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Modulation of oxidative phosphorylation (OXPHOS) by radiation- induced biophotons

Michelle Le[a], Fiona E. McNeill[a], Colin B. Seymour[a], Andrej Rusin[a], Kevin Diamond[a], Andrew J. Rainbow[a], James Murphy[b], Carmel E. Mothersill[a,*]

[a] McMaster University, 1280, Main Street West, Hamilton, Ontario, Canada L8S 4K1
[b] Institute of Technology Sligo, F91 YW50, Ireland

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ABSTRACT

Radiation-induced biophotons are an electromagnetic form of bystander signalling. In human cells, biophoton signalling is capable of eliciting effects in non-irradiated bystander cells. However, the mechanisms by which the biophotons interact and act upon the bystander cells are not clearly understood. Mitochondrial energy production and ROS are known to be involved but the precise interactions are not known. To address this question, we have investigated the effect of biophoton emission upon the function of the complexes of oxidative phosphorylation (OXPHOS). The exposure of bystander HCT116 p53 +/+ cells to biophoton signals emitted from β-irradiated HCT116 p53 +/+ cells induced significant modifications in the activity of Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase) such that the activity was severely diminished compared to non-irradiated controls. The enzymatic assay showed that the efficiency of NADH oxidation to NAD+ was severely compromised. It is suspected that this impairment may be linked to the photoabsorption of biophotons in the blue wavelength range (492–455 nm). The photobiomodulation to Complex I was suspected to contribute greatly to the inefficiency of ATP synthase function since it resulted in a lower quantity of H^+ ions to be available for use in the process of chemiosmosis. Other reactions of the ETC were not significantly impacted. Overall, these results provide evidence for a link between biophoton emission and biomodulation of the mitochondrial ATP synthesis process. However, there are many aspects of biological modulation by radiation-induced biophotons which will require further elucidation.

1. Introduction

The biological effects of low-dose ionizing radiation is an area of research which is very widely studied due to its relevance in every day practice in occupational and clinical settings. Yet, there is still much uncertainty surrounding the biological implications of low doses as there are many conflicting effects which have been observed in the low dose region of the dose response curve, whether they are observations of hyper-radiosensitivity (Marples and Joiner, 1993) or hormetic responses (Calabrese et al., 2008; Scott, 2007). With that being said, the linear non-threshold model has been challenged in the scientific research community due to the suggestion that it does not accurately represent the risk of biological effects at low doses (Cohen, 2008; Tubiana et al., 2009). Among the phenomena which have contributed to the challenging of the LNT at low doses is the radiation-induced bystander effect (RIBE). The RIBE is a phenomenon whereby cells which have not been directly exposed to ionizing radiation, but which have received bystander signals from cells that have been directly irradiated, exhibit characteristics resembling the effects of having been irradiated. RIBE manifest as a result of inter-cellular signalling via the transfer of soluble factors through possibly related mechanisms involving gap-junction intercellular communication (GJIC) (Azzam et al., 1998) and medium transfer experiments (Mothersill and Seymour, 1997), or via a physical mechanism characterized by electromagnetic signalling (Ahmad et al., 2013; Le et al., 2015). The latter mechanism of bystander effect mediation (biophoton-mediated bystander effects) is the focus of the present study.

The emission of weak photon fluences from biological material (ranging from 10 to 10^3 photons cm^{-2} s^{-1} (Rajewsky, 1931)) is referred to as biophoton emission and is a well-established phenomenon in the field of biophotonic research (Popp et al., 1984; Volodyaev and Beloussov, 2015). Emission occurs both spontaneously (Bajpai et al., 2013) and as a result of exogenous perturbation by triggers such as visible light (Niggli, 1996), UV light (Niggli, 1993; Niggli et al., 2008),

* Corresponding author.
E-mail address: mothers@mcmaster.ca (C.E. Mothersill).

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chemical stress (Popp et al., 1984; Devaraj et al., 1991), and mechanical stress (Kobayashi et al., 1997; Slawinski et al., 1992; Bajpai et al., 1991). Moreover, investigation of the properties of biophoton emission using sensitizers and quenching agents have revealed excited species involved in cellular metabolism as a potential source of biophotons (Cadenas et al., 1980). The quantification of biophoton emission from whole organisms and tissues has since been established as a method of non-invasively characterising the oxidative status of a given system (Kobayashi et al., 1999; Tilbury, 1992; Kataoka et al., 2001). The action of biophotons as a means of intercellular communication was identified in 1980 when Kaznacheev demonstrated the induction of significant adverse effects in a fibroblast cell population that was optically-coupled, but not chemically associated, with a fibroblast culture which was treated with the Coxsackie A13 virus (Kaznacheev et al., 1980). This effect has since been corroborated by multiple supporting studies citing evidence for intercellular communication via a signal that is electromagnetic in nature (Galantsev et al., 1993; Albrecht-Buehler, 1992; Shen et al., 1994; Fels et al., 2009). We have recently investigated this mechanism of communication following exposure of in vitro cell cultures to ionizing β-radiation. Our investigations have demonstrated that radiation-induced biophoton signalling is able to induce clonogenic cell death in bystander cells and have demonstrated that the magnitude of the effect is dependent upon the function of the bystander cells' p53 proteins (Le et al., 2017a). Intercellular biophoton signalling also has a profound effect upon mitochondria as the signals were effective in inducing mitochondrial membrane depolarization (Le et al., 2017b). The ability of the biophoton signal to modulate mitochondrial membrane potential leads to the suggestion that the mechanism for electromagnetic signals in driving bystander responses could be linked to mitochondrial function. Mitochondria have been identified as an integral participant in the RIBE whether as an extra-nuclear target of direct irradiation (Azzam et al., 2012) or as a recipient of bystander signals (Murphy et al., 2005; Lyng et al., 2000). The modification of mitochondrial physiology following chemically-mediated bystander signalling has been documented (Lyng et al., 2000, 2002; Acheva et al., 2008). However, it has not been studied in the context of biophoton-mediated bystander effects. The interest in assessing mitochondrial function following receipt of biophoton bystander signals stems from the involvement of redox reactions in regulating the mitochondrial functions responsible for cellular metabolism. It is hypothesized that the input of energy carried by biophotons into the mitochondrial electron transport chain (ETC) may act either to drive or inhibit the redox reactions involved in electron shuttling. We anticipate that both upregulation or downregulation of electron transport chain activities leading to a modulation in ATP production could have profound effects upon cellular response. In this study, we aimed to address this question by assessing the activity of the electron transport chain (ETC) complexes along with ATP synthase also known as the enzyme complexes of OXPHOS in response to biophoton exposure.

