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A melatonin-based fluorescence method for the measurement of mitochondrial complex III function in intact cells

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Abstract

Mitochondrial complex III (MC-3) plays a pivotal role in electron transfer and oxidative phosphorylation. Impaired MC-3 functions may contribute to a variety of diseases by interrupting normal bioenergetics and increasing reactive oxygen production and oxidative stress. Currently, MC-3 function is assessed by measuring the cytochrome c reductase activity spectrophotometrically in isolated mitochondria or MC-3. The cytoplasmic microenvironment critical for mitochondrial complex functions may be depleted during these isolation processes. The development of a reliable method to measure MC-3 activities in intact cells or tissues is highly desirable. This report describes a novel fluorescence-based method to assess MC-3 functions, i.e. Qi site electron transfer, in the intact cells. Human mesangial and teratocarcinoma NT2 cells were used to demonstrate that melatonin induced oxidation of 2',7'-dichlorodihydrofluorescein (H₂DCF) was inhibited by antimycin A, the MC-3 Qi site specific inhibitor; but not by myxothiazol, the MC-3 Qo site specific inhibitor, nor rotenone, the mitochondrial complex I inhibitor. These results indicate that melatonin induced oxidation of H₂DCF is reflecting MC-3 Qi site electron transfer activities. Modifying structures of the side groups at the R3 and R5 positions of the indole ring of melatonin diminished its efficacy for inducing H₂DCF oxidation, suggesting a specific interaction of melatonin with the MC-3 Qi site. These results suggest that the fluorogenic property of melatonin-induced H₂DCF oxidation provides a MC-3 Qi site electron transfer specific measurement in intact cells. Interestingly, by using this method, the Qi site electron transfer activity in transformed or immortalized cells was found to be significantly higher than the non-transformed cells.

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Keywords

mitochondrial complex III; Qi site; electron transfer; melatonin; H₂DCF; cancer

Introduction

The mitochondrial complex III (MC-3) or *bc1* complex catalyzes the transfer of electrons from ubiquinol to cytochrome *c* and the concomitant pumping of protons from the mitochondrial matrix to the intermembrane space. The MC-3 is composed of multiple subunits and contains two distinct quinone-binding sites (i.e., the ubiquinol oxidation site [Qo] and the ubiquinone reduction site [Qi]), which are located on opposite sides of the inner mitochondrial membrane. The transfer of electrons from ubiquinol to cytochrome (cyt) *c* involves multiple single-electron steps and ubiquinol/semiubiquinone transition, and is accomplished by a process termed Q cycle. Following binding of ubiquinol in MC-3, the electron transfer at the Qo site occurs in a bifurcated manner between cyt *c*₁ and cyt *b*, mediated by the motion of Rieske iron sulfur proteins. The electrons transferred to cyt *c*₁ lead to reduction of cyt *c* whereas electrons transferred to cyt *b* at the b_L and b_H hemes reduce the semiubiquinone at the Qi site to further drive the Q cycle [1,2]. Electron transfer at the Qo site is inhibited by myxothiazol and stigmatellin while at the Qi site is specifically blocked by antimycin A and other inhibitors [3].

Impaired electron transfer of mitochondria resulting in deficiencies in bioenergetics and overproduction of reactive oxygen species (ROS) has been implicated in the pathogenesis of various human diseases, including metabolic syndrome, accelerated aging, neurodegenerative disorders, diabetes, cardiovascular disorders, and cancer [4–6]. Impairment of mitochondrial electron transfer may result from dysfunction of the individual complex or a combination of complexes of the respiratory chain. For example, we have previously demonstrated the concurrent upregulation of complex I and diminution of complex III in renal mitochondria from db/db mice with nephropathy [7]. The evaluation of individual mitochondrial complexes is thus essential to unlock the mechanisms involved with mitochondrial dysfunction in diseases.

