A Proposed Enzyme-Linked Hydrogen Peroxide Radical Assay System (HORAS)

by

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ABSTRACT

Reactive Oxygen Species (ROS) are chemically reactive molecules that contain oxygen. ROS are naturally generated as a byproduct during mitochondrial oxidative metabolism as well as by cellular responses to a variety of inflammatory stimuli. Intracellularly formed ROS plays an important role in maintaining homeostasis and in cell signaling but, ROS are challenging to quantify. Phagocytic cells such as macrophages may produce H_2O_2 during the action of bacterial engulfment. Here UV-Vis versus LC-ESI-MS detection methods for an enzyme-linked, cellular assay of H_2O_2 production in cultured macrophages are compared. In the presence of Horseradish Peroxidase (HRP), Amplex Red (AR) reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin that can be measured by UV/Vis at an absorbance of 570 nm or by LC-ESI-MS at 214 m/z $[\text{M}+\text{H}]^+$. RAW 264.7 macrophages were stimulated by microscopic foreign particles, with the addition of 0.1mM of Amplex Red substrate and 10 ng/mL of HRP to the cellular media to enzymatically detect H_2O_2 production. The oxidation product resorufin can be detected by the colorimetric method as low as 50 pmol while liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS) was able to detect as little as 0.2 pmol in vitro. Thus, it was possible to measure low levels of H_2O_2 released by cells using an enzyme coupled cellular assay with LC-ESI-MS.

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LIST OF SELECTED ABBREVIATIONS

ADS Antioxidant defense systems

AR Amplex red

ECL Enhanced chemiluminescence

ESI Electro spray ionization

ESR Electron spin resonance

FAD Flavin adenine dinucleotide

GPx Glutathione peroxidase

GSH γ -glutamylcysteinylglycine

HRP Horseradish peroxidase

H₂O₂ Hydrogen peroxide

IgG Immunoglobulin G

LOQ Safe Limit detection and quantification

MS Mass spectrometry

NADPH Nicotinamide adenine dinucleotide phosphate

NOX NADPH oxidases

oxLDL Oxidized low density lipoprotein

PBS phosphate buffered saline

PMA Phorbol 12-myristate 13-acetate

ROS Reactive oxygen species

SOD Superoxide dismutase

Tris Tris (hydroxymethyl) aminomethane

SD Standard Deviation

SE Standard Error

CHAPTER 1: Introduction

1.1 Reactive Oxygen Species

Atomic oxygen is inclined to accept electrons and form reactive oxygen species due to the unpaired electrons in the outer electron shell. There are six different kinds of reactive oxygen species (ROS): Oxygen, superoxide, peroxide, hydrogen peroxide, hydroxyl radical, and hydroxyl ion. Molecular oxygen is crucial for the survival of aerobic organisms. Aerobic energy metabolism is dependent on complete reduction of a molecule of oxygen to water which requires four electrons and several sequential intermediates. These intermediates are considered as ROS, and ROS are highly reactive compared to molecular O₂. The most common ROS in cells are Superoxide, Hydrogen peroxide, and Hydroxyl radical.

1.1.1 Oxidant Sources

Mitochondria

ROS include several reactive molecules and free radicals derived from one or more unpaired electron oxygen molecules. All aerobic cells produce ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical in various enzymes and organelles within the cell. Cells produce ROS as a byproduct of many metabolic reactions in the mitochondria, peroxisomes, the endoplasmic reticulum (ER) and particularly in the setting of ER stress (Halliwell & Gutteridge, 1984). Mitochondria may be a major site of ROS production in response to physiological reactions under aerobic conditions (Chance *et al.*, 1979). During mitochondrial oxidative phosphorylation, Complex I (NADH-ubiquinone oxidoreductase) and Complex III (Q-cytochrome c oxidoreductase) transfer a single electron to the oxygen molecule. Thus, superoxide anion (O₂⁻) is initially formed by the electron transfer chain in inner mitochondrial membrane (Turrens *et al.*, 1985). Superoxide anion can be converted to H₂O₂ by an enzyme called super oxide dismutase (SOD) which is located

in the intermembrane space of mitochondria (McCord & Fridovich, 1969 and Weisiger & Fridovich, 1973). About 0.1% to 0.5% of mitochondrial O₂ consumption leads to H₂O₂ production enzymatically or spontaneously (Bayne *et al.*, 2005 and Boveris *et al.*, 1972).

NADPH oxidases

Reactive oxygen species were initially considered as unwanted by-products of other biological reactions since they were known to cause cell membrane and DNA damage. However, Sbarra and Karnovsky (1995) first observed a sudden increase in oxygen consumption during phagocytosis which was dependent on glucose metabolism. The NADPH oxidase complex is the primary enzyme system which is responsible for respiratory bursts (Rossi & Zatti, 1964). Superoxide anion (O₂⁻) production occurs during phagocytosis via the NOX2 complex, also known as gp91^{phox}, as part of their essential role in host defense (Babior *et al.*, 1973 and Teahan *et al.*, 1987).

All NADPH oxidases (NOX) enzymes are composed of six highly conserved transmembrane domains which contain two hemes, NADPH and FAD binding regions at the COOH terminus (Bedard & Krause, 2007). In phagocytes, NOX2 and p22^{phox} are found in both intracellularly and in plasma membranes with four cytosolic proteins (p47^{phox}, p67^{phox}, p40^{phox}, and Rac). During phagocytosis, NOX2 assembles by recruiting cytosolic proteins, exchanging GDP to GTP on Rac, to become an active enzyme complex (Holmström & Finkel, 2014). Phosphorylation of the cytosolic p47^{phox} allows interaction with p22^{phox} and leads to the recruitment of other cytoplasmic subunits, p67^{phox} and p40^{phox}, to form the active NOX2 enzyme complex. The active form of NOX2 transports electron from cytosolic NADPH to extracellular or phagosomal oxygen to generate superoxide anions which is also known as the respiratory burst (Bedard & Krause, 2007).

Other Enzymes

Mitochondria and NOX enzymes are well characterized intracellular sources of ROS. Studies have shown various enzymes such as xanthine oxidase, nitric oxide synthase, cyclooxygenases, cytochrome p450 enzymes, and lipoxygenases can also produce trace amount of ROS (Holmström & Finkel, 2014). Similarly, peroxisomes and endoplasmic reticulum (ER) may also produce ROS depending on cell type.

1.1.2 Antioxidant Defense Systems

Different intracellular concentrations of ROS may have various biological effects. ROS may serve as signaling molecules. For example, concentrations of 10^{-8}M of H_2O_2 is involved in growth signaling while concentrations of 10^{-4}M of H_2O_2 leads to apoptosis (Stone and Yang, 2006). ROS homeostasis is essential since imbalanced ROS may damage macromolecules including; DNA, protein, and lipids. This damage may cause cell damage or death. In order to counterbalance the production of ROS, cells have Antioxidant Defense Systems (ADS) to scavenge excessively produced ROS. ADS can be divided by enzymatic antioxidants and non-enzymatic antioxidants.

