The use of site-specific suppressors to measure the relative contributions of different mitochondrial sites to skeletal muscle superoxide and hydrogen peroxide production

Renata L.S. Goncalves[1,3], Mark A. Watson[3], Hoi-Shan Wong[3], Adam L. Orr[2], Martin D. Brand[*]

Buck Institute for Research on Aging, 8001 Redwood Blvd, Novato, CA, 94945, USA

A B S T R A C T

Reactive oxygen species are important signaling molecules crucial for muscle differentiation and adaptation to exercise. However, their uncontrolled generation is associated with an array of pathological conditions. To identify and quantify the sources of superoxide and hydrogen peroxide in skeletal muscle we used site-specific suppressors (S1QELs, S3QELs and NADPH oxidase inhibitors). We measured the rates of hydrogen peroxide release from isolated rat muscle mitochondria incubated in media mimicking the cytosol of intact muscle. By measuring the extent of inhibition caused by the addition of different site-specific suppressors of mitochondrial superoxide/hydrogen peroxide production (S1QELs for site I_Q and S3QELs for site III_{Qo}), we determined the contributions of these sites to the total signal. In media mimicking resting muscle, their contributions were each 12–18%, consistent with a previous method. In C2C12 myoblasts, site I_Q contributed 12% of cellular hydrogen peroxide production and site III_{Qo} contributed about 30%. When C2C12 myoblasts were differentiated to myotubes, hydrogen peroxide release increased five-fold, and the proportional contribution of site I_Q doubled. The use of S1QELs and S3QELs is a powerful new way to measure the relative contributions of different mitochondrial sites to muscle hydrogen peroxide production under different conditions. Our results show that mitochondrial sites I_Q and III_{Qo} make a substantial contribution to superoxide/hydrogen peroxide production in muscle mitochondria and C2C12 myoblasts. The total hydrogen peroxide release rate and the relative contribution of site I_Q both increase substantially upon differentiation to myotubes.

1. Introduction

Different cellular compartments and different sources within those compartments can generate superoxide and hydrogen peroxide (H_2O_2) under diverse conditions [2,6]. Historically, mitochondria were considered the main cellular source of superoxide and hydrogen peroxide, particularly during muscle contraction and exercise [21,27,28]. However, more recent evidence also implicates NADPH oxidases (NOXs) as important sources [10,18,27].

Mitochondria generate superoxide and/or H_2O_2 from multiple sites whose contributions vary with metabolic state. Under contrived conditions of substrate supply and inhibition of normal electron transport, electrons have been shown to escape from at least 11 sites to reduce oxygen to superoxide and/or H_2O_2 (Fig. 1A). These sites have different maximum rates, also described as different capacities (Fig. 1B). Under less artificial conditions, these sites will operate below their maximum capacities (Fig. 1B native *ex vivo* rate and red stacked bars). In intact cells and tissues, where multiple substrates are oxidized simultaneously, the overall mitochondrial superoxide/H_2O_2 production is the summed rate from all sites. However, a major roadblock in our understanding of the physiological and pathological roles of specific sites of superoxide/H_2O_2 production has been a lack of tools to unequivocally identify the relative contributions of individual sites within the total. Different approaches have been used to assess the sites generating superoxide/H_2O_2 but each presents varying degrees of specificity and experimental and analytical complexity.

Interruption of cellular respiration, using classical inhibitors of the electron transport chain and genetic interventions, is now routine.

Fig. 1. Mitochondrial sites of superoxide and/or H_2O_2 production. **(A)** Sites associated with electron transport that generate superoxide/H_2O_2 (red circles). Sites are associated with either the NAD (orange) or the ubiquinone (Q) pool (green). In the former, superoxide/H_2O_2 is produced at the flavin binding site (F) of the dehydrogenase complexes of branched chain 2-oxoacids (BCOADH, site B_F), 2-oxoadipate (OADH, site A_F), pyruvate (PDH, site P_F) and 2-oxoglutarate (OGDH, site O_F); these complexes feed electrons to the NAD pool. The second group uses Q as electron acceptor and superoxide/H_2O_2 is produced at the Q binding sites of complex III (site III_{Qo}), mitochondrial glycerol 3-phosphate dehydrogenase (GPDH, site G_Q) and dihydroorotate dehydrogenase (DHODH, site D_Q) and also at the flavin binding sites of complex II (site II_F) and the electron transfer flavoprotein and ETF:Q oxidoreductase (ETF:QOR) system, (site E_F). Complex I generates superoxide/H_2O_2 from the flavin (I_F) and Q sites (I_Q). Enzymes using the same acceptor are in orange (NAD^+) or green (Q). **(B)** Maximum capacities and native rates of the sites in isolated skeletal muscle mitochondria (data from Refs. [3,10]). Capacities were defined using saturating substrate concentrations and pharmacologically restricting electron flux out of the targeted site. For comparison, the native rates of superoxide/H_2O_2 release from skeletal muscle mitochondria *ex vivo* at "rest" and "exercise" [10] are plotted on the same scale. Stacked red bars adjacent to the left axis represent the contribution of each site *ex vivo* in relation to its maximum capacity. **(C)**

