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Research Paper

Production of superoxide and hydrogen peroxide in the mitochondrial matrix is dominated by site I_Q of complex I in diverse cell lines

Jingqi Fang, Hoi-Shan Wong [1], Martin D. Brand [*]

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

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ABSTRACT

Understanding how mitochondria contribute to cellular oxidative stress and drive signaling and disease is critical, but quantitative assessment is difficult. Our previous studies of cultured C2C12 cells used inhibitors of specific sites of superoxide and hydrogen peroxide production to show that mitochondria generate about half of the hydrogen peroxide released by the cells, and site I_Q of respiratory complex I produces up to two thirds of the superoxide and hydrogen peroxide generated in the mitochondrial matrix. Here, we used the same approach to measure the engagement of these sites in seven diverse cell lines to determine whether this pattern is specific to C2C12 cells, or more general. These diverse cell lines covered primary, immortalized, and cancerous cells, from seven tissues (liver, cervix, lung, skin, neuron, heart, bone) of three species (human, rat, mouse). The rate of appearance of hydrogen peroxide in the extracellular medium spanned a 30-fold range from HeLa cancer cells (3 pmol/min/mg protein) to AML12 liver cells (84 pmol/min/mg protein). The mean contribution of identified mitochondrial sites to this extracellular hydrogen peroxide signal was 30 ± 7% SD; the mean contribution of NADPH oxidases was 60 ± 14%. The relative contributions of different sites in the mitochondrial electron transport chain were broadly similar in all seven cell types (and similar to published results for C2C12 cells). 70 ± 4% of identified superoxide/hydrogen peroxide generation in the mitochondrial matrix was from site I_Q; 30 ± 4% was from site III_{Qo}. We conclude that although absolute rates vary considerably, the relative contributions of different sources of hydrogen peroxide production are similar in nine diverse cell types under unstressed conditions *in vitro*. Identified mitochondrial sites account for one third of total cellular hydrogen peroxide production (half each from sites I_Q and III_{Qo}); in the mitochondrial matrix the majority (two thirds) of superoxide/hydrogen peroxide is from site I_Q.

1. Introduction

Mitochondria produce ATP but also generate superoxide and hydrogen peroxide. Leaks of electrons from the electron transport chain and associated metabolic enzymes cause one-electron reduction of oxygen to form superoxide or two-electron reduction to form hydrogen peroxide [1]. At least eleven sites in mammalian mitochondria can generate superoxide and/or hydrogen peroxide, either in the matrix or on the cytosolic side of the inner membrane [2–4]. Techniques to quantify their contributions under physiologically-relevant conditions have been developed [5–7]. Using isolated muscle mitochondria incubated in media mimicking the cytosol of resting skeletal muscle, use of endogenous reporters established that superoxide/hydrogen peroxide was produced mainly by sites I_Q and I_F of complex I, site II_F of complex II, and site III_{Qo} of complex III [5]. Subsequently, inhibitors of specific sites were used to establish their contributions in C2C12 myoblasts [6] and myotubes [7].

Suppressors of site I_Q electron leak (S1QELs) and suppressors of site III_{Qo} electron leak (S3QELs) [8–10] specifically suppress production of superoxide/hydrogen peroxide from site I_Q and site III_{Qo}, respectively, without inhibiting electron transport, affecting oxidative phosphorylation, or causing cytotoxicity at their effective concentrations [9,10]. They can be used to delineate the relative contributions of superoxide/hydrogen peroxide production from these specific mitochondrial sites to total intracellular levels of hydrogen peroxide by measuring their inhibition of hydrogen peroxide spillage to the medium [6].

NADPH oxidases (NOXs) generate superoxide as their primary function. Seven mammalian NOX homologs and six NOX subunits are

* Corresponding author.
E-mail addresses: jfang@buckinstitute.org (J. Fang), helen@calicolabs.com (H.-S. Wong), mbrand@buckinstitute.org (M.D. Brand).
[1] Current address: Calico Life Sciences LLC, 1170 Veterans Blvd, South San Francisco, CA 94080, U.S.A.