Enzymatic Antioxidants

Enzymatic antioxidants work by breaking down and removing free radicals. The major antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase that maintain the cellular reductive potential and each antioxidant has distinct functions. Two superoxide molecules are converted to H_2O_2 and O_2 by SOD. Three different isoforms of SOD; CuZn-SOD, Mn-SOD, and EC-SOD have been identified (Zelko *et al.*, 2002). CuZn-SOD is a copper and zinc-containing homodimer found in intracellular cytoplasmic spaces of mammalian cells. The tetramer form of

Mn-SOD is exclusively found in the mitochondrial spaces of aerobic cells while EC-SOD was first identified in the extracellular spaces of human plasma (Zelko *et al.*, 2002).

Catalase is a well-recognized antioxidant enzyme that is found in nearly all aerobic organisms. Catalase is mainly localized in peroxisomes but is also found in the cytosol of human neutrophils and alveolar macrophages to catalyze the composition of H₂O₂ to water and oxygen (Davies *et al.*, 1979).

Another enzyme that responsible for the reduction of H₂O₂ is glutathione peroxidase (GPx). GPx are a family of tetrameric enzymes that dependent on the micronutrient selenium (Se) at the active site and uses low-molecular-weight thiols, such as γ -glutamylcysteinylglycine (GSH), to reduce H₂O₂ via the following reactions (Imai & Nakagawa, 2003):

Glutathione Peroxidase (GPx): $2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$

Non-Enzymatic Antioxidants

Non-enzymatic antioxidants work by interrupting free radical chain reactions. A few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, and GSH. The water-soluble antioxidants including vitamin C (ascorbic acid) are present in cellular fluids such as the cytosol, or cytoplasmic matrix by scavenging oxygen free radicals (Birben *et al.*, 2012). The lipid-soluble antioxidant vitamin E is found in cell membranes and protects against oxidant induced membrane injury. Vitamin C and vitamin E can be activated by GSH which is localized in all compartments. GSH is the major soluble antioxidant, and can function as an antioxidant in numerous ways (Nimse & Pal, 2015). GSH reduces hydrogen peroxide to water and oxygen. In addition, GSH is a cofactor for detoxifying enzymes such as GPx. The oxidized form of glutathione (GSSG) is reduced, and so recycled, by glutathione reductase (GRD) enzymes, with NADPH as a reductant:

GRD: $\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$

1.2 H₂O₂ Production in Macrophages

Macrophages

Macrophages are mononuclear cells capable of phagocytosis which play a significant role in immunity and immune responses (Epelman *et al.*, 2015). They are distributed throughout mammalian organs and their morphology varies depending on their state of activity.

Phagocytosis

Cells have a variety of ways to internalize particle and solutes such as pinocytosis, receptor mediated endocytosis and phagocytosis. Phagocytosis, first observed in 1884 by Ilyich Metchnikoff, is the uptake large particles ($>0.5 \mu\text{m}$) into cells by actin-dependent mechanisms (Aderem & Underhill, 1999). Phagocytosis occurs by sequential interactions between macrophages surface receptors and opsonic ligand on surfaces of particles.

Oxidized Low-Density Lipoprotein produces Reactive Oxygen Species in Macrophages

Oxidized Low-Density Lipoprotein (oxLDL) is generated from native LDL that plays a role as a cholesterol transporter and accumulates in atherosclerotic lesions in humans (Yia-Herttuala *et al.*, 1989). The accumulation of oxLDL in atherosclerotic lesion causes atherosclerosis (Liu *et al.*, 2006). Macrophages scavenger receptors such as CD36 bind and engulf accumulated oxLDL and become foam cells that induce inflammatory reactions within the arterial walls which, also contributes to atherosclerosis (Yang *et al.*, 2007 and Itabe, 2009 and Han & Nicholson, 1998). A previous study showed that an 8 hour treatment with $20 \mu\text{g/ml}$ of oxLDL increased ROS generation in RAW264.7 cells causing an increase in NOX2 mRNA expression and protein levels (Lee *et al.*, 2014). Thus, production of intracellular ROS by a signaling cascade through NOX family NADPH

oxidases may act as a second messenger in the inflammatory response. Studies have shown that oxLDL stimulates phagocytosis in macrophages causing activation of inflammation signaling molecules (Bae *et al.*, 2009 and Lee *et al.*, 2014).

Physiological Role of Hydrogen Peroxide

All aerobic cells have various enzymes to eliminate H₂O₂ since it is toxic to cells and damages macromolecules. Despite its toxicity, cells produce H₂O₂ for a variety of functions in cells. Only phagocytic cells use H₂O₂ as a defense mechanism since phagocytic cells may depend on H₂O₂ to help degrade engulfed particles (Baehner *et al.*, 1975). Recent studies have shown that H₂O₂ is also produced in response to activation at the cell surface, which may function as an intracellular messenger in cells. Unlike other messenger molecules, H₂O₂ is a ubiquitous intracellular messenger that oxidizes critical residues of effector protein to activate various function in cells (Woo *et al.*, 2010). In general, H₂O₂ responds to different signaling pathways depending on the cell type, the magnitude of the dose, and the duration of the cell exposures. When H₂O₂ concentration is 10⁻⁸ to 10⁻⁷ M, cells proliferate (Giorgio *et al.*, 2007). 10⁻⁷ to 10⁻⁵ M of H₂O₂ can cause either temporary or permanent growth arrest, while oxidative stress (10⁻⁴ M of H₂O₂) causes cell death by apoptotic or necrotic mechanisms. Oxidative stress is highly studied since it may cause diseases such as atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, arthritis, and aging (Finkel & Holbrook, 2000). Thus, it is important to quantify and track the small diffusible H₂O₂ molecules in live cells.

1.3 Hydrogen Peroxide Detection Method

To clearly understand cell signaling events, it is important to accurately detect and quantify ROS like H_2O_2 . Several techniques and analytical approaches such as fluorescence and chemiluminescence have been used to detect H_2O_2 .

Fluorescent probes

There are many methods that have been examined to measure H_2O_2 in real-time. An effective way to measure H_2O_2 is using Amplex Red (AR) in the presence of Horseradish Peroxidase (HRP). Non-fluorescent molecules of AR are converted to highly fluorescent resorufin by reacting with H_2O_2 at a stoichiometry of 1:1 when HRP catalyzes an H_2O_2 dependent oxidation (Figure 1.1). This assay is sensitive to 2 pmol of H_2O_2 (Mohanty *et al.*, 1997). HRP has a high catalysis rate and yield but, it is also one of the least specific enzymes of the peroxidase family thus HRP could utilize many cellular compounds including thiols for its substrate.

Figure 1.1 Non-fluorescent Amplex red is oxidized by the horseradish peroxidase and hydrogen peroxide to highly fluorescent Resorufin (Dikalov, Griendling, & Harrison, 2007)

Dichlorodihydrofluorescein diacetate (DCFH-DA) is also commonly used to detect intracellular H_2O_2 and oxidative stress. DCFH-DA is a cell permeable non-fluorescent probe that can be hydrolyzed by intracellular esterases (Kalyanaraman *et al.*, 2010). Two electron oxidation

of DCFH forms the fluorescent product dichlorofluorescein (DCF) (Figure 1.2). DCF can be monitored by several fluorescence-based techniques such as confocal microscopy and flow cytometry which allows for the measurement of H_2O_2 concentration over time. However, the intracellular redox chemistry of DCFH is complex and the DCF assay has several limitations to measure intracellular H_2O_2 . As Figure 1.2 shows, DCFH does not directly react with H_2O_2 to form DCF, and therefore cannot be used as direct measurement of H_2O_2 . In addition, redox active metals such as Fe^{2+} with presence of H_2O_2 can oxidize DCFH to form DCF results in amplification of fluorescent signal intensity (Karlsson *et al.*, 2010).