Relative contributions of mitochondrial sites to superoxide/H_2O_2 production *ex vivo*. During the oxidation of a complex mixture of substrates mimicking the cytosol of skeletal muscle at rest and during exercise different sites contribute to the total superoxide/H_2O_2 measured. At "rest" sites II_F, I_Q and I_F are major contributors, generating 24%, 23% and 20%, respectively. Sites III_{Qo} and perhaps E_F are minor contributors, accounting for 15% and 13% respectively. During "exercise" total H_2O_2 is significantly decreased and site I_F dominates, generating 44% of total superoxide/H_2O_2. Data from Ref. [10]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

However, the effects of such manipulations on superoxide/H_2O_2 production are very difficult to interpret unambiguously since they invariably alter electron and metabolite fluxes and thus alter superoxide/H_2O_2 production from more than one site. For example, when a particular step in electron transport is inhibited, upstream sites will become more reduced and tend to increase their superoxide/H_2O_2 production, whereas downstream sites will become more oxidized and tend to decrease their superoxide/H_2O_2 production. These effects fatally confound interpretation of direct effects at an intended target site with secondary effects at other sites connected by electron or metabolite flows [3].

An accurate, though more labor-intensive, approach is the use of endogenous reporters to assess the contributions from sites I_F and III_{Qo}. Previously, we demonstrated that the rate of superoxide/H_2O_2 generation at site I_F in complex I can be calibrated to the reduction state of matrix NAD(P)H [22,24]. Similarly, the rate of superoxide generation at site III_{Qo} in complex III can be calibrated to the reduction state of cytochrome b_{566} [24]. Therefore, NAD(P)H and cytochrome b_{566} can be used as endogenous reporters to enable estimation of the rates of superoxide/H_2O_2 production from sites I_F and site III_{Qo}, respectively, under diverse conditions [22,24]. This approach also allows estimation of the rates from other sites by using appropriate inhibitors of those sites and correcting for secondary changes in the redox states of NAD(P)H and cytochrome b_{655} induced by the inhibitors [10,19,23]. Using isolated mitochondria incubated in complex media designed to mimic skeletal muscle cytosol *in vivo*, these methods revealed for the first time the rate of superoxide/H_2O_2 formation from distinct sites *ex vivo*. Under conditions mimicking resting muscle, sites I_Q and II_F generated the majority of superoxide/H_2O_2, followed by sites III_{Qo} and I_F, with a possible minor contribution from site E_F (Fig. 1C). In medium mimicking exercise, the contributions of most sites decreased and site I_F became the main source of superoxide/H_2O_2 [10]. Because the rates of superoxide/H_2O_2 production from each site can increase or decrease independently under different conditions, it is reasonable to speculate that one or more sites will alter their superoxide/H_2O_2 production during cellular differentiation or during the transition from a healthy to a disease state.

An alternative way to assess the relative contribution of an individual site is to selectively suppress its ability to leak electrons to oxygen, and measure the decrease in total superoxide/H_2O_2 release. We have discovered small molecule site-selective suppressors of mitochondrial superoxide/H_2O_2 production [4,16,17] and pioneered their use for this purpose. S1QELs (Suppressors of site I_Q Electron Leak; "Cycles") and S3QELs (Suppressors of site III_{Qo} Electron Leak; "Sequels") are distinct from classical electron transport inhibitors and antioxidants. At appropriate concentrations they inhibit superoxide/H_2O_2 production from a single site (I_Q and III_{Qo}, respectively), yet do not block electron flow or substrate utilization and do not disturb membrane potential or energy transduction [4,17]. These compounds not only prevent superoxide/H_2O_2 production in isolated mitochondria, but also show protective effects *in situ* and *in vivo* [4,17]. Addition of S3QELs, S1QELs and NADPH oxidase inhibitors (GKT136901 or ML171) to C2C12 myoblasts demonstrated that site III_{Qo}, site I_Q and NADPH oxidases contribute 30%, 15% and 40%, respectively, to the total rate of H_2O_2 release to the extracellular medium [32]. This finding

supported previous evidence that NOXs are a major source of H_2O_2 in muscle [10,21,27,28] and suggested that mitochondria are equally important in muscle myoblasts.

