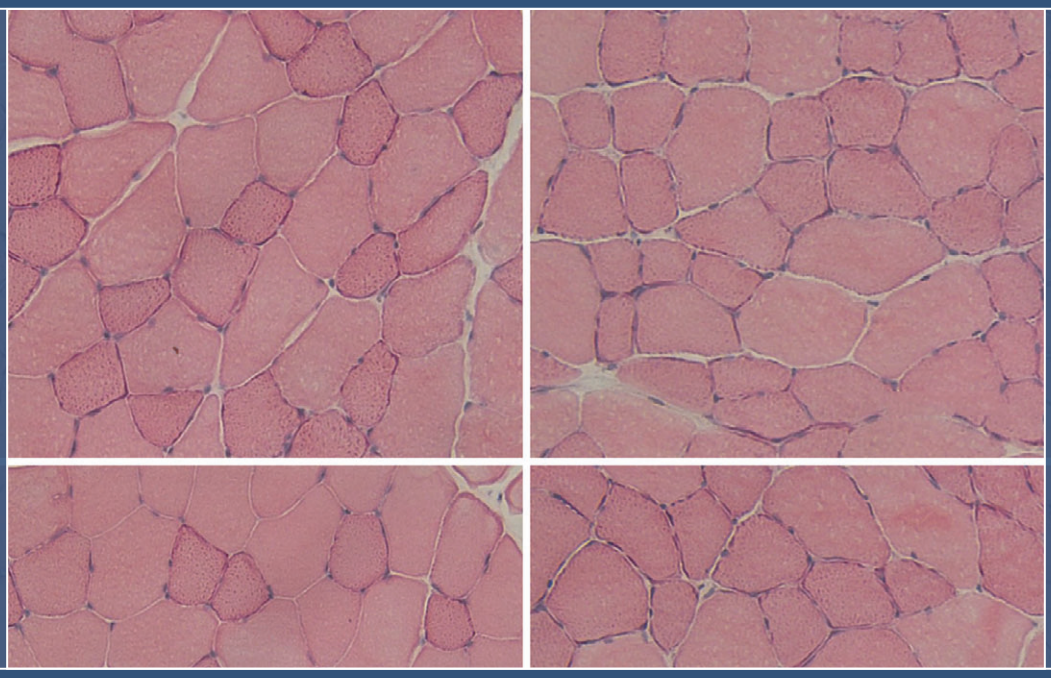
www.actaphysiol.org

December 2015 • Volume 215 • Number 4

Acta Physiologica

OFFICIAL JOURNAL OF THE FEDERATION OF EUROPEAN PHYSIOLOGICAL SOCIETIES



PUBLICATION HISTORY

Acta Physiologica 2006–

Acta Physiologica Scandinavica 1940–2005

Skandinavisches Archiv für Physiologie 1889–1939

Acta Physiologica

AIMS AND SCOPE

Acta Physiologica is an important forum for the publication of high quality original research in physiology and related areas by authors from all over the world. Acta Physiologica is a leading journal in all aspects of the science of physiology. The Journal publishes full length original articles on important new observations as well as reviews and commentaries. *Acta Physiologica* is the official journal of the Federation of European Physiological Societies.
Manuscripts should be submitted online at http://mc.manuscriptcentral.com/aph, see instructions on the inside back cover. Other material should be sent to the Chief Editor, Pontus B. Persson, Acta Physiologica, Hessische Str. 3-4, 10115 Berlin, Germany.

DISCLAIMER

The Publisher, Society and Editors cannot be held responsible for errors or any consequences arising from the use of information contained in this journal; the views and opinions expressed do not necessarily reflect those of the Publisher, Society and Editors, neither does the publication of advertisements constitute any endorsement by the Publisher, Society and Editors of the products advertised.

COPYRIGHT AND COPYING

INFORMATION FOR SUBSCRIBERS

Acta Physiologica is published in 12 issues per year. Subscription prices for 2015 are: Institutional: €837 (Europe), $1250 (The Americas), $1462 (Rest of World). Prices are exclusive of tax. Australian GST, Canadian GST/HST and European VAT will be applied at the appropriate rates. For more information on current tax rates, please go to wileyonlinelibrary.com/tax-vat. The price includes online access to the current and all online back files to January 1st 2011, where available. For other pricing options, including access information and terms and conditions, please visit wileyonlinelibrary.com/access

DELIVERY TERMS AND LEGAL TITLE

Where the subscription price includes print issues and delivery is to the recipients address, delivery terms are Delivered at Place (DAP); the recipient is responsible for paying any import duty or taxes. Title to all issues transfers FOB our shipping point, freight prepaid. We will endeavour to fulfill claims for missing or damaged copies within six months of publication, within our reasonable discretion and subject to availability.

BACK ISSUES

Single issues from current and recent volumes are available at the current single issue price from cs-journals@wiley.com. Earlier issues may be obtained from Periodicals Service Company, 11 Main Street, Germantown, NY 12526, USA. Tel: +1 518 537 4700, Fax: +1 518 537 5899, Email: psc@periodicals.com

PUBLISHER

Acta Physiologica is published by John Wiley & Sons Ltd, 9600 Garsington Road, Oxford OX4 2DQ, UK, Tel: +44 1865 776868, Fax: +44 1865 714591

Journal Customer Services: For ordering information, claims and any enquiry concerning your journal subscription please go to www.wileycustomerhelp.com/ask or contact your nearest office:
Americas: Email: cs-journals@wiley.com; Tel: +1 781 388 8598 or 1 800 835 6770 (Toll free in the USA & Canada)
Europe, Middle East and Africa: Email: cs-journals@wiley.com; Tel: +44(0) 1865 778 315
Asia Pacific: Email: cs-journals@wiley.com; Tel: +65 6511 8000
Japan: For Japanese speaking support, Email: cs-japan@wiley.com; Tel: (toll-free): 005 316 50 480.
Visit our Online Customer Help available in 7 languages at www.wileycustomerhelp.com
This journal is available online at Wiley Online Library. Visit wileyonlinelibrary.com to search the articles and register for table of contents email alerts.

ONLINEOPEN

Acta Physiologica accepts articles for Open Access publication. Please see www.actaphysiol.org and select author guidelines for further information about OnlineOpen.

Access to this journal is available free online within institutions in the developing world through the AGORA initiative with the FAO and the HINARI initiative with the WHO. For information, visit www.aginternetwork.org, www.who.int/hinari/en/

Wiley's Corporate Citizenship initiative seeks to address the environmental, social, economic and ethical challenges faced in our business and which are important to our diverse stakeholder groups. We have made a long-term commitment to standardize and improve our efforts around the world to reduce our carbon footprint. Follow our progress at www.wiley.com/go/citizenship.

ABSTRACTING AND INDEXING SERVICES

The Journal is indexed by Biobase, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Calcium and Calcified Tissue Abstracts (Online Edition), Chemical Abstracts, Current Contents, Derwent Biotechnology Abstracts, EMBASE, Excerpta Medica Abstract Journals MEDLINE, Nutrition Abstracts and Reviews, Science Citation Index, SCOPUS.

ISSN (Online) 1748-1716

For submission instructions, subscription and all other information visit: www.actaphysiol.org

COVER

Tibialis anterior mean cross-sectional area and fibre distribution 28 days (n = 4–7) post-bupivacaine injection. *Acta Physiol (Oxf)* 215:44-55.

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 159–160

ExActa

Transcription

If asked to give a short introduction to the concept of gene transcription and the role of transcription factors therein, would you be able to? Sure, we thought – and started reading.

Gene expression, the process necessary to transmit information from genetically encoded information into functional processes within a living organism, is where it all starts. Basic principles are followed, which have proved so successful that they are used by prokaryotes and eukaryotes alike.

Of the several steps involved in this complex process, let us focus on the first: transcription. Or, more precisely, eukaryotic transcription, which, although they resemble one another, differs from its prokaryotic analogue. Transcription describes the process of copying the information stored within a DNA sequence into RNA, which is basically carried out by RNA polymerases. These enzymes act as readers of double-helical DNA and produce a complementary single-stranded RNA, the primary transcript. Only if the transcribed gene encodes for a protein, the resulting RNA is called mRNA (messenger RNA). Other kinds of non-coding RNA are known which have different functions. mRNA works as a template for protein synthesis through translation, proteins being the functional players enabling dynamic eukaryotic life.

The mechanism of transcription is divided into five substeps: pre-initiation, initiation, promotor clearance, elongation and termination. Transcription starts with pre-initiation. For this, generally, a promotor region is needed. Promoters are localized upstream of the transcription start site. This is when transcription factors appear onstage, which are able to bind DNA, activate or repress the promotor and, consequently, exert a critical effect on transcription. Once transcription factors have bound to the promotor sequence, together with RNA polymerase, a transcription initiation complex is formed.

Following synthesis of the first bond, RNA polymerase must clear the promoter. In both pro- and eukaryotic transcription, RNA polymerase often releases the RNA transcript and produces truncated transcripts in a process called abortive initiation (Goldman *et al.* 2009). Why does that happen? – We do not know for sure, although certain functions of these truncated nucleotide sequences have been discussed (Lee *et al.* 2010). During elongation, true replication happens: RNA polymerase reads the DNA template strand from its 3′ to 5′ endings, forming a complementary RNA strand (5′→3′) which is an exact copy of the coding strand. RNA polymerase functions in this step also as a control instance. While proofreading, incorrectly inserted bases are replaced so that potential mistakes are eliminated directly. Replication ends with termination/polyadenylation, which is still less well understood in eukaryotes. The whole process is incredibly complex, and results from many different pathophysiological contexts and functional studies contribute to our understanding not only of what happens, but why; and what can go wrong, and why.

Physiology, the very one we publish about in Acta Physiologica, evolves. It does so just as, say, personalized medicine does in the wakes of the genetic revolution. Although our manuscripts not usually focus on subcellular mechanisms alone, let us have a look at what is new here.

A recent publication from Hansen and colleagues takes a careful yet comprehensive look into the role of iron in diabetes pathophysiology: during the primary inflammatory assault on the pancreatic islet cells, iron generates reactive oxygen species, leading to oxidative damage and apoptosis – and activation of β-cellular transcription factors, a multitude of which is activated by ROS, leading to β-cell dedifferentiation, loss of function and apoptosis (Hansen *et al.* 2014). For example, both the NF-κB and JNK pathways in β-cells are affected, which leads to apoptosis and a JNK-mediated translocation of pancreatic and duodenal homeobox-1 (Pdx-1), the transcription factor which β-cell differentiation and maintenance of insulin secretion critically depend on (Kaneto *et al.* 2010).

Developmental physiologists deal not only with individual transcription factors, but usually with systems and networks of these, activated in a time-dependent or hierarchical manner (Rana *et al.* 2013). Even if transcription and transcription factors are not among the usual suspects you would round up in your daily research routine, you will have heard of the myriad roles of famous hypoxia-inducible factor (HIF), meticulously studied mostly due to its role in the pathogenesis of our civilizations' greatest scourges, be it cancer or obstructive vascular disease. Recently, prolyl 4-hydroxylases have moved to the centre of

 doi: 10.1111/apha.12597

attention due to their role as regulators of the eukaryotic organism's concerted defence mechanisms which are activated in case of a shortage of oxygen (Myllyharju 2013). The Von Hippel–Lindau (VHL) complex controls physiologic HIF ubiquitination and degradation. Organ-specific VHL knockout models have been developed and may serve as useful tools to study hypoxia adaptation (Mathia *et al.* 2013). The kidney, given its vulnerability to hypoxic insults, especially of the medullary region, is just one example (Seeliger *et al.* 2012; Dietrich *et al.* 2013).

Wherever you look in biomedical journals, transcription, transcriptional control of gene expression and the role of transcription factors therein are everywhere, illustrating how old interdepartmental and interdisciplinary barriers are becoming irrelevant in the face of the ever-accelerating genetic revolution. In exciting times, expect to be surprised.

Conflict of interest

None.

The authors wish to thank Professor Holger Scholz for critical reading and discussion of the manuscript.

P. B. Persson and M. Mueller
Institute of Vegetative Physiology, Charité-Universitaetsmedizin Berlin,
Berlin, Germany
E-mail: pontus.persson@charite.de

References

Dietrich, A., Mathia, S., Kaminski, H., Mutig, K., Rosenberger, C., Mrowka, R., Bachmann, S. & Paliege, A. 2013. Chronic activation of vasopressin V2 receptor signalling lowers renal medullary oxygen levels in rats. *Acta Physiol* **207**, 721–731.

Goldman, S.R., Ebright, R.H. & Nickels, B.E. 2009. Direct detection of abortive RNA transcripts *in vivo*. *Science* **324**, 927–928.

Hansen, J.B., Moen, I.W. & Mandrup-Poulsen, T. 2014. Iron: the hard player in diabetes pathophysiology. *Acta Physiol* **210**, 717–732.

Kaneto, H., Katakami, N., Matsuhisa, M. & Matsuoka, T.A. 2010. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators Inflamm* **2010**, 453892.

Lee, S., Nguyen, H.M. & Kang, C. 2010. Tiny abortive initiation transcripts exert antitermination activity on an RNA hairpin-dependent intrinsic terminator. *Nucleic Acids Res* **38**, 6045–6053.

Mathia, S., Paliege, A., Koesters, R., Peters, H., Neumayer, H.H., Bachmann, S. & Rosenberger, C. 2013. Action of hypoxia-inducible factor in liver and kidney from mice with Pax8-rtTA-based deletion of von Hippel-Lindau protein. *Acta Physiol* **207**, 565–576.

Myllyharju, J. 2013. Prolyl 4-hydroxylases, master regulators of the hypoxia response. *Acta Physiol* **208**, 148–165.

Rana, M.S., Christoffels, V.M. & Moorman, A.F. 2013. A molecular and genetic outline of cardiac morphogenesis. *Acta Physiol* **207**, 588–615.

Seeliger, E., Sendeski, M., Rihal, C.S. & Persson, P.B. 2012. Contrast-induced kidney injury: mechanisms, risk factors, and prevention. *Eur Heart J* **33**, 2007–2015.

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 161–162

Editorial

Acta Physiologica in 2015

For Acta Physiologica, the last issue of the year has traditionally become a place to thank our readers, contributors, reviewers, editors, publishers and the Scandinavian Physiological Society for the outstanding efforts which have made our journal what it is – a source of knowledge, a place for scientific discourse, and a medium which, in spite of the high degree of individual differentiation, unites all those interested in Physiology around the globe. Moreover, this editorial will highlight novel developments in biomedical publishing important to authors, reviewers and editors alike.

Acta Physiologica: Current developments

Manuscripts and submissions

The current positive development of Acta Physiologica's impact factor has continued in 2015. This has been reflected in a steady rise in the number of manuscripts submitted (Persson 2014a), which remains at around the historical peak value. All manuscripts submitted to Acta are handled at no cost for authors, and all efforts of the editorial team, reviewers and associate editors are invested into providing each author with a first decision on their manuscript no later than 14 days after submission. Free reading, that is *open access*, is available for all submissions for a common fee. Most of our authors, however, choose the free-to-publish option, meaning that these publications come at no cost for the authors.

Regions of interest

Acta Physiologica, as the journal of the Scandinavian Physiological Society, has, over the last years, not only established itself firmly as an international scientific publication organ, but has steadily been climbing the rank list of established journals in Physiology. As such, it attracts a worldwide readership and receives submissions from all continents. Europe and North America continue to account for the majority of downloads. These two regions each account for roughly 35% of all downloads. As before, Europe is leading in terms of contributions (Persson 2013b), while, however, Scandinavia remains on the rise (Persson 2014b). The increasing worldwide availability of Acta Physiologica, especially in emerging economies, has been made possible through numerous philanthropic initiatives, and we are very glad to report the rising numbers of submissions from these countries, supported and encouraged by the journal's strict 'free-to-publish-in' policy (Persson 2013b). Our philanthropic initiatives together with Wiley allow for extensive availability in less favoured regions.

Sense and sensibility: Assessing and quantifying scholarly impact

Scientists are decidedly tired of discussing the value of the impact factor (IF) for assessing quality – it's an old hat. Yet it continues to be used for just that purpose. For Acta Physiologica, the development of the IF has been encouraging, reflected in the number and quality of manuscripts submitted (Persson 2014c). This year's impact factor is 4.38 and places us among the top three journals publishing original articles in all fields of physiology. Discussions about alternative measures of both scientific quality and impact do, however, not stop at our doorstep: Benchmarking, the systematic comparison of our performance to that of other journals, has become standard procedure, but relies on performance metrics that serve as *understandable* and *useful* performance indicators (Kulakowski & Chronister 2006). Altmetrics, short for 'alternative scholarly impact metrics', have been proposed as an alternative, although, originally, did not refer to citations or citation counts (Fenner 2014). On the Open Science (www.openscience.org) platform, supporting the cause of altmetrics, Martin Fenner cites Richard Rhodes, the Pulitzer Prize-winning author of 'Making of the Atomic Bomb':

'Good science, original work, always went beyond the body of received opinion, always represented a dissent from orthodoxy. However, then, could the orthodox fairly assess it?' (Rhodes 1986).

In a world in which 'orthodox' seems to have acquired a negative connotation, what is behind this alternative, then? Is it, actually, more 'fair'? Altmetrics, an umbrella term for a multitude of metrics originating with *#altmetrics*, comprise measurements of article (a) views, (b) discussions, (c) downloads, (d) citations and (e) recommendations (Lin & Fenner 2013). Altmetrics can be gamed and bought, lack information about quality (imagine calculating altmetrics scores of Andrew Wakefield's Lancet article on the MMR/autism link, which took twelve years to be retracted) and fail when it comes to comparing articles

 doi: 10.1111/apha.12605

from different publication dates (try calculating the scores for the key publications of the last Nobel laureates) (Beall 2013). Impact metrics are needed – but the Philosopher's Stone has not been found. Let us keep looking, keep discussing, stay sceptical, stay humble – and always curious. It is, after all, what science is all about. Thank you, everyone.

Conflict of interest

None.

P. B. Persson
Institute of Vegetative Physiology, Charité-Universitaetsmedizin Berlin,
Berlin, Germany
E-mail: pontus.persson@charite.de

References

Beall, J. (2013). Article-Level Metrics: An Ill-Conceived and Meretricious Idea. [online] Available from: http://scholarlyoa.com/2013/08/01/article-level-metrics/ [Accessed September 8, 2015]

Fenner, M. (2014) Altmetrics and Other Novel Measures for Scientific Impact. In: S. Bartling & S. Friesike (eds) *Opening Science*, pp. 179–189. Springer, Heidelberg.

Kulakowski, E.C. & Chronister, L.U. 2006. *Research Administration and Management*. Jones and Bartlett, Sudbury.

Lin, J. & Fenner, M. 2013. Altmetrics in evolution: defining and redefining the ontology of article-level metrics. *Inform Stand Q* **25**, 20.

Persson, P.B. 2013b. Who are our readers? *Acta Physiol (Oxf)* **208**, 137.

Persson, P.B. 2014a. Submissions bode well for 2014. *Acta Physiol (Oxf)* **210**, 1.

Persson, P.B. 2014b. Welcome home Scandinavia!. *Acta Physiol (Oxf)* **210**, 701.

Persson, P.B. 2014c. Downloading-reading-citing? Some thoughts on our bibliometrics. *Acta Physiol (Oxf)* **211**, 249–250.

Rhodes, R. 1986. *The Making of the Atomic Bomb*. Simon and Schuster, New York.

  doi: 10.1111/apha.12605

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 163–164

Editorial

Good publication practice in physiology 2015

Revised Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals

Acta Physiologica adheres to the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals, issued by the International Committee of Medical Journal Editors (ICMJE). All authors, reviewers and editors are encouraged to regularly consult these guidelines, to ensure compliance with the respective current version (available online from: http://www.ICMJE.org). In brief, the last amendment made in December 2014 affects the following issues:

Purpose

Reproducibility has been added as a main objective of every medical journal article published in adherence to the ICMJE guidelines. To this end, it is now explicitly required that the Methods section must 'aim to be sufficiently detailed such that others with access to the data would be able to reproduce the results' (ICMJE 2014).

Conflict of Interest

ICMJE now explicitly recommends authors to avoid 'entering in to agreements with study sponsors [...] that interfere with authors' access to all of the study's data or [...] their ability to analyse and interpret the data and to prepare and publish manuscripts independently when and where they choose' (ICMJE 2014).

Protection of Research Participants

In addition to the required reference to (a) approval obtained from the responsible institutional or national review committee and (b) compliance with the Helsinki Declaration in its current (latest) version, the revised guidelines stress the role of the editor, who is encouraged to cast another critical glance, as 'approval by a responsible review committee does not preclude editors from forming their own judgment whether the conduct of the research was appropriate' (ICMJE 2014) (see also: Structure and Reporting of Experimental Data - Human Subjects, below). Any concerns raised by institutional or regulatory authorities regarding the research project, as well as any corrective action in this regard, need to be declared upon submission of the manuscript and must be available to the handling editor.

Overlapping/duplicate publication

As registration of clinical trials is mandatory (see also: Structure and Reporting of Experimental Data - Human Subjects, below), this entails the prior publication of trial data in clinical trial registries, which is not considered as 'prior publication', given that ICMJE recommendations on word limits and structure are complied with, and that the issue is sufficiently declared, including the name and specifics of the repository (ICMJE 2014).

Fees

In the latest revision of the ICMJE guidelines, fees associated with publishing in biomedical journals are given special focus, which might be due to the current exponential growth in predatory publishers and the increasing number of victims (Xia *et al.* 2015). ICMJE strictly requires full transparency regarding 'revenue streams': 'Any fees or charges that are required for manuscript processing and/or publishing materials in the journal shall be clearly stated in a place that is easy for potential authors to find prior to submitting their manuscripts [...]'(ICMJE 2014). Notably, Acta Physiologica remains free to publish in.

Contracted research

If any part of the research reported in a manuscript has been outsourced to a paid collaborator, this must be clearly stated in the Methods section.

Citations and references

It seems obvious, yet it apparently needs clarification: ICMJE now requires that neither authors, editors nor peer reviewers use references to promote self-interests.

Structure and Reporting of Special Experimental Data

In addition to the uniform requirements for original research articles, Acta Physiologica's author guidelines

 doi: 10.1111/apha.12606

outline special prerequisites for certain data sets, as highlighted below.

High-throughput data

For microarray data, Acta Physiologica requires compliance with the MIAME criteria and the provision of an accession number at the time of manuscript submission. Novel nucleotide or protein sequences must be uploaded to an ICMJE-endorsed database and made publicly available at the time of acceptance of the manuscript (Persson 2013).

Human Subjects

Studies involving subjects require registration, ethical conductance and guideline-compliant reporting: before participant enrolment, clinical trials, that is any research project in which participants are prospectively assigned to any intervention, must be registered in a public trials registry as a prerequisite for the manuscript to be considered for publication. Thus, Acta Physiologica, in line with ICMJE requirements, aims at (a) preventing selective reporting and unnecessary duplication of research and (b) meeting transparency requirements (ICMJE 2014). All research projects involving human or animal subjects are to be conducted in accordance with national and international guidelines, and this must be documented in the manuscript for it to be eligible for consideration for Acta Physiologica. For human studies, this involves, for example, obtaining written informed consent prior to subject enrolment, approval from the respective national or institutional ethics committee, full observance of the Declaration of Helsinki and a declaration of whether the data collected are sex- or ethnicity-specific. For detailed requirements, please consult Acta Physiologica's Author Guidelines and ICMJE's Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals in due time. Acta Physiologica adheres to and encourages consultation of the International Initiative for Enhancing the Quality and Transparency of Health Research (www.equator-network.org) and, in case of randomized controlled trials, the CONSORT statement (www.consort-statement.org).

Animal data

All animal studies must be conducted in accordance with the 3R principles (Russell & Burch 1959) and appropriate national and international legal and administrative regulations and understanding. Examples are national laws, the European directive 2010/63/EU, the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the ARRIVE guidelines (Kilkenny *et al.* 2010). All manuscripts submitted to Acta Physiologica must state the authority from which approval of animal experiments was obtained and the permission number. Species, strain and number of animals used are to be stated. Whenever an invasive method is used, dosage, route and frequency of premedication, anaesthesia, analgesia and/or tranquilizers must be specified to make the manuscript eligible for consideration for publication in Acta Physiologica.

Conflict of Interest

None.

P. B. Persson
Institute of Vegetative Physiology,
Charité-Universitaetsmedizin Berlin,
Berlin, Germany
E-mail: pontus.persson@charite.de

References

International Committee of Medical Journal Editors (2014) Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals. [online]. Available from: http://www.ICMJE.org. [Accessed September 8, 2015]

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M. & Altman, D.G. 2010. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol* **8**, e1000412.

Persson, P.B. 2013. Good publication practice in physiology 2013: Revised author guidelines for Acta Physiologica. *Acta Physiol*, **209**, 250–253.

Russell, W.M.S. & Burch, R.L. 1959. *The Principles of Humane Experimental Technique*. Methuen, London.

Xia, J., Harmon, J.L., Connolly, K.G., Donnelly, R.M., Anderson, M.R. & Howard, H.A. 2015. Who publishes in "predatory" journals? *J Assoc Inf Sci Technol* **66**, 1406–1417.

  doi: 10.1111/apha.12606

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 165–166

Editorial

Did you know that your cravings might be microbes controlling your mind?

Perched on a high stool in my kitchen, I found myself pondering my current, somewhat peculiar activity: I was drinking olive brine out of the jar. While never in my life having craved a bar of chocolate, the craving for green olive brine is reoccurring. Is this craving rather bodily greed than need? What are cravings and are they bad?

As olive brine consists of sodium chloride and water, I therefore assume that the craving I experienced was related to salt. Humans lose water and sodium through several processes such as transpiration, respiration, diarrhoea and vomiting (Hurley & Johnson 2015, and references therein), and this loss can evoke a sodium appetite to make up for the lost electrolytes. Nonetheless, the average person in the western world consumes more sodium than necessary for normal physiological function (Soleimani 2011). Therefore, the salt hunger most of us feel is 'salt gluttony': we do not need it (Schulkin 1986). This 'salt gluttony' has been suggested to originate from, for example, learned preferences, could be a stress-induced intake (for a review: Leshem 2013) or is evolutionary (Smith 1937).

But what about my periodic craving for pancakes? Only hormonal or is there more to the story? A rather new aspect of our eating behaviour has emerged over the past few years, namely the interplay between the gut microbiota, and us as hosts. This 'microbial organ' carries out many functions for the host such as aiding in nutrient acquisition and also immune system development (Alcock *et al.* 2014, and references therein). Nonetheless, several studies indicate that there might be a gut host–parasite/symbiont resource allocation conflict (Alcock *et al.* 2014, and references therein). The gut microbiota can modify host eating behaviour so as to satisfy their needs but in the process resulting in a reduction of host fitness. The phenomenon of host behaviour modification has long been known for more obvious parasitic relationships, such as the exceedingly common protozoan parasite *Toxoplasma gondii,* where the rat, as infected intermediate host, displays reduced predator aversion to aid in the transmission of the parasite (Hari Dass & Vyas 2014). The gut microbiota has been suggested to affect host eating behaviour through various means such as production of neurotransmitters and short chain fatty acids such as butyric acid, shown to have a mood-altering effect in mice as well as inducing changes in taste and pain sensitivity (Alcock *et al.* 2014, and references therein). This creates a mixed symbiotic/parasitic relationship with the host, with increasing parasitic capacity with decreasing microbial diversity since fewer resources have to be spent competing with other microbes (Alcock *et al.* 2014, and references therein). These findings have led to an assumption that a more diverse gut microbiota will lead to healthier eating habits due to increased host fitness as the microbial community tips in favour of a symbiotic relationship rather than a parasitic one.

Different gut microbes have been shown to grow differentially well on different substrates: for example, Bacteroidetes grows well on fats and *Prevotella* on carbohydrates. Interestingly, microbes able to digest some seaweed-specific compounds have been isolated from human guts in populations where seaweed often is consumed as well as cellulose-digesting microbes in children raised on cellulose-rich sorghum (Alcock *et al.* 2014, and references therein) exemplifying the complex interplay between our diets and our gut microbiota.

These findings lead me to conclude that the carbohydrate cravings I sometimes feel might not only be hormonal in origin; they might not even be my own, but my system being hijacked by my gut microbiota. The complexity of human cravings is vast – an intricate interplay between actual physiological needs and psychological, hormonal as well as microbial factors. I am having pancakes for dinner.

Conflict of interest

None.

A. Zakrisson
Institute of Vegetative Physiology,
Charité-Universitaetsmedizin Berlin,
Berlin, Germany
E-mail: a.zakrisson.01@cantab.net

References

Alcock, J., Maley, C.C. & Aktipis, C. 2014. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *BioEssays* **36**, 940–949.

Hari Dass, S.A. & Vyas, A. 2014. *Toxoplasma gondii* infection reduces predator aversion in rats through

 doi: 10.1111/apha.12593

epigenetic modulation in the host medial amygdala. *Mol Ecol* **23**, 6114–6122.

Hurley, S.W. & Johnson, A.K. 2015. The biopsychology of salt hunger and sodium deficiency. *Pflügers Arch* **467**, 445–456.

Leshem, M. 2013. *The Human Penchant for Deranged Salt Balance. Neurobiology of body Fluid Homeostasis: Transduction and Integration (series IV: Frontiers in Neuroscience)*, pp. 1–22. Taylor and Francis, Boca Raton, FL.

Schulkin, J. 1986. The evolution and expression of salt appetite. In: G. de Caro, A.N. Epstein & M. Massi (eds) *The Physiology of Thirst and Sodium Appetite*, pp. 491–496. Springer US, New York.

Smith, H.W. 1937. *The Physiology of the Kidney*. Oxford University Press, Oxford.

Soleimani, M. 2011. Dietary fructose, salt absorption and hypertension in metabolic syndrome: towards a new paradigm. *Acta Physiol* **201**, 55–62.

  doi: 10.1111/apha.12593

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 167–176

Antihypertensive and cardioprotective effects of the dipeptide isoleucine–tryptophan and whey protein hydrolysate

M. Martin,[1] I. Kopaliani,[1] A. Jannasch,[2] C. Mund,[1] V. Todorov,[3] T. Henle[4] and A. Deussen[1]

1 Institute of Physiology, Medical Faculty, TU Dresden, Germany

2 Cardiac Surgery, Heart Center Dresden at TU Dresden, TU Dresden, Germany

3 Division of Nephrology, Department of Internal Medicine III, University Hospital Carl Gustav Carus at TU Dresden, TU Dresden, Germany

4 Institute of Food Chemistry, Faculty of Science, TU Dresden, Germany

Received 13 May 2015, revision requested 16 July 2015, accepted 24 July 2015

Correspondence: M. Martin, Institute of Physiology, Medical Faculty Carl Gustav Carus, TU Dresden, Fetscherstr. 74, Germany
E-mail: Melanie.Martin@tu-dresden.de

Abstract

Aims: Angiotensin-converting enzyme inhibitors are treatment of choice in hypertensive patients. Clinically used inhibitors exhibit a structural similarity to naturally occurring peptides. This study evaluated antihypertensive and cardioprotective effects of ACE-inhibiting peptides derived from food proteins in spontaneously hypertensive rats.

Methods and Results: Isoleucine–tryptophan (*in vitro* IC_{50} for ACE = 0.7 μM), a whey protein hydrolysate containing an augmented fraction of isoleucine–tryptophan, or captopril was given to spontaneously hypertensive rats (n = 60) over 14 weeks. Two further groups, receiving either no supplement (Placebo) or intact whey protein, served as controls. Systolic blood pressure age-dependently increased in the Placebo group, whereas the blood pressure rise was effectively blunted by isoleucine–tryptophan, whey protein hydrolysate and captopril (-42 ± 3, -38 ± 5, -55 ± 4 mm Hg vs. Placebo). At study end, myocardial mass was lower in isoleucine–tryptophan and captopril groups but only partially in the hydrolysate group. Coronary flow reserve (1 μM adenosine) was improved in isoleucine–tryptophan and captopril groups. Plasma ACE activity was significantly decreased in isoleucine–tryptophan, hydrolysate and captopril groups, but in aortic tissue only after isoleucine–tryptophan or captopril treatment. This was associated with lowered expression and activity of matrix metalloproteinase-2. Following isoleucine–tryptophan and captopril treatments, gene expression of renin was significantly increased indicating an active feedback within renin–angiotensin system.

Conclusion: Whey protein hydrolysate and isoleucine–tryptophan powerfully inhibit plasma ACE resulting in antihypertensive effects. Moreover, isoleucine–tryptophan blunts tissue ACE activity, reduces matrix metalloproteinase-2 activity and improves coronary flow reserve. Thus, whey protein hydrolysate and particularly isoleucine–tryptophan may serve as innovative food additives with the goal of attenuating hypertension.

