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Nox4 in Sport-induzierter Angiogenese

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INHALTSVERZEICHNIS

1 ZUSAMMENFASSUNG

Bewegung und sportliche Aktivität fördern die Gesundheit des Organismus und senken das Risiko chronischer Krankheiten. Sie bewirken dabei eine Vielzahl von physiologischen und biochemischen Veränderungen in der Skelettmuskulatur, insbesondere Muskelfasertyp-Transformation, Änderungen des Muskelmetabolismus und der Angiogenese. Unter basalen Bedingungen spielen *reactive oxygen species* (ROS) eine essentielle Rolle für die normale Muskelfunktion. Die Sport-induzierte Produktion von ROS erweist sich als wichtige physiologische Funktion für die Regulierung der Muskelkraft und der Anpassungsreaktion der Muskelfasern auf das Training. Eine der wichtigsten Quellen von ROS im kardiovaskulären System sowie in der Skelettmuskulatur ist die Familie der NADPH-Oxidasen (Nox). Im Unterschied zu anderen NADPH-Oxidasen ist Nox4 konstitutiv aktiv und produziert Wasserstoffperoxid (H_2O_2), welches in diversen zellulären Signalkaskaden involviert ist. Gleichzeitig gibt es zahlreiche Hinweise, dass Nox4 über die ROS-Produktion an Sport-induzierten Anpassungsprozessen in Skelettmuskeln beteiligt ist. Vor diesem Hintergrund wurde die Hypothese aufgestellt, dass Nox4 die Sport-induzierte Transformation von langsam- zu schnellkontrahierenden Muskelfasern, die Änderungen des Muskelstoffwechsels sowie die Sport-induzierte und die retinale Angiogenese beeinflusst. Die Untersuchung der Sport-induzierten Fasertyptransformation zeigte, dass die relative Zusammensetzung der Muskelfasern in Nox4-*Knockout*- und Wildtyp-Mäusen sehr ähnlich und somit von Nox4 unabhängig war. Obwohl das Training die Expression von PGC1α und GLUT4 sowie die AMPK-Aktivierung steigerte, hatte Nox4 nur eine geringe, nicht konstitutive Auswirkung auf den Muskelmetabolismus. Außerdem zeigte die vorliegende Studie, dass Nox4 die Sport-induzierte Angiogenese fördert. Nox4 führte zu einer erhöhten *Stretch*- und Hypoxie-induzierten Expression von VEGF in Myoblasten, die aus C2C12-Zellen und Satellitenzellen differenziert wurden. Als Folge des Nox4-*Knockout*s wurde nicht nur eine Reduktion der VEGF-Expression, sondern auch eine Steigerung der Expression von Angiopoietin 1 (Ang1) nachgewiesen, welches die Sport-induzierte Angiogenese hemmte. Das Fehlen von Nox4 schützte außerdem vor der retinalen Neoangiogenese und trug zu einer schnelleren Heilung nach der *Oxygen-induced retinopathy* (OIR) bei, indem das Netzwerk neuer Gefäße mittels Ang1 stabilisiert wurde. Somit führt Nox4 zur Sport- und Hypoxie-induzierten Angiogenese durch einen Doppelmechanismus der Induktion und Aufrechterhaltung der VEGF Expression und der Hemmung von Ang1.

2 SUMMARY

Exercise and physical activity promote fitness and health of the organism. They initiate multiple physiological and biochemical events in skeletal muscles, particularly fiber type transformation, changes in muscle metabolism and angiogenesis. Under basal conditions, the reactive oxygen species (ROS) play an essential role for the normal muscle function. Contraction-induced ROS generation has an important physiological function for the regulation of muscle force and the adaptive response of the muscle fibers to exercise training. One of the main sources of ROS in the cardiovascular system and in skeletal muscle is the family of NADPH oxidases (Nox). Different to other NADPH oxidases, Nox4 is constitutive active and produces hydrogen peroxide (H_2O_2), which is involved in diverse cellular signaling cascades. Simultaneously, there are many indications that Nox4 participates via ROS generation in exercise-induced adjustment processes in skeletal muscles. Against this background, it was hypothesized that Nox4 contributes to exercise-induced slow- to fast-twitch muscle fiber transformation and changes in muscle metabolism as well as exercise-induced and retinal angiogenesis. Analysis of exercise-induced muscle fiber switch showed that the relative composition of muscle fibers in Nox4 knockout and wild type mice were unaltered and thus independent from Nox4. Although exercise increased the expression of GLUT4 or PGC1α and AMPK activation, Nox4 had only minor, not constitutive effects on the muscle metabolism. Furthermore, this study showed that Nox4 promotes exercise-induced angiogenesis. Nox4 led to increased stretch- and hypoxia-induced VEGF expression in myoblasts, which were differentiated from C2C12 and satellite cells. As a consequence of Nox4 knockout, not only the reduction of VEGF-expression was observed, but also the increase in the expression of angiopoietin 1 (Ang1), which inhibited exercise-induced angiogenesis. Moreover, deletion of Nox4 protected from retinal neo-angiogenesis and promoted healing after oxygen induced retinopathy (OIR) by stabilizing the network of regrown vessels via Ang1. Thus, Nox4 contributes to exercise- and hypoxia-induced angiogenesis through a dual mechanism of induction and maintenance of VEGF and prevention of Ang1 expression.

3 ABKÜRZUNGSVERZEICHNIS

AMPK	AMP aktivierte Proteinkinase
Ang	Angiopoietin
DPI	Diphenyleneiodonium
DR	Diabetische Retinopathie
GLUT4	Glukosetransporter 4
H_2O_2	Wasserstoffperoxid
Hif1α	*Hypoxia-inducible factor 1-alpha*
MEF2	*Myocyte enhancer factor 2*
MHC	Skelettmuskel-Myosin schwere Kette
mRNA	*Messenger* RNA
NADPH	Nicotinamidadenindinukleotidphosphat
NFAT	*Nuclear factor of activated T-cells*
Nox	NADPH Oxidase
OIR	*Oxygen-induced retinopathy*
PGC1α	*Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*
ROS	*Reactive oxygen species* bzw. reaktive Sauerstoffspezies
VEGF	*Vascular Endothelial Growth Factor*

4 ÜBERGREIFENDE ZUSAMMENFASSUNG

4.1 Einleitung

Sport führt zu einer Verbesserung der körperlichen Leistung, was viele gesundheitliche Vorteile, vor allem für das kardiovaskuläre System mit sich bringt[1]. Eine regelmäßige körperliche Belastung erhöht das maximale Herzzeitvolumen, führt zu einer höheren Kapillardichte und zur Anpassung des aeroben Metabolismus von Muskeln, was wiederum die Sauerstoffaufnahme in der Peripherie und die Sauerstoffverwertung verbessert. Sportliche Aktivität ist ein extrem komplexer Stimulus für den Organismus, der durch die Interaktion von vegetativem Nervensystem, motorischer Innervation und Belastung sowie lokalen Faktoren, wie z.B. Hypoxie sowie *reactive oxygen species* (ROS), eine Vielzahl von physiologischen und biochemischen Veränderungen im Muskel einleitet. Dazu gehören insbesondere die Muskelfasertyp-Transformation, die Änderungen des Muskelmetabolismus und die Angiogenese.

Reaktive Sauerstoffspezies (ROS) fungieren als *second messenger*, beeinflussen eine Reihe von Wachstumsfaktoren und sind demzufolge in vielen zellulären Signalkaskaden involviert. Gleichzeitig ist die erhöhte Produktion von ROS die Ursache für oxidativen Stress und wird mit pathologischen Zuständen, wie z. B. vaskulären Erkrankungen, in Verbindung gebracht[2]. ROS werden unter basalen Bedingungen in Skelettmuskeln produziert und sind essentiell für die physiologische Muskelfunktion[3]. Körperliche Belastung führt im Muskel zur Steigerung der Produktion von ROS in Abhängigkeit von der Intensität der Muskelkontraktion[4,5]. Gleichzeitig aktivieren ROS auch Mechanismen, die die Zellen vor oxidativem Stress schützen[6]. So wirken Enzyme wie z. B. die Superoxid Dismutase und die Katalase den potentiell negativen Auswirkungen erhöhter ROS-Produktion entgegen[7,8]. Die Sport-induzierte Produktion von ROS erweist sich auch als wichtige physiologische Funktion für die Regulierung der Muskelkraft und der Anpassungsreaktion der Muskelfasern an das Training[9].

Die Skelettmuskeln sind aus Muskelfasern zusammengesetzt, die anhand ihrer funktionellen, metabolischen sowie kontraktilen Eigenschaften in verschiedene Typen unterteilt werden[10]. Beim Menschen werden zwischen TypI-, TypIIa- und TypIIx-Muskelfasern unterschieden[1], wohingegen bei Nagetieren TypI, TypIIa, TypIIb sowie andere phänotypische und funktionelle Variationen wie z.B. TypIIx/d Fasern definiert werden. Die langsam-kontrahierenden TypI-Fasern haben eine hohe Kapillardichte, eine

hohe oxidative und eine niedrige glykolytische Kapazität. Sie sind ermüdungsresistent und somit für das Ausdauertraining von herausragender Bedeutung. Die schnell-kontrahierenden Muskelfasern vom TypIIb haben dagegen eine niedrige Kapillardichte, aber eine hohe glykolytische und eine niedrige oxidative Kapazität und sind somit für anaerobe Leistung, wie sie z. Bsp. beim Sprinten erbracht werden muss, unentbehrlich. Sie bilden bei Kraftsportlern einen erheblichen Teil der Muskelmasse. Die schnell-kontrahierenden TypIIa-Fasern besetzen intermediäre Positionen. Sie verstoffwechseln Glukose und haben eine hohe oxidative Kapazität[11]. Neben der genetischen Vorbestimmung des Muskelfasermusters beeinflussen die neuromuskuläre Aktivität[12], die mechanische Belastung[13] und die damit verbundene erhöhte ROS-Produktion die Zusammensetzung der Muskelfasern. Auch ein verändertes Hormonprofil[14] sowie das Altern[15] spielen dabei eine wichtige Rolle. Ein intensives Ausdauertraining führt zur Fasertyp-änderung von TypIIb zu TypIIa[16], wohingegen eine eingeschränkte Bewegung den Anteil an TypI-Fasern in der Beinmuskulatur verringert[17]. Muskelbelastung führt zur Aktivierung einer Reihe von Signalkaskaden, die die Genexpression beeinflussen und mit einer Änderung im ATP-Gleichgewicht bzw. -Umsatz in der Zelle sowie mit einer Zunahme an freiem intrazellulären Calcium verbunden sind[18]. ROS beeinflussen wiederum die Calciumfreisetzung aus dem sarcoplasmatischen Retikulum[19,20]. Calmodulin bindet freies Calcium und aktiviert Calcineurin, welches eine selektive NFAT/MEF2-vermittelte Transkription der Gene von langsam-kontrahierenden Fasern einleitet[21]. Die Erhöhung der intrazellulären Ca^{2+}-Konzentration mit Ionophor Ionomycin in kultivierten primären Muskelzellen führt zur Bildung von langsam-kontrahierenden Fasern und erhöht die mitochondriale Aktivität[22]. Studien zeigen, dass die Expression von *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (PGC1α), einem Schlüsselmediator der Mitochondrienbiogenese, durch Sport beeinflusst wird und in die Muskelfaser-Transformation involviert ist[11,23–26]. Die PGC1α-abhängigen Faktoren, wie der Glukosetransporter-4 (GLUT4)[27] sowie der wichtigste Energiesensor der Zelle, die AMP-aktivierte Proteinkinase (AMPK)[28], spielen eine wichtige Rolle im Muskelmetabolismus und werden durch Sport und ROS-Produktion beeinflusst[29–31].

Wie schon erwähnt, unterscheiden sich Muskelfasern in ihrer Kapillardichte, wobei Sport zu einer Fasertyp-abhängigen angiogenen Antwort führt[32]. Als Angiogenese wird die Neubildung der Blutgefäße aus bereits vorhandenen Blutgefäßen bezeichnet. Es ist ein komplexer, multizellulärer Prozess, der von einer Vielzahl lokaler, wie auch organ-

spezifischer Faktoren abhängt[33,34]. Angiogenese wird durch einen entzündungsähnlichen Zustand eingeleitet, der durch eine unzureichende Blutgefäßversorgung und Sauerstoffmangel zur Aktivierung von Endothelzellen und Makrophagen, sowie zur Ausschüttung von Zytokinen und Wachstumsfaktoren und letztlich zur Änderung des Kapillarnetzwerks führt. Die proangiogenen Wachstumsfaktoren wie *Vascular Endothelial Growth Factor* (VEGF) und Semaphorin spielen eine wichtige Rolle bei der Aktivierung einiger weniger naszierender Endothelzellen, welche sich durch eine Reihe weiterer Differenzierungen zu Tip- und Stalkzellen entwickeln und die Bildung des neuen Gefäßes ermöglichen[34]. Die physiologische Angiogenese ist beim Erwachsenen stark eingeschränkt. Sie wird durch eine Vielzahl von pro- und anti-angiogenen Faktoren kontrolliert und tritt vor allem beim Wachstum von weiblichen Reproduktionsorganen[34] oder in Verbindung mit Remodelingprozessen wie Wundheilung[35] und sportlicher Aktivität[33] auf. Zur pathophysiologischen Angiogenese zählen die diabetische Retinopathie und die Retinopathie von Frühgeborenen. Die diabetische Retinopathie (DR) ist die am weitesten verbreitete mikrovaskuläre Komplikation bei Diabetes und die häufigste Ursache für Erblindung bei Erwachsenen. Unabhängig von dem Diabetes Typ ist das Risiko einer Retinopathie sehr hoch[36]. Nicht proliferative DR, die sich durch kleine Gefäßveränderungen und retinale Blutungen auszeichnet, führt zur Unterversorgung und somit zur relativen Ischämie der Netzhautareale. Dies kann wiederum zum unkontrollierten Wachstum neuer Blutgefäße und somit zur Entstehung einer proliferativen Retinopathie führen[37]. Die Hemmung der Angiogenese und die Gefäßstabilisierung ist deshalb das Ziel von vielen Therapieansätzen bei angioproliferativen Netzhauterkrankungen des Menschen[38]. Die pathologische Angiogenese sowie die Entwicklung einer Retinopathie werden mit dem Anstieg der ROS-Produktion in Verbindung gebracht[39–41]. Es gibt viele Hinweise, dass ROS eine wichtige Rolle bei der Angiogenese spielen; der zugrundeliegende molekulare Mechanismus ist jedoch unbekannt.

Die Produktion von ROS betrifft verschiedene Kompartimente der Zelle wie Mitochondrien, sarcoplasmatischen Retikulum, Sarcolemma und Cytosol. Allerdings werden Mitochondrien nicht als die primäre Quelle der ROS-Produktion während der Muskelfaserkontraktion betrachtet[42–44]. In den letzten Jahren hat sich die Familie der NADPH-Oxidasen (Nox) als eine der wichtigsten Quellen von ROS im kardiovaskulären System sowie in der Skelettmuskulatur herauskristallisiert. Eine große Anzahl von Publikationen liefert Beweise dafür, dass die spezifischen Nox-Isoformen über ROS die

endotheliale Zellmigration[45,46] und die Angiogenese[47–49] fördern. Aktuell sind sieben Mitglieder dieser Familie bekannt: Nox1 bis Nox5, DUOX1 und DUOX2. Von großer Bedeutung für die Gefäßneubildung sind Nox1, Nox2 und Nox4, was am Modell der Hinterbein-Ischämie gezeigt wurde [48,49]. Das Nox4-Protein wird in einer Vielzahl von Zellen exprimiert, vor allem in Endothel- und Muskelzellen sowie in Adipozyten und ist eine Ausnahme in der Familie der NADPH-Oxidasen. Im Unterschied zu den anderen Nox-Familienmitgliedern produziert Nox4 kein Superoxid-Anion (O^{2-}), sondern direkt Wasserstoffperoxid (H_2O_2)[50]. H_2O_2 ist die langlebigste ROS, welche in diversen zellulären Signalkaskaden involviert ist[42,51]. Nox4 ist konstitutiv aktiv und wird durch die Expression und den Sauerstoffpartialdruck im Gewebe reguliert. Die ROS-Produktion durch Nox4 kann als eine Reaktion auf den zellulären Sauerstoffgehalt betrachtet werden, wobei Nox4 als ein Sauerstoffsensor fungiert[52]. Die Lokalisation von Nox4 ist auf die intrazelluläre Membrankompartimente begrenzt[53–55]. Das von Nox4-produzierte H_2O_2 reguliert unter anderem im sarcoplasmatischen Retikulum den Ca^{2+}-abhängigen Ryanodin-Rezeptor und ist direkt für die intrazelluläre Ca^{2+}- Konzentration während einer tetanischen Muskelkontraktion verantwortlich[55]. Dies stellt eine mögliche Verbindung zwischen Nox4 und der Muskelfaserdifferenzierung dar. Vor diesem Hintergrund ist die Hypothese, dass Nox4 die Sport-induzierte Transformation von langsam- zu schnell-kontrahierenden Muskelfasern sowie die damit verbundenen Änderungen des Muskelstoffwechsels beeinflusst, logisch. Bis heute ist die Rolle des Nox4-Proteins in der Sport-induzierten sowie retinalen Angiogenese nicht ausreichend untersucht worden, obwohl Nox4 offensichtlich in den adaptiven Signalkaskaden der Angiogenese involviert ist. So stimuliert VEGF die Migration von Endothelzellen durch den Ca^{2+}-Zustrom, der sowohl von Nox4 als auch von H_2O_2 abhängig ist[46,56]. Die Ligatur der Femoralarterie führt zu einer Ischämie, die die Angiogenese in Nox4-defizienten Mäusen verlangsamt[48]. Es wurde außerdem gezeigt, dass Hypoxie als ein starker angiogener Faktor die Expression von Nox4 steigert[57]. Auf dieser Basis beruhte die Hypothese, dass Nox4 eine wichtige Rolle in der Sport-induzierten Angiogenese sowie in der Netzhaut-Neovaskularisierung spielt.