Here we show that S1QELs and S3QELs suppress superoxide/H_2O_2 generation in isolated mitochondria suspended in a complex mixture of substrates mimicking skeletal muscle at rest. Importantly, the S1QEL- and S3QEL-sensitive rates are indistinguishable from measurements made using endogenous reporters, validating the use of S1QELs and S3QELs in such assays. We extend the use of S1QELs, S3QELs and GKT136901 in cells [32] to estimate the contributions of site I_Q, site III_{Qo} and NOXs to the total extracellular H_2O_2 released over the time course of differentiation of C2C12 myoblasts to myotubes. We conclude that S1QELs and S3QELs, if titrated carefully, reliably quantify the relative contributions of sites I_Q and III_{Qo} to cellular H_2O_2 production in complex model systems and that the contribution of site I_Q increases disproportionately during C2C12 cell differentiation.

2. Materials and methods

Animals, Mitochondria, and Reagents – Female Wistar rats were from Charles River Laboratories, age 5–10 weeks, and fed chow *ad libitum* with free access to water. Mitochondria were isolated from hind limb skeletal muscle at 4 °C in Chappell-Perry buffer (CP1; 100 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.4, at 4 °C) by standard procedures and kept on ice until use (up to ~5 h). Protein was measured by the biuret method. The animal protocol was approved by the Buck Institute Animal Care and Use Committee in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Reagents were from Sigma except when otherwise stated. S1QELs and S3QELs were from the sources described previously [4,17,32].

Oxygen Consumption and Superoxide/H_2O_2 Release – Skeletal muscle mitochondria (0.3 mg of protein $\cdot$ ml^{-1}) were incubated at 37 °C for 10 min in "basic medium" (described below) [10]. ATP (6 mM) was added into the chamber, followed after 1 min by the "complex substrate mix" to mimic the cytosol of skeletal muscle during rest [10]. Rates of superoxide/H_2O_2 release were measured collectively as rates of H_2O_2 production as two superoxide molecules are dismutated by endogenous or exogenous superoxide dismutase to yield one H_2O_2. H_2O_2 was detected using 5 U ml^{-1} horseradish peroxidase and 50 μM Amplex UltraRed in the presence of 25 U ml^{-1} superoxide dismutase in a Varian Cary Eclipse spectrofluorometer (excitation 560 nm, emission 590 nm) with constant stirring and calibrated with known amounts of H_2O_2 in the presence of all relevant additions (because some reagents quenched fluorescence). For the experiments performed with S3QEL2.2, S3QEL3, S1QEL1.1 and S1QEL2.1, mitochondria were incubated in basic medium for 10 min at 37 °C, then added to a black 96-well plate with clear flat bottom (VWR, Radnor, PA; Cat No. 89091-012) containing the basic medium, complex substrate mix, and different S1QEL/S3QEL concentrations or DMSO. The rate of H_2O_2 release was measured by the change in fluorescence signal (excitation 540 nm, emission 590 nm) for 30 min at 37 °C using a PHERAStar FS platereader.
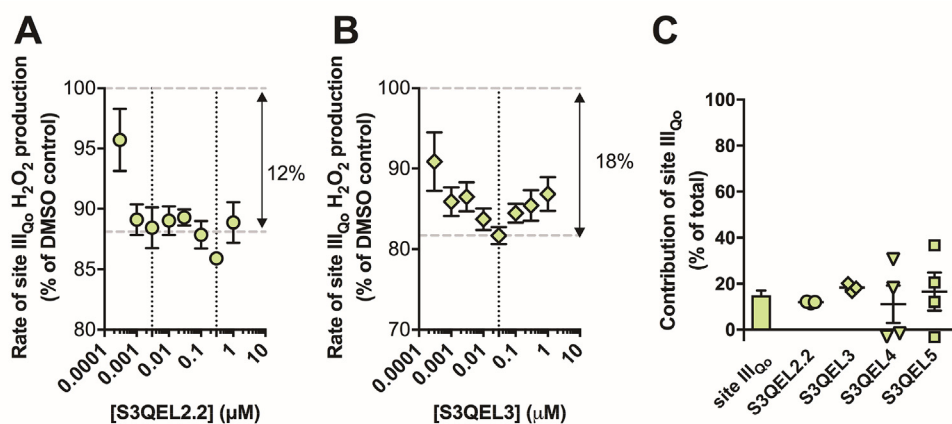
Basic medium – 40 mM taurine, 3 mM KH_2PO_4, 4 mM NaCl, 52.85 mM KCl, 5.46 mM $MgCl_2$ (targeted free Mg^{2+} concentration 600 μM), 0.214 mM $CaCl_2$ (targeted Ca^{2+} concentration 0.05 μM), 10 mM HEPES, 2 mM EGTA, 0.3% w/v fatty acid-free bovine serum albumin, 1 μg ml^{-1} oligomycin (to prevent oxidative phosphorylation of ADP generated by contaminating extramitochondrial ATPases, and to keep the mitochondria in the required "resting" state), pH 7.1. Targeted Na^+ concentration was 16 mM and total K^+ concentration was 80 mM. The medium had K^+ and Cl^- adjusted to give an osmolarity of 290 mosM. Total Mg^{2+} and Ca^{2+} concentrations to give the targeted free values were calculated using the software MaxChelator [10]. Physiological concentrations of bicarbonate were omitted for technical reasons.