These investigations encompass an analysis of enzymatic activity of the various complexes of the ETC in HCT116 p53 +/+ bystander cells in response to biophoton exposure. Spectrometry in the UV and visible wavelength ranges are also employed to characterize the biophoton emission resultant to direct cellular irradiation with β-emitter, tritium (^{3}H). The primary objective of this work is to further clarify the mechanism by which biophotons induce modifications in cells that are recipients of the bystander signal.

2. Materials & methods

2.1. Cell culture

Human colon carcinoma cell line, HCT116 p53 +/+, was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin sulphate. Cultures were incubated at 37 °C at 95% humidity and 5% CO_2. Cells were cultured in 75 cm^2 flasks and passaged when the cells reached 70–80% confluence. Cells were dissociated from the flask substrate by incubating them with a 1:1 solution of 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 3 min. The trypsinzed cells in solution were the neutralized with 7 mL of complete growth medium and subsequently plated into new flasks. 24 h prior to an experiment, the cells received full-volume medium changes with complete growth medium. All reagents used for cell culture were obtained from Gibco/Life Technologies unless otherwise specified.

For experimental set-up in the case of enzymatic assay experiments, cells destined to receive electromagnetic bystander signals were seeded in100 mm diameter petri dishes containing a 5 mL volume of growth medium, at a density of 2.1×10^6 cells per flask (2.8×10^4 cells/cm^2), and allowed to grow for 72 h before being harvested for mitochondrial isolation. At this point cultures were confluent with approximately 2.2×10^5 cells per cm^2. Cells destined to receive direct irradiation from beta(β)-particles, cells were seeded at a density of 2000 cells/cm^2 in 75c^2 flasks containing 10 mL of growth medium.

2.2. Cell irradiation

Cells were either exposed directly to β-particles from tritium (^{3}H) or exposed to the electromagnetic bystander signals that were emitted from the β-particle-exposed cells. For cells destined to receive direct irradiation from ^{3}H, 857.5 μCi of tritiated water was added to the cell culture and incubated with the cells for 24 h to achieve a dose of 0.5 Gy. For cells destined to receive the electromagnetic bystander signals, cells in a 100 mm diameter dish were placed superior to the direct-β-irradiated cell population such that the two monolayers were 15 mm apart. The bystander cells were exposed to the electromagnetic bystander signals for 24 h.

2.3. Mitochondrial isolation

Following exposure of bystander cells to the electromagnetic bystander signals, mitochondria were isolated from the bystander cell populations following a protocol adapted from O'Dowd and colleagues (O'Dowd et al., 2009). Mitochondrial isolation was accomplished by first washing the confluent bystander cell monolayer with 5 mL of phosphate-buffered saline (PBS) 2 times. The washing PBS was then discarded and 10 mL of fresh PBS was added to the flask and the bystander cell monolayer was dissociated from the flask substrate using a cell scraper. The cell suspension was then centrifuged at 1000*g* at 4 °C for 10 min in a ThermoScientific Sorvall ST40R benchtop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 400 μL ice-cold mitochondrial isolation buffer (adapted from O'Dowd et al., 2009). The cells were transferred to a 1.5 mL centrifuge tube and homogenized on ice using a handheld VWR homogenizer with a polybutylene terephthalate pestle. The cells were homogenized by breaking the cells with 40 strokes while the homogenizer rotational speed reached 12,000 rotations per minute (rpm). Large debris was pelleted at 2000*g* at 4 °C for 10 min in a ThermoScientific Sorvall Legend Micro21R benchtop centrifuge. The supernatant was collected and transferred to a new 1.5 mL centrifuge tube and mitochondrial were pelleted at 10,000*g* at 4 °C for 10 min. The supernatant was discarded and the pellet was resuspended in another 400 μL of ice-cold mitochondrial isolation buffer. The mitochondria were pelleted a second time at 10,000*g* at 4 °C for 10 min and the supernatant was discarded again. The remaining mitochondrial pellet was resuspended in 200 μL of 10% glycerol-PBS solution and the sample was frozen at −80 °C until needed for future use to assess enzymatic activity.

2.4. Protein quantification

To quantify the amount of mitochondrial protein in each of the isolated mitochondria samples, 15 μL of the mitochondrial sample was

added to 15 µL 2% CHAPS-TBS solution (3-[(3-**ch**olamidopropyl)dimethyl**a**mmonio]−1-**p**ropane**s**ulfonate supplied as a solution with tris buffered saline (TBS) by Thermo-Scientific). and the sample was vortexed for 1 min. The mitochondria were then centrifuged at 10,000*g* for 2 min and the supernatant was analyzed using the ThermoScientific BCA protein assay kit (cat no: 23227).