Das Ziel dieser Arbeit ist es, ein besseres Verständnis der komplexen Regulation der Aktivierung und Inhibition des Nox4 Signalweges in Sport-induzierten Prozessen sowie in der Netzhaut-Vaskularisierung zu entwickeln und daraus eine mögliche therapeutische Anwendung abzuleiten.

4.2 Diskussion

Die Untersuchung der Sport-induzierten Fasertyptransformation widerlegte die aufgestellte Hypothese und zeigte, dass die relative Zusammensetzung der Muskelfasern in Nox4-*Knockout*- und Wildtyp-Mäusen sehr ähnlich und somit von Nox4 unabhängig ist. Sportliche Aktivität steigerte zwar die Expression von PGC1α und GLUT4 sowie die AMPK-Aktivierung, jedoch hatte Nox4 nur eine geringe, nicht konstitutive Auswirkung auf den Metabolismus im Skelettmuskel. Die Sport-induzierte Angiogenese wurde aber deutlich durch Nox4 beeinflusst. So wurde die das Wachstum der Kapillaren in Wildtyp- aber nicht in Nox4-*Knockout*-Mäusen beobachtet. Es konnte gezeigt werden, dass Nox4 zu einer erhöhten *Stretch*- und Hypoxie-induzierten Expression von VEGF in Myoblasten führt. Als Folge des Nox4-*Knockout* wurde nicht nur die Reduktion der VEGF-Expression, sondern auch die Steigerung der Expression von Angiopoietin 1 (Ang1) beobachtet, was die Sport-induzierte Angiogenese hemmte. Das Fehlen von Nox4 schützte außerdem vor der retinalen Neoangiogenese und trug zur schnelleren Heilung nach der *Oxygen-induced retinopathy* (OIR) bei, in dem das Netzwerk der nachgewachsenen Gefäße mittels Ang1 stabilisiert wurde. Im Gegensatz dazu haben Nox4 sowie Nox1 und Nox2 keine Auswirkungen auf die physiologische Angiogenese bei der Netzhautentwicklung.

4.2.1 Sport-induzierte Transformation der Muskelfaser ist von Nox4 unabhängig

Sportliches Training auf einem Laufband oder in einem Laufrad ist ein geeignetes, etabliertes Modell für die Untersuchung der Muskelfasertransformation sowie der stimulierten Angiogenese[32,58]. So wurden Nox4-Knockout- und Wildtyp-Mäuse einem akuten zehntägigen sowie siebenwöchigen Lauftraining auf einem Laufband unterzogen. In einem weiteren Versuch wurde den Mäusen durch einen uneingeschränkten Zugang zum Laufrad ein freiwilliger Lauf ermöglicht, wobei die Nox4-*Knockout*-Mäuse und die Wildtypen ähnlich lange Strecken liefen. Dies deutete daraufhin, dass Nox4-*Knockout* zumindest keinen Einfluss auf die Kondition der Mäuse hatte. Mittels ATPase-Färbung konnten unterschiedliche Muskelfasern differenziert werden. Die relative Zahl der langsam-kontrahierenden Fasern in Nox4-*Knockout*-Tieren war leicht höher als in den Kontroll-Tieren. Dies deutete darauf hin, dass der Nox4-*Knockout* unter basalen Bedingungen zu mehr langsam-kontrahierende Fasern im Muskel führt. Weder kurzes noch lan-

ges Laufbandtraining hatte einen Einfluss auf die Verteilung der verschiedenen Muskelfasern. Im Gegensatz dazu erhöhte das freiwillige Training die relative Anzahl von langsam-kontrahierenden und intermediären Fasern auf Kosten der schnellkontrahierenden Fasern. Dies steht im Einklang mit dem Konzept, dass sich durch das Training die schnell-kontrahierenden Fasern zu intermediären Fasern oder auch weiter zu langsam-kontrahierenden Fasern transformieren. Die Änderung der relativen Zusammensetzung der Muskelfasern war jedoch zwischen den Nox4-*Knockout*- und den Wildtyp-Mäusen sehr ähnlich. Der Unterschied zwischen akutem und freiwilligem Lauf ist eine mögliche Folge von mehreren Faktoren, wie Dauer, Intensität und dazugehöriger Stress. Die Expression aller muskelfaserspezifischen mRNA-Isoformen der Skelettmuskel-Myosin schweren Kette (MHC)[59] war nach dem freiwilligen Lauf hochreguliert, was auf eine Hypertrophie der Muskelfasern hindeutete. Diese relativen Änderungen waren jedoch zwischen Nox4-*Knockout*- und Wildtyp-Mäusen vergleichbar. Die nachhaltige Veränderung der Muskelfaserzusammensetzung wurde nur durch das Laufradtraining induziert, dabei hatte Nox4 keine Auswirkungen auf die Sport-induzierte Faserspezifikation.

4.2.2 Sport-induzierte Änderung der Energiebilanz ist unabhängig von Nox4

Die Analyse des relativen mitochondrialen Cytochrom b DNA-Gehalts zeigte, dass Nox4 keinen Einfluss auf die mitochondriale Dichte sowohl bei kurzer als auch bei langer, siebenwöchiger sportlicher Belastung hatte. Es ist mindestens ein sechswöchiges Ausdauertraining erforderlich, um einen höheren stationären Mitochondriengehalt zu erreichen. Dies ist sowohl vom Muskelfasertyp als auch von der Häufigkeit, Intensität und Dauer des Trainings abhängig[18]. Die charakteristische Laufart der Mäuse, welche sich in einem Wechsel von schnellem Kurzstreckenlauf und einer kurzen Pause widerspiegelt, ähnelt einem Krafttraining mit der Rekrutierung von schnell-kontrahierenden Muskelfasern. Sehr hohe Intensität und geringe Dauer des Trainings induzieren stärker die Proteinsynthese, die zur myofibrillären Muskelhypertrophie führen kann, wobei der Mitochondriengehalt in vergrößerten Muskelfasern "verdünnt" wird[18]. Ein Mitochondrium beinhaltet jedoch mehrere Kopien der mitochondrialen DNA, weshalb auch weitere Marker des Energiestoffwechsels untersucht wurden. Zwar führte der freiwillige Lauf schon nach wenigen Tagen zu einer starken Induktion der PGC1α Expression, hatte aber kaum einen positiven Effekt auf die PGC1α Expression nach vier Wochen. Sowohl die

Sport-induzierte als auch die basale PGC1α Expression waren zwischen den beiden Mäuse-Stämmen vergleichbar hoch und somit Nox4-unabhängig. Die durch das chronische Laufradtraining induzierte GLUT4-Expression war ebenfalls von Nox4 unabhängig. Auffällig war allerdings, dass das akute Training am Laufband die PGC1α-Expression sowie die GLUT4-Expression in Wildtyp-Mäusen, aber nicht in Nox4-*Knockout*-Mäusen erhöhte. Das höhere Verhältnis von langsam- zu schnell-kontrahierenden Muskelfasern in Nox4-defizienten Mäusen unter basalen Bedingungen könnte schnelle Anpassungen im Energiestoffwechsel nicht erforderlich machen, da keine wirkliche Energieänderung von AMPK erfasst wird. Diese Kinase wird von AMP aktiviert und durch Kreatinphosphat allosterisch gehemmt und reagiert deshalb sehr sensitiv auf den Energiestatus des Muskels[60]. *In vitro* Untersuchungen zeigen, dass die PGC1α-Expression von der H_2O_2-Konzentration abhängig ist. Das hohe H_2O_2-Niveau induziert indirekt über die AMPK-Aktivierung die PGC1α-Transkription[61]. Die beeinträchtigte Reaktion auf die Änderung des Energiestoffwechsels könnte allerdings auch eine Folge der abgeschwächten AMPK-Aktivierung sein. Dennoch erhöhte sich die AMPK-Phosphorylierung sowohl beim einmaligen akuten, als auch beim zehntägigen Laufbandtraining unabhängig von Nox4. Welche Rolle PGC1α in der Sport-induzierten Mitochondrienbiogenese und in der Fasertyptransformation spielt, ist umstritten. Es ist beschrieben, dass PGC1α für die Sport-induzierte Mitochondrienbiogenese und die Fasertyptransformation irrelevant ist[62,63]. Die Sport-induzierte GLUT4-Expression wird durch AMPK-Aktivierung reguliert, durch den Muskelfasertyp und den Skelettmuskel bestimmt und ist stark von der Art und Intensität der Belastung abhängig[29,64,65]. Dabei ist die basale GLUT4-Proteinexpression bei langsam-kontrahierenden Muskelfasern höher als bei schnell-kontrahierenden Muskelfasern[66,67]. Zusammengenommen legen diese Daten nahe, dass Nox4 nur eine geringe, nicht konstitutive Auswirkung auf den Metabolismus im Skelettmuskel hat.

4.2.3 Sport-induzierte Angiogenese ist durch Nox4 vermittelt

Das Lauftraining, sowohl auf dem Laufband als auch im Laufrad, erhöhte das Verhältnis von Endothelzellen zu Muskelfasern in Wildtyp-Mäusen, wohingegen dieser Effekt in Nox4-*Knockout*-Mäusen nicht beobachtet wurde. Dieser Befund deutete darauf hin, dass Nox4 für die Sport-induzierte Angiogenese erforderlich ist. Austrainierte Muskeln zeigen eine weitaus höhere Sauerstoffaustauschkapazität als untrainierte Muskeln. Reguläres Training verbessert deren Sauerstoffaufnahmekapazität und fördert die

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Angiogenese[68,69]. Das Training führt über die relative Hypoxie und einer zellulären inflammatorischen Aktivierung zur Stabilität des Hypoxie-induzierbaren Faktor 1α (Hif1α) und folglich zu einem Anstieg der VEGF-Expression[57]. Dabei spielt vor allem das von der Skelettmuskulatur produzierte VEGF eine zentrale Rolle in der Sport-induzierten Angiogenese und bei der Anpassung der Muskeln in Mäusen[70,71].

4.2.4 Nox4 reguliert *Stretch*- und Hypoxie-vermittelte Expression von VEGF

Die spezifische Auswirkung von Nox4 auf die VEGF-Expression wurde in den Myoblasten analysiert, die aus C2C12 Zellen und Satellitenzellen differenziert wurden, wobei die Satellitenzellen aus dem Skelettmuskel der Wildtyp- und Nox4-*Knockout*-Mäuse isoliert wurden. Parallel zu einem Anstieg der Proteinexpression von Hif1α und Nox4 unter Hypoxie (2% O_2, $8\,h$) wurde eine verstärkte Induktion der mRNA-Expression von VEGF in den C2C12 Myoblasten gemessen. Diphenyleneiodonium (DPI) reduzierte den Anstieg der VEGF-Expression durch die Hemmung der ROS-Produktion von NADPH Oxidasen und von Flavoproteinen[72]. Dagegen erhöhte eine Stimulation mit H_2O_2 die VEGF-Expression in den C2C12 Myoblasten. Darüber hinaus rief auch *Stretch,* als eine Simulation der Bewegung durch den zyklischen mechanischen Stress, einen Anstieg der VEGF-Expression in den C2C12 Myoblasten hervor, wobei auch hier DPI oder das H_2O_2-abbauende Enzym Katalase hemmend wirkten. Diese Befunde zeigten deutlich, dass ROS und speziell H_2O_2 eine wichtige Rolle in Hypoxie- und *Stretch*-induzierter VEGF-Expression spielen. Die spezifische Beteiligung von Nox4 in diesem ROS-abhängigen Prozess wurde in den Myoblasten aus differenzierten Satellitenzellen untersucht. Sowohl Hypoxie als auch *Stretch* führten zur Induktion der VEGF-Expression in den Satellitenzellen, jedoch war die Auswirkung auf die VEGF Expression in Nox4-defizienten Satellitenzellen deutlich kleiner als in Wildtyp-Satellitenzellen. Das legte den Schluss nahe, dass Nox4 *Stretch*- und Hypoxie-vermittelte Expression von VEGF in den Satellitenzellen reguliert. Verschiedene Studien zeigen außerdem, dass Nox4 als Sauerstoffsensor agiert[52]. Dabei wird Hif1α durch H_2O_2 stabilisiert und aktiviert[52,73], was wiederum zur Bindung von Hif1α an das *Hypoxia-responsive element* (HRE) des Nox4-Promoters und dem zufolge zum Anstieg der Nox4-Expression führen kann[57].

4.2.5 Die Sport-induzierte Expression von VEGF und Ang1 im Skelettmuskel sind unterschiedlich durch Nox4 beeinflusst

Ein Anstieg der VEGF-Expression ist die Folge der zellulären Signalkaskade als Reaktion auf die Muskelkontraktion und tritt auch nach einem einzigen Laufbandtraining auf[74]. Die Analyse der VEGF-mRNA-Expression in murinem Muskelgewebe von Wildtyp-Tieren nach dem Training ergab, dass die VEGF-Expression nach zehntägigem akutem Training, jedoch nicht nach vierwöchigem Lauf, signifikant erhöht war. Die VEGF-abhängigen Gene Semaphorin 6A und 6D[75] wiesen ein ähnliches Expressionsmuster wie VEGF auf, dabei wurde auch hier die Genexpression in Nox4-*Knockout*-Tieren durch das Training nicht beeinflusst. Dennoch war nach vierwöchigem freiwilligem Lauf mehr Neovaskularisierung in den Wildtyp- als in den Nox4-defizienten Mäusen zu beobachten. Das deutete darauf hin, dass eine Anpassung der VEGF-Expression an einen neuen Gleichgewichtszustand stattfand. Immer mehr Hinweise bestätigen die Ansicht, dass Sport nicht nur pro-angiogene Faktoren wie VEGF induziert, sondern auch anti-angiogene Faktoren reguliert[76]. Die Angiopoietin (Ang) / Tie2-Rezeptor Signaltransduktion ist ebenfalls in die Sport-induzierten Angiogenese involviert. Das akute Training ergab keine wesentliche Änderung in der Expression von Angiopoietinen und Tie2 in der Skelettmuskulatur beider Stämme. Der freiwillige Lauf erhöhte dagegen die mRNA Expression von Ang1 und Tie2, wobei die Ang1 Expression in Nox4-Knockout-Mäusen deutlich höher als in Wildtyp-Mäusen war. Ang1 wird durch perivaskuläre Zellen exprimiert und fördert die Reifung und Stabilisierung der Gefäße[77]. Dagegen wirkt Ang2 als Antagonist von Ang1 destabilisierend und fördert die angiogene Sprossung[78]. Das Verhältnis von Ang1 zu Ang2 bestimmt die regulatorische Antwort in verschiedenen Stadien der Angiogenese und somit der Stabilität der neugebildeten Gefäße[79,80]. Außerdem ist der Anstieg in der Tie2-Expression ein Marker für die vaskuläre Stabilität und die Reifung des Kapillarnetzwerkes. Zusammen mit der Tatsache, dass nur Wildtyp-Mäuse mit einer Zunahme der Kapillardichte beim freiwilligen Lauf reagierten, bedeutet dies, dass die Ang1-Signaltransduktion in Abwesenheit von Nox4 verbessert werden konnte.