Currently, MC-3 function is assessed by measuring the cyt *c* reductase activity in isolated mitochondria [8], a technically sensitive but cumbersome measurement [9]. Another drawback of this measurement is that MC-3 function is not evaluated in intact cells because of the limited permeability of cyt *c* and interferences from other cellular chromophores. The assessment of MC-3 function via cyt *c* reductase activity or other spectrometric methods in isolated mitochondria without the cytoplasmic microenvironment niche, where the important regulatory mechanisms of mitochondrial function reside, may not truly reflect cellular MC-3 functions. For example, recent attempts to measure the reduction of cyt *b* at the b_L and b_H hemes by ubiquinol showed that in isolated MC-3 the electron transfer is neither inhibited by antimycin A nor myxothiazol, two highly effective blockers of MC-3 function in intact mitochondria or intact cells [10]. In our previous studies, we have shown in isolated mitochondria that the melatonin-induced oxidation of 2',7'-dichlorodihydrofluorescein (H₂DCF) was specifically inhibited by antimycin A, but not myxothiazol or rotenone [7,11],

suggesting that the action of melatonin is largely dependent on the Qi site function of MC-3. In the current study, we have developed a novel method to measure MC-3 function in intact cells based on the melatonin-induced oxidation of H₂DCF. This method overcomes the limitations of the currently available methods and allows assessing MC-3 function in situ without isolation of mitochondria.

Materials and Methods

Materials

Melatonin, 5-methoxyindole, indole and gramine were purchased from Sigma (St. Louis, MO, USA) and dissolved in 100% ethanol at 100mM, stored at -20°C in the dark, and further diluted with ethanol if needed. 2',7'-dichlorodihydrofluorescein (H₂DCF) diacetate was purchased from Invitrogen (Eugene, OR, USA). During the measurement, the final concentration of ethanol in samples is 0.1%, which does not affect H₂DCF oxidation. Antimycin A, myxothiazol, rotenone and other chemicals were from Sigma. The kits (MC3-Qi kit) for MC-3 Qi site electron transfer measurement were acquired from QTK Technologies, Inc (San Antonio, TX). Human teratocarcinoma NT2 cell line was obtained from ATCC (Manassas, VA). Human mesangial cells (HMC) were kindly provided by Dr. Hanna E Abboud [Department of Medicine, University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, Texas]. Human bone marrow mesenchymal stem cells (MSC) were kindly provided by Dr. Xiao-Dong Chen (UTHSCSA) and cultured following the procedure established [12]. ParC5 cell line was a gift from Dr. David O Quissel (School of Dentistry, University of Colorado Health Science Center, Denver, CO), originally derived from freshly isolated rat parotid gland acinar cells following transformation with simian virus 40, and exhibited morphological, biochemical, and functional characteristics similar to acinar cells [13]. The HSY cell line was originally established by Yanagawa *et al.* [14] and was kindly provided by Dr. James Turner (NIDCR, National Institutes of Health, Bethesda, MD).

Preparation of rat parotid acinar cells

Parotid gland acinar cells were prepared from 3-month old male Sprague-Dawley rats following the procedure described previously by us [15]. Briefly, parotid glands were dissected, finely minced and digested with collagenase (96 U/ml, Wathington Biochemical Corp, Lakewood, NJ) and hyaluronidase (0.19mg/ml, Sigma) in Hank's balance salt solution containing 33 mM HEPES, pH 7.4 (HBSS) at 37°C for 60 min with vigorous agitation of 300 rpm. The digestive mixture was oxygenated every 10 min. At the end, the digestive mixture was passed through a 100 µm nylon cell strainer and the acinar cells were collected and washed with HBSS by centrifugation at 150 g for 5 min. The freshly isolated rat parotid acinar cells were labeled and used in the subsequent experiments for assessing MC-3 Qi site electron transfer activities. All procedures involving animals were approved by the South Texas Veterans Health Care System Institutional Animal Care and Use Committee and performed in compliance with the guidelines.