Complex substrate mix – 100 μM acetoacetate (Sigma Cat No. A8509), 300 μM 3-hydroxybutyrate (cat No. 54965), 2500 μM alanine (cat No. A7627), 500 μM arginine (cat No. A5006), 1500 μM aspartate (Cat No. 11189), 1500 μM glutamate (cat No. 49621), 6000 μM glutamine (cat No. G3126), 7000 μM glycine (cat No. G8898), 150 μM isoleucine (cat No. I2752), 200 μM leucine (cat No. L8000), 1250 μM lysine (cat No. L5501), 500 μM proline (cat No. P1847), 2000 μM serine (cat No. S4500), 300 μM valine (cat No. V0500), 100 μM citrate (cat No. C0759), 200 μM malate (cat No. M1750), 30 μM 2-oxoglutarate (cat No. 75890), 100 μM pyruvate (cat No. P2256), 200 μM succinate (cat No. S3674), 100 μM glycerol-3-phosphate (cat No. G6501), 50 μM dihydroxyacetone phosphate (cat No. 37442), 1000 μM carnitine (cat No. C0158), 500 μM acetylcarnitine (cat No. A6706), 10 μM palmitoylcarnitine (cat No. 4509) [10]. 6000 μM ATP (cat No. A7699) was also added to this mix for PHERAStar assays.

C2C12 myoblasts – C2C12 cells (C3H mouse skeletal muscle-derived myoblasts) from the American Type Culture Collection were cultured under 5% (v/v) CO_2 in air at 37 °C in growth medium (Dulbecco's modified Eagle's medium (Corning, Corning, NY; Cat No. 10–013), supplemented with 10% v/v fetal bovine serum (Gemini Bio Products, Sacramento, CA), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). The differentiation of C2C12 myoblasts to myotubes was induced by replacing growth medium with differentiation medium (Dulbecco's modified Eagle's medium (Corning, Corning, NY; Cat No. 10–013), supplemented with 2% v/v horse serum (Gemini Bio Products, Sacramento, CA), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). C2C12 myoblasts were seeded at a density of 10,000 to 12,000 cells/well and allowed to attach for 24 h in a 96-well microtiter plate (black, flat bottom). Growth medium was then switched to differentiation medium for 1–4 days to induce C2C12 differentiation. Differentiation medium was changed daily. Myogenic differentiation was confirmed by creatine kinase activity [5,9] determined in cell lysates using an NADPH-coupled assay following the protocol supplied by Sigma-Aldrich (Cat. #MAK116; St Louis, MA). Results are expressed as Units (U) per mg protein in the cell lysate.

