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short report

A Proposed Model of Apoptosis of Pancreatic Insulin-Secreting Beta-Cells

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Introduction

Diabetes mellitus type 1(T1DM), or insulin dependent diabetes, results from autoimmune beta-cell destruction.[1] Beta-cells secrete insulin, the primary regulator of glucose homeostasis. When beta-cells die, it results in a decrease in insulin secretory capacity leading to harmful increases in blood glucose levels. The Ca^{2+}-independent phospholipase A2 (iPLA2β) enzyme participates in beta-cell death by a programmed pathway, or apoptosis, that involves generation of ceramides.[2] Ceramides are lipids, which are known to cause apoptosis in several cell systems.[3] The established pathway of ceramide generation is via the hydrolysis of sphingomyelins (SMs) by sphingomyelinase (SMase). It is believed that the neutral form of sphingomyelinase (NSMase) plays a key role in the apoptosis of beta-cells. Figure 1 describes a proposed mechanism for beta-cell apoptosis.[2] In this model, endoplasmic reticulum (ER) stress, induced by depletion of ER Ca^{2+} stores by thapsigargin, activates iPLA2β.

When activated, the iPLA2β enzyme hydrolyzes the sn-2 substituent in membrane phospholipids in the absence of Ca^{2+}.[4] As shown in Figure 2, activation of iPLA2β leads to the hydrolysis of the fatty acid arachidonic acid (AA) and a product devoid of an sn-2 substituent. These are designated as lysophospholipids (LPCs). When they contain choline as the head group in the sn-3 position, they are referred to as lysophosphatidylcholines (LPCs).