Measurement of melatonin-induced oxidation of H₂DCF

Primary human mesangial cells were cultured in RPMI medium supplemented with 15% fetal bovine serum for 3 days at 37°C in a humidified 5% CO₂/95% air incubator. The NT2 cell line was cultured in 100-mm dishes in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FBS) for 3 days till confluence. The cultured cells were then harvested with EDTA-Trypsin, suspended in phosphate buffered saline supplemented with 1 mM MgSO₄ and 1 mM CaCl₂ (PBS), and labeled with H₂DCF diacetate (1 μM) at 37°C for 30 min with agitation. Labeled cells were washed with 10 volumes of fresh PBS and suspended in PBS at a density of 2.5-3×10⁵ cells/ml or as specified. The oxidation of H₂DCF in these labeled cells was monitored by fluorescence with excitation wavelength of 506 and emission of 529 nm in a cuvette using a PTI QM-6 spectrofluorometer equipped with a magnetic mixer. Following recording of the baseline fluorescence intensity at room temperature for 2-4 min, melatonin (20 μM or as specified) was added to trigger the MC-3 Qi site dependent H₂DCF oxidation. Change in fluorescence was continuously monitored for another 5 min or more. To test the effect of MC-1 and MC-3 inhibitors on melatonin-induced H₂DCF oxidation, different concentrations of antimycin A, myxothiazol or rotenone were incubated with cells in the cuvette for 5 min prior to melatonin addition. The rates of fluorescence change following melatonin addition in the presence of these inhibitors were normalized to control (measured in the absence of MC-1 or -3 inhibitors).

Statistical analysis

Results presented as mean±S.E and Student's t-test was used to evaluate the differences between two groups. Differences were considered statistically significant at $P < 0.05$.

Results

Oxidation of H₂DCF increases fluorescence emission. Application of melatonin in suspensions of human teratocarcinoma NT2 and mesangial cells labeled with H₂DCF induced a steady increase in fluorescence, indicating rapid cellular oxidation of H₂DCF following the application of melatonin (Fig. 1). The rate of the melatonin-induced H₂DCF oxidation was constant and dependent on the concentrations of melatonin (0-20 μM), as shown in NT2 cells (Fig. 1A). Similar results were observed in primary mesangial cells (dose-response data not shown) except for the slope of melatonin (20 μM) induced H₂DCF oxidation was less than in NT2 cells (Fig. 1B). At the highest concentration of melatonin tested (100 μM), the rate of the melatonin-induced H₂DCF oxidation remained linear and no saturation was observed, similar to the dose-response relationship found previously in isolated mitochondria [7]. The rate of change in H₂DCF fluorescence in response to melatonin (20 μM) was not altered in NT2 cells with cell densities varying from 1 to 0.13 × 10⁶ cells/ml (data not shown). The human mesangial cells were considered non-transformed cells and used for the subsequent characterization of the melatonin induced oxidation of H₂DCF. In these experiments, melatonin and other analogs (5-methoxyindole, indole and gramine) were tested at 20 μM to produce good demonstrable signal to noise ratio.

We next tested the melatonin-induced H₂DCF fluorescence in intact mesangial cells in the presence of various specific mitochondrial complex inhibitors. Following pre-exposure of mesangial cells to the MC-3 Qi site specific inhibitor antimycin A, the melatonin-induced H₂DCF fluorescence increases were inhibited in a concentration-dependent manner with complete inhibition at 5 μ M (Fig. 2A). In contrast, the Qo site specific inhibitor myxothiazol had no effect (Fig. 2B) even at concentrations (5 μ M) that had been shown to inhibit Qo site electron transfer and ATP synthesis significantly in other systems [16]. The MC-1 specific inhibitor rotenone did not affect the melatonin-induced oxidation of H₂DCF at concentrations lower than 2 μ M; at high concentration (5 μ M), it slightly inhibited the melatonin response (~20%, Fig. 2C). These results indicate that the main effect of melatonin on H₂DCF oxidation in human mesangial cells is dependent on the Qi site electron transfer and the measurement of melatonin-induced H₂DCF oxidation provides an assessment of MC-3 activity in intact cells.

