Video Article

Measuring Trans-Plasma Membrane Electron Transport by C2C12 Myotubes

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Abstract

Trans-plasma membrane electron transport (tPMET) plays a role in protection of cells from intracellular reductive stress as well as protection from damage by extracellular oxidants. This process of transporting electrons from intracellular reductants to extracellular oxidants is not well defined. Here we present spectrophotometric assays by C2C12 myotubes to monitor tPMET utilizing the extracellular electron acceptors: water-soluble tetrazolium salt-1 (WST-1) and 2,6-dichlorophenolindophenol (DPIP or DCIP). Through reduction of these electron acceptors, we are able to monitor this process in a real-time analysis. With the addition of enzymes such as ascorbate oxidase (AO) and superoxide dismutase (SOD) to the assays, we can determine which portion of tPMET is due to ascorbate export or superoxide production, respectively. While WST-1 was shown to produce stable results with low background, DPIP was able to be re-oxidized after the addition of AO and SOD, which was demonstrated with spectrophotometric analysis. This method demonstrates a real-time, multi-well, quick spectrophotometric assay with advantages over other methods used to monitor tPMET, such as ferricyanide (FeCN) and ferricytochrome c reduction.

Video Link

The video component of this article can be found at https://www.jove.com/video/57565/

Introduction

The ability of purified plasma membranes to reduce electron acceptors has led to the view that the plasma membrane has an inherent redox capacity[1]. Previously seen in fungi, plants, and animals, tPMET is a process common to multiple organisms[2,3,4,5]. Specifically, this process has been demonstrated in *Saccharomyces cerevisiae*, carrot cells, erythrocytes, lymphocytes, osteosarcoma, melanoma, macrophages, skeletal muscle, and neutrophils[2,3,4,5,6,7]. In a process that transports electrons across the plasma membrane to reduce extracellular oxidants, tPMET is involved in many cellular functions including: cell growth[5,8], cell viability[9], iron metabolism[10], cell signaling[11,12,13], and protection from reactive oxygen species[12,14,15]. Due to tPMET's involvement in many cellular functions, an imbalance of tPMET has been hypothesized to contribute to the development of some serious health conditions, including cancer[16], cardiovascular disease[17], and metabolic syndrome[18].

There are multiple ways to monitor the transfer of electrons across the plasma membrane, but the most widely used technique is to assess the reduction of extracellular electron acceptors through colorimetric assays. Commonly used extracellular electron acceptors are tetrazolium salts, DPIP, FeCN, and ferricytochrome c[19,20]. The most commonly used tetrazolium salt is a second-generation salt known as WST-1[19]. This compound is easier to utilize in colorimetric assays compared to first generation tetrazolium salts due to two sulfonate groups, which increase its water solubility[21]. WST-1, in conjunction with the intermediate electron acceptor 1-methoxy-phenazine methosulfate (mPMS), is reduced in two single-electron transfer events. This reduction changes the weakly-colored oxidized form of WST-1 to a more intense, yellow formazan[20,22]. WST-1 has a high molar extinction coefficient of 37×10^3 $M^{-1}cm^{-1}$, leading to a high assay sensitivity[21,22]. DPIP is also utilized as an extracellular electron acceptor to monitor tPMET. It has been shown that DPIP can be reduced extracellularly by tPMET without the aid of intermediate electron acceptors[23,24]. Due to the lack of intermediate electron acceptors, DPIP can directly pick-up electrons from the plasma membrane, unlike WST-1[24]. Similar to DPIP, FeCN has been shown to be reduced extracellularly to ferrocyanide by tPMET without the aid of intermediate electron acceptors[19,24]. Unlike WST-1 and DPIP, FeCN has a low molar extinction coefficient leading to a lower assay sensitivity[9]. Another commonly used extracellular electron acceptor to monitor tPMET is ferricytochrome c. Similar to WST-1, ferricytochrome c reduction increases with the use of intermediate electron acceptor, mPMS[22]. Unlike WST-1 though, the ferricytochrome c method is less sensitive due to a high background and a low molar extinction coefficient[22].