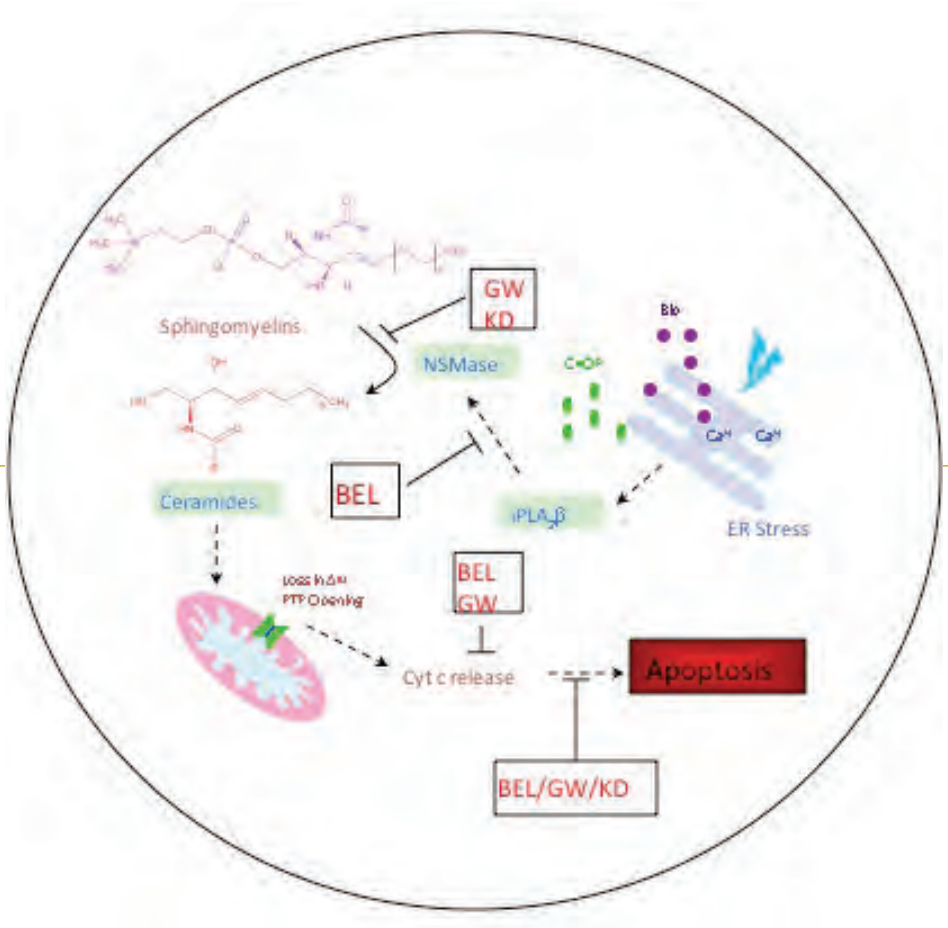

Figure 1. Proposed Model by which iPLAβ/ Ceramides Induce Beta-Cell Apoptosis

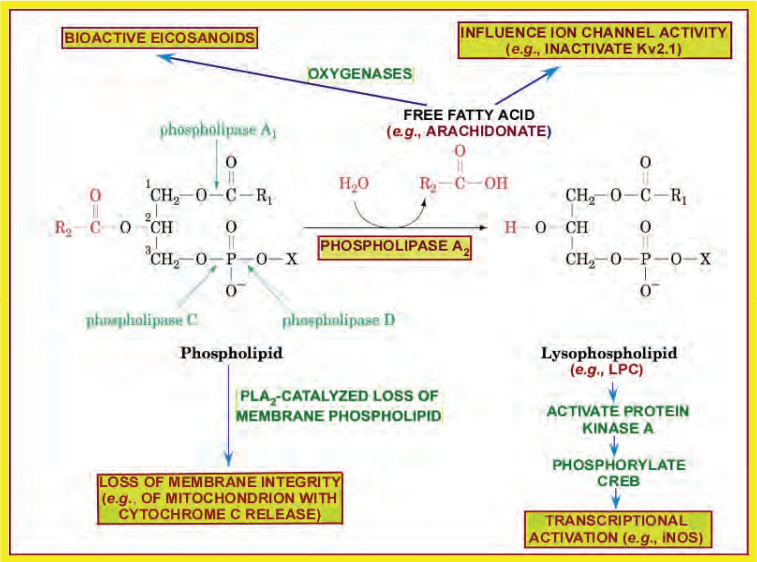

Figure 2. Summary of Phospholipase A2 Activities

Objective

The objective of this project was to identify which lipid products of iPLA2β activation impact NSMase expression and at what concentrations. In initial experiments, two variables were considered: the concentration of each lipid and the duration of exposure. INS-1 insulonoma cells that over expressed for iPLA2β were exposed to one of three products of iPLA2β activation: arachidonic acid (AA), lysophosphatidylcholine 18:0 (LPC 18:0), or lysophosphatidylcholine 16:0 (LPC 16:0). It should be noted that LPC 18:0 and LPC 16:0 are lysolipids that contain different fatty acid substituents at the sn-1 position. Thus, LPC- 18:0 contains stearate with a carbon chain length of 18 and zero double bonds and LPC-16:0 contains palmitate with a carbon chain length of 16 and zero double bonds The differing ratios describe the carbon chain length and number of double bonds. LPC 18:0, also referred to as pomitate, contains an eighteen carbon chain with zero double bonds; LPC 16:0, also referred to as stearate, contains a 16 carbon chain with zero doubles. Preliminary findings suggested that LPC and AA induce NSMase within 6 hours in a concentration-dependent manner. Thus, in this experiment, the cells were exposed to AA, LPC 18:0, or LPC 16:0 for a 6 hour interval at 37°C.

Methods

The experiment involved isolating RNA from the INS-1 cells treated with the three lipids. From these RNA samples, cDNA was generated and then processed for Real-Time PCR to quantitate NSMase mRNA expression of NSMase. The following set of procedures was repeated weekly:

Three 6-well plates were seeded with over-expressing INS-1 cells, an insulinoma cell line that is widely used to study beta-cell function. Each well contained 999 µl of growth medium and 1 µl of lipid solution, totaling to a volume of 1 mL in each well. The control, or vehicle, used was 0.01% dimethyl sulphoxide (DMSO).