Figure 1.2 Cell permeable DCFH-DA is cleaved by intracellular esterase to DCFH and oxidized by hydrogen peroxide to the fluorescent molecule (Dikalov *et al.*, 2007).

Chemiluminescent probes

Commonly used chemiluminescence probes are lucigenin, luminol, MCLA [2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazin-3-one, hydrochloride] and coelenterazine. To measure superoxide, lucigenin-enhanced chemiluminescence (LEC) may be used since it is reasonably specific for $\text{O}_2^{\cdot -}$ and its signal is linear. LEC are also easily detected by a scintillation counter or a luminometer (Dikalov *et al.*, 2007 and Rost *et al.*, 1998). Lucigenin-amplified chemiluminescence reactions are as follows (Faulkner & Fridovich, 1993):

Reaction. 1: $\text{O}_2^{\cdot-} + \text{LC}^{2+} \rightarrow \text{LC}^{\cdot+} + \text{O}_2$

Reaction. 2: $\text{LC}^{\cdot+} + \text{O}_2^{\cdot-} \rightarrow \text{LCO}_2$

Reaction. 3: $\text{LCO}_2 \rightarrow 2N\text{-methylacridone} + h\nu$

The lucigenin radical can react with $\text{O}_2^{\cdot-}$ to form NMA and a photon (Figure 1.3). However, $\text{O}_2^{\cdot-}$ production can be overestimated by redox cycling (Rost *et al.*, 1998). In addition, chemiluminescence based assays are easy and specific for superoxide but other ROS can only be measured by use of inhibitors.

Figure 1.3 $\text{O}_2^{\cdot-}$ reduces lucigenin (LC^{2+}) to its cation radical ($\text{LC}^{\cdot+}$). Cation radical reacts with a second $\text{O}_2^{\cdot-}$ to form the energy-rich dioxetane molecule (LCO_2) and results in emitting of a photon (Vladimirov & Proskurnina, 2009).

The enhanced chemiluminescence luminol dependent (ECL) assay is another attractive analytical method to measure H_2O_2 since it is highly sensitive with a fast reaction time. The chemiluminescence of luminol can be catalyzed by different catalyst compounds such as copper (II) and hexacyanoferrate (III) in hydrogen peroxide assays (Steijger *et al.*, 1996 and Yoshida *et al.*, 1999). Several studies have found that ECL has a great potential to measure H_2O_2 by providing

more intense, prolonged, and stable light emission proportional to H_2O_2 concentration. In basic media in the presence of catalyzers and oxidants with the addition of an electron rich compound (enhancer), luminol emits light at 425nm (Bhandari *et al.*, 2010; Ramos *et al.*, 2001; Navas *et al.*, 1996) (Figure 1.4).

Figure 1.4 Luminol oxidized by hydrogen peroxide with presence of HRP to the fluorescent molecule. The amount of light detected is proportional to the hydrogen peroxide concentration.

Electron Spin Resonance (ESR)

Electron spin resonance (ESR) or also known as electron paramagnetic resonance (EPR) spectroscopy is formed by a microwave generator, a resonator, and electrical magnets (Figure 1.5). ESR is an analytical technique based on absorption of microwave radiation stimulated by an electromagnetic field in molecules such as free radicals and transition metal ions with unpaired electrons (Dikalove *et al.*, 2007).

Figure 1.5 A typical ESR spectrometer is formed by a resonator cavity surrounded by a pair of electrical magnets and a microwave generator that emits radiation towards the cavity (Dikalove *et al.*, 2007).

The unpaired electrons are excited to a high-energy state under the magnetic field by the absorption of microwave radiation ((Dikalove *et al.*, 2007 and Konhno, 2010 and Lee *et al.*, 2012). The excited electron changes its direction of spin and relaxes to ground state by emitting energy. The transition between two different energy levels takes place by absorbing a quantum of radiation of frequency in the microwave region. Microwave absorption is measured as a function of the magnetic field by ESR spectrometer. Although ESR is extremely sensitive and specific it is extremely expensive and requires certain probes such as cyclic hydroxylamines (Lee, *et al.*, 2012). In addition, an ESR spectrometer requires an extra antioxidant or inhibitor experiment to verify presence of H₂O₂ since some other reaction may yield the radical products or oxidant which result in amplification of the ESR signal.

New Methods for H₂O₂ Detection

Previous studies have shown that oxidation of AR by HRP produced fluorescent resourfin that can be detected by liquid chromatography with electrospray ionization and tandem mass spectrometry

(LC-ESI-MS) (Florentinus-mefailoski, Safi, & Marshall, 2013). Resorufin was analyzed by HPLC-MS and SIM at 214m/z results in the log linear dilution curve as low as at 200 fmol on column. The combination of enzyme-linked H₂O₂ radical assay and LC-ESI-MS can yield sensitivity to the fmol range with reduction of quantification and detection limit.

Rationale and Objective

Reactive oxygen species detection in macrophages has always been challenging due to the lack of techniques that are sensitive for low-level detection at the cellular level. The objective of this work was to apply liquid chromatography, electrospray ionization and mass spectrometry in order to monitor H₂O₂ concentrations in cultured macrophages in response to inflammatory signals. Oxidized AR products can be detected by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI/MS) at 214 m/z [M+H]. Thus, the mass spectral detection method to an enzyme coupled cellular assay may provide greater sensitivity and simplicity to measure H₂O₂ in live cells.

CHAPTER 2: MATERIALS and METHODS

2.1 Materials

Horseradish peroxidase (HRP), H₂O₂, Ampliflu red (10-acetyl-3,7 dihydroxyphenoxazine), resorufin, luminol, 4-Iodophenylboronic acid (4-IPBA), Sodium orthovanadate, PMA, Ionomycin, superoxide dismutase, catalase were purchased from SigmaAldrich. Tris-HCl were purchased from BioShop. MaxiSorp 96well plates were obtained from Nunc (ThermoFisher Scientific). Cell culture flasks and 6well culture plates were obtained from Greiner Bio One. PVDF membrane was obtained from Millipore (Billerica, MA, USA). The solvents of optical grade were obtained from Caledon Laboratories (Georgetown ON, Canada). The LTQ ion trap was obtained from Thermo Fisher Scientific (Thermo Electron Corporation, Waltham MA, USA). The HPLC was from Agilent Technologies (Santa Clara CA, USA).

