Detecting Changes in Cholesterol Activity (Accessibility) at the Membrane Surface Using Perfringolysin O Mutants

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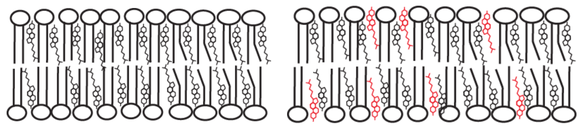
Introduction

Cholesterol is an essential component of mammalian cell membranes and it is important to regulate the structure and function of lipid bilayers. Changes in cholesterol levels are involved in many physiological and pathological events such as the formation of arterial plaques, viral entry into cells, sperm capacitation, and receptor organization. Determination of cholesterol trafficking and distribution is essential for understanding how cells regulate cholesterol activity. A cholesterol probe capable of distinguishing changes in cholesterol chemical activity within membranes would facilitate investigations in this area.

Perfringolysin O (PFO) is a cytolysin secreted by *Clostridium perfringens* that requires cholesterol in the target cell membrane for binding.(1) The specificity of PFO for high levels of active cholesterol makes the toxin a potential tool for the detection of cholesterol distribution and trafficking.(3) We have recently shown that introducing point mutations in the membrane-interacting domain 4 loops can altered the threshold of cholesterol concentration required in model membranes to trigger binding.(4) Using this we hope to develop a probe to detect membrane cholesterol activity.

Cholesterol Activity

The cholesterol activity of a membrane is related to the ability of cholesterol molecules to interact with other molecules at the membrane surface (escape tendency)(2).



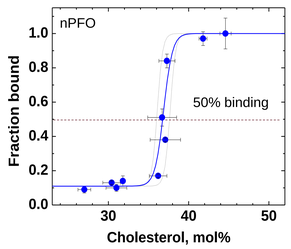
Low Cholesterol Activity
(30% Mol Cholesterol)

High Cholesterol Activity
(35% Mol Cholesterol)

Measuring Cholesterol activity

Recently it has proposed that cholesterol activity and not overall cholesterol level is responsible for many physiological interactions including cholesterol homeostasis. Current cholesterol probes like filipin measure overall cholesterol levels and as a result are ill suited to measure cholesterol activity.

1 Binding of Native PFO is Cholesterol Dependent and Highly Cooperative

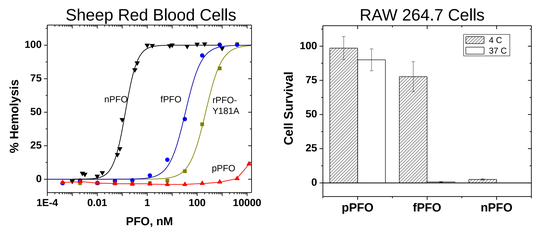


PFO binding is completely cholesterol dependent, but it binds only high cholesterol activity. It has a very steep sigmodal binding curve. This could be used in cells to detect high cholesterol activity in an on off manner.

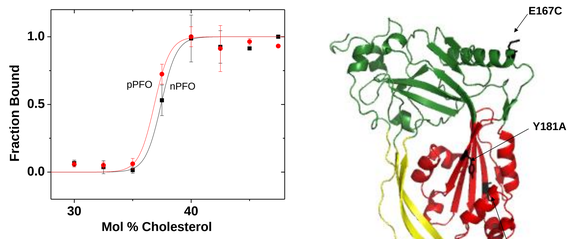
Binding of nPFO (final concentration of 0.2 μM) was determined using intrinsic tryptophan fluorescence on liposomes of varying cholesterol content and containing POPC,POPE and Sphingomyelin in a constant 1:1:1 ratio (total lipid concentration of 0.2 mM)(3).

The mol % of cholesterol of liposomes was determined by individual quantification of cholesterol and total phospholipids. Cholesterol was quantified using the Amplex® Red Cholesterol Assay Kit (Invitrogen) and total phospholipids by phosphate determination after acid hydrolysis. The horizontal dotted line represents 50% binding which for nPFO is ~ 37 mol%. (4)

2 Engineered A Non-Lytic PFO Derivative



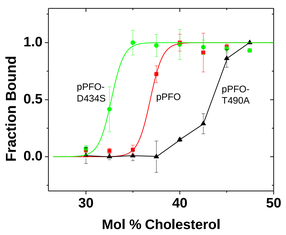
Hemolytic activity of PFO derivatives was determined using sheep red blood cells and RAW 264.7 cells. Red blood cells were incubated with varied concentrations of PFO at 37°C of 20 min. and hemolysis was quantified by measuring the absorbance of released hemoglobin. Death of Raw cells was determined by counting with trypan blue before and after incubation at the indicated temperature for 20 min. with 1μM of the indication PFO derivative.