The set up was as follows:

Vehicle	Vehicle	1.1-AA
1.2-AA	2.1-AA	2.2-AA

Vehicle	*0.01% DMSO*
Vehicle	*0.01% DMSO*
1.1-AA	*5 µM AA*
1.2-AA	*5 µM AA*
2.1-AA	*100 µM AA*
2.2-AA	*100 µM AA*

Vehicle	1-LPC18:0	2-LPC18:0
3-LPC18:0	4-LPC18:0	5-LPC18:0

Vehicle	*0.01% DMSO*
1-LPC18:0	*5 µM LPC 18:0*
2-LPC18:0	*10 µM LPC 18:0*
3LPC18:0	*25 µM LPC 18:0*
4LPC18:0	*50 µM LPC 18:0*
5LPC18:0	*100 µM LPC 18:0*

Vehicle	1-LPC16:0	2-LPC16:0
3-LPC16:0	4-LPC16:0	5-LPC16:0

Vehicle	*0.01% DMSO*
1-LPC16:0	*5 µM LPC 16:0*
2-LPC16:0	*10 µM LPC 16:0*
3LPC16:0	*25 µM LPC 16:0*
4LPC16:0	*50 µM LPC 16:0*
5LPC16:0	*100 µM LPC 16:0*

Figure 3. Six-Well Plate Set Up for Treatment

RNA was harvested from the cells using the Qiagen RNeasy Mini Kit and Protocol. The RNA samples were then quantified using a spectrophotometer. Results were obtained from duplicates of each RNA sample. If the average 260/280 ratio for each RNA sample was within the range of 1.9 and 2.1, the samples were prepared for cDNA synthesis. If not, the experiment was then repeated with a fresh batch of INS-1 cells. Next, in preparation for Real Time-PCR, first-strand cDNA was generated. A 96-well RT-PCR plate was set up in quadruplets. The first set of the wells were used to determine mRNA expression of 18S, a house-keeping gene which serves as a control. The remaining set of wells was used to determine mRNA expression of NSMase.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	A	A	B	B	B	B	C	C	C	C
B	D	D	D	D	E	E	E	E	F	F	F	F
C	C1 through D12 are empty wells.											
D												
E	A	A	A	A	B	B	B	B	C	C	C	C
F	D	D	D	D	E	E	E	E	F	F	F	F
G	G1 through H12 are empty wells.											
H												

Legend	
18 S mRNA expression	
NSMase mRNA expression	
A	*Vehicle (DMSO)*
B	*Vehicle (DMSO)*
C	*5 μM AA*
D	*5 μM AA*
E	*100 μM AA*
F	*100 μM AA*

	1	2	3	4	5	6	7	8	9	10	11	12
A	16A	16A	16A	16A	16B	16B	16B	16B	16C	16C	16C	16C
B	16D	16D	16D	16D	16E	16E	16E	16E	16F	16F	16F	16F
C	18A	18A	18A	18A	18B	18B	18B	18B	18C	18C	18C	18C
D	18D	18D	18D	18D	18E	18E	18E	18E	18F	18F	18F	18F
E	16A	16A	16A	16A	16B	16B	16B	16B	16C	16C	16C	16C
F	16D	16D	16D	16D	16E	16E	16E	16E	16F	16F	16F	16F
G	18A	18A	18A	18A	18B	18B	18B	18B	18C	18C	18C	18C
H	18D	18D	18D	18D	18E	18E	18E	18E	18F	18F	18F	18F

Legend	
18 S mRNA expression	
NSMase mRNA expression	
16A	*Vehicle (DMSO)*
16B	*5 μM LPC 16:0*
16C	*10 μM LPC 16:0*
16D	*25 μM LPC 16:0*
16E	*50 μM LPC 16:0*
16F	*100 μM LPC 16:0*
18A	*Vehicle (DMSO)*
18B	*5 μM LPC 18:0*
18C	*10 μM LPC 18:0*
18D	*25 μM LPC 18:0*
18E	*50 μM LPC 18:0*
18F	*100 μM LPC 18:0*

Figure 4. 96-Well Plate Set Up for RT-PCR

Results

Figure 5 portrays the curve of the data collected and analyzed. Real-time Polymerase Chain Reaction (RT-PCR) allows for the detection of PCR amplification during the early phases of a reaction and collects data as the reaction is proceeding. By doing so, RT-PCR allows for easier and more precise quantitation of DNA and RNA. As can be seen in Figure 5, a PCR curve can be broken up into three phases. During the first phase, an exact doubling of product is accumulating at every cycle. At the second phase, the reaction components are consumed and the reaction begins to slow down as products start to degrade. In the last phase, the reaction has stopped and no more products are being made.