We next elucidated the effects of modifying the side groups at positions R3 and R5 on melatonin-induced H₂DCF oxidation in intact mesangial cells. The molecular structure of melatonin includes an indole ring with a methoxy group at position 5 (R5) and an acylaminoethyl side-chain at position 3 (R3) [17]. Melatonin and 5-methoxyindole both have the R5 methoxy group with 5-methoxyindole containing a hydrogen at R3. Indole is a compound with hydrogen at positions R3 and R5, and gramine with hydrogen at R5 and a dimethylaminomethyl group at R3. The potency of inducing H₂DCF oxidation in mesangial cells is significantly diminished for 5-methoxyindole and indole as compared to melatonin (Fig. 3 A, B and D), indicating that both the acylaminoethyl side-chain at R3 and the methoxy group at R5 were important structure requirements for effective induction of H₂DCF oxidation in intact mesangial cells and replacement of these side chains with hydrogen affected the efficacy. Gramine did not show any detectable effect on oxidation of H₂DCF, indicating that replacing the acylaminoethyl side-chain with a dimethylaminomethyl group at R3 and having the hydrogen at R5 completely eliminated the activity for inducing H₂DCF oxidation through the MC-3 Qi site (Fig. 3 C and D). The attenuated response of indole is completely inhibited by antimycin A (Fig. 3 E), but not myxothiazol (Fig. 3 F), further indicating that the H₂DCF oxidation within mesangial cells in response to melatonin is mediated by MC-3 Qi site electron transfer. These results suggest the side groups at the positions R3 and R5 of melatonin are essential for cellular H₂DCF oxidation through a MC-3 Qi site specific process.

Since the rate of Qi site electron transfer in NT2 cells is much higher than that of human mesangial cells (Fig. 1), we examined several other cell types to determine whether there is a gain of function in the Qi site electron transfer in transformed or immortalized cells using the MC-3Qi Kit based on the method described. In Fig. 4, we showed that the freshly isolated rat parotid acinar cells, the non-transformed human mesangial and MSC cells had significantly lower Qi site electron transfer activities than transformed or immortalized NT2, HSY and ParC-5 cells. Interestingly, compared to freshly isolated cells, SV40 immortalized rat parotid cells ParC-5 had dramatically increased MC-3 Qi site electron transfer activity (Fig. 4). These results suggested alteration of MC-3 function between non-transformed and transformed/immortalized cells.

Discussion

The method of measuring MC-3 function in intact cells has not been reported previously. We have demonstrated an innovative method to assess the MC-3 Qi site electron transfer in intact cells based on the measurement of melatonin-induced cellular H₂DCF oxidation. The oxidation of H₂DCF induced by melatonin is specifically dependent on the function of MC-3 Qi site electron transport and the increased fluorescence of H₂DCF oxidation by melatonin provides a measurement of MC-3 activity at the Qi site.

H₂DCF is oxidized by a variety of oxidants, such as peroxynitrite and hydrogen peroxide, which results in green fluorescence production [18]. The reaction between H₂DCF and ROS occurs via a nonenzymatic chemical reaction, leading to formation of a highly conjugated tricyclic system that can accommodate single electrons and is also highly fluorescent. Unlike the oxidation of H₂DCF by ROS, the melatonin-induced cellular oxidation of H₂DCF is dependent on the function of complex III of the mitochondrial respiratory chain and is specifically inhibited by the Qi site blocker antimycin A (Fig. 2). The melatonin-induced oxidation of H₂DCF is generally not affected by the complex I inhibitor rotenone, the MC-3 Qo site inhibitor myxothiazol (Fig. 2), the uncoupling reagent carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μ M) and the complex V (ATP synthase) inhibitor oligomycin A (5 μ M) in human renal mesangial cells (data not shown). The steady rates of melatonin-induced oxidation of H₂DCF are unsaturable in the ranges of melatonin concentrations tested (0.5-100 μ M, data not shown). Additionally, the rates of the melatonin responses remain linear without detectable decay over a period up to 20 min, even with very low melatonin concentration (0.5 μ M, Fig. 1). These features of the melatonin-induced oxidation of H₂DCF in intact mesangial cells are consistent with our previous observations in isolated mitochondria [11] but distinct from those of ROS mediated H₂DCF oxidation [19]. The melatonin side groups at positions R3 and R5 play an essential role in the MC-3 dependent H₂DCF oxidation since its analogues gramine, indole and 5-methoxyindole induced H₂DCF oxidation were either completely blocked or attenuated (Fig. 3). These results imply specific interactions between melatonin and the MC-3 Qi site although further studies are required to understand these interactions. The constant rate of H₂DCF oxidation observed under low melatonin concentration (0.5 μ M) for extended period (>20 min) suggests that the concentration of melatonin inside the cells and/or mitochondria may not alter during H₂DCF oxidation. Further studies will be needed to determine the fate of melatonin following binding or interaction with the MC-3 Qi site.