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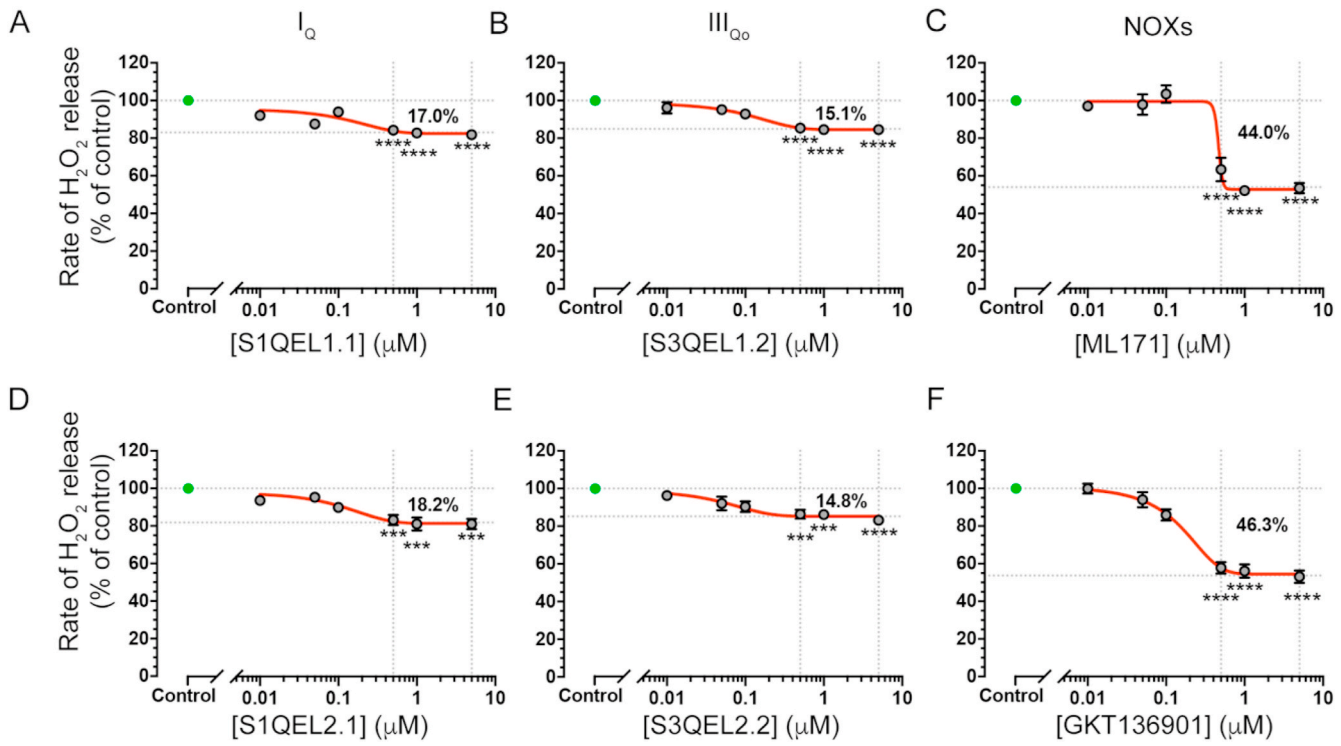

Fig. 1. Effects of S1QELs, S3QELs and NOX inhibitors on hydrogen peroxide release from AML12 hepatocytes. Cellular hydrogen peroxide release was assessed in the presence of two series of S1QELs (S1QEL1.1 (A) and S1QEL2.1 (D)), two series of S3QELs (S3QEL1.2 (B) and S3QEL2.2 (E)) and two series of NOX inhibitors (ML171 (C) and GKT136901 (D)). Vertical dotted lines indicate the range of inhibitor concentrations judged to give maximum inhibition; horizontal dotted lines indicate uninhibited and maximally inhibited rates. Lines were fit using "log(inhibitor) vs. response – Variable slope" in GraphPad Prism, excluding the points at zero inhibitor. Values are means ± SEM (N ≥ 3 independent biological replicates, each the mean of 3 technical replicates.Asterisks show significance of differences from control for points judged to give maximal inhibition.

known, with various tissue distributions and activation mechanisms [11]. NOXs transport electrons across membranes to reduce oxygen [12]. The immediate product is superoxide; hydrogen peroxide is rapidly generated by spontaneous and enzymatic dismutation. Specific NOX inhibitors, including ML171 [13] and GKT136901 [14,15], can be used to delineate the relative contribution of NOXs [6].