Keywords angiotensin-converting enzyme, arterial hypertension, cardiac hypertrophy, matrix metalloproteinases, whey proteins.

 doi: 10.1111/apha.12578

High blood pressure may be regarded as a pandemic disease affecting more than 50% of the population above an age of 50 years worldwide (Bonita & Reddy 2003, Rogers 2003). Hypertension is associated with vessel stiffening, arterial remodelling, peripheral arterial occlusive disease, coronary heart disease, cardiac infarction, cardiac hypertrophy, heart failure and stroke (European Society of Hypertension-European Society of Cardiology Guidelines 2003). The renin–angiotensin–aldosterone system (RAAS) is known to play a pivotal role in blood pressure regulation and salt/fluid homoeostasis (Johnston *et al.* 1992). Thus, pharmaceutical agents blocking RAAS activity, for example renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers or aldosterone antagonists, have proven useful in the treatment of hypertension and associated diseases. With respect to ACE inhibition, it is of interest to note that several food peptides have been determined that show a great potential for effective RAAS inhibition (Nakamura *et al.* 1995a, Hata *et al.* 1996, Seppo *et al.* 2003). Such peptides could potentially be used as functional food ingredients to delay age-dependent changes of structure and function in the cardiovascular system. Evidence for the effectiveness of such an approach is provided by the finding that isoleucine–proline–proline (IPP) and valine–proline–proline (VPP) contained in a sour milk product decreased blood pressure in hypertensive patients after 8 weeks (Nakamura *et al.* 1995a, Hata *et al.* 1996). In screening experiments, we have isolated the dipeptide isoleucine–tryptophan (IW) from whey protein which exhibited a significantly lower inhibitory constant for ACE than IPP and VPP (IC_{50}: IW 0.7 μM, IPP 5 μM, VPP 9 μM). This prompted us to critically test the ACE-inhibitory potential and blood pressure-lowering and cardioprotective effects of IW in spontaneously hypertensive rats (SHR), a well-established model of experimental hypertension. In addition, we tested an enzymatically prepared hydrolysate of whey protein which contained an elevated fraction of the dipeptide. The results obtained fully support the concept that IW powerfully inhibits plasma and tissue ACE similar to captopril. Thus, IW may be suggested as an innovative food additive which may allow delay or even prevention of hypertension and associated cardiovascular diseases.

Methods

Reagents and samples

Captopril was purchased from Sigma-Aldrich (Seelze, Germany) in analytical quality. Hippuryl-histidyl-leucine and IW (>99%) were purchased from Bachem (Weil am Rhein, Germany). All other chemicals were of analytical grade. Whey protein was from Milei (Leutkirch, Germany). Hydrolysates of whey protein were prepared enzymatically using a combination of alcalase and trypsin as described recently (Lunow *et al.* 2013). Rat food pellets were prepared by Ssniff (Soest, Germany) by cold-pressing after adding the different supplements.

Animals

Sixty male spontaneously hypertensive rats (SHR) were used in the study. For details, please see Data S1. The procedures were approved by the local authorities (permission no.: 24-9168.11-1/2009-46) and are in accordance with NIH regulations.

Experimental groups

Animals were randomly assigned to five groups. The Placebo group (n = 12) received standard food pellets without any supplement and served as a time control for the development of elevated blood pressure and related cardiac and vascular alterations in this experimental model. The captopril (CAP) group (n = 12) received 69 mg kg^{-1} body weight (bw) per day and served as the positive control for the effects of strong ACE inhibition. The isoleucine–tryptophan (IW) group (n = 12) received the chemically pure dipeptide at a daily dose of 19 mg kg^{-1} bw. This group served to test for the effects of the isolated dipeptide. The hydrolysate (HYD) group (n = 12) received enzymatically prepared hydrolysate from whey protein containing an enriched fraction of IW. The applied dose was 770 mg hydrolysate kg^{-1} bw per day, corresponding to 3 mg IW kg^{-1} bw. This group served to test for potential ACE-blocking effects of the peptide mix hydrolysed from whey protein. Another group of rats (n = 12) received the intact whey protein (WProt) at a dose of 770 mg kg^{-1} body mass. This group served to test for potential *in vivo* hydrolysis of the whey protein to peptides with ACE-inhibitory action. Rats were weighed every 2 weeks. The intake of freely accessible food pellets was monitored weekly.

Blood pressure measurements

Systolic arterial blood pressure (SBP) was assessed by the tail-cuff method with an electro-sphygmomanometer (FMI, Seeheim, Germany) in awake rats twice weekly from week 10 to week 28 post-natal (pn). Measurements reported started in week 14 pn and the treatment of rats started at week 14.5 pn. They were performed between 8 and 11:30 a.m. to avoid circadian variability. Values reported for each time point

are based on averaging five separate blood pressure measurements for each rat. Heart rate was obtained from the blood pressure signal.

Echocardiography

Echocardiography (Echo) was conducted as described by others (Pawlush *et al.* 1993) under light chloral aldehyde anaesthesia (300 mg kg^{-1}, i.p.) using the M-mode. Echo measurements were performed at weeks 13, 19 and 27 pn. Each measurement was averaged over 3–5 consecutive cardiac cycles. From recorded signals, stroke volume (SV), cardiac output (CO), cardiac index (CI) and left ventricular fractional shortening (FS) were calculated as detailed in the Data S1.

Isolated heart perfusion

At the end of the feeding period, the hearts were isolated and subjected to isolated perfusion. Following assessment of coronary dilator reserve, coronary ACE inhibition was quantified by assessing the vasoconstrictive effects of angiotensin I and angiotensin II. For details, see Data S1.

ACE activity in plasma and aortic tissue

ACE activity in blood plasma and aortic tissue homogenate was measured using the substrate hippuryl-histidyl-leucine as described in the Data S1.

Western blot and gelatin zymography

Latent 72-kDa pro-MMP2 and active 62-kDa MMP2 as well as TIMP2 protein in heart and aorta were quantified using Western blot. Protein content in the samples was determined by BCA protein assay (Thermo Fischer Scientific, Schwerte, Germany). To assess 62-kDa MMP2 activity, zymography was performed as described by Todt and Fridman (Toth & Fridman 2001) with some modifications. Details are given in the Data S1.

RNA isolation, reverse transcription and quantitative PCR

Isolation of total RNA from rat kidney tissue was performed after automated disruption and homogenization using the MagNa Lyser Instrument (Roche Diagnostics, Mannheim, Germany). Cleared lysate was used for further RNA purification (RNAeasy Mini kit; QIAGEN, Hilden, Germany). Subsequently, 1 μg of total RNA was reverse-transcribed to cDNA using a reverse transcription system (Applied Biosystems, Darmstadt, Germany). The amplification of the cDNA templates for quantification was carried out using the SensiMix SYBR & Fluorescein Kit (Bioline, Luckenwalde, Germany). All kits were applied according to the manufacturer's instructions. Details are given in the Data S1.

Statistics

Data are given as mean ± SEM except for blood pressure measurements which are reported as mean ± SD. Values obtained with different supplements were compared to Placebo (or WProt) using analysis of variance and were tested for significant differences using the unpaired, two-tailed *t*-test. To analyse multiple treatment groups, ANOVA with *post hoc* least significant difference was used. A value of $P < 0.05$ was considered to indicate a significant difference.

Results

Systolic blood pressure

Body mass, heart rate and SBP did not differ significantly between groups at the start of the feeding period. Table 1 displays the average daily intake of compound, body mass, heart rate and SBP at the end of the feeding period. There was no significant difference with respect to body mass and heart rate between groups. However, SBP development was different. SBP increased over 14 weeks in Placebo and WProt groups by 30 ± 2 and 35 ± 5 mm Hg respectively. In contrast, SBP did not change significantly in groups treated with IW or HYD. SBP even fell in the CAP group by 24 ± 1 mm Hg. Thus, IW, HYD and CAP effectively prevented the increase in SBP. The kinetics of age-dependent changes of SBP is shown in Figure 1. During the 14-week feeding period, SBP almost linearly increased with age in the WProt and Placebo groups indicating that supplementation of the non-hydrolysed protein did not have any effect on blood pressure development in this model. This age-dependent linear increase in SBP is completely set off in the IW and HYD groups. Both IW and HYD were less effective than CAP given at a maximally effective dose of 69 mg kg^{-1} bw.

Echocardiography

Heart echo was obtained in weeks 13, 19 and 27 pn. Because there were no significant differences in any echo parameter between weeks 13 and 19 pn, Table 2 shows representative data for weeks 13 and 27. Heart rate and fractional shortening were similar in all groups at all times. However, after 14 weeks of CAP treatment, SV and CO were increased ($P < 0.01$). Cardiac index (CI) decreased in the Placebo and WProt

Table 1 Average daily intake of compounds, heart rate and systolic blood pressure

	IW	HYD	WProt	CAP	Placebo
Daily intake, mg kg^{-1} bw	19	770	770	69	–
Body weight, g	385 ± 29	398 ± 34	398 ± 16	401 ± 37	404 ± 25
Heart rate, bpm	428 ± 36	424 ± 26	436 ± 22	442 ± 31	439 ± 18
SBP, mm Hg†	−42 ± 3*	−38 ± 5*	−12 ± 6	−55 ± 4*	–
SBP, mm Hg‡	−7 ± 1*	−5 ± 2*	+35 ± 5*	−24 ± 1*	+30 ± 2*

IW, isoleucine–tryptophan; HYD, whey protein hydrolysate; WProt, intact whey protein; CAP, captopril; SBP, systolic blood pressure; bw, body weight; wk28, week 28 (end of study); wk14, week 14 (begin of study), †difference vs. Placebo, ‡difference wk28 vs. wk14, *$P < 0.05$.

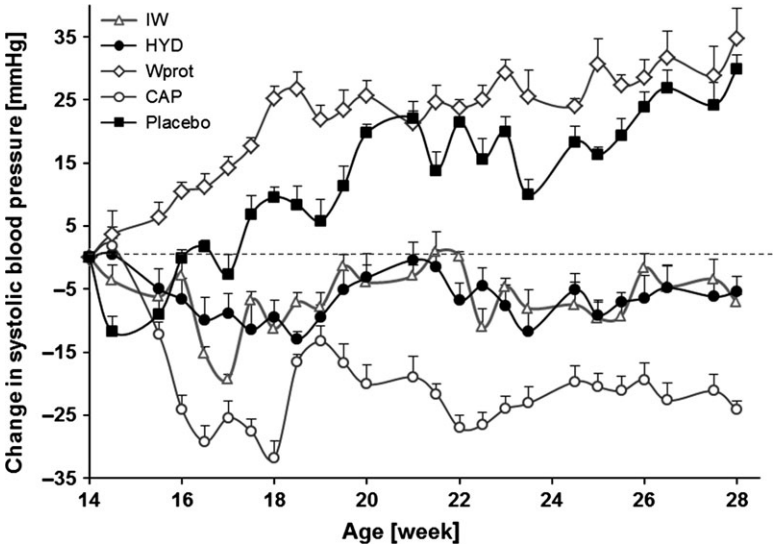


Figure 1 Change of systolic blood pressure in spontaneously hypertensive rats during the 14-week application of isoleucine–tryptophan (IW), whey protein hydrolysate (HYD), intact whey protein (WProt), captopril (CAP) or none (Placebo), $n = 12$ per group.

groups during the feeding period by 18 and 14% respectively ($P < 0.01$). In contrast, a fall of CI was absent in groups IW, HYD and CAP.

Heart mass

Heart mass indices of all animals at the end of study are given in Figure 2. Whole heart mass per unit body mass was significantly lower in the IW and CAP groups as compared to Placebo and WProt ($P < 0.05$). Left ventricular/body mass ratio was lower in IW and CAP groups. The HYD group showed a trend towards a decrease in the left ventricular/body mass ratio (not significant) but no change in whole heart/body mass ratio (Fig. 2).

Coronary flow reserve

Coronary flow reserve assessed by the reactive flow overshoot after a 20-s complete flow stop (Fig. 2c) and the flow increase during intracoronary adenosine infusion (Fig. 2d) gave similar results. Highest coronary flows were measured in the CAP group (augmentation 38 ± 5% and 80 ± 17% versus Placebo, $P < 0.05$) followed by the IW group (augmentation 25 ± 3% and 48 ± 15% vs. Placebo, $P < 0.05$). Also in the HYD group, coronary flow reserve was improved (significant vs. WProt group, not significant vs. Placebo). Hearts of the WProt group showed the lowest flow reserve (not significantly different from Placebo).

ACE activity in plasma, aortic tissue and coronary vessels

Figure 3 displays ACE activities assessed in plasma (a) and aorta (b). Relative to the Placebo group, plasma ACE activity was decreased in IW (23 ± 4%), HYD (20 ± 7%) and CAP (41 ± 7%)-treated SHR (all $P < 0.01$). Plasma ACE activity of the WProt group was similar to that of Placebo (Fig. 3a). Aortic tissue ACE activity was significantly lower in groups IW (24 ± 3%) and CAP (25 ± 3%) vs. Placebo. Aortic ACE activity in the HYD group was similar to those of Placebo and WProt groups (Fig. 3b).

  doi: 10.1111/apha.12578

Table 2 Echocardiographic parameters

	IW		HYD		WProt		CAP		Placebo	
Week pn	13	27	13	27	13	27	13	27	13	27
HR, bpm	359 ± 20	379 ± 31	381 ± 26	372 ± 35	365 ± 42	338 ± 37	385 ± 38	378 ± 29	350 ± 33	340 ± 35
SV, μL	146 ± 7	146 ± 11	136 ± 5	145 ± 12	152 ± 9	150 ± 12	143 ± 5	164 ± 10*	155 ± 9	145 ± 8
CO, ml min^{-1}	52 ± 5	57 ± 7	50 ± 3	55 ± 8	54 ± 4	53 ± 3	53 ± 2	64 ± 4*	51 ± 4	50 ± 3
FS, %	70.4 ± 2.8	71.1 ± 4.0	70.4 ± 3.0	71.1 ± 2.7	71.2 ± 3.5	72.1 ± 3.7	70.2 ± 4.4	68.4 ± 4.3	69.2 ± 3.5	69.7 ± 2.9
CI, μL min^{-1} g^{-1}	150 ± 26	148 ± 27	140 ± 11	137 ± 28	154 ± 19	132 ± 11*	155 ± 10	162 ± 18	151 ± 19	124 ± 11*

HR, heart rate; SV, stroke volume; CO, cardiac output; FS, fractional shortening; CI, cardiac index; data are given as mean ± SD, n = 12 per group, *$P < 0.05$ vs. week 13 pn. For abbreviations, see legend to Table 1.

In addition, it was tested whether the different compounds supplemented with the daily food *in vivo* had a significant effect on coronary vessel tone regulation *ex vivo*. Thus, the effect of AngI on coronary flow of isolated perfused hearts served as an index of ACE activity. Coronary infusion of AngII caused a similar flow reduction in all groups (average 36%). The decrease in coronary flow in response to AngI infusion was severely blunted in the groups that had received IW or CAP during the feeding period ($P < 0.05$). A smaller inhibition of the AngI-induced coronary constriction was seen in the HYD group ($P = 0.056$ vs. Placebo and $P < 0.05$ vs. WProt). Figure 3c displays the ratio of flow decreases during AngI vs. those during AngII. For Placebo and WProt, this ratio is 1.04 ± 0.12 and 1.15 ± 0.09 respectively. The ratio was significantly decreased for groups IW, CAP and HYD. Because compounds were added with the food pellets, the results clearly indicate effective *in vivo* ACE inhibition which persisted after heart isolation.

Kidney renin mRNA levels

Compared to rats of the Placebo group, kidney renin mRNA levels of the CAP group were increased more than 4.5 times. Also, in the IW group, a smaller but significant increase in renin mRNA was observed (1.5-times; $P < 0.05$; Figure 4). Despite a similarly increased numerical value, mRNA level was not significantly elevated in the HYD group due to a larger variance.

MMP2 and TIMP2

Figure 5 and Table 3 demonstrate changes in expression and activity of MMP2, as well as TIMP2 protein in response to various supplements. Both IW and CAP significantly ($P < 0.05$) decreased pro-MMP2 expression, as well as MMP2 activity in heart and aorta. Even though TIMP2 expression remained unchanged, there was a significant ($P < 0.05$) decrease in the MMP2/TIMP2 ratio. No significant differences were observed in the HYD group as compared to Placebo.

Discussion

The present study convincingly shows that IW, a dipeptide present in whey protein (Sienkiewicz 1981) and an enzymatically prepared hydrolysate from whey protein may fully attenuate the age-associated SBP rise in SHR similar to CAP (Fig. 1). However, while IW closely mimics all effects of CAP on heart mass (Fig. 2a & b), coronary flow reserve (Fig. 2c & d), ACE activity in plasma and aorta (Fig. 3a & b), coronary *ex vivo* ACE activity (Fig. 3c) and expression

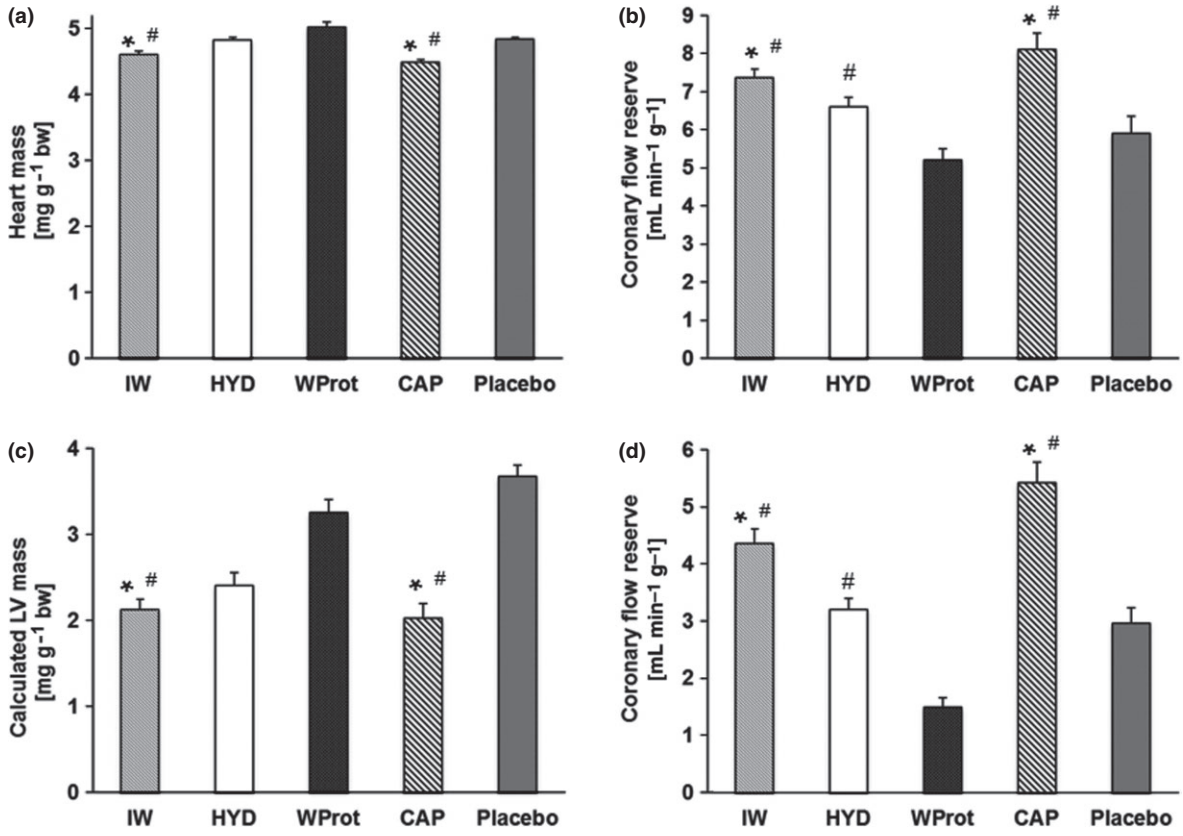


Figure 2 Heart weight determined gravimetrically post-mortem (a) and left ventricular mass determined by echo normalized to body weight (b) after feeding of different compounds for 14 weeks. For abbreviations, see legend to Figure 1. $n = 12$ for each group. Coronary flow reserve determined as maximal flow (c) after a 20-s flow stop ($n = 7–11$) and (d) during intracoronary adenosine infusion (1 μM, $n = 7–11$). For further information, see legend to Figure 1. $^{*}P < 0.05$ vs. Placebo, $^{\#}P < 0.05$ vs. WProt.

and activity of MMP2 (Table 3), this is achieved only in part with HYD. Furthermore, ACE inhibitors IW and CAP cause an increase in renin mRNA expression in the kidney (Fig. 4).

The pronounced blood pressure-lowering effect of IW (Fig. 1) clearly documents that this peptide is effectively absorbed in the gastrointestinal tract and reaches plasma and tissue concentrations high enough to evoke substantial inhibition of ACE (Fig. 3). While IW was given at a molar dose of approximately 60 μmol kg^{-1} bw per day, CAP was applied at a dose of 318 μmol kg^{-1} bw per day. This is a maximally effective CAP dose chosen to document the maximal effect which may be achieved by ACE inhibition in our model. The blood pressure-lowering effect seen with CAP (-24 ± 1 mm Hg) is similar to that reported by others (Wu & Ding 2002). The IW dose of 60 μmol kg^{-1} bw per day was chosen, because this dose appears realistic with respect to the yield obtained with optimized whey protein hydrolysis technology (Henle *et al.* 2009). It is expected that with an equimolar concentration of IW, the difference towards the effect of CAP might decrease. Most notably, neither Ile nor Trp (as single amino acids) exerts any inhibitory effect on ACE as determined in separate *in vitro* experiments (see Data S1).

Because IW is present in whey protein, a hydrolysate was prepared which contained an enriched IW fraction. In the preparation used, 3 mg IW was contained in the daily dose of 770 mg hydrolysate kg^{-1} bw. Thus, in the HYD group, IW was substituted at a molar dose of approximately 9.5 μmol kg^{-1} bw per day. Despite this rather low dose of IW, the SBP was lowered similarly as in the IW group which received a 6.3-fold higher dose of IW. It seems unlikely that the entire blood pressure-lowering effect in the HYD group was brought about by the IW fraction. Rather, in whey hydrolysate, other peptides are contained which may inhibit ACE activity, for example WL and GY (Martin *et al.* 2008, Khedr *et al.* 2015). Furthermore, precursor peptides could be contained in this hydrolytic mix which may be activated *in vivo* (e.g. by gastrointestinal digest or tissue peptidases). A nutritive effect of protein supplementation on SBP can be ruled out, because the identical amount of protein/peptide supplied in the WProt group was ineffective (Figs 1, 2

  doi: 10.1111/apha.12578

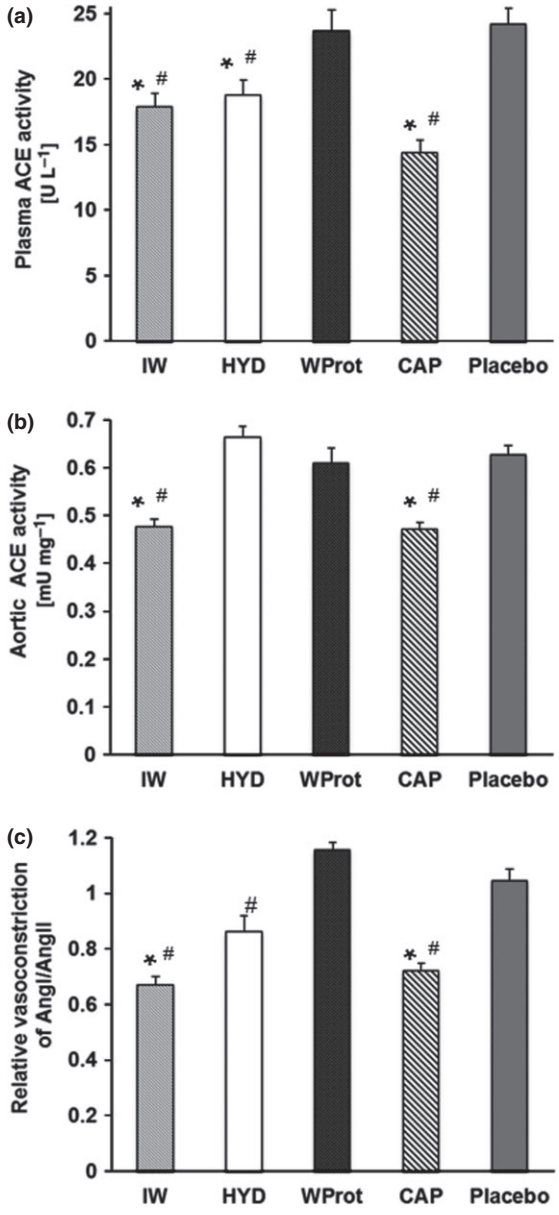


Figure 3 ACE activity in (a) plasma, (b) aorta and (c) coronary vessels. Coronary ACE activity is expressed as the ratio of vasoconstrictive effects of AngI and AngII. A decreased ratio reflects ACE inhibition. For abbreviations, see legend to Figure 1. n = 7–12 per group, $^{*}P < 0.05$ vs. Placebo, $^{\#}P < 0.05$ vs. WProt.

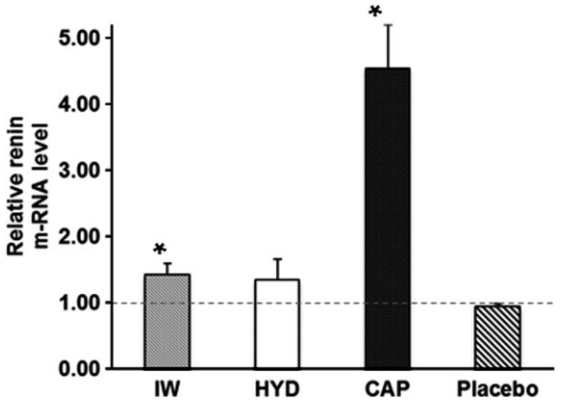


Figure 4 Relative levels of renin mRNA expression in kidney of SHR. For abbreviations, see legend to Figure 1. n = 12 for each group, $^{*}P < 0.05$ vs. Placebo.

and 3). This excludes that the full-length whey protein is hydrolysed quantitatively during gastrointestinal digest resulting in ACE-blocking and blood pressure-lowering peptides.

Previous experimental studies have shown a blood pressure-lowering effect of peptides from hydrolysates of food proteins. Eight-week oral administration of sour milk containing the tripeptides IPP and VPP (10 mg kg^{-1} bw) decreased SBP of SHR by 14 mm Hg as compared to a control group (Kim *et al.* 2008). This effect is smaller than those observed in the present study. IW lowered SBP by 42 ± 3 mm Hg and HYD decreased SBP by 38 ± 5 mm Hg (Table 1). In a clinical study with hypertensive patients, Hata *et al.* (1996) demonstrated that a daily intake of 95 mL of a sour milk preparation containing peptides IPP and VPP for 8 weeks caused a significant decrease in SBP by 14 mm Hg. The daily dose used was equivalent to 0.033 mg VPP and 0.025 mg IPP kg^{-1} bw. Thus, the dose applied to humans was considerably lower than that used in rat studies (Kim *et al.* 2008). Nonetheless, a significant antihypertensive effect was reported (Nakamura *et al.* 1995b). Because IW has a considerably lower IC_{50} for ACE inhibition than IPP or VPP and because IW lowered SBP (Fig. 1, Table 1) to a greater extent than IPP and VPP (Kim *et al.* 2008), it seems likely that IW or an IW-containing hydrolysate when applied in humans may have a great potential to lower blood pressure.

Despite the similar antihypertensive effect in the HYD and IW groups, there is a remarkable difference with respect to tissue ACE activities and also (at least quantitatively) with respect to heart mass, coronary flow reserve, coronary ACE activity, renin mRNA expression and tissue MMP activity. These quantitatively different effects are probably best explained by the differential efficacy on plasma ACE and local tissue ACE (De Mello & Frohlich 2014). Plasma ACE activity was similarly blunted in HYD and IW groups. In both groups, also the increase in blood pressure was blunted to a similar extent (Fig. 1). However, HYD was less effective in lowering aortic ACE and MMP2 activities (Figs 3 and 5, Table 3), lowering heart mass (Fig. 2) and causing feedback inhibition of renin mRNA expression (Fig. 4). These divergent results are probably best explained by assuming that

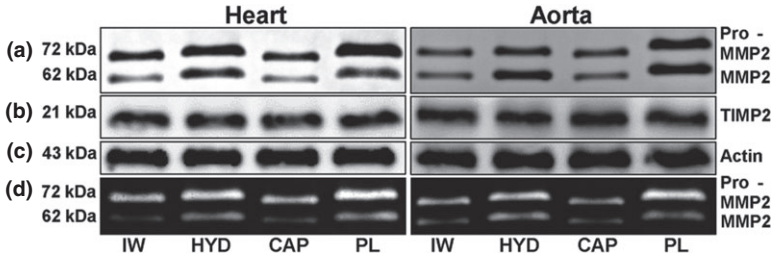


Figure 5 (a) Western blots of 72-kDa pro-MMP2, 62-kDa MMP2, (b) 21-kDa TIMP2 protein and (c) actin (loading control). (d) Zymography of MMP2 activity. $n = 5$ per group. For quantitative data and abbreviations, see Table 3.

Table 3 MMP2 and TIMP2 in heart and aorta of SHR after 14 weeks of treatment

	IW	HYD	CAP	Placebo
72-kDa Pro-MMP2 protein (%)				
Heart	73 ± 10*	90 ± 11	67 ± 12*	100
Aorta	66 ± 7*	88 ± 6	62 ± 9*	100
62-kDa MMP2 protein (%)				
Heart	67 ± 12*	91 ± 9	62 ± 7*	100
Aorta	64 ± 14*	90 ± 11	61 ± 12*	100
Total MMP2/TIMP2 ratio				
Heart	0.67 ± 0.1*	0.89 ± 0.11	0.61 ± 0.12*	1
Aorta	0.59 ± 0.07*	0.85 ± 0.06	0.54 ± 0.09*	1
62-kDa MMP2 activity (%)				
Heart	69 ± 7*	93 ± 7	64 ± 8*	100
Aorta	66 ± 8*	91 ± 6	60 ± 9*	100

MMP2, matrix- metalloproteinase-2; TIMP2, tissue inhibitor of metalloproteinase-2; data are given as mean ± SD relative to Placebo, $n = 5$ per group, $^*P < 0.05$ vs. Placebo. For abbreviations, see legend to Table 1.

blocking of plasma and endothelial ACE activity had a clear impact on blood pressure (CAP, IW and HYD). However, tissue ACE inhibition and prevention of MMP activation was required to achieve the reduction of myocardial hypertrophy and the improvement of coronary flow reserve (CAP and IW). This interpretation is fully compatible with the notion that the vascular ACE (plasma and endothelial ACE) greatly determines blood pressure, but that the local tissue ACE plays a major role in tissue remodelling (Rosenthal *et al.* 1984, Bader & Ganten 2008, Bader 2010).

The disease-related hypertrophy of SHR develops around the 40th week of life (Conrad *et al.* 1995). Because our study started in week 14 pn, this probably explains why no effects of cardiac remodelling were observed during the first 6 weeks of treatment, whereas significant effects on cardiac remodelling were seen over the full period of the study (Table 2, Fig. 2a & b). It is likely that effects would even be larger at an age beyond 40 weeks. Tissue ACE is strongly inhibited by IW similar to CAP, whereas the whey hydrolysate was inferior in inhibiting aortic ACE activity (Fig. 3c). In addition to the assessment of coronary ACE activity *ex vivo* in the isolated heart model, it might have been desirable to also measure myocardial ACE activity. However, because the hearts were subjected to isolated organ perfusion and this might have interfered with myocardial ACE activity, we decided not to perform consecutive ACE activity measurement in myocardial homogenate.

The powerful effects of IW objectified in the current study in SHR may differ from those in humans, for example, due to differences in GI peptide absorption or a lower efficacy on human ACE. In a still ongoing study on human volunteers, we found that plasma levels of IW steeply increase within 30 min after a single oral ingestion of 50 mg of IW. This was paralleled by a 32% decrease of plasma ACE activity (Martin *et al.* 2015). Thus, when applied to humans, IW evokes effects similar to those observed in SHR.

Perspective

The present study provides evidence for substantial antihypertensive effects of IW and an IW-containing hydrolysate from whey protein. IW mimics all effects determined in a positive control employing the established ACE inhibitor captopril. Thus, IW which consists of two essential amino acids might serve as a powerful food supplement with the aim of blocking vascular and tissue ACE. Previous experimental studies have shown that RAAS inhibition at young age may delay, decrease or even completely block hypertension at higher age (Harrap *et al.* 1990, Brower *et al.* 2007, Ishiguro *et al.* 2009, Neglia *et al.* 2011). This protective effect was seen even if the treatment was discontinued (Ishiguro *et al.* 2009). Because the application of functional food ingredients appears most interesting for preventive measures, future studies must address the effects of whey peptides in normotensive/pre-hypertensive subjects with respect to age-dependent blood pressure development.