4.2.6 Nox4-Defizienz verbessert den Heilungsprozess nach der OIR

Die postnatale Entwicklung der Mausretina ermöglicht die Untersuchung der Entwicklung der retinalen Angiogenese[81]. Diese zeigte in Nox1-, Nox2- und Nox4-*Knockout*-

Mäusen sowie in den Kontroll-Tieren einen ähnlichen Verlauf. Es konnte keine postnatale Änderung in der Ausbildung des Kapillarnetzes festgestellt werden. Somit scheinen die Nox-Proteine für die physiologische Entwicklung der Netzhaut irrelevant zu sein. Die NOX4-Expression in retinalen Endothelzellen ist während der retinalen Gefäßentwicklung unbeeinflusst, während diese und die Neovaskularisierung im OIR-Ratte-Modell erhöht ist[82]. Im OIR-Maus-Modell fördert Ang1-Überexpression die Bildung eines gesunden Gefäßnetzwerkes durch die Hemmung übermäßiger Neoangiogenese[83]. Mit Hilfe eines geeigneten Maus-Modells konnten wir zeigen, dass Nox4-*Knockout* zu einer deutlichen Abnahme der Neovaskularisierung führt und die Heilung der Retina verbessert. Es wurde gezeigt, dass nur Nox1, aber weder Nox2 noch Nox4 an der Neovaskularisierung im OIR-Modell beteiligt sind[84]. Allesdings ist Nox4 die häufigste und die Nox2 zweithäufigste Nox-Isoform in primären humanen retinalen microvasculären Endothelzellen sowie in anderen Endothelzellen. *In vitro* Nox4-*Knockdown* reduziert dabei die ROS-Produktion[82], die Migration der Endothelzellen sowie die *Tube Formation*[85]. *Scavenging* von Nox4-produziertem H_2O_2 hemmt die retinale *Tube Formation*. Dabei schwächt der *in vivo Knockdown* von retinalen Nox4 deutlich die retinale Neovaskularisierung im OIR-Modell[85]. In einer weiteren Studie wurde gezeigt, dass Nox2-*Knockout* im OIR-Modell zur Abnahme der Neoangiogenese führt[88]. Dusting GJ et al. führte auf, dass Nox4-*Knockout* die retinale Neovaskularisierung reduziert[89]. Somit scheint es, dass die VEGF-induzierte Neoangiogenese in der Nox4-defizienten Retina beeinträchtigt ist, während die durch Ang1 induzierte Stabilisierung der Gefäße die Heilung der Netzhaut bei der Sauerstoff-induzierten Retinopathie verbessert.

4.2.7 Ausblick

Sport ist eine der wichtigsten und effektivsten nicht-medikamentösen Maßnahmen gegen verschiedene Krankheiten, wie periphere arterielle Verschlusskrankheit oder Diabetes. Die vorliegende Studie zeigte, dass die von Nox4-produzierten ROS für die Sport-induzierte Angiogenese essentiell sind. Daher könnten Antioxidantien den Effekt von sportlicher Aktivität auf die Angiogenese des Skelettmuskels beeinträchtigen.[86,87] Im Gegensatz dazu könnte die Hemmung von Nox4 bei retinalen Gefäßerkrankungen wie z. B. bei der diabetischen Retinopathie den Heilungsprozess fördern, da ein solcher Ansatz eine überschüssige VEGF-Expression verhindern und dadurch auch vor anomaler Neoangiogenese schützen würde.

5 ÜBERSICHT DER PUBLIKATIONEN

- **Vogel J**, Kruse C, Zhang M, Schröder K. Nox4 supports proper capillary growth in exercise and retina neo-vascularization. *The Journal of physiology*. 2015;593(9):2145-2154.

- **Vogel J**, Figueiredo de Rezende F, Rohrbach S, Zhang M, Schröder K. Nox4 Is Dispensable for Exercise Induced Muscle Fibre Switch. *PloS one*. 2015;10(6):e0130769.

Nox4 supports proper capillary growth in exercise and retina neo-vascularization

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Key points

- We provide evidence for two distinct functions of the NADPH oxidase Nox4 in angiogenesis using Nox4 knockout mice.
- First, Nox4 maintains vascular endothelial growth factor expression and prevents an increase in angiopoietin 1 expression, thereby contributing to angiogenesis in exercise.
- Second, deletion of Nox4, via an enhanced angiopoietin 1 expression, contributes to stabilization of new formed vessels and prevents an exacerbated neo-angiogenesis in oxygen-induced retinopathy.
- By contrast, Nox4 does not influence developmental angiogenesis.

Abstract By producing H_2O_2, the NADPH oxidase Nox4 is involved in hypoxia-induced angiogenesis, as present in vascular remodelling of the hypertrophic heart or blood flow recovery after hind limb ischaemia. In the present study, we hypothesized that Nox4 contributes to proper capillary growth in the retina and in exercised muscles and investigated this in wild-type and $Nox4^{-/-}$ mice. Exercise, as induced by voluntary running in a running wheel or forced running on a treadmill, stimulated capillary growth in wild-type but not $Nox4^{-/-}$ mice. As an underlying mechanism, we identified both vascular endothelial growth factor (VEGF) expression to be reduced and angiopoietin 1 (Ang1) expression to be increased in response to Nox4 knockout. To differentiate the two factors, oxygen-induced retinopathy was investigated. In this model, deletion of Nox4 protected from neo-angiogenesis and stabilized the network of regrown vessels, which is a typical feature of Ang1. However the angiogenesis in the developing retina was similar between $Nox4^{-/-}$ and wild-type mice. Thus, Nox4 contributes to exercise- and hypoxia-induced angiogenesis through a dual mechanism of maintaining VEGF and preventing Ang-1 expression, whereas the developmental angiogenesis is Nox4 independent.

Introduction

Angiogenesis is a major prerequisite for the proper development and adaptation of tissue to changes in demand or nutrient supply. The start signal for angiogenesis is an inflammation-like state, with endothelial cell activation, macrophage recruitment and cytokine production at the site of needed changes in the capillary network. Activated endothelial cells start to form tip cells that migrate toward

where the new vessel is needed and, subsequently, by the formation of stalk cells that follow the tip cell, proliferate and form the lumen of the new vessel (Herbert, Stainier & Didier, 2011).

In earlier work, we reported that Nox4 is the predominant isoform of NADPH oxidases in endothelial cells and that genetic deletion of Nox4 attenuates angiogenesis in response to ischaemia after femoral artery ligation (Schröder *et al.* 2012). Among the Nox enzymes, Nox4 is an exception. Different from other NADPH oxidases, Nox4 is constitutively active and produces H_2O_2 (Brandes *et al.* 2014). Furthermore hypoxia as the main force for the induction of angiogenesis increases Nox4 expression (Diebold *et al.* 2010). These features enable Nox4 to elicit the long lasting and adaptive signalling processes involved in differentiation or angiogenesis.

Developmental angiogenesis has been studied extensively in the murine retina because mice are born blind and retina vascularization develops after birth. In humans, retina angiogenesis is complete shortly before normal term birth (Stahl *et al.* 2009) and dysfunctional retinal angiogenesis is a frequent problem in preterm infants. Angiogenesis in the eye of adults can be a consequence of hyperglycaemia in diabetes, thrombosis in vein occlusions or developmental delays in retinopathy of prematurity. Under such conditions, the problem is not non-functional angiogenesis, but rather an excessive, unlimited angiogenesis. Therefore, reducing angiogenesis and promoting vessel stabilization in adult eye disease are the goals of many therapeutic approaches (Todorich *et al.* 2014).

A situation of physiological angiogenesis is exercise. In addition to adaptation to a more demanding muscle workload, angiogenesis in skeletal muscle is also an efficient therapy for peripheral artery disease. Acute exercise obviously increases the energy consumption of the muscle. Consequently, blood flow during exercise increases 15- to 20-fold, enabling an adequate supply with nutrients and oxygen. In accordance with the Hagen–Poiseulle law, a minor increase in diameter is sufficient to considerably raise the blood flow in the target organ or muscle because vessel resistance is a function of the radius to the power of four. In muscle, this is reflected by the high flow reserve. Oxygen supply to muscle is not limited by the dilator capacity of the resistance vessel but instead by perfusion and thus the number of capillaries surrounding a muscle fibre. Increased capillarization results in a longer mean transit time and improved diffusion conditions. The formation of new capillaries by angiogenesis is therefore an essential step in the adaptive response to exercise.

We hypothesize that Nox4 plays an important role in angiogenesis of the retina and in exercised skeletal muscle and investigated this using knockout mice.

Methods

Animals

All animal experiments were conducted in accordance with the German Animal Protection Act and were approved by the District Government of Darmstadt (approval numbers V54-19c20/15-F28/31 and -F28/23), Germany. All adult animals in the present study were killed by cervical dislocation after isoflurane anaesthesia (Forene®; AbbVie, Ludwigshafen am Rhein, Germany), whereas pups were de-capitated under anaesthesia. C57/BL6J Nox4$^{-/-}$ mice have been described previously (Schröder *et al.* 2012). Animals had been backcrossed for 10 generations onto a C57BL6/J background and C57BL/6J mice served as controls. Nox2$^{y/-}$ mice were obtained from Charles Rivers and Nox1$^{y/-}$ mice were kindly provided by Karl-Heinz Krause, Geneva (Gavazzi *et al.* 2006). All exercise experiments were initiated at a mouse age of 6–8 weeks and only male animals were used. Mice were housed in a specified pathogen-free facility under a 12:12 h light/dark cycle with free access to chow and water.

Animal models

Serum vascular endothelial growth factor (VEGF) level was measured by MyRiad RBM (Austin, TX, USA) using the RodentMAP, version 2.0, antigen panel (MyRiad RBM). Treadmill exercise training was performed on a four-chamber running belt system (TSE Systems GmbH, Bad Homburg, Germany). For repeated forced endurance exercise, mice were trained daily for 1 h with additional warm-up and cool-down phase. The 10 days of training was performed initially at 10 m min^{-1} and a 5% incline with a gradual increase to 15 m min^{-1} and 10% incline equal for all mice. Mice in the control groups remained in their cages in the treadmill room throughout the exercise bouts. For the voluntary running experiment, mice randomly assigned to the 4 weeks running group ($n = 6$–8) were provided with a running wheel equipped with an activity counter (running distance). It would be an oversimplification to assume that treadmill running and voluntary running in a running wheel only differ in the intensity of exercise. Numerous other factors are of relevance: wheel running is a burst exercise, which occurs throughout the whole night; it is not associated with the psychological stress of the treadmill and takes place during the maximum physiological circadian activity of the mice. At the end of the experiments, mice were sacrificed immediately after the last training and muscles were quickly excised, rinsed with ice-cold phosphate-buffered saline (PBS), blotted dry, snap-frozen and stored in liquid nitrogen or Tissue-Tek (Sakura, Heppenheim, Germany) for subsequent analyses.

The murine model of oxygen-induced retinopathy (OIR) was performed as described previously (Connor *et al.* 2009). Briefly, at postnatal day 7 (P7), pups with their mothers were transferred into the hyperoxia system (Biospherix, Lacona, NY, USA) and exposed to 75 ± 2% oxygen for 5 days (P7–P12) followed by a subsequent return to normoxia (room air). Pups were killed at day 12, 14 or 17 and retinas were stained with fluorescein isothiocyanate *Griffonia* (*Bandeiraea*) *simplicifolia* BS-I lectin (1:100) in 1% Triton X-100 (both Sigma-Aldrich, St Louis, MO, USA) in 0.1 M PBS overnight, washed and mounted with mounting media (DakoCytomation, Glostrup, Denmark). Images were taken with the aid of a digital microscope (Carl Zeiss, Oberkochen, Germany). Image J software (National Institutes of Health, Bethesda, MD, USA), together with the appropriate plug-ins and macros, was used to analyse vessel regrowth and neo-angiogenesis (Stahl *et al.* 2009). Very similar developmental retina angiogenesis was analysed as described previously (Pitulescu *et al.* 2010). The formation of the superficial vascular plexus was analysed at days 3, 5.5 and 7 using whole mount staining with a CD31 antibody.

Histochemical analysis of skeletal muscle

To determine capillary density, cryostate cross-sections of the gastrocnemius and soleus muscles embedded in Tissue-Tek were used. After fixation in phosphate-buffered formalin (4% in PBS), the tissue was blocked with 1% Rotiblock (Carl Roth GmbH, Karlsruhe, Germany) and permeabilized with 0.5% Triton X-100, followed by incubation with directly labelled anti-CD31 (BD Pharmingen, Heidelberg, Germany) and anti-laminin antibodies (Abcam, Cambridge, UK), and imaged by confocal microscopy on a LSM 510 META (Carl Zeiss).

Analysis of mRNA expression

Total RNA was extracted from the muscle tissue with TRIzol in accordance with the manufacturer's instructions (Qiagen, Hildenberg, Germany). From 1 μg of RNA, cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers; semiquantitative real-time PCR was performed with Fast Plus EvaGreen Master Mix for quantitative PCR w/Low ROX (2x, 100 rxn) (Biotium, Hayward, CA, USA) in a Mx3005 cycler (Stratagene, La Jolla, CA, USA) with the indicated primers. We attempted to use several standard housekeeping genes, such as EF, GAPDH or β-actin, and all of them were regulated upon exercise. Eventually, we found B2M to be stably expressed in all forms of exercise performed by the mice. Relative expressions of target genes were normalized using B2M

Table 1. Primers used in the present study

Target	Sequence (5′ to 3′)
mB2M fw	GTCTTTCTGGTGCTTGTCTC
mB2M rev	GTATGTTCGGCTTCCCATTC
mSema6d fw	TGACGTGGAGGTCCAGACAG
mSema6d rev	CTGCACATCGGGTTGAAAGC
mSema6a fw	CTAGACAGGCTGACGTAGAC
mSema6a rev	CCAAGGTATCGACCCTGTAG
h,m,r VEGF A fw	GTGGACATCTTCCAGGAGTA
h,m,r VEGF A rev	GCTGTAGGAAGCTCATCTCT
mAngiopoietin1 fw	GTATAAAATGGGTTTTGGGAATCC
mAngiopoietin1 rev	TTGCCTGCTGTCCCTGTGTGACC
mAngiopoetin2 fw	GGGAAGGCAACGAGGCGCATT
mAngiopoetin2 rev	CGCGGTCCCCGTGAGTCCTG
mTie2 fw	ATGGCTCAGGCATTCCAGAACAG
mTie2 rev	TGGCCTTCCTGTTAAGGGCCAGA

as a housekeeping gene, analysed by the $\Delta\Delta C_t$ method and given as a ratio compared to control experiments. The primers used are listed in Table 1.

Cell culture

C2C12 cells were obtained from ATCC and kept in non-confluent undifferentiated culture. Satellite cells were isolated from 5- to 6-week-old male mice using a protocol similar to that established by Danoviz & Yablonka-Reuveni (2012). In brief, muscle tissue was minced into small pieces, digested with 0.1% pronase, triturated with a 10 ml pipette, filtered and directly plated onto collagen coated dishes. Differentiation was monitored by the expression of myosin heavy chain isoforms (data not shown). Cells were kept in culture at 37°C and 5% CO_2 in proliferation medium consisting of Dulbecco's modified Eagle's medium, 1% penicillin–streptomycin, 4 mM glutamine, 1.5 g L^{-1} sodium bicarbonate, 1 mM sodium pyruvate and 20% fetal calf serum. For the experiments, cells were allowed to reach confluence and were differentiated for 7 days in differentiation medium containing Dulbecco's modified Eagle's medium, 1% penicillin–streptomycin, 4 mM glutamine, 1.5 g L^{-1} sodium bicarbonate, 1 mM sodium pyruvate and 4% horse serum (all Invitrogen).

Cyclic stretch and hypoxia

Cyclic stretch was performed as described previously (Fisslthaler *et al.* 2001). Differentiated cells were seeded on flexible-bottomed six-well culture plates coated with collagen (BioFlex; Flexcell International Corp., Hillsborough, NC, USA). After 7 days of differentiation, the cells were mounted onto loading plates in a FlexerCell FX-3000 strain unit (Flexcell International Corp.) and

placed in an incubator. Cells were stretched with an average strain of 6% at a rate of 1 Hz, and static control experiments were performed on cells on stretch plates not exposed to cyclic strain.

For hypoxia, differentiated cells were incubated for 24 h at 1% O_2 in a hypoxic incubator (Invivo2 400; Ruskinn Technology, Leeds, UK).

Statistical analysis

Unless otherwise indicated, data are provided as the mean ± SEM. Statistical analysis for multiple groups was performed by ANOVA, followed by the Bonferroni least significant difference *post hoc* test and, for two group comparisons, by a two-tailed *t* test for normally distributed values. Not normally distributed values were analysed by the Mann–Whitney *U* test. $P < 0.05$ was considered statistically significant.