As revealed in Figure 6A, NSMase message expression increased as the concentration of arachidonic acid was increased. Additionally, although there was minimal expression observed with concentrations below 10 μM, higher concentrations of LPC 18:0 correlated with increased levels of NSMase expression (figure 6B). However, as the concentration of LPC 18:0 was increased from 10 μM towards 100 μM, NSMase expression decreased. It is suspected that after a certain point, high concentrations of LPC 18:0 have a toxic effect on NSMase. To further elucidate the correlation between the concentration of LPC 18:0 and the mRNA expression of NSMase, future experiments are likely to be focused on concentrations between 10 μM and 25μM. Figure 6B also indicates that lysophosphatidylcholine 16:0 does not appear to have an effect on NSMase expression. Since the activation of iPLA2β leads to release of the fatty acid arachidonic acid and a lysolipid such as LPC, our experiments reveal that a relationship exists between iPLA2β activation and NSMase message expression. Figure 6 portrays a validation of the data collected and analyzed in Figure 5.

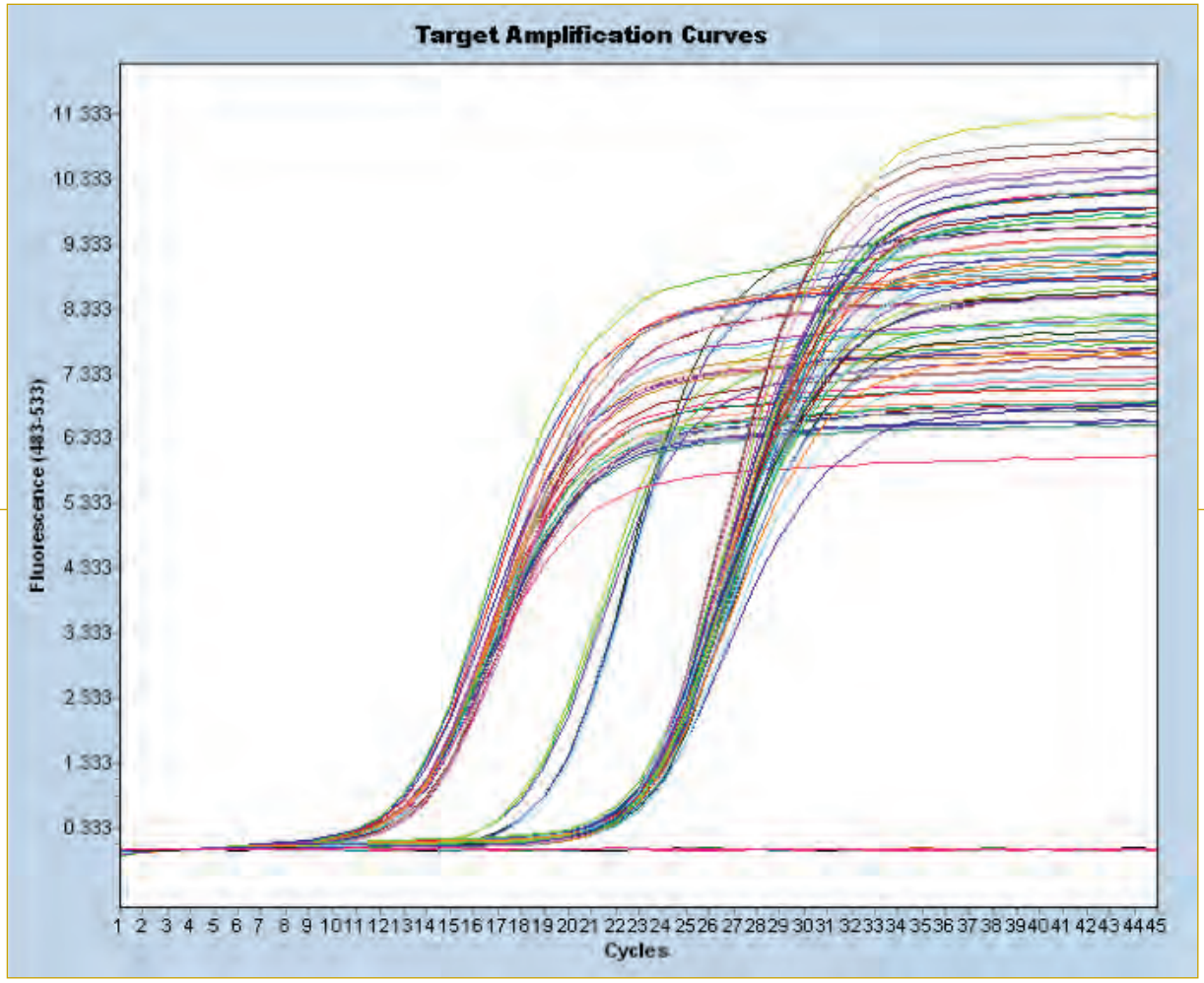

Figure 5. PCR Target Amplification Curves

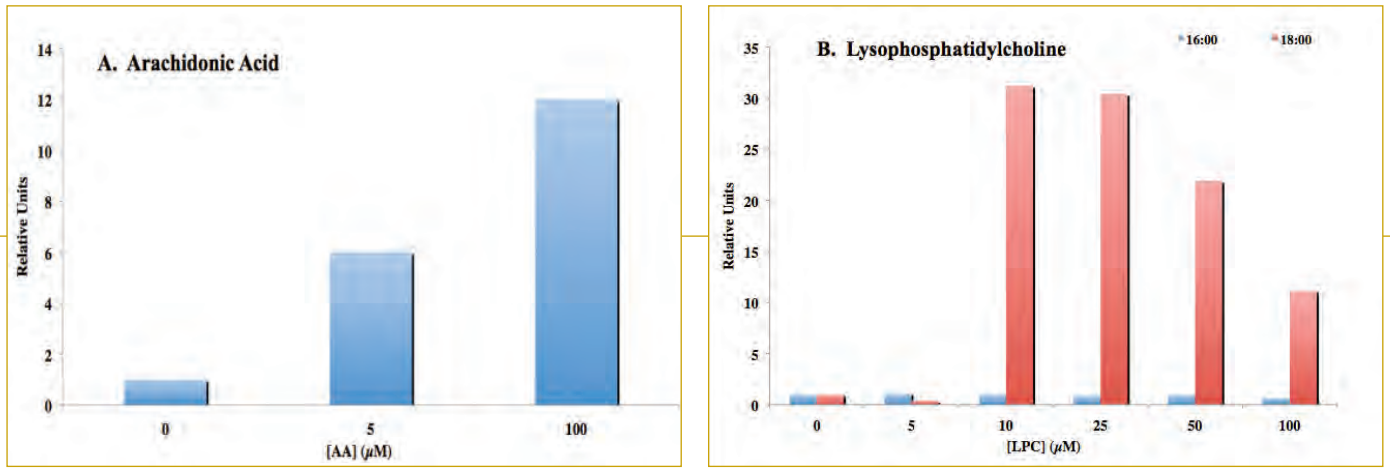

Figure 6. Effects of AA and LPC on NSMase Message Expression ß-Cells

Conclusion

The study shows that arachidonic acid and lysophosphatidylcholine induce NSMase expression. Interestingly, LPC 18:0 induced NSMase whereas LPC 16:0 did not. Thus, it appears that ceramide generation via NSMase-induced hydrolysis of SMs is facilitated by certain, but not all, products of iPLA2β activation in beta-cells. Further studies are needed to confirm these findings and establish statistical significance.

Acknowledgements

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