We thank Dr. Stephan Wiedemann and Dr. Aljoscha Brandt (Department of Cardiology; TU Dresden) for the introduction to rat heart echocardiography and for their willingness to offer the use of the ultrasonic device. We also thank Peter Lachmann, Silke Brillof and Prof. Dr. Norbert Weiss

  doi: 10.1111/apha.12578

(Division of Nephrology and University Center for Vascular Medicine, Department of Internal Medicine III, University Hospital Carl Gustav Carus at TU Dresden) for the introduction and for the use of the RT-PCR measurements.

Funding

This work was supported by a grant-in-aid from the Federal Ministry of Education and Research of Germany, BMBF [grant number 0315473A].

Conflict of interest

The authors declare that they have no conflict of interest.

References

Bader, M. 2010. Tissue renin-angiotensin-aldosterone systems: targets for pharmacological therapy. *Annu Rev Pharmacol Toxicol* **50**, 439–465.

Bader, M. & Ganten, D. 2008. Update on tissue renin-angiotensin systems. *J Mol Med (Berl)* **86**, 615–621.

Bonita, R. & Reddy, S. 2003. *The World Health Report 2003 - Shaping the Future*. Neglected Global Epidemics: three growing threats, pp. 85–87. World Health Organization, Geneva.

Brower, G.L., Levick, S.P. & Janicki, J.S. 2007. Inhibition of matrix metalloproteinase activity by ACE inhibitors prevents left ventricular remodeling in a rat model of heart failure. *Am J Physiol Heart Circ Physiol* **292**, H3057–H3064.

Conrad, C.H., Brooks, W.W., Hayes, J.A., Sen, S., Robinson, K.G. & Bing, O.H. 1995. Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. *Circulation* **91**, 161–170.

De Mello, W.C. & Frohlich, E.D. 2014. Clinical perspectives and fundamental aspects of local cardiovascular and renal Renin-Angiotensin systems. *Front Endocrinol (Lausanne)* **5**, 16.

European Society of Hypertension-European Society of Cardiology Guidelines, C. 2003. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens* **21**, 1011–1053.

Harrap, S.B., Van der Merwe, W.M., Griffin, S.A., Macpherson, F. & Lever, A.F. 1990. Brief angiotensin converting enzyme inhibitor treatment in young spontaneously hypertensive rats reduces blood pressure long-term. *Hypertension* **16**, 603–614.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y. & Takano, T. 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am J Clin Nutr* **64**, 767–771.

Henle, T., Deussen, A. & Martin, M. & TU-Dresden. 2009. Whey protein hydrolysate containing tryptophan peptide consisting of alpha lactalbumin and the use thereof. Patent EP 2 320 751 B1. Filing date: 02.07.2009.

Ishiguro, K., Hayashi, K., Sasamura, H., Sakamaki, Y. & Itoh, H. 2009. "Pulse" treatment with high-dose angiotensin blocker reverses renal arteriolar hypertrophy and regresses hypertension. *Hypertension* **53**, 83–89.

Johnston, C.I., Burrell, L.M., Perich, R., Jandeleit, K. & Jackson, B. 1992. The tissue renin-angiotensin system and its functional role. *Clin Exp Pharmacol Physiol Suppl* **19**, 1–5.

Khedr, S., Martin, M. & Deussen, A. 2015. Inhibitory efficacy and biological variability of tryptophan containing dipeptides on human plasma angiotensin converting enzyme activity. *J Hypertens* **04**, 2–6.

Kim, S.M., Kim, J.Y. & Choue, R.W. 2008. Effects of IPP and VPP supplementation on blood pressure, ACE activity and no production in spontaneously hypertensive rats. *FASEB J* **22**, 1112–1113.

Lunow, D., Kaiser, S., Brückner, S., Gotsch, A. & Henle, T. 2013. Selective release of ACE-inhibiting tryptophan-containing dipeptides from food proteins by enzymatic hydrolysis. *Eur Food Res Technol* **237**, 27–37.

Martin, M., Wellner, A., Ossowski, I. & Henle, T. 2008. Identification and quantification of inhibitors for Angiotensin-converting enzyme in hypoallergenic infant milk formulas. *J Agric Food Chem* **56**, 6333–6338.

Martin, M., Dieterich, P., Kaiser, S. & Deussen, A. 2015. Poster presentations. *Acta Physiol* **213**, 68–199.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S. & Takano, T. 1995a. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J Dairy Sci* **78**, 777–783.

Nakamura, Y., Yamamoto, N., Sakai, K. & Takano, T. 1995b. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J Dairy Sci* **78**, 1253–1257.

Neglia, D., Fommei, E., Varela-Carver, A., Mancini, M., Ghione, S., Lombardi, M., Pisani, P., Parker, H., D'Amati, G., Donato, L. & Camici, P.G. 2011. Perindopril and indapamide reverse coronary microvascular remodelling and improve flow in arterial hypertension. *J Hypertens* **29**, 364–372.

Pawlush, D.G., Moore, R.L., Musch, T.I. & Davidson, W.R. Jr. 1993. Echocardiographic evaluation of size, function, and mass of normal and hypertrophied rat ventricles. *J Appl Physiol*, **74**, 2598–2605.

Rogers, A. 2003. *The World Health Report 2002 - Reducing Risks, Promoting Healthy Life. Chapter 4: quantifying Selected Major Risks to Health*, pp. 57–58. World Health Organization, Geneva.

Rosenthal, J.H., Pfeifle, B., Michailov, M.L., Pschorr, J., Jacob, I.C. & Dahlheim, H. 1984. Investigations of components of the renin-angiotensin system in rat vascular tissue. *Hypertension* **6**, 383–390.

Seppo, L., Jauhiainen, T., Poussa, T. & Korpela, R. 2003. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *Am J Clin Nutr* **77**, 326–330.

Sienkiewicz, T. 1981. Nomenclature and some properties of whey proteins. II. Alpha-lactalbumin, immunoglobulins,

proteose-peptones, minor proteins and enzymes. *Nahrung* **329**, 335–343.

Toth, M. & Fridman, R. 2001. Assessment of Gelatinases MMP-2 and MMP-9 by Gelatin Zymography. *Methods Mol Med* **57**, 163–174.

Wu, J. & Ding, X. 2002. Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Res Int* **35**, 367–375.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Martin *et al.*: Antihypertensive and Cardioprotective Effects of the Dipeptide Isoleucine-Tryptophan and Whey Protein Hydrolysate.

  doi: 10.1111/apha.12578

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 177–190

The effect of exercise mode on the acute response of satellite cells in old men

J. P. Nederveen,[1] S. Joanisse,[1] C. M. L. Séguin,[1] K. E. Bell,[1] S. K. Baker,[2] S. M. Phillips[1] and G. Parise[1,3]

1 Department of Kinesiology, McMaster University, Hamilton, ON, Canada

2 Department of Medicine, McMaster University, Hamilton, ON, Canada

3 Department of Medical Physics & Applied Radiation Sciences, McMaster University, Hamilton, ON, Canada

Received 5 May 2015,
revision requested 8 June 2015,
revision received 3 September 2015,
accepted 7 September 2015
Correspondence: G. Parise,
Departments of Kinesiology and Medical Physics and Applied Radiation Sciences,
McMaster University, Hamilton, Ontario, Canada L8S 4L8.
E-mail: pariseg@mcmaster.ca

Abstract

Aim: A dysregulation of satellite cells may contribute to the progressive loss of muscle mass that occurs with age; however, older adults retain the ability to activate and expand their satellite cell pool in response to exercise. The modality of exercise capable of inducing the greatest acute response is unknown. We sought to characterize the acute satellite cell response following different modes of exercise in older adults.

Methods: Sedentary older men (n = 22; 67 ± 4 years; 27 ± 2.6 kg*m^{-2}) were randomly assigned to complete an acute bout of either resistance exercise, high-intensity interval exercise on a cycle ergometer or moderate-intensity aerobic exercise. Muscle biopsies were obtained before, 24 and 48 h following each exercise bout. The satellite cell response was analysed using immunofluorescent microscopy of muscle cross sections.

Results: Satellite cell expansion associated with type I fibres was observed 24 and 48 h following resistance exercise only ($P < 0.05$), while no expansion of type II-associated satellite cells was observed in any group. There was a greater number of activated satellite cells 24 h following resistance exercise (pre: 1.3 ± 0.1, 24 h: 4.8 ± 0.5 Pax7 + /MyoD+cells/100 fibres) and high-intensity interval exercise (pre: 0.7 ± 0.3, 24 h: 3.1 ± 0.3 Pax7 + /MyoD+cells/100 fibres) ($P < 0.05$). The percentage of type I-associated SC co-expressing MSTN was reduced only in the RE group 24 h following exercise (pre: 87 ± 4, 24 h: 57 ± 5%MSTN+ type I SC) ($P < 0.001$).

Conclusion: Although resistance exercise is the most potent exercise type to induce satellite cell pool expansion, high-intensity interval exercise was also more potent than moderate-intensity aerobic exercise in inducing satellite cell activity.

Keywords ageing, exercise, satellite cells.

A predictable characteristic of ageing is the loss of muscle mass and strength collectively referred to as sarcopenia (Snijders *et al.* 2009). The gradual loss of muscle is thought to begin as early as the fifth decade of life (Hughes *et al.* 2001) and ultimately results in impaired functional capacity, increased incidence of injury and loss of independence (Roubenoff & Hughes 2000, Snijders *et al.* 2009). The precise mechanisms underlying the progression of sarcopenia are not completely understood; however, the loss of satellite cell (SC) number and function may be contributing factors (Snijders *et al.* 2009, McKay *et al.* 2012, 2013, Verdijk *et al.* 2012).

 doi: 10.1111/apha.12601

Although the notion that ageing is associated with a reduction in SC number is popular, the evidence is equivocal in humans, with some studies suggesting a reduced SC pool (Kadi *et al.* 2004, Sajko *et al.* 2004) in older adults, while others report no change as a function of ageing (Roth *et al.* 2001, Dreyer *et al.* 2006). More recent work suggests that there may be a specific reduction in type II-associated SC in older adults (Verdijk *et al.* 2007, McKay *et al.* 2012). Importantly, ageing is not only associated with a reduction in SC pool size but also in the ability of the SC pool to respond following a bout of exercise (McKay *et al.* 2012, 2013). This may be a particularly relevant fact since the extent of the SC response following acute exercise is associated with the degree of muscle growth following training in young males (Bellamy *et al.* 2014); however, whether this relationship exists in older adults has yet to be determined.

Various forms of exercise like resistance training (Mackey *et al.* 2007, Verdijk *et al.* 2009, Leenders *et al.* 2013) and even endurance training (Charifi *et al.* 2003, Verney *et al.* 2008) lead to increases in muscle fibre size in the elderly and may be an effective countermeasure in slowing the inevitable loss of muscle mass associated with ageing. More specifically, increases were observed in type IIa fibres following endurance training (Charifi *et al.* 2003, Verney *et al.* 2008) and in type II fibres following resistance exercise (Verdijk *et al.* 2009 Leenders *et al.* 2013). Resistance exercise in older adults has been shown to lead to an acute increase in the number of SC associated with type I fibres, but this response is delayed (Snijders *et al.* 2014) or not observed in type II fibres (McKay *et al.* 2012) depending on the time at which the last biopsy was taken. This is in contrast to what is observed in young adults. Expansion of the SC pool is associated with an increase in muscle mass in younger adults (Kadi & Thornell 2000, Verdijk *et al.* 2009, Bellamy *et al.* 2014) and in type II muscle fibre size in older adults (Verdijk *et al.* 2014). Only one study has reported an increase in fibre CSA without an expansion of the SC pool. These authors show an increase in CSA of both type I and II fibres with an expansion of the SC pool associated with only type I fibres following 12 weeks of aerobic training (Fry *et al.* 2014). The magnitude of SC expansion following resistance exercise is lower in older adults as compared to young (Dreyer *et al.* 2006, Snijders *et al.* 2009, McKay *et al.* 2012), and the impact of satellite cell expansion on promoting adaptation appears highly relevant in young adults. Importantly, it has not yet been determined whether SC expansion in older adults is related to long-term muscle adaptation; however, given the potential importance of the SC response in promoting adaptation, and the delayed SC response observed in older adults, a characterization of SC expansion following different modes of activity may be of interest.

Myostatin (MSTN) is a transforming growth factor β family member and is a negative regulator of skeletal muscle growth (McPherron *et al.* 1997). In older adults, MSTN serum levels are inversely correlated with muscle mass (Yarasheski *et al.* 2002). Research has also shown elevated protein (Léger *et al.* 2008) and elevated gene expression of MSTN in older adults compared to young controls (Raue *et al.* 2006, Léger *et al.* 2008) at rest. MSTN has also been shown to impair myoblast proliferation *in vitro* (McCroskery *et al.* 2003), and our laboratory has recently demonstrated an impaired reduction in MSTN co-expression with SC following a single bout of resistance exercise in older adults (McKay *et al.* 2012). Taken together, these data suggest that MSTN may play a role in muscle wasting associated with ageing. Identifying modes of exercise that maximize the reduction in MSTN co-expression with SC may allow for maximal muscle gains in older adults.

Resistance (Verney *et al.* 2008, Verdijk *et al.* 2009, Leenders *et al.* 2013) and endurance (Charifi *et al.* 2003, Verney *et al.* 2008) exercise training have been shown to improve muscle mass and strength, and improve performance capacity in older adults. Recently, there has been great interest in determining the effectiveness of different forms of aerobic exercise like high-intensity interval training and how its health benefits compare to more traditional endurance exercise. High-intensity interval training is a more time-efficient alternative to traditional endurance exercise with similar health benefits, similar adherence during the training intervention, and is well tolerated by a large range of healthy and diseased populations (Currie *et al.* 2013, Terada *et al.* 2013, Gillen & Gibala 2014). The aim of this study was to examine and compare the acute SC response in older adults following resistance exercise (RE), high-intensity interval exercise (HIT) and moderate-intensity aerobic exercise (AE). We hypothesized that both the RE and the HIT would have the greatest acute SC response and that this may in part be mediated by a greater reduction in MSTN co-expression with SC.

Materials and methods

Ethical approval

This study was approved by the Hamilton Integrated Research Ethics Board (HIREB). All procedures conformed to the most recent revision of the Declaration of Helsinki on the use of human subjects as research participants. Furthermore, subjects gave their

  doi: 10.1111/apha.12601

informed written consent prior to enrolling in the study.

Subjects

Twenty-two untrained healthy older males (67 ± 7 years) were recruited from the Hamilton area and its surrounding communities. Subjects were not involved in any type of exercise training programme for ≥4 months prior to the study and were required to complete a routine screening and health questionnaires. Prospective subjects were excluded based on the following criteria: evidence of heart disease, respiratory disease, uncontrolled hypertension, diabetes, any major orthopaedic disability, the use of non-steroidal anti-inflammatory drugs (NSAIDs) as their use may affect SC activation as previously shown (Mackey *et al.* 2007) and smoking. Additionally, prospective subjects were required to complete an exercise stress test prior to participation in order to ensure they were in good health and physically capable of performing the tasks required in the study. Specifically, the stress test consisted of an incremental cycling test to volitional fatigue on a cycle ergometer, providing values for maximum oxygen uptake (VO_2 peak), maximum heart rate (HR_{max}) and peak power for each subject. Subjects were then placed in one of three exercise groups; each group was matched for age, weight, BMI, VO_2 peak, strength and lean mass tissue. Each group would perform a single bout of RE, AE or HIT.

Baseline testing and familiarization

One week prior to any acute bout of exercise, subjects visited the laboratory for the following: a body composition assessment using dual-energy X-ray absorptiometry (DEXA; GE Healthcare, Toronto, ON, Canada), a familiarization session with the equipment and procedures involved in the study and a preliminary strength test. Both the familiarization session and preliminary strength test were also used for baseline testing. Subjects who were selected for the AE and HIT groups were required to cycle on a cycle ergometer at predicted workloads to ensure that their power output produced the appropriate physiological response – as determined by their heart rate. The power output for subjects in the AE group was required to elicit 70% of a subject's HR_{max} (60% of their VO_2 peak), while the power output for subjects in the HIT group was required to elicit 90–95% of a subject's HR_{max} (90% of their VO_2 peak). For those subjects chosen for the RE group, the preliminary strength test was not only used to determine strength but also the workload assigned for their exercise day. The preliminary strength test consisted of a bilateral 10 repetition maximum (10-RM) test for both leg press and knee extension. Leg muscle strength (10-RM) was estimated on weight machines for leg extension (Atlantis Precision Series Leg Extension C-105, Laval QC) and leg press (HUR 3545 Leg Press Incline: HUR, Northbrook, IL, USA). In brief, subjects performed familiarization sessions with the machines, to ensure correct lifting technique was demonstrated and practiced. Following this demonstration, participants performed a warm-up of 10 repetitions at a very light load (~60–70% 10-RM). The weight was then increased per set, and participants completed up to 10 repetitions. Between each 10-RM attempt, participants were given 3 min of rest and no more than three attempts were required to determine 10-RM. Additionally, it is important to eliminate the effects of differences in diet (i.e. protein intake) when assessing anabolic responses. As such, subjects consumed a standardized, weight maintenance diet beginning 2 days prior to the acute exercise bout and lasting until the final day of testing (48 h post-exercise). The diet was composed of roughly 55% carbohydrate, 30% fat and 15% protein. Subjects arrived fasted for each muscle biopsy and were provided with a standardized, mixed macronutrient snack that delivered a suboptimal protein dose (5–7 g protein) immediately following exercise by a member of the research team. Subjects consumed their prescribed breakfasts upon returning home from the laboratory on days where they received biopsies.

Acute exercise protocol

Subjects arrived at the laboratory in the morning and completed a single acute bout of exercise which was unique to each subject depending on what group he was selected for (AE, HIT or RE). Subjects in the AE group were required to complete 30 min of cycling at a moderate intensity on a cycle ergometer. The intensity was set at a heart rate that corresponded to 55–60% of the individual's baseline VO_2 peak. Subjects in the HIT group instead performed 10 × 60 sec bouts of high-intensity cycling at a heart rate that corresponded to 90–95% of the individual's baseline VO_2 peak. These 60-s bouts of high-intensity cycling were interspersed by 60-s of low-intensity cycling (50W) for recovery. Subjects in the RE group were required to complete three sets of bilateral leg press and knee extension at approximately 95% of their 10-RM with 2 min of rest in between each sets. Participants completed leg press followed by leg extension. The first two sets required subjects to complete 10 repetitions of each, while the 3rd and final set required subjects to complete as many repetitions of

each until volitional failure (Mean ± SEM, leg extension: 13 ± 2 reps; leg press is 19 ± 2).

Blood measures

A resting serum and plasma sample was obtained prior to the intervention (baseline) and 24 h post-exercise from the antecubital vein. Serum samples were analysed for creatine kinase (CK) activity as per the instructions provided by the manufacturer using a commercially available kit (Pointe Scientific, Canton, MI, USA).

Muscle sample collection

A total of four percutaneous needle biopsies were taken from the mid-portion of the *vastus lateralis* under local anaesthetic using a custom-modified 5-mm Bergstrom needle adapted for manual suction under local anaesthesia (2% xylocaine). Subjects came into the laboratory during the morning of the intervention, and 2 biopsies were taken prior to exercise for baseline analysis (Pre). This study is part of a larger, previously published study where measurements of muscle protein synthesis were taken (Bell *et al.* 2015). Four biopsies were necessary to measure muscle fractional synthetic rate; specifically, 2 pre-exercise biopsies were needed (Pre1 and Pre2). The other biopsies were obtained at 24 h and 48 h post-exercise. Incisions and biopsies taken on the same leg were spaced by approximately 5 cm to minimize any effect of the previous biopsy. Biopsies were taken alternately of each leg (2 biopsies/leg). Diurnal variation was controlled for to the best of our abilities by taking all biopsies in the morning between the hours of 6 am and 9 am following an overnight fast. Upon excision, muscles samples were immediately divided into smaller pieces that were snap-frozen in liquid nitrogen for gene expression analysis and mounted in optimal cutting temperature (OCT) for immunohistochemistry.

Immunohistochemistry

Muscle cross sections (7 μm) were prepared from OCT-embedded samples and allowed to air-dry for approximately 30 min and subsequently stored at −80 °C. Tissue sections were brought to room temperature fixed in 2% paraformaldehyde (PFA) for 10 min, washed and then blocked for 90 min (in PBS containing 2% bovine serum albumin [BSA], 5% foetal bovine serum, 0.02% Triton X-100, 0.1% sodium azide and 5% goat serum [GS]).

For fibre-type-specific SC quantification, slides were incubated with primary antibodies against Pax7, laminin, MHCI and MHCII and appropriate secondary antibodies. To determine Pax7 and MyoD co-expression, slides were incubated with primary antibodies against Pax7, laminin and MyoD1 followed by appropriate secondary antibodies. For Pax7 and MSTN co-expression, slides were incubated with primary antibodies against Pax7, laminin, MHCI and MSTN, followed by appropriate secondary antibodies. All secondary antibodies and streptavidin were from Invitrogen; the biotinylated secondary antibody was from Vector Canada. Detailed antibody information is found in Table 2.

Nuclei were visualized with 4′,6-diamidino-2 phenylindole (DAPI, 1:20 000; Sigma-Aldrich, Oakville, ON, Canada) prior to coverslipping slides with fluorescent mounting media (DAKO, Burlington, ON, Canada). Images were observed with a Nikon Eclipse 90i microscope at a magnification of 20X and captured with a Photometrics Cool SNAP HQ2 fluorescent camera (Nikon Instrument, Melville, NY, USA). Analysis was completed using the Nikon NIS-Elements AR software (Nikon Instruments) on a large-scale image. The following are the number of fibres quantified for various analyses: fibre-type-specific SC response (total fibres, 313 ± 44; type I, 118 ± 35; type II, 186 ± 41); activity of the SC pool (total fibres, 200 ± 74); and SC-specific MSTN expression (total fibres, 250 ± 80; type I, 96 ± 41; type II, 153 ± 58). All areas selected for analysis were free of freeze-fracture artefact, and care was taken so that areas where longitudinal fibres were present were not used in the analysis. Muscle fibres on the periphery of muscle cross sections were not used in the analysis. All immunofluorescent analyses were completed in a blinded fashion.

RNA isolation

RNA was isolated from muscle tissue approximately 15–30 mg in size using the Trizol/RNeasy method. All samples were homogenized using 1 mL of Trizol® reagent (Life Technologies, Burlington, ON, Canada) in Lysing D Matrix tubes (MP Biomedicals, Solon, OH, USA) with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) at a speed of 6.0 m/s for 40 s. Homogenized samples were then mixed with 200 μL of chloroform (Sigma-Aldrich), agitated vigorously for 15 s and centrifuged at 12000 ***g*** for 10 min. The upper aqueous (RNA) phase was purified using E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) as per the instructions provided by the manufacturer. RNA concentration (ng/μL) and purity (A260/A280) was deduced using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA). RNA integrity as represented

by RNA integrity numbers (RIN scale of 0–10) was discovered using the Agilent 2100 Bioanalyzer (Agilent Technologies, Toronto, ON, Canada). RNA samples were then reverse-transcribed using commercially available high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as described by manufacturer and using an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Mississauga, ON, Canada) to obtain cDNA for quantitative gene analysis.

Quantitative real-time RT-PCR

All individual qRT-PCRs were run in duplicate 25 μL reactions containing RT Sybr® Green qPCR Master Mix (Qiagen Sciences, Valencia, CA, USA). Each 25 μL reaction was prepared with the epMotion 5075 Eppendorf automated pipetting system (Eppendorf). Primer sequence for MSTN was as follows: forward: 5′-TGGTCATGATCTTGCTGTAACCTT-3′ and reverse: 5′-TGTCTGTTACCTTGACCTCTAAAA-3′. Primers for SMAD3 were designed by SABioscience (SABioscience, Mississauga, ON, Canada; cat. no. PPH01921C).

Statistical analysis

Statistical analysis was completed using SigmaStat 3.1.0 analysis software (Systat Software, Chicago, IL, USA). A two-way repeated-measures ANOVA with one factor for time (Pre, 24 h, 48 h) and exercise type (AE, HIT, RE) was conducted to search for statistical differences in the SC response, the extent of Pax7 and MyoD co-expression and the extent of Pax7 and MSTN co-expression. The SC response and the Pax7 and MSTN co-expression analyses were analysed independently in type I and II fibres. To determine whether a difference existed with CSA, myonuclear domain and number of nuclei/fibre, a two-way ANOVA (factor for fibre type and factor for group) was used. Any significant interactions or main effects were analysed using the Tukey's *post hoc* test across the different modalities of exercise. A one-way ANOVA was used to determine whether differences existed between groups for the following variables: age, height, weight, BMI, body fat percentage, lean tissue mass, VO_2 peak, 10-RM leg press and 10-RM knee extension. Statistical significance was accepted at $P \leq 0.05$. All results were presented as means ± standard error of the mean (SEM).

Results

Subject characteristics

There were no differences in age, BMI, body fat percentage and lean tissue mass between groups. In addition, each group shared similar fitness levels and lower body strength. No differences were observed in fibre cross-sectional area (CSA), number of myonuclei per fibre or myonuclear domain. Complete subject characteristics are reported in Table 1.

Table 1 Subject characteristics

Variable	AE; $n = 7$	HIT; $n = 8$	RE; $n = 7$
Age (years)	68 ± 3	67 ± 5	66 ± 3
Height (cm)	1.74 ± 0.07	1.78 ± 0.06	1.75 ± 0.07
Weight (kg)	82.6 ± 4.7	86.0 ± 8.6	82.3 ± 12.3
BMI (kg/m^2)	27.3 ± 1.8	27.0 ± 2.8	26.7 ± 3.5
Body fat %	25.6 ± 4.2	23.9 ± 5.9	23.9 ± 4.9
Lean tissue mass (kg)	60.1 ± 4.7	63.4 ± 6.4	60.3 ± 6.5
VO_2 Peak (mL/kg/min)	30.7 ± 5.5	31.9 ± 6.8	27.3 ± 6.3
10-RM leg press (kg)	61.3 ± 8.0	59.8 ± 9.9	48.6 ± 19.2
10-RM knee extension (kg)	52.4 ± 5.0	54.3 ± 13.5	52.3 ± 14.8
CSA of type I fibre (μm^2)	5888 ± 1039*	6141 ± 738*	5706 ± 1199*
CSA of type II fibre (μm^2)	4901 ± 823	5109 ± 714	4863 ± 941
Myonuclei per type I fibre	4.0 ± 0.8*	4.4 ± 1.0*	4.1 ± 0.6*
Myonuclei per type II fibre	3.3 ± 0.6	3.2 ± 0.7	2.8 ± 0.5
Myonuclear domain of type I fibre (μm^2/nuclei)	1491 ± 305	1447 ± 281	1425 ± 431
Myonuclear domain of type II fibre (μm^2/nuclei)	1514 ± 156	1550 ± 159	1680 ± 165

Each value (Mean ± SD).
Baseline characteristics of subjects in the AE, HIT and RE group.
*Indicates a significant difference compared to type II.

Table 2 Antibody information

Antibody	Species	Source	Clone	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	Neat	Alexa 594, 488 goat anti-mouse 1:500
Antilaminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488, 647 goat anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 Slow isoform	Neat	Alexa Fluor 488 goat anti-mouse, 1:500
Anti-MHCII	Rabbit	Abcam	ab91506 Fast isoform	1:1000	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Mouse	Dako	5.8A	1:30	Goat anti-mouse biotinylated secondary antibody, 1:200; streptavidin-594 fluorochrome, 1:250
Anti-MSTN	Rabbit	Abcam	Ab98337	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500

Detailed information on primary and secondary antibodies used for immunofluorescence of muscle cross sections.

Serum creatine kinase (CK)

CK activity was determined in serum prior to and 24 h following either an acute bout of AE, HIT or RE. CK activity was increased 24 h post-RE (pre: 40.8 ± 6.1 U/L, 24 h: 141 ± 37 U/L; $P < 0.001$) and HIT (pre: 46 ± 10 U/L, 24 h: 90 ± 12 U/L) ($P < 0.05$) but not AE (pre: 42 ± 4 U/L, 24 h: 68 ± 5 U/L). Serum CK activity 24 h post-intervention was greater following RE than AE ($P < 0.05$), and there was a trend for higher activity following RE than HIT ($P = 0.081$).

Fibre-type-specific SC response

There was a significant increase in the SC pool in the RE group with an increase of 63% in the number of Pax7 + cells associated with type I fibres 24 h and a 45% increase 48 h following exercise ($P < 0.05$) (Fig. 1f). The number of Pax7 + cells associated with

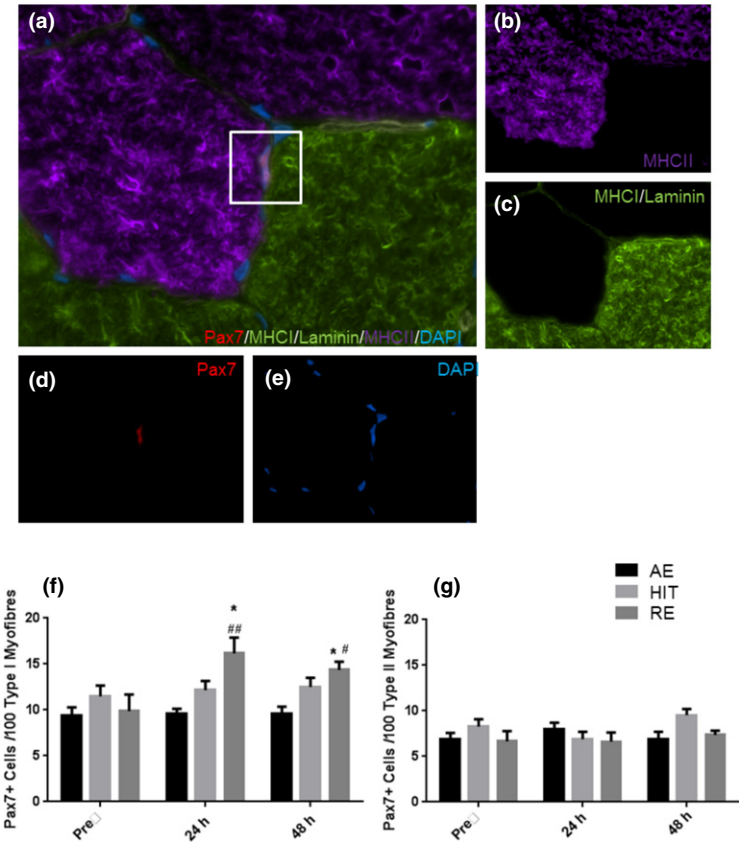


Figure 1 Acute fibre-type-specific SC response 24 and 48 h following AE, HIT and RE. (a) Representative image of a Pax7/MHCI/laminin/MHCII/DAPI stain of a muscle cross section, square represents a SC associated with a type II fibre. Single channel views of (b) MHCII purple, (c) MHCII/laminin green, (d) Pax7 red, (e) DAPI (nuclei) blue. (f) Acute response of SC associated with type I and (g) type II fibres. Bars represent means ± SEM, $^{*}P < 0.05$ vs. Pre, $^{\#\#}P < 0.05$ vs. AE and HIT $^{\#}P < 0.05$ vs. AE.

type I fibres was greater in RE at 24 h compared to AE and HIT (24 h: RE: 16 ± 2, AE: 10 ± 1, HIT: 12 ± 1 Pax7 + cells/100 type I fibre) ($P < 0.05$) (Fig. 1f). At 48 h, the number of Pax7 + cells associated with type I fibres was still greater in RE compared to AE (48 h: RE: 14 ± 1, AE: 10 ± 1 Pax7 + cells/100 type I fibre) ($P < 0.05$) but not compared to HIT ($P > 0.05$) (Fig. 1f). There was no appreciable increase in Pax7 + cells associated with type II fibres following acute exercise in either of the exercise groups ($P > 0.05$) (Fig. 1g).

Activity of the SC pool

To further describe the SC response following three different modes of exercise, we determined the proportion of cells expressing MyoD, one of two primary MRFs expressed during early (proliferation) and later (differentiation) stages of the myogenic programme. SC-expressing MyoD was identified as active. Following acute exercise, there was an increase in both the RE and HIT groups 24 h following exercise (HIT: Pre 0.7 ± 0.3; 24 h: 3.1 ± 0.3; RE: Pre: 1.3 ± 0.1; 24 h: 4.8 ± 0.5 Pax7 + /MyoD+ cells/100 fibres) ($P < 0.001$), and this increase was observed across all three groups at 48 h following exercise (AE: Pre: 0.9 ± 0.3; 48 h: 3.1 ± 0.8; HIT: Pre: 0.7 ± 0.3; 48 h: 4.7 ± 0.3; RE: Pre: 1.3 ± 0.1 48 h: 4.9 ± 0.4; Pax7 + /MyoD+ cells/100 fibres) ($P < 0.05$) (Fig. 3c). At 24 h following exercise, the number of active SC was greater in the RE group (4.8 ± 1.4 Pax7 + /MyoD+ cells/100 fibres) than both the AE (1.9 ± 0.5 Pax7 + /MyoD+ cells/100 fibres) and the HIT (3.1 ± 0.9 Pax7 + /MyoD+ cells/100 fibres) groups ($P < 0.05$), whereas at 48 h, the number of active SC was greater in both the HIT (4.7 ± 0.3 Pax7 + /MyoD+ cells/100 fibres) and RE (4.9 ± 0.4 Pax7 + /MyoD+ cells/100 fibres) compared to the AE (3.1 ± 0.8 Pax7 + /MyoD+ cells/100 fibres) group ($P < 0.05$) (Fig. 3c).