Establishing the proportion of total superoxide/hydrogen peroxide produced by specific sites in cells is crucial for understanding cellular behavior and signaling, and is a prerequisite for investigating superoxide/hydrogen peroxide production in physiology and pathology. Our previous studies showed that hydrogen peroxide released from C2C12 myoblasts arises ~40% from NOXs, 30% from site III_{Qo} and 15% from site I_Q [6]. However, it is unknown whether this pattern is specific to C2C12 cells, or more general. Here, we survey the contributions of superoxide/hydrogen peroxide production from site I_Q, site III_{Qo} and NOXs in seven diverse cultured cell lines.

2. Materials and methods

2.1. Reagents

Reagents were from the sources in Ref. [6].

2.2. Cells

AML12 (mouse liver), HeLa (human cervix epithelial), BJ-1 (human foreskin fibroblasts), H9c2 (rat heart myoblasts), A-549 (human lung epithelial), and U-2OS (human bone epithelial cells) from ATCC, and N27a (rat dopaminergic neural cells) from Millipore Sigma, were cultured under 5% (v/v) CO_2 in air at 37 °C in the different media recommended by the vendors containing the different glucose concentrations listed in Supplementary Table 1.

2.3. Hydrogen peroxide release

Measured as described [6] with slight modifications. 7500–12,000 cells/well were seeded in 96-well black microtiter plates and grown for 48 h until confluent. Medium was switched to Krebs Ringer Modified Buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 20 mM HEPES and glucose (concentrations in Supplementary Table 1), pH 7.4 at 37 °C) with 0.1% w/v bovine serum albumin (KRB-BSA) at 37 °C for 30 min. Measurement of hydrogen peroxide release was initiated by switching to assay medium containing 25 μM Amplex UltraRed, 5 U/ml HRP and 25 U/ml SOD1 in KRB-BSA. Fluorescence (Ex 540/Em 590) was monitored for 30 cycles using a PHERAStar FS(X) platereader. The contributions of NOXs, site I_Q and site III_{Qo} to hydrogen peroxide release were assessed by supplementing the medium with NOX inhibitors, S1QELs or S3QELs in the same volume of solvent (1 μl/mL DMSO) as added to the control. NOX inhibitors decreased assay sensitivity, so H_2O_2 calibrations included inhibitor. Cells were lysed using 0.1% (v/v) Triton X-100 and protein content assessed using a Bio-Rad protein assay kit. Rates of H_2O_2 production were normalized to cell protein in each well.

2.4. Mitochondrial respiration

Measured as described [6] with slight modifications. 5000 AML12 hepatocytes/well were seeded into XFe96 microplates, grown for 48 h until confluent, washed with KRB-BSA, then incubated for 30 min in KRB-BSA in air at 37 °C. Mitochondrial respiration was assessed using an XFe96 extracellular flux analyzer (Agilent) after subtracting rates with rotenone (2 μM) plus antimycin A (2 μM) in each well. Basal rates were calculated from the last point before addition of oligomycin (2 μM). Maximal oxygen consumption rate was induced by 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and calculated as the mean of three timepoints. Protein was assessed as in section 2.3.