2.5. Enzymatic activity assays for electron transport chain

2.5.1. Complexes

2.5.1.1. Complex I: NADH dehydrogenase. The activity of Complex I was assessed in mitochondria isolated from non-irradiated control cells and in mitochondria isolated from bystander cells exposed to electromagntic bystander signals. The protocol used was adapted from that developed by Spinazzi et al. (2012). The reaction (shown below) was initiated by adding Coenzyme Q1 (Ubiquinone 1) to a final concentration of 0.1 mM to the Complex I reaction buffer (50 mM potassium phosphate buffer pH 7.5, 3 mg/mL fatty acid-free BSA, 0.3 mM KCN, 0.1 mM NADH, 30 µg of mitochondrial protein).

Reaction 7.1:

$$NADH + H^+ + UQ1 \xrightarrow[I]{} NAD^+ + UQ1H_2$$

The assay was conducted in a total volume of 200 µL using a glass-bottom 96-well plate (MatTek) and absorbance measurements were taken at 340 nm, at 30 °C, for 2 min using a Tecan Infinite 200 Pro plate reader to assess the disappearance of NADH (NADH extinction coeffcient: 6.2 $mmol^{-1}$ cm^{-1}). In parallel, another well was set up whereby the reaction buffer was treated with Complex I inhibitor (Rotenone) to a final concentration of 10 µM, so that the specific Complex I activity could be isolated. Prior to any measurements, the Complex I reaction buffer was incubated at 30 °C for 10 min to allow the reagents to equilibrate. All chemicals used in enzymatic assays were obtained from Sigma-Aldrich unless otherwise specified. 2-min baseline measurements were also taken for each well whereby absorbance (340 nm) of the reaction mixture was measured prior to the initiation of the reaction by the addition of Coenzyme Q1. The baselines were subtracted from absorbance data acquired in the presence of Coenzyme Q1 to eliminate any effects attributed to evaporation of the reaction buffer.

2.5.1.2. Complex II-III: succinate dehydrogenase, ubiquinol cytochrome c oxidoreductase. Complex II and III activity were assessed using the protocol developed by Spinazzi et al. (2012). The 200 µL volume of reaction buffer (20 mM potassium phosphate buffer pH 7.5, 300 µM KCN, 10 mM succinate, 20 µg of mitochondrial sample) was incubated at 37 ° C for 10 min to allow for full activation of the enzyme. Following incubation, baseline measurements were recorded for 3 min (measurements taken at 10-second intervals) at 550 nm using a Tecan Infinite 200 Pro plate reader. 50 µM of oxidized cytochrome c was then added to the buffer to initiate the reaction shown below and the absorbance at 550 nm was measured for an additional 3 min to assess the reduction of cytochrome c (extinction coeffcient for reduced cytochrome c: 18.5 $mmol^{-1}$ cm^{-1}). In parallel, another well containing the reaction buffer was treated with 10 mM of the Complex II inhibitor, malonate, in order to assess the specific activity.

Reaction 7.2:

$$succinate + oxidizedcytochromec \xrightarrow{II-III} malate + reducedcytochromec$$

$$C_4H_6O_4 + C_{42}H_{52}FeN_8O_6S_2 \xrightarrow{II-III} C_4H_6O_5 + C_{42}H_{52}FeN_8O_5S_2$$

2.5.1.3. Complex IV: cytochrome c oxidase. The activity of cytochrome c oxidase (complex IV) was assessed in mitochondrial samples isolated from non-irradiated control cells and cells exposed to electromagnetic bystander signals emitted from cells directly-irradiated with tritium. The protocol used for the assay was adapted from the protocol developed by Spinazzi et al. Spinazzi et al. (2012) whereby 20 µg of mitochondrial sample was added into the reaction buffer containing 50 mM of potassium phosphate (KH_2 PO_4) buffer (pH 7.0) and 60 µM reduced cytochrome c. The reaction mixture was incubated at 37 ° C for 10 min. Prior to the addition of the mitochondrial sample, the baseline activity was assessed by measuring the absorbance of the reaction mixture at 550 nm for 3 min (10-second intervals). The absorbance at 550 nm was measured for an additional 3 min immediately following the addition of the mitochondrial sample to determine to rate of cytochrome c oxidation (extinction coefficient for reduced cytochrome c: 18.5 $mmol^{-1}$ cm^{-1}). The reaction (shown below) was assessed for complex IV activity.

Reaction 7.3:

$$reducedcytochromec + \frac{1}{2}O_2 + 2H + 2e^- \xrightarrow{IV} oxidizedcytochromec + H_2O$$

$$C_{42}H_{52}FeN_8O_6S_2 + \frac{1}{2}O_2 + 2H + 2e^- \xrightarrow{IV} C_{42}H_{52}FeN_8O_6S_2 + H_2O$$

In parallel, the specific activity of Complex IV was investigated via the addition of 0.3 mM of the Complex IV inhibitor, potassium cyanide (KCN), into the reagent mixture prior to the initiation of the reaction.