Here we present a method for real-time analysis of tPMET through spectrophotometric assays. The method utilized the extracellular electron acceptors WST-1 and DPIP, as they both have a high molar extinction coefficient while being less expensive compared to the other commonly used extracellular electron acceptors such as ferricytochrome c. We utilized phenazine methosulfate (PMS) instead of mPMS they have a similar chemical makeup and PMS is far less expensive. mPMS is photochemically stable which is an important characteristic for a commercial kit that needs a long shelf life. However, we make PMS fresh for each assay, so stability should not be an issue. We also present a method to evaluate possible enzymatic interactions (see **Figure 1**) between the extracellular electron acceptor and enzymes that may be utilized to further

characterize the process of tPMET. Specifically, the enzymes AO and SOD can be used determine which portion of tPMET is due to ascorbate transport or extracellular superoxide release, two common methods for electrons to be transported across the plasma membrane.

Protocol

NOTE: See **Figure 1** for a schematic overview of key steps.

1. WST-1 Reduction Assay

1. Grow and differentiate C2C12 adherent cells using standard cell culture procedures[7] in a 96-well plate utilizing rows A-F.
   1. Use a differentiation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% horse serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Incubate the cells at 37 °C with 5% CO_2.
   2. When monitoring ascorbate involvement in tPMET, supplement differentiation media with 100 µM ascorbic acid. Allow cells to incubate in differentiation media for ~24-48 h.

2. Prepare stock WST-1 and PMS solutions.
   1. To make a 10 mM stock of WST-1, dissolve 0.033 g of WST-1 (Formula Weight (FW): 651.34 g/mol) in 5 mL of phosphate buffered saline (PBS). Store at 4 °C.
   2. To make a 5 mM stock of PMS, dissolve 0.0023 g of PMS (FW: 306.34 g/mol) in 1.5 mL of deionized H_2O (diH_2O). Store at -20 °C and protect from light.

3. Add 0.0108 g of glucose for a final concentration of 5 mM to 11.4 mL of PBS or HEPES buffered saline (HBS; 20 mM HEPES sodium salt, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$). Add 480 µL of 10 mM WST-1 for a final concentration of 400 µM and 48 µL of 5 mM PMS for a final concentration of 20 µM.
4. When monitoring ascorbate involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 6 µL of diH_2O and to the other aliquot add 6 µL of 2 kU/mL AO for a final concentration of 2 U/mL.
5. When monitoring superoxide involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 55 µL of 0.1 M KPO_4 buffer and to the other aliquot add 55 µL of 6.5 kU/mL SOD for a final concentration of 60 U/mL.
6. Wash the cells with PBS. Aspirate the media and add 150 µL PBS. Then aspirate the PBS.
   NOTE: The assay is done with plated, attached cells. Thus, there is no centrifugation or use of trypsin in the washing process.
7. Add 100 µL of WST-1 solution (-) SOD or (-) AO to columns 1-6 and add 100 µL of WST-1 solution (+) SOD or (+) AO to columns 7-12 in the 96-well plate. Use rows G and H as background controls (*i.e.*, to monitor change in absorbance in the reagent alone in wells without cells).
8. Measure the absorbance values using the spectrophotometer every 10 min for 1 h at 438 nm.
9. After reading, aspirate the media and wash each well with 150 µL of PBS. Then aspirate the PBS.
10. Calculate the change in absorbance for each well by subtracting the initial absorbance for a well from the absorbance at any time point for that well. Correct this change for the change in absorbance (if any) observed in the background wells (*i.e.*, wells with assay solution but no cells).
11. For analysis, normalize the absorbance data to the 60 min control measurement or wash wells with PBS or HBS and then perform a bicinchoninic acid (BCA) protein assay.
12. Add 2 mg/mL bovine serum albumin (BSA) standard (range from 0.5-2 µL for the standard curve, depending on the degree of confluence of the cells) to the empty wells (rows G and H), then add the BCA reagent to all wells.
13. Quantify the data via nmol of WST-1 reduction per µg of protein. Use 37 mM^{-1} cm^{-1}[22] as the extinction coefficient for reduced WST-1 at 438 nm.