Melatonin or N-acetyl-5-methoxytryptamine is a compound naturally found in animals, plants, and microbes with powerful antioxidant activities [20]. Many *in vivo* effects of melatonin are mediated by the melatonin receptors. Numerous studies have documented that melatonin is a highly effective endogenous scavenger of ROS due to its unique antioxidant cascade reactions [21,22]. However, there are a few recent studies by us and others, which demonstrate that melatonin stimulates production of ROS in some cells and in isolated mitochondria at pharmacological concentrations [7,11,23,24]. While others have shown that inhibitors of phospholipase A2 and lipoxygenase largely blocked the melatonin (1 mM)-stimulated ROS production [24], our previous results demonstrated that the melatonin-stimulated ROS production was inhibited by antimycin A, but not myxothiazol and rotenone

in isolated mitochondria [7,11]. These findings indicate that the melatonin response is likely mediated by the MC-3 Qi site electron transfer. The concentrations of mitochondrial complex inhibitors used in our previous and current studies were in the range that block mitochondrial respiration [25]. In this current work, we provide further evidence that melatonin-induced oxidation of H₂DCF is inhibited specifically by antimycin A, but not rotenone and myxothiazol in intact cells (Fig. 2). The observation that FCCP, an uncoupling reagent, had no effect on melatonin-induced oxidation of H₂DCF further suggests that the MC-3 Qi site mediated H₂DCF oxidation occurs independent of mitochondrial membrane potential. Most interestingly, our current results provide a quick and reliable method, through melatonin-induced H₂DCF oxidation, to specifically evaluate MC-3 Qi site electron transfer activities in the intact normal or pathogenic/metabolic altered cells.

We have used this newly described MC-3 analytic method to survey MC-3 activities in several transformed/immortalized cells or non-transformed cells. Intriguingly, transformed or immortalized cells (ParC-5, NT2, and HSY) were found to have significantly higher activities at the MC-3 Qi site electron transfer in response to melatonin (Figs. 1 and 4). The significance of these results requires further studies to delineate the mechanisms underlying the differences in basal MC-3 activities between normal and transformed cells and whether these observations are associated with cell transformation. It has been reported recently that a gain of function in mitochondrial oxidative phosphorylation is associated with Li-Fraumeni syndrome carrying the germline *TP53* mutations [26]. It is well established that p53 functions as a tumor suppressor and regulates various aspects of many essential cellular functions, such as cell proliferation, DNA repair, apoptosis and mitochondrial respiration [27]. As MC-3 is a major site of superoxide generation in mitochondria [28] and markers of oxidative stress are often increased in a variety of cancer [29–31], it is tempting to speculate that the higher MC-3 function as we detected in immortalized or transformed cells may contribute to the elevated oxidative stress seen in cancer. Whether the gain of function in the Qi site electron transfer activity in MC-3 observed in ParC-5, NT2 and HSY cells is a phenomenon applicable to other tumor cells as well as physiological significance of the increased Qi site electron transfer activity in MC-3 requires further investigations.