2.5.1.4. Complex V: ATP synthase. The activity of ATP synthase (Complex V) was assessed using a coupled enzyme assay adapted from a protocol received through personal communication with Dr. James Murphy (IT Sligo, Sligo, Ireland). 190 µL of the reaction mixture containing 50 mM Tris buffer (pH 8.0), 1 mg fatty acid-free BSA, 20 mM $MgCl_2$, 50 mM KCl, 5 µM antimycin A, 10 mM phosphoenol pyruvate (PEP), 2.5 mM MgATP, 4 units of Lactate dehydrogenase (LDH), 4 units pyruvate kinase (PK), and 400 µM NADH, was first incubated at 37 ° C. Baseline absorbance measurements at 340 nm were taken for a 3 min duration following incubation and thereafter, reaction 7.4 was started by the addition of 20 µg of mitochondrial protein (isolated from non-irradiated control cells or cells which were exposed to electromagnetic bystander signals). The ADP produced from the complex V-driven reaction then interacted with PEP in the presence of pyruvate kinase (PK) to initiate reaction 7.5 producing ATP and pyruvate. The pyruvate product from the prior reaction then oxidized NADH in the presence of LDH to produce NAD^+ (reaction 7.6). The rate of NADH oxidation (NADH extinction coefficient: 6.2 $mmol^{-1}$ cm^{-1}) was subsequently determined by measuring the absorbance at 340 nm every 10 s for a 3-min duration. To assess the specific activity of complex V, 2.5 µM of the inhibitor oligomycin A was added into the reagent mixture prior to the addition of mitochondrial protein.

Reactions 7.4, 7.5, 7.6:

$$ATP \xrightarrow{V} ADP + phosphate$$

$$C_{10}H_{16}N_5O_{13}P_3 \xrightarrow{V} C_{10}H_{15}N_5O_{10}P_2 + P$$

$$ATP + phosphoenolpyruvate \xrightarrow{PK} ATP + pyruvate$$

$$Pyruvate + NADH \xrightarrow{LDH} lactate + NAD^+$$

2.5.1.5. Citrate synthase. The activity of citrate synthase, a 51.7 kDa mitochondrial matrix enzyme, was measured and used as a marker representative of overall mitochondrial mass in a sample. Citrate synthase activity was measured as a control (similar to the way that actin is used as a loading control in western blots) to ensure that the mitochondrial mass was similar between control and treatment samples. The ability of citrate synthase (CS) to catalyze the reaction between Acetyl CoA (AcCoA) and oxaloacetic acid (reaction 7.7) was assessed by first measuring the baseline absorbance of the reaction buffer (100 mM Tris Triton X-100 buffer pH 8.0, 0.1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 0.3 mM Ac CoA, 20 µg of mitochondrial sample) at 37 °C and 412 nm for 3 min. The reaction was then initiated

Table 7.1
Substrates measured in the investigation of enzymatic activity.

System Assessed	Substrate	Concentration [μM]	Extinction coefficient [$mmol^{-1}\ cm^{-1}$]
Complex I	NADH	100	6.2
Complex II-III	Cytochrome c (reduced)	50	18.5
Complex IV	Cytochrome c (reduced)	60	18.5
Complex V	NADH	400	6.2
Citrate Synthase	DTNB	100	13.6

by adding oxaloacetic acid to the reaction mixture to a final concentration of 0.5 mM. Thereafter, the absorbance at 412 nm was measured for an additional 3 min. The extinction coefficient of DTNB is 13.6 $mmol^{-1}\ cm^{-1}$.

Reaction 7.7:

$$AcCoA + oxaloaceticacid + H_2O \xrightarrow{CS} citrate + CoA - SH$$

2.6. Statistical analysis

Samples were assessed using three biological replicates which were assessed via three technical repeats to achieve a final sum of 9 data points per experimental permutation. Calculated enzyme activity values were compared using a 2-way analysis of variance (ANOVA).

Quantitative assessment of enzymatic activity was determined using equation 7.8 adapted from Spinazzi and colleagues (Spinazzi et al., 2012) where the substrates used and their corresponding extinction coefficients are specified in Table 7.1. In equation 7.8, v_A defines the rate of consumption of a reactant and v_Z defines the rate of formation of a product in units of nmol $min^{-1}\ mg^{-1}$, $\frac{\Delta A}{t}$ is the change in absorbance per minute, ε is the extinction coefficient of the substrate specified in Table 7.1, V is the volume of the mitochondrial sample in mL, and C is the concentration of mitochondrial protein in the sample in mg mL^{-1}.

Specific activity or the degree of activity that is certainly attributed to complex function was calculated by subtracting the activity of the complex with inhibitor from the activity of the complex without the inhibitor. From this, sensitivity of the assay for a given complex can be determined by taking the ratio of the specific activity and the activity of the complex without the inhibitor.

Reaction 7.8:

$$\upsilon_A \text{ or } \upsilon_z = \frac{\frac{\Delta A}{t} \bullet 1000}{\varepsilon \bullet V \bullet C}$$

3. Results

3.1. Oxidative phosphorylation enzymatic activity

3.1.1. Complex I activity

The ability of Complex I to oxidize NADH to NAD+ was assessed in mitochondrial isolates extracted from non-irradiated controls cells and bystander cells exposed to electromagnetic bystander signals (Fig. 1). The specific activity of Complex I was assessed by treating the reaction mixture with the Complex I inhibitor, Rotenone, such that any NADH oxidation observed that was non-specific to Complex I could be identified. For the experimental permutation containing mitochondria from non-irradiated (control) cells and absent of rotenone, the concentration of NADH decreased over the 2-min measurement at a rate of 40.80 ± 14.50 nmol $min^{-1}\ mg^{-1}$ of mitochondrial protein. In contrast, the reaction whereby rotenone was added into the mixture containing mitochondria from control cells demonstrated an enzymatic activity rate of 5.36 ± 1.30 $min^{-1}\ mg^{-1}$ of mitochondrial protein. The