2. DPIP Reduction Assay

1. Grow and differentiate C2C12 adherent cells with the same procedure as step 1.1.
2. Prepare stock 10 mM DPIP solution as follows. Dissolve 0.029 g of DPIP (FW: 290.08 g/mol) in 10 mL of diH_2O. Confirm the concentration of DPIP by measuring absorbance at 600 nm with a spectrophotometer. Use 1 mM^{-1} cm^{-1}[23] as the extinction coefficient for reduced DPIP at 600 nm. Store at 4 °C.
3. Add 0.0108 g of glucose to 11.880 mL of PBS for a final concentration of 5 mM. Add 120 µL of 10 mM DPIP for a final concentration of 100 µM.
4. When monitoring ascorbate involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 6 µL of diH_2O and to the other aliquot add 6 µL of 2 kU/mL AO for a final concentration of 2 U/mL.
5. When monitoring superoxide involvement in tPMET, divide the solution into two 6 mL aliquots. To one aliquot, add 55 µL of 0.1 M KPO_4 buffer and to the other aliquot add 55 µL of 6.5 kU/mL SOD for a final concentration of 60 U/mL.
6. Aspirate the media and wash cells in 150 µL of PBS. Aspirate the PBS and add 100 µL of DPIP solution (-) SOD or (-) AO to columns 1-6 and add 100 µL of DPIP solution (+) SOD or (+) AO to columns 7-12 in the 96-well plate. Use rows G and H will as background controls (*i.e.*, to monitor change in absorbance in the reagent alone in wells without cells).
7. Measure the absorbance at 600 nm using spectrophotometer every 10 min for 1 h. Quantify the change in absorbance relative to the control at 60 min similar to steps 1.9-1.13.

3. Determination of Whether Reduced Electron Acceptors Are Substrates for AO or SOD

1. To 5.436 mL of PBS or HBS, add 240 µL of 10 mM WST-1 for a final concentration of 400 µM and 24 µL of 5 mM PMS for a final concentration of 20 µM.
   1. For DPIP, add 60 µL of 10 mM DPIP, for a final concentration of 100 µM, to 5.940 mL of PBS or HBS.

2. Add 100 µL of solution to each well in a flat-bottom 96-well plate in the absence of cells and measure the absorbance in a spectrophotometer at 438 nm, for WST-1, or 600 nm, for DPIP.
3. Add 1 µL of 10 mM ascorbate to half of the wells for a final concentration of 100 µM. Monitor the absorbance until it stabilizes.
4. Upon stabilization, add 1 µL of 200 U/mL AO for a final concentration of 2 U/mL to each well or add 1 µL of 6 kU/mL SOD for a final concentration of 60 U/mL to each well and monitor the absorbance for 1 h.

Representative Results

Statistics were performed with ANOVA with repeated measures using RStudio statistical software[25]. Sample sizes are indicated in the figure legends.

To monitor tPMET, C2C12 myotubes were utilized along with extracellular electron acceptors, WST-1 and DPIP. AO was used to determine which portion of WST-1 and DPIP reduction was due to ascorbate efflux and SOD was used to determine which portion of WST-1 reduction was due to extracellular superoxide release. As shown in **Figure 2**, C2C12 myotubes are capable of tPMET as seen by the reduction of WST-1. With the addition of AO, WST-1 reduction was suppressed by ~30% indicating that ~30% of tPMET was due to the export of ascorbate (**Figure 2A**). With the addition of SOD, WST-1 reduction was suppressed by ~70%, indicating that ~70% of tPMET was due extracellular superoxide release (**Figure 2B**). When DPIP was utilized as an extracellular electron acceptor with the addition of AO, DPIP reduction was suppressed over 100% (**Figure 3**). This raised questions regarding the validity of using DPIP with AO to assess contribution of ascorbate.

To look into whether reduced WST-1 is a substrate for AO or SOD, WST-1 was reduced with ascorbic acid. AO or SOD was added, and the absorbance was monitored. After 1 h, there was no change in absorbance between the reduced form of WST-1 without AO or SOD and the reduced form of WST-1 with AO or SOD (**Figure 4A, B**). Therefore, the reduced WST-1 is not a substrate for these enzymes, and the use of AO or SOD in conjunction with WST-1 is appropriate for assessment of roles of ascorbate or superoxide, respectively.