2.2 Method

Substrate reactions

HRP (1mg/ml) was made to 50% glycerol and stored at -20 °C. Amplex red (AR) 5 mg was dissolved in dry dimethyl sulfoxide (DMSO) and aliquots were stored in the dark at -20 °C. Amplex red was mixed in the reaction buffer (Tris 20 mM, pH 8.8) with a final substrate concentration of 0.1 mM. A total of 10 ng/ml of the HRP was added to reactions for time course measurements. The substrate and enzyme were mixed just prior to the addition of the H₂O₂ solution. For enhanced chemiluminescence (ECL), luminol was dissolved in dry DMSO and stored at -20 °C until dissolved in Tris buffer (pH 8.8) and reacted in the presence of 4-iodophenylboronic acid and 10 ng/ml of the HRP with addition of different concentration of H₂O₂ mixed just prior to the detection.

Cell Culture

RAW 264.7 cells were grown to 50% confluence in 75 cm flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were plated at 25% confluence in 6-well plates for 72 hours prior to assay; 1mL of ~50% confluent cell culture per well. Prior to the experiment to reduce the presence of extraneous growth medium proteins, the cells were washed with PBS for three times. Isotonic experimental medium (20 mM HEPES, 5 mM glucose, 140 mM NaCl, and 1mM of each CaCl_2 and MgCl_2) were added.

Lipoprotein Isolation and Oxidation

A total of 0.4451g KBr and 0.9 mL of normal human plasma were mixed into a Beckman 3 mL ultracentrifuge tube (McFarlane *et al.*, 2005). A 0.196M solution of NaCl was gently run down the side onto the top of the plasma without disturbing the layers that occurred. Ultracentrifugation was performed at 4°C, at 100,000 rpm for 1 hour with an acceleration and deceleration setting of six. After ultracentrifugation, LDL was transferred to a 2 ml O-ring tube using 21G needle and, placed into the 0.5mM EDTA containing PBS dialyzed at 4°C for overnight (Shen *et al.*, 2008). Oxidation was performed using isolated LDL placed in PBS containing 5 μM CuSO_4 at 37°C for 18 hours. To terminate the reaction 0.3 mM EDTA and 40 μM butylated hydroxytoluene was incubated and dialyzed at 4°C for overnight. Modified LDL was dialyzed extensively at 4°C against PBS containing 0.5 mM EDTA. Prepared oxLDL (~ 0.0125 mg/ μl) was stored in the dark at 4°C.

Preparation of IgG coated beads

15 μL of 10 mg/mL human IgG dissolved in PBS was combined with 1.5 μL of 2 μm particles made of melamine resin (Fluka, Sigma-Aldrich, Product of Germany), diluted in 500 μL of 1 x phosphate buffered saline (PBS), and incubated for 30 min prior to washing three times after

pelletting IgG coated beads at 148,000 rpm, and after removing the supernatant containing unbound IgG. IgG coated beads were re-suspended in 50uL of PBS.

Preparation of oxLDL coated beads

The same procedure was used to produce oxLDL coated beads as the IgG coated beads however the oxLDL coated beads were incubated in the dark due to light sensitivity.

Enzyme reactions in 6 wells

AR (0.1 mM) and 10 ng/ml of HRP were added into 6 well plates where cells were grown. There were different treatments: beads, beads coated with IgG, beads coated with oxLDL, oxLDL coated beads with 500 units/ml SOD and 1000 units/ml catalase, 1 μ M PMA, 1 μ M ionomycin, 1 mM sodium orthovanadate, and H₂O₂. Sodium orthovanadate, SOD (500 unit/ml) and Catalase (1000 unit/ml) were added 30 minutes prior to addition of 0.1mM AR and 10 ng/ml of HRP. Each treatment was incubated for 30 minutes and stored at 37°C and 5% CO₂ in the dark. After 30 minutes, reactions were quenched by placing onto ice.

Colorimetric Detection

The oxidation of non-fluorescent AR by HRP in the presence of H₂O₂ was monitored by UV/Vis spectroscopy. AR reacts with H₂O₂ in 1:1 stoichiometric ratio to produce resorufin that can be detected colorimetrically. The plate was read on the microplate fluorimeter (Multiskan spectrum, Model number 354, Thermo labsystems) at 570 nm after 30 minutes of incubation by transferring 200 uL of supernatants into 96 wells.

Enhanced chemiluminescent (ECL) detection

In the case of ECL, the limit of sensitivity for H₂O₂ was measured by adding 10 ng/ml of HRP and ECL solution (1.25 mM of luminol and 2.5 mM of enhancer) into 96 well plate with different

concentrations of H₂O₂ and detected by Bio-Rad gel doc system as well as microplate reader (Synergy HTX, BioTek Instruments, Inc, Winooski, USA). The amount of generated light is proportional to H₂O₂ concentration. To test limit of sensitivity using ECL, dot blotting 10 ng/μl of HRP onto methanol-activated PVDF in the presence of 1.25 mM luminol and the enhancing agent 2.5 mM of 4-iodophenylboronic acid and with detection by BIO-RAD gel doc (Haan & Behrmann, 2007).

Liquid Chromatography-Electrospray Ionization Mass spectrometry

A 1ml volume of 0.1mM AR reacted with H₂O₂ at a stoichiometry of 1:1 with 10 ng/ml of HRP to yield oxidation product, resorufin. To concentrate and desalt the resorufin from the media, 200 μL of supernatant was collected by zip-tipping. To equilibrate the C18 ziptip (EMD Millipore) was pipette up the 65% acetonitrile (ACN) with 5% FA buffer for ten times and washed in 5% formic acid (FA) for ten times. To collect resorufin, the 200 μL supernatant was pushed through the resin bed 20 times before washing in 5% FA. Resorufin was eluted in 65% ACN with 5% FA in 2 μL. The resorufin was diluted 10-fold in 5% formic acid. The LC-ESI-MS analysis was performed by manual injection of 20 μL of the sample via an Agilent 1100 high pressure liquid chromatography (HPLC) pump set at 70% acetonitrile and 30% water to a final of 0.1% acetic acid as the ionization buffer flowing at 10 μL per minute at 5 kV over 15 cm x 300 um of normal phase column.

Statistics

Data shown are the means of blank ± 3Standard deviation (SD) for limit of detection or means ± SE of at least three independent experiments performed in triplicate as indicated in the figure legends. Comparison between control and treatment was made using t test ($P \leq 0.05$) or comparison

between groups by one-way ANOVA ($P \leq 0.05$) followed by a Tukey test or two-way ANOVA ($P \leq 0.05$) was used to compare difference of each treatment of H_2O_2 production by the statistical analysis system R. A value of $P < 0.05$ was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Determination of Optimal conditions for Amplex Red Assay

Before using a colorimetric method, the reaction conditions were optimized and demonstrated linearity with respect to time, HRP, and H₂O₂ standard curves *in vitro* (Figure 3.1, Figure 3.2, Figure 3.3). The lowest detection limit for HRP was 10 pg and safe detection limit was above 50 pg (Figure 3.2). Similarly, using the optimized reaction, H₂O₂ concentration was measured from 0.25 μ M to 1 mM and a linear relationship was found between 0.25 μ M to 100 μ M of H₂O₂ concentration and absorbance at 570 nm, with a correlation coefficient of 0.99. Using the AR and HRP reaction, 0.05 nmol of H₂O₂ can be measured (Figure 3.3). The limit of safe detection for quantification (LOQ) was computed using blank of Mean+3SD=0.061. The lowest safe detection limit was 0.5 nmol H₂O₂.

