (80%) of citrate synthase at 1 hour after muscle contraction induced by electrical stimulation in rat soleus muscles.[8,16] Increased expression of SOD, catalase, and glutathione peroxidase was also found. These observations indicate that muscle contraction, even for a very short time, increases activity and expression of important proteins involved in energy production and resistance to fatigue.[8,16]

To validate the results of the macroarray analysis, six genes were selected for confirmation of changes using RT-PCR. Expression of HSP60, HSP70, HSP90, SOD2, catalase, and MEK1 was increased by electrical stimulation in the ES and CL muscles. These proteins have constitutive roles in skeletal muscle and play an important part in maintaining muscle function. Most of these processes (e.g., protein synthesis, metabolism, and stress response) are highly interconnected and form a complex signaling network during muscle recovery from injury.[5] ES increased expression of several heat shock proteins, in particular HSP27, HSP60, HSP70, and HSP90, as observed in the macroarray results. This latter effect has been associated with various components of the cytoskeleton and steroid hormone receptors.[19] HSP70 is known to mediate repair and degradation of altered or denaturated proteins.[5] Increased HSP expression due to a single session of exercise has been described.[14] Venojarvi et al. tested the effects of 4-week immobilization and subsequent remobilization on HSP expression and oxidative stress in the lateral gastrocnemius and plantaris muscles of rats.[20] They found that expression of HSPs was upregulated during the recovery from immobilization, particularly in the lateral gastrocnemius muscle. Following immobilization, both intensive and spontaneous exercise upregulated HSP expression in the lateral gastrocnemius and partly in the plantaris muscle.[20]

Expression of the genes of oxidative stress was also raised by muscle contraction. SOD and CAT play an important role in the intracellular redox balance. Recently, we have shown increased expression of SOD1, SOD2, and catalase in the ES soleus muscle, suggesting that these antioxidant enzymes are important signaling components for maintaining muscle function.[8] ES also increased expression of ribosomal proteins S3, S19, L6, and L13A by at least tenfold. Ribosomal proteins have the complex task of coordinating protein biosynthesis, yet regulation of their expression remains unknown. Skeletal muscle hypertrophy occurs in part due to an increase in protein mass per fiber. This accumulation of protein results from a net increase in protein synthesis in relation to protein breakdown. Increased rates of protein synthesis (translation) have been reported in several models of resistance exercise.[21,22]

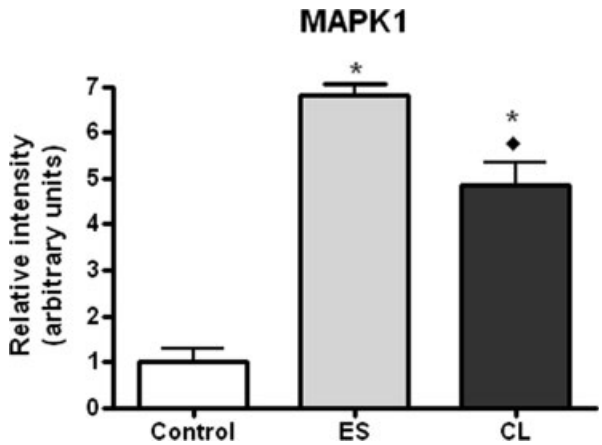

FIGURE 6. mRNA levels of MAP kinase kinase 1 (MEK1) in control (white bars), electrically stimulated (ES) (light gray bars), and contralateral (CL) (dark gray bars) soleus muscle at 1 hour after electrical stimulation. The analysis was performed by real-time RT-PCR. *$P < 0.001$ compared with values from control; ◆$P < 0.05$ compared with values from electrically stimulated muscle.

MEK1 has been described as a potent activator of mitogen-activated protein kinases (MAPKs), including extracellular signal–related kinase (ERK), Jun kinase (JNK), and p38 MAPK. These proteins exert important signaling effects on metabolism and adaptive responses in skeletal muscle.[23,24] MAPK8 (or JNK) pathway activation by muscle contraction per se has been associated with mitochondrial biogenesis.[25] In this study, an increase of 5.8-, 4.8-, and 5.5-fold in expression of MEK1, MAPK6, and MAPK8, respectively, was found.

A marked increase in expression of anti-apoptotic genes, such as defender against cell death 1, nucleophosmine, and, as mentioned earlier, ribosomal proteins S3, S19, L6, and L13A, was observed in the ES and CL muscles. There has been no previous study on expression of these genes after a single exercise session.

In conclusion, evidence has been presented herein that high-intensity muscle contraction promotes increased expression of genes (mainly those related to protein synthesis and metabolism) in both ES and CL soleus muscles. These observations strongly suggest that exercise training in the CL leg of injured subjects may avoid the expected decrease in muscle functionality in both the injured and non-injured leg.

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