MSTN expression

To determine whether MSTN expression was differentially affected following three different modes of exercise, whole-muscle MSTN and SMAD3, a downstream target of MSTN, gene expression and SC-specific MSTN levels were determined following acute exercise. A significant reduction in the percentage of SC co-expressing MSTN specifically associated with type I fibres in the RE group 24 h following exercise (Pre: 87 ± 4; 24 h: 57 ± 5%MSTN+ type I SC) ($P < 0.001$) (Fig. 4f). Additionally, the observed reduction was greater in the RE group at 24 h (57 ± 5%MSTN+ type I SC) compared to the HIT group (79 ± 5%MSTN+ type I SC) ($P < 0.05$) and a strong trend ($P = 0.06$) compared to the AE group (67 ± 4%MSTN+ type I SC) (Fig. 4f). At 48 h following acute exercise, the percentage of type I-associated SC co-expressing MSTN was significantly reduced in each exercise group (AE: Pre: 84 ± 6, 48 h: 67 ± 4; HIT: Pre: 92 ± 3, 48 h: 52 ± 7; RE Pre: 87 ± 4, 48 h: 67 ± 4%MSTN+ type I SC) ($P < 0.05$) (Fig. 4f). A main effect for time was observed 48 h following acute exercise with a significant reduction in the percentage of SC co-expressing MSTN associated with type II fibres (Fig. 4g) ($P < 0.05$).

Similar to what was observed with type I SC-specific levels of MSTN, a main effect for time was

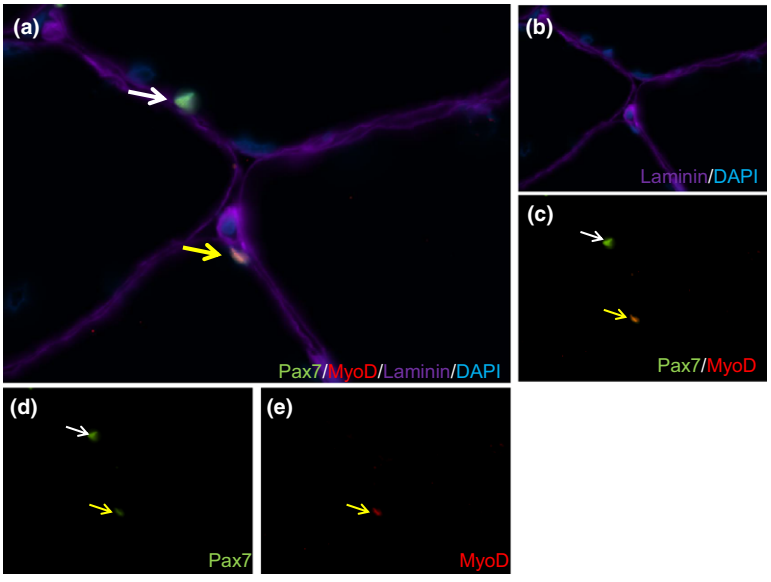


Figure 2 MyoD expression within a SC. (a) Representative image of a Pax7/MyoD/laminin/DAPI stain of a muscle cross section, white arrow represents a Pax7 + /MyoD- SC, yellow arrow represents a Pax7 + /MyoD+ SC. Single channel views of (b) laminin purple, and DAPI (nuclei) blue, (c) Pax7 green and MyoD red, (d) Pax7 green and (e) MyoD red.

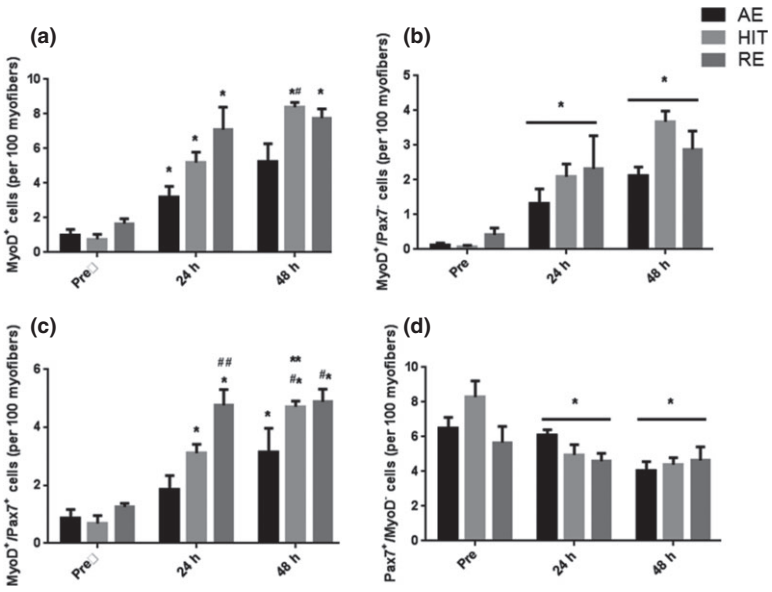


Figure 3 The activation status of SC following acute exercise. (a) The number of MyoD+ cells, (b) MyoD+/Pax7- (differentiating), (c) MyoD+/Pax7 + (activated) and (d) Pax7 + /MyoD- (proliferating) SC. Bars represent means ± SEM, $^{**}P < 0.05$ vs. 24, $^{\#\#}P < 0.05$ vs. HIT and AE, $^{\#}P < 0.05$ vs. AE, $^{*}P < 0.05$ vs. Pre.

observed at 24 and 48 h following acute exercise with a reduction in MSTN whole-muscle mRNA expression (Fig. 4h). Interestingly, a main effect for group was observed for SMAD3 gene expression in whole muscle, and expression was significantly lower in the RE group compared to the AE and HIT groups (Fig. 4i).

Discussion

The primary novel finding of the current study was that HIT was nearly as effective as RE in increasing the number of active SC following an acute bout of exercise. Additionally, all three modes of exercise led to significant decreases in the proportion of SC co-expressing MSTN following acute exercise.

The development of successful therapeutic strategies to promote the maintenance of muscle mass in older adults requires an understanding of the underlying mechanisms of sarcopenia and the processes that control muscle growth. It has been consistently reported that the loss of muscle mass with ageing is primarily accounted for by the loss of total muscle fibres (Lexell *et al.* 1988) and atrophy of type II fibres (Lexell *et al.* 1988, Kim *et al.* 2005, Dreyer *et al.* 2006, Kosek *et al.*2006, Petrella *et al.* 2006, Nilwik *et al.* 2013). Furthermore, type II fibre atrophy is associated with a specific reduction in the SC pool associated with type II fibres (Verdijk *et al.* 2007, 2012, McKay *et al.* 2012, Mackey *et al.* 2014). Although the reduction and dysfunction of the SC pool in older individuals does not directly contribute to loss of functional capacity, the compromised SC pool is likely a contributing factor to the loss of muscle mass with age (Snijders *et al.* 2009). Our data are consistent with previous studies reporting a reduced CSA of type II fibres as compared to type I fibres (Table 1).

The resistance training-induced increase in muscle fibre area has been associated with an expansion of the total SC pool (Petrella *et al.* 2008) and more recently has been shown to be associated with an increase in the SC pool associated specifically with type I fibres in young adults (Bellamy *et al.* 2014). Additionally, 12 weeks of resistance training in older adults resulted in an increase in type II muscle fibre size and was also associated with an expansion of the type II fibre-associated SC pool (Verdijk *et al.* 2014). It has been demonstrated that individuals who gained the most muscle mass following resistance exercise training were the only ones in which a significant expansion of the SC pool was also observed (Petrella *et al.* 2008). Further, we have recently demonstrated that the acute type I fibre-associated SC response following a single bout of resistance exercise is related to marked long-term increases in muscle mass following resistance training in young healthy males (Bellamy *et al.* 2014). Therefore, the acute expansion of the SC pool would appear to be important in determining increases in muscle mass, at least with resistance training in a young population. Although this association has not yet been established in an older population, determining which mode of exercise stimulates the most potent SC response may help in prescribing the optimal exercise programme to minimize muscle loss associated with ageing.

Exercise training is a known therapeutic strategy capable of attenuating the effects of sarcopenia by inducing skeletal muscle hypertrophy (McPherron & Lee 1997, Verney *et al.* 2008, Verdijk *et al.* 2009,

  doi: 10.1111/apha.12601

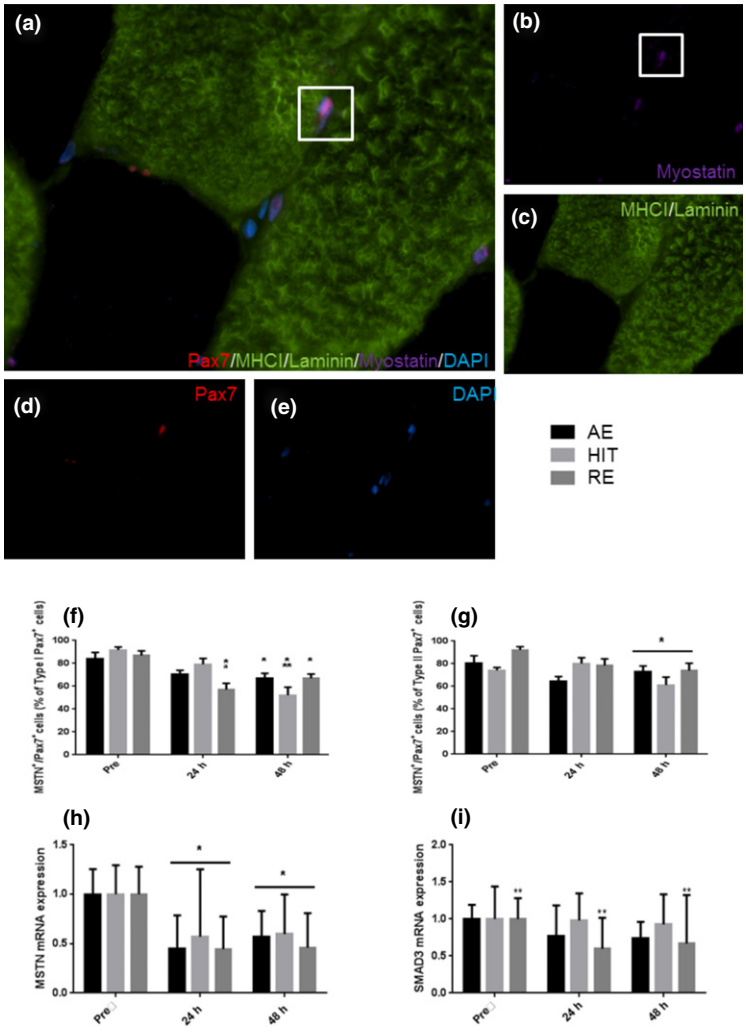


Figure 4 Effect of exercise on MSTN co-expression to SC. (a) Representative image of a Pax7/MSTN/MHCI/laminin/DAPI stain of a muscle cross section, white square represents a Pax7 + / MSTN+ SC. Single channel views of (b) MSTN purple, (c) MHCI/laminin green, (d) Pax7 red, (e) DAPI (nuclei) blue. The percentage of type I-associated SC-expressing MSTN (f) and type II-associated SC-expressing MSTN (g). Whole-muscle mRNA expression of (h) MSTN and (i) SMAD3 expressed as a fold change from pre. A $P < 0.05$ vs. HIT, $^{*}P < 0.05$ vs. Pre, $^{**}P < 0.05$ vs. 24, $^{***}P < 0.05$ group effect vs. AE and HIT.

Leenders *et al.* 2013). Resistance exercise has been shown to effectively induce SC activity (Dreyer *et al.* 2006, Verdijk *et al.* 2009, McKay *et al.* 2012, Leenders *et al.* 2013), which was consistent with the findings of the current study. We demonstrated a 63% and a 45% increase in the number of SC associated with type I fibres 24 and 48 h, respectively, following RE (Fig. 1). Consistent with previous reports, no appreciable expansion of SC associated with type II fibres was observed in any of the exercise groups following acute exercise (Fig. 1) (McKay *et al.* 2012). We observed that the increase in type I-associated SC content following a bout of RE is greater than HIT and AE at 24 h. At 48 h, while the type I-associated SC content was still significantly elevated in the RE group as compared to the AE group, there was no significant difference compared to the HIT group. Subjects were required to complete preliminary strength tests and familiarization sessions at least 1 week prior to pre-biopsy. Although these tests may be a significant insult to untrained skeletal muscle, other studies have also collected muscle biopsies 1 week after the completion of strength tests (Verdijk *et al.* 2009) and as little as 4 days following the completion of strength tests (Leenders *et al.* 2013). We also acknowledge that the 10-RM baseline testing (i.e. leg extension and leg press) may sufficiently perturb the muscle so that there is an adaptive response and may affect the SC response in subsequent bouts of exercise. This adaptive response may reflect the 'repeated bout phenomenon', whereby bouts of exercise may protect against subsequent bouts (McHugh 2003). Given that the working muscle group (i.e. the quadriceps) does not need to be exercised in the same manner as the original bout (Eston *et al.* 1996), this protective effect may exist regardless of modality.

Additionally, no differences were observed between groups prior to exercise indicating that any response induced by the familiarization sessions returned to baseline.

Previous studies have described an expansion in the total SC pool (Charifi *et al.* 2003) and more recently

specifically to the SC pool associated with type II fibres (Verney *et al.* 2008) in older adults following extended interval-type training, while more traditional endurance exercise training did not lead to an expansion of the SC pool in obese older type 2 diabetes patients (Snijders *et al.* 2011). However, Fry *et al.* 2014 recently described an expansion of the SC pool associated with type I fibres following 12 weeks of traditional aerobic exercise training in sedentary adults. These studies highlight the potential importance of exercise mode and intensity in mediating progression of the SC through the myogenic programme leading to an expansion of the SC pool. Taken together, these data suggest that while RE may provide sufficient stimuli to cause expansion of the SC pool, HIT may also elicit an expansion of the SC pool later as compared to a bout of AE. We did not observe an expansion of SC associated with type I fibres following AE and HIT. Furthermore, there was no expansion of SC associated with type II fibres following any type of exercise during the time period at which biopsies were taken. However, given the relatively short time course (i.e. 48 h post-exercise), we may have missed this expansion if it did occur. Indeed, the lower intensity of both AE and HIT compared to RE may also have resulted in a slower expansion of the SC pool.

Recent work has demonstrated an increase in MyoD+ SC 24, 48 and 72 h following an acute bout of RE in older men. This increase was accompanied by an increase in the number of SC associated with type II fibres 72 h after an acute bout of RE in older men (Snijders *et al.* 2014). Unfortunately, we were unable to measure the response of the SC pool at 72 h; however, the increase in MyoD observed 48 h following RE and HIT could translate to expansion of the SC pool at later time points. In recent years, HIT has been described as a more time-efficient alternative to traditional AE (Gibala *et al.* 2012). HIT has been shown to be beneficial to a large range of populations including healthy individuals (Gibala *et al.* 2012), type 2 diabetes patients (Little *et al.* 2011, Terada *et al.* 2013) and patients with cardiovascular diseases (Currie *et al.* 2013). Importantly, similar benefits are derived following HIT and AE across either healthy or diseased populations (Currie *et al.* 2013, Terada *et al.* 2013, Gillen & Gibala 2014). During training interventions, adherence to either HIT or AE appears to be similar (Currie *et al.* 2013, Terada *et al.* 2013). If designing an exercise programme to counter the loss of muscle mass observed with ageing, a focus should be placed on RE as it is a known anabolic stimulus in older adults (Mackey *et al.* 2006, Verdijk *et al.* 2009, Leenders *et al.* 2013); however, the benefits of aerobic exercise in this population should also be considered. Due to the similarities in health outcomes and adherence, between both training methods despite a much lower time commitment for HIT, it may be of a greater benefit to incorporate HIT rather than traditional AE when prescribing an exercise prescription to older adults.

The progression of SC through the myogenic programme from activation, proliferation to terminal differentiation is dictated by a network of transcription factors referred to as myogenic regulatory factors (MRFs). MyoD is a primary MRF and plays a role in SC activation/proliferation (Megeney *et al.* 1996, Kitzmann *et al.* 1998) and continues to be expressed during the early stages of differentiation (Rudnicki *et al.* 2008). We have previously demonstrated an attenuated SC response in older men following a bout of resistance exercise evidenced by fewer MyoD+ cells (fewer active SC (MyoD+/Pax7 +) when compared to young men. The increase in active SC was only observed 48 h following resistance exercise in old men, whereas an increase was observed as early as 3 h post-exercise in young men (McKay *et al.* 2012). Here, we report an increase in the total number of cells expressing MyoD at 24 and 48 h in every group (Fig. 3a). More specifically, we describe a delayed response in SC activation following AE (Fig. 3c). We observed an increase in active SC at 24 h, which persisted until 48 h following either RE or HIT, but this increase was only observed at 48 h following AE. Additionally, the number of active SC at 24 h was greatest in the RE group; however, at 48 h following exercise, this number was greater in both the RE and the HIT when compared to the AE group. Together, these data suggest that HIT and RE lead to a greater activation of SC compared to AE. This finding further supports the importance of exercise mode and intensity in promoting SC activation.

Previous studies have shown that MSTN negatively regulates SC activation and self-renewal (McCroskery *et al.* 2003), especially in aged muscle (McKay *et al.* 2012). Downregulation of MSTN expression following exercise may regulate the response to hypertrophic stimuli. Furthermore, previous work from our laboratory has reported an attenuated reduction in MSTN signalling in older men following RE as compared to young. Similarly, we observe a time effect at 48 h with a reduced number of type II-associated SC co-expressing MSTN (Fig. 4g). In type I fibres, a significant decrease was observed at 24 h in the RE group only, but this reduction was observed in all three groups 48 h following acute exercise. Additionally, in the HIT group, there were significantly fewer SC co-expressing MSTN at 48 h than at 24 h (Fig. 4f). Whole-muscle MSTN gene expression was also measured and was significantly reduced 24 and 48 h following acute

exercise. These data suggest that the SC pool may be able to respond more quickly to exercise that is known to elicit an anabolic response like RE than other modes of exercise due to the reduction in MSTN expression observed at 24 h following RE only.

Serum CK activity was increased in both RE and HIT groups 24 h following exercise. Additionally, CK activity was greater 24 h following RE compared to AE, and a strong trend was observed for greater activity 24 h following RE when compared to HIT. Although circulating CK activity is a suboptimal proxy measure of muscle damage, its activity has been reported to be increased following exercise (Newham *et al.* 1986, Nosaka & Clarkson 1996). Taken together, muscle damage induced by RE may be greater than that induced by either AE or HIT based on serum CK activity 24 h following exercise, which may explain the more pronounced SC response following RE as compared to AE or HIT. Furthermore, the more rapid reduction in MSTN co-expression with SC observed following RE may be a response to damage and the initiation of repair, whereas the reduction in co-expression observed 48 h following AE and HIT may represent a remodelling/adaptive process without the same need for repair as compared to the RE group.

Interestingly, we observed a lower mRNA expression of SMAD3 at the whole-muscle level following RE compared to the other two groups. MSTN is believed to bind to the serine/threonine kinase receptor ActRIIb that phosphorylates receptor-regulated SMAD2 and SMAD3 (Huang *et al.* 2011). Phosphorylated SMAD2/SMAD3 forms complexes that translocate to the nucleus, where it is thereby involved in regulating the transcription of target genes, ultimately leading to the suppression of myogenesis. More specifically, upregulation of SMAD3 expression via MSTN inhibits MyoD expression leading to impaired differentiation in human (McFarlane *et al.* 2011) and murine (Langley *et al.* 2002) myoblasts. We have previously demonstrated that SMAD3 mRNA expression is greater in older men compared to young men at rest and 3 and 24 h following RE (McKay *et al.* 2012). The greater expression of SMAD3 was accompanied by a greater MSTN protein expression in older men. Following 48 h of RE, SMAD3 mRNA expression and MSTN protein expression in older men were reduced to that of young men (McKay *et al.* 2012). SMAD3 mRNA expression seems to closely mimic MSTN protein expression. Measuring the expression of phosphorylated SMAD3 as a downstream target of MSTN signalling would have been ideal, but we were not able to do so. Therefore, we determined the mRNA expression of SMAD3 as a downstream target of MSTN. The lower expression of SMAD3 observed in the RE group could in part explain the greater proportion of active SC ($MyoD^+/Pax7^+$) observed following the completion of a bout of RE. While the reduction of MSTN expression at the level of whole muscle across time may indicate that all modes of exercise are capable of this physiological response, it is possible that RE provides the most temporally effective reduction, which may indicate the need for a more immediate response for repair/adaptation/remodelling following RE.

The recruitment of fibres during these various modes of exercise may lend insight into the responses observed in the SC pool. The widely accepted Henneman size principle (Mendell 2005) postulates that while some fibres are activated at much lower thresholds (i.e. lower intensity of exercise), both a greater recruitment and a recruitment of larger fibres are activated when greater activation intensity is required. Recruitment of 'higher order' fibres during RE typically follows the Henneman size principal; predominately, type I fibres are recruited first, and as more contractions occur, higher threshold fibres are recruited (Sale 1987). However, there is likely a continuum of contractile and metabolic capacity and likely a considerable overlap of the abilities of each fibre 'type' across the spectrum (Staron 1997). In aerobic exercise of similar intensity (~60% VO_2 peak), there is a greater decrease in muscle glycogen detected in type I fibres as compared to type II fibres (Ball-Burnett *et al.* 1991). However, there is nearly a 30% decrease in muscle glycogen in type II fibres, suggesting that recruitment of 'higher order' fibres even within the moderate-intensity workload (Ball-Burnett *et al.* 1991). In line with this, work performed with higher intensity aerobic exercise has supported the notion that indicates that type II fibres are indeed active at much heavier workloads (Gollnick *et al.* 1974, Vollestad *et al.* 1984, Beelen & Sargeant 1993). Taken together, these data indicate that while there is some recruitment of 'higher order' fibres during traditional type AE and, to a greater extent, HIT-style exercise, limitations do exist in comparison with RE. Given the notion that there is either a reduced (McKay *et al.* 2012) or delayed (Snijders *et al.* 2014) response in the SC pool in type II fibres, it becomes paramount to properly select exercise modalities that will facilitate this SC response. Our findings suggest that RE stimulates a greater reduction of MSTN and it may be a reflection of the greater recruitment of type II fibres (and the associated SC pool). Moreover, the SC-specific reduction in MSTN along with a reduction in whole-muscle MSTN may further support the benefits of RE as the most potent mode of exercise in increasing muscle mass.

Data from this study reinforce current dogma that RE is the exercise mode able to elicit the greatest SC response even in older adults. In line with this, Bell *et al.* 2015 reported an increase in myofibrillar protein fractional synthetic rate (FSR) in both the RE and HIT groups 24 and 48 h following exercise; however, this increase was greater following RE. Together, these data suggest that RE may be the exercise modality able to stimulate the greatest anabolic response. Despite the observation that all three modes of exercise resulted in a reduction in SC-specific co-expression of MSTN, HIT and RE led to an increase in SC activation more rapidly than that observed following AE. Although we demonstrate a reduction in the percentage of type I-associated SC co-expressing MSTN in RE compared to other groups, a reduction was observed 48 h following exercise in the percentage of type II-associated SC co-expressing MSTN in all three exercise groups. Type II fibres are known to be more affected by sarcopenia, and eliminating or slowing the atrophy process of type II fibres observed with ageing would be ideal, but maintaining or maximizing CSA of type I fibres may also be an effective way in slowing the age-related loss of muscle mass. A reduction in type I-associated SC co-expressing MSTN following exercise may be indicative of an anabolic response.

We have recently demonstrated that the acute SC response associated with type I fibres is associated with the greatest increases in muscle mass following resistance exercise training (Bellamy *et al.* 2014). Although this study was a study in young adults, it highlights that in some instances, the acute SC response may be related to long-term skeletal muscle adaptation. However, SC function is impaired in older adults and therefore determining whether the acute SC response in either fibre type is associated with long-term gains in muscle mass in this population, as it does in a healthy young population, should also be determined. The current study allowed us to determine which type of exercise resulted in the greatest SC response in older adults. The findings of this study demonstrate that RE is the mode of exercise that results in the greatest and most efficient SC response. However, incorporating HIT instead of AE into an exercise training programme for older adults where the primary goal is to slow the process of sarcopenia may be justified.

Conflict of interest

There are no conflict of interests.

The Pax7 hybridoma cells developed by Dr. A. Kawakami and the A4.951 developed by Dr. H. Blau were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Dr. G Parise was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Grant (1455843) and by funding from the Labarge Optimal Aging Initiative from McMaster University. JP Nederveen was supported by a NSERC Canadian Graduate Scholarship (CGS-D).

References

Ball-Burnett, M., Green, H.J. & Houston, M.E. 1991. Energy metabolism in human slow and fast twitch fibres during prolonged cycle exercise. *J Physiol* **437**, 257–267.

Beelen, A. & Sargeant, A.J. 1993. Effect of prior exercise at different pedalling frequencies on maximal power in humans. *Eur J Appl Physiol Occup Physiol* **66**, 102–107.

Bell, K.E., Séguin, C., Parise, G., Baker, S.K. & Phillips, S.M. 2015. Day-to-Day changes in muscle protein synthesis in recovery from resistance, aerobic, and high-intensity interval exercise in older men. *J Gerontol A Biol Sci Med Sci* **70**, 1024–1029.

Bellamy, L.M., Joanisse, S., Grubb, A., Mitchell, C.J., McKay, B.R., Phillips, S.M., Baker, S. & Parise, G. 2014. The acute satellite cell response and skeletal muscle hypertrophy following resistance training. *PLoS ONE* **9**, 10.

Charifi, N., Kadi, F., Féasson, L. & Denis, C. 2003. Effects of endurance training on satellite cell frequency in skeletal muscle of old men. *Muscle Nerve* **28**, 87–92.

Currie, D.K., Dubberley, B.J., Mckelvie, S.R. & Macdonald, J.M. 2013. Low-volume, high-intensity interval training in patients with CAD. *Med Sci Sports Exerc* **45**, 1436–1442.

Dreyer, H.C., Blanco, C.E., Sattler, F.R., Schroeder, E.T. & Wiswell, R.A. 2006. Satellite cell numbers in young and older men 24 hours after eccentric exercise. *Muscle Nerve* **33**, 242–253.

Eston, R.G., Finney, S., Baker, S. & Baltzopoulos, V. 1996. Muscle tenderness and peak torque changes after downhill running following a prior bout of isokinetic eccentric exercise. *J Sports Sci* **14**, 291–299.

Fry, C.S., Noehren, B., Mula, J., Ubele, M.F., Westgate, P.M., Kern, P.A. & Peterson, C.A. 2014. Fiber type-specific satellite cell response to aerobic training in sedentary adults. *J Physiol* **592**, 2625–2635.

Gibala, M.J., Little, J.P., Macdonald, M.J. & Hawley, J.A. 2012. Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol* **590**, 1077–1084.

Gillen, J.B. & Gibala, M.J. 2014. Is high-intensity interval training a time-efficient exercise strategy to improve health and fitness? *Appl Physiol Nutr Metab* **39**, 409–412.

Gollnick, P.D., Piehl, K. & Saltin, B. 1974. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol* **241**, 45–57.

Huang, Z., Chen, X. & Chen, D. 2011. Myostatin: a novel insight into its role in metabolism, signal pathways, and expression regulation. *Cell Signal* **23**, 1441–1446.

Hughes, V.A., Frontera, W.R., Wood, M., Evans, W.J., Dallal, G.E., Roubenoff, R. & Fiatarone Singh, M.A. 2001.

Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. *J Gerontol A Biol Sci Med Sci* **56**, B209–B217.

Kadi, F. & Thornell, L.E. 2000. Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. *Histochem Cell Biol* **113**, 99–103.

Kadi, F., Charifi, N., Denis, C. & Lexell, J. 2004. Satellite cells and myonuclei in young and elderly women and men. *Muscle Nerve* **29**, 120–127.

Kim, J., Kosek, D.J., Petrella, J.K., Cross, J.M. & Bamman, M.M. 2005. Resting and load-induced levels of myogenic gene transcripts differ between older adults with demonstrable sarcopenia and young men and women. *J Appl Physiol (1985)* **99**, 2149–2158.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N.J. & Fernandez, A. 1998. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol* **142**, 1447–1459.

Kosek, D.J., Kim, J.-S., Petrella, J.K., Cross, J.M. & Bamman, M.M. 2006. Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *J Appl Physiol (1985)* **101**, 531–544.

Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S. & Kambadur, R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem* **277**, 49831–49840.

Leenders, M., Verdijk, L.B., Van Der Hoeven, L., Van Kranenburg, J., Nilwik, R. & van Loon, L.J. 2013. Elderly men and women benefit equally from prolonged resistance-type exercise training. *J Gerontol A Biol Sci Med Sci* **68**, 769–779.

Léger, B., Derave, W., De Bock, K., Hespel, P. & Russell, A.P. 2008. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. *Rejuvenation Res* **11**, 163–175.

Lexell, J., Taylor, C.C. & Sjostrom, M. 1988. What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. *J Neurol Sci* **84**, 275–294.

Little, J.P., Gillen, J.B., Percival, M., Safdar, A., Tarnopolsky, M.A., Punthakee, Z., Jung, M.E. & Gibala, M.J. 2011. Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. *J Appl Physiol (1985)* **111**, 1554–1560.

Mackey, A.L., Esmarck, B., Kadi, F., Koskinen, S.O.A., Kongsgaard, M., Sylvestersen, A., Hansen, J.J., Larsen, G. & Kajer, M. 2006. Enhanced satellite cell proliferation with resistance training in elderly men and women. *Scand J Med Sci Sports* **17**, 34–42.

Mackey, A.L., Kajer, M., Dandanell, S., Mikkelsen, K.H., Holm, L., Døssing, S., Kadi, F., Koskinen, S.O., Jensen, C.H., Schrøder, H.D. & Langberg, H. 2007. The influence of anti-inflammatory medication on exercise-induces myogenic precursor cell responses in humans. *J Appl Physiol (1985)* **103**, 425–431.

Mackey, A.L., Karlsen, A., Couppé, C., Mikkelsen, U.R., Nielsen, R.H., Magnusson, S.P. & Kjaer, M. 2014. Differential satellite cell density of type I and II fibres with lifelong endurance running in old men. *Acta Physiol (Oxf)* **210**, 612–627.

McCroskery, S., Thomas, M., Maxwell, L., Sharma, M. & Kambadur, R. 2003. Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* **162**, 1135–1147.

McFarlane, C., Hui, G.Z., Amanda, W.Z.W., Lau, H.Y., Lokireddy, S., Xiaojia, G., Mouly, V., Butler-Browne, G., Gluckman, P.D., Sharma, M. & Kambadur, R. 2011. Human myostatin negatively regulates human myoblast growth and differentiation. *Am J Physiol Cell Physiol* **301**, 195–203.

McHugh, M.P. 2003. Recent advances in the understanding of the repeated bout effect: the protective effect against muscle damage from a single bout of eccentric exercise. *Scand J Med Sci Sports* **13**, 88–97.

McKay, B.R., Ogborn, D.I., Bellamy, L.M., Tarnopolsky, M.A. & Parise, G. 2012. Myostatin is associated with age-related human muscle stem cell dysfunction. *FASEB J* **26**, 2509–2521.

McKay, B.R., Ogborn, D.I., Baker, J.M., Toth, K.G., Tarnopolsky, M.A. & Parise, G. 2013. Elevated SOCS3 and altered IL-6 signaling is associated with age-related human muscle stem cell dysfunction. *Am J Physiol Cell Physiol* **304**, 717–728.

McPherron, A.C. & Lee, S.J. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci USA* **94**, 12457–12461.

McPherron, A.C., Lawler, A.M. & Lee, S.J. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83–90.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E. & Rudnicki, M.A. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev* **10**, 1173–1183.

Mendell, L.M. 2005. The size principle: a rule describing the recruitment of motoneurons. *J Neurophysiol* **93**, 3024–3026.

Newham, D.J., Jones, D.A. & Edwards, R.H. 1986. Plasma creatine kinase changes after eccentric and concentric contractions. *Muscle Nerve* **9**, 59–63.

Nilwik, R., Snijders, T., Leenders, M., Groen, B.B.L., van Kranenburg, J., Verdijk, L.B. & van Loon, L.J. 2013. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. *Exp Gerontol* **48**, 492–498.

Nosaka, K. & Clarkson, P.M. 1996. Changes in indicators of inflammation after eccentric exercise of the elbow flexors. *Med Sci Sports Exerc* **28**, 953–961.

Petrella, J.K., Kim, J., Cross, J.M., Kosek, D.J. & Bamman, M.M. 2006. Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am J Physiol Endocrinol Metab* **291**, 937–946.