It is well documented that melatonin is synthesized from serotonin by the sequential action of arylalkylamine N-acetyltransferase and hydroxyindole-O-methyltransferase [32]. The pineal gland is considered as the main site for melatonin production in mammals [33]. It has been shown that N-acetyltransferase or hydroxyindole-O-methyltransferase, the enzymes critical to melatonin synthesis, are not detected in the kidney [34], which contains mesangial and other renal cell types. Therefore, the potential interference from endogenous melatonin should be small and negligible for mesangial cells. However, caution should be taken for using this method to assess MC-3 Qi site electron transfer activities in pinealocytes or cells that may produce high concentrations of endogenous melatonin.

In summary, our current studies have established an easy and effective method to assess MC-3 function in intact cells based on the fluorogenic property of melatonin-induced H₂DCF oxidation.

Acknowledgments

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Fig 1.

Melatonin-induced fluorescence of 2',7'-dichlorodihydrofluorescein (H₂DCF) in intact human teratocarcinoma NT2 and primary mesangial cells. Fluorescence in H₂DCF labeled cells was recorded at 506 nm excitation and 529 nm emission for 2-4 min *prior to* melatonin addition (arrow) to measure the baseline. Following addition of 0-20 μM of melatonin as indicated by the arrows in NT2 (A) or mesangial cells (B), the fluorescence was continuously measured for at least 5 min. The rate of increase in fluorescence was linear and the melatonin concentration dependent.

Fig 2.

Effects of mitochondrial complex inhibitors on melatonin-induced fluorescence of 2',7'-dichlorodihydrofluorescein (H₂DCF). Following recording the baseline fluorescence intensity at room temperature for 2-4 min, melatonin (20 μ M) was added to start the melatonin-induced H₂DCF oxidation. To test the effect of inhibitors of mitochondrial complexes I and III, different concentrations of antimycin A (A), myxothiazol (B) or rotenone (C) were incubated with cells in the cuvette for 5 min prior to melatonin addition. The rates of H₂DCF oxidation as indexed by fluorescence change following melatonin addition in the presence of these inhibitors were normalized to controls (measured in the absence of any inhibitors). The values in the graphs were percentage of the control rate of melatonin-induced H₂DCF oxidation in the presence of different inhibitors of complexes I and III, respectively, and represent results from a typical experiment.

Fig 3.

Structure of the side groups at positions R3 and R5 alters the efficacy of compounds in inducing 2',7'-dichlorodihydrofluorescein (H_2DCF) oxidation. Compounds with similar structures to melatonin, i.e. possessing an indole ring with different side groups at positions R3 and R5, were compared for their efficacy in inducing H_2DCF oxidation in labeled human mesangial cells. Cellular H_2DCF oxidation were monitored as described in Fig 1 and started by addition of melatonin (20 μM , A), the structural analogs 5-methoxyindole (20 μM , D) or indole (20 μM , B), which have either methoxy or hydrogen at R5, respectively and hydrogen at positions R3, or gramine (20 μM , C), which has a hydrogen at R5 and a dimethylaminomethyl group at R3. The values in D are mean $\pm$ SE rates of melatonin, 5-methoxyindole, indole, and gramine-induced H_2DCF oxidation from three experiments, * $P < 0.01$ vs melatonin. Despite reduced potency compared to melatonin, the responses of indole were completely abolished by antimycin A (2 μM , E), but not by myxothiazol (2 μM ,

F). The arrows in the figure indicate the addition of melatonin, 5-methoxyindole, indole, gramine or mitochondrial complex III inhibitors, respectively.

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Fig 4.

Comparison of MC-3 Qi site electron transfer between normal and cancerous or immortalized cells. The rates of melatonin (20 μ M) induced H_2DCF oxidation were measured in human mesangial cells (HMC), human bone marrow mesenchymal stem cells (MSC), freshly isolated rat parotid gland acinar cells (PG), ParC-5, HSY and human teratocarcinoma NT2 as described in Methods. The values in the graphs were mean $\pm$ SE from 3-6 measurements, * P <0.01 vs transformed or immortalized cells.