Results

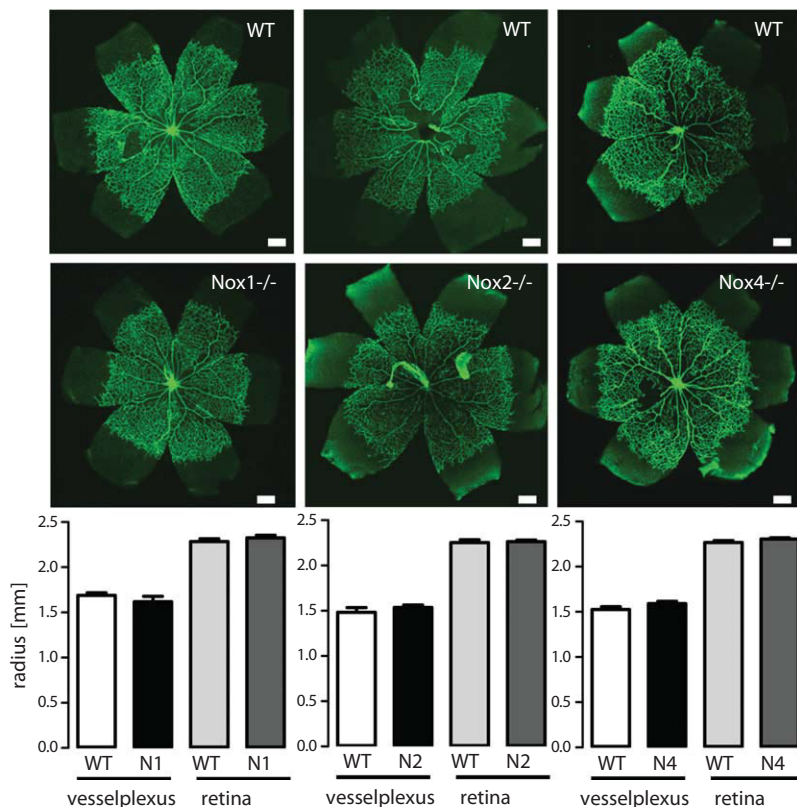### Physiological retina angiogenesis is not mediated by NADPH oxidases

In C57BL/6J mice, the superficial vascular plexus forms during the first week after birth by radial outgrowth of vessels. Within 8 days of birth, the radial vessels reach

the edge of the retina. Although basal VEGF levels in adult mice are slightly but significantly reduced in the absence of Nox4 (wild-type: 118 ± 4 *vs.* Nox4$^{-/-}$: 104 ± 5 pg ml^{-1}; $P < 0.05$), we did not find a reduced formation of the vascular plexus as shown, for example, for day 5.5 after birth (Fig. 1) or any other vascularization of the retina (data not shown). Similarly, developmental angiogenesis was similar between wild-type and Nox1 and Nox2 knockout mice, respectively (Fig. 1). Thus, at least global constitutive knockout models do not suggest that Nox1, Nox2 and Nox4 are indispensable for developmental retina angiogenesis.

Exercise-induced angiogenesis is mediated by Nox4

Next, exercise was studied as a model of stimulated angiogenesis. This was performed with two different protocols: 10 days of forced exercise on a tread mill and 4 weeks of voluntary running. In the voluntary group, mice had free access to running wheels and both strains ran similar distances (wild-type: 5646 ± 930 m *vs.* Nox4$^{-/-}$: 4352 ± 955 m, $n = 6$, not significant).

Both 10 days of tread mill exercise or 4 weeks of voluntary running increased the endothelial to muscle fibre ratio in wild-type mice. Importantly, this effect was not observed in Nox4 knockout mice (Fig. 2*A, C, D* and *F*).


Figure 1. Retina angiogenesis is not mediated by NADPH oxidases
Representative images are shown of retinas from wildtype (WT), Nox1$^{y/-}$, Nox2$^{y/-}$ or Nox4$^{-/-}$ mice at day 5.5 after birth, stained for endothelial cells with CD31 (green) to show capillaries in whole flat mounts. Statistics are provided below the images. Scale bar = 200 μm ($n > 5$).

Nox4 contributes to stretch and hypoxia mediated expression of VEGF

Exercise-induced angiogenesis is driven by VEGF, which is induced in response to hypoxia and stresses such as increases in stretch. To explore the impact of Nox4 on VEGF expression under more controlled conditions, we first utilized C2C12 satellite cells, which, prior to the experiments, were differentiated into myofibroblasts. When these cells were exposed to hypoxia with 2% O_2 for 8 h, hypoxia inducible factor 1α (Hif1α) protein abundance increased, as did Nox4 mRNA expression (Fig. 3*A*). Consequently, hypoxia induced the expression of VEGF mRNA and this was inhibited by the flavoprotein inhibitor diphenyleneiodonium (DPI), which blocks most reactive oxygen species (ROS) sources. By

contrast, the addition of H_2O_2 even potentiated VEGF expression (Fig. 3*B*). Similar results were obtained when the cells were exposed to cyclic stretch to simulate one aspect of exercise. VEGF mRNA expression increased and this was inhibited when ROS were reduced either by DPI or the H_2O_2-decomposing enzyme catalase (Fig. 3*C*). Taken together, these experiments indicate a role for H_2O_2 in hypoxia- and stretch-induced VEGF expression. To study the specific involvement of Nox4, satellite cells were isolated from wild-type and Nox4$^{-/-}$ mice. Hypoxia, as well as stretch, induced an increase in VEGF mRNA in both wild-type and Nox4$^{-/-}$. However, the effect was significantly smaller in Nox4-deficient cells compared to wild-type cells. Thus, Nox4 contributes to hypoxia- and stretch-induced VEGF-A expression in satellite cells.
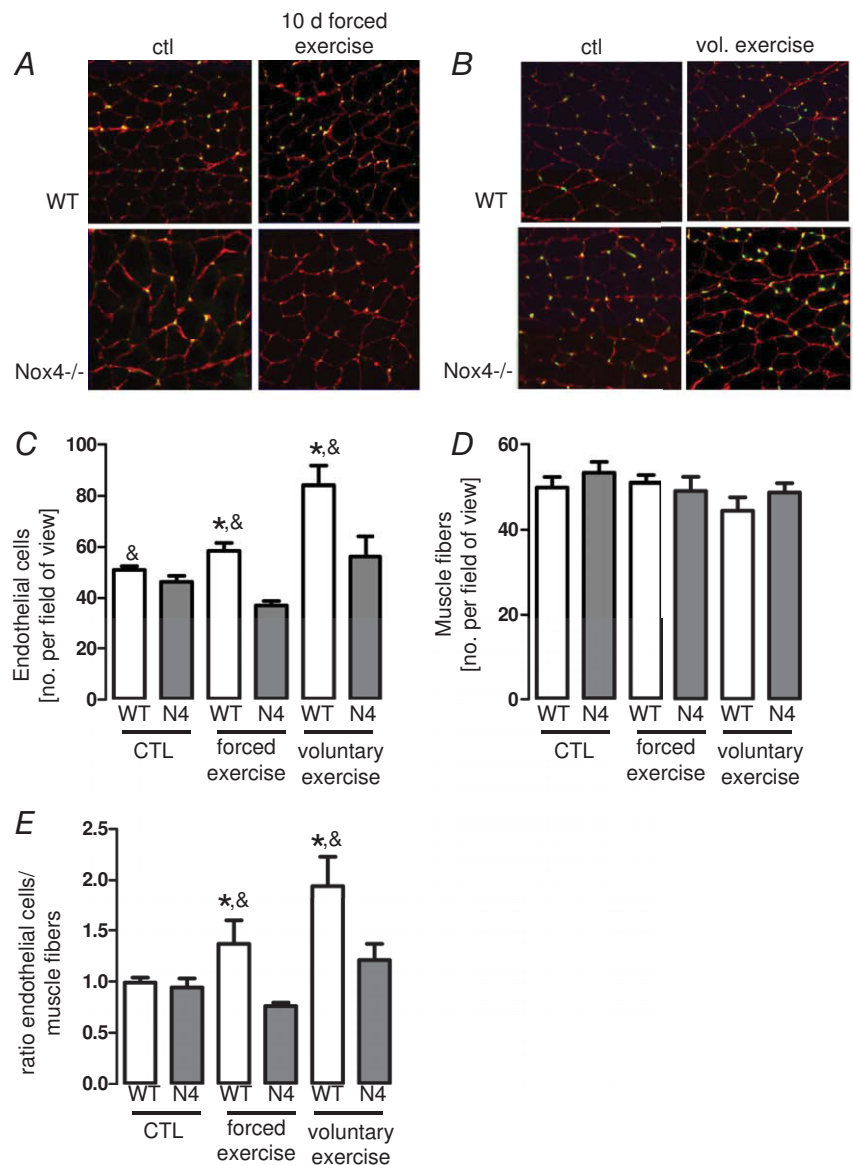

Figure 2. Exercise-induced capillarization is mediated by Nox4
A and *B*, representative images of gastrocnemius muscle from sedentary (ctl) and exercised (Ex.) wild-type (WT) or Nox4$^{-/-}$ mice, stained for CD31 (green) to show capillaries, as well as laminin (red) to define muscle fibres (see the online version for colours). Sections were made after 10 days (*A*) and after 4 weeks of voluntary (*B*) exercise. *C–E*, statistics. *C*, endothelial cells per field of view. *D*, muscle fibre per field of view. *E*, ratio: endothelial cells per muscle fibre. (*n* > 5). *$P < 0.05$ (ctl *vs.* Ex.); &$P < 0.05$ (WT *vs.* Nox4$^{-/-}$).

Exercise induced VEGF and Ang1 expression in skeletal muscle is differentially modified by Nox4

To seek *in vivo* confirmation of the cell culture experiments, VEGF mRNA expression was analysed in murine muscle tissue after exercise. VEGF mRNA expression was increased only in the initiation phase of exercise and later returned to baseline (Fig. 4A). To confirm these findings, the expression of the VEGF A-dependent genes semaphorin 6A and 6D (Segarra *et al.* 2012) was determined, which was found to parallel that of VEGF.
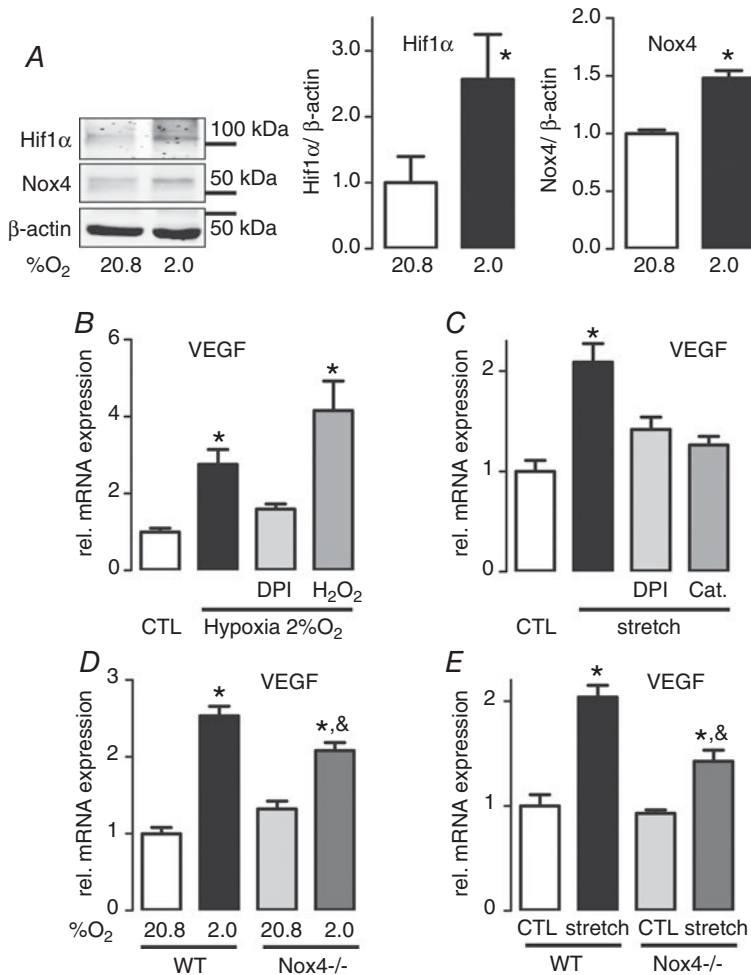
Figure 3. Nox4 contributes to stretch- and hypoxia-induced expression of VEGF
A, Nox4 and Hif1α protein expression analysed by western blotting. B and C, quantitative PCR analysis for VEGF mRNA expression. After 3 days of differentiation from myoblasts to myotubes, C2C12 cells were treated with or without DPI (1 μM), catalase (500 U ml⁻¹) or H_2O_2 (400 μM) for 8 h in 2% O_2 hypoxia (B) or subjected to cyclic stretch with the Flexcell system for 1 h (C). Mean ± SEM ($n > 3$) *$P < 0.05$. VEGF expression in satellite cells isolated from skeletal muscle of wild-type (WT) or Nox4$^{-/-}$ mice with or without 2% O_2 hypoxia (D) or 1 h cyclic stretch with the Flexcell system (E). Mean ± SEM ($n > 5$ with each n = cells from one mouse) *$P < 0.05$ (ctl *vs.* Ex.); &$P < 0.05$ (WT *vs.* Nox4$^{-/-}$). Ctl, control; Ex., exercised.
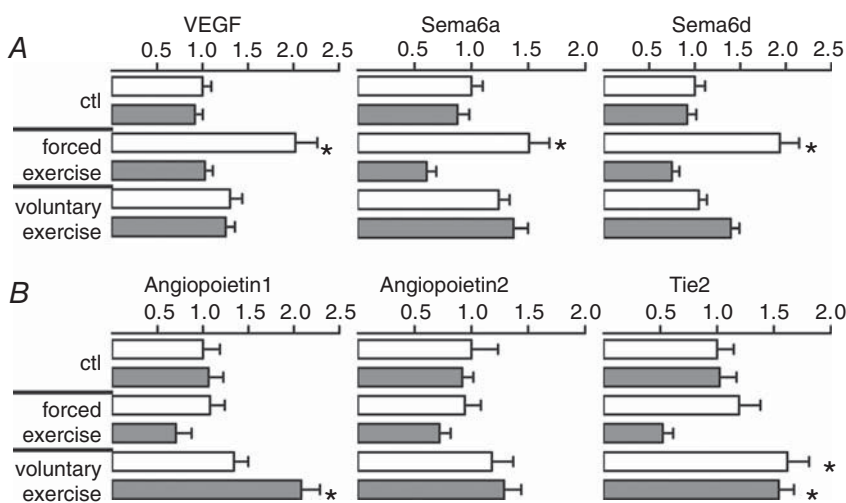
Figure 4. Exercise-induced VEGF and Ang1 expression are differentially modified by Nox4
Quantitative PCR for the genes indicated performed from muscle of mice subjected to 10 days or 4 weeks of voluntary exercise. Mean ± SEM ($n > 5$); *$P < 0.05$ (ctl *vs.* Ex.). Ctl, control; Ex., exercised.

Importantly, the effects on VEGF and semaphorins were restricted to wild-type animals, whereas no exercise-induced increase of these genes was observed in Nox4$^{-/-}$ mice.

As a second HIF1α- and angiogenesis-associated system, the angiopoietins (Ang1 and 2) were analysed (Fig. 4B). Ang1 is expressed by perivascular cells and promotes vessel maturation, quiescence, migration and survival of endothelial cells. By contrast, Ang2 is expressed in endothelial cells and promotes angiogenic sprouting (Eklund & Saharinen, 2013). Both angiopoietins bind to the same receptor Tie2. In our analyses, no significant changes in angiopoietin expression occur in forced exercise. However, in skeletal muscles of voluntarily running mice, Ang1 and Tie2 mRNA expression was increased and Ang1 mRNA expression in Nox4$^{-/-}$ mice was higher than in wild-type mice. Taken together with the finding that only wild-type animals respond with an increase in capillary density in voluntary exercise, this indicates that Ang1 signal transduction may be enhanced in the absence of Nox4.

Nox4 deficiency improves healing after oxygen-induced retinopathy

To confirm the findings reported above, we analysed a second model of angiogenesis regulated by Ang1, namely the OIR model of mice. In this model, Ang1 was described to promote healthy vascular network formation by inhibiting abnormal neo-angiogenesis (Lee *et al.* 2013). Indeed, we found that late healing after OIR was enhanced in Nox4-deficient mice (Fig. 5). This effect was accompanied by fewer regions of neo-angiogenesis. Thus, it appears that VEGF induced neo-angiogenesis is impaired in Nox4-deficient retinas, whereas Ang1 induced stabilization of the vessels improved the rescue of the retinal vessels from oxygen-induced retinopathy.