Petrella, J.K., Kim, J.-S., Mayhew, D.L., Cross, J.M. & Bamman, M.M. 2008. Potent myofiber hypertrophy during

resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physiol (1985)* **104**, 1736–1742.

Raue, U., Slivka, D., Jemiolo, B., Hollon, C. & Trappe, S. 2006 Myogenic gene expression at rest and after a bout of resistance exercise in young (18-30 yr) and old (80-89 yr) women. *J Appl Physiol (1985)* **101**, 53–59.

Roth, S.M., Martel, G.F., Ivey, F.M., Lemmer, J.T., Tracy, B.L., Metter, E.J., Hurley, B.F. & Rogers, M.A. 2001. Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training. *J Gerontol A Biol Sci Med Sci* **56**, 240–247.

Roubenoff, R. & Hughes, V.A. 2000. Sarcopenia: current concepts. *J Gerontol A Biol Sci Med Sci* **55**, 716–724.

Rudnicki, M.A., Le Grand, F., McKinnell, I. & Kuang, S. 2008. The molecular regulation of muscle stem cell function. *Cold Spring Harb Symp Quant Biol* **73**, 323–331.

Sajko, S., Kubínová, L., Cvetko, E., Kreft, M., Wernig, A. & Erzen, I. 2004. Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. *J Histochem Cytochem* **52**, 179–185.

Sale, D.G. 1987. Influence of exercise and training on motor unit activation. *Exerc Sport Sci Rev* **15**, 95–151.

Snijders, T., Verdijk, L.B. & van Loon, L.J. 2009. The impact of sarcopenia and exercise training on skeletal muscle satellite cells. *Ageing Res Rev* **8**, 328–338.

Snijders, T., Verdijk, L.B., Hansen, D., Dendale, P. & van Loon, L.J. 2011. Continuous endurance-type exercise training does not modulate satellite cell content in obese type 2 diabetes patients. *Muscle Nerve* **43**, 393–401.

Snijders, T., Verdijk, L.B., Smeets, J.S., McKay, B.R., Senden, J.M., Hartgens, F., Parise, G., Greenhaff, P. & van Loon, L.J. 2014. The skeletal muscle satellite cell response to a single bout of resistance-type exercise is delayed with aging in men. *Age (Dordr)* **36**, 9699.

Staron, R.S. 1997. Human skeletal muscle fiber types: delineation, development, and distribution. *Can J Appl Physiol* **22**, 307–327.

Terada, T., Friesen, A., Chahal, B.S., Bell, G.J., McCargar, L.J. & Boulé, N.G. 2013. Feasibility and preliminary efficacy of high intensity interval training in type 2 diabetes. *Diabetes Res Clin Pract* **99**, 120–129.

Verdijk, L.B., Koopman, R., Schaart, G., Meijer, K., Savelberg, H.H.C.M. & van Loon, L.J. 2007. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Endocrinol Metab* **292**, 151–157.

Verdijk, L.B., Gleeson, B.G., Jonkers, R.A.M., Meijer, K., Savelberg, H.H.C.M., Dendale, P. & van Loon, L.J. 2009. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type-specific increase in satellite cell content in elderly men. *J Gerontol A Biol Sci Med Sci* **64**, 332–339.

Verdijk, L.B., Dirks, M.L., Snijders, T., Prompers, J.J., Beelen, M., Jonkers, R.A.M., Thijssen, D.H.J., Hopman, M.T.E. & van Loon, L.J. 2012. Reduced satellite cell numbers with spinal cord injury and aging in humans. *Med Sci Sports Exerc* **44**, 2322–2330.

Verdijk, L.B., Snijders, T., Drost, M., Delhaas, T., Kadi, F. & van Loon, L.J. 2014. Satellite cells in human skeletal muscle; from birth to old age. *Age (Dordr)* **36**, 545–557.

Verney, J., Kadi, F., Charifi, N., Féasson, L., Saafi, M.A., Castells, J., Piehl-Aulin, K. & Denis, C. 2008. Effects of combined lower body endurance and upper body resistance training on the satellite cell pool in elderly subjects. *Muscle Nerve* **38**, 1147–1154.

Vollestad, N.K., Vaage, O. & Hermansen, L. 1984. Muscle glycogen depletion patterns in type I and subgroups of type II fibres during prolonged severe exercise in man. *Acta Physiol Scand* **122**, 433–441.

Yarasheski, K.E., Bhasin, S., Sinha-Hikim, I., Pak-Loduca, J. & Gonzalez-Cadavid, N.F. 2002. Serum myostatin-immunoreactive protein is increased in 60–92 year old women and men with muscle wasting. *J Nutr Health Aging* **6**, 343–348.

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 177–190

The effect of exercise mode on the acute response of satellite cells in old men

J. P. Nederveen,[1] S. Joanisse,[1] C. M. L. Séguin,[1] K. E. Bell,[1] S. K. Baker,[2] S. M. Phillips[1] and G. Parise[1,3]

1 Department of Kinesiology, McMaster University, Hamilton, ON, Canada

2 Department of Medicine, McMaster University, Hamilton, ON, Canada

3 Department of Medical Physics & Applied Radiation Sciences, McMaster University, Hamilton, ON, Canada

Received 5 May 2015,
revision requested 8 June 2015,
revision received 3 September 2015,
accepted 7 September 2015
Correspondence: G. Parise,
Departments of Kinesiology and Medical Physics and Applied Radiation Sciences,
McMaster University, Hamilton, Ontario, Canada L8S 4L8.
E-mail: pariseg@mcmaster.ca

Abstract

Aim: A dysregulation of satellite cells may contribute to the progressive loss of muscle mass that occurs with age; however, older adults retain the ability to activate and expand their satellite cell pool in response to exercise. The modality of exercise capable of inducing the greatest acute response is unknown. We sought to characterize the acute satellite cell response following different modes of exercise in older adults.

Methods: Sedentary older men (n = 22; 67 ± 4 years; 27 ± 2.6 kg*m^{-2}) were randomly assigned to complete an acute bout of either resistance exercise, high-intensity interval exercise on a cycle ergometer or moderate-intensity aerobic exercise. Muscle biopsies were obtained before, 24 and 48 h following each exercise bout. The satellite cell response was analysed using immunofluorescent microscopy of muscle cross sections.

Results: Satellite cell expansion associated with type I fibres was observed 24 and 48 h following resistance exercise only ($P < 0.05$), while no expansion of type II-associated satellite cells was observed in any group. There was a greater number of activated satellite cells 24 h following resistance exercise (pre: 1.3 ± 0.1, 24 h: 4.8 ± 0.5 Pax7 + /MyoD+cells/100 fibres) and high-intensity interval exercise (pre: 0.7 ± 0.3, 24 h: 3.1 ± 0.3 Pax7 + /MyoD+cells/100 fibres) ($P < 0.05$). The percentage of type I-associated SC co-expressing MSTN was reduced only in the RE group 24 h following exercise (pre: 87 ± 4, 24 h: 57 ± 5%MSTN+ type I SC) ($P < 0.001$).

Conclusion: Although resistance exercise is the most potent exercise type to induce satellite cell pool expansion, high-intensity interval exercise was also more potent than moderate-intensity aerobic exercise in inducing satellite cell activity.

Keywords ageing, exercise, satellite cells.

A predictable characteristic of ageing is the loss of muscle mass and strength collectively referred to as sarcopenia (Snijders *et al.* 2009). The gradual loss of muscle is thought to begin as early as the fifth decade of life (Hughes *et al.* 2001) and ultimately results in impaired functional capacity, increased incidence of injury and loss of independence (Roubenoff & Hughes 2000, Snijders *et al.* 2009). The precise mechanisms underlying the progression of sarcopenia are not completely understood; however, the loss of satellite cell (SC) number and function may be contributing factors (Snijders *et al.* 2009, McKay *et al.* 2012, 2013, Verdijk *et al.* 2012).

 doi: 10.1111/apha.12601

Although the notion that ageing is associated with a reduction in SC number is popular, the evidence is equivocal in humans, with some studies suggesting a reduced SC pool (Kadi *et al.* 2004, Sajko *et al.* 2004) in older adults, while others report no change as a function of ageing (Roth *et al.* 2001, Dreyer *et al.* 2006). More recent work suggests that there may be a specific reduction in type II-associated SC in older adults (Verdijk *et al.* 2007, McKay *et al.* 2012). Importantly, ageing is not only associated with a reduction in SC pool size but also in the ability of the SC pool to respond following a bout of exercise (McKay *et al.* 2012, 2013). This may be a particularly relevant fact since the extent of the SC response following acute exercise is associated with the degree of muscle growth following training in young males (Bellamy *et al.* 2014); however, whether this relationship exists in older adults has yet to be determined.

Various forms of exercise like resistance training (Mackey *et al.* 2007, Verdijk *et al.* 2009, Leenders *et al.* 2013) and even endurance training (Charifi *et al.* 2003, Verney *et al.* 2008) lead to increases in muscle fibre size in the elderly and may be an effective countermeasure in slowing the inevitable loss of muscle mass associated with ageing. More specifically, increases were observed in type IIa fibres following endurance training (Charifi *et al.* 2003, Verney *et al.* 2008) and in type II fibres following resistance exercise (Verdijk *et al.* 2009 Leenders *et al.* 2013). Resistance exercise in older adults has been shown to lead to an acute increase in the number of SC associated with type I fibres, but this response is delayed (Snijders *et al.* 2014) or not observed in type II fibres (McKay *et al.* 2012) depending on the time at which the last biopsy was taken. This is in contrast to what is observed in young adults. Expansion of the SC pool is associated with an increase in muscle mass in younger adults (Kadi & Thornell 2000, Verdijk *et al.* 2009, Bellamy *et al.* 2014) and in type II muscle fibre size in older adults (Verdijk *et al.* 2014). Only one study has reported an increase in fibre CSA without an expansion of the SC pool. These authors show an increase in CSA of both type I and II fibres with an expansion of the SC pool associated with only type I fibres following 12 weeks of aerobic training (Fry *et al.* 2014). The magnitude of SC expansion following resistance exercise is lower in older adults as compared to young (Dreyer *et al.* 2006, Snijders *et al.* 2009, McKay *et al.* 2012), and the impact of satellite cell expansion on promoting adaptation appears highly relevant in young adults. Importantly, it has not yet been determined whether SC expansion in older adults is related to long-term muscle adaptation; however, given the potential importance of the SC response in promoting adaptation, and the delayed SC response observed in older adults, a characterization of SC expansion following different modes of activity may be of interest.

Myostatin (MSTN) is a transforming growth factor β family member and is a negative regulator of skeletal muscle growth (McPherron *et al.* 1997). In older adults, MSTN serum levels are inversely correlated with muscle mass (Yarasheski *et al.* 2002). Research has also shown elevated protein (Léger *et al.* 2008) and elevated gene expression of MSTN in older adults compared to young controls (Raue *et al.* 2006, Léger *et al.* 2008) at rest. MSTN has also been shown to impair myoblast proliferation *in vitro* (McCroskery *et al.* 2003), and our laboratory has recently demonstrated an impaired reduction in MSTN co-expression with SC following a single bout of resistance exercise in older adults (McKay *et al.* 2012). Taken together, these data suggest that MSTN may play a role in muscle wasting associated with ageing. Identifying modes of exercise that maximize the reduction in MSTN co-expression with SC may allow for maximal muscle gains in older adults.

Resistance (Verney *et al.* 2008, Verdijk *et al.* 2009, Leenders *et al.* 2013) and endurance (Charifi *et al.* 2003, Verney *et al.* 2008) exercise training have been shown to improve muscle mass and strength, and improve performance capacity in older adults. Recently, there has been great interest in determining the effectiveness of different forms of aerobic exercise like high-intensity interval training and how its health benefits compare to more traditional endurance exercise. High-intensity interval training is a more time-efficient alternative to traditional endurance exercise with similar health benefits, similar adherence during the training intervention, and is well tolerated by a large range of healthy and diseased populations (Currie *et al.* 2013, Terada *et al.* 2013, Gillen & Gibala 2014). The aim of this study was to examine and compare the acute SC response in older adults following resistance exercise (RE), high-intensity interval exercise (HIT) and moderate-intensity aerobic exercise (AE). We hypothesized that both the RE and the HIT would have the greatest acute SC response and that this may in part be mediated by a greater reduction in MSTN co-expression with SC.

Materials and methods

Ethical approval

This study was approved by the Hamilton Integrated Research Ethics Board (HIREB). All procedures conformed to the most recent revision of the Declaration of Helsinki on the use of human subjects as research participants. Furthermore, subjects gave their

  doi: 10.1111/apha.12601

informed written consent prior to enrolling in the study.

Subjects

Twenty-two untrained healthy older males (67 ± 7 years) were recruited from the Hamilton area and its surrounding communities. Subjects were not involved in any type of exercise training programme for ≥4 months prior to the study and were required to complete a routine screening and health questionnaires. Prospective subjects were excluded based on the following criteria: evidence of heart disease, respiratory disease, uncontrolled hypertension, diabetes, any major orthopaedic disability, the use of non-steroidal anti-inflammatory drugs (NSAIDs) as their use may affect SC activation as previously shown (Mackey *et al.* 2007) and smoking. Additionally, prospective subjects were required to complete an exercise stress test prior to participation in order to ensure they were in good health and physically capable of performing the tasks required in the study. Specifically, the stress test consisted of an incremental cycling test to volitional fatigue on a cycle ergometer, providing values for maximum oxygen uptake (VO_2 peak), maximum heart rate (HR_{max}) and peak power for each subject. Subjects were then placed in one of three exercise groups; each group was matched for age, weight, BMI, VO_2 peak, strength and lean mass tissue. Each group would perform a single bout of RE, AE or HIT.

Baseline testing and familiarization

One week prior to any acute bout of exercise, subjects visited the laboratory for the following: a body composition assessment using dual-energy X-ray absorptiometry (DEXA; GE Healthcare, Toronto, ON, Canada), a familiarization session with the equipment and procedures involved in the study and a preliminary strength test. Both the familiarization session and preliminary strength test were also used for baseline testing. Subjects who were selected for the AE and HIT groups were required to cycle on a cycle ergometer at predicted workloads to ensure that their power output produced the appropriate physiological response – as determined by their heart rate. The power output for subjects in the AE group was required to elicit 70% of a subject's HR_{max} (60% of their VO_2 peak), while the power output for subjects in the HIT group was required to elicit 90–95% of a subject's HR_{max} (90% of their VO_2 peak). For those subjects chosen for the RE group, the preliminary strength test was not only used to determine strength but also the workload assigned for their exercise day. The preliminary strength test consisted of a bilateral 10 repetition maximum (10-RM) test for both leg press and knee extension. Leg muscle strength (10-RM) was estimated on weight machines for leg extension (Atlantis Precision Series Leg Extension C-105, Laval QC) and leg press (HUR 3545 Leg Press Incline: HUR, Northbrook, IL, USA). In brief, subjects performed familiarization sessions with the machines, to ensure correct lifting technique was demonstrated and practiced. Following this demonstration, participants performed a warm-up of 10 repetitions at a very light load (~60–70% 10-RM). The weight was then increased per set, and participants completed up to 10 repetitions. Between each 10-RM attempt, participants were given 3 min of rest and no more than three attempts were required to determine 10-RM. Additionally, it is important to eliminate the effects of differences in diet (i.e. protein intake) when assessing anabolic responses. As such, subjects consumed a standardized, weight maintenance diet beginning 2 days prior to the acute exercise bout and lasting until the final day of testing (48 h post-exercise). The diet was composed of roughly 55% carbohydrate, 30% fat and 15% protein. Subjects arrived fasted for each muscle biopsy and were provided with a standardized, mixed macronutrient snack that delivered a suboptimal protein dose (5–7 g protein) immediately following exercise by a member of the research team. Subjects consumed their prescribed breakfasts upon returning home from the laboratory on days where they received biopsies.

Acute exercise protocol

Subjects arrived at the laboratory in the morning and completed a single acute bout of exercise which was unique to each subject depending on what group he was selected for (AE, HIT or RE). Subjects in the AE group were required to complete 30 min of cycling at a moderate intensity on a cycle ergometer. The intensity was set at a heart rate that corresponded to 55–60% of the individual's baseline VO_2 peak. Subjects in the HIT group instead performed 10 × 60 sec bouts of high-intensity cycling at a heart rate that corresponded to 90–95% of the individual's baseline VO_2 peak. These 60-s bouts of high-intensity cycling were interspersed by 60-s of low-intensity cycling (50W) for recovery. Subjects in the RE group were required to complete three sets of bilateral leg press and knee extension at approximately 95% of their 10-RM with 2 min of rest in between each sets. Participants completed leg press followed by leg extension. The first two sets required subjects to complete 10 repetitions of each, while the 3rd and final set required subjects to complete as many repetitions of

each until volitional failure (Mean ± SEM, leg extension: 13 ± 2 reps; leg press is 19 ± 2).

Blood measures

A resting serum and plasma sample was obtained prior to the intervention (baseline) and 24 h post-exercise from the antecubital vein. Serum samples were analysed for creatine kinase (CK) activity as per the instructions provided by the manufacturer using a commercially available kit (Pointe Scientific, Canton, MI, USA).

Muscle sample collection

A total of four percutaneous needle biopsies were taken from the mid-portion of the *vastus lateralis* under local anaesthetic using a custom-modified 5-mm Bergstrom needle adapted for manual suction under local anaesthesia (2% xylocaine). Subjects came into the laboratory during the morning of the intervention, and 2 biopsies were taken prior to exercise for baseline analysis (Pre). This study is part of a larger, previously published study where measurements of muscle protein synthesis were taken (Bell *et al.* 2015). Four biopsies were necessary to measure muscle fractional synthetic rate; specifically, 2 pre-exercise biopsies were needed (Pre1 and Pre2). The other biopsies were obtained at 24 h and 48 h post-exercise. Incisions and biopsies taken on the same leg were spaced by approximately 5 cm to minimize any effect of the previous biopsy. Biopsies were taken alternately of each leg (2 biopsies/leg). Diurnal variation was controlled for to the best of our abilities by taking all biopsies in the morning between the hours of 6 am and 9 am following an overnight fast. Upon excision, muscles samples were immediately divided into smaller pieces that were snap-frozen in liquid nitrogen for gene expression analysis and mounted in optimal cutting temperature (OCT) for immunohistochemistry.

Immunohistochemistry

Muscle cross sections (7 μm) were prepared from OCT-embedded samples and allowed to air-dry for approximately 30 min and subsequently stored at −80 °C. Tissue sections were brought to room temperature fixed in 2% paraformaldehyde (PFA) for 10 min, washed and then blocked for 90 min (in PBS containing 2% bovine serum albumin [BSA], 5% foetal bovine serum, 0.02% Triton X-100, 0.1% sodium azide and 5% goat serum [GS]).

For fibre-type-specific SC quantification, slides were incubated with primary antibodies against Pax7, laminin, MHCI and MHCII and appropriate secondary antibodies. To determine Pax7 and MyoD co-expression, slides were incubated with primary antibodies against Pax7, laminin and MyoD1 followed by appropriate secondary antibodies. For Pax7 and MSTN co-expression, slides were incubated with primary antibodies against Pax7, laminin, MHCI and MSTN, followed by appropriate secondary antibodies. All secondary antibodies and streptavidin were from Invitrogen; the biotinylated secondary antibody was from Vector Canada. Detailed antibody information is found in Table 2.

Nuclei were visualized with 4′,6-diamidino-2 phenylindole (DAPI, 1:20 000; Sigma-Aldrich, Oakville, ON, Canada) prior to coverslipping slides with fluorescent mounting media (DAKO, Burlington, ON, Canada). Images were observed with a Nikon Eclipse 90i microscope at a magnification of 20X and captured with a Photometrics Cool SNAP HQ2 fluorescent camera (Nikon Instrument, Melville, NY, USA). Analysis was completed using the Nikon NIS-Elements AR software (Nikon Instruments) on a large-scale image. The following are the number of fibres quantified for various analyses: fibre-type-specific SC response (total fibres, 313 ± 44; type I, 118 ± 35; type II, 186 ± 41); activity of the SC pool (total fibres, 200 ± 74); and SC-specific MSTN expression (total fibres, 250 ± 80; type I, 96 ± 41; type II, 153 ± 58). All areas selected for analysis were free of freeze-fracture artefact, and care was taken so that areas where longitudinal fibres were present were not used in the analysis. Muscle fibres on the periphery of muscle cross sections were not used in the analysis. All immunofluorescent analyses were completed in a blinded fashion.

RNA isolation

RNA was isolated from muscle tissue approximately 15–30 mg in size using the Trizol/RNeasy method. All samples were homogenized using 1 mL of Trizol® reagent (Life Technologies, Burlington, ON, Canada) in Lysing D Matrix tubes (MP Biomedicals, Solon, OH, USA) with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) at a speed of 6.0 m/s for 40 s. Homogenized samples were then mixed with 200 μL of chloroform (Sigma-Aldrich), agitated vigorously for 15 s and centrifuged at 12000 ***g*** for 10 min. The upper aqueous (RNA) phase was purified using E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) as per the instructions provided by the manufacturer. RNA concentration (ng/μL) and purity (A260/A280) was deduced using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA). RNA integrity as represented

by RNA integrity numbers (RIN scale of 0–10) was discovered using the Agilent 2100 Bioanalyzer (Agilent Technologies, Toronto, ON, Canada). RNA samples were then reverse-transcribed using commercially available high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as described by manufacturer and using an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Mississauga, ON, Canada) to obtain cDNA for quantitative gene analysis.

Quantitative real-time RT-PCR

All individual qRT-PCRs were run in duplicate 25 μL reactions containing RT Sybr® Green qPCR Master Mix (Qiagen Sciences, Valencia, CA, USA). Each 25 μL reaction was prepared with the epMotion 5075 Eppendorf automated pipetting system (Eppendorf). Primer sequence for MSTN was as follows: forward: 5′-TGGTCATGATCTTGCTGTAACCTT-3′ and reverse: 5′-TGTCTGTTACCTTGACCTCTAAAA-3′. Primers for SMAD3 were designed by SABioscience (SABioscience, Mississauga, ON, Canada; cat. no. PPH01921C).

Statistical analysis

Statistical analysis was completed using SigmaStat 3.1.0 analysis software (Systat Software, Chicago, IL, USA). A two-way repeated-measures ANOVA with one factor for time (Pre, 24 h, 48 h) and exercise type (AE, HIT, RE) was conducted to search for statistical differences in the SC response, the extent of Pax7 and MyoD co-expression and the extent of Pax7 and MSTN co-expression. The SC response and the Pax7 and MSTN co-expression analyses were analysed independently in type I and II fibres. To determine whether a difference existed with CSA, myonuclear domain and number of nuclei/fibre, a two-way ANOVA (factor for fibre type and factor for group) was used. Any significant interactions or main effects were analysed using the Tukey's *post hoc* test across the different modalities of exercise. A one-way ANOVA was used to determine whether differences existed between groups for the following variables: age, height, weight, BMI, body fat percentage, lean tissue mass, VO_2 peak, 10-RM leg press and 10-RM knee extension. Statistical significance was accepted at $P \leq 0.05$. All results were presented as means ± standard error of the mean (SEM).

Results

Subject characteristics

There were no differences in age, BMI, body fat percentage and lean tissue mass between groups. In addition, each group shared similar fitness levels and lower body strength. No differences were observed in fibre cross-sectional area (CSA), number of myonuclei per fibre or myonuclear domain. Complete subject characteristics are reported in Table 1.

Table 1 Subject characteristics

Variable	AE; $n = 7$	HIT; $n = 8$	RE; $n = 7$
Age (years)	68 ± 3	67 ± 5	66 ± 3
Height (cm)	1.74 ± 0.07	1.78 ± 0.06	1.75 ± 0.07
Weight (kg)	82.6 ± 4.7	86.0 ± 8.6	82.3 ± 12.3
BMI (kg/m^2)	27.3 ± 1.8	27.0 ± 2.8	26.7 ± 3.5
Body fat %	25.6 ± 4.2	23.9 ± 5.9	23.9 ± 4.9
Lean tissue mass (kg)	60.1 ± 4.7	63.4 ± 6.4	60.3 ± 6.5
VO_2 Peak (mL/kg/min)	30.7 ± 5.5	31.9 ± 6.8	27.3 ± 6.3
10-RM leg press (kg)	61.3 ± 8.0	59.8 ± 9.9	48.6 ± 19.2
10-RM knee extension (kg)	52.4 ± 5.0	54.3 ± 13.5	52.3 ± 14.8
CSA of type I fibre (μm^2)	5888 ± 1039*	6141 ± 738*	5706 ± 1199*
CSA of type II fibre (μm^2)	4901 ± 823	5109 ± 714	4863 ± 941
Myonuclei per type I fibre	4.0 ± 0.8*	4.4 ± 1.0*	4.1 ± 0.6*
Myonuclei per type II fibre	3.3 ± 0.6	3.2 ± 0.7	2.8 ± 0.5
Myonuclear domain of type I fibre (μm^2/nuclei)	1491 ± 305	1447 ± 281	1425 ± 431
Myonuclear domain of type II fibre (μm^2/nuclei)	1514 ± 156	1550 ± 159	1680 ± 165

Each value (Mean ± SD).
Baseline characteristics of subjects in the AE, HIT and RE group.
*Indicates a significant difference compared to type II.

Table 2 Antibody information

Antibody	Species	Source	Clone	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	Neat	Alexa 594, 488 goat anti-mouse 1:500
Antilaminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488, 647 goat anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 Slow isoform	Neat	Alexa Fluor 488 goat anti-mouse, 1:500
Anti-MHCII	Rabbit	Abcam	ab91506 Fast isoform	1:1000	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Mouse	Dako	5.8A	1:30	Goat anti-mouse biotinylated secondary antibody, 1:200; streptavidin-594 fluorochrome, 1:250
Anti-MSTN	Rabbit	Abcam	Ab98337	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500

Detailed information on primary and secondary antibodies used for immunofluorescence of muscle cross sections.

Serum creatine kinase (CK)

CK activity was determined in serum prior to and 24 h following either an acute bout of AE, HIT or RE. CK activity was increased 24 h post-RE (pre: 40.8 ± 6.1 U/L, 24 h: 141 ± 37 U/L; $P < 0.001$) and HIT (pre: 46 ± 10 U/L, 24 h: 90 ± 12 U/L) ($P < 0.05$) but not AE (pre: 42 ± 4 U/L, 24 h: 68 ± 5 U/L). Serum CK activity 24 h post-intervention was greater following RE than AE ($P < 0.05$), and there was a trend for higher activity following RE than HIT ($P = 0.081$).

Fibre-type-specific SC response

There was a significant increase in the SC pool in the RE group with an increase of 63% in the number of Pax7 + cells associated with type I fibres 24 h and a 45% increase 48 h following exercise ($P < 0.05$) (Fig. 1f). The number of Pax7 + cells associated with

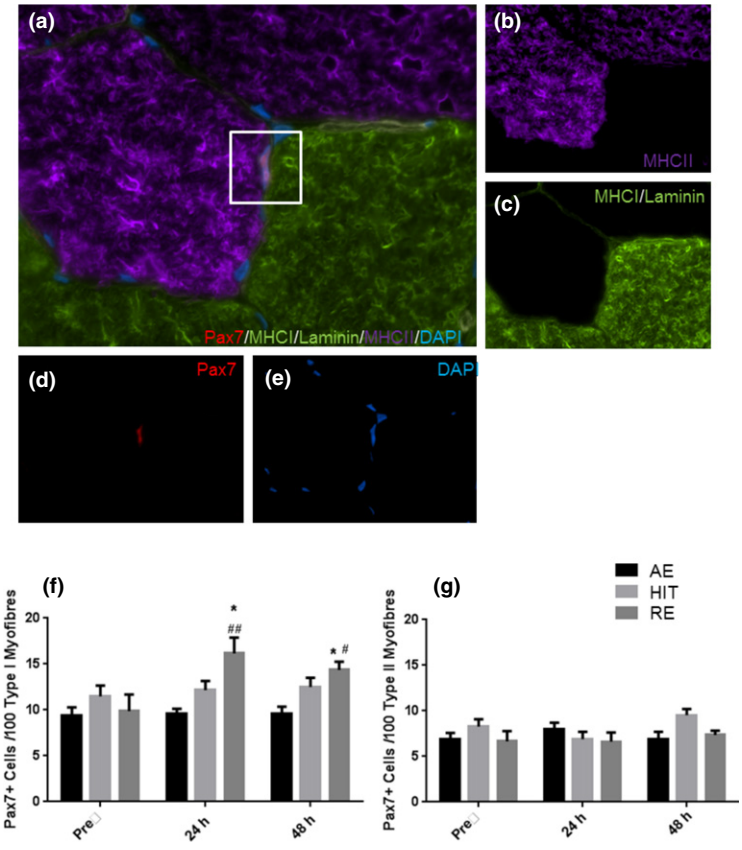


Figure 1 Acute fibre-type-specific SC response 24 and 48 h following AE, HIT and RE. (a) Representative image of a Pax7/MHCI/laminin/MHCII/DAPI stain of a muscle cross section, square represents a SC associated with a type II fibre. Single channel views of (b) MHCII purple, (c) MHCII/laminin green, (d) Pax7 red, (e) DAPI (nuclei) blue. (f) Acute response of SC associated with type I and (g) type II fibres. Bars represent means ± SEM, $^{*}P < 0.05$ vs. Pre, $^{\#\#}P < 0.05$ vs. AE and HIT $^{\#}P < 0.05$ vs. AE.

type I fibres was greater in RE at 24 h compared to AE and HIT (24 h: RE: 16 ± 2, AE: 10 ± 1, HIT: 12 ± 1 Pax7 + cells/100 type I fibre) ($P < 0.05$) (Fig. 1f). At 48 h, the number of Pax7 + cells associated with type I fibres was still greater in RE compared to AE (48 h: RE: 14 ± 1, AE: 10 ± 1 Pax7 + cells/100 type I fibre) ($P < 0.05$) but not compared to HIT ($P > 0.05$) (Fig. 1f). There was no appreciable increase in Pax7 + cells associated with type II fibres following acute exercise in either of the exercise groups ($P > 0.05$) (Fig. 1g).

Activity of the SC pool

To further describe the SC response following three different modes of exercise, we determined the proportion of cells expressing MyoD, one of two primary MRFs expressed during early (proliferation) and later (differentiation) stages of the myogenic programme. SC-expressing MyoD was identified as active. Following acute exercise, there was an increase in both the RE and HIT groups 24 h following exercise (HIT: Pre 0.7 ± 0.3; 24 h: 3.1 ± 0.3; RE: Pre: 1.3 ± 0.1; 24 h: 4.8 ± 0.5 Pax7 + /MyoD+ cells/100 fibres) ($P < 0.001$), and this increase was observed across all three groups at 48 h following exercise (AE: Pre: 0.9 ± 0.3; 48 h: 3.1 ± 0.8; HIT: Pre: 0.7 ± 0.3; 48 h: 4.7 ± 0.3; RE: Pre: 1.3 ± 0.1 48 h: 4.9 ± 0.4; Pax7 + /MyoD+ cells/100 fibres) ($P < 0.05$) (Fig. 3c). At 24 h following exercise, the number of active SC was greater in the RE group (4.8 ± 1.4 Pax7 + /MyoD+ cells/100 fibres) than both the AE (1.9 ± 0.5 Pax7 + /MyoD+ cells/100 fibres) and the HIT (3.1 ± 0.9 Pax7 + /MyoD+ cells/100 fibres) groups ($P < 0.05$), whereas at 48 h, the number of active SC was greater in both the HIT (4.7 ± 0.3 Pax7 + /MyoD+ cells/100 fibres) and RE (4.9 ± 0.4 Pax7 + /MyoD+ cells/100 fibres) compared to the AE (3.1 ± 0.8 Pax7 + /MyoD+ cells/100 fibres) group ($P < 0.05$) (Fig. 3c).

MSTN expression

To determine whether MSTN expression was differentially affected following three different modes of exercise, whole-muscle MSTN and SMAD3, a downstream target of MSTN, gene expression and SC-specific MSTN levels were determined following acute exercise. A significant reduction in the percentage of SC co-expressing MSTN specifically associated with type I fibres in the RE group 24 h following exercise (Pre: 87 ± 4; 24 h: 57 ± 5%MSTN+ type I SC) ($P < 0.001$) (Fig. 4f). Additionally, the observed reduction was greater in the RE group at 24 h (57 ± 5%MSTN+ type I SC) compared to the HIT group (79 ± 5%MSTN+ type I SC) ($P < 0.05$) and a strong trend ($P = 0.06$) compared to the AE group (67 ± 4%MSTN+ type I SC) (Fig. 4f). At 48 h following acute exercise, the percentage of type I-associated SC co-expressing MSTN was significantly reduced in each exercise group (AE: Pre: 84 ± 6, 48 h: 67 ± 4; HIT: Pre: 92 ± 3, 48 h: 52 ± 7; RE Pre: 87 ± 4, 48 h: 67 ± 4%MSTN+ type I SC) ($P < 0.05$) (Fig. 4f). A main effect for time was observed 48 h following acute exercise with a significant reduction in the percentage of SC co-expressing MSTN associated with type II fibres (Fig. 4g) ($P < 0.05$).