Figure 3.1 Time course of the change in fluorescence of oxidation product of AR. A 2 mM of H_2O_2 was added into 0.1 mM of AR and 10 ng/ml of HRP in total volume of 200 mL mixture to determine the optimal reaction time. Reactions were completed after ~30 min. Absorbance was measured using a microplate fluorimeter at 570 nm. Data points were the mean of triplicate measurements with the standard error bars.

Figure 3.2 To optimize the reaction conditions by producing HRP standard curves *in vitro* reaction. The HRP concentration response to 0.05 ng/ml in total volume of 200 μ L in the presence of 0.1 mM AR and 2 mM H₂O₂ into 96 wells. The plate was read on the microplate fluorimeter at 570 nm after 15 min of incubation in dark. To 0.05ng/ml of HRP can be detected, but the LOQ was 0.25 ng/ml (Mean of blank+3SD=0.051, N=3). After 5 ng/ml, reaction curve reached a plateau. The relationship between HRP and absorbance at 570 nm from 0.05 ng/ml to 5 ng/ml. Data points are the mean of triplicate measurements and some of the standard error (S.E.) are not visible due to small deviations.

Figure 3.3 H₂O₂ standard curve *in vitro* assay. The effect of different concentrations of H₂O₂ on UV/Vis absorbance at 570 nm, with AR as the HRP substrate. The HRP concentration was 10 ng/ml with 0.1 mM of AR in total volume of 200 μ L into 96 wells. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation. After addition of above 0.1 mM H₂O₂, reaction curve reached a plateau. The lowest detection level of H₂O₂ was 0.05 nmol while LOQ was 0.5 nmol per 200 μ l (Mean of blank+3SD=0.061). Data points are the mean of triplicate measurements and S.E. are not visible due to small deviations.

3.2 Detection of H₂O₂ production in RAW 264.7

After determining the optimal concentration of AR, HRP, and H₂O₂ for experimentation, it was applied the optimized conditions to measure H₂O₂ in RAW264.7 cells. A phagocytic cell can be stimulated by microscopic foreign particles such as aggregated oxLDL and Immunoglobulin G (IgG) opsonized bacteria or by soluble stimulants such as PMA or chemotactic peptides such as N-formly-met-leu-phe. Different treatments were used to elicit an oxidative response including: melamine beads, beads coated in IgG, beads coated in oxLDL, ionomycin, PMA, cytochalasin B & N-fromyl- met-leu-phe, and sodidum orthovanadate to investigate whether the cells could be stimulated to release measurable amounts of resorufin by fluorescence at 570 nm.

When RAW 264.7 cells stimulated with PMA for 5min incubation, it showed significant increase of H₂O₂ production. Since catalase and SOD are the main mechanisms for conversion of super oxide to H₂O₂ and then H₂O, SOD and catalase were added in 30min prior to addition of the treatment (oxLDL coated beads). When macrophages were treated with oxLDL coated beads and PMA, they produced the highest absorbance of resorufin compared to oxLDL alone, beads alone, and sodium orthovanadate (Figure 3.10). The confidence intervals using the Tukey method for oxLDL coated beads and oxLDL coated beads with SOD and catalase comparisons yield showed biologically significant difference (Figure A. 1). Figure 3.10 demonstrated that results are biologically significant using R. One Way-ANOVA test resulted in $F(5,83)=10.91$, $p=4.35e^{-0.85}$. Multy-way ANOVA test resulted in $F(5,83)=10.911$, $p=4.351e^{-08}$. However, blank of mean + 3SD was 0.0574 and all treatment was not above LOQ except treatment with PMA. Using the line of the best fit ($y = 19.206x + 0.0187$) generated from the H₂O₂ standard curve, the yield of H₂O₂ in each treatment was computed (Figure 3.11).

Figure 3.4 A colorimetric cellular HRP assay measured by UV/Vis spectrometry. The production of H_2O_2 (ROS) by RAW 264.7 macrophages in response to ligand coated beads was measured by an enzyme linked cellular assay where the substrate Amplex red (AR) and the enzyme Horse radish peroxidase (HRP) were exogenously supplied to yield resorufin in a H_2O_2 dependent reaction. The reaction mixture contained 10 ng/ml of HRP and 0.1 mM of AR in isotonic experimental media with different conditions: melanin beads, IgG coated beads and 1 μ M PMA. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation by transferring 200 μ L of supernatants into 96 wells. Each data point was representative of three experiments.

Figure 3.5 A colorimetric cellular HRP assay measured by UV/Vis spectrometry. The yield of resorufin in a H_2O_2 dependent reaction by RAW cells in response to ligand coated beads and ionomycin in the presence of Amplex Red (AR) and the enzyme Horse radish peroxidase (HRP). The reaction mixture contained 10ng/ml of HRP and 0.1 mM of AR in isotonic experimental media with different conditions: Beads, IgG coated beads and 2 μ M ionomycin. The plate was read on the microplate fluorimeter at 570nm after 15 min of incubation by transferring 200 μ L of supernatants into 96 wells. Computed mean of blank+3SD was 0.121 when N=4. Absorbance of each treatment was lower than 0.121 indicated that it is not above LOQ.

Figure 3.6 A colorimetric cellular HRP assay measured by UV/Vis spectrometry. The yield of resorufin in a H_2O_2 dependent reaction by RAW cells in response to different ligand coated beads in the presence of Amplex Red (AR) and the enzyme Horse Radish Peroxidase (HRP). The reaction mixture contained 10ng/ml of HRP and 0.1 mM of AR were exogenously added to the isotonic experimental media were different conditions to measured production of resorufin. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation by transferring 200 μL of supernatants into 96 wells. Each data point was representative of five independent experiments. (Mean of blank+3SD=0.0469, N=5). Each treatment was not above the LOQ.

Figure 3.7 Production of resorufin from the activation of the macrophage respiratory burst measured by enzyme-catalyzed oxidation of AR assay. Ten ng/ml of HRP and 0.1 mM of AR in isotonic experimental media with different conditions. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation by transferring 200 μ L of supernatants into 96 wells. Each data point was representative of four independent experiments. Average absorbance was corrected by subtracting from the average absorbance of blank (Mean of blank+3SD=0.0485).

Figure 3.8 A cellular HRP assay measured effect of beads coated oxLDL on antioxidant enzymes were measured by UV/Vis absorbance at 570 nm. The reaction mixture contained 10 ng/ml of HRP and 0.1 mM of AR were exogenously added to the isotonic experimental media were different conditions to measured production of resorufin. Catalase (1000 units/ml) and SOD (500 units/ml) was incubated 30 min prior to addition of 0.1 mM AR and 10 ng/ml of HRP. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation by transferring 200 μ L of supernatants into 96 wells. Each data point was representative of four independent experiments. (Mean of blank+3SD=0.0637, N=4)

Figure 3.9 To test enzyme-catalyzed oxidation of AR using beads by measuring absorbance at 570 nm. Cells were incubated on ice to reduce the production of H_2O_2 for 30 min after addition of each treatment. The plate was read on the microplate fluorimeter at 570 nm after 30 min of incubation by transferring 200 μ L of supernatants into 96 wells. After centrifugation at 14800 rpm for 5 min, absorbance has decreased compared to when supernatant was not centrifuged. To compare treatment between oxLDL coated beads and oxLDL coated Beads after centrifugation, paired sample t-test used ($p=1.71972E^{-08}<0.05$). Each data point was representative of three independent experiments.

