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Cell Plasma Membrane Cholesterol as a Diagnostic

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Abstract

Cholesterol is a tightly regulated major structural component of the cell plasma membrane (PM) where it forms stoichiometric complexes with phospholipids and sphingolipids. The amount of cholesterol in the PM exhibits a regulatory role in basal activity of several biomolecular processes by direct binding to proteins and by indirect local environmental effects within the PM that are also coupled to overall cellular cholesterol homeostasis. The term “active cholesterol” refers to PM cholesterol not complexed to lipids, a cholesterol state that arises above a threshold mole fraction of cholesterol in the PM. Active cholesterol level in the PM provides a control mechanism for cellular cholesterol homeostasis through its recognition by membrane bound proteins that activate genes of cholesterol synthesis enzymes. Uptake of LDL, production and release of HDL as well as reversible storage of cholesterol in the cytosol by covalent modification are also regulated and dependent on PM cholesterol (thermodynamic) activity: active cholesterol. A number of human disease states have been found to have associated alterations in PM cholesterol and thus a method for its determination is described.

Introduction

The understanding of cellular cholesterol processing is historical and of increasing importance in understanding disease states [1–3]. An experimental aim of this research

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group has been the ability to monitor PM cholesterol thermodynamic activity at live cells and tissues without significant perturbation of the native physiological state [4–10]. In particular, the active cholesterol pool is easily depleted leading to its physiological determination by traditional assays being out of reach [11]. Our approach is to measure the rate of cholesterol diffusion from the PM at the point-of-contact of a microelectrode [4,6]. The enzyme-modified electrode consumes the sparingly-soluble aqueous-cholesterol adjacent to the PM thus perturbing the equilibrium between aqueous- and PM-cholesterol resulting in diffusion of cholesterol from the PM only at the site of electrode contact. That is, the enzyme modified electrode creates a demand for cholesterol at the site of contact and measures the diffusional response. The rate of cholesterol diffusion (extraction) is related to the thermodynamic activity of cholesterol (which is controlled by the active cholesterol concentration) in the PM by classic kinetic theory [12]. The transition state is cholesterol that has exited the PM and has not yet formed a solvent cage. In our conceptual model, the thermodynamic activity of cholesterol in the PM is reporting on the concentration of active (not complexed) cholesterol which is the only state able exit the PM. Figure 1 depicts the most relevant cellular processing pathways involved in PM cholesterol homeostasis and electrode signal.

Work on this project began with demonstrating the functionality of cholesterol oxidase immobilized on a solid electrode. Devadoss et al. [13] showed that the enzyme spontaneously inserts in the lipid bilayer membrane formed on indium-tin oxide semiconductor electrodes. The lipid membrane is formed in two steps where the electrode surface is initially modified with a lipid containing a thiol functionality at the polar head group end. Vesicle fusion is used to cast the outer lipid leaflet. Signal transduction is achieved by electro-reduction of hydrogen peroxide produced from enzymatic cholesterol oxidation by molecular oxygen. While this system showed a concentration dependence for flow-injection of buffered cholesterol solutions, a challenge was instability of the baseline current due to electro-reduction of molecular oxygen at the potential required for electro-reduction of hydrogen peroxide.

To circumvent the baseline stability problem platinum electrodes are used because of the ability to catalytically electro-oxidize hydrogen peroxide at a moderate potential by heterogeneous electron transfer. Bokoch et al. [14] demonstrated lipid bilayer deposition and enzyme insertion at platinum electrodes using the same protocol previously developed for immobilizing enzyme at indium-tin oxide electrodes. Repeatable flow-injection responses to buffered cholesterol solutions of various concentration were reported as well as remarkable signal and baseline stability for repeated continuous exposure over hours. Additionally, detection of cholesterol carried by LDL particles is possible and significant degradation of electrode performance presumably from adsorption of the LDL particles to the electrode surface occurs.

The PM Cholesterol Switch

In work for single cell and animal tissue analysis of cholesterol efflux, capillary platinum microelectrodes 10 μm in diameter are constructed in-house and modified with the lipid bilayer membrane containing the oxidase enzyme. Characterization of the electrode

performance in quantification of cholesterol solution was followed by application to diffusion of cholesterol from the lipid bilayer membrane of giant vesicles, as a cell and PM model system [5]. Repositioning of the electrode is used for signal quantification where the response for contact between the vesicle membrane with the electrode is referenced to the baseline current with the electrode removed from the vesicle membrane. A nonlinear dependence of electrode response on cholesterol content of the lipid bilayer membrane is observed. The data clearly support the existence of an active cholesterol pool not complexed to phospholipids. Vesicles containing 33 % cholesterol (mol/mol) do not show an electrode response because this amount is below the stoichiometric threshold required for existence of a measurable active cholesterol concentration. Vesicles containing 50 % cholesterol showed moderate efflux and those containing 66 % show an increase above the 50 % vesicles that is larger than the increase between 33 % and 50 % cholesterol. The data set reflects the nonlinear growth of the active cholesterol pool with increasing mole fraction of cholesterol and is direct support of work by the McConnell group on model lipid membrane systems [12] and the Steck group on cells that show a role of stoichiometric binding between cholesterol and phospholipids lipids in regulation of cholesterol homeostasis [3].