Rate of H_2O_2 release from C2C12 myoblasts – Rates of H_2O_2 release were assessed using the fluorescent probe Amplex UltraRed (Invitrogen) as described [32]. The measurement is based on the horseradish peroxidase (HRP)-mediated oxidation of Amplex UltraRed (non-fluorescent) to Amplex UltroxRed (fluorescent) by H_2O_2. Any extracellular release of superoxide was also captured by the addition of excess exogenous superoxide dismutase 1 (SOD1) to convert superoxide to H_2O_2. We assume that intracellular superoxide/H_2O_2 production is irreversible, so lowering extracellular H_2O_2 levels by the added HRP will not alter production rates. On the day of experiment, cells were incubated with pre-warmed Krebs Ringer Modified Buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM HEPES, 1 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4 at 37 °C) supplemented with 0.1% w/v bovine serum albumin (KRB-BSA) at 37 °C for 30 min. The BSA content in KRB-BSA was determined empirically to optimize signal-to-noise ratio. The measurement of H_2O_2 release was initiated by changing KRB-BSA to a pre-warmed assay buffer containing 25 μM Amplex UltraRed, 5 U ml^{-1} HRP and 25 U ml^{-1} SOD1 in KRB-BSA. Changes in fluorescence signal (excitation 540 nm, emission 590 nm; top optics) were monitored for 60 cycles (~68 min) at 37 °C using a PHERAStar FS platereader. After the measurement, the reaction mixture was aspirated and the cells were washed once with phosphate-buffered saline-A (PBS-A). The cells were lysed using 0.1% (v/v) Triton X-100 and the protein content was assessed using a Bio-Rad protein assay kit. Measured fluorescence in the operational range was linearly dependent on H_2O_2 added during signal calibration; fluorescence values were converted to pmol H_2O_2 using plate-matched calibration curves. The measured H_2O_2 release rate was constant between 20 and 40 min from the start of the measurement, and was calculated as the slope of a plot of measured H_2O_2 content against time after subtraction of the small rate of H_2O_2 release in the absence of cells in parallel wells for each experiment. The rates of H_2O_2 release were then normalized to the

Fig. 2. Use of S3QELs to assess the relative contribution of site III_{Qo} to the rate of superoxide/H_2O_2 production by skeletal muscle mitochondria *ex vivo* at "rest". Mitochondrial H_2O_2 release rate was measured in a complex buffer mimicking the cytosol of skeletal muscle at rest in the presence of different concentrations of **(A)** S3QEL2.2 and **(B)** S3QEL3. Horizontal dashed lines indicate uninhibited and maximally suppressed rates. Vertical dotted lines define the range of S3QEL concentrations used to calculate maximum suppression. Values are means ± SEM (N = 3 independent experiments). **(C)** Contribution of site III_{Qo} at "rest" comparing the rate reported by the reduction state of cytochrome b_{566} calibrated against the rate of H_2O_2 production from site III_{Qo} (green bar, data from Ref. [10], SEM calculated by error propagation) with the rates obtained using S3QELs. The concentrations of S3QEL2.2 and S3QEL3 used to define the contribution of site III_{Qo} were 0.003–0.3 μM and 0.03 μM, respectively; S3QEL4 and S3QEL5 were used at 10 μM. The contributions estimated using each individual S3QEL in (*C*) were not significantly different (P > 0.89 by one-way ANOVA and Dunnett's post-test) from the value reported in Ref. [10]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cell protein content of each well and expressed as pmol $H_2O_2 \cdot min^{-1} \cdot$ mg protein^{-1}. The contributions of NOXs, site I_Q and site III_{Qo} to total H_2O_2 release were assessed by supplementing the assay buffer with NOX inhibitor (GKT136901), S1QEL or S3QEL. The NOX inhibitor decreased the response of the HRP-Amplex UltraRed assay to added H_2O_2; this effect was corrected by calibrating in the presence of inhibitor.

3. Results and discussion

S3QELs report superoxide production from the outer ubiquinone binding site of complex III, site III_{Qo} – We first used four structurally distinct S3QELs [24] to assess the contribution of site III_{Qo} in mitochondria isolated from rat skeletal muscle. The mitochondria were incubated in a complex medium mimicking rat muscle cytosol at rest (see Materials and Methods for composition of the medium) [10]. Despite their structural differences, S3QELs consistently suppressed 11–18% of the total rate of superoxide/H_2O_2 release under this condition (Fig. 2A–C). This is statistically indistinguishable from the 15 ± 2% assigned previously to site III_{Qo} [10] under the same conditions using cytochrome b_{566} as an endogenous reporter (Fig. 2C green bar). The increase in superoxide/H_2O_2 release measured at higher S3QEL3 concentrations is likely due to off-target effects and emphasizes the need for careful titration of S3QELs; the values at these higher concentrations were excluded from estimates.