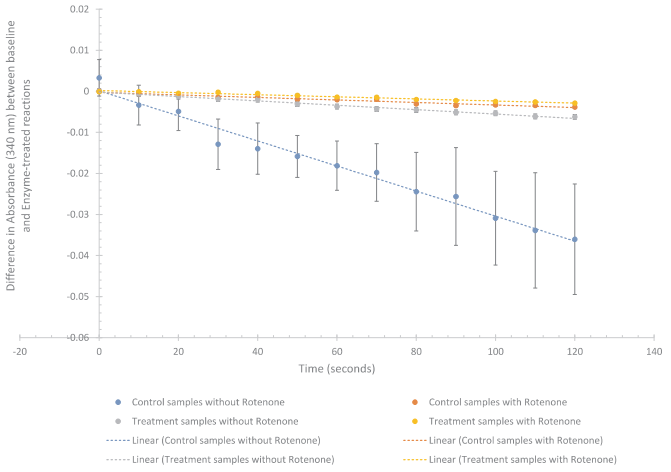

Fig. 1. Complex I activity. Oxidation of NADH demonstrated by a decrease in absorbance at 340 nm over a 2 min duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) obtained from Human colon carcinoma cells with wild type p53 (HCT-p53+/+) tested in triplicate (3 technical replicates). Errors bars represent standard error for n = 9.

presence of the complex I inhibitor demonstrates a significant reduction in enzyme activity elicited by the control mitochondria ($p < 0.0001$). From analyzing the degree of oxidation of NADH upon Complex I inhibition with that observed in the uninhibited sample, the specific activity of Complex I can be reported as 35.44 ± 13.63 nmol $min^{-1}\ mg^{-1}$ protein and the sensitivity of Complex I is 81.22 ± 11.76%.

When Complex I in bystander cell-extracted mitochondria was assessed in the absence of rotenone, the enzyme activity was 7.56 ± 2.67 nmol $min^{-1}\ mg^{-1}$ protein. When enzyme activity was assessed in bystander cell-extracted mitochondria in the presence of the inhibitor rotenone, the enzyme activity was 3.88 ± 1.15 nmol min-1 mg-1 protein. It is apparent that the function of Complex I has been compromised in mitochondria extracted from the cells which were exposed to the electromagnetic bystander signals to the extent that the enzyme activity exhibited in these samples does not differ significantly from the permutation in which Complex I function was inhibited (bystander with rotenone $p = 0.929$; control with rotenone $p = 0.983$). Most importantly, the enzyme activity observed in the bystander cell mitochondria (no inhibitor) was significantly weaker than that observed in the control cell mitochondria (no inhibitor) ($p < 0.0001$). This result suggests that the bystander signal was effective in eliciting a modification in the activity of Complex I such that it is less efficient at receiving electrons from NADH. From this observation, it can be deduced that Complex I in the bystander mitochondria are less effective at moving electrons and subsequently pumping protons into the intermembrane space.

3.1.2. Complex II-III activity

The activity of succinate dehydrogenase and ubiquinol cytochrome c oxidoreductase were assessed in mitochondrial samples that were isolated from non-irradiated control cells and bystander cells ex-posed to electromagnetic bystander signals (Fig. 2). When non-irradiated control mitochondria were assessed for their Complex II-III activity, it was observed that the oxidized cytochrome c was reduced at a rate of 71.64 ± 14.64 nmol $min^{-1}\ mg^{-1}$ mitochondrial protein. In parallel, treatment of the reaction mixture with malonate was used to determine the specific activity of Complex II-III such that any reduction of cytochrome c observed in its presence could be attributed to factors extraneous to Complex II-III themselves. When malonate was added to the reaction mixture containing non-irradiated control mitochondria, the rate of cytochrome c reduction was 10.16 ± 2.76 nmol $min^{-1}\ mg^{-1}$ protein. The addition of the competitive inhibitor, mal-onate, was effective in significantly diminishing the observed enzyme activity ($p =$

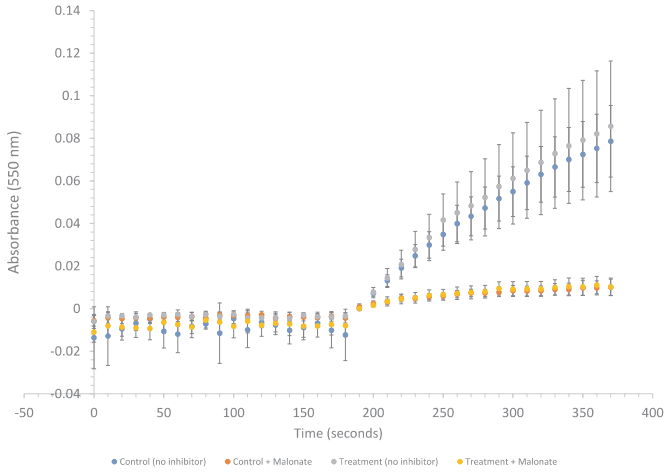

Fig. 2. Complex II-III activity. Reduction of cytochrome c demonstrated by an increase in absorbance at 550 nm over a 3 min duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) obtained from Human colon carcinoma cells with wild type p53 (HCT-p53+/+) tested in triplicate (3 technical replicates). Errors bars represent standard error for n = 9.

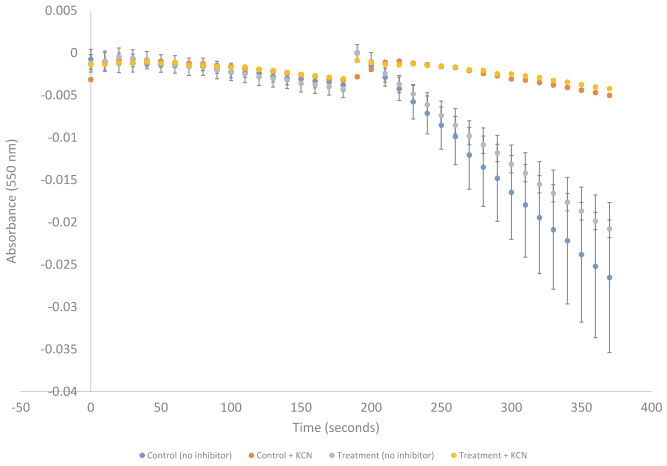

Fig. 3. Complex IV activity. Oxidation of cytochrome c demonstrated by a decrease in absorbance at 550 nm over a 3 min duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) obtained from Human colon carcinoma cells with wild type p53 (HCT-p53+/+) tested in triplicate (3 technical replicates). Errors bars represent standard error for n = 9.