Single Cell Interrogation

The capillary platinum microelectrodes modified with a lipid bilayer membrane containing cholesterol oxidase can also be implemented for extraction and detection of cholesterol diffusion from the Oocyte PM [4]. In this work it is demonstrated that the aqueous layer thickness between the electrode (enzyme) and the PM directly influenced response magnitude. It was also shown that modulating the PM cholesterol level by solution extraction and subsequent redelivery from solution can be tracked by electrode response. That is, electrode response magnitude reflects the amount of (active) cholesterol in the PM because diffusion rate is increased at higher concentrations as predicted by classic kinetic theory. A shortcoming of the electrode-supported lipid bilayer membrane containing inserted oxidase enzyme is that it is not stable at physiological temperature disallowing experiments on functioning mammalian cells and tissues.

A thiol chemistry linking scheme was used to attach cholesterol oxidase to 4 μm diameter platinum disk electrodes and, through collaboration with Jonathan Smith at the Cleveland Clinic, studies on single macrophage cells showed evaluated for PM cholesterol. Three distinct levels of PM cholesterol were demonstrated [6]. In this work, 50 fA electrode signals were resolved. Native macrophages showed increased PM cholesterol diffusion after differentiation to the foam cell state that is induced by uptake of acetylated LDL. This result indicated that some of the excess cholesterol exists in the PM, not only as stored ester-linked lipid deposits in the cytosol. Furthermore, an additional increase in PM cholesterol is shown for the foam cell state when the enzymatic pathway for storage is chemically blocked. It is noted that a host of methods used to lower the macrophage PM cholesterol, such as cholesterol extraction and statin treatments to slow biosynthesis of cholesterol, resulted in no diffusional response at the electrodes indicating that the active cholesterol pool had been significantly depleted.

Applications in Cystic Fibrosis and the Prospect of Human Analysis

The opportunity to analyze cystic fibrosis (CF) samples arose in a collaboration with Thomas Kelley at Case Western Reserve University who had discovered elevated cellular cholesterol for the CF disease state [15–20]. Jiang et al. [7] compared mouse trachea tissue from CF and control samples and showed a clear increase in cholesterol diffusion at the CF tissue. The CF phenotype can be induced in wild-type cells by chemical inhibition of the chloride ion channel that causes CF when mutated. Interestingly, induction of the CF phenotype showed in initial decrease in PM cholesterol during the first 24 hours and a subsequent increase in PM cholesterol after 48 hours [18]. This result presented the notion that the PM cholesterol increase in CF is a secondary response to lost chloride ion channel activity. The postulate is that cholesterol synthesis is up-regulated to reestablish lowered PM cholesterol and that an over-correction occurs leading to elevated PM cholesterol and over-accumulation of cholesterol inside the cell. We note this result, in particular, as it had an impact on the CF research community in terms of their understanding of CF cell biology.

The results on CF samples led to the goal of tracking PM cholesterol in the nasal passage of CF human subjects as a means of gauging disease progression, severity, and intervention treatments. The initial aim is to test the hypothesis that cholesterol is elevated in airway tissue of humans suffering from CF. Confirmation of this hypothesis would afford increased relevance of cell-line and animal models to the human disease state, thus significantly advancing our understanding of the disease. Additionally, with the advent of new drugs targeted directly to restore activity of disease causing mutant forms of the chloride ion channel, there is a clear need to test the efficacy of these compounds on various aspects of CF disease progression. Modern assessments focus on broad clinical outcomes that evaluate disease management, but give little insight into what disease processes are being influenced at the cellular level. There is currently no method to obtain a non-invasive assessment of intracellular effects for dysfunction of the chloride ion channel.

The prospect of conducting electrochemical cholesterol diffusion experiments at human tissue, however, presented analytical challenges in terms of signal-to-noise and data acquisition method. In the cell and tissue work, two cell or tissue types (for example CF vs wild-type or native vs. drug treated) can be compared using the same electrode and the signal ratio can be reported. This scheme is not possible for human studies and better control of sensor repeatability is needed. Additionally the signal is in the low pA range requiring faraday shielding of the experiment which is an obstacle for screening many subjects.

Overcoming Traditional Challenges

To solve these challenges in detection of cholesterol diffusion from human tissue, *in vivo*, a double potential pulse data acquisition scheme was developed. Initial experiments were conducted at single cells using a micro-cavity electrode geometry [8]. The optimized method uses disk platinum microelectrodes that are held in contact with tissue while hydrogen peroxide from enzymatic cholesterol oxidation is allowed to accumulate between the electrode surface and the tissue surface. At 5 second intervals hydrogen peroxide that has accumulated is electro-oxidized by a potential pulse. A second replicate pulse conducted 0.5

s after the initial pulse provides a background charge for subtraction and difference charge acquisition. Repeating the waveform allows averaging of data that report the cholesterol diffusion rate. Figure 2 shows the CF increase at mouse trachea tissue over wild-type control samples confirming the earlier amperometric result [7] using the double potential pulse method.