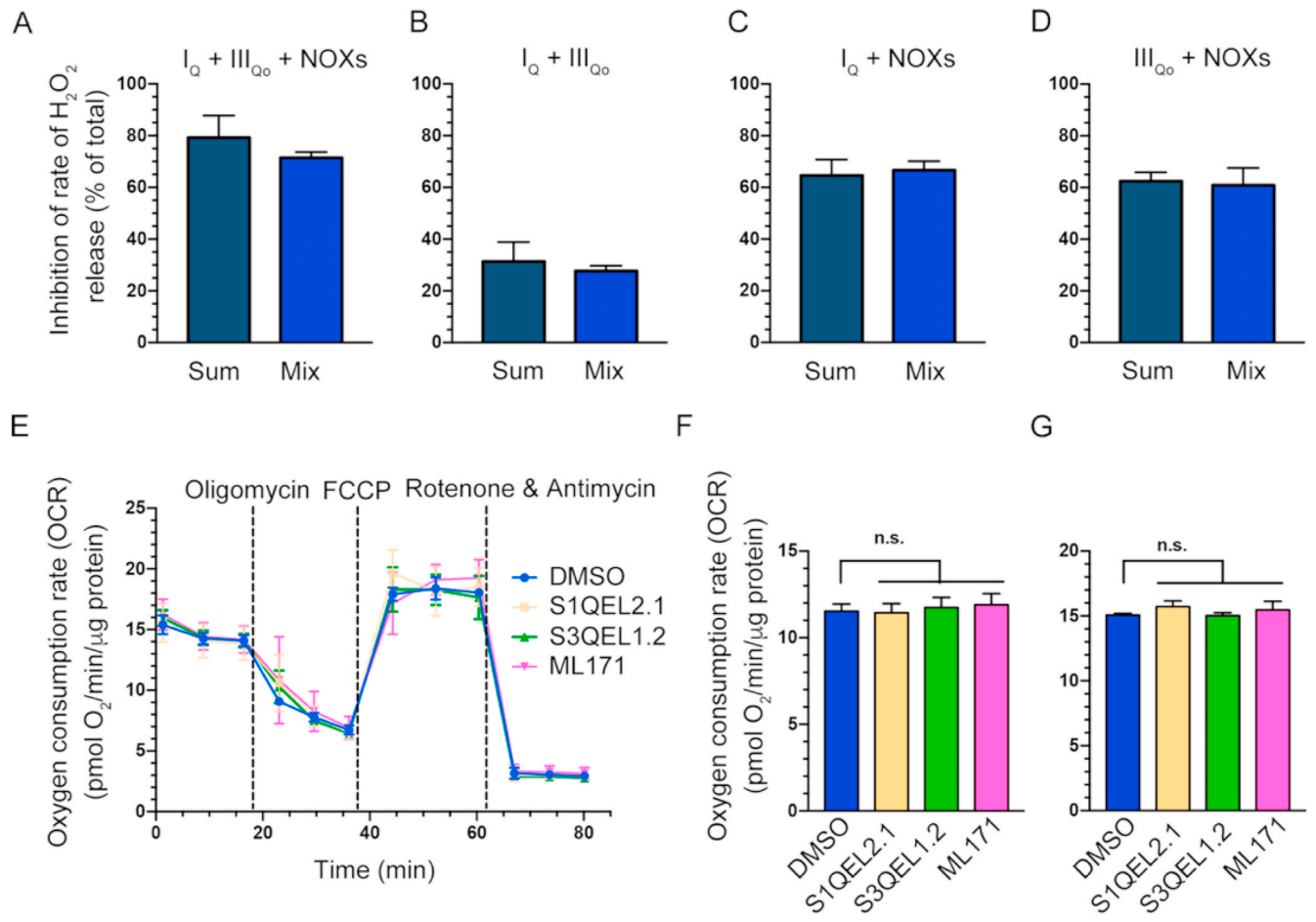

Fig. 2. Effects of S1QELs, S3QELs and NOX inhibitors on hydrogen peroxide release and respiration in AML12 hepatocytes. A–D, Effects of different combinations as indicated of S1QEL2.1 (0.5 μM – site I_Q suppressor), S3QEL1.2 (0.5 μM – site III_{Qo} suppressor) and ML171 (1 μM – NOX inhibitor) on rates of hydrogen peroxide release were measured either separately in different runs and then added ("Sum") or mixed together in a single run ("Mix"). The concentrations of inhibitor and suppressors were selected based on the minimum concentration giving maximum inhibition of hydrogen peroxide release in Fig. 1. E–G, Effects of suppressors and inhibitors (0.5 μM S1QEL2.1, 0.5 μM S3QEL1.2, 1 μM ML171) on respiration rates of AML12 cells. Values in A–D are means ± range of 3 biological repeats (because of the way data were compiled from separate experiments, no standard statistical test was appropriate); values in E–G are means ± SEM (N = 3 independent biological replicates, each the mean of 3 technical replicates). n.s., not significant via one-way ANOVA.