Figure 3.10 A hydrogen peroxide and reactive oxygen species cellular assay was measured by UV/Vis absorbance at 570 nm. Ten ng/ml of HRP and 0.1 mM of AR in isotonic experimental media were incubated with ligand coated oxLDL for 30 minutes and PMA was incubated for 5 minutes. Catalase (1000 units/ml) and SOD (500 units/ml) were incubated 30 minutes prior to addition of 0.1 mM AR and 10 ng/ml of HRP. The supernatant was centrifuged at 14800 rpm for 5 minutes to remove excess melanin beads. Mean of absorbance in each treatment was plotted in bar graph. Each data point was representative of five independent experiments. (Mean of blank +3SD=0.0574, N=5). One Way-ANOVA test resulted in $F(5,83)=10.91$, $p=4.35e^{-0.85}$. Multy-way ANOVA test resulted in $F(5,83)=10.911$, $p=4.351e^{-08}$ Using R. While the results are biologically significant, it was not above the LOQ. The confidence intervals using the Tukey method for each of the ten comparisons yields are shown **Figure A. 1**.

Figure 3.11 The production of H₂O₂ by RAW 264.7 macrophages in response to microbeads. Using the line of the best fit equation: $y = 0.096x + 0.0187$ from the standard curve of H₂O₂, **Figure 3.3**, it was found that the mmol amount of H₂O₂ production in each treatment.

3.3 Determination of Optimal Conditions for ECL Experimentation

Enhanced chemiluminescence (ECL) is a simple and highly sensitive method to measure H_2O_2 . To conduct an ECL experiment in order to investigate sensitivity compared to enzyme-catalyzed oxidation of AR assay, three different methods were compared to test the sensitivity of ECL measurement. When the ECL method was used, the lowest detection limit for H_2O_2 and HRP were 10 nmol of H_2O_2 per 200 μL and 2 ng/ml HRP compared to Amplex red-HRP assay which allowed us to detect 0.2 nmol of H_2O_2 per 200 μL . The ECL method measured by Bio-Rad gel doc or Bio-Tek microplate was not very sensitive. In addition, immobilized HRP was detected by the ECL method using PVDF and measured by Bio-Rad gel doc showed a much higher ECL signal. Up to 10 pg of HRP could be detected, but using the ECL method only 0.1 mM H_2O_2 was detected (Figure 3.15). The ECL method is a simple and sensitive method to measure HRP in the presence of excess H_2O_2 but was not sensitive at low concentrations of hydrogen peroxide.

Figure 3.12 The detection of HRP and H₂O₂ using the ECL method. Panels: A, HRP standard curves *in vitro* reaction when HRP concentration responses up to 0.5 ng/ml in total volume of 200 μL, with presence of 100 mM Tris-HCl, 1.25 mM Luminol, and 2 mM 4-IPBA and 5.3 mM H₂O₂. Each well indicates ng/ml of HRP respectively; 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 2.5, 5, 7.5, 10, 20, 25, 50, 75; B, the H₂O₂ standard curve in the 10 ng/ml HRP, 100 mM Tris-HCl, 1.25 mM Luminol, and 2 mM 4-IPBA. With μM of H₂O₂ respectively; 0, 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100; C, indicated the same procedures as B but, with different concentration of H₂O₂ in High-Binding 96 well plates to be measured by Bio-Rad geldoc.

Figure 3.13 The image analysis of the HRP standard curves quantified using Image Lab software (see Figure 3.12.A). HRP standard curves *in vitro* reaction with presence of 100 mM Tris-HCl, 1.25 mM Luminol, and 2 mM 4-IPBA and 5.3 mM H₂O₂. As little as 0.1 ng/ml of HRP can be detected but LOQ was 0.75 ng/ml (Mean of Blank+3SD=6611.97). After 10 ng/ml of HRP, pixel intensity was fully saturated. Each data point was representative of three experiments and some of the error bars are not visible due to small deviations.

Figure 3.14 The oxidation of luminol in the presence of increasing H₂O₂ was measured by Bio-Tek microplate reader. In vitro reaction with presence of 100 mM Tris-HCl, 1.25 mM Luminol, and 2 mM 4-IPBA and 5.3 mM H₂O₂. Enhanced chemiluminescent was tested to measure the LOD for H₂O₂. Luminol was oxidized by HRP using H₂O₂ in 1:1 stoichiometric to produce aminophthalate with light emission detectable to 0.2 nmol level. The LOD for H₂O₂ using ECL was 10 nmol of H₂O₂ per 200 μ l (Mean of blank+3SD=1436.08). Each data point was representative of three experiments and some of the error bars are not visible due to small deviations. The ECL method was not judged to be significantly better than UV/Vis.

A.

B.

Figure 3.15 The ECL reaction measured on methanol activated PVDF with increasing HRP and H₂O₂. Different concentration of HRP and H₂O₂ onto methanol-activated PVDF with detection by gel doc after a minute incubation in ECL solution (5.3 mM H₂O₂, 1.25 mM luminol, and 2.5 mM enhancing agent of 4-iodophenylboronic acid in total volume of 1 ml). Panel; A, the dilution curve of HRP showed that as little as 0.1 ng/μl can be detected. Panel; B, the dilution curve of H₂O₂ showed the sensitivity of 0.1 mM.

3.4 Detection of H₂O₂ production in RAW 264.7 using LC-ESI-MS

To determine the sensitivity of resorufin, the liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS) at 214 m/z [M+H] was used. The concentration of resorufin from 0 nM or from 100 nM to 10 μ M was injected through a 2 μ L loop into a mobile phase of 70% acetonitrile and 30% water to a final concentration of 0.1% acetic acid as the ionization buffer flowing at 10 μ L per minute at 5 kV over 15 cm and 300 μ m ID normal phase (300Å) column.

LC-ESI-MS allowed for the detection of 0.2 pmol amounts of resorufin (

Figure 3.16). Relative abundance computed to obtain the line of the best fit ($y=6939.9x+602.91$, $R^2=0.997$) and the LOQ was 0.2 pmol (Blank of $\text{mean}+3\text{SD}=2.53\text{E}+04$) (Figure 3.17). In order to avoid an extra step of ziptipping for sample clean-up, EtOH extraction was tested and showed that resorufin recovery was less than 50% (Figure 3.18). Before injecting resorufin from cellular media into LC-ESI-MS, resorufin was collected using a ziptip. A 1ml volume of 0.1 mM AR reacted with H₂O₂ at a stoichiometry 1:1 with 10ng/ml of HRP to yield the oxidation product, resorufin. The yield of resorufin in an H₂O₂ dependent reaction by RAW cells in response to different ligand coated beads in the presence of Amplex Red (AR) and the enzyme Horse Radish Peroxidase (HRP). To concentrate and desalted the resorufin from the supernatant, 200 μ L of supernatant was zip-tipped and 2 μ l of eluent were diluted 10 folds and injected via a 20 μ L loop for analysis by LC-ESI-MS (Figure 3.20). The relative abundance shown by bar graph and blank of $\text{mean}+3\text{SD}$ was 479,240 and the treatments were not above the LOQ. One Way-ANOVA resulted in $F(6,21)=1.969$, $p=0.116$. Multy-way ANOVA resulted in $F(6,21)=1.969$, $p=0.1163$ using R (Figure 3.21). Relative abundance computed to bar graph by using the line of the best fit generated from the resorufin standard curve and quantify the yield of resorufin in each treatment (Figure 3.22).