Similar to what was observed with type I SC-specific levels of MSTN, a main effect for time was

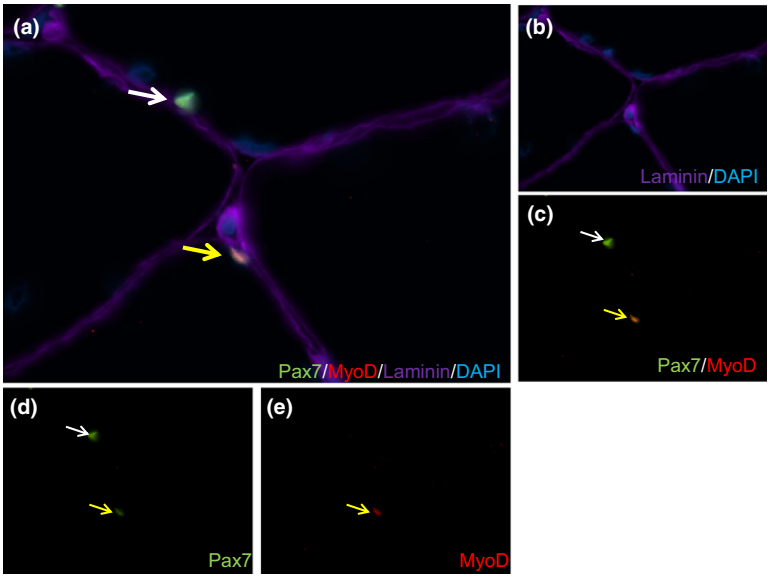


Figure 2 MyoD expression within a SC. (a) Representative image of a Pax7/MyoD/laminin/DAPI stain of a muscle cross section, white arrow represents a Pax7 + /MyoD- SC, yellow arrow represents a Pax7 + /MyoD+ SC. Single channel views of (b) laminin purple, and DAPI (nuclei) blue, (c) Pax7 green and MyoD red, (d) Pax7 green and (e) MyoD red.

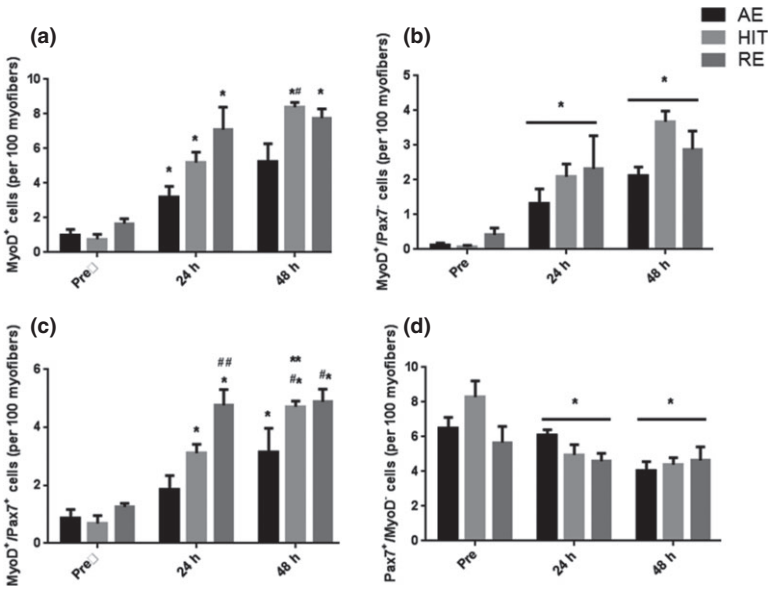


Figure 3 The activation status of SC following acute exercise. (a) The number of MyoD+ cells, (b) MyoD+/Pax7- (differentiating), (c) MyoD+/Pax7 + (activated) and (d) Pax7 + /MyoD- (proliferating) SC. Bars represent means ± SEM, $^{**}P < 0.05$ vs. 24, $^{\#\#}P < 0.05$ vs. HIT and AE, $^{\#}P < 0.05$ vs. AE, $^{*}P < 0.05$ vs. Pre.

observed at 24 and 48 h following acute exercise with a reduction in MSTN whole-muscle mRNA expression (Fig. 4h). Interestingly, a main effect for group was observed for SMAD3 gene expression in whole muscle, and expression was significantly lower in the RE group compared to the AE and HIT groups (Fig. 4i).

Discussion

The primary novel finding of the current study was that HIT was nearly as effective as RE in increasing the number of active SC following an acute bout of exercise. Additionally, all three modes of exercise led to significant decreases in the proportion of SC co-expressing MSTN following acute exercise.

The development of successful therapeutic strategies to promote the maintenance of muscle mass in older adults requires an understanding of the underlying mechanisms of sarcopenia and the processes that control muscle growth. It has been consistently reported that the loss of muscle mass with ageing is primarily accounted for by the loss of total muscle fibres (Lexell *et al.* 1988) and atrophy of type II fibres (Lexell *et al.* 1988, Kim *et al.* 2005, Dreyer *et al.* 2006, Kosek *et al.*2006, Petrella *et al.* 2006, Nilwik *et al.* 2013). Furthermore, type II fibre atrophy is associated with a specific reduction in the SC pool associated with type II fibres (Verdijk *et al.* 2007, 2012, McKay *et al.* 2012, Mackey *et al.* 2014). Although the reduction and dysfunction of the SC pool in older individuals does not directly contribute to loss of functional capacity, the compromised SC pool is likely a contributing factor to the loss of muscle mass with age (Snijders *et al.* 2009). Our data are consistent with previous studies reporting a reduced CSA of type II fibres as compared to type I fibres (Table 1).

The resistance training-induced increase in muscle fibre area has been associated with an expansion of the total SC pool (Petrella *et al.* 2008) and more recently has been shown to be associated with an increase in the SC pool associated specifically with type I fibres in young adults (Bellamy *et al.* 2014). Additionally, 12 weeks of resistance training in older adults resulted in an increase in type II muscle fibre size and was also associated with an expansion of the type II fibre-associated SC pool (Verdijk *et al.* 2014). It has been demonstrated that individuals who gained the most muscle mass following resistance exercise training were the only ones in which a significant expansion of the SC pool was also observed (Petrella *et al.* 2008). Further, we have recently demonstrated that the acute type I fibre-associated SC response following a single bout of resistance exercise is related to marked long-term increases in muscle mass following resistance training in young healthy males (Bellamy *et al.* 2014). Therefore, the acute expansion of the SC pool would appear to be important in determining increases in muscle mass, at least with resistance training in a young population. Although this association has not yet been established in an older population, determining which mode of exercise stimulates the most potent SC response may help in prescribing the optimal exercise programme to minimize muscle loss associated with ageing.

Exercise training is a known therapeutic strategy capable of attenuating the effects of sarcopenia by inducing skeletal muscle hypertrophy (McPherron & Lee 1997, Verney *et al.* 2008, Verdijk *et al.* 2009,

  doi: 10.1111/apha.12601

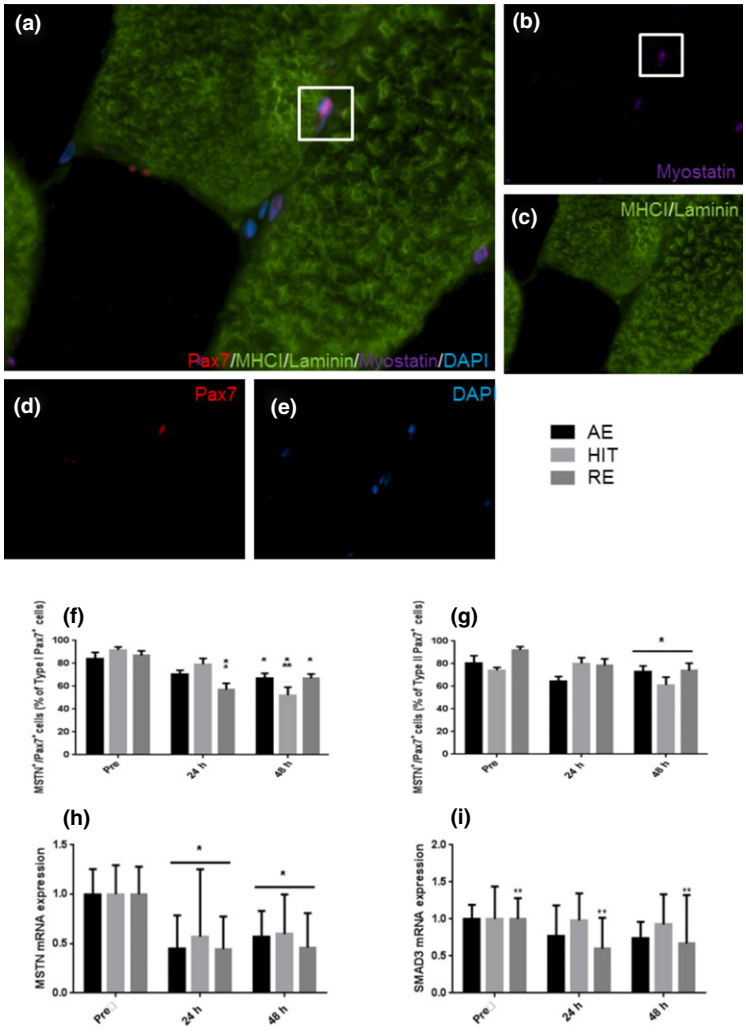


Figure 4 Effect of exercise on MSTN co-expression to SC. (a) Representative image of a Pax7/MSTN/MHCI/laminin/DAPI stain of a muscle cross section, white square represents a Pax7 + / MSTN+ SC. Single channel views of (b) MSTN purple, (c) MHCI/laminin green, (d) Pax7 red, (e) DAPI (nuclei) blue. The percentage of type I-associated SC-expressing MSTN (f) and type II-associated SC-expressing MSTN (g). Whole-muscle mRNA expression of (h) MSTN and (i) SMAD3 expressed as a fold change from pre. A $P < 0.05$ vs. HIT, $^{*}P < 0.05$ vs. Pre, $^{**}P < 0.05$ vs. 24, $^{***}P < 0.05$ group effect vs. AE and HIT.

Leenders *et al.* 2013). Resistance exercise has been shown to effectively induce SC activity (Dreyer *et al.* 2006, Verdijk *et al.* 2009, McKay *et al.* 2012, Leenders *et al.* 2013), which was consistent with the findings of the current study. We demonstrated a 63% and a 45% increase in the number of SC associated with type I fibres 24 and 48 h, respectively, following RE (Fig. 1). Consistent with previous reports, no appreciable expansion of SC associated with type II fibres was observed in any of the exercise groups following acute exercise (Fig. 1) (McKay *et al.* 2012). We observed that the increase in type I-associated SC content following a bout of RE is greater than HIT and AE at 24 h. At 48 h, while the type I-associated SC content was still significantly elevated in the RE group as compared to the AE group, there was no significant difference compared to the HIT group. Subjects were required to complete preliminary strength tests and familiarization sessions at least 1 week prior to pre-biopsy. Although these tests may be a significant insult to untrained skeletal muscle, other studies have also collected muscle biopsies 1 week after the completion of strength tests (Verdijk *et al.* 2009) and as little as 4 days following the completion of strength tests (Leenders *et al.* 2013). We also acknowledge that the 10-RM baseline testing (i.e. leg extension and leg press) may sufficiently perturb the muscle so that there is an adaptive response and may affect the SC response in subsequent bouts of exercise. This adaptive response may reflect the 'repeated bout phenomenon', whereby bouts of exercise may protect against subsequent bouts (McHugh 2003). Given that the working muscle group (i.e. the quadriceps) does not need to be exercised in the same manner as the original bout (Eston *et al.* 1996), this protective effect may exist regardless of modality.

Additionally, no differences were observed between groups prior to exercise indicating that any response induced by the familiarization sessions returned to baseline.

Previous studies have described an expansion in the total SC pool (Charifi *et al.* 2003) and more recently

specifically to the SC pool associated with type II fibres (Verney *et al.* 2008) in older adults following extended interval-type training, while more traditional endurance exercise training did not lead to an expansion of the SC pool in obese older type 2 diabetes patients (Snijders *et al.* 2011). However, Fry *et al.* 2014 recently described an expansion of the SC pool associated with type I fibres following 12 weeks of traditional aerobic exercise training in sedentary adults. These studies highlight the potential importance of exercise mode and intensity in mediating progression of the SC through the myogenic programme leading to an expansion of the SC pool. Taken together, these data suggest that while RE may provide sufficient stimuli to cause expansion of the SC pool, HIT may also elicit an expansion of the SC pool later as compared to a bout of AE. We did not observe an expansion of SC associated with type I fibres following AE and HIT. Furthermore, there was no expansion of SC associated with type II fibres following any type of exercise during the time period at which biopsies were taken. However, given the relatively short time course (i.e. 48 h post-exercise), we may have missed this expansion if it did occur. Indeed, the lower intensity of both AE and HIT compared to RE may also have resulted in a slower expansion of the SC pool.

Recent work has demonstrated an increase in MyoD+ SC 24, 48 and 72 h following an acute bout of RE in older men. This increase was accompanied by an increase in the number of SC associated with type II fibres 72 h after an acute bout of RE in older men (Snijders *et al.* 2014). Unfortunately, we were unable to measure the response of the SC pool at 72 h; however, the increase in MyoD observed 48 h following RE and HIT could translate to expansion of the SC pool at later time points. In recent years, HIT has been described as a more time-efficient alternative to traditional AE (Gibala *et al.* 2012). HIT has been shown to be beneficial to a large range of populations including healthy individuals (Gibala *et al.* 2012), type 2 diabetes patients (Little *et al.* 2011, Terada *et al.* 2013) and patients with cardiovascular diseases (Currie *et al.* 2013). Importantly, similar benefits are derived following HIT and AE across either healthy or diseased populations (Currie *et al.* 2013, Terada *et al.* 2013, Gillen & Gibala 2014). During training interventions, adherence to either HIT or AE appears to be similar (Currie *et al.* 2013, Terada *et al.* 2013). If designing an exercise programme to counter the loss of muscle mass observed with ageing, a focus should be placed on RE as it is a known anabolic stimulus in older adults (Mackey *et al.* 2006, Verdijk *et al.* 2009, Leenders *et al.* 2013); however, the benefits of aerobic exercise in this population should also be considered. Due to the similarities in health outcomes and adherence, between both training methods despite a much lower time commitment for HIT, it may be of a greater benefit to incorporate HIT rather than traditional AE when prescribing an exercise prescription to older adults.

The progression of SC through the myogenic programme from activation, proliferation to terminal differentiation is dictated by a network of transcription factors referred to as myogenic regulatory factors (MRFs). MyoD is a primary MRF and plays a role in SC activation/proliferation (Megeney *et al.* 1996, Kitzmann *et al.* 1998) and continues to be expressed during the early stages of differentiation (Rudnicki *et al.* 2008). We have previously demonstrated an attenuated SC response in older men following a bout of resistance exercise evidenced by fewer MyoD+ cells (fewer active SC (MyoD+/Pax7 +) when compared to young men. The increase in active SC was only observed 48 h following resistance exercise in old men, whereas an increase was observed as early as 3 h post-exercise in young men (McKay *et al.* 2012). Here, we report an increase in the total number of cells expressing MyoD at 24 and 48 h in every group (Fig. 3a). More specifically, we describe a delayed response in SC activation following AE (Fig. 3c). We observed an increase in active SC at 24 h, which persisted until 48 h following either RE or HIT, but this increase was only observed at 48 h following AE. Additionally, the number of active SC at 24 h was greatest in the RE group; however, at 48 h following exercise, this number was greater in both the RE and the HIT when compared to the AE group. Together, these data suggest that HIT and RE lead to a greater activation of SC compared to AE. This finding further supports the importance of exercise mode and intensity in promoting SC activation.

Previous studies have shown that MSTN negatively regulates SC activation and self-renewal (McCroskery *et al.* 2003), especially in aged muscle (McKay *et al.* 2012). Downregulation of MSTN expression following exercise may regulate the response to hypertrophic stimuli. Furthermore, previous work from our laboratory has reported an attenuated reduction in MSTN signalling in older men following RE as compared to young. Similarly, we observe a time effect at 48 h with a reduced number of type II-associated SC co-expressing MSTN (Fig. 4g). In type I fibres, a significant decrease was observed at 24 h in the RE group only, but this reduction was observed in all three groups 48 h following acute exercise. Additionally, in the HIT group, there were significantly fewer SC co-expressing MSTN at 48 h than at 24 h (Fig. 4f). Whole-muscle MSTN gene expression was also measured and was significantly reduced 24 and 48 h following acute

exercise. These data suggest that the SC pool may be able to respond more quickly to exercise that is known to elicit an anabolic response like RE than other modes of exercise due to the reduction in MSTN expression observed at 24 h following RE only.

Serum CK activity was increased in both RE and HIT groups 24 h following exercise. Additionally, CK activity was greater 24 h following RE compared to AE, and a strong trend was observed for greater activity 24 h following RE when compared to HIT. Although circulating CK activity is a suboptimal proxy measure of muscle damage, its activity has been reported to be increased following exercise (Newham *et al.* 1986, Nosaka & Clarkson 1996). Taken together, muscle damage induced by RE may be greater than that induced by either AE or HIT based on serum CK activity 24 h following exercise, which may explain the more pronounced SC response following RE as compared to AE or HIT. Furthermore, the more rapid reduction in MSTN co-expression with SC observed following RE may be a response to damage and the initiation of repair, whereas the reduction in co-expression observed 48 h following AE and HIT may represent a remodelling/adaptive process without the same need for repair as compared to the RE group.

Interestingly, we observed a lower mRNA expression of SMAD3 at the whole-muscle level following RE compared to the other two groups. MSTN is believed to bind to the serine/threonine kinase receptor ActRIIb that phosphorylates receptor-regulated SMAD2 and SMAD3 (Huang *et al.* 2011). Phosphorylated SMAD2/SMAD3 forms complexes that translocate to the nucleus, where it is thereby involved in regulating the transcription of target genes, ultimately leading to the suppression of myogenesis. More specifically, upregulation of SMAD3 expression via MSTN inhibits MyoD expression leading to impaired differentiation in human (McFarlane *et al.* 2011) and murine (Langley *et al.* 2002) myoblasts. We have previously demonstrated that SMAD3 mRNA expression is greater in older men compared to young men at rest and 3 and 24 h following RE (McKay *et al.* 2012). The greater expression of SMAD3 was accompanied by a greater MSTN protein expression in older men. Following 48 h of RE, SMAD3 mRNA expression and MSTN protein expression in older men were reduced to that of young men (McKay *et al.* 2012). SMAD3 mRNA expression seems to closely mimic MSTN protein expression. Measuring the expression of phosphorylated SMAD3 as a downstream target of MSTN signalling would have been ideal, but we were not able to do so. Therefore, we determined the mRNA expression of SMAD3 as a downstream target of MSTN. The lower expression of SMAD3 observed in the RE group could in part explain the greater proportion of active SC ($MyoD^+/Pax7^+$) observed following the completion of a bout of RE. While the reduction of MSTN expression at the level of whole muscle across time may indicate that all modes of exercise are capable of this physiological response, it is possible that RE provides the most temporally effective reduction, which may indicate the need for a more immediate response for repair/adaptation/remodelling following RE.

The recruitment of fibres during these various modes of exercise may lend insight into the responses observed in the SC pool. The widely accepted Henneman size principle (Mendell 2005) postulates that while some fibres are activated at much lower thresholds (i.e. lower intensity of exercise), both a greater recruitment and a recruitment of larger fibres are activated when greater activation intensity is required. Recruitment of 'higher order' fibres during RE typically follows the Henneman size principal; predominately, type I fibres are recruited first, and as more contractions occur, higher threshold fibres are recruited (Sale 1987). However, there is likely a continuum of contractile and metabolic capacity and likely a considerable overlap of the abilities of each fibre 'type' across the spectrum (Staron 1997). In aerobic exercise of similar intensity (~60% VO_2 peak), there is a greater decrease in muscle glycogen detected in type I fibres as compared to type II fibres (Ball-Burnett *et al.* 1991). However, there is nearly a 30% decrease in muscle glycogen in type II fibres, suggesting that recruitment of 'higher order' fibres even within the moderate-intensity workload (Ball-Burnett *et al.* 1991). In line with this, work performed with higher intensity aerobic exercise has supported the notion that indicates that type II fibres are indeed active at much heavier workloads (Gollnick *et al.* 1974, Vollestad *et al.* 1984, Beelen & Sargeant 1993). Taken together, these data indicate that while there is some recruitment of 'higher order' fibres during traditional type AE and, to a greater extent, HIT-style exercise, limitations do exist in comparison with RE. Given the notion that there is either a reduced (McKay *et al.* 2012) or delayed (Snijders *et al.* 2014) response in the SC pool in type II fibres, it becomes paramount to properly select exercise modalities that will facilitate this SC response. Our findings suggest that RE stimulates a greater reduction of MSTN and it may be a reflection of the greater recruitment of type II fibres (and the associated SC pool). Moreover, the SC-specific reduction in MSTN along with a reduction in whole-muscle MSTN may further support the benefits of RE as the most potent mode of exercise in increasing muscle mass.

Data from this study reinforce current dogma that RE is the exercise mode able to elicit the greatest SC response even in older adults. In line with this, Bell *et al.* 2015 reported an increase in myofibrillar protein fractional synthetic rate (FSR) in both the RE and HIT groups 24 and 48 h following exercise; however, this increase was greater following RE. Together, these data suggest that RE may be the exercise modality able to stimulate the greatest anabolic response. Despite the observation that all three modes of exercise resulted in a reduction in SC-specific co-expression of MSTN, HIT and RE led to an increase in SC activation more rapidly than that observed following AE. Although we demonstrate a reduction in the percentage of type I-associated SC co-expressing MSTN in RE compared to other groups, a reduction was observed 48 h following exercise in the percentage of type II-associated SC co-expressing MSTN in all three exercise groups. Type II fibres are known to be more affected by sarcopenia, and eliminating or slowing the atrophy process of type II fibres observed with ageing would be ideal, but maintaining or maximizing CSA of type I fibres may also be an effective way in slowing the age-related loss of muscle mass. A reduction in type I-associated SC co-expressing MSTN following exercise may be indicative of an anabolic response.

We have recently demonstrated that the acute SC response associated with type I fibres is associated with the greatest increases in muscle mass following resistance exercise training (Bellamy *et al.* 2014). Although this study was a study in young adults, it highlights that in some instances, the acute SC response may be related to long-term skeletal muscle adaptation. However, SC function is impaired in older adults and therefore determining whether the acute SC response in either fibre type is associated with long-term gains in muscle mass in this population, as it does in a healthy young population, should also be determined. The current study allowed us to determine which type of exercise resulted in the greatest SC response in older adults. The findings of this study demonstrate that RE is the mode of exercise that results in the greatest and most efficient SC response. However, incorporating HIT instead of AE into an exercise training programme for older adults where the primary goal is to slow the process of sarcopenia may be justified.

Conflict of interest

There are no conflict of interests.

The Pax7 hybridoma cells developed by Dr. A. Kawakami and the A4.951 developed by Dr. H. Blau were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Dr. G Parise was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Grant (1455843) and by funding from the Labarge Optimal Aging Initiative from McMaster University. JP Nederveen was supported by a NSERC Canadian Graduate Scholarship (CGS-D).

References

Ball-Burnett, M., Green, H.J. & Houston, M.E. 1991. Energy metabolism in human slow and fast twitch fibres during prolonged cycle exercise. *J Physiol* **437**, 257–267.

Beelen, A. & Sargeant, A.J. 1993. Effect of prior exercise at different pedalling frequencies on maximal power in humans. *Eur J Appl Physiol Occup Physiol* **66**, 102–107.

Bell, K.E., Séguin, C., Parise, G., Baker, S.K. & Phillips, S.M. 2015. Day-to-Day changes in muscle protein synthesis in recovery from resistance, aerobic, and high-intensity interval exercise in older men. *J Gerontol A Biol Sci Med Sci* **70**, 1024–1029.

Bellamy, L.M., Joanisse, S., Grubb, A., Mitchell, C.J., McKay, B.R., Phillips, S.M., Baker, S. & Parise, G. 2014. The acute satellite cell response and skeletal muscle hypertrophy following resistance training. *PLoS ONE* **9**, 10.

Charifi, N., Kadi, F., Féasson, L. & Denis, C. 2003. Effects of endurance training on satellite cell frequency in skeletal muscle of old men. *Muscle Nerve* **28**, 87–92.

Currie, D.K., Dubberley, B.J., Mckelvie, S.R. & Macdonald, J.M. 2013. Low-volume, high-intensity interval training in patients with CAD. *Med Sci Sports Exerc* **45**, 1436–1442.

Dreyer, H.C., Blanco, C.E., Sattler, F.R., Schroeder, E.T. & Wiswell, R.A. 2006. Satellite cell numbers in young and older men 24 hours after eccentric exercise. *Muscle Nerve* **33**, 242–253.

Eston, R.G., Finney, S., Baker, S. & Baltzopoulos, V. 1996. Muscle tenderness and peak torque changes after downhill running following a prior bout of isokinetic eccentric exercise. *J Sports Sci* **14**, 291–299.

Fry, C.S., Noehren, B., Mula, J., Ubele, M.F., Westgate, P.M., Kern, P.A. & Peterson, C.A. 2014. Fiber type-specific satellite cell response to aerobic training in sedentary adults. *J Physiol* **592**, 2625–2635.

Gibala, M.J., Little, J.P., Macdonald, M.J. & Hawley, J.A. 2012. Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol* **590**, 1077–1084.

Gillen, J.B. & Gibala, M.J. 2014. Is high-intensity interval training a time-efficient exercise strategy to improve health and fitness? *Appl Physiol Nutr Metab* **39**, 409–412.

Gollnick, P.D., Piehl, K. & Saltin, B. 1974. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol* **241**, 45–57.

Huang, Z., Chen, X. & Chen, D. 2011. Myostatin: a novel insight into its role in metabolism, signal pathways, and expression regulation. *Cell Signal* **23**, 1441–1446.

Hughes, V.A., Frontera, W.R., Wood, M., Evans, W.J., Dallal, G.E., Roubenoff, R. & Fiatarone Singh, M.A. 2001.

Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. *J Gerontol A Biol Sci Med Sci* **56**, B209–B217.

Kadi, F. & Thornell, L.E. 2000. Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. *Histochem Cell Biol* **113**, 99–103.

Kadi, F., Charifi, N., Denis, C. & Lexell, J. 2004. Satellite cells and myonuclei in young and elderly women and men. *Muscle Nerve* **29**, 120–127.

Kim, J., Kosek, D.J., Petrella, J.K., Cross, J.M. & Bamman, M.M. 2005. Resting and load-induced levels of myogenic gene transcripts differ between older adults with demonstrable sarcopenia and young men and women. *J Appl Physiol (1985)* **99**, 2149–2158.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N.J. & Fernandez, A. 1998. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol* **142**, 1447–1459.

Kosek, D.J., Kim, J.-S., Petrella, J.K., Cross, J.M. & Bamman, M.M. 2006. Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *J Appl Physiol (1985)* **101**, 531–544.

Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S. & Kambadur, R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem* **277**, 49831–49840.

Leenders, M., Verdijk, L.B., Van Der Hoeven, L., Van Kranenburg, J., Nilwik, R. & van Loon, L.J. 2013. Elderly men and women benefit equally from prolonged resistance-type exercise training. *J Gerontol A Biol Sci Med Sci* **68**, 769–779.

Léger, B., Derave, W., De Bock, K., Hespel, P. & Russell, A.P. 2008. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. *Rejuvenation Res* **11**, 163–175.

Lexell, J., Taylor, C.C. & Sjostrom, M. 1988. What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. *J Neurol Sci* **84**, 275–294.

Little, J.P., Gillen, J.B., Percival, M., Safdar, A., Tarnopolsky, M.A., Punthakee, Z., Jung, M.E. & Gibala, M.J. 2011. Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. *J Appl Physiol (1985)* **111**, 1554–1560.

Mackey, A.L., Esmarck, B., Kadi, F., Koskinen, S.O.A., Kongsgaard, M., Sylvestersen, A., Hansen, J.J., Larsen, G. & Kajer, M. 2006. Enhanced satellite cell proliferation with resistance training in elderly men and women. *Scand J Med Sci Sports* **17**, 34–42.

Mackey, A.L., Kajer, M., Dandanell, S., Mikkelsen, K.H., Holm, L., Døssing, S., Kadi, F., Koskinen, S.O., Jensen, C.H., Schrøder, H.D. & Langberg, H. 2007. The influence of anti-inflammatory medication on exercise-induces myogenic precursor cell responses in humans. *J Appl Physiol (1985)* **103**, 425–431.

Mackey, A.L., Karlsen, A., Couppé, C., Mikkelsen, U.R., Nielsen, R.H., Magnusson, S.P. & Kjaer, M. 2014. Differential satellite cell density of type I and II fibres with lifelong endurance running in old men. *Acta Physiol (Oxf)* **210**, 612–627.

McCroskery, S., Thomas, M., Maxwell, L., Sharma, M. & Kambadur, R. 2003. Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* **162**, 1135–1147.

McFarlane, C., Hui, G.Z., Amanda, W.Z.W., Lau, H.Y., Lokireddy, S., Xiaojia, G., Mouly, V., Butler-Browne, G., Gluckman, P.D., Sharma, M. & Kambadur, R. 2011. Human myostatin negatively regulates human myoblast growth and differentiation. *Am J Physiol Cell Physiol* **301**, 195–203.

McHugh, M.P. 2003. Recent advances in the understanding of the repeated bout effect: the protective effect against muscle damage from a single bout of eccentric exercise. *Scand J Med Sci Sports* **13**, 88–97.

McKay, B.R., Ogborn, D.I., Bellamy, L.M., Tarnopolsky, M.A. & Parise, G. 2012. Myostatin is associated with age-related human muscle stem cell dysfunction. *FASEB J* **26**, 2509–2521.

McKay, B.R., Ogborn, D.I., Baker, J.M., Toth, K.G., Tarnopolsky, M.A. & Parise, G. 2013. Elevated SOCS3 and altered IL-6 signaling is associated with age-related human muscle stem cell dysfunction. *Am J Physiol Cell Physiol* **304**, 717–728.

McPherron, A.C. & Lee, S.J. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci USA* **94**, 12457–12461.

McPherron, A.C., Lawler, A.M. & Lee, S.J. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83–90.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E. & Rudnicki, M.A. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev* **10**, 1173–1183.

Mendell, L.M. 2005. The size principle: a rule describing the recruitment of motoneurons. *J Neurophysiol* **93**, 3024–3026.

Newham, D.J., Jones, D.A. & Edwards, R.H. 1986. Plasma creatine kinase changes after eccentric and concentric contractions. *Muscle Nerve* **9**, 59–63.

Nilwik, R., Snijders, T., Leenders, M., Groen, B.B.L., van Kranenburg, J., Verdijk, L.B. & van Loon, L.J. 2013. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. *Exp Gerontol* **48**, 492–498.

Nosaka, K. & Clarkson, P.M. 1996. Changes in indicators of inflammation after eccentric exercise of the elbow flexors. *Med Sci Sports Exerc* **28**, 953–961.

Petrella, J.K., Kim, J., Cross, J.M., Kosek, D.J. & Bamman, M.M. 2006. Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am J Physiol Endocrinol Metab* **291**, 937–946.

Petrella, J.K., Kim, J.-S., Mayhew, D.L., Cross, J.M. & Bamman, M.M. 2008. Potent myofiber hypertrophy during

resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physiol (1985)* **104**, 1736–1742.

Raue, U., Slivka, D., Jemiolo, B., Hollon, C. & Trappe, S. 2006 Myogenic gene expression at rest and after a bout of resistance exercise in young (18-30 yr) and old (80-89 yr) women. *J Appl Physiol (1985)* **101**, 53–59.

Roth, S.M., Martel, G.F., Ivey, F.M., Lemmer, J.T., Tracy, B.L., Metter, E.J., Hurley, B.F. & Rogers, M.A. 2001. Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training. *J Gerontol A Biol Sci Med Sci* **56**, 240–247.

Roubenoff, R. & Hughes, V.A. 2000. Sarcopenia: current concepts. *J Gerontol A Biol Sci Med Sci* **55**, 716–724.

Rudnicki, M.A., Le Grand, F., McKinnell, I. & Kuang, S. 2008. The molecular regulation of muscle stem cell function. *Cold Spring Harb Symp Quant Biol* **73**, 323–331.

Sajko, S., Kubínová, L., Cvetko, E., Kreft, M., Wernig, A. & Erzen, I. 2004. Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. *J Histochem Cytochem* **52**, 179–185.

Sale, D.G. 1987. Influence of exercise and training on motor unit activation. *Exerc Sport Sci Rev* **15**, 95–151.

Snijders, T., Verdijk, L.B. & van Loon, L.J. 2009. The impact of sarcopenia and exercise training on skeletal muscle satellite cells. *Ageing Res Rev* **8**, 328–338.