To determine if reduced DPIP is a substrate for AO or SOD, DPIP was reduced with ascorbic acid. After 20 min, AO was added, and the absorbance was monitored. Reduced DPIP was shown to be a substrate for AO, as seen in **Figure 5A**, where DPIP returns to its oxidized form within 40 min. When SOD was added, DPIP was also re-oxidized (**Figure 5B**). Therefore, reduced DPIP is a substrate for these enzymes and not an appropriate extracellular electron acceptor to be utilized with these enzymes.
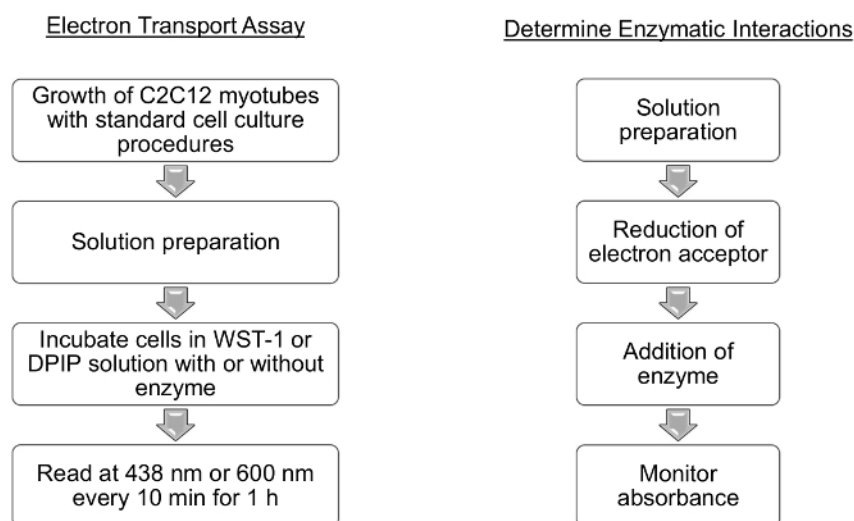

Figure 1: A schematic representation of the key steps of the electron transport assays and determination of enzymatic interactions with the extracellular electron acceptors. Please click here to view a larger version of this figure.
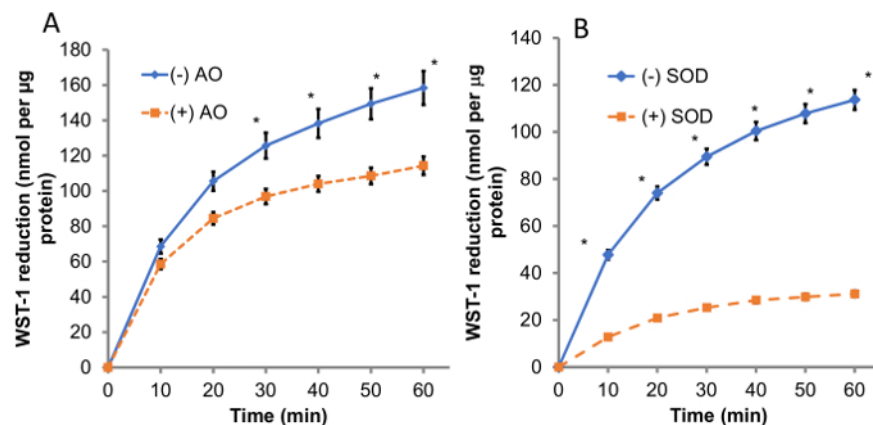
Figure 2: The addition of AO and SOD suppresses WST-1 reduction by C2C12 myotubes. (**A**) tPMET was analyzed for 60 min using 400 μM WST-1 and 20 μM PMS with the addition of 2 U/mL AO or diH$_2$O (N = 18/group). (**B**) tPMET was analyzed for 60 min with the addition of 60 U/mL SOD or 0.1 M KPO$_4$ buffer (N = 36/group). *$p \geq$ 0.05 at the indicated time point. Error bars represent standard error of the mean and may be too small to visualize. Please click here to view a larger version of this figure.
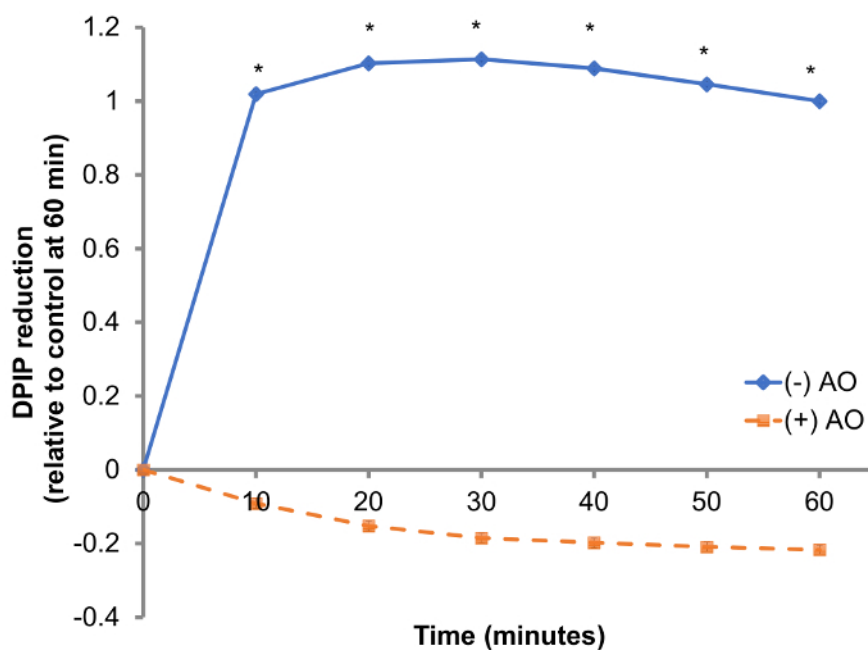