Discussion

NADPH oxidases have been shown to impact on angiogenesis in several disease models. Nox1 has been implicated in tumour vascularization (Garrido-Urbani
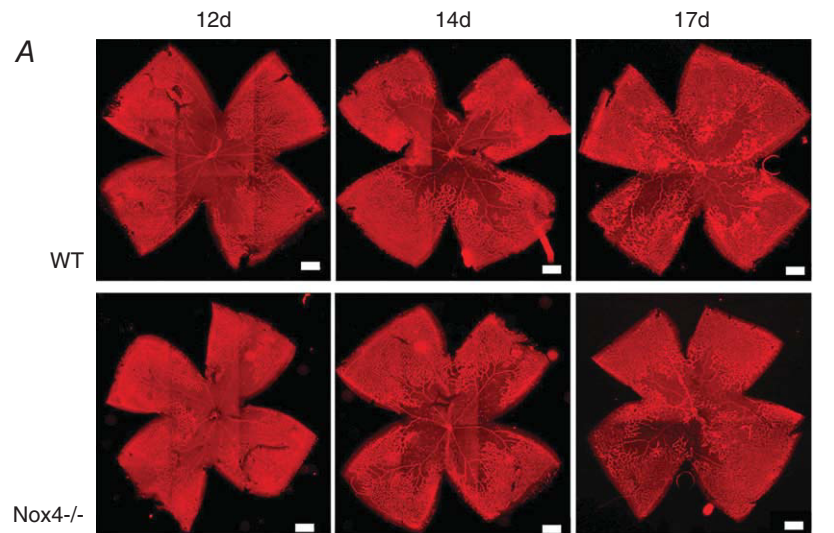
Figure 5. Nox4 deficiency promotes healing after oxygen-induced retinopathy
A, representative images of retinas from wild-type (WT) or Nox4$^{-/-}$ mice exposed to oxygen-induced retinopathy, stained for endothelial cells with lectin (red) to show capillaries. Whole flat mounts were made from pups at age 12, 14 and 17 days. *B* and *C*, statistics of the relative avascular area (*B*) and the area of neo-angiogenesis (*C*) (*n* > 5) *P* < 0.05 (WT *vs.* Nox4$^{-/-}$).

_et al._ 2011) and, after hindlimb ischaemia, Nox1, Nox2 and Nox4 have all been reported as being relevant for vessel regrowth (Tojo _et al._ 2005; Schröder _et al._ 2012). To date, the role of Nox enzymes for developmental angiogenesis has not been studied in depth and the data on spontaneous retina angiogenesis reported in the present study suggest that Nox enzymes are dispensable for this process.

The present study provides evidence indicating that Nox4 is required for exercise-induced angiogenesis. Trained muscles exhibit an enhanced oxygen exchange capacity and repetitive training promotes angiogenesis (Richardson _et al._ 1999; Tesch, 1988). The training-induced formation of new vessels is probably a consequence of a greater abundance of growth factors such as VEGF and exercise endurance capacity has previously been shown to depend on VEGF expression in the muscle (Olfert _et al._ 2009). Recently, it was reported that especially skeletal muscle derived VEGF plays a pivotal role in exercise-induced angiogenesis and muscle-adaptation in mice (Delavar _et al._ 2014). Accordingly, in the early phase of forced repeated exercise, an induction of VEGF expression along with an increase in neo-angiogenesis was observed in wild-type mice but, interestingly, not in Nox4$^{-/-}$ mice in the present study. Up-regulation of VEGF is a consequence of cell signalling in response to muscle contraction and occurs even after a single treadmill run (Annex _et al._ 1998; Tang _et al._ 2010). Moreover, exercise by numerous mechanisms, including relative hypoxia, increased nitric oxide formation, exercise-induced cellular inflammatory activation and alterations in protein stability, can lead to Hif1α stabilization and, consequently, to increases in VEGF expression (Diebold _et al._ 2010). We have shown previously that Nox4 maintains a proper expression of Hif1α and other studies have reported that Nox4 is an oxygen sensor (Nisimoto _et al._ 2014; Zhang _et al._ 2010). Potentially, Nox4-deficient cells recognize the drop in O$_2$-tension less efficiently than wild-type cells and this is also a consequence of attenuated Hif1α expression. In the present study, we observed that both stretch and hypoxia stimulate the expression of VEGF in an ROS- and Nox4-dependent manner. After long-term voluntary exercise, we found no significant increase in VEGF mRNA expression but still more neo-vascularization in wild-type than in Nox4-deficient mice, which suggests that there is adaptation to a new steady state. Indeed, growing evidence supports the view that exercise not only induces pro-angiogenic factors such as VEGF, but also regulates anti-angiogenic factors (Olenich _et al._ 2013). In the present study, we found that the expression of Ang1 was increased in muscle from Nox4$^{-/-}$ compared to wild-type mice. In the vascular system, pericytes, which stabilize vessels, produce Ang1 (Ribatti _et al._ 2011). Pericytes and Ang1 work together to prevent the formation of new vessels. They stabilize the vessel

and thereby inhibit neo-angiogenesis. Endothelial cells produce platelet-derived growth factor that recruits pericytes, whereas TGFβ contributes to the differentiation of pericytes into myofibroblasts (Humphreys, 2012). Importantly, TGFβ is one of the most potent inducers of Nox4 and Nox4 is involved in the differentiation of other mesenchym-derived cells such as adipocytes or myofibroblasts (Hecker _et al._ 2009; Schröder _et al._ 2009). Therefore, it is likely that the pericyte to myofibroblast transition also is regulated by Nox4, which, however, still needs to be established and is beyond the scope of the present study. VEGF negatively regulates pericyte function and vessel maturation (Greenberg _et al._ 2008). Although highly speculative, it is possible that more pericytes are present on the vessels of Nox4-deficient mice as a result of less myocyte differentiation and a lower VEGF level in Nox4$^{-/-}$ mice. Indeed, we found that Nox4-deficient mice, when allowed to perform voluntary exercise, express more Ang1 in skeletal muscles than wild-type mice. Lee _et al._ (2013) found that Ang1 overexpression, as well as Ang1 supplementation, improved vessel regrowth and prevented neo-angiogenesis in a model of oxygen-induced retinopathy. Using the same model, we found that Nox4-deficiency reduced the number of neo-vascularization spots without preventing vessel regrowth. At least in part, this is in agreement with the recent finding showing that only Nox1, and neither Nox2 nor Nox4, is involved in vessel regrowth in the OIR model (Wilkinson-Berka _et al._ 2014). We conclude that pericytes and Ang1 play an important role in Nox4-regulated angiogenesis. A major shortcoming of our work is that we measure Ang1, Tie2, VEGF-A and HIF1a mainly on the mRNA and not at the protein level. Given that several of these factors adhere to matrix and that the protein and mRNA levels are often different, the results of mRNA measurements should not be over-interpreted. Although they provide an important impact of Nox4 on the mRNA of the cytokines measured, they cannot demonstrate any causal link between differences in mRNA and protein-mediated functional consequences.

Nevertheless, the present study provides evidence for a role of Nox4 as a double-edged sword in angiogenesis. As a result of its contribution to VEGF-expression, Nox4 supports exercise-induced angiogenesis. By contrast, Nox4 deficiency may contribute to vessel stabilization in rethinopathy via an enhanced expression of Ang1 and thereby prevents neo-vascularization of the retina.

References

Annex BH, Torgan CE, Lin P, Taylor DA, Thompson MA, Peters KG & Kraus WE (1998). Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. _Am J Physiol Heart Circ Physiol_ **274**, H860–H8607.

Brandes RP, Weissmann N & Schröder K (2014). Nox family NADPH oxidases: molecular mechanisms of activation. *Free Radic Biol Med* **76**, 208–226.

Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, Sapieha P, Stahl A, Willett KL & Smith, Lois EH (2009). Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc* **4**, 1565–1573.

Danoviz ME & Yablonka-Reuveni Z (2012). Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol Biol* **798**, 21–52.

Delavar H, Nogueira L, Wagner PD, Hogan MC, Metzger D & Breen EC (2014). Skeletal myofiber VEGF is essential for the exercise training response in adult mice. *Am J Physiol Regul Integr Comp Physiol* **306**, R586–R595.

Diebold I, Petry A, Hess J & Görlach A (2010). The NADPH oxidase subunit NOX4 is a new target gene of the hypoxia-inducible factor-1. *Mol Biol Cell* **21**, 2087–2096.

Eklund L & Saharinen P (2013). Angiopoietin signaling in the vasculature. *Special Issue: Endothelial Biology* **319**, 1271–1280.

Fisslthaler B, Popp R, Michaelis UR, Kiss L, Fleming I & Busse R (2001). Cyclic stretch enhances the expression and activity of coronary endothelium-derived hyperpolarizing factor synthase. *Hypertension* **38**, 1427–1432.

Garrido-Urbani S, Jemelin S, Deffert C, Carnesecchi S, Basset O, Szyndralewiez C, Heitz F, Page P, Montet X, Michalik L, Arbiser J, Ruegg C, Krause KH & Imhof BA (2011). Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPARalpha mediated mechanism. *PLoS One* **6**, e14665.

Gavazzi G, Banfi B, Deffert C, Fiette L, Schappi M, Herrmann F & Krause K (2006). Decreased blood pressure in NOX1-deficient mice. *FEBS Lett* **580**, 497–504.

Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Murphy E, Huang J, Scheppke L, Stockmann C, Johnson RS, Angle N & Cheresh DA (2008). A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**, 809–813.

Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, Pennathur S, Martinez FJ & Thannickal VJ (2009). NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat Med* **15**, 1077–1081.

Herbert SP, Stainier & Didier YR (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* **12**, 551–564.

Humphreys BD (2012). Targeting pericyte differentiation as a strategy to modulate kidney fibrosis in diabetic nephropathy. *Semin Nephrol* **32**, 463–470.

Lee J, Kim KE, Choi D, Jang JY, Jung J, Kiyonari H, Shioi G, Chang W, Suda T, Mochizuki N, Nakaoka Y, Komuro I, Yoo O & Koh GY (2013). Angiopoietin-1 guides directional angiogenesis through integrin $\alpha v\beta 5$ signaling for recovery of ischemic retinopathy. *Sci Transl Med* **5**, 203ra127.

Nisimoto Y, Diebold BA, Constentino-Gomes D & Lambeth JD (2014). Nox4: a hydrogen peroxide-generating oxygen sensor. *Biochemistry* **53**, 5111–5120.

Olenich SA, Gutierrez-Reed N, Audet GN & Olfert IM (2013). Temporal response of positive and negative regulators in response to acute and chronic exercise training in mice. *J Physiol* **591**, 5157–5169.

Olfert IM, Howlett RA, Tang K, Dalton ND, Gu Y, Peterson KL, Wagner PD & Breen EC (2009). Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. *J Physiol* **587**, 1755–1767.

Pitulescu ME, Schmidt I, Benedito R & Adams RH (2010). Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nat Protoc* **5**, 1518–1534.

Ribatti D, Nico B & Crivellato E (2011). The role of pericytes in angiogenesis. *Int J Dev Biol* **55**, 261–268.

Richardson RS, Grassi B, Gavin TP, Haseler LJ, Tagore K, Roca J & Wagner PD (1999). Evidence of O2 supply-dependent VO2 max in the exercise-trained human quadriceps. *J Appl Physiol (1985)* **86**, 1048–1053.

Schröder K, Wandzioch K, Helmcke I & Brandes RP (2009). Nox4 acts as a switch between differentiation and proliferation in preadipocytes. *Arterioscler Thromb Vasc Biol* **29**, 239–245.

Schröder K, Zhang M, Benkhoff S, Mieth A, Pliquett R, Kosowski J, Kruse C, Luedike P, Michaelis UR, Weissmann N, Dimmeler S, Shah AM & Brandes RP (2012). Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. *Circ Res* **110**, 1217–1225.

Segarra M, Ohnuki H, Maric D, Salvucci O, Hou X, Kumar A, Li X & Tosato G (2012). Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. *Blood* **120**, 4104–4115.

Stahl A, Connor KM, Sapieha P, Willett KL, Krah NM, Dennison RJ, Chen J, Guerin KI & Smith, L E H (2009). Computer-aided quantification of retinal neovascularization. *Angiogenesis* **12**, 297–301.

Strobel NA, Peake JM, Matsumoto A, Marsh SA, Coombes JS & Wadley GD (2011). Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. *Med Sci Sports Exerc* **43**, 1017–1024.

Tang K, Xia FC, Wagner PD & Breen EC (2010). Exercise-induced VEGF transcriptional activation in brain, lung and skeletal muscle. *Respir Physiol Neurobiol* **170**, 16–22.

Tesch PA (1988). Skeletal muscle adaptations consequent to long-term heavy resistance exercise. *Med Sci Sports Exerc* **20**, S132–S134.

Todorich B, Yiu G & Hahn P (2014). Current and investigational pharmacotherapeutic approaches for modulating retinal angiogenesis. *Expert Rev Clin Pharmacol* **7**, 375–391.

Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N & Alexander RW (2005). Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. *Circulation* **111**, 2347–2355.

Venditti P, Napolitano G, Barone D & Di Meo S (2014). Vitamin E supplementation modifies adaptive responses to training in rat skeletal muscle. *Free Radic Res*, 1–32.

Wilkinson-Berka JL, Deliyanti D, Rana I, Miller AG, Agrotis A, Armani R, Szyndralewiez C, Wingler K, Touyz RM, Cooper ME, Jandeleit-Dahm KA & Schmidt, Harald HHW (2014). NADPH oxidase, NOX1, mediates vascular injury in ischemic retinopathy. *Antioxid Redox Signal* **20**, 2726–2740.

Zhang M, Brewer AC, Schröder K, Santos, Celio X C, Grieve DJ, Wang M, Anilkumar N, Yu B, Dong X, Walker SJ, Brandes RP & Shah AM (2010). NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. *Proc Natl Acad Sci U S A* **107**, 18121–18126.

Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

J.V. and C.K. were responsible for the conception and design of the experiments, as well as collection, analysis and interpretation of data. M.Z. was responsible revising the manuscript critically for important intellectual content. K.S. was responsible for the conception and design of the experiments, as well as collection, analysis and interpretation of data, and drafting the article. All authors approved the final version of the manuscript; all persons designated as authors qualify for authorship; and all those who qualify for authorship are listed.

Translational perspective

Exercise is the most important non-medical treatment for several diseases such as peripheral arterial occlusive disease or even diabetes. In the present study, we provide evidence that Nox4-derived ROS are required for exercise-induced angiogenesis. Therefore, anti-oxidants should be supplemented with caution because they may impair the training effect of skeletal muscle (Strobel *et al.* 2011; Venditti *et al.* 2014). By contrast, in retinal vascular diseases such as proliferative diabetic retinopathy and retinopathy of prematurity, the inhibition of Nox4 might promote recovery because such an approach would prevent an overshoot in VEGF expression and thereby exacerbated neo-angiogenesis.

RESEARCH ARTICLE

Nox4 Is Dispensable for Exercise Induced Muscle Fibre Switch

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Abstract

Introduction

By producing H_2O_2, the NADPH oxidase Nox4 is involved in differentiation of mesenchymal cells. Exercise alters the composition of slow and fast twitch fibres in skeletal. Here we hypothesized that Nox4 contributes to exercise-induced adaptation such as changes in muscle metabolism or muscle fibre specification and studied this in wildtype and Nox4-/- mice.

Results

Exercise, as induced by voluntary running in a running wheel or forced running on a treadmill induced a switch from fast twitch to intermediate fibres. However the induced muscle fibre switch was similar between Nox4-/- and wildtype mice. The same held true for exercise-induced expression of PGC1α or AMPK activation. Both are increased in response to exercise, but with no difference was observed between wildtype and Nox4-/- mice.

Conclusion

Thus, exercise-induced muscle fibre switch is Nox4-independent.

Introduction

Exercise increases the formation of reactive oxygen species (ROS). Contraction-induced ROS generation has been shown to be an important physiological function for the regulation of both muscle force production and contraction-induced adaptive responses of muscle fibres to exercise training [1]. One important source of ROS in cells is the family of NADPH oxidases, which comprises seven members: Nox1 through 5 and DUOX1 and 2. Among the Nox enzymes Nox4 is an exception. Different to other NADPH oxidases, Nox4 is constitutively active and produces H_2O_2 [2]. These features enable Nox4 to elicit long lasting and adaptive signalling processes as involved in differentiation or angiogenesis.

Table 1.