S1QELs report superoxide/H_2O_2 production from the ubiquinone binding site of complex I, site I_Q – Complex I produces superoxide/H_2O_2 from two distinct sites [30]: site I_F and site I_Q. Site I_F has a lower capacity for superoxide/H_2O_2 generation than site I_Q (Fig. 1B), which is active during reverse electron transfer through complex I [8,11,12,26,30]. Site I_Q has emerged as an important source of superoxide not only in skeletal muscle mitochondria under conditions mimicking resting muscle [10], but also in unstimulated C2C12 myoblasts [32], ischemia-reperfusion [7], immune signaling [15] and aging [29]. S1QELs have proven useful for defining the contribution of site I_Q under physio-pathological conditions in isolated mitochondria, *in situ* and *in vivo* [4,10,31,32].

To determine the contribution of site I_Q to the total rate of superoxide/H_2O_2 release at "rest", three distinct S1QELs from structurally different families [4,16] were employed. CN-POBS, S1QEL1.1 and S1QEL2.1 suppressed 23%, 13% and 15% of the total superoxide/H_2O_2 release, respectively (Fig. 3A–C). Nigericin, which suppresses superoxide/H_2O_2 production at site I_Q by dissipating the mitochondrial pH gradient [12] also inhibited 24% of the total signal (Fig. 3C). The
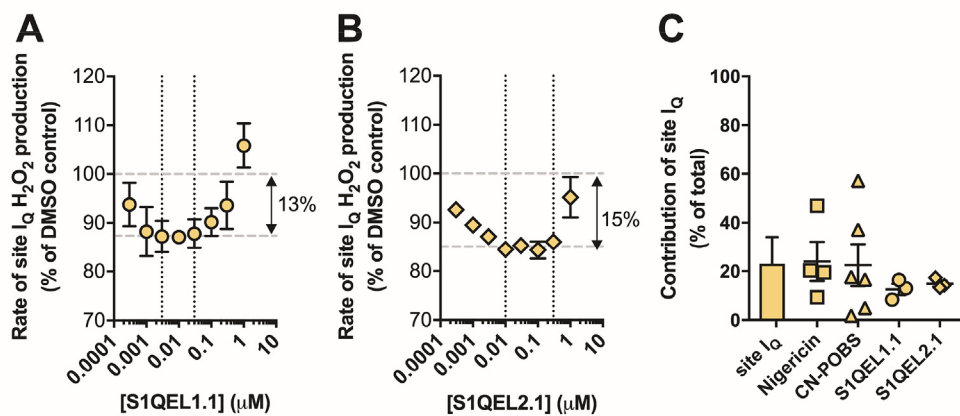
relative contribution of site I_Q to the total superoxide/H_2O_2 produced at "rest" defined using S1QELs or nigericin is statistically indistinguishable from the 23 ± 11% assigned previously to site I_Q in this condition [10] using rotenone after correcting for the redox changes caused in sites I_F and III_{Qo} (Fig. 3C orange bar). The increase in superoxide/H_2O_2 release measured at higher S1QEL concentrations is likely due to off-target effects and emphasizes the need for careful titration of S1QELs; the values at these higher concentrations were excluded from estimates.

Taken together, S1QELs and S3QELs reliably report the contributions from sites I_Q and III_{Qo} when isolated mitochondria oxidize a complex mixture of substrates.

Relative contributions of NOXs and mitochondrial sites I_Q and III_{Qo} to H_2O_2 production during C2C12 differentiation to myotubes – During muscle differentiation, mitochondrial biogenesis and bioenergetic remodeling culminate in greater mitochondrial density as well as increased total superoxide/H_2O_2 production [14,25]. This boost in superoxide/H_2O_2 output is considered an essential regulator of this process since antioxidants impair muscle differentiation [13,14,20]. In undifferentiated C2C12 myoblasts, NOXs (mostly NOX4, functionally cytosolic) are major contributors to H_2O_2 production (~40% of the total) [32], as are mitochondria, which account for 45% of the total. The major mitochondrial sources are sites I_Q and III_{Qo}, which contribute ~15% and 30% of total H_2O_2 respectively [32]. The remaining 15% originates from undefined sources [32].