Figure 3: The addition of AO suppresses DPIP reduction by C2C12 myotubes. tPMET was analyzed for 60 min using 100 μM DPIP with or without the addition of 2 U/mL AO (N = 36/group). *$p \geq$ 0.05 at the indicated time point. Error bars represent standard error of the mean and may be too small to visualize. Please click here to view a larger version of this figure.

Figure 4: Reduced WST-1 is not a substrate for AO or SOD. (**A**) WST-1 was reduced with ascorbate. After 45 min, AO was added, and the absorbance was monitored for 1 h. No change in absorbance was observed. (**B**) The procedure is the same as above, except SOD was added after 45 min. No change in absorbance was observed (N = 24/group). Error bars represent standard error of the mean and may be too small to visualize. Please click here to view a larger version of this figure.
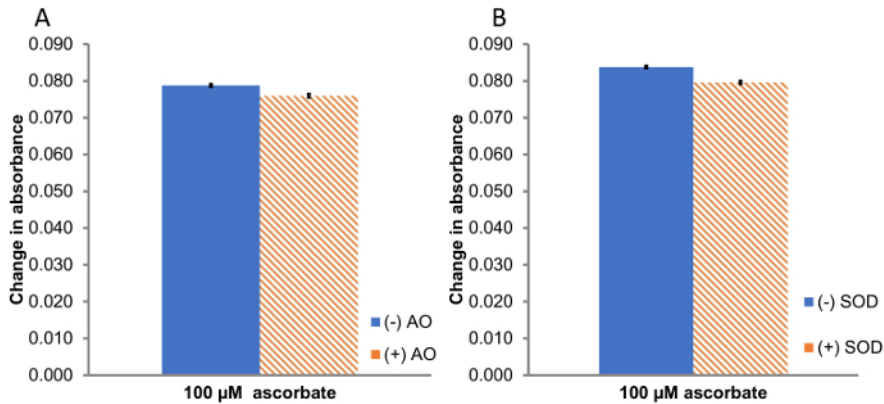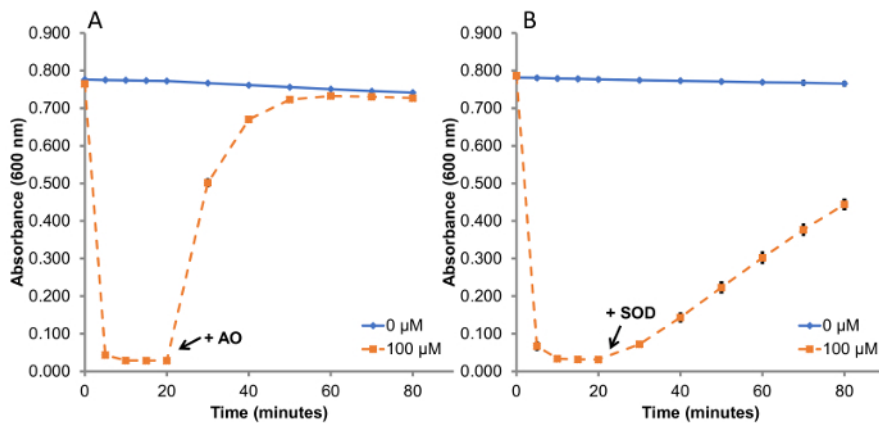