Snijders, T., Verdijk, L.B., Hansen, D., Dendale, P. & van Loon, L.J. 2011. Continuous endurance-type exercise training does not modulate satellite cell content in obese type 2 diabetes patients. *Muscle Nerve* **43**, 393–401.

Snijders, T., Verdijk, L.B., Smeets, J.S., McKay, B.R., Senden, J.M., Hartgens, F., Parise, G., Greenhaff, P. & van Loon, L.J. 2014. The skeletal muscle satellite cell response to a single bout of resistance-type exercise is delayed with aging in men. *Age (Dordr)* **36**, 9699.

Staron, R.S. 1997. Human skeletal muscle fiber types: delineation, development, and distribution. *Can J Appl Physiol* **22**, 307–327.

Terada, T., Friesen, A., Chahal, B.S., Bell, G.J., McCargar, L.J. & Boulé, N.G. 2013. Feasibility and preliminary efficacy of high intensity interval training in type 2 diabetes. *Diabetes Res Clin Pract* **99**, 120–129.

Verdijk, L.B., Koopman, R., Schaart, G., Meijer, K., Savelberg, H.H.C.M. & van Loon, L.J. 2007. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Endocrinol Metab* **292**, 151–157.

Verdijk, L.B., Gleeson, B.G., Jonkers, R.A.M., Meijer, K., Savelberg, H.H.C.M., Dendale, P. & van Loon, L.J. 2009. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type-specific increase in satellite cell content in elderly men. *J Gerontol A Biol Sci Med Sci* **64**, 332–339.

Verdijk, L.B., Dirks, M.L., Snijders, T., Prompers, J.J., Beelen, M., Jonkers, R.A.M., Thijssen, D.H.J., Hopman, M.T.E. & van Loon, L.J. 2012. Reduced satellite cell numbers with spinal cord injury and aging in humans. *Med Sci Sports Exerc* **44**, 2322–2330.

Verdijk, L.B., Snijders, T., Drost, M., Delhaas, T., Kadi, F. & van Loon, L.J. 2014. Satellite cells in human skeletal muscle; from birth to old age. *Age (Dordr)* **36**, 545–557.

Verney, J., Kadi, F., Charifi, N., Féasson, L., Saafi, M.A., Castells, J., Piehl-Aulin, K. & Denis, C. 2008. Effects of combined lower body endurance and upper body resistance training on the satellite cell pool in elderly subjects. *Muscle Nerve* **38**, 1147–1154.

Vollestad, N.K., Vaage, O. & Hermansen, L. 1984. Muscle glycogen depletion patterns in type I and subgroups of type II fibres during prolonged severe exercise in man. *Acta Physiol Scand* **122**, 433–441.

Yarasheski, K.E., Bhasin, S., Sinha-Hikim, I., Pak-Loduca, J. & Gonzalez-Cadavid, N.F. 2002. Serum myostatin-immunoreactive protein is increased in 60–92 year old women and men with muscle wasting. *J Nutr Health Aging* **6**, 343–348.

ACTA PHYSIOLOGICA

Acta Physiol 2015, **215**, 191–203

Deficiency of heat shock transcription factor 1 suppresses heat stress-associated increase in slow soleus muscle mass of mice

Y. Ohno,[1] T. Egawa,[2,3] S. Yokoyama,[1] A. Nakai,[4] T. Sugiura,[5] Y. Ohira,[6] T. Yoshioka[7] and K. Goto[1,2]

1 Laboratory of Physiology, School of Health Sciences, Toyohashi SOZO University, Toyohashi, Japan
2 Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University, Toyohashi, Japan
3 Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan
4 Department of Molecular Biology, Graduate School of Medicine, Yamaguchi University, Ube, Japan
5 Faculty of Education, Yamaguchi University, Yamaguchi, Japan
6 Graduate School of Health and Sports Science, Doshisha University, Kyotanabe, Japan
7 Hirosaki Gakuin University, Hirosaki, Japan

Received 1 July 2015, revision requested 20 July 2015, revision received 29 July 2015, accepted 3 September 2015
Correspondence: K. Goto, PhD, Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University, 20-1 Matsushita, Ushikawa, Toyohashi, Aichi 440-8511, Japan. E-mail: gotok@sepia.ocn.ne.jp

Abstract

Aim: Effects of heat shock transcription factor 1 (HSF1) deficiency on heat stress-associated increase in slow soleus muscle mass of mice were investigated.

Methods: Both HSF1-null and wild-type mice were randomly assigned to control and heat-stressed groups. Mice in heat-stressed group were exposed to heat stress (41 °C for 60 min) in an incubator without anaesthesia.

Results: Significant increase in wet and dry weights, and protein content of soleus muscle in wild-type mice was observed seven days after the application of the heat stress. However, heat stress had no impact on soleus muscle mass in HSF1-null mice. Neither type of mice exhibited much effect of heat stress on HSF mRNA expression (HSF1, HSF2 and HSF4). On the other hand, heat stress upregulated heat shock proteins (HSPs) at the mRNA (HSP72) and protein (HSP72 and HSP110) levels in wild-type mice, but not in HSF1-null mice. The population of Pax7-positive nuclei relative to total myonuclei of soleus muscle in wild-type mice was significantly increased by heat stress, but not in HSF1-null mice. Furthermore, the absence of HSF1 gene suppressed heat stress-associated phosphorylation of Akt and p70 S6 kinase (p-p70S6K) in soleus muscle.

Conclusion: Heat stress-associated increase in skeletal muscle mass may be induced by HSF1 and/or HSF1-mediated stress response that activates muscle satellite cells and Akt/p70S6K signalling pathway.

Keywords heat stress, heat shock proteins, heat shock transcription factor 1, satellite cell, skeletal muscle.

Skeletal muscle exhibits a greater plasticity to various cellular stimuli, such as mechanical loading (Goldspink 1999). Increase in mechanical loading induced by exercise is a typical stimulus for skeletal muscle hypertrophy (Goldspink 1999). Akt, so-called protein kinase B, is known as a key mediator of protein synthesis and hypertrophy in skeletal muscle (Bodine *et al.* 2001, Sugiura *et al.* 2005). Overexpression of constitutively active form of Akt induces muscle hypertrophy and prevents muscle atrophy (Bodine

 doi: 10.1111/apha.12600

et al. 2001). Therefore, it is generally accepted that mechanical loading induces muscle hypertrophy via the activation of Akt signalling pathway (Bodine *et al.* 2001, Sasai *et al.* 2010). On the other hand, it has been proposed that muscle satellite cells, skeletal muscle-specific stem cells, play a role in muscle hypertrophy (Hawke & Garry 2001, Fry *et al.* 2014) even though recent studies demonstrated that muscle satellite cells may not be essential for the loading-associated hypertrophy (McCarthy *et al.* 2011) and regrowth (Jackson *et al.* 2012) of skeletal muscles. Loading-associated increase in the population of Pax7-positive satellite cells is observed during regrowth of normal and atrophied soleus muscles (Morioka *et al.* 2008, Matsuba *et al.* 2009). Therefore, the population of satellite cells may be increased in response to hypertrophic stimuli.

Previous studies showed that heat stress is also one of the hypertrophic stimuli for skeletal muscle in not only rodents (Uehara *et al.* 2004, Kobayashi *et al.* 2005, Ohno *et al.* 2010, 2012) but also humans (Goto *et al.* 2007, 2011). Heat stress activates Akt and its downstream p70 S6 kinase (p70S6K), which stimulates muscle protein synthesis via increasing mRNA translation through the phosphorylation of ribosomal protein components (Bodine *et al.* 2001, Latres *et al.* 2005, Sugiura *et al.* 2005, Wang & Proud 2006, Fujita *et al.* 2007), in a temperature-dependent manner in rat skeletal muscle (Yoshihara *et al.* 2013). In addition, the population of Pax7-positive satellite cells is increased in heat stress-associated hypertrophied skeletal muscles (Kojima *et al.* 2007, Ohno *et al.* 2010). However, the molecular mechanisms for heat stress-associated skeletal muscle hypertrophy remain unclear.

Heat stress induces stress response (Welch 1992, Craig *et al.* 1993, McArdle *et al.* 2006, Hayes *et al.* 2009) mediated by heat shock transcription factors (HSFs) and then upregulates heat shock proteins (HSPs) in various cells via binding to heat shock element located at the upstream region of HSP genes (Sarge *et al.* 1993, Morimoto 1998, Santoro 2000, Akerfelt *et al.* 2010, Sakurai & Enoki 2010). Among three HSFs (HSF1, HSF2 and HSF4) in mammals, HSF1 plays a central role in induction of HSPs (Zhang *et al.* 2002, McArdle *et al.* 2006). HSPs, which act as molecular chaperones, play a part of the tightly regulated systems for maintenance of cellular homeostasis during the normal cell growth and for survival in response to various environmental stresses, including heat stress (Hartl 1996, Fink 1999). HSP72, inducible 70-kDa HSP (HSP70), as well as HSP25, is highly sensitive to cellular stresses (Welch 1992, Latchman 2001, Oishi *et al.* 2005, O'Neill *et al.* 2006, Huey *et al.* 2007). In fact, an application of heat stress upregulates HSP25 and HSP72 in mammalian skeletal muscle (Goto *et al.* 2003, Uehara *et al.* 2004, Kobayashi *et al.* 2005, Selsby & Dodd 2005, Oishi *et al.* 2009, Ohno *et al.* 2010, 2011, 2012). However, physiological roles of stress response as well as heat stress-induced upregulations of HSPs in skeletal muscle are also still unclear. In addition, there is no report regarding the response of HSP110 in skeletal muscle to heat stress, even though HSP110 belongs to a subfamily of the HSP70 and also acts as a molecular chaperone (Mattoo *et al.* 2013).

Recent studies investigated a physiological role of HSF1 in a plasticity of mouse skeletal muscle (Yasuhara *et al.* 2011, Koya *et al.* 2013, Nishizawa *et al.* 2013). Absence of HSF1 depressed the expression of HSPs in soleus muscle (Yasuhara *et al.* 2011, Koya *et al.* 2013, Nishizawa *et al.* 2013). Suppression of the population of Pax7-positive satellite cells in both regenerating and overloaded soleus muscle was observed in HSF1-null mice (Koya *et al.* 2013, Nishizawa *et al.* 2013). Furthermore, the injury-associated regeneration and the loading-associated regrowth of soleus muscles in HSF1-null mice were also depressed, compared with wild-type mice (Yasuhara *et al.* 2011, Koya *et al.* 2013, Nishizawa *et al.* 2013). These observations suggest that HSF1 plays a role in skeletal muscle plasticity. However, the effects of HSF1 deficiency on heat stress-associated hypertrophy of skeletal muscle are still unknown. Therefore, in this study, we investigated the effects of HSF1 deficiency on heat stress-associated upregulation of HSPs and increase in skeletal muscle mass using HSF1-null mice. The effects of heat stress on the activation level of Akt and p70S6K and the number of muscle satellite cells in HSF1-null mice were also investigated.

Materials and methods

Animals and grouping

All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD) and were approved by the Animal Use Committee of Toyohashi SOZO University. Male HSF1-null and wild-type (ICR) mice were prepared as described previously (Inouye *et al.* 2004). Mice aged 10–20 weeks were used in this experiment ($n = 84$). Both HSF1-null ($n = 39$) and wild-type mice ($n = 45$) were randomly assigned to untreated control and heat-stressed groups. All mice were housed in a home cage 20 × 31 cm and 13.5 cm height in a clean room controlled at approximately 23 °C with a 12-/12-h light–dark cycle. Solid diet and water were provided *ad libitum*.

  doi: 10.1111/apha.12600

Exposure to heat stress

Heat-stressed groups in both HSF1-null and wild-type mice were exposed to environmental heat stress (41 °C for 60 min) in a heat stress chamber without anaesthesia. The protocol of heat stress application in this study caused an increase in colonic temperature up to 41 °C and induced the upregulation of HSPs in mammalian skeletal muscle (Naito *et al.* 2000, Kobayashi *et al.* 2005, Ohno *et al.* 2012). During heat stress, mice freely accessed to diet and water in the cage. No abnormalities of moving and motion of mice were observed during as well as after heat stress. In addition, no mice died during and after heat stress in this study. After the heat stress procedure, all mice were housed in a room maintained ~23 °C.

Sampling

Soleus muscles of heat-stressed group in both HSF1-null and wild-type mice were dissected from both hindlimbs 1 (Day 1), 3 (Day 3) and 7 (Day 7) days after application of the heat stress. Soleus muscles of untreated control group in each type of mice were also dissected before heat stress (Pre) and at Day 7. The responses of soleus muscle to various external stimuli have been extensively investigated (Allen *et al.* 1996, Kawano *et al.* 2004, Yasuhara *et al.* 2011). Furthermore, our previous studies have demonstrated heat stress-associated increase of soleus muscle in rats and mice (Uehara *et al.* 2004, Kobayashi *et al.* 2005, Ohno *et al.* 2010, 2012). In the present study, therefore, slow soleus muscle, but not fast plantaris muscle and gastrocnemius muscle, was used to investigate the effect of HSF1 deficiency on the heat stress-associated increase in skeletal muscle mass. After the experimental period, the animals of each group were killed by cervical dislocation. Soleus muscles were excised from both hindlimbs. Dissected soleus muscles were rapidly trimmed of excess fat and connective tissues, wet-weighed on a microbalance and frozen in isopentane cooled by liquid nitrogen. The samples were stored at −80 °C until analyses.

Muscle dry weight and muscle protein content

Soleus muscles at Day 7 (n = 9–11 in untreated control and heat-stressed groups, respectively) were used for the measurement of muscular dry weight and protein content. Left frozen muscles, which were wet-weighed, were placed in a freeze dryer (−45 °C) under vacuum for 72 h (Naito *et al.* 2000, Kobayashi *et al.* 2005). Muscle tissues were then reweighed on a microbalance after 72 h. Right muscles were homogenized in 1 mL of tissue lysis reagent (CelLytic™-MT, Sigma-Aldrich, St. Louis, MO, USA) and completely solubilized by alkaline treatment with 2 N NaOH at 37 °C for 1 h (Ohno *et al.* 2010, 2012, Yasuhara *et al.* 2011). Protein content of the tissue lysate was determined using the Bradford technique (protein assay kit; Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma-Aldrich) as the standard. Then, absolute protein content in soleus muscle was calculated.

Real-time reverse transcription PCR

Left soleus muscles at Pre, Day 1, Day 3 and Day 7 (n = 5–6 in both groups each day) were cross-sectionally cut into halves at the mid-belly region. In this study, the mRNA expressions of HSFs including HSF1, HSF2, and HSF4, and HSPs including HSP25, heat shock cognate (HSC) 70, HSP72, and HSP110 were assessed by real-time reverse transcription PCR (RT-PCR).

Total RNA was extracted from the proximal portion of left soleus muscle using the miRNeasy Mini kit (Qiagen, Hiden, Germany) according to the manufacturer's protocol. Samples (~10 ng of RNA) were reverse-transcribed using the first-strand cDNA Synthesis kit according to the manufacturer's instructions [PrimeScript RT Master Mix (Perfect Real Time) for mRNA, Takara Bio, Otsu, Japan]. Synthesized cDNA was applied to real-time RT-PCR (Thermal Cycler Dice Real Time System II MRQ, Takara Bio) using Takara SYBR Premix Ex Taq II for mRNA, and analysed with Takara Thermal Cycler Dice Real Time System Software Ver. 4.00 according to the manufacturer's instructions. The real-time cycle conditions were 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s for mRNA. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

The primers were designed using the Takara Bio Perfect Real Time Support System (Takara Bio). Primers used for detection of mouse cDNA were as follows: HSF1, 5′-ACAGTGTCACCCGGCTGTTG-3′ (forward) and 5′-GACTGCACCAGTGAGATGAGGA A-3′ (reverse); HSF2, 5′-GCAGTGTTGTTCAACAT GTGTCAG-3′ (forward) and 5′-AGTTCCCATCCAG GAATGCAAG-3′ (reverse); HSF4, 5′-TGATGGATC TGGACATGGAGTTG-3′ (forward) and 5′-CTAG CATGAGTGGAGTTCCCAGTG-3′ (reverse); HSP25, 5′-TCCCTGGACGTCAACCACTTC-3′ (forward) and 5′-AGAGATGTAGCCATGTTCGTCCTG-3′ (reverse); HSC70, 5′-AGCTGCCTGGCATTTGTGTG-3′ (forward) and 5′-GTGCGGTTACCCTGGTCATTG-3′ (reverse); HSP72, 5′-CAAGAACGCGCTCGAATCCT A-3′ (forward) and 5′-TCCTGGCACTTGTCCAGC

AC-3′ (reverse); HSP110, 5′-ACTCATGAAATCAGA GCGAAGGTC-3′ (forward) and 5′-TCAATATTTGG GCCATTTGGAG-3′ (reverse); and GAPDH, 5′-TGT GTCCGTCGTGGATCTGA-3′ (forward) and 5′-TTG CTGTTGAAGTCGCAGGAG-3′ (reverse).

Western blotting and densitometry

The distal portion of left soleus muscle at Pre, Day 1, Day 3 and Day 7 was homogenized in isolation buffer (0.1 mL mg^{-1} muscle wet weight) of tissue lysis reagent (CelLytic^{TM}-MT, Sigma-Aldrich) with 10% (v/v) protease inhibitor cocktail (P8340, Sigma-Aldrich) and 1% (v/v) phosphatase inhibitor cocktail (524625, Calbiochem, San Diego, CA, USA) and centrifuged at 12 000 ***g*** (4 °C for 10 min), and then, the supernatant was collected. Protein content of the supernatant was determined using the Bradford technique (protein assay kit; Bio-Rad) and bovine serum albumin (Sigma-Aldrich) as the standard. After the determination of protein content, the supernatant samples were mixed with sodium dodecyl sulphate (SDS) sample buffer [30% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris–HCl, 0.05% (w/v) bromophenol blue and pH 6.8] and were boiled for 5 min. The SDS–polyacrylamide gel electrophoresis (PAGE) was carried out on 8 or 12% polyacrylamide containing 0.5% SDS at a constant current of 20 mA for 120 min. Equal amounts of protein (6 μg) were loaded on each gel. Molecular weight markers (Bio-Rad Precision Markers) were applied to both sides of gel as the internal controls for transfer process or electrophoresis.

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Hybond-P, GE Healthcare, Buckinghamshire, UK) using a Bio-Rad mini trans-blot cell at a constant voltage of 100 V for 60 min at 4 °C. After the transfer, the membranes were blocked for 1 h using a blocking buffer (RPN418, GE Healthcare). Then, the membranes were incubated for 2 h with a primary antibody: HSP25 (ADI-SPA-801, Enzo Life Sciences, Farmingdale, NY, USA), HSC70 (ADI-SPA-816, Enzo Life Sciences), HSP72 (ADI-SPA-812, Enzo Life Sciences), phosphorylated Akt at Ser^{473} residue (p-Akt: 9271, Cell Signaling Technology, Danvers, MA, USA), total Akt (t-Akt: 9272, Cell Signaling Technology), phosphorylated p70S6K at Thr^{389} residue (p- p70S6K: 9234, Cell Signaling Technology), total p70S6K (t-p70S6K: 2708, Cell Signaling Technology), GAPDH (G9545, Sigma-Aldrich) and then reacted with a secondary antibody (anti-rabbit immunoglobulin G conjugate to horseradish peroxidase; Cell Signaling Technology) for 2 h. To detect HSP110, we generated antiserum HSP110 by immunizing rabbit, as described previously (Fujimoto *et al.* 2004). After the final wash, protein bands were visualized using chemiluminescence (GE Healthcare), and signal density was measured using Light-Capture (AE-6971) with CS Analyzer version 2.08b (ATTO Corporation, Tokyo, Japan). In the present study, the expression of GAPDH was evaluated to ensure the equal loading. In addition, the expression levels of p-Akt and p-p70S6K were evaluated using the value relative to the expression levels of t-Akt and t-p70S6K respectively.

Immunohistochemical analyses

Frozen right soleus muscles at Pre, Day 1 and Day 3 were cut cross-sectionally into halves. Serial transverse cryosections (8 μm thick) of the proximal portion of soleus muscles were cut at −20 °C and mounted on the slide glasses. The sections were air-dried and stained to analyse the profiles of Pax7-positive nuclei by the standard immunohistochemical technique (Ohno *et al.* 2010).

Monoclonal anti-Pax7 antibody (undiluted tissue culture supernatant of hybridoma cells obtained from the Developmental Studies Hybridoma Bank, Iowa, IA, USA) was used for the detection of muscle satellite cells (Seale *et al.* 2000). Cross sections were fixed with 4% paraformaldehyde and then were post-fixed in ice-cold methanol. After blocking using a reagent (1% Roche blocking reagent, Roche Diagnostic, Penzberg, Germany), samples were incubated with the primary antibodies for Pax7 and rabbit polyclonal anti-laminin (Z0097, DakoCytomation, Glostrup, Denmark). Sections were also incubated with the second antibodies for Cy3-conjugated anti-mouse IgG (diluted 1 : 100; Jackson Immuno Research, West Grove, PA, USA) and with fluorescein isothiocyanate-conjugated anti-rabbit IgG (diluted 1 : 200; Sigma-Aldrich). Then, nuclei were stained in a solution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg mL^{-1}; Sigma-Aldrich). The images of muscle sections were incorporated into a personal computer (DP-BSW version 02.02, Olympus, Tokyo, Japan) using a microscope (IX81 with DP70, Olympus). The population of Pax7-positive nuclei located within the laminin-positive basal membrane relative to the total number of DAPI-positive nuclei in the whole transverse section of approximately 400 muscle fibres in each animal was calculated.

Statistical analysis

All values were expressed as means ± SEM. Statistically significant level of HSF1 mRNA (Fig. 1) was analysed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test. Statistical

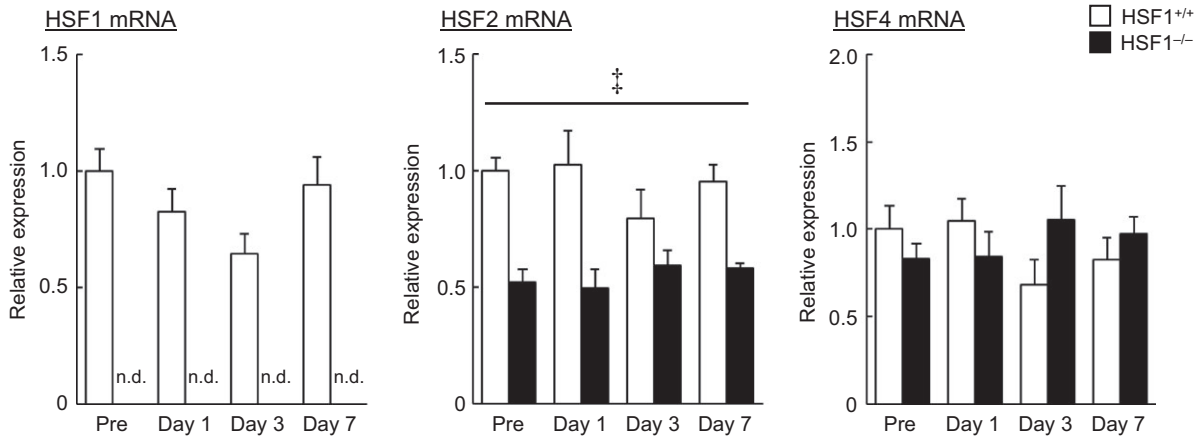


Figure 1 Expressions of heat shock transcription factor (HSF) mRNAs in response to heat stress. $HSF1^{+/+}$: wild-type mice; $HSF1^{-/-}$: heat shock transcription factor 1 (HSF1)-null mice; Pre: before heat stress; Day 1, Day 3 and Day 7: 1, 3 and 7 days after heat stress; n.d.: not detected. ‡: Significant main effect of mice, $P < 0.05$. The mRNA levels of HSFs are shown as relative to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are expressed relative to the value at Pre in $HSF1^{+/+}$ (1.0). Values are means ± SEM. $n = 5$–6 per group at each day.

significance for the response of soleus muscle mass of HSF1-null and wild-type mice to heat stress was analysed by unpaired Student's *t*-test or Welch's *t*-test following F-test. All other statistically significant levels were analysed using a two-way [mice and treatment (Table 1) or mice and time (Figs 1–5)] ANOVA for multiple comparisons followed by the Tukey–Kramer test. When a significant interaction between two main effects was observed, one-way ANOVA followed by the Tukey–Kramer test was performed. When a significant main effect of mice was observed, unpaired Student's *t*-test or Welch's *t*-test following *F*-test (Table 1) or one-way ANOVA followed by the Tukey–Kramer test (Figs 1–5) was performed in each type of mice. The significance level was accepted at $P < 0.05$.

Results

Effects of heat stress on muscle mass

First of all, we investigated whether HSF1 deficiency impacts on heat stress-associated increase in skeletal muscle mass at Day 7, as previous studies showed that heat stress-associated increase in skeletal muscle mass in wild-type mice and rats were observed 7 days after heat stress (Kobayashi *et al.* 2005, Ohno *et al.* 2010, 2012). Table 1 shows the body weight, absolute soleus muscle mass and muscle mass relative to body weight in wild-type and HSF1-null mice 7 days after the application of heat stress. Previous reports demonstrated that HSF1-deficient mice exhibit growth retardation (Xiao *et al.* 1999, Hashimoto-Torii *et al.* 2014). In our previous studies, the growth retardation in HSF-null mice was also observed (Yasuhara *et al.* 2011, Koya *et al.* 2013, Nishizawa *et al.* 2013). On the other hand, it is generally accepted that there are age-associated changes in skeletal muscle properties of developing mice (Rowe & Goldspink 1969, Chen *et al.* 2011, Neal *et al.* 2012). In the present study, therefore, we have investigated the effects of heat stress on skeletal muscle responses using HSF1-null mice, compared with age-matched (10–20 weeks) wild-type mice. There was a significant main effect of mice on body weight ($P < 0.05$). The body weight in HSF1-null mice was significantly lower than that in wild-type mice ($P < 0.05$). No significant effects of heat stress on body weight were observed in the two types of mice.

In wild-type mice, the absolute soleus muscle mass and muscle mass relative to body weight was significantly increased by heat stress ($P < 0.05$). Although soleus muscle mass in HSF1-null mice was significantly higher than that in wild-type mice ($P < 0.05$), no significant differences in relative muscle weight between control and heat-stressed groups were observed in HSF1-null mice. There was a significant difference of heat stress-induced changes in muscle mass between HSF1-null and wild-type mice ($P < 0.05$). Heat stress-associated increase in muscle mass was observed in wild-type mice ($P < 0.05$), but not in HSF1-null mice.

Effects of heat stress on HSFs and HSPs

As we confirmed that heat stress-associated increase in soleus muscle mass was observed in wild-type mice 7 days after the heating, but not in HSF-null mice, the responses of HSFs and HSPs to heat stress were investigated in both types of mice. Changes in mRNA

Table 1 Effects of heat stress on body weight and soleus muscle mass in HSF1-null and wild-type mice

	$HSF1^{+/+}$			$HSF1^{-/-}$		
	Control	Heat	Fold change (Heat/Control)	Control	Heat	Fold change (Heat/Control)
Body weight (g)‡	40.2 ± 0.80	39.0 ± 0.48	0.97 ± 0.01	36.2 ± 1.06	35.7 ± 1.16	0.99 ± 0.03
Absolute muscle wet weight (mg)‡	7.49 ± 0.26	8.43 ± 0.18*	1.13 ± 0.02	8.96 ± 0.44	8.72 ± 0.33	0.97 ± 0.04§
Absolute muscle dry weight (mg)	1.85 ± 0.05	2.17 ± 0.05*	1.17 ± 0.02	2.36 ± 0.13*	2.23 ± 0.09	0.95 ± 0.04§
Absolute muscle protein content (mg)‡	1.18 ± 0.02	1.32 ± 0.02*	1.12 ± 0.01	1.39 ± 0.07	1.36 ± 0.05	0.98 ± 0.04§
Relative muscle wet weight (mg $g^{-1} \times 10^{-2}$)	18.7 ± 0.56	21.7 ± 0.51*	1.16 ± 0.03	24.7 ± 0.87*	24.5 ± 0.79†	0.99 ± 0.03§
Relative muscle dry weight (mg $g^{-1} \times 10^{-2}$)	4.60 ± 0.11	5.57 ± 0.12*	1.21 ± 0.03	6.50 ± 0.29*	6.27 ± 0.23	0.96 ± 0.03§
Relative muscle protein content (mg $g^{-1} \times 10^{-2}$)	2.95 ± 0.06	3.40 ± 0.05*	1.15 ± 0.02	3.84 ± 0.15*	3.84 ± 0.15†	1.00 ± 0.04§

$HSF1^{+/+}$: wild-type mice; $HSF1^{-/-}$: heat shock transcription factor 1 (HSF1)-null mice; Control: untreated age-matched control group; Heat: heat-stressed group; relative muscle mass: soleus muscle mass relative to body weight. Values are means ± SEM. n = 9–11 in each group.

*,†Significant different from Control and Heat in $HSF1^{+/+}$, respectively, $P < 0.05$.

‡Significant main effect of mice, $P < 0.05$.

§Significant different from $HSF1^{+/+}$, $P < 0.05$.

expression of HSF1, HSF2 and HSF4 in soleus muscle following heat stress are shown in Fig. 1. HSF1 mRNA was not detected in HSF1-null mice, and the basal expression level of HSF2 and HSF4 in HSF1-null mice was ~50% and ~85% of wild-type mice. Although no significant effect of heat stress on HSF1, HSF2 and HSF4 mRNA expression was observed in wild-type and HSF1-null mice, a significant main effect of mice on the mRNA expression of HSF2 was observed ($P < 0.05$). The expression level of HSF2 mRNA in HSF1-null mice was lower than that in wild-type mice during the experimental period ($P < 0.05$).

Figure 2 shows the changes in mRNA expression of HSP25, HSC70, HSP72 and HSP110 in soleus muscle in response to heat stress. The basal mRNA expression level of HSP25, HSC70, HSP72 and HSP110 in HSF1-null mice was lower than that in wild-type mice. The expression level of HSP25 and HSC70 mRNA in HSF1-null mice was also significantly lower than that in wild-type mice at Day 7 ($P < 0.05$). Heat stress-associated decrease in mRNA expression of HSC70 in wild-type mice was observed at Day 1 and Day 3, compared with the level at Pre ($P < 0.05$). On the other hand, a significant increase in HSP72 mRNA in wild-type mice was observed at Day 7 ($P < 0.05$). There was a significant difference in the expression of HSP72 mRNA between wild-type and HSF1-null mice at Day 7 ($P < 0.05$). A significant main effect of mice on the mRNA expression of HSP110 was observed ($P < 0.05$). Lower expression level of HSP110 mRNA in HSF1-null mice was observed, compared with wild-type mice during the experimental period ($P < 0.05$).