In differentiation medium (see Materials and Methods), C2C12 myoblasts differentiated to myotubes in 3–4 days (Fig. 4A, black line indicating creatine kinase activity). To understand the dynamics and the sources of H_2O_2 generated during differentiation, we measured extracellular H_2O_2 release rates at different stages of differentiation in the presence or absence of the NOX inhibitor (GKT136901), S3QEL1.2 or S1QEL2.1. Basally (day 0), C2C12 myoblasts released 12 pmol H_2O_2 min^{-1} mg protein^{-1} (Fig. 4), which is consistent with our previous observations [32]. After 24 h in differentiation medium, the rate of H_2O_2 release increased by ~50% (to 18 pmol H_2O_2 min^{-1} mg protein^{-1}). Fully differentiated C2C12 myotubes released H_2O_2 at 54–60 pmol H_2O_2 min^{-1} mg protein^{-1}, 5-fold faster than C2C12 myoblasts. In agreement with our previous report [32], in undifferentiated C2C12 myoblasts NOXs were a major source of H_2O_2 (~32% of H_2O_2 released), and mitochondria contributed at least 38% (site III_{Qo} 27% and site I_Q 12%) with the remaining 30% generated by undefined sources. The rates of H_2O_2 release attributed to NOXs, site III_{Qo} and site I_Q were each significantly increased in fully differentiated myotubes compared to myoblasts (Fig. 4A, blue, green and orange

Fig. 3. Use of S1QELs to assess the relative contribution of site I_Q to the rate of superoxide/H_2O_2 production by skeletal muscle mitochondria *ex vivo* at "rest". Mitochondrial H_2O_2 release rate was measured in a complex buffer mimicking the cytosol of skeletal muscle at rest in the presence of different concentrations of **(A)** S1QEL1.1 and **(B)** S1QEL2.1. Horizontal dashed lines indicate uninhibited and maximally suppressed rates. Vertical dotted lines define the range of S1QEL concentrations used to calculate maximum suppression. Values are means ± SEM (N = 3 independent experiments) **(C)** Contribution of site I_Q at "rest" comparing the rate inhibited by 4 μM rotenone after correction for changes in sites I_F and III_{Qo} using the calibration curves previously reported (orange bar; data from Ref. [10], SEM calculated by error propagation) with the rates obtained using nigericin and S1QELs. The concentrations used to define the contribution of site I_Q were 0.003–0.03 μM for S1QEL1.1 and 0.01–0.3 μM for S1QEL2.1. Nigericin was used at 0.1 μM and CN-POBS at 2.5 μM (CN-POBS data was corrected for partial inhibition, and is taken from Ref. [10]). The contributions estimated using nigericin, CN-POBS, S1QEL1.1 and S1QEL2.1 in (C) were not significantly different (P > 0.92 by one-way ANOVA and Dunnett's post-test) from the value reported in Ref. [10]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
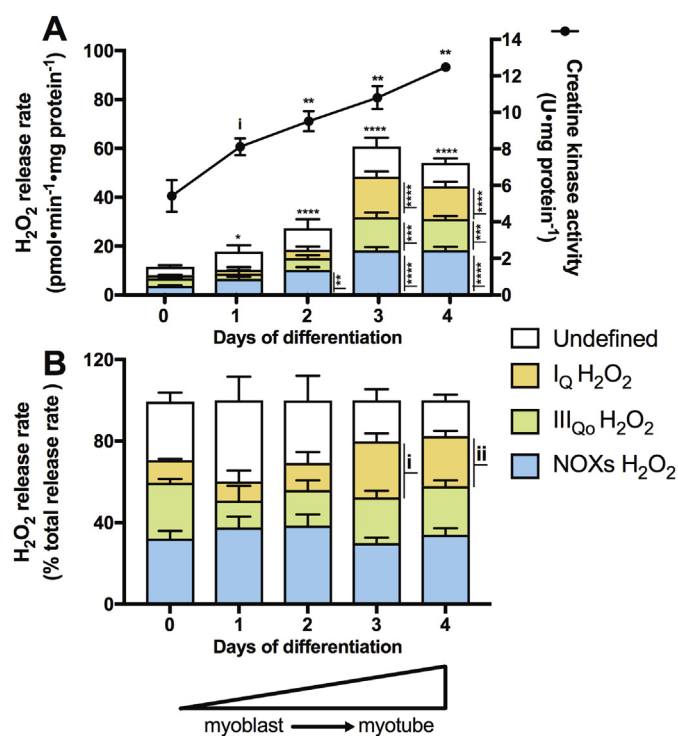
Fig. 4. Sources of H_2O_2 release from C2C12 myoblasts at different times during differentiation to myotubes. The contributions of NOXs, site I_Q and site III_{Qo} were determined as the differences between the rates of H_2O_2 release in the absence and presence of NOX inhibitor (10 μM GKT136901), S1QEL (0.03 μM S1QEL2.1) and S3QEL (10 μM S3QEL1.2) [32]. In **(A)** the total bar height shows the rates in the absence of inhibitors or suppressors and the stacked bars show the contributions of each individual site; "undefined" sources were calculated by difference. Creatine kinase activity reports differentiation to myotubes. **(B)** Contribution from each site in (A) expressed as % of total uninhibited rate on each day after differentiation. Values are means ± SEM (N = at least 3 independent experiments). Changes in creatine kinase activity (A) and in the absolute (A) or relative (B) rates of H_2O_2 release during differentiation were analyzed by one-way ANOVA and Dunnett's post-test with day 0 as control. *p < 0.05; ***p < 0.001; ****p < 0.0001, i, p = 0.054 and ii, p = 0.094. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stacked bars). Strikingly, the relative contribution from site I_Q increased > 2-fold during C2C12 differentiation (Fig. 4B, orange bars) whereas the relative contributions from NOXs and site III_{Qo} did not change. In differentiated myotubes, mitochondria were the major contributors to the rate of H_2O_2 production, accounting for nearly half the total, with sites III_{Qo} and I_Q contributing ~24% each. NOXs contributed ~34%, with the remaining 18% originating from unidentified sources (Fig. 4B).