Target	Sequence 5'-NNN-3'	reference, if applicable
mMyHCI fw	GCCTGGGCTTACCTCTCTATCAC	[18]
mMyHCI rev	CTTCTCAGACTTCCGCAGGAA	
mMyHCIIa fw	CAGCTGCACCTTCTCGTTTG	
mMyHCIIa rev	CCCGAAAACGGCCATCT	
mMyHCIIx fw	GGACCCACGGTCGAAGTTG	
mMyHCIIx rev	CCCGAAAACGGCCATCT	
mMyHCIIb fw	CAATCAGGAACCTTCGGAACAC	
mMyHCIIb rev	GTCCTGGCCTCTGAGAGCAT	
mB2M fw	GTCTTTCTGGTGCTTGTCTC	
mB2M rev	GTATGTTCGGCTTCCCATTC	
mPGC1alpha fw	ACAGCTTTCTGGGTGGATTG	
mPGC1alpha rev	TGTCTCTGTGAGAACCGCTA	
mGLUT4 fw	ATGGCTGTCGCTGGTTTCTC	[19]
mGLUT4 rev	ACCCATGCCGACAATGAAGT	
mCytochrom B fw	CAATCGTTCACCTCCTCTTC	
mCytochrom B rev	TCTGGGTCTCCTAGTATGTC	

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Besides changes in angiogenesis adult skeletal muscle adapts to work load with hypertrophy / atrophy and muscle fibre switch. Depending on their capilarization and metabolic and contractile properties, muscle fibres group into three major categories: Slow twitch type I fibres with high capillary density and high oxidative capacity adapted to endurance exercise, fast twitch fibres type IIb fibres with low capillary density and low oxidative capacity ideal for sprint and anaerobic performance and type IIa fibres, which have an intermediate position. These muscle can work for up to 30 minutes, have intermediate capillary density and high oxidative capacity. The three fibre types differ in their type of myosin which defines the ATPase activity of the muscle. Slow type I fibres express MHCIb and within the two fast types, type IIA expresses MHCIIa, type IID MHCIIx, and type IIb express MHCIIb [3]. Since long, it is debated on whether or not the fiber pattern within one muscle is genetically determined. A landmark study comparing fiber types in monozygotic and dizygotic twins provided strong support for a genetic determination of muscle fiber composition in humans [4]. Nevertheless, conversion of type IIB into type hA fibers with intensive endurance training has been demonstrated [5] and leg immobilization decreases the percentage in type I fibers [6]. Moreover, in addition to the standard fiber type nomenclature, a variety of hybrid fibers can be distinguished, and their phenotypic variation is less well studied as they are not covered by current categorization. Thus, genetic determination as well as demand impact on the fiber composition. Indeed muscle fibres are capable of altering their phenotype in response to changes in demand, e.g., increased or decreased neuromuscular activity [7], mechanical loading or unloading [8], altered hormonal profiles (especially of the thyroid hormones [9]), and aging [10]. Already some training units are sufficient to induce a reduction in type IIb fibres and a corresponding increase in type IIa fibres together with a switch in MHC isoforms [11,12].

Exercise-induced gene expression is at least in part a consequence of an increase in free intracellular Ca^{2+} as a consequence of more frequent neural stimulation. Fibre-type-specific gene expression in skeletal muscles has been described to be controlled by the calcium-regulated serine/threonine phosphatase calcineurin. Activation of calcineurin in skeletal myocytes selectively up-regulates slow-fibre-specific gene promoters, while inhibition of calcineurin

promotes slow-to-fast fibre transformation. Transcriptional activation of slow-fibre-specific transcription appears to be mediated by a combinatorial mechanism involving NFAT and MEF2 [13]. In a previous work we found that Nox4 contributes to the increase in intracellular Ca^{2+} in the course of osteoclast differentiation [14]. Others found that in skeletal muscle Nox4--derived H_2O_2 directly controls the cytosolic calcium concentration during tetanic contraction providing a potential link between Nox4 and muscle adaptation [15]. On this basis, we hypothesize that Nox4 contributes to the switch of fast to slow muscle fibres in response to exercise.

Utilizing three different regimens of exercise herein we analysed the contribution of Nox4 to muscle fibre switch in wildtype and Nox4-/- mice.

Material and Methods

Animals

All animal experiments were conducted in accordance with the German Animal Protection Act and were approved by the District Government of Darmstadt (approval numbers V54-19c20/15-F28/31 and-F28/23) Germany. Animals in this study where killed by cervical dislocation after isofluran (Forene, AbbVie) anaesthesia. C57/BL6 Nox4$^{-/-}$ mice have been previously described [16]. Animals had been backcrossed for 10 generations onto the C57BL6/J background and C57BL/6J mice served as controls. All experiments were initiated at a mouse age of 6–8 weeks and only male animals were used. Mice were housed in a specified pathogen-free facility with 12/12 hours day/night cycle and free access to chow and water. Body weight was monitored at least at the beginning and at the end of the experiments.

Animal models

Treadmill exercise training was performed on a 4-chamber running belt system (TSE). For repeated forced endurance exercise mice were trained daily for 1h with additional warm-up and cool-down phase. Two different protocols were used: A short, more severe and a longer, more moderate one: The 10 days training was performed initially at 10 m/min and a 5% incline with a gradual increase to 15m/min and 10% incline equal for all mice. The 7 weeks training was performed 5 days/week followed by 2 days break during the weekend. Within the first two weeks treadmill speed was gradually increased from 10 m/min with 5% incline to 15m/min and 10% incline. The rational for having 10 days vs. 7 weeks treadmill was to have an extreme early time point and a time point that for sure will represent a phenotype of regular training induced changes. Mice in the control groups remained in their cages in the treadmill room throughout the exercise bouts. For the voluntary running experiment, mice randomly assigned to the 4 weeks running group (n = 6–8) were provided with a running wheel equipped with an activity counter (running distance). It would be an oversimplification to assume that treadmill running and voluntary running in a running wheel only differ in the intensity of exercise. Numerous other factors are of relevance here: Wheel running is a burst exercise, which occurs throughout the whole night, it is not associated with the psychological stress of the treadmill and happens at the physiological circadian activity maximum of the mice. At the end of the experiments, mice were sacrificed immediately after the last training and muscles were quickly excised, rinsed with ice-cold PBS (phosphate buffered saline), blotted dry, snap-frozen, and stored in liquid nitrogen or TissueTek for later analyses.

Histochemical analysis of skeletal muscle

To determine the muscle fiber-type composition, myofibrillar adenosine-triphosphatase (mATPase) histochemistry was performed following the method of Brooke and Kaiser [17].

Briefly 10μm thick sections were pre-incubated at pH 4.3 in Na-Acetate/ KCl buffer, 40 mmol/ L each. ATPase reaction was allowed at pH 9.4 with ATP (adenosine triphosphate, 1.6g/l) followed by sequential incubation with 1% Ca^{2+}, 1% $CoCl_2$ and eventually staining with 1% $(NH_4)_2S$. As a result type 1 (slow) fibres appear darkest, type IIb (fast) intermediate, and type IIa lightest.

Analysis of mRNA expression

Total RNA was extracted from the muscle tissue with TRIzol according to the manufacturer's instructions (Qiagen). From 1 μg of RNA cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers; semi-quantitative real-time PCR was performed with Fast Plus EvaGreen Master Mix for qPCR w/ Low ROX (2x, 100 rxn) (Biotium, Hayward, CA, USA) in a Mx3005 cycler (Stratagene) with the indicated primers. We tried several standard housekeeping genes like EF (eukaryontic elongation factor), GAPDH (glycerinaldehyd-3-phosphat-dehydrogenase) or β-actin and all of them where regulated upon exercise. Eventually we found B2M (beta-2-microglobulin) to be stable expressed in all forms of exercise performed by the mice. Relative expressions of target genes were normalized using B2M as housekeeping gene, analysed by the delta-delta-CT method and given as ratio compared to control experiments. The following primers were used:

Statistical analysis

Unless otherwise indicated, data are given as means ± standard error of mean (SEM). Statistical analysis for multiple groups was performed by analysis of variance (ANOVA) followed by Bonferroni LSD-post-test and for two group comparisons by two-tailed T-test for normally distributed values. Not normally distributed values were analysed by Mann-Whitney-Test. A probability value < 0.05 was considered significant.

Results

Exercise-induced muscle fibre switch is independent of Nox4

Fibre distribution was analysed by ATPase staining. As shown in Fig 1, the relative number of slow twitch fibres was slightly higher in sedentary Nox4-/- animals when compared to wildtype mice. Although the numeral difference in muscle composition was rather small, it appears that Nox4 deficiency may lead to greater expression of slow fibre type muscle under sedentary conditions (Fig 1). Exercise in mice was performed with three different protocols: 10 days forced exercise, 7 weeks forced exercise and 4 weeks voluntary running. In the voluntary group mice had free access to running wheels and both strains ran similar distances (WT 5646±930m vs. Nox4-/- 4352±955m, n = 6, p = ns). Neither short term nor long term repeated forced exercise had an effect on the distribution of the different muscle fibres (Fig 1A, 1B, 1D, 1E & 1F). In contrast, voluntary exercise increased the relative number of slow twitch and intermediate fibres on the cost of fast twitch fibres in the skeletal muscle of both, wildtype and Nox4-/- mice (Fig 1C–1F). Importantly the portion of fast fibres decreased much more than the fraction of slow fibres increased and thus the number of intermediate fibres increased with exercise to a higher extent than the slow fibres (Fig 1F). This is in line with the concept that fast twitch fibres through the intermediate fibre type trans-differentiate into slow fibres or remain at the stage of intermediate fibre type upon exercise. Importantly, the basal difference in fibre composition between wildtype and Nox4-/- animals disappeared upon voluntary exercise. However, the change in the relative composition of muscle fibres was similar between wildtype and Nox4-/-
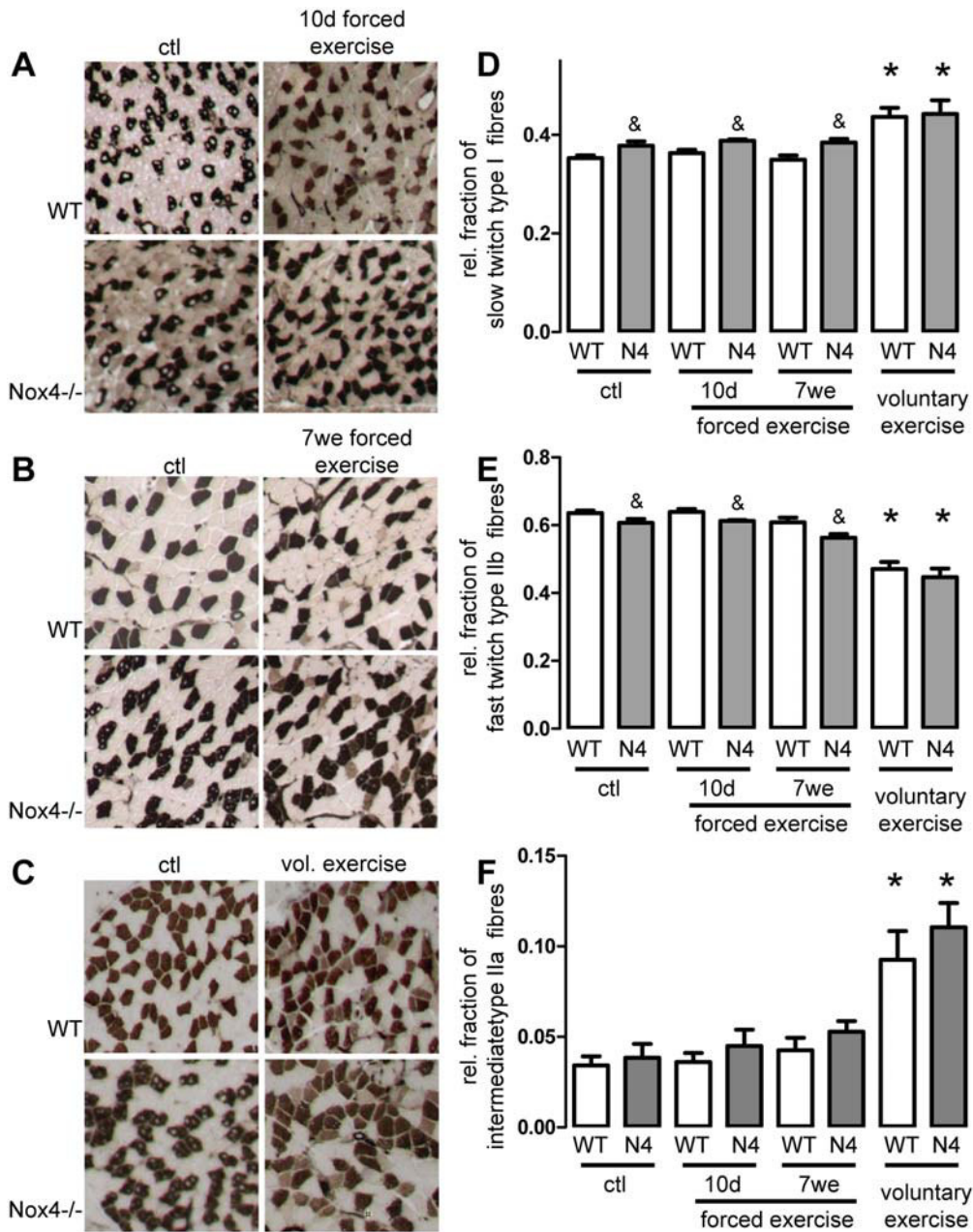
Fig 1. Exercise-induced muscle fibre switch is independent of Nox4. (A-C) Representative images of sedentary and exercised soleus muscle stained for myosin ATPase to determine fibre-type distribution. (D-F): Quantification of slow (dark), fast (light) and intermediate fibres ratio per field of view. mean±SEM (n>5). *p <0.05 (ctl vs. Ex.); &p<0.05 (WT vs. Nox4-/-)

mice, indicating that exercise-induced changes in muscle fibre composition occur independently of Nox4.

ATPase staining as the only way to determine fibre specification is insufficient. Therefore also skeletal muscle myosin heavy chain (MHC) mRNA isoform expression was determined as the expression of the MHC isoforms serves as marker for muscle fibre specification [3]. All MHC isoforms were up-regulated after voluntary running indicating hypertrophy of the
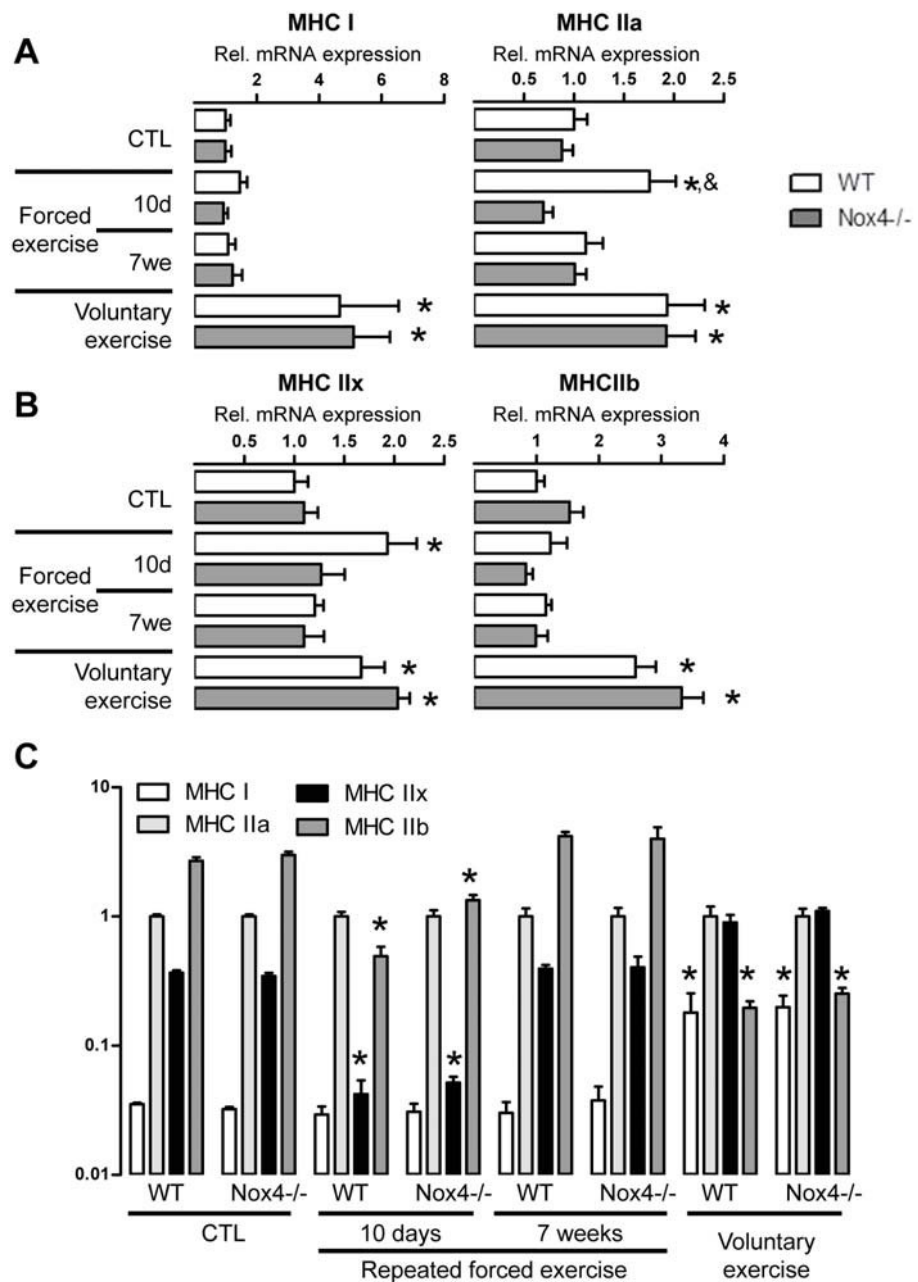
Fig 2. Nox4 has no impact on MHC expression pattern. (A;B) Quantitative PCR for the genes indicated after 10 days and 7 weeks of repeated forced or 4 weeks of voluntary exercise relative to the wildtype controls. (C): Statistics of MHC isoform expression relative to MHCIIa on a logarhythmic scale. mean±SEM (n>5). *p <0.05 (ctl vs. Ex.); &p<0.05 (WT vs. Nox4-/-)