2.5. Statistics

Data are mean ± SEM within a cell line and mean ± SD between cell lines, and except where indicated were analyzed by one-way ANOVA, with inter-group differences detected by Dunnett's test when $p < 0.05$. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

3. Results and discussion

3.1. Contributions of site I_Q, site III_{Qo} and NOXs to hydrogen peroxide release from AML12 hepatocytes

Previous work validated the specificities of S1QELs, S3QELs and NOX inhibitors by showing that S1QELs with unrelated chemical structures (S1QEL1.1 and S1QEL2.1) each suppressed hydrogen peroxide release equally in C2C12 myoblasts, as did two unrelated S3QELs (S3QEL1.2 and S3QEL2.2), and two unrelated NOX inhibitors (ML171 and GKT136901) [6]. We tested the same pairs of inhibitors using AML12 hepatocytes (Fig. 1). Despite their structural differences, S1QEL1.1 and S1QEL2.1 suppressed hydrogen peroxide release equally, by 17–18% (Fig. 1A, D). S3QEL1.2 and S3QEL2.2 also had comparable effects, ~15% (Fig. 1B, E). Although the mechanism of inhibition of NOXs by ML171 and GKT136901 is different [13–15], each inhibited by 44–46% (Fig. 1C, F). These results validate the specificity of the suppressors and inhibitors in a second cell type, and show that site I_Q (18%), site III_{Qo} (15%) and NOXs (45%) all contribute to hydrogen peroxide release from AML12 cells.

3.2. Independence of S1QELs, S3QELs and NOX inhibitors

To define the contributions of different sites using suppressors and inhibitors, their effects should be independent. To evaluate independence, we mixed them together and compared their combined suppression to the sum of their separate effects. There was no difference between the sum of individual effects and the mixture effect for all three sites (Fig. 2A) or for any two sites (Fig. 2B–D). There were also no effects of these compounds on respiration (Fig. 2E–G). Thus, the actions of the suppressors and inhibitors, and the production of superoxide/hydrogen peroxide from site I_Q, site III_{Qo} and NOXs, are independent, consistent with results using C2C12 myoblasts [6].

3.3. Contributions of I_Q, III_{Qo} and NOXs to hydrogen peroxide release in six further cell lines

To address our central question, whether the pattern of contributions of different sites to cellular hydrogen peroxide production in C2C12 myocytes [6] and myotubes [7] is typical or differs by cell type, we