0.001). This diminished activity shows that the Specific Activity of Complex II-III in control mitochondria is 61.48 ± 13.23 nmol min^{-1} mg^{-1} and the sensitivity of Complex II-III is 85.48 ± 2.82%. This suggests that approximately 14% of the enzyme activity that was observed is not attributed to Complex II-III.

Enzyme activity was also assessed in mitochondria isolated from electromagnetic-irradiated cells. These mitochondrial samples expressed cytochrome c reduction at a rate of 89.66 ± 31.73 nmol min^{-1} mg^{-1} mitochondrial protein. When comparing the mitochondrial enzyme activity of the biophoton-irradiated samples to the control samples, we find that there is no significant difference among the two populations (p = 0.599). That is to say that the exposure of cells to the electromagnetic bystander signal did not alter the enzyme activity of complexes II and III significantly compared to controls which were not exposed to the bystander signal. For the mitochondria extracted from bystander signal-exposed cells, we also assessed the enzyme activity following incubation with the inhibitor, malonate, in order to confirm that most of the activity observed was indeed attributed to complex II and III. In the presence of the inhibitor (malonate), cytochrome c was reduced at a rate of 12.83 ± 3.81 nmol min^{-1} mg^{-1} mitochondrial protein. The sensitivity of Complex II and III activity in the bystander signal-exposed samples was therefore 82.46 ± 7.4%.

3.1.3. Complex IV activity

The activity of cytochrome c oxidase was measured in non-irradiated (control) mitochondrial samples and in mitochondrial samples isolated from cells exposed to electromagnetic bystander signals (Fig. 3). The activity found in control mitochondrial samples was 22.75 ± 7.22 nmol min^{-1} mg^{-1}. The activity in control samples was not significantly different from the activity in mitochondrial samples exposed to the bystander signal (20.58 ± 4.50 nmol min^{-1} mg^{-1}, p = 0.926). The lack of statistical difference between the complex activity between treatment and control samples suggests that the bystander signal was not effective in altering the ability of complex IV to oxidize cytochrome c.

When the complex IV inhibitor, potassium cyanide (KCN), was added into the reaction mixture and it was found that the activity for control samples and treatment

samples were 4.73 ± 0.55 nmol min^{-1} mg^{-1} and 4.84 ± 0.99 nmol min^{-1} mg^{-1}, respectively. From the inhibitor-treated samples it can be concluded that the specific activity of Complex IV in the control samples was 18.01 ± 7.22 nmol min^{-1} mg^{-1} and 15.75 ± 3.76 nmol min^{-1} mg^{-1} in the treatment samples. The sensitivity of complex IV was therefore found to be 74.05 ± 7.27% for the control samples and 75.90 ± 3.34% for the treatment samples.

3.1.4. Complex V activity

The activity of ATP synthase was determined by measuring the rate of NADH oxidation via tracking absorbance at 340 nm (Fig. 4). When mitochondrial from non-irradiated control cells were assayed, the activity of complex V was found to be 96.70 ± 26.07 nmol min^{-1} mg^{-1} of mitochondrial protein. In contrast, the activity of complex V that was exhibited by mitochondria isolated from bystander signal-exposed cells was significantly lower (p < 0.0001) at 19.03 ± 6.33 nmol min^{-1} mg^{-1}. The activity expressed by the bystander mitochondrial was similar to the activity that was found upon incubation of the reaction mixture with the complex V inhibitor, Oligomycin A (activity of control samples with inhibitor: 11.85 ± 6.39 nmol min^{-1} mg^{-1}, p = 0.920; activity of bystander-exposed samples with inhibitor: 8.59 ± 2.00 nmol min^{-1} mg^{-1}, p = 0.792). With the use of the inhibitor, we can see that the sensitivity of complex V's activity was 87.20 ± 4.30% and that the rest of the NADH oxidation being observed was background activity that is not attributed to the action of complex

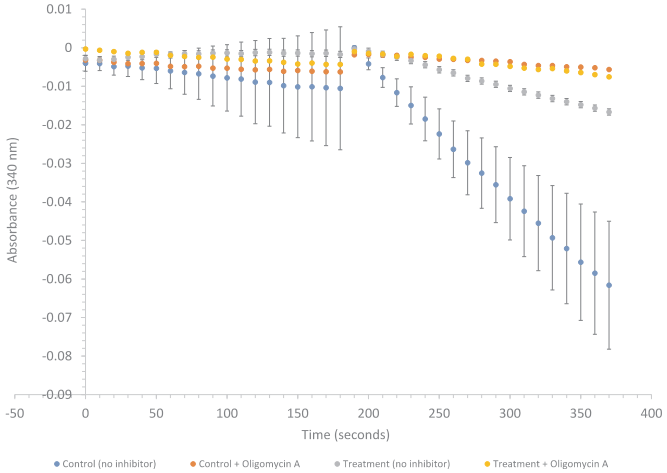

Fig. 4. Complex V activity. Oxidation of NADH demonstrated by a decrease in absorbance at 340 nm over a 3 min duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) obtained from Human colon carcinoma cells with wild type p53 (HCT-p53+/+) tested in triplicate (3 technical replicates). Errors bars represent standard error for n = 9.