Figure 3.16 Standard curve of resorufin by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS). Resorufin was detected by liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS) at 214 m/z [M+H]. Each peak indicated pmol amount of resorufin and y-axis was relative intensity while x-axis was time. Total ion current trace of the open mass window.

Figure 3.17 The Resorufin standard curve as measured by LC-ESI-MS. Different amount of increasing resorufin was injected and detected by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS) at 214 m/z [M+H]. The lowest detection limit for resorufin was at 0.2 pmol while safe detection limit and quantification (LOQ) was 0.2 pmol as well (Mean of blank+3SD= 2.53E+04).

Figure 3.18 Resorufin recovery test using EtOH extraction. Cultured RAW 264.7 cells in 6 well for 48 hours and added isotonic experiment media (pH 7.4). 10 µM of resorufin was incubated for 30 minutes at 37°C. Total 1ml of each sample was dried using a speed-vac to vacuum dry to remove solvent and leave resorufin. One ml of EtOH were added into fully dried samples and dried again. The same procedure was repeated for three times and 200 µL was transferred to 96 wells to be measured by UV/Vis at 570 nm. Resorufin recovery was less than 50%.

Figure 3.19 Resorufin recovery test: direct injection of resorufin versus zip-tipping 200 μl of resorufin sample and detected by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS). Different concentration of resorufin was injected through 20 μl loop. Right side of resorufin standard curve was generated by ziptipping sample before injecting into 20 μl loop by a liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS) at 214 m/z [M+H]. Each peak indicated pmol amount of resorufin and y-axis was relative intensity while x-axis was time. Total ion current trace of the open mass window

Figure 3.20 The production of H_2O_2 by Raw 264.7 cells in response to oxLDL coated beads and stimulator was measured by LC-ESI-MS. AR and HRP were exogenously supplied to yield resorufin in a H_2O_2 dependent reaction. To concentrate and desalted the resorufin from the 200 μL of supernatant zip-tipped. Resorufin was detected by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS) at 214 m/z $[\text{M}+\text{H}]$ by injecting through a 2 μL loop into a mobile phase of 70% acetonitrile and 30% water to a final of 0.1% acetic acid as the ionization buffer flowing at 10 μL per minute over 15 cm and 300 μm ID normal phase (300 $\text{\AA}$) column. First 5 peaks indicated Blank samples. Each three peaks indicated different treatments. 5min PMA

Figure 3.21 The production of H₂O₂ by Raw 264.7 cells in response to oxLDL coated beads and stimulator was measured by LC-ESI-MS was computed to bar graph using Figure 3.20. A total of 20 μ L of experimental media was concentrated and injected. Mean of blank+3SD was 479,23978 when N=4. Although error is analytical but treatments were not above the LOQ. Using R, One Way-ANOVA test resulted in $F(6,21)=1.969$, $p=0.116$. Multy-way ANOVA test resulted in $F(6,21)=1.9686$, $p=0.1163$. Confidence intervals used the Tukey method for each of the ten comparisons yields are shown **Figure A. 2**.

Figure 3.22 The production of H_2O_2 by Raw 264.7 cells in response to ligand coated beads and stimulator was measured by LC-ESI-MS calculated to find moles of resorufin using the resorufin standard curve (see Figure 3.17). A total of 20 μL of experimental media was concentrated and injected. Using the equation: $y = 6939.9x + 602.91$ from the standard curve of resorufin, and computed resorufin production in each treatment.

CHAPTER 4: DISCUSSION

The aim of this research was to determine whether H_2O_2 in live cultured cells could be monitored by a combination of enzyme-linked H_2O_2 radical assay UV/Vis, ECL and LC-ESI-MS. An enzyme coupled reaction used the exogenous addition of the enzyme HRP and the substrate AR to detect trace amounts of H_2O_2 by macrophages during rest versus phagocytosis. In the presence of HRP, AR reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce the red-fluorescent oxidation product, resorufin. The large amount of ROS produced by human neutrophils in response to N-fMLP or PMA may be measured by the oxidation of AR. Hence, the oxidation of AR by HRP should produce a direct measure of H_2O_2 . Previous studies have shown that barely detectable amounts of H_2O_2 are produced by macrophages by the oxidation of AR. Five replicates of the experiment were conducted where PMA, Sodium Orthovanadate, and oxLDL coated beads were presented to RAW 264.7 cells to compare the mean absorbance at 570nm. The comparison of AR, hydrogen peroxide/peroxidase UV/Vis assays ruled out future use of ECL that was not sensitive to low concentrations of hydrogen peroxide. The yield, sensitivity, quantification and detection limit of the enzyme-linked H_2O_2 assay and LC-ESI-MS of were compared to AR and the hydrogen peroxide/peroxidase assay *in vitro* as well as *in vivo*. The UV/Vis versus LC-ESI-MS showed both qualitative and quantitative differences with respect to measured oxidation of AR in response to PMA.

4.1 Characterization of Sensitivity for Detection of H_2O_2

The enzyme-catalyzed oxidation of AR assay is a simple and sensitive method for measurement of the H_2O_2 production from RAW264.7 cells. This method allows detection of 5pmol H_2O_2 per 100 μ l in a 96-well fluorescence microplate *in vitro* assay (Zhou *et al.*, 1997). Although enzyme-catalyzed oxidation of AR assay is a sensitive method for measurement of H_2O_2 , the signals were

not strong and were typically below the safe limit of detection and quantification (LOQ). The lowest concentration of H₂O₂ that can be detected may give a reading not clearly resolved from the baseline. Our experiment showed lower than 0.5 nmol of H₂O₂ per 200 µl was below the LOQ to safely quantify yield of H₂O₂ in live cells. In addition, the LOQ for H₂O₂ using ECL was 1nmol of H₂O₂ per 200 µl. As it was expected, the limit of sensitivity for the detection of H₂O₂ can be achieved by as little as 0.2 pmol per 2 µL using LC-ESI-MS.