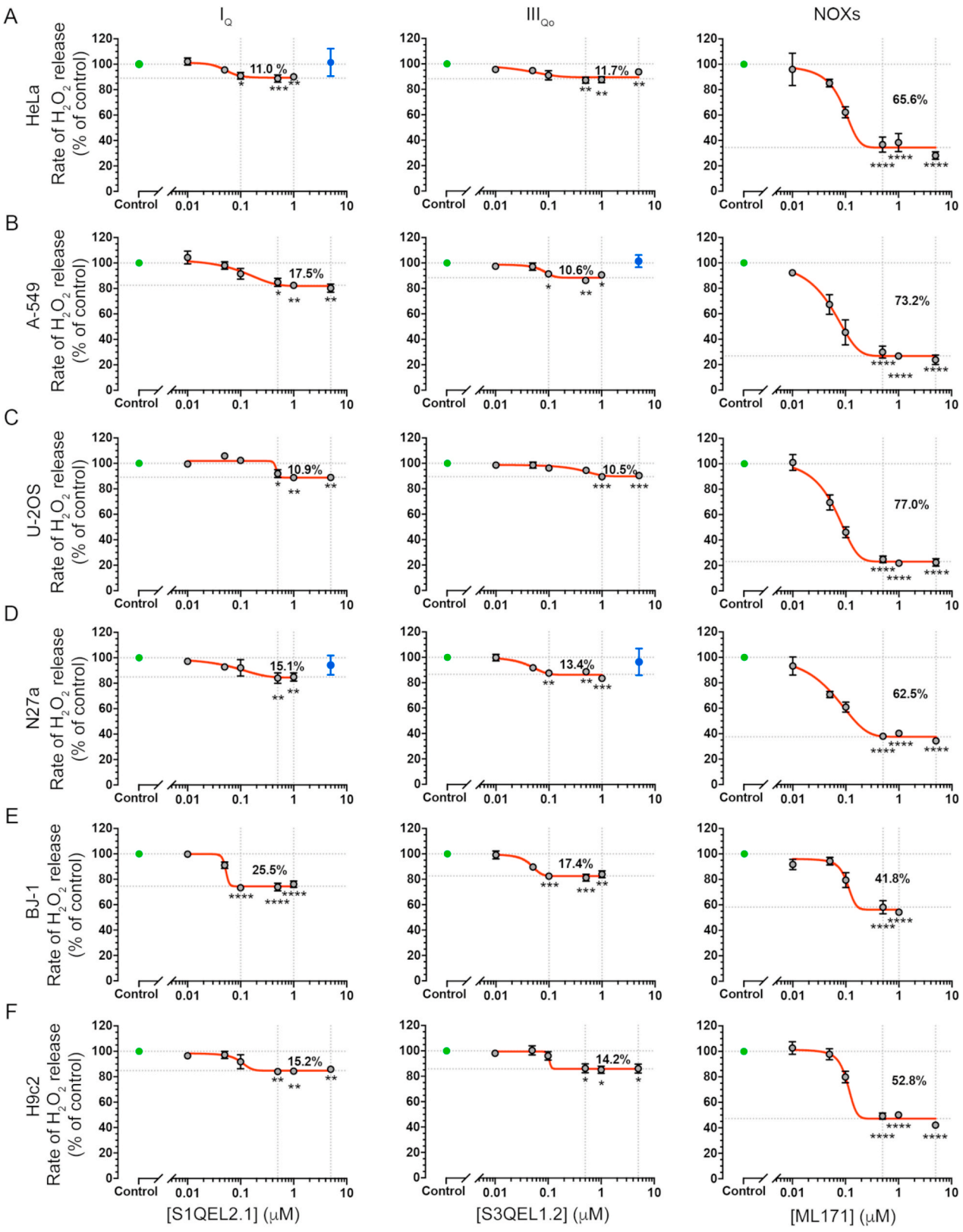

Fig. 3. Effects of S1QEL2.1, S3QEL1.2 and the NOX inhibitor ML171 on hydrogen peroxide release from six cell lines. A–F, different cell lines as indicated. Cellular hydrogen peroxide release was assessed in the presence of S1QEL 2.1 (left), S3QEL 1.2 (middle) and NOX inhibitor ML171 (right). Vertical dotted lines indicate the range of inhibitor concentrations judged to give maximum inhibition; horizontal dotted lines indicate uninhibited and maximally-inhibited rates. Lines were fit using "log(inhibitor) vs. response – Variable slope" in GraphPad Prism, excluding the points at zero inhibitor and at higher concentrations judged to give off-target effects. Values are means ± SEM ($N \geq 3$ independent experiments, each the mean of three biological replicates. Asterisks show significance of differences from control for points judged to give maximal inhibition.