Proof-of-concept data for the *in vivo* human measurement followed where the ease of access of the oral mucosa served the experimental tissue. Yu et al. [10] found that a portion of the human *in vivo* signal for double potential pulse detection of cholesterol diffusion from the mucosa constitutes a background portion from adsorption of species to the electrode during the time allowed for accumulation of hydrogen peroxide. After initially working with platinum disk microelectrodes, she determined that 10 μm diameter carbon disk microelectrodes modified with a sparse coverage of electrodeposited platinum particles of nanometer dimensions showed a smaller background and retained the required ability to attached the oxidase enzyme and electro-oxidize hydrogen peroxide.

Because of the small size of the electrode in combination with the double potential pulse data acquisition scheme, the method is selective to only hydrogen peroxide that accumulates at the electrode contact site and is blind to any hydrogen peroxide that may be present in bulk tissue near the contact site [9]. Moreover, the method negates all faradiac steady-state current from diffusional species that react at the electrode. All capacitive charge is also removed avoiding common signal drift limitations in electrochemical sensing. The practical utility of the double potential pulse method as a general scheme for biosensing at cellular surfaces is that the approach masks physical differences between individual microelectrodes as well as the large increase in faradaic background observed at the human mucosa membrane. Figure 3 shows data for the total charge that passes during the first potential pulse for control electrodes and enzyme-modified electrodes in buffer (panel A) and at the mucosa (panel B). Panels C and D show the double potential pulse difference charge for the same electrodes in buffer and at the mucosa, respectively. Only from the difference charge data at the mucosa can the signal from accumulated hydrogen peroxide be resolved. Also, the approach does not require puncture of the skin (sensor or reference potential electrode) which facilitated IRB approval and participant volunteering.

Concluding Remarks and Future Direction

Through Collaboration with Minchul Shin at Georgia Southern University, ongoing work for human cholesterol sensing is using nano/micro manufacturing to produce sensor chips containing well-spaced regions of SiO_2 that are circular with a 10–20 μm diameter for attachment of oxidase. The small size of the SiO_2 regions prevents cholesterol depletion and allows for signal selectivity as noted above. The SiO_2 patches containing covalently attached oxidase enzyme have 1–5 circular platinum electrodes that are 2 μm in diameter and that are evenly spaced for hydrogen peroxide electro-oxidation (Figure 4). These batch-produced, single-use sensor chips are expected to increase signal-to-noise ratio as well as signal-to-background such that human measurements may be conducted without the use of faraday shielding. This general approach to noninvasive human sensing could serve as a platform for screening children for hypercholesterolemia and for other markers such as glucose.

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Highlights

- Active plasma membrane cholesterol serves as a switch controlling storage and biosynthesis of cholesterol inside the cell.
- Airway tissue of the cystic fibrosis diseased state exhibits elevated plasma membrane cholesterol and accumulated cholesterol inside the cell.
- The ability to trigger and monitor plasma membrane cholesterol diffusion using enzyme-modified microelectrodes is reported.
- A noninvasive means of evaluating plasma membrane cholesterol in the airway tissue of human cystic fibrosis patients is needed and progress towards this achievement is described.

Fig 1. Cellular processing and trafficking of cholesterol, green arrows show trafficking directions to and from the plasma membrane as well as storage in lipid droplets and synthesis in the ER, cholesterol is purple rectangle. Diffusion from the plasma membrane is caused and monitored by contacting the plasma membrane with an enzyme modified microelectrode.

Fig 2.

Difference charge traces (pulse two subtracted from pulse one) for an enzyme modified electrode (Enzyme) in contact with trachea tissue from s cystic fibrosis mouse (CF) and a wild-type mouse (WT). Control (Bare) platinum microelectrode responses showing no difference between CF and WT mouse trachea tissue. Electrodes are 50 μm diameter platinum disk microelectrodes manufactured in-house. Control and enzyme electrodes are matched for similar difference charge response in buffer solution.

Fig 3.

Total charge for first pulse at control (blue) and enzyme-modified (red) electrodes in buffer (A) and at the mucosa (B). Double potential pulse difference charge for the same control (blue) and enzyme-modified (red) electrodes in buffer (C) and at the mucosa (D). Error bars (A and B marked with stars) are SD of three consecutive, stabilized (first) potential pulse charge measurements and error bars (C and D) are SD for three consecutive double potential pulse difference charge measurements. Supplemental information for ref. 10.

Fig 4. SEM image of one of five sensors of the array on the chip. The large circle is a 20 μm diameter of SiO₂ for enzyme attachment, smaller inset circles are three 2 μm diameter platinum disk electrodes for hydrogen peroxide oxidation, surrounding material is polyamide insulator.