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muscle fibres, and the extent of the response was similar between wildtype and Nox4-/- mice (Fig 2A & 2B). Different to histology, mRNA expression analyses revealed, that 10 days treadmill exercise induced a significant increase in MHCIIa and IIx mRNA expression in muscles from wildtype mice, which was not observed in Nox4-deficient animals. For a better visualization of relative changes in MHC isoforms we calculated the expression of the MHC isoforms
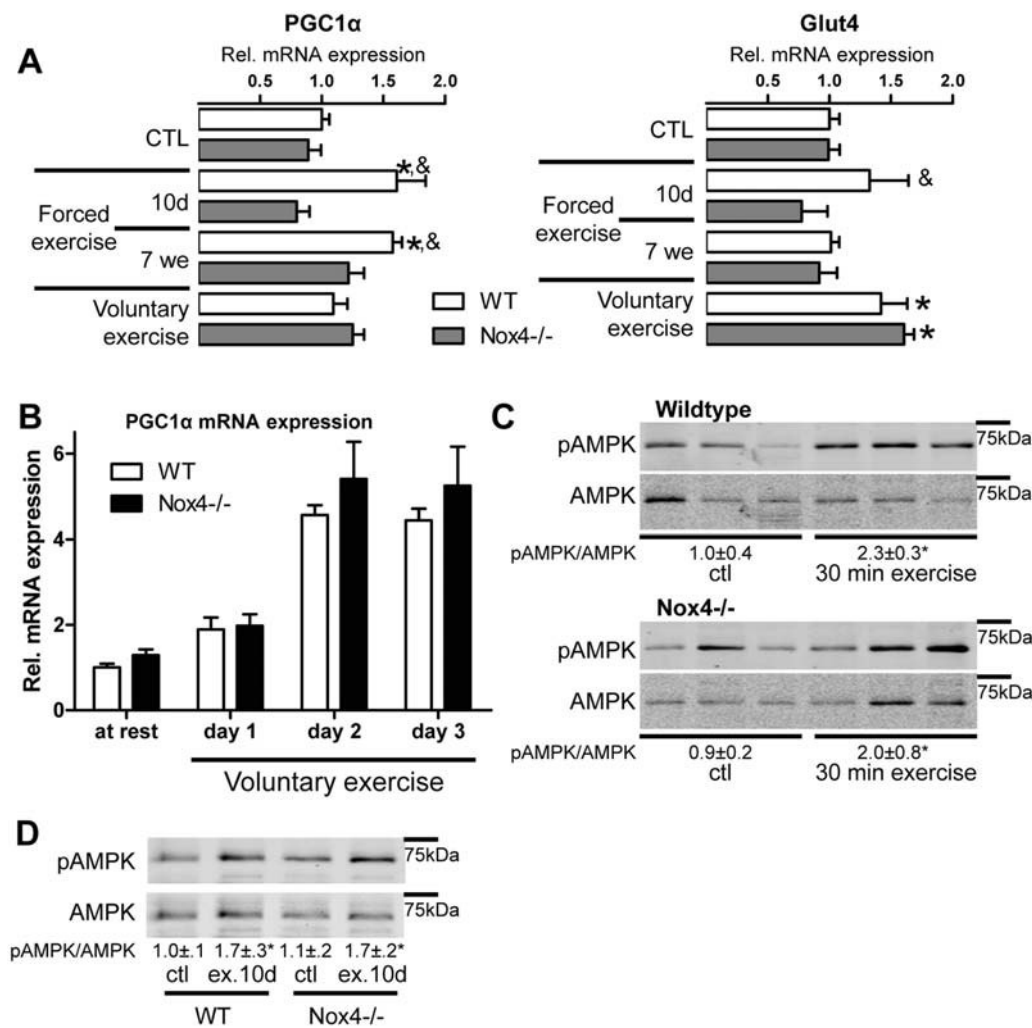
Fig 3. Exercise-induced switch in muscle energy metabolism is Nox4 independent. (A) Quantitative PCR for the genes indicated after 10 days and 7 weeks of repeated forced or 4 weeks of voluntary exercise. (B) Timeline of a quantitative PCR for PGC1α in musculus soleus tissue with voluntary exercise. mean±SEM (n>5). *p <0.05 (ctl vs. exercise); &p<0.05 (WT vs. Nox4-/-) (C&D) Western blot of musculus soleus tissue after (C) 30 of min single or (D) 10 day of repeated forced exercise. Numbers below the blots indicate the ratio of pAMPK and AMPK revealed by densitometry, mean±SEM (n>3). *p <0.05 (ctl vs. Ex.)

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relative to MHC2a. MHC-ratios were similar between wildtype and Nox4-/- mice ([Fig 2C](#)). Collectively, the data suggest that only the voluntary exercise protocol induced sustained changes in muscle fibre composition and that Nox4 does not have an impact on exercise-induced fibre specification.

Exercise induced switch in energy consumption is independent of Nox4

Exercise results in metabolic adaptation of the muscle to promote energy supply or-utilization. Energy metabolism and ATP production mainly depend on mitochondria. Interestingly, mitochondrial content as measured by mitochondrial cytochrome B DNA was not different between wildtype and Nox4-/- animals ([Fig 3C](#)). However, since one mitochondrion comprises several copies of mitochondrial DNA, we analysed additional markers of energy metabolism and focused on PGC1α. This protein acts as a key mediator of mitochondrial biogenesis in a

calcium/ calmodulin-dependent protein kinase IV-dependent manner [20]. As shown in Fig 3A, PGC1α expression was increased upon repeated forced exercise in wildtype but not Nox4-/- mice. This effect was not seen after 4 weeks of voluntary exercise. To further analyse this, we determined PGC1α expression early in voluntary running. PGC1α expression greatly increased after the onset of running but the effect was similar between wildtype and Nox4-/- (Fig 3B). To obtain information about the activity of PGC1α, glucose transporter 4 (GLUT4) mRNA expression was measured, which is under the control of this transcription factor [21]. Repeated forced exercise for 10 days increased GLUT4 expression in wildtype but not in Nox4-/- mice, while the voluntary exercise induced increase in GLUT4 expression was independent of Nox4 (Fig 3A). Next, we analysed AMPK which activates PGC1α. Both 30 min as well as 10 day of repeated exercise increased AMPK phosphorylation independently of Nox4 (Fig 3D & 3E). Thus, Nox4 only has a minor, non-consistent impact on skeletal muscle metabolism control.

Discussion

Here we provide evidence that Nox4 is dispensable for the exercise-induced muscle fibre switch. Our study exclusively focused on mice and the number of identified fiber types of mice and human is different as well as their distribution and relative contribution to the muscle as large. Thus, caution has to be execute when transferring the current data to the human situation. Nox4 influences muscle fibre composition during development, but this difference is lost after voluntary exercise whereas it is maintained during forced exercise for a short period of 10 days as well as in a long term training over 7 weeks. This also indicates that there is a difference between repeated forced exercise and repeated voluntary running. This is a potential consequence of several factors: The duration, the intensity and the associated stress. Probably, forced exercise reflects submaximal intensity short term load, while voluntary exercise corresponds more to prolonged but moderate training. Although pausing rate on the running belt was identical between the two mouse strains and voluntary running distance was similar, we cannot exclude that the small differences in muscle adaptation are a consequence of minor differences in exercise capacity or intensity. To exclude this point, oxygen update / CO_2 excretion during exercise should have been measured, what is, unfortunately beyond our capacity. Mitochondria are the central source of energy for muscle contraction, but Nox4 had no influence on mitochondrial density in exercise. Indeed it is known, that at least six weeks of endurance training is required to reach a new, higher steady-state mitochondrial content, dependent on the fiber type being recruited as well as exercise specifications like frequency, intensity and duration [22]. Mice running differs from human running, as mice run is rather an interval running with very fast short distance running followed by a pause. Such kind of running is similar to resistance training, which recruits fast-fibers, does not lead to a mitochondrial adaptation. It rather appears that the very high intensity and low duration of such resistance training represents a strong stimulus for the synthesis of myofibrillar proteins leading to muscle hypertrophy and eventually the mitochondrial content within enlarged muscle fibers may even be "diluted" within the cell [22]. In our experiments there was no difference in the mRNA expression of the key molecule of mitochondrial biogenesis and muscle fibre type determination—PGC1α [23] under basal conditions between the two strains. Upon repeated forced exercise PGC1α, however, was induced in wildtype, but not in Nox4-deficient mice. This effect might be explained by the higher ratio of slow to fast fibers observed under basal conditions in Nox4 deficient mice. Such differences under basal constitution might make fast adaptations in energy metabolism unnecessary as no real deficiency is detected by the sensors. Putative signals coupling muscle activity with gene expression probably arise from combinations of accelerations in ATP

turnover or imbalances between mitochondrial ATP synthesis, cellular ATP demand and Ca^{2+} fluxes [22]. In cell culture depletion of intracellular Ca^{2+} stores with ionomycin contributes to the formation of slow fibers and increases mitochondrial activity [24]. Indeed Nox4 regulates ryanodine receptor Ca^{2+} release and thereby maintains intracellular Ca^{2+} level [15]. However, impaired energy sensing could also be a consequence of attenuated AMPK activation as this kinase is one of the most important energy sensors [25]. The AMP activated protein kinase (AMPK) is inhibited allostericly by creatinine phosphate and therefore sensitive to the energy status of the muscle fibre. AMPK induced genes include muscle GLUT-4, hexokinase, uncoupling protein 3, and some of the mitochondrial enzymes of oxidative phosphorylation [26]. However, no differences were found between wildtype and Nox4-/- in the phosphorylation of AMPK or the expression of Glut4. In conclusion, under sedentary conditions Nox4 deficient mice have slightly more slow that fast twitch fibres but no difference in exercise induced muscle fibre switch was obvious between wildtype and Nox4-/- animals.

Supporting Information

S1 Fig. (A;B) Heart and body weight from mice under basal conditions and after 10 days or 7 weeks of repeated forced or 4 weeks of voluntary exercise. (C): Statistics of heart/ body weight. mean±SEM (n>5). *p <0.05 (ctl vs. Ex.); &p<0.05 (WT vs. Nox4-/-)
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: JV FR KS. Performed the experiments: JV FR. Analyzed the data: JV FR KS. Contributed reagents/materials/analysis tools: SR MZ. Wrote the paper: KS.

References

1. Powers SK, Nelson WB, Hudson MB (2011) Exercise-induced oxidative stress in humans: cause and consequences. Free Radic Biol Med 51 (5): 942–950. doi: 10.1016/j.freeradbiomed.2010.12.009 PMID: 21167935

2. Brandes RP, Weissmann N, Schröder K (2014) Nox family NADPH oxidases: Molecular mechanisms of activation. Free Radic. Biol. Med.

3. Pette D, Staron RS (2000) Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech 50 (6): 500–509. PMID: 10998639

4. Komi PV, Viitasalo JH, Havu M, Thorstensson A, Sjodin B, Karlsson J (1977) Skeletal muscle fibres and muscle enzyme activities in monozygous and dizygous twins of both sexes. Acta Physiol Scand 100 (4): 385–392. PMID: 199045

5. Howald H (1982) Training-induced morphological and functional changes in skeletal muscle. Int J Sports Med 3 (1): 1–12. PMID: 7040262

6. Haggmark T, Jansson E, Eriksson E (1981) Fiber type area and metabolic potential of the thigh muscle in man after knee surgery and immobilization. Int J Sports Med 2 (1): 12–17. PMID: 6460706

7. Gorza L, Gundersen K, Lomo T, Schiaffino S, Westgaard RH (1988) Slow-to-fast transformation of denervated soleus muscles by chronic high-frequency stimulation in the rat. J Physiol 402: 627–649. PMID: 3236251

8. Desaphy J, Pierno S, Liantonio A, de Luca A, Didonna MP, Frigeri A et al. (2005) Recovery of the soleus muscle after short- and long-term disuse induced by hindlimb unloading: effects on the electrical properties and myosin heavy chain profile. Neurobiol Dis 18 (2): 356–365. PMID: 15686964

9. Nwoye L, Mommaerts WF (1981) The effects of thyroid status on some properties of rat fast-twitch muscle. J Muscle Res Cell Motil 2 (3): 307–320. PMID: 6457058

10. Adamo ML, Farrar RP (2006) Resistance training, and IGF involvement in the maintenance of muscle mass during the aging process. Ageing Res Rev 5 (3): 310–331. PMID: 16949353

11. Campos G E R, Luecke TJ, Wendeln HK, Toma K, Hagerman FC, Murray TF et al. (2002) Muscular adaptations in response to three different resistance-training regimens: specificity of repetition maximum training zones. Eur J Appl Physiol 88 (1–2): 50–60.

12. Hostler D, Schwirian CI, Campos G, Toma K, Crill MT, Hagerman GR et al. (2001) Skeletal muscle adaptations in elastic resistance-trained young men and women. Eur J Appl Physiol 86 (2): 112–118. PMID: 11822469

13. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM et al. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev 12 (16): 2499–2509. PMID: 9716403

14. Goettsch C, Babelova A, Trummer O, Erben RG, Rauner M, Rammelt S et al. (2013) NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis. J Clin Invest 123 (11): 4731–4738. PMID: 24216508

15. Sun Q, Hess DT, Nogueira L, Yong S, Bowles DE, Eu J et al. (2011) Oxygen-coupled redox regulation of the skeletal muscle ryanodine receptor-Ca2+ release channel by NADPH oxidase 4. Proc. Natl. Acad. Sci. U.S.A. 108 (38): 16098–16103. doi: 10.1073/pnas.1109546108 PMID: 21896730

16. Schröder K, Zhang M, Benkhoff S, Mieth A, Pliquett R, Kosowski J et al. (2012) Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. Circ. Res. 110 (9): 1217–1225. doi: 10.1161/CIRCRESAHA.112.267054 PMID: 22456182

17. Brooke MH, Kaiser KK (1970) Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. J Histochem Cytochem 18 (9): 670–672. PMID: 4249441

18. Yuan Y, Shi X, Liu Y, Yang G (2011) FoxO1 regulates muscle fiber-type specification and inhibits calcineurin signaling during C2C12 myoblast differentiation. Mol. Cell. Biochem. 348 (1–2): 77–87. doi: 10.1007/s11010-010-0654-8 PMID: 21086023

19. Miura S, Kai Y, Tadaishi M, Tokutake Y, Sakamoto K, Bruce CR et al. (2013) Marked phenotypic differences of endurance performance and exercise-induced oxygen consumption between AMPK and LKB1 deficiency in mouse skeletal muscle: changes occurring in the diaphragm. Am. J. Physiol. Endocrinol. Metab. 305 (2): E213–29. doi: 10.1152/ajpendo.00114.2013 PMID: 23695215

20. Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R et al. (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science 296 (5566): 349–352. PMID: 11951046

21. Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL (1998) Myocyte Enhancer Factor 2 (MEF2)-Binding Site Is Required forGLUT4 Gene Expression in Transgenic Mice: REGULATION OF MEF2 DNA BINDING ACTIVITY IN INSULIN-DEFICIENT DIABETES. Journal of Biological Chemistry 273 (23): 14285–14292. PMID: 9603935

22. Hood DA (2001) Invited Review: Contractile activity-induced mitochondrial biogenesis in skeletal muscle. Journal of Applied Physiology 90 (3): 1137–1157. PMID: 11181630

23. Lin J, Wu H, Tarr PT, Zhang C, Wu Z, Boss O et al. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418 (6899): 797–801. PMID: 12181572

24. Kubis HP, Haller EA, Wetzel P, Gros G (1997) Adult fast myosin pattern and Ca2+-induced slow myosin pattern in primary skeletal muscle culture. Proc Natl Acad Sci U S A 94 (8): 4205–4210. PMID: 9108130

25. Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13 (4): 251–262. doi: 10.1038/nrm3311 PMID: 22436748

26. Winder WW (2001) Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. J Appl Physiol (1985) 91 (3): 1017–1028. PMID: 11509493

7   DARSTELLUNG DES EIGENEN ANTEILS

- **Nox4 supports proper capillary growth in exercise and retina neo-vascularization**

  Ich war verantwortlich für die Planung, Durchführung und Datenerfassung, Auswertung und Interpretation sämtlicher Experimente, mit Ausnahme der kompletten Analyse von Gefäßstrukturen an der Retina der Maus (Präparation, Färbung, Bilderfassung, Auswertung).