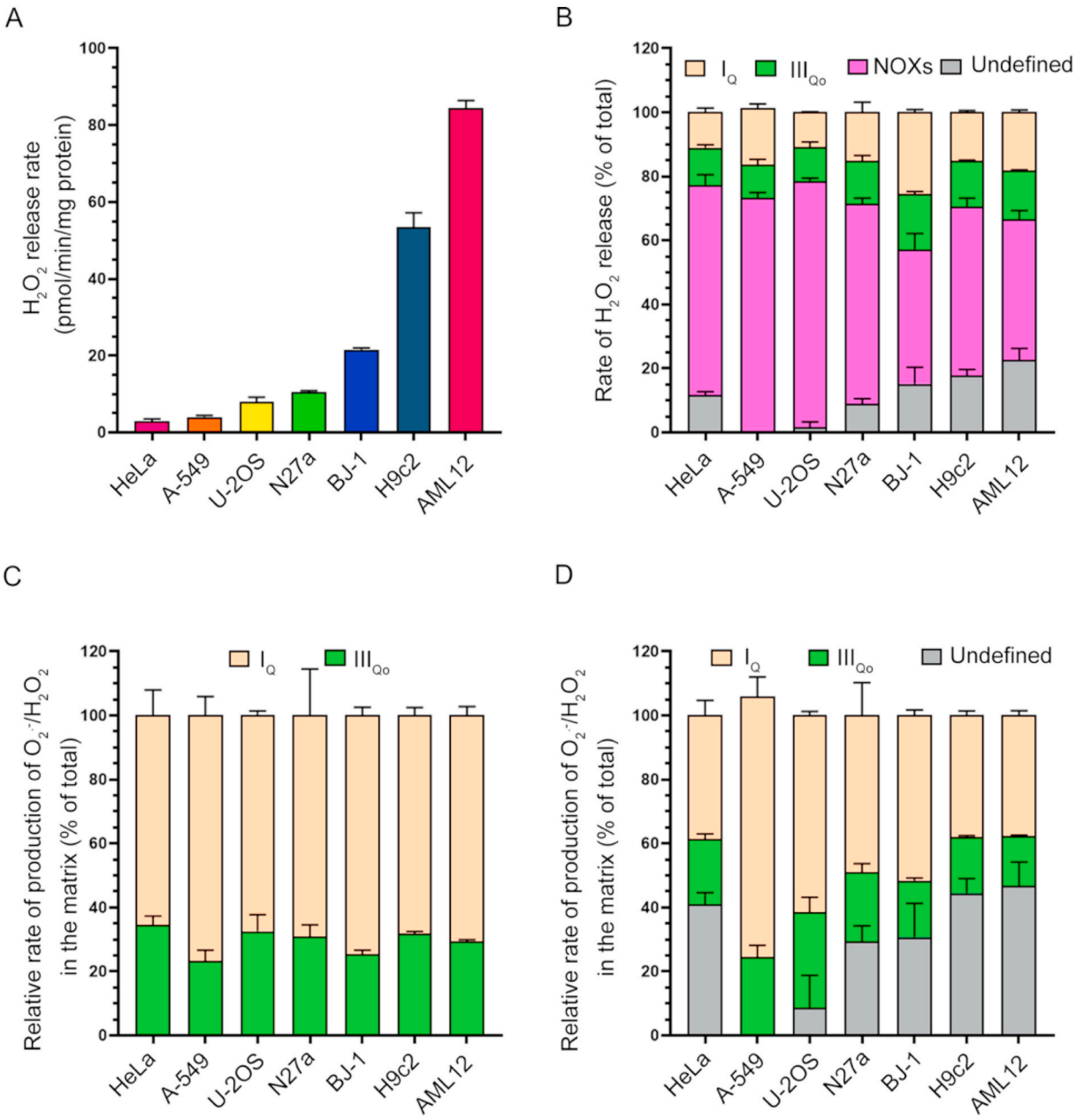

Fig. 4. Rates of release of hydrogen peroxide from seven different cell lines and relative contributions of different sites in whole cells and in the mitochondrial matrix. (A) Absolute rates of hydrogen peroxide release to the medium. (B) Relative contributions of site I_Q, site III_{Qo} and NOXs to rates of hydrogen peroxide release to the medium. (C) Calculated maximum contributions of superoxide and/or hydrogen peroxide production from site I_Q and site III_{Qo} in the mitochondrial matrix, assuming unidentified sources are not mitochondrial. (D) Calculated minimum contributions of superoxide and/or hydrogen peroxide production from site I_Q and site III_{Qo} in the mitochondrial matrix, assuming unidentified sources are entirely mitochondrial. Values in (B, C, D) were calculated from the data in Figs. 1 and 3 (shown in full in Supplementary Tables 2–4). Values are means ± SEM (N ≥ 3 independent experiments each the mean of three biological replicates).

surveyed a further six diverse cell lines. Including AML12 cells, they were of three different types (primary, immortalized, cancerous), from three different species (human, mouse, rat), and seven different tissues (liver, cervix, lung, skin, neuron, heart, bone). If C2C12 cells are atypical, some of these disparate cell lines might show a different pattern. We employed single suppressors of each site since Fig. 1 and [6] showed that different suppressors of a site gave similar results. To reduce the variables, we used the same simple medium for all lines, but used glucose at the concentration in the recommended culture media (Supplementary Table 1). Fig. 3 and Supplementary Table 2 show the results. In each cell line, S1QEL2.1, S3QEL1.2 and ML171 at their effective concentrations each significantly suppressed hydrogen peroxide release. In a few cases the highest suppressor concentration possibly stimulated the rates, presumably by off-target effects [6]; these points were disregarded.

Absolute cellular hydrogen peroxide production rates differed 30-fold, from HeLa cells (2.9 pmol/min/mg protein) and other epithelial cancerous cells (A-549 and U-2OS) to AML12 liver cells (84.3 pmol/min/mg protein) (Fig. 4A). Neural N27a cells also had low rates. Primary BJ-1 fibroblasts and immortalized H9c2 myoblasts had intermediate rates similar to those of C2C12 myoblasts [6]. The differences between lines may reflect different rates of superoxide and hydrogen peroxide production, intracellular scavenging, or both. Different cells can have very different rates of oxygen utilization, depending on cell type, function, cell size, metabolic rate and the species' body mass

[16–18]. The underlying differences in mitochondrial abundance may contribute to different rates of extracellular hydrogen peroxide production. A growing body of evidence indicates that cancer cells have increased antioxidant ability to counterbalance oxidizing conditions and improve their survival [19,20]. Increased antioxidant defenses and lower mitochondrial abundance of cancer cells (HeLa, A-549 and U-2OS) may contribute to the relatively low rate of extracellular hydrogen peroxide release compared to the other cell lines (Fig. 4A).