Complex I protein content is known to increase disproportionally during muscle differentiation [13,14,25]. Site I_Q is active during reverse electron transport, when the ubiquinone reduction state and mitochondrial proton motive force are elevated [26,30], and superoxide/H_2O_2 production through reverse electron transport is important for muscle differentiation [13]. Our results corroborate these findings and indicate that site I_Q becomes more active during the differentiation of C2C12 myoblasts into myotubes.

Conclusion – In the present paper we show that, when properly titrated, S1QELs and S3QELs are reliable and convenient tools to measure the relative contributions of mitochondrial sites I_Q and III_{Qo} to superoxide and H_2O_2 production in different biological systems.

In isolated skeletal muscle mitochondria incubated in a complex medium mimicking muscle cytosol at rest, the contribution of site I_Q to superoxide/H_2O_2 production measured using three structurally distinct S1QELs (CN-POBS, S1QEL1.1 and S1QEL2.1) was 13–23%, and the contribution of site III_{Qo} measured using four structurally distinct S3QELs (S3QEL2.2, S3QEL3. S3QEL4 and S3QEL5) was 11–18%. These values are indistinguishable from corresponding values (15% and 23% respectively) previously reported using rotenone and myxothiazol after labor-intensive corrections for their secondary effects on sites I_F and III_{Qo} using NAD(P)H and cytochrome b_{566} calibration curves [10].

A NOX inhibitor, a S1QEL and a S3QEL were used to estimate the relative contributions of NOXs and mitochondria, specifically sites I_Q and III_{Qo}, to H_2O_2 production in C2C12 cells. During the differentiation of C2C12 myoblasts to myotubes, the rate of H_2O_2 appearance in the medium (i.e. the steady-state concentration of H_2O_2 in the cytosol) increased five-fold. Strikingly, although the relative contribution of NOXs was similar throughout C2C12 differentiation, the contribution of mitochondrial site I_Q more than doubled in fully differentiated myotubes.

The mechanisms of action of S3QELs and S1QELs have not been fully explored [4,17]. It has been proposed that S1QELs suppress superoxide/H_2O_2 production at site I_Q by binding to the ND1 subunit of complex I and inhibiting reverse electron flow [1]. However, such

inhibition occurs only at high concentrations of particular S1QELs, and we have shown that inhibition of reverse electron flow and suppression of superoxide/H_2O_2 production are not generally related [33]. Instead we propose that S1QELs suppress superoxide/H_2O_2 production at site I_Q by inducing structural changes in the ubiquinone-binding pocket [33]. Future work will investigate precise mechanisms in more detail and determine the physiological importance of specific sites of superoxide and H_2O_2 production in myotube differentiation by chronic application of S1QELs and S3QELs in culture and *in vivo*.

We conclude that S3QELs and S1QELs, when properly titrated in each system and medium, are accurate and convenient tools to identify, quantify, and manipulate the relative contributions of mitochondrial sites III_{Qo} and I_Q to superoxide and H_2O_2 production in complex biological systems.

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Declaration of competing interest

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