Figure 5: Reduced DPIP is a substrate for AO and SOD. (**A**) DPIP was reduced with ascorbate. After 20 min, AO was added, and the absorbance was monitored. Within 40 min, the samples returned to their original absorbance, indicating that reduced DPIP is a substrate for AO. (**B**) DPIP was reduced with ascorbate. After 20 min, SOD was added, and the absorbance was monitored. Over time, the reduced DPIP was re-oxidized, indicating that reduced DPIP is a substrate for SOD (N = 24/group). Error bars represent standard error of the mean and may be too small to visualize. Please click here to view a larger version of this figure.
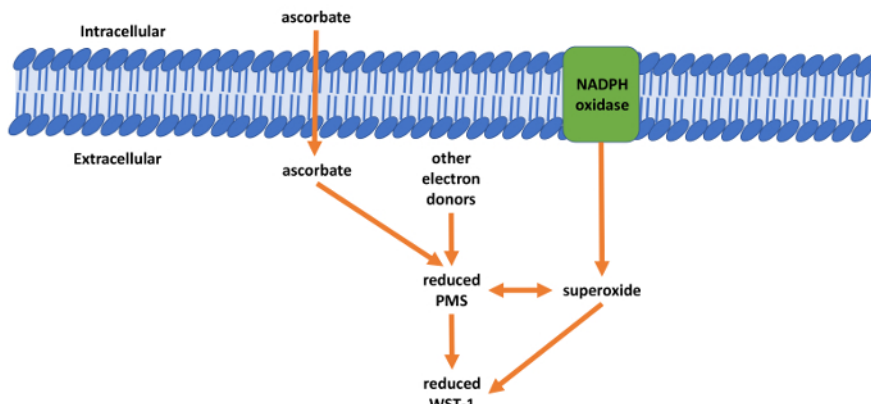

Figure 6: Schematic depicting tPMET. In this model of tPMET, ascorbate (or another electron carrier) exported by the cell can reduce extracellular PMS. In addition, NADPH oxidases generate extracellular superoxide, which can reduce WST-1 directly or reduce it indirectly via reduction of PMS. Electrons from other plasma membrane donors can also reduce PMS, which can donate electrons to either WST-1 or O_2 with subsequent WST-1 reduction. Please click here to view a larger version of this figure.

Discussion

We have presented two methods for utilizing extracellular electron acceptors, WST-1 and DPIP, in spectrophotometric assays to monitor tPMET in C2C12 myotubes. With the growth of cell lines in standard culture procedures and a spectrophotometer plate reader, it is possible to monitor tPMET with these electron acceptors in a simple microplate assay. WST-1 reduction is reproducible from well-to-well within an assay, but there is day-to-day variability. The day-to-day coefficient of variation (CV) utilizing PBS as the buffer is 0.18 and the CV utilizing HBS as the buffer is 0.23.

From previous literature utilizing mPMS and WST-1, we have modified this protocol, specifically, for the use of PMS. Thus, troubleshooting included determining the appropriate concentrations of PMS and WST-1 for use with the myotubes described. We also determined that temperature (in a range of 23-37 °C) is not a critical component for the assay, while shaking the plate prior to each absorbance reading is important. However, these issues should be investigated for other cell lines to which the assay is applied. The findings regarding the ability of SOD and AO to directly oxidize reduced WST-1 suggest that screening of new assay components for this property is a key aspect of troubleshooting.