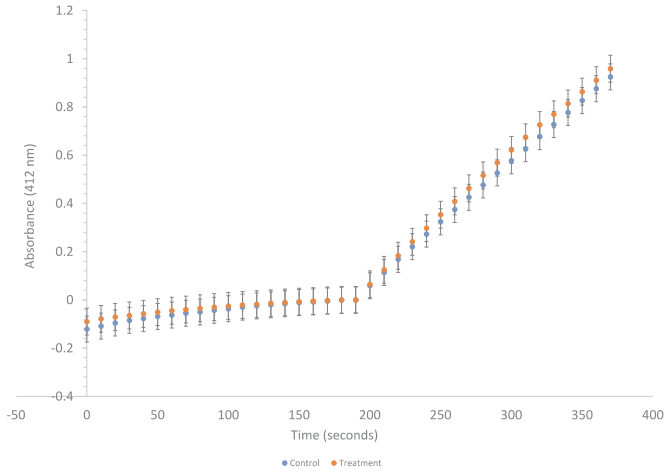

Fig. 5. Citrate Synthase activity. The formation of citric acid demonstrated by an increase in absorbance at 412 nm over a 3 min duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) obtained from Human colon carcinoma cells with wild type p53 (HCT-p53+/+) tested in triplicate (3 technical replicates). Errors bars represent standard error for n = 9.

V. The results illustrated here also suggest that the electromagnetic bystander signal is effective in compromising mitochondrial Complex V activity given that the activity observed in the bystander samples was considerably lower than that observed in the control samples. It can be suggested from these observations that the bystander signal could be responsible for compromised ATP and energy production by the mitochondria.

3.1.5. Citrate synthase activity

The citrate synthase activity found for control samples was $672.74 \pm 76.58\ \text{nmol min}^{-1}\ \text{mg}^{-1}$ and $682.92 \pm 32.04\ \text{nmol min}^{-1}\ \text{mg}^{-1}$ for treatment samples. The activity between these samples was not significantly different ($p = 0.834$). Because the citrate synthase activities between control and treatment samples were similar, we can be confident that the two different types of samples did not differ in terms of the mitochondrial content. Thus, any differences in activity observed in complexes I through V are attributed to modifications induced by the treatment itself, as opposed to differences in mitochondrial quantity. Citrate Synthase activity for control and treatment samples is illustrated in Fig. 5.

4. Discussion

The enzymatic activity experiments presented in this paper demonstrated an impairment in the function of Complex I, also known as NADH dehydrogenase or NADH: ubiquinone oxidoreductase, such that NADH exhibited a significantly diminished ability to become oxidized into NAD+. We have previously shown (Le et al., 2017c) that the emitted biophoton intensity that is emitted from ^{3}H irradiated cells is related to ^{3}H activity i.e. as the ^{3}H activity increases biophoton increases. Published intensity measurements were conducted at 340 ± 5 nm, but biophotons are emitted at wavelengths outside of this range. Recent preliminary data from our laboratory suggest that photons that range in wavelength from UV through visible to infra red are emitted and the intensity across these wavelength ranges changes with ^{3}H activity. It is possible therefore that blue light (450–495 nm) emitted by the ^{3}H-irradiated HCT116 p53 +/+ cells triggered a modification in the biological activity of the electron transfer flavoprotein (ETF) responsible for shuttling the 2 electrons from NADH to the flavin mononucleotide (FMN) (Gautier et al., 2014). When light induces chromophore isomerization in proteins which possess "optical actuators", such as flavoproteins, the protein undergoes a conformational or chemical change resulting in the modifycation of the protein's activity whereby the modification in activity can manifest as an activation or deactivation (Conrad et al., 2014). It is suggested that the absorption of biophotons in the blue wavelength range emitted from ^{3}H-irradiated cells is able to induce a modification in ETF such that its ability to accept electrons from NADH is impaired thus leading to a low rate of NADH oxidation into NAD+. This is possible since photons are known to reduce flavoproteins (Penzer and Radda, 1968); that being said, the photoreduction of ETF would render it unable to accept electrons from NADH since it already carries an electron or 2 electrons as a result of the photoreduction. The relative inefficiency in electron acceptance by ETF will lead to fewer electrons being donated to FMN and subsequently, fewer electrons being sent downstream to iron-sulfur clusters (Fe-S) and finally to ubiquinone (Q) to become ubiquinol (QH_2). Ultimately, the diminished ability for electron transport through Complex I will result in an overall lower rate of proton (H^+) pumping into the intermembrane space. As basal complex 1 activity was also observed to be the lowest of the complexes evaluated in this study, any loss in activity would therefore have a more pronounced effect on proton pumping by the ETC. In addition to ETF, electron transfer flavoprotein: ubiquinone oxidireductase (ETF-QO) was also initially considered as a candidate to explain the effects observed in Complex I since its activity is also triggered or diminished by blue light wavelengths. However, its role in the observed reduction in activity cannot be confirmed because the endpoint for measuring Complex I activity was the oxidation of NADH. Since ETF-QO participates in the shuttling of electrons through the series of Fe-S clusters to ubiquinone, we did not have a direct means of measuring its role as we did not measure the activity of this component of Complex I.