The protein expression level of HSP25, HSC70, HSP72 and HSP110 in both wild-type and HSF1-null mice was shown in Fig. 3. The basal protein expression level of HSP25, HSC70, HSP72 and HSP110 in HSF1-null mice was lower than that in wild-type mice. There was a significant main effect of mice on the protein expression of HSP25 and HSC70 in soleus muscle ($P < 0.05$). The protein expression level of HSP25 and HSC70 in HSF1-null mice was significantly less than that in wild-type mice during experimental period ($P < 0.05$). There was also a significant main effect of time on the protein expression of HSP25 ($P < 0.05$). Significant increase in HSP25 protein was observed at Day 3 in wild-type mice ($P < 0.05$, vs. Day 1), and Day 7 in wild-type ($P < 0.05$, vs. Pre and Day 1) and HSF1-null mice ($P < 0.05$, vs. Day 1). On the other hand, a significant increase in HSP72 in wild-type mice was

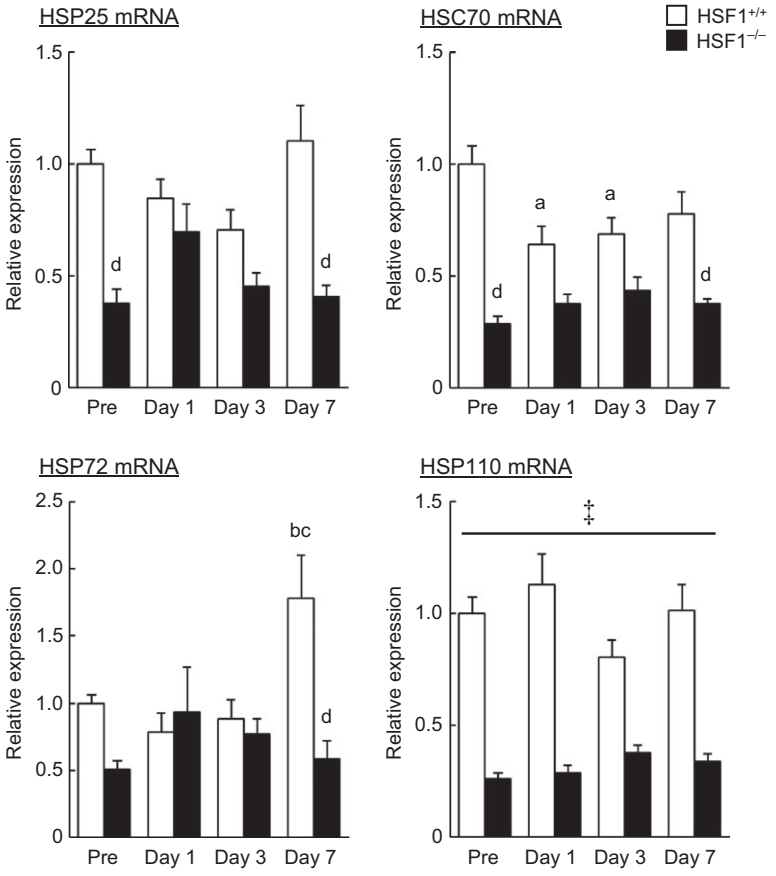


Figure 2 Expressions of heat shock protein (HSP) mRNAs in response to heat stress. HSC70: heat shock cognate 70. (a, b, c and d): Significant different from Pre, Day 1, Day 3 in each type of mice, and the time-matched HSF1$^{+/+}$, respectively, $P < 0.05$. See Fig. 1 for other abbreviations, statistics and symbols. The mRNA levels of HSPs are shown as relative to the mRNA level of GAPDH. Values are expressed relative to the value at Pre in HSF1$^{+/+}$ (1.0). Values are means ± SEM. n = 5–6 per group at each day.

observed at Day 1 ($P < 0.05$) and the level returned towards the initial level (Pre) at Day 7. The higher protein expression level of HSP72 in wild-type mice was observed, compared with HSF1-null mice at Day 1 and Day 3 ($P < 0.05$). Changes in the protein expression level of HSP110 after heat stress were similar to those of HSP72. In wild-type mice, heat stress significantly upregulated HSP110 protein at Day 1 ($P < 0.05$). At Day 1 and Day 3, the expression level of HSP110 in HSF1-null mice was significantly less than that in wild-type mice ($P < 0.05$). Impact of heat stress on overall changes in the protein expression of HSP72 and HSP110 in HSF1-null mice was smaller than that in wild-type mice.

Effects of heat stress on Pax7-positive nuclei

Figure 4 shows the changes in the population of Pax7-positive nuclei relative to total muscle nuclei in response to heat stress. There was no significant difference in the relative population of Pax7-positive nuclei of soleus muscle between wild-type and HSF1-null mice at Pre. In wild-type mice, the relative population of Pax7-positive nuclei of soleus muscle in wild-type mice gradually increased until Day 3 (8% at Day 1 and 24% at Day 3). A significant increase in the population of Pax7-positive nuclei in wild-type mice was observed at Day 3, compared with Pre ($P < 0.05$). On the other hand, there was no significant change in the population of Pax7-positive nuclei in HSF1-null mice. The population of Pax7-positive nuclei in HSF1-null mice was significantly less than that in wild-type mice after heat stress (Day 1 and Day 3; $P < 0.05$).

Effects of heat stress on Akt and p70S6K

Figure 5 shows the representative protein level of p-Akt, t-Akt, p-p70S6K and t-p70S6K in soleus muscle of both wild-type and HSF1-null mice. Relative expression level of p-Akt and p-p70S6K in HSF1-null mice was higher than that in wild-type mice. In wild-type mice, the application of heat stress significantly upregulated the relative expression level of p-Akt at Day 3 and Day 7 ($P < 0.05$). However, a significant decrease in the relative expression level of p-Akt in HSF1-null mice was observed at Day 1. At Day 7, the p-Akt level in HSF1-null mice was significantly less than that in wild-type mice ($P < 0.05$). Upregulation of the relative expression level of p-p70S6k in wild-type mice was observed at Day 7 ($P < 0.05$). The relative expression level of p-p70S6K in wild-type mice

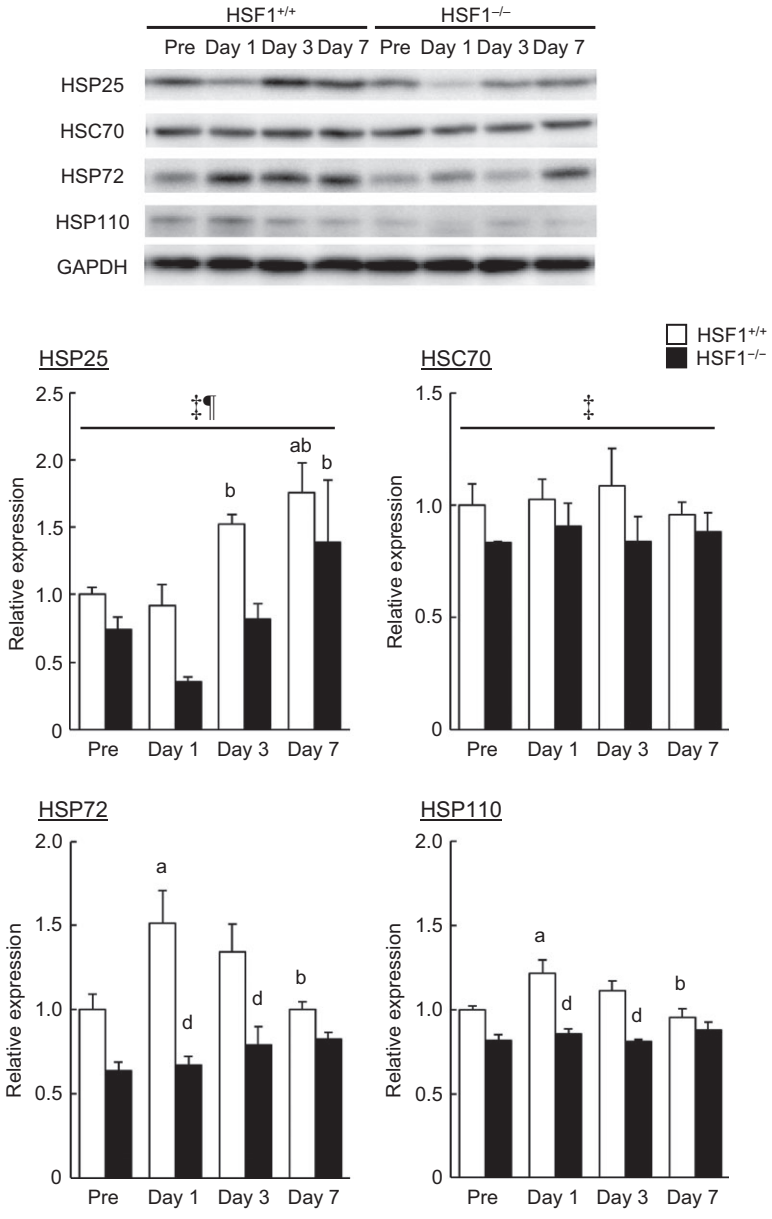


Figure 3 Representative expression patterns and mean expression levels of heat shock protein (HSP) 25, heat shock cognate (HSC) 70, HSP72 and HSP110 in response to heat stress. ¶: Significant main effect of time (Pre vs. Day 7, Day 1 vs. Day 3, and Day 1 vs. Day 7), $P < 0.05$. See Figs 1 and 2 for other abbreviations, statistics and symbols. Values are expressed relative to the value at Pre in HSF1$^{+/+}$ (1.0). Values are means ± SEM. n = 5–6 per group at each day.

was significantly higher than that in HSF1-null mice at Day 7 ($P < 0.05$).

Discussion

The present study demonstrated that the application of heat stress caused to increase in soleus muscle mass in wild-type mice, but not in HSF1-null mice. Heat stress had no effect on the mRNA expression levels of HSFs (HSF1, HSF2 and HSF4) regardless of the genotype. On the other hand, the expression of HSPs (mRNA of HSP72, and protein of HSP72 and HSP110) was upregulated by heat stress in wild-type mice, but not in HSF1-null mice. The population of Pax7-positive nuclei and the phosphorylation level of Akt and p70S6K in wild-type mice were increased by heat stress, but not in HSF1-null mice.

HSF1 gene and heat stress-associated increase in skeletal muscle mass

In the present study, soleus muscle mass was increased 7 days after the application of heat stress in wild-type mice. This result is consistent with the results from rats (Uehara *et al.* 2004, Kobayashi *et al.* 2005, Ohno *et al.* 2010) and mice (Ohno *et al.* 2012). However, in HSF1-null mice, heat stress had no effect on soleus muscle mass. Previous studies showed that the absence of HSF1 gene attenuated loading-associated muscle regrowth of adult soleus muscle (Yasuhara *et al.*

2011, Koya *et al.* 2013). Therefore, HSF1 gene plays a role in heat stress-associated regrowth of skeletal muscle.

HSF1 gene and heat stress-associated upregulations of HSPs

In the present study, the absence of HSF1 gene depressed heat stress-associated upregulation of HSP72 protein. This is the first report showing the effects of heat stress on the expression level of HSP72 in skeletal muscle of HSF1-null mice. It has been also reported that the application of heat stress on skeletal muscle as well as C2C12 myotubes upregulates the expression of HSPs, such as HSP72 (Uehara *et al.* 2004, Kobayashi *et al.* 2005, Ohno *et al.* 2010, 2011, 2012). Therefore, heat stress-associated upregulation of HSP72 in skeletal muscle may be attributed to HSF1-mediated stress response. On the other hand, the upregulation of HSP72 in soleus muscles of HSF1-null mice was also induced by loading (Yasuhara *et al.* 2011, Koya *et al.* 2013). Loading-associated upregulation of HSPs in skeletal muscle may be attributed to unknown mechanism(s).

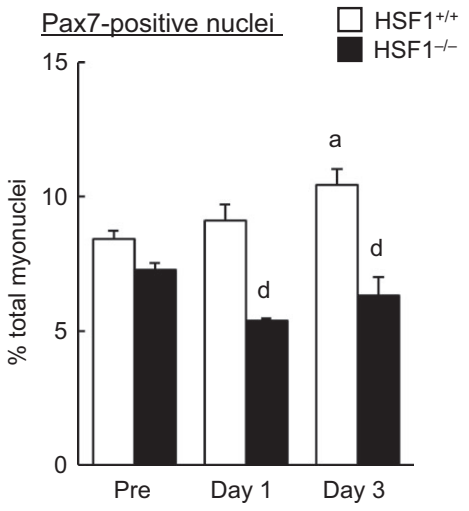


Figure 4 Relative population of Pax7-positive nuclei to total muscle nuclei in response to heat stress. See Figs 1 and 2 for other abbreviations, statistics and symbols. Values are means ± SEM. n = 5–6 per group at each day.

In the present study, heat stress-associated upregulation of HSP110 protein in soleus muscle was observed in wild-type mice, but not in HSF1-null mice. There are no reports about the expression level and a physiological role of HSP110 in skeletal muscle. Although HSP110 has a role of molecular chaperones like HSP70 and acts in synergy with HSP70 in *E. coli* DH5α cells (Mattoo *et al.* 2013), a physiological role of HSP110 protein in skeletal muscle remains unclear. Additional experiments should be needed to reveal this issue.

In the present study, the responses of protein expression level of HSPs following heat stress were inconsistent with those in mRNA expression level of HSPs. This result is consistent with the previous observations that showed the changes in mRNA and

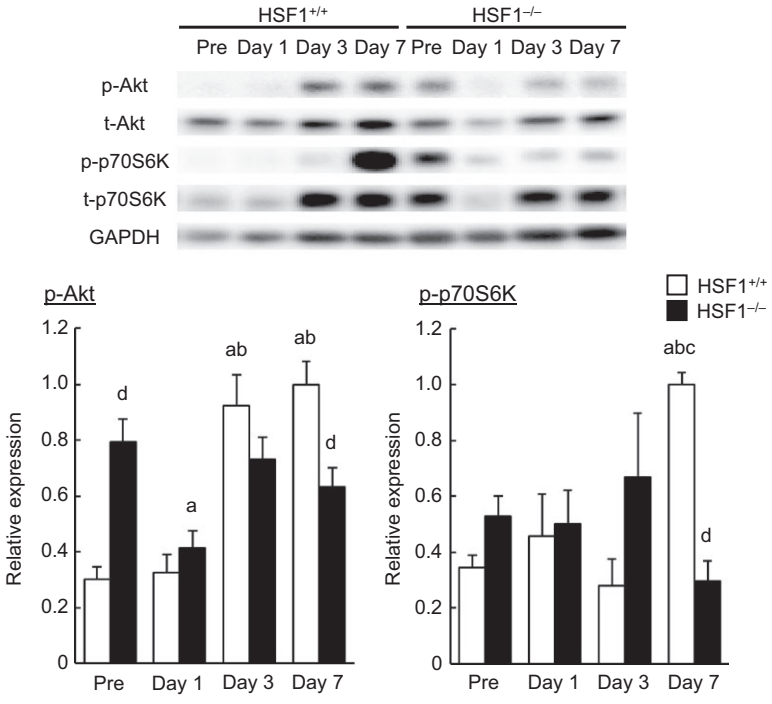


Figure 5 Representative expression patterns and mean expressions of phosphorylated Akt/total Akt and phosphorylated p70 S6 kinase (p70S6K)/total p70S6K in response to heat stress. p-Akt: phosphorylated Akt; t-Akt: total Akt, p-p70S6K: phosphorylated p70S6K; t-p70S6K: total p70S6K. See Figs 1 and 2 for other abbreviations, statistics and symbols. Values are expressed as relative to the value at Day 7 in HSF1$^{+/+}$ (1.0). Values are means ± SEM. n = 5–6 per group at each day.

protein expressions of HSPs in skeletal muscle by mechanical loading (Liu *et al.* 2004, Ogata *et al.* 2009, Yasuhara *et al.* 2011, Koya *et al.* 2013). Although the protein expression level of HSPs in skeletal muscle may be modulated by post-translational regulation (Mizzen & Welch 1988, Petersen & Lindquist 1989, Liu *et al.* 2004, 2006, Ogata *et al.* 2009), we have no clear explanation for regulatory mechanisms of HSPs in skeletal muscle following heat stress at present.

Possible mechanism(s) of HSF1 gene on heat stress-associated increase in skeletal muscle mass

In the present study, there was no significant difference in the basal population level of Pax7-positive satellite cells between wild-type and HSF1-null mice. This result is consistent with our previous studies (Koya *et al.* 2013, Nishizawa *et al.* 2013). Furthermore, heat stress-associated increase in the population of Pax7-positive satellite cells was observed at Day 3 in wild-type mice, but not in HSF1-null mice. Even though it has been reported that heat stress enhanced the proliferation of Pax7-positive nuclei of soleus muscle in wild-type rats (Kojima *et al.* 2007, Ohno *et al.* 2010), this is the first report showing the effects of heat stress on muscle satellite cells in HSF1-null mice. Molecular mechanism of heat stress-associated as well as loading-associated increase in muscle satellite cells remains unclear. Previous study reported that the overexpression of HSP70 prevented the decrease in muscle satellite cells due to the decrease in mechanical loading (Miyabara *et al.* 2012). In addition, overloading-associated increase in Pax7-positive satellite cells was inhibited in HSF1-null mice (Koya *et al.* 2013). Therefore, HSF1 and/or HSF1-mediated stress response may play a role in the increase of muscle satellite cells via unknown mechanism(s). Although there are arguments regarding a role of muscle satellite cells in skeletal muscle hypertrophy (Hawke & Garry 2001, McCarthy *et al.* 2011, Jackson *et al.* 2012), HSF1 and/or HSF1-mediated stress response might contribute to heat stress-associated increase in skeletal muscle mass, namely hypertrophy, via the activation of muscle satellite cells.

The present study also demonstrated that a significant increase in phosphorylated form of Akt and p70S6K in soleus muscle following heat stress in wild-type mice. Heat stress-associated activation of Akt/p70S6K was also observed in rat soleus muscle (Yoshihara *et al.* 2013). On the other hand, the absence of HSF1 inhibited heat stress-induced activation of Akt/p70S6K, suggesting that HSF1 and/or HSF1-mediated stress response might play a role in heat stress-associated activation of Akt/p70S6K pathway. As Akt/p70S6K pathway plays a key role in intracellular signalling of protein synthesis in skeletal muscle (Bodine *et al.* 2001, Latres *et al.* 2005, Sugiura *et al.* 2005, Wang & Proud 2006, Fujita *et al.* 2007), heat stress-associated phosphorylation of Akt and p70S6K may contribute to the increase in skeletal muscle mass via stimulation of protein synthesis.

In the present study, a higher phosphorylation level of Akt of soleus muscle in HSF-null mice was observed, compared with wild-type mice. In addition, the level in HSF1-null mice was transiently decreased by heat stress, but not in wild-type mice. On the other hand, the absence of HSF1 had no effect on the phosphorylated level of p70S6K. We have no clear explanation regarding this reduction at present.

In conclusion, this study demonstrated that HSF1 deficiency suppressed heat stress-associated increase in slow soleus muscle mass in mice. In addition, heat stress-mediated upregulation of HSP72 and HSP110 in mouse soleus muscle was attenuated by the absence of HSF1. Not only heat stress-associated increase in muscle satellite cells but also heat stress-associated activation of Akt/p70S6K pathway was also depressed by HSF1 deficiency. HSF1 and/or HSF1-mediated stress response may be essential for heat stress-associated increase in skeletal muscle mass.

Conflict of interest

The authors have declared that no conflict of interests exists.

Authors thank Dr. L. L. Tang of Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University for his technical assistance. This study was supported, in part, by Grants-in-Aid for Young Scientists (B, 23700647, Y. Ohno), Grants-in-Aid for challenging Exploratory Research (26560372, KG) and Grants-in-Aid for Scientific Research (C, 25350641, Y. Ohno; C, 26350818, T.Y.) from the Japan Society for the Promotion of Science, the Uehara Memorial Foundation (K.G.), the Naito Foundation (K.G.) and Graduate School of Health Sciences, Toyohashi SOZO University (K.G.).

References

Akerfelt, M., Morimoto, R.I. & Sistonen, L. 2010. Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* **11**, 545–555.

Allen, D.L., Yasui, W., Tanaka, T., Ohira, Y., Nagaoka, S., Sekiguchi, C., Hinds, W.E., Roy, R.R. & Edgerton, V.R. 1996. Myonuclear number and myosin heavy chain expression in rat soleus single muscle fibers after spaceflight. *J Appl Physiol* **81**, 145–151.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. & Yancopoulos, G.D. 2001.

Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat Cell Biol* **3**, 1014–1019.

Chen, D., Wang, W., Guo, W., Yu, Q., Burnstock, G., He, C., Xiang, Z. & Zheng, H. 2011. Expression of $P2Y_6$ receptors in the developing mouse skeletal muscle and after injury and repair. *J Anat* **218**, 643–651.

Craig, E.A., Gambill, B.D. & Nelson, R.J. 1993. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol Rev* **57**, 402–414.

Fink, A.L. 1999. Chaperone-mediated protein folding. *Physiol Rev* **79**, 425–449.

Fry, C.S., Lee, J.D., Jackson, J.R., Kirby, T.J., Stasko, S.A., Liu, H., Dupont-Versteegden, E.E., McCarthy, J.J. & Peterson, C.A. 2014. Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy. *FASEB J* **28**, 1654–1665.

Fujimoto, M., Izu, H., Seki, K., Fukuda, K., Nishida, T., Yamada, S., Kato, K., Yonemura, S., Inouye, S. & Nakai, A. 2004. HSF4 is required for normal cell growth and differentiation during mouse lens development. *EMBO J* **23**, 4297–4306.

Fujita, S., Abe, T., Drummond, M.J., Cadenas, J.G., Dreyer, H.C., Sato, Y., Volpi, E. & Rasmussen, B.B. 2007. Blood flow restriction during low-intensity resistance exercise increases S6K1 phosphorylation and muscle protein synthesis. *J Appl Physiol (1985)*, **103**, 903–910.

Goldspink, G. 1999. Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload. *J Anat* **194**, 323–334.

Goto, K., Okuyama, R., Sugiyama, H., Honda, M., Kobayashi, T., Uehara, K., Akema, T., Sugiura, T., Yamada, S., Ohira, Y. & Yoshioka, T. 2003. Effects of heat stress and mechanical stretch on protein expression in cultured skeletal muscle cells. *Pflugers Arch* **447**, 247–253.

Goto, K., Oda, H., Morioka, S., Naito, T., Akema, T., Kato, H., Fujiya, H., Nakajima, Y., Sugiura, T., Ohira, Y. & Yoshioka, T. 2007. Skeletal muscle hypertrophy induced by low-intensity exercise with heat-stress in healthy human subjects. *Jpn J Aerospace Environ Med* **44**, 13–18.

Goto, K., Oda, H., Kondo, H., Igaki, M., Suzuki, A., Tsuchiya, S., Murase, T., Hase, T., Fujiya, H., Matsumoto, I., Naito, H., Sugiura, T., Ohira, Y. & Yoshioka, T. 2011. Responses of muscle mass, strength and gene transcripts to long-term heat stress in healthy human subjects. *Eur J Appl Physiol* **111**, 17–27.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. *Nature* **381**, 571–579.

Hashimoto-Torii, K., Torii, M., Fujimoto, M., Nakai, A., El Fatimy, R., Mezger, V., Ju, M.J., Ishii, S., Chao, S.H., Brennand, K.J., Gage, F.H. & Rakic, P. 2014. Roles of heat shock factor 1 in neuronal response to fetal environmental risks and its relevance to brain disorders. *Neuron* **82**, 560–572.

Hawke, T.J. & Garry, D.J. 2001. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* **91**, 534–551.

Hayes, D., Napoli, V., Mazurkie, A., Stafford, W.F. & Graceffa, P. 2009. Phosphorylation dependence of hsp27 multimeric size and molecular chaperon function. *J Biol Chem* **284**, 18801–18807.

Huey, K.A., McCall, G.E., Zhong, H. & Roy, R.R. 2007. Modulation of HSP25 and TNF-alpha during the early stages of functional overload of a rat slow and fast muscle. *J Appl Physiol* **102**, 2307–2314.

Inouye, S., Izu, H., Takaki, E., Suzuki, H., Shirai, M., Yokota, Y., Ichikawa, H., Fujimoto, M. & Nakai, A. 2004. Impaired IgG production in mice deficient for heat shock transcription factor 1. *J Biol Chem* **279**, 38701–38709.

Jackson, J.R., Mula, J., Kirby, T.J., Fry, C.S., Lee, J.D., Ubele, M.F., Campbell, K.S., McCarthy, J.J., Peterson, C.A. & Dupont-Versteegden, E.E. 2012. Satellite cell depletion does not inhibit adult skeletal muscle regrowth following unloading-induced atrophy. *Am J Physiol Cell Physiol* **303**, C854–C861.

Kawano, F., Ishihara, A., Stevens, J.L., Wang, X.D., Ohshima, S., Horisaka, M., Maeda, Y., Nonaka, I. & Ohira, Y. 2004. Tension- and afferent input-associated responses of neuromuscular system of rats to hindlimb unloading and/or tenotomy. *Am J Physiol Regul Integr Comp Physiol* **287**, R76–R86.

Kobayashi, T., Goto, K., Kojima, A., Akema, T., Uehara, K., Aoki, H., Sugiura, T., Ohira, Y. & Yoshioka, T. 2005. Possible role of calcineurin in heating-related increase of rat muscle mass. *Biochem Biophys Res Commun* **331**, 1301–1309.

Kojima, A., Goto, K., Morioka, S., Naito, T., Akema, T., Fujiya, H., Sugiura, T., Ohira, Y., Beppu, M., Aoki, H. & Yoshioka, T. 2007. Heat stress facilitates the regeneration of injured skeletal muscle in rats. *J Orthop Sci* **12**, 74–82.

Koya, T., Nishizawa, S., Ohno, Y., Goto, A., Ikuta, A., Suzuki, M., Ohira, T., Egawa, T., Nakai, A., Sugiura, T., Ohira, Y., Yoshioka, T., Beppu, M. & Goto, K. 2013. Heat shock transcription factor 1-deficiency attenuates overloading-associated hypertrophy of mouse soleus muscle. *PLoS ONE* **8**, e77788.

Latchman, D.S. 2001. Heat shock proteins and cardiac protection. *Cardiovasc Res* **51**, 637–646.

Latres, E., Amini, A.R., Amini, A.A., Griffiths, J., Martin, F.J., Wei, Y., Lin, H.C., Yancopoulos, G.D. & Glass, D.J. 2005. Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. *J Biol Chem* **280**, 2737–2744.

Liu, Y., Lormes, W., Wang, L., Reissnecker, S. & Steinacker, J.M. 2004. Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. *Eur J Appl Physiol* **91**, 330–335.

Liu, Y., Gampert, L., Nething, K. & Steinacker, J.M. 2006. Response and function of skeletal muscle heat shock protein 70. *Front Biosci* **11**, 2802–2827.

Matsuba, Y., Goto, K., Morioka, S., Naito, T., Akema, T., Hashimoto, N., Sugiura, T., Ohira, Y., Beppu, M. & Yoshioka, T. 2009. Gravitational unloading inhibits the regenerative potential of atrophied soleus muscle in mice. *Acta Physiol (Oxf)* **196**, 329–339.

Mattoo, R.U., Sharma, S.K., Priya, S., Finka, A. & Goloubinoff, P. 2013. Hsp110 is a bona fide chaperone using ATP to unfold stable misfolded polypeptides and reciprocally collaborate with Hsp70 to solubilize protein aggregates. *J Biol Chem* **288**, 21399–21411.

McArdle, A., Broome, C.S., Kayani, A.C., Tully, M.D., Close, G.L., Vasilaki, A. & Jackson, M.J. 2006. HSF expression in skeletal muscle during myogenesis: implications for failed regeneration in old mice. *Exp Gerontol* **41**, 497–500.

McCarthy, J.J., Mula, J., Miyazaki, M., Erfani, R., Garrison, K., Farooqui, A.B., Srikuea, R., Lawson, B.A., Grimes, B., Keller, C., Van Zant, G., Campbell, K.S., Esser, K.A., Dupont-Versteegden, E.E. & Peterson, C.A. 2011. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* **138**, 3657–3666.

Miyabara, E.H., Nascimento, T.L., Rodrigues, D.C., Moriscot, A.S., Davila, W.F., AitMou, Y., deTombe, P.P. & Mestril, R. 2012. Overexpression of inducible 70-kDa heat shock protein in mouse improves structural and functional recovery of skeletal muscles from atrophy. *Pflugers Arch* **463**, 733–741.

Mizzen, L.A. & Welch, W.J. 1988. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J Cell Biol* **106**, 1105–1116.

Morimoto, R.I. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Gen Dev* **12**, 3788–3796.

Morioka, S., Goto, K., Kojima, A., Naito, T., Matsuba, Y., Akema, T., Fujiya, H., Sugiura, T., Ohira, Y., Beppu, M., Aoki, H. & Yoshioka, T. 2008. Functional overloading facilitates the regeneration of injured soleus muscles in mice. *J Physiol Sci* **58**, 397–404.

Naito, H., Powers, S.K., Demirel, H.A., Sugiura, T., Dodd, S.L. & Aoki, J. 2000. Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J Appl Physiol* **88**, 359–363.

Neal, A., Boldrin, L. & Morgan, J.E. 2012. The satellite cell in male and female, developing and adult mouse muscle: distinct stem cells for growth and regeneration. *PLoS ONE* **7**, e37950.

Nishizawa, S., Koya, T., Ohno, Y., Goto, A., Ikuita, A., Suzuki, M., Ohira, T., Egawa, T., Nakai, A., Sugiura, T., Ohira, Y., Yoshioka, T., Beppu, M. & Goto, K. 2013. Regeneration of injured skeletal muscle in heat shock transcription factor 1-null mice. *Physiol Rep* **1**, e00071.

Ogata, T., Oishi, Y., Higashida, K., Higuchi, M. & Muraoka, I. 2009. Prolonged exercise training induces long-term enhancement of HSP70 expression in rat plantaris muscle. *Am J Physiol Regul Integr Comp Physiol* **296**, R1557–R1563.

Ohno, Y., Yamada, S., Sugiura, T., Ohira, Y., Yoshioka, T. & Goto, K. 2010. A possible role of NF- kappaB and HSP72 in skeletal muscle hypertrophy induced by heat stress in rats. *Gen Physiol Biophys* **29**, 234–242.

Ohno, Y., Yamada, S., Sugiura, T., Ohira, Y., Yoshioka, T. & Goto, K. 2011. Possible role of NF-κB signals in heat stress-associated increase in protein content of cultured C2C12 cells. *Cells Tissues Organs* **194**, 363–370.

Ohno, Y., Yamada, S., Goto, A., Ikuta, A., Sugiura, T., Ohira, Y., Yoshioka, T. & Goto, K. 2012. Effects of heat stress on muscle mass and the expression levels of heat shock proteins and lysosomal cathepsin L in soleus muscle of young and aged mice. *Mol Cell Biochem* **369**, 45–53.

Oishi, Y., Ogata, T., Ohira, Y., Taniguchi, K. & Roy, R.R. 2005. Calcineurin and heat shock protein 72 in functionally overloaded rat plantaris muscle. *Biochem Biophys Res Commun* **330**, 706–713.

Oishi, Y., Hayashida, M., Tsukiashi, S., Taniguchi, K., Kami, K., Roy, R.R. & Ohira, Y. 2009. Heat stress increases myonuclear number and fiber size via satellite cell activation in rat regenerating soleus fibers. *J Appl Physiol* **107**, 1612–1621.

O'Neill, D.E., Aubrey, F.K., Zeldin, D.A., Michel, R.N. & Noble, E.G. 2006. Slower skeletal muscle phenotypes are critical for constitutive expression of Hsp70 in overloaded rat plantaris muscle. *J Appl Physiol* **100**, 981–987.

Petersen, R.B. & Lindquist, S. 1989. Regulation of HSP70 synthesis by messenger RNA degradation. *Cell Regul* **1**, 135–149.

Rowe, R.W. & Goldspink, G. 1969. Muscle fibre growth in five different muscles in both sexes of mice. *J Anat* **104**, 519–530.

Sakurai, H. & Enoki, Y. 2010. Novel aspects of heat shock factors: DNA recognition, chromatin modulation and gene expression. *FEBS J* **277**, 4140–4149.

Santoro, M.G. 2000. Heat shock factors and the control of the stress response. *Biochem Pharmacol* **59**, 55–63.

Sarge, K.D., Murphy, S.P. & Morimoto, R.I. 1993. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Mol Cell Biol* **13**, 1392–1407.

Sasai, N., Agata, N., Inoue-Miyazu, M., Kawakami, K., Kobayashi, K., Sokabe, M. & Hayakawa, K. 2010. Involvement of PI3K/Akt/TOR pathway in stretch-induced hypertrophy of myotubes. *Muscle Nerve* **41**, 100–106.

Seale, P., Sabourin, L.A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. & Rudnicki, M.A. 2000. Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777–786.

Selsby, J.T. & Dodd, S.L. 2005. Heat treatment reduces oxidative stress and protects muscle mass during immobilization. *Am J Physiol Regul Integr Comp Physiol* **289**, R134–R139.

Sugiura, T., Abe, N., Nagano, M., Goto, K., Sakuma, K., Naito, H., Yoshioka, T. & Powers, S.K. 2005. Changes in PKB/Akt and calcineurin signaling during recovery in atrophied soleus muscle induced by unloading. *Am J Physiol Regul Integr Comp Physiol* **288**, R1273–R1278.

Uehara, K., Goto, K., Kobayashi, T., Kojima, A., Akema, T., Sugiura, T., Yamada, S., Ohira, Y., Yoshioka, T. & Aoki, H. 2004. Heat-stress enhances proliferative potential in rat soleus muscle. *Jpn J Physiol* **54**, 263–271.

Wang, X. & Proud, C.G. 2006. The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* **21**, 362–369.

Welch, W.J. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* **72**, 1063–1081.

Xiao, X., Zuo, X., Davis, A.A., McMillan, D.R., Curry, B.B., Richardson, J.A. & Benjamin, I.J. 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO J* **18**, 5943–5952.

Yasuhara, K., Ohno, Y., Kojima, A., Uehara, K., Beppu, M., Sugiura, T., Fujimoto, M., Nakai, A., Ohira, Y., Yoshioka, T. & Goto, K. 2011. Absence of heat shock transcription factor 1 retards the regrowth of atrophied soleus muscle in mice. *J Appl Physiol* **111**, 1142–1149.

Yoshihara, T., Naito, H., Kakigi, R., Ichinoseki-Sekine, N., Ogura, Y., Sugiura, T. & Katamoto, S. 2013. Heat stress activates the Akt/mTOR signalling pathway in rat skeletal muscle. *Acta Physiol (Oxf)* **207**, 416–426.

Zhang, Y., Huang, L., Zhang, J., Moskophidis, D. & Mivechi, N.F. 2002. Targeted disruption of hsf1 leads to lack of thermotolerance and defines tissue-specific regulation for stress-inducible Hsp molecular chaperones. *J Cell Biochem* **86**, 376–393.