Our data suggests that PMS should be utilized in conjunction with WST-1 in order to elicit optimal WST-1 reduction. We found that in a 1 mL volume, 100 µM ascorbate reduces 322 nmol of WST-1 per minute in the presence of PMS, which is required for direct reduction of WST-1 by ascorbate. In a 1 mL volume, 2 U/mL of AO oxidizes ascorbate at a rate of 4,400 nmol/s, or 800 times faster than the reduction of WST-1 by ascorbate. Clearly, enzymatic activity of AO is much faster than direct reduction of PMS/WST-1 by ascorbate. We expect that the same would follow for superoxide and SOD, though we do not have a system for measuring the absolute rate of WST-1 reduction by superoxide.

To determine whether inclusion of PMS affects the reduction of WST-1 by superoxide, we used a xanthine oxidase (4.5 mU/mL)/hypoxanthine (0.1 mM) superoxide generating system as previously described[26,27]. Inclusion of PMS approximately doubles the rate of WST-1 reduction by this system. Thus, it appears that PMS mediates transfer of electrons from superoxide to WST-1.

To further evaluate the requirement of PMS for cellular WST-1 reduction, we assayed reduction in the presence or absence of PMS and WST-1, and found that PMS is required for cellular WST-1 reduction. When cells are incubated in assay reagent containing PMS alone (in the absence of WST-1), there is an increase in absorbance at 438 nm. This likely reflects an accumulation of the semiquinone form of PMS (PMS-SQ) that has a peak absorbance at 440 nm[28]. Accumulation of PMS-SQ suggests that PMS is being reduced faster than it can pass electrons to O_2 to produce superoxide. Thus, the accumulation of PMS-SQ is consistent with the reported slow reduction of O_2 by PMS-SQ compared to the rate for fully-reduced PMS[28].

We have also presented a protocol to determine if the electron acceptors are substrates for the enzymes that may be utilized to monitor tPMET. By monitoring the reduction of the extracellular electron acceptors in a spectrophotometer followed by the addition of the enzyme of interest, it is possible to determine if the electron acceptor is a substrate for these enzymes. Through this method, we have shown that reduced DPIP is a substrate for AO and SOD indicating that it is not a suitable electron acceptor for monitoring ascorbate efflux and superoxide export.

The procedure for testing whether or not assay components such as SOD or AO create artifacts is a significant improvement over existing methods. For example, reduced DPIP is a substrate for SOD, suggesting that published data obtained with use of DPIP and SOD should be interpreted accordingly. Our findings support previous research conducted by Dayan and Dawson in which the absorbance of leuco 2,6-dichloroindophenol was monitored after the addition of AO: DPIP was oxidized indicating that it is a substrate for AO[29]. However, our findings add context to research conducted by Tan and Berridge[24], as well as Bellavite et al.[30], that utilized SOD to inhibit DPIP reduction. In a study conducted by Tan and Berridge comparing the electron acceptors of WST-1, DPIP, and FeCN in HeLa cells, they saw that the reduction of some of the electron acceptors was suppressed in the presence of SOD. The reduction of WST-1, in conjunction with mPMS, was inhibited ~80% in the presence of SOD while the reduction of DPIP was inhibited by 40%. The reduction of FeCN, in conjunction with an alternative intermediate electron acceptor, was not inhibited in the presence of SOD[24]. When evaluating the mode of electron exchange between NADPH oxidases and DPIP or cytochrome c, Bellavite et al. found that DPIP and cytochrome c are reduced by NADPH oxidases and that this reduction is significantly inhibited by SOD[30]. Berridge and Tan have also demonstrated that SOD can inhibit WST-1 reduction by 80% in mouse cells lines of 32Dcl23, WEHI3B, and J774 as well as human cell lines of Jurkat, MALME3M, and 143B[6,31]. With human primary neutrophil cells, WST-1 reduction was inhibited 95% in the presence of SOD[6,32].