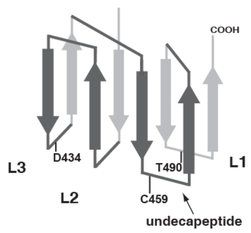


The created pPFO scaffold for our probe contains 4 mutation. The C459A and E167C mutations move the sole cysteine in the protein out of the binding domain for labeling.(4) Individually and combined Y181A and F318A eliminated pore formation ability on model membranes.(5) However single amino acid substitutions are still hemolyticly active at high concentrations. Both mutations are required to abolish hemolytic activity.

rPFO=PFO-^{C459A}, fPFO=rPFO$^{E167C-F318A}$, pPFO=rPFO$^{E167C-F318A-Y181A}$

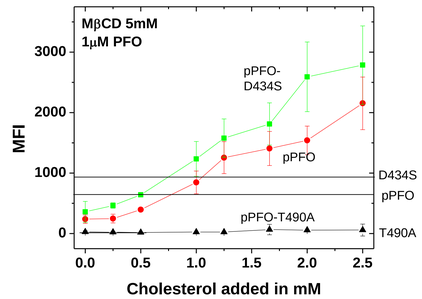
3 Modification of Domain 4 Loops Alters the Binding Threshold of pPFO Derivatives





Cholesterol-dependent binding of the two pPFO mutants selected for cellular studies compared to the parental pPFO. Binding measurements were taken as described in section 2. Data show a more than 5% difference in the mol % cholesterol required for binding. (left panel) Diagram of PFO domain 4 with the loops and the modified residues marked. (right panel)

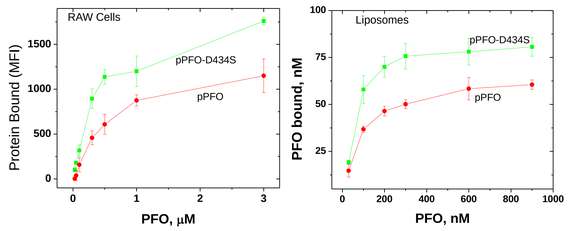
4 PFO Probes Binding to Live RAW Cells



Cholesterol content on macrophage-like cells was varied by treatment with β-methyl-cyclodextrin (MβCD)-cholesterol complexes. Binding of Alexa-labeled pPFO derivatives was quantified using flow cytometry as indicated below. The cholesterol activity required for pPFO-T490A binding was not achieved in live cells with this treatment. The differential binding of pPFO and pPFO-D434S to live cells containing various cholesterol levels suggests that the cholesterol:MβCD treatment modified the total amount of cholesterol in the plasma membrane, but the cholesterol activity at the membrane surface remained similar.

Binding of PFO derivates to Raw 264.7 macrophages-like cells treated with MβCD : cholesterol complexes. The horizontal lines indicate the binding of probes to untreated cells. The experiment was done as follows. Aliquots of 10^6 cells were washed 2 times in PBS 1% FCS and then grown on minimal media for 1 hr. The cells were then washed 2 more time and incubated at 37 °C for 30 min in 0.5 ml of PBS 1% FCS with 5 mM MβCD complexed with the indicated concentration of cholesterol. The cells were then washed two more times and incubated at 4 °C for 20 min with 0.5 μM of the indicated PFO derivative in 100 μL of PBS 1% FCS. After washing unbound protein the mean fluorescence intensity (MFI) of the cells was determined by flow cytometry.

5 Membranes With Identical Cholesterol Content Bind Different Amount of PFO Derivatives



Binding curves for pPFO and pPFO-D434S in RAW cells (left panel) and model membranes (right panel). Both pPFO derivative shows different maximal binding to the same nature or model membrane

Aliquots of 10^6 RAW cells were washed 2 times in PBS 1% FCS and then grown on minimal media for 1 hr. The cells were then washed two more times and incubated at 4 °C for 20 min with indicated concentration of the PFO derivative in 100 μL of PBS 1% FCS. After washing away unbound protein, the (MFI) of the cells was determined by flow cytometry.

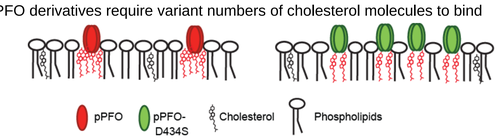
The binding of varied concentration of the PFO derivatives to liposomes containing 38% cholesterol was determined using intrinsic tryptophan fluorescence as indicated in section 2.

6 Binding of pPFO Decreases Cholesterol Activity

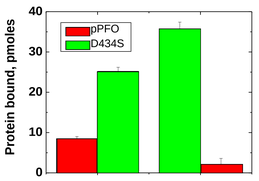
Why is the total binding of pPFO derivatives to identical membranes not the same?

Binding of PFO derivatives decreases cholesterol activity on the membranes surface

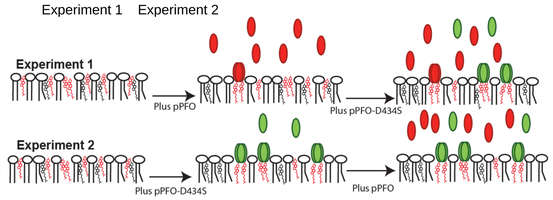
PFO derivatives require variant numbers of cholesterol molecules to bind



To differentiate between these two possible explanations we analyzed the sequential binding of pPFO derivatives



pPFO and pPFO^{D434S} binding was determined to 36 mol% cholesterol liposomes in subsequent binding assays to the same set of liposomes. Binding was quantified using intrinsic tryptophan fluorescence. The initial fluorescence for the protein added second was determined in a separate cuvette. This showed that PFO derivatives with low cholesterol thresholds can bind to liposomes that have already bound the maximum amount of a derivative with a higher cholesterol threshold.



Conclusions

1. We have engineered a non-lytic PFO parental derivative (pPFO) that has similar properties as the native toxin.
2. We obtained pPFO derivatives that bind differentially to membranes of varied cholesterol activity
3. Binding of PFO to membranes reduced the overall cholesterol activity.

Acknowledgments

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