Complex IV (cytochrome c oxidase) activity in the cells exposed to radiation-induced biophotons was increased slightly above controls, however, the difference was not significant. It is possible that Complex IV was not influenced by the biophotons at all. Alternatively, it is also possible that the observed effect resulted from the interaction of a multiple biophoton wavelengths to produce the net effect observed. It is well document that red and infrared light acts as a photomodulator upon cytochrome c oxidase to ultimately stimulate an increase in ATP production (Wong-Riley et al., 2005; Karu, 2010; Brunori et al., 2005). Photons ranging from 600 to 850 nm are used to stimulate cytochrome c oxidase activity by taking advantage of the absorbance profiles of 4 redox centres (Cu_A, Cu_B, heme a, heme a_3) (Karu, 2010) belonging to cytochrome c oxidase. These redox centres are responsible for transferring electrons from cytochrome c to dioxygen (reducing to water), to drive the proton pumping function of Complex IV. The acceptance of incoming red photons has been shown to accelerate the process of electron transfer by the redox centres (Brunori et al., 2005; Marcus and Sutin, 1985). Photons in this range can act to stimulate both Cu_A and Cu_B activity (Karu, 2010) contributing to what would be observed as an increase in Complex IV activity above that exhibited by the control. However, while Complex IV demonstrated an increase in activity in the current study, it was not significantly different from the activity of Complex IV in control cells. It is suggested that the stimulatory effect induced by the incidence of light at red wavelengths must compete with the absorption of blue photons by cytochrome c oxidase (Peak absorption by cytochrome c oxidase occurs between 400 and 440 nm (Chen et al., 1992; Lubart et al., 1992) which induces photochemical destruction to heme a_3, also known as cytochrome a_3 resulting in an impairment of the overall function of cytochrome c oxidase (Epel and Butler, 1969; Ninnemann et al., 1970)).

In the current study, Complex V, or ATP synthase, activity demonstrated significantly diminished activity in cells exposed to biophotons compared to control cells. Such an effect means that overall ATP production by biophoton-exposed cells is compromised. This effect can be explained by a reduction in the strength of the electrochemical gradient across the inner mitochondrial membrane. Since fewer electrons were able to be shuttled from NADH to Ubiquinone in Complex I, the proton pumping function of Complex I did not have as much energy available

to carry out its proton pumping function. This results in the presence of fewer H^+ ions in the intermembrane space and consequently a weaker electrochemical gradient. The electrochemical gradient is quite strongly dependent upon the function of Complex I because NADH (involved in Complex I) is better at donating electrons than is FADH (involved in Complex II). Because of this, Complex I actually pumps protons across the membrane whereas Complex II does not. Subsequent to this step, the electrons from both Complexes I and II are carried to Complex III by Ubiquinol. Subsequently, the electrons from Complex III are carried to Complex IV by cytochrome c. While there may be a relatively lower quantity of electrons being input into Complexes III and IV, their actual functional capacities are not affected by upstream impairments. To elaborate, the rate at which they can oxidize or reduce cytochrome c remains unchanged. The result of the input of fewer electrons into the system is simply a lower overall quantity of oxidized or reduced cytochrome c molecules. In contrast, the function of ATP synthase is mediated by the concentration of H^+ ions in the intermembrane space because H^+ ions act to turn the ATP synthase "turbine" in the process of chemiosmosis. Due to the dependence of chemiosmosis upon the H^+ ion concentration, ATP synthase function is affected by upstream impairments that result in compromised proton pumping. The impairment of NADH's electron donor efficiency results in a reduction in the intermembrane space's proton concentration by up to 63%. This is because transfer of NADH electrons along the ETC leads to the pumping of approximately 10 protons from the matrix to the intermembrane space, whereas transport of $FADH_2$ electrons along the ETC drives the pumping of only approximately 6 protons (Ripple et al., 2013). Thus, in the case where the oxidation of NADH is completely inhibited, there will be 63% fewer protons than expected in the intermembrane space available for use in ATP production by ATP synthase.

Compromised ATP synthesis characterizes a state of mitochondrial dysfunction which can lead to impairments in biological function. On a cellular level, when a cell is severely deficient in ATP to the extent where it does not have sufficient energy to sustain processes required for viability, the cell can undergo apoptotic death or necrotic cell death which does not require regulation via the input of energy (Kushnareva and Newmeyer, 2010; Nicotera and Melino, 2004). On the whole-organism level, mitochondrial dysfunction has been shown to exhibit a strong correlation with the severity of fatigue in humans (Myhill et al., 2009; Filler et al., 2014). In a study by Myhill et al., venous blood samples were taken from patients experiencing fatigue and from healthy volunteers to show that ATP concentration in neutrophils and the efficiency of oxidative phosphorylation was significantly diminished in the participants experiencing fatigue compared to healthy controls (Myhill et al., 2009). Moreover, the assessment of mitochondrial enzyme activity in fatigue patients with known mitochondrial disorders found reductions in complex I, III and IV activities compared to controls (Smits et al.). This literature in concert with the current study's findings provide support for a possible role of biophoton bystander signalling in the induction of fatigue.

5. Conclusions

Biophotons emitted from human cell lines exposed to ionizing radiation possess the capability of modulating the activity of the electron transport chain to ultimately modify the mitochondrial ATP production process. In the particular cell line investigated in the current study (HCT116 p53 +/+), the biophotons emitted as a result of β-irradiation were effective in reducing the activity of Complex I which consequently affected the ability of ATP synthase to produce ATP due to a deficiency of H^+ in the intermembrane space. The impairment of ATP synthesis by radiation-induced biophoton signalling suggests a possible etiological role for radiation in driving fatigue in whole organisms. While these results provide evidence to support the ability of biophotons to modulate biological functions, the biophoton spectrum of emission is probably very complex and is likely not isolated to eliciting effects upon a single biological system. Further investigation will be required to further elucidate the effects that biophotons can have upon bystander populations.

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Roles of each author

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