The data here suggest that it is important to verify that enzymes used to assess specific contributions of exported molecules to tPMET cannot re-oxidize the reduced form of the extracellular electron acceptor. We found that (data not shown) ferrous chloride and ferrous sulfate were not soluble in PBS. In HBS and phenol red-free DMEM, they were rapidly oxidized, demonstrating incompatibility with HBS or DMEM as assay media. In contrast, AO and SOD do not use potassium ferrocyanide as a substrate, suggesting that it is a suitable extracellular electron acceptor if its low extinction coefficient is not an issue.

One of the main limitations of this assay is that PMS/WST-1 and DPIP can be reduced by multiple electron donors, such as NAD(P)H, ascorbate, and flavonoids such as quercetin and myricetin[19,33,34]. However, this can be overcome by the addition of enzymes (e.g., SOD, AO) or inhibitors to determine specific contributors to PMS/WST-1 or DPIP reduction. Another limitation is that DPIP can act as a substrate for AO and SOD. Thus, these enzymes should not be utilized in conjunction with DPIP to monitor tPMET. An additional important limitation of this study is the ability of PMS and other redox cyclers to produce superoxide[26,35,36]. This is illustrated in **Figure 6**. On the other hand, PMS itself reportedly has no direct effect on tPMET[32]. Additionally, Tan and Berridge[32] have shown that the NADPH oxidase (NOX) inhibitor, diphenyleneiodinium chloride (DPI), can suppress the majority of WST-1 reduction, suggesting that DPI-sensitive tPMET can be a specific measure of NOX contribution to tPMET. Another limitation is that we see small changes in absorbance when utilizing WST-1. We have found that when WST-1 is made fresh and cells are confluent, the greatest change in absorbance is seen. Previous studies conducted in P388 cell lines have shown that the amount of formazan produced positively correlates with cell number[21]. Therefore, the more confluent the cells are then the greater the reduction in WST-1. This can be corrected for by normalizing to microgram of protein for each well.

One caveat regarding this assay is that it is primarily designed to assess global tPMET. Although it can be manipulated to monitor multiple forms of tPMET, such as the example of ascorbate efflux, more specific assays might be more appropriate when the goal does not require measurements in the context of tPMET. Fox example, if superoxide is the main interest, there are a number of other avenues to monitor superoxide, such as use of probes including fluorescent impermeable probes, aconitase, and nitroblue tetrazolium[37].

Existing probes to monitor tPMET such as DPIP, FeCN and ferricytochrome *c*, present disadvantages. For example, reduced DPIP is a substrate for AO and SOD, FeCN has a low molar extinction coefficient that limits its utility in real-time assays, and cytochrome *c* is costly. Additionally, some of these probes can produce high background leading to low sensitivity when measuring a change in absorbance[32]. WST-1 has been shown to not be a substrate for enzymes such as AO and SOD. Additionally, WST-1 has a high molar extinction coefficient and a low background reaction, creating a more sensitive tPMET assay. Moving forward, this assay can be utilized with a wide range of inhibitors to better understand the process of tPMET, map the path of electron transport in a given cell line, and lead to better understanding of how a cell's redox environment is maintained.

Critical steps of this protocol include determining that the cells are ready for experimentation (~70% confluent) as well as making the assay reagents, specifically WST-1 and PMS, fresh for each assay. It is also critical to wash the cells before the addition of assay reagents. The DMEM utilized in this protocol does not contain ascorbate, although we supplement differentiation medium with ascorbate. A number of available media do contain ascorbate. However, the cells are washed before the addition of the assay reagents, which are ascorbate-free, to remove any residual ascorbate from the medium. As a portion of tPMET is attributable to molecular efflux (*e.g.,* ascorbate export), it is important to start spectrophotometric readings immediately after the addition of assay reagent so as not to miss the first moments of efflux. Also, it is critical to include background wells for each individual reagent in an experiment. The assay constituents described in this paper caused negligible background change in absorbance. However, some additives (*e.g.,* phloretin) can promote a substantial background reaction. Thus, it is important that the background is subtracted out when the data are analyzed to eliminate any confounding effects that the reagents themselves may produce.

In summary, the protocol presented here provides a means of assessing tPMET and suggests a number of caveats and aspects of the protocol that should be taken into account when applying the assay to different cell lines and/or assessing specific contributors (ascorbate and superoxide used here) to tPMET.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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