- **Nox4 is dispensable for exercise induced muscle fibre switch**

  Ich war verantwortlich für die Planung, Durchführung und Datenerfassung, Auswertung und Interpretation sämtlicher Experimente.

8 LITERATURVERZEICHNIS

1. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab. 2013;17(2):162-184.
2. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. Molecular and cellular biochemistry. 2004;264(1-2):85-97.
3. Niess AM, Simon P. Response and adaptation of skeletal muscle to exercise--the role of reactive oxygen species. Frontiers in bioscience : a journal and virtual library. 2007;12:4826-4838.
4. Reid MB. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. Journal of applied physiology (Bethesda, Md. : 1985). 2001;90(2):724-731.
5. Reid MB, Khawli FA, Moody MR. Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. Journal of applied physiology (Bethesda, Md. : 1985). 1993;75(3):1081-1087.
6. Dröge W. Free radicals in the physiological control of cell function. Physiological Reviews. 2002;82(1):47-95.
7. McArdle A, Pattwell D, Vasilaki A, Griffiths RD, Jackson MJ. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. American journal of physiology. Cell physiology. 2001;280(3):7.
8. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. The Journal of clinical investigation. 2000;105(11):1631-1639.
9. Powers SK, Nelson WB, Hudson MB. Exercise-induced oxidative stress in humans: cause and consequences. Free radical biology & medicine. 2011;51(5):942-950.
10. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiological Reviews. 2011;91(4):1447-1531.
11. Yan Z, Okutsu M, Akhtar YN, Lira VA. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. J. Appl. Physiol. 2011;110(1):264-274.
12. Gorza L, Gundersen K, Lømo T, Schiaffino S, Westgaard RH. Slow-to-fast transformation of denervated soleus muscles by chronic high-frequency stimulation in the rat. The Journal of physiology. 1988;402:627-649.
13. Desaphy J-F, Pierno S, Liantonio A, et al. Recovery of the soleus muscle after short- and long-term disuse induced by hindlimb unloading: effects on the electrical properties and myosin heavy chain profile. Neurobiology of disease. 2005;18(2):356-365.
14. Nwoye L, Mommaerts WF. The effects of thyroid status on some properties of rat fast-twitch muscle. Journal of muscle research and cell motility. 1981;2(3):307-320.
15. Adamo ML, Farrar RP. Resistance training, and IGF involvement in the maintenance of muscle mass during the aging process. Ageing research reviews. 2006;5(3):310-331.
16. Howald H. Training-induced morphological and functional changes in skeletal muscle. International journal of sports medicine. 1982;3(1):1-12.
17. Häggmark T, Jansson E, Eriksson E. Fiber type area and metabolic potential of the thigh muscle in man after knee surgery and immobilization. International journal of sports medicine. 1981;2(1):12-17.

18. Hood DA. Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. Journal of applied physiology (Bethesda, Md. : 1985). 2001;90(3):1137-1157.

19. Kawakami M, Okabe E. Superoxide anion radical-triggered Ca2+ release from cardiac sarcoplasmic reticulum through ryanodine receptor Ca2+ channel. Molecular pharmacology. 1998;53(3):497-503.

20. Cherednichenko G, Zima AV, Feng W, Schaefer S, Blatter LA, Pessah IN. NADH oxidase activity of rat cardiac sarcoplasmic reticulum regulates calcium-induced calcium release. Circulation research. 2004;94(4):478-486.

21. Chin ER, Olson EN, Richardson JA, et al. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes & Development. 1998;12(16):2499-2509.

22. Kubis HP, Haller EA, Wetzel P, Gros G. Adult fast myosin pattern and Ca2+-induced slow myosin pattern in primary skeletal muscle culture. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(8):4205-4210.

23. Lin J, Wu H, Tarr PT, et al. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature. 2002;418(6899):797-801.

24. Olesen J, Kiilerich K, Pilegaard H. PGC-1alpha-mediated adaptations in skeletal muscle. Pflugers Archiv : European journal of physiology. 2010;460(1):153-162.

25. Yan Z. Exercise, PGC-1alpha, and metabolic adaptation in skeletal muscle. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2009;34(3):424-427.

26. Yan Z, Lira VA, Greene NP. Exercise training-induced regulation of mitochondrial quality. Exercise and sport sciences reviews. 2012;40(3):159-164.

27. Thai MV, Guruswamy S, Cao KT, Pessin JE, Olson AL. Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. The Journal of biological chemistry. 1998;273(23):14285-14292.

28. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature reviews. Molecular cell biology. 2012;13(4):251-262.

29. Richter EA, Hargreaves M. Exercise, GLUT4, and Skeletal Muscle Glucose Uptake. Physiological Reviews. 2013;93(3):993-1017.

30. Mungai PT, Waypa GB, Jairaman A, et al. Hypoxia Triggers AMPK Activation through Reactive Oxygen Species-Mediated Activation of Calcium Release-Activated Calcium Channels. Molecular and Cellular Biology. 2011;31(17):3531-3545.

31. O'Neill HM. AMPK and Exercise: Glucose Uptake and Insulin Sensitivity. Diabetes Metab J. 2013;37(1):1.

32. Waters RE, Rotevatn S, Li P, Annex BH, Yan Z. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. Am. J. Physiol., Cell Physiol. 2004;287(5):8.

33. Bloor CM. Angiogenesis during exercise and training. Angiogenesis. 2005;8(3):263-271.

34. Clapp C, Thebault S, Jeziorski MC, Martínez De La Escalera, Gonzalo. Peptide hormone regulation of angiogenesis. Physiol. Rev. 2009;89(4):1177-1215.

35. DiPietro LA. Angiogenesis and scar formation in healing wounds. Current opinion in rheumatology. 2013;25(1):87-91.

36. Wu Y, Tang L, Chen B. Oxidative stress: implications for the development of diabetic retinopathy and antioxidant therapeutic perspectives. Oxidative medicine and cellular longevity. 2014;2014:752387.

37. Kowluru RA, Chan P-S. Oxidative stress and diabetic retinopathy. Experimental diabetes research. 2007;2007:43603.

38. Todorich B, Yiu G, Hahn P. Current and investigational pharmacotherapeutic approaches for modulating retinal angiogenesis. Expert review of clinical pharmacology. 2014;7(3):375-391.

39. Okuno Y, Nakamura-Ishizu A, Otsu K, Suda T, Kubota Y. Pathological neoangiogenesis depends on oxidative stress regulation by ATM. Nat Med. 2012;18(8):1208-1216.

40. Wilkinson-Berka JL, Rana I, Armani R, Agrotis A. Reactive oxygen species, Nox and angiotensin II in angiogenesis: Implications for retinopathy. Clin. Sci. 2013;124(10):597-615.

41. Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX. Inhibition of Reactive Oxygen Species by Lovastatin Downregulates Vascular Endothelial Growth Factor Expression and Ameliorates Blood-Retinal Barrier Breakdown in db/db Mice: Role of NADPH Oxidase 4. Diabetes. 2010;59(6):1528-1538.

42. Powers SK, Talbert EE, Adhihetty PJ. Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. The Journal of physiology. 2011;589(9):2129-2138.

43. Jackson MJ. Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise. Philosophical Transactions of the Royal Society B: Biological Sciences. 2005;360(1464):2285-2291.

44. Stahl A, Connor KM, Sapieha P, et al. Computer-aided quantification of retinal neovascularization. Angiogenesis. 2009;12(3):297-301.

45. Petry A, Djordjevic T, Weitnauer M, Kietzmann T, Hess J, Görlach A. NOX2 and NOX4 mediate proliferative response in endothelial cells. Antioxidants & redox signaling. 2006;8(9-10):1473-1484.

46. Pendyala S, Gorshkova IA, Usatyuk PV, et al. Role of Nox4 and Nox2 in Hyperoxia-Induced Reactive Oxygen Species Generation and Migration of Human Lung Endothelial Cells. Antioxidants & redox signaling. 2009;11(4):747-764.

47. Datla SR, Peshavariya H, Dusting GJ, Mahadev K, Goldstein BJ, Jiang F. Important Role of Nox4 Type NADPH Oxidase in Angiogenic Responses in Human Microvascular Endothelial Cells In Vitro. Arteriosclerosis, thrombosis, and vascular biology. 2007;27(11):2319-2324.

48. Schröder K, Zhang M, Benkhoff S, et al. Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. Circulation research. 2012;110(9):1217-1225.

49. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. Circulation. 2005;111(18):2347-2355.

50. Brandes RP, Weissmann N, Schröder K. Nox family NADPH oxidases: Molecular mechanisms of activation. Free radical biology & medicine. 2014;76:208-226.

51. Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. Molecular cell. 2007;26(1):1-14.

52. Nisimoto Y, Diebold BA, Cosentino-Gomes D, Constentino-Gomes D, Lambeth JD. Nox4: a hydrogen peroxide-generating oxygen sensor. Biochemistry. 2014;53(31):5111-5120.

53. Helmcke I, Heumüller S, Tikkanen R, Schröder K, Brandes RP. Identification of Structural Elements in Nox1 and Nox4 Controlling Localization and Activity. Antioxidants & redox signaling. 2009;11(6):1279-1287.

54. Chen K, Kirber MT, Xiao H, Yang Y, Keaney JF. Regulation of ROS signal transduction by NADPH oxidase 4 localization. The Journal of cell biology. 2008;181(7):1129-1139.

55. Sun Q-A, Hess DT, Nogueira L, et al. Oxygen-coupled redox regulation of the skeletal muscle ryanodine receptor-Ca2+ release channel by NADPH oxidase 4. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(38):16098-16103.

56. Evangelista AM, Thompson MD, Bolotina VM, Tong X, Cohen RA. Nox4- and Nox2-dependent oxidant production is required for VEGF-induced SERCA cysteine-674 S-glutathiolation and endothelial cell migration. Free Radical Biology and Medicine. 2012;53(12):2327-2334.

57. Diebold I, Petry A, Hess J, Görlach A. The NADPH oxidase subunit NOX4 is a new target gene of the hypoxia-inducible factor-1. Molecular biology of the cell. 2010;21(12):2087-2096.

58. Narkar VA, Downes M, Yu RT, et al. AMPK and PPARdelta agonists are exercise mimetics. Cell. 2008;134(3):405-415.

59. Pette D, Staron RS. Myosin isoforms, muscle fiber types, and transitions. Microscopy research and technique. 2000;50(6):500-509.

60. Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. The American journal of physiology. 1996;270(2 Pt 1):304.

61. Irrcher I, Ljubicic V, Hood DA. Interactions between ROS and AMP kinase activity in the regulation of PGC-1 transcription in skeletal muscle cells. AJP: Cell Physiology. 2008;296(1):C116-C123.

62. Geng T, Li P, Okutsu M, et al. PGC-1 plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. AJP: Cell Physiology. 2010;298(3):C572-C579.

63. Rowe GC, El-Khoury R, Patten IS, Rustin P, Arany Z, Dzeja P. PGC-1α is Dispensable for Exercise-Induced Mitochondrial Biogenesis in Skeletal Muscle. PloS one. 2012;7(7):e41817.

64. Gallagher PM, Touchberry CD, Teson K, McCabe E, Tehel M, Wacker MJ. Effects of an acute bout of resistance exercise on fiber-type specific to GLUT4 and IGF-1R expression. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2013;38(5):581-586.

65. M Lehnen A. Changes in the GLUT4 Expression by Acute Exercise, Exercise Training and Detraining in Experimental Models. J Diabetes Metab. 2013;01(S10).

66. Daugaard, JR, Nielsen JN, Kristiansen S, Andersen JL, Hargreaves M, Richter EA. Fiber type-specific expression of GLUT4 in human skeletal muscle: influence of exercise training. Diabetes. 2000;49(7):1092-1095.

67. Stuart CA, Howell ME, Baker JD, et al. Cycle training increased GLUT4 and activation of mammalian target of rapamycin in fast twitch muscle fibers. Medicine and science in sports and exercise. 2010;42(1):96-106.

68. Richardson RS, Grassi B, Gavin TP, et al. Evidence of O2 supply-dependent VO2 max in the exercise-trained human quadriceps. Journal of applied physiology (Bethesda, Md. : 1985). 1999;86(3):1048-1053.

69. Tesch PA. Skeletal muscle adaptations consequent to long-term heavy resistance exercise. Medicine and science in sports and exercise. 1988;20(5 Suppl):4.

70. Delavar H, Nogueira L, Wagner PD, Hogan MC, Metzger D, Breen EC. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. American journal of physiology. Regulatory, integrative and comparative physiology. 2014;306(8):95.

71. Olfert IM, Howlett RA, Tang K, et al. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. The Journal of physiology. 2009;587(Pt 8):1755-1767.

72. Pullar JM. Diphenyleneiodonium Triggers the Efflux of Glutathione from Cultured Cells. Journal of Biological Chemistry. 2002;277(22):19402-19407.

73. Zhang M, Brewer AC, Schröder K, et al. NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(42):18121-18126.

74. Tang K, Xia FC, Wagner PD, Breen EC. Exercise-induced VEGF transcriptional activation in brain, lung and skeletal muscle. Respiratory physiology & neurobiology. 2010;170(1):16-22.

75. Segarra M, Ohnuki H, Maric D, et al. Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. Blood. 2012;120(19):4104-4115.

76. Olenich SA, Gutierrez-Reed N, Audet GN, Olfert IM. Temporal response of positive and negative regulators in response to acute and chronic exercise training in mice. The Journal of physiology. 2013;591(Pt 20):5157-5169.

77. Ribatti D, Nico B, Crivellato E. The role of pericytes in angiogenesis. The International journal of developmental biology. 2011;55(3):261-268.

78. Eklund L, Saharinen P. Angiopoietin signaling in the vasculature. Experimental cell research. 2013;319(9):1271-1280.

79. Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes & Development. 1999;13(9):1055-1066.

80. Hoier B, Nordsborg N, Andersen S, et al. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. The Journal of physiology. 2012;590(Pt 3):595-606.

81. Stahl A, Connor KM, Sapieha P, et al. The mouse retina as an angiogenesis model. Invest. Ophthalmol. Vis. Sci. 2010;51(6):2813-2826.

82. Wang H, Yang Z, Jiang Y, Hartnett ME. Endothelial NADPH oxidase 4 mediates vascular endothelial growth factor receptor 2-induced intravitreal neovascularization in a rat model of retinopathy of prematurity. Molecular vision. 2014;20:231-241.

83. Lee J, Kim KE, Choi D-K, et al. Angiopoietin-1 guides directional angiogenesis through integrin αvβ5 signaling for recovery of ischemic retinopathy. Science translational medicine. 2013;5(203):203.

84. Wilkinson-Berka JL, Deliyanti D, Rana I, et al. NADPH oxidase, NOX1, mediates vascular injury in ischemic retinopathy. Antioxidants & redox signaling. 2014;20(17):2726-2740.

85. Li J, Wang JJ, Zhang SX. NADPH oxidase 4-derived H_2O_2 promotes aberrant retinal neovascularization via activation of VEGF receptor 2 pathway in oxygen-induced retinopathy. Journal of diabetes research. 2015;2015:963289.

86. Strobel NA, Peake JM, Matsumoto A, Marsh SA, Coombes JS, Wadley GD. Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. Medicine and science in sports and exercise. 2011;43(6):1017-1024.

87. Venditti P, Napolitano G, Barone D, Di Meo S. Vitamin E supplementation modifies adaptive responses to training in rat skeletal muscle. Free radical research. 2014;48(10):1179-1189.
88. Chan EC, van Wijngaarden P, Liu G-S, Jiang F, Peshavariya H, Dusting GJ. Involvement of Nox2 NADPH oxidase in retinal neovascularization. Investigative ophthalmology & visual science. 2013;54(10):7061-7067.
89. Dusting GJ, Peshavariya H, Chan E, van Wijngaarden P, Liu G-S. NADPH Oxidase Signaling Crucial for Neovascularization in Oxygen-induced Retinopathy in Mice: Nox2, Nox4 and VEGF Signaling. Investigative ophthalmology & visual science. 2014;55(13):1265.

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10 SCHRIFTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Nox4 in Sport-induzierter Angiogenese

in dem Institut für Physiologie I (Kardiovaskuläre Physiologie) unter Betreuung und Anleitung von Prof. Dr. Katrin Schröder ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

Vogel J, Kruse C, Zhang M, Schröder K, Nox4 supports proper capillary growth in exercise and retina neo-vascularization, The journal of physiology, 593(9), 2145-2154, 2015

Vogel J, Figueiredo de Rezende F, Rohrbach S, Zhang M, Schröder K, Nox4 is dispensable for exercise induced muscle fibre switch, PloS one, 10(6), e0130769, 2015

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      (Ort, Datum)   (Unterschrift)