From the data in Figs. 1 and 3, we calculated the relative contributions of site I_Q, site III_{Qo} and NOXs to total hydrogen peroxide release from each cell line (Fig. 4B and Supplementary Table 2). Although lines differed quantitatively, the general pattern was similar. In all cells, mitochondria were important contributors; the sum of the two identified mitochondrial sites was 30 ± 7% (SD) (range 21–43%). In all cells the contributions of I_Q and III_{Qo} were similar: 16 ± 5% (SD) (range 11–26%) from I_Q and 13 ± 3% (SD) (range 11–17%) from III_{Qo}. In all cells, NOXs were the single greatest contributor; 60 ± 14% (SD) (range 42–77%). The unidentified sources of hydrogen peroxide were low, 11 ± 9% (SD) (range -1 to 23%). In C2C12 cells the unidentified contribution does not increase in parallel with identified mitochondrial sites following impairment of mitochondrial antioxidant defenses [6], so unidentified sources are probably mostly cytosolic. Therefore, although absolute rates of total cellular hydrogen peroxide release differ 30-fold, the relative contributions of different sources are broadly similar in seven diverse cell types under nominally unstressed conditions *in vitro*.

3.4. Site I_Q dominates superoxide/hydrogen peroxide production in the mitochondrial matrix

Superoxide in the mitochondrial matrix drives many pathologies [21], making it important to define the relative importance of different sites of superoxide production in the matrix. Site I_Q generates superoxide/hydrogen peroxide entirely in the matrix [2,6,22]. Site III_{Qo} generates only superoxide [22,23], about half of it in the matrix [2,6,23,24]. NOXs generate superoxide and hydrogen peroxide in the cytosol and directly to the extracellular medium [12,25]. As mentioned above, undefined sources are probably cytosolic [6]. These considerations allow calculation of total matrix production of superoxide/hydrogen peroxide as the proportion of cellular hydrogen peroxide release sensitive to S1QELs plus half of that sensitive to S3QELs, giving a default estimate. If instead we assume that undefined sources are entirely mitochondrial, they are also added, giving a conservative estimate of total matrix production. The contributions of I_Q and III_{Qo} to total matrix superoxide/hydrogen peroxide production are then calculated separately as percentages of the default or conservative estimates of total matrix production.

Fig. 4C and Supplementary Table 3 show the relative contributions of sites I_Q and III_{Qo} to matrix superoxide/hydrogen peroxide production under the default assumption that unidentified reactions are cytosolic, giving maximum estimates of the contributions of I_Q and III_{Qo}. In all cell lines, I_Q dominated matrix production of superoxide/hydrogen peroxide from identified sites, accounting for 70 ± 4% (SD) (range 65–77%), with III_{Qo} responsible for the remainder, 30 ± 4% (SD) (range 23–35%). Fig. 4D and Supplementary Table 4 show the relative contributions of I_Q and III_{Qo} to matrix superoxide/hydrogen peroxide production under the conservative assumption that unidentified reactions are entirely in the matrix, giving minimum estimates of their contributions. Across all cell lines, I_Q generated 51 ± 16% (SD) (range 38–81%) of the total, III_{Qo} generated 21 ± 5% (SD) (range 16–30%), and unidentified sites generated the remainder.

We conclude that although absolute rates vary considerably, the relative contributions of different sources of hydrogen peroxide are similar in nine diverse cell types under unstressed conditions *in vitro*. Identified mitochondrial sites account for one third of total cellular hydrogen peroxide production (half each from sites I_Q and III_{Qo}); in the mitochondrial matrix the majority (two thirds) of superoxide/hydrogen peroxide arises from site I_Q. Using site-specific suppressors provides a customized strategy to explore the roles of superoxide and/or hydrogen peroxide production in specific physiologies and pathologies.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101722.

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