4.2 Measurement of H₂O₂ Production

Macrophages are scavenger cells capable of phagocytosis. The measurement of the H₂O₂ production during phagocytosis in RAW 264.7 cells was measured with the Amplex Red Hydrogen Peroxide/peroxidase assay. The powerful carcinogenic substance PMA can directly activate protein kinase C (PKC) to activate NAPDPH oxidase (Swindle *et al.*, 2002). When we RAW 264.7 cells were stimulated with 1µM of PMA for 5 min, a high level of H₂O₂ (Mean±SE: $819 \pm 5.26E^{-05}$ µmol) was produced. Swindle, Hunt, & Coleman (2002) identified superoxide as the major source of ROS after 3 to 7 minutes incubation with PMA using pholasin-dependent chemiluminescence. The inflammatory ligand oxLDL may activate macrophages and increase H₂O₂ by macrophage-derived foam cells (Karunakaran *et al.*, 2016 and Rajagopalan *et al.*, 1996). The treatment of macrophages with oxLDL induced the production of H₂O₂ 350 µmol/L to 450 µmol/L (Sukhanov *et al.*, 2006). Similarly, our result showed that when macrophages were incubated with oxLDL, they produced up to 170 µmol (Mean±SE: $170 \pm 2.09E^{-05}$ µmol) of H₂O₂ per 200 µL. Solid aggregates of oxLDL can activate macrophages and trigger inflammatory phagocytic activation leading to the release of free radicals and create more oxLDL. Microbeads were coated with oxLDL to and used in a phagocytosis assay (Vance *et al.*, 2016). Solid aggregates

of oxLDL can activate macrophage engulfment and produce H₂O₂ (Mean± SE: 250 ± 2.15E⁻⁰⁵ μmol) as measured by the production and accumulation of resorufin. The signal was specific as demonstrated by the addition of catalase and SOD. The SOD and catalase enzymes lowers superoxide concentration by catalyzing the superoxide into hydrogen peroxide and oxygen while catalase promotes the degradation of H₂O₂ to O₂ and H₂O. Addition of SOD and Catalase prior to addition of oxLDL coated beads, macrophages decreased production of H₂O₂ (Mean±SE: 0.185 μmol ± 8.33E⁻⁰⁶). When macrophages were incubated with oxLDL for 2 hours, intracellular catalase activity increased while activity of SOD remain consistent (Sukhanov *et al.*, 2006 and Hultén *et al.*, 2007). Macrophages upregulate catalase activity to restore ROS balance in response to oxidative stress (Hultén *et al.*, 2007).

4.3 Luminol Dependent Chemiluminescent

ECL is sensitive and quantitatively detects H₂O₂ since luminol oxidized by HRP using H₂O₂ in 1:1 stoichiometric to produce aminophthalate with light emission detectable to the 0.2 nmol level. The ECL assay allowed detection of HRP up to 0.01 ng and the LOQ for H₂O₂ was 10 nmol of H₂O₂ per 200 μl. It is clear from our data that ECL is not as sensitive as by mass spectrometry combined enzyme-catalyzed oxidation of AR assay. Although some studies suggested another enhancer such as Diogenes to increase sensitivity, it did not mention the LOQ to be comparable to our method (Yamazaki *et al.*, 2011).

4.4 Mass spectrometry

Fluorescent resorufin can be detected by applying liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS) at 214 m/z [M+H] to an *in vitro* reaction (Florentinus-mefailoski *et al.*, 2013). Resorufin from the cellular media in 0.1% formic acid

produced an intense peak at 214 m/z by LC-ESI-MS using isocratic HPLC and SIM. The collection of resorufin from 20 μ L of cellular media analyzed by LC-ESI-MS at 214 m/z [M+H] was not above LOQ. The resorufin from a larger volume of cellular media may have to be concentrated to provide a signal that is well resolved from baseline.

Tyrosine phosphorylation was involved in the activation of the macrophage respiratory burst (Bassal *et al.*, 1997). Sodium orthovanadate is a phosphor-tyrosine phosphatase (PTP) inhibitor that enhance the respiratory burst in neutrophils or macrophages (Green, Hamilton, & Phillips, 1992). Studies showed that tyrosine kinase activity increased by PMA which is associated with the respiratory burst (Bassal *et al.*, 1997). Previously, the addition of vanadate prior to triggering the respiratory burst, enhanced the effect of the tyrosine kinases activity resulting in an increase in respiratory burst but had no effect in our experiments. There was an apparent increase in absorbance at 570 nm in response to treatment with PMA even after centrifugation (Figure 3.10). but no effect of PMA was observed by LC-ESI-MS. Many studies have showed that PMA induced accumulation of RNA and enzymes that are related to oxygen-dependent metabolism and apoptotic cells (Dolgushin *et al.*, 2013). Thus, it was proposed that PMA induced cell secretion that cannot be removed by centrifugation at 14800 rpm and may interfere the absorbance of the resorufin while measuring intensity at 214 m/z using LC-ESI-MS was not effected by PMA induced cell secretion. Thus, further experiments are required to determine the source of the discrepancy between UV/Vis versus LC-ESI-MS.

CHAPTER 5: CONCLUSION

If the ROS generated in cells can be measured and analyzed precisely, it can be employed to evaluate immune function in response to ligands and drugs. In this study, a novel enzyme linked cellular assay to detect H_2O_2 produced by cultured macrophages during the phagocytosis of oxLDL coated beads by the exogenous provision of Amplex Red and HRP monitored by LC-ESI-MS. In the presence of HRP and Amplex red, the H_2O_2 produced by the cells reacted in a 1:1 stoichiometric ratio to produce the red fluorescent oxidation product, resorufin that may be quantified by LC-ESI-MS. Resorufin can be detected by fluorescence using excitation of 570 nm and emission of 585 nm at 0.5 nmol to 1 nmol level that showed a safe detection limit similar to ECL. The small amount of H_2O_2 produced by cultured macrophages, while biologically significant, was below the limit of safe detection and quantification by UV/Vis methods. The quantification of resorufin by LC-ESI-MS at 214 m/z [M+H] allowed for detection as low as 0.2 pmol level. Although, quantified H_2O_2 production level by macrophages in response to the inflammatory ligands from 20 μL were not above the LOQ, there was a trend to greater H_2O_2 production with inflammatory stimulation. The mass spectral detection method to an enzyme couples cellular assay is a useful tool for sensitive detection of H_2O_2 in cells. Thus, it can be widely applied in biochemical research and in clinical trials for H_2O_2 detection techniques.

Future Work

LC-ESI-MS is a very sensitive method to detect as low as 0.2 pmol of H_2O_2 . However, zip-tipping 20 μL of resorufin from cellular media with 20 μL injection into LC-ESI-MS is not sufficient. It is believed that increasing the volume to 1ml of cellular media collected over C18 with 20 μL injection loop will allow for the quantification of H_2O_2 above the LOQ.

APPENDIX

Figure A. 1 95% family-wise confidence level for simultaneous measurement of H_2O_2 production using the combination of enzyme-linked H_2O_2 radical assay. The confidence intervals using the Tukey method for each of the ten comparisons yields are shown. The pairwise comparisons indicated that OxLDL-beads are significantly different from the control. In addition, SOD and Catalase quench the signal from OxLDL-beads are also significantly different.

Figure A. 2 95% family-wise confidence level for simultaneous measurement of H_2O_2 production analyzed by LC-ESI-MS. The confidence intervals using the Tukey method for each of the ten comparisons yields are shown. The pairwise comparisons indicated that there no significantly different from the control.

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