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The Role of Saturated Fatty Acids in Connexin Expression on Endothelial Cells and Eosinophils

Kurt Anthony Ashack

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Health Science

Department of Biomedical Sciences

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Abstract

Eotaxin is a potent chemoattractant for eosinophils that becomes elevated in the tissues in response to inflammation and increased adiposity. Therefore, eosinophil migration into adipose tissue should increase in diet-induced obese individuals, yet it does not. This suggested to us that something within the cellular microenvironment of diet-induced obese individuals might be altering the endothelial leukocyte adhesion molecules (ELAM) such as VCAM-1 or selectins on the endothelial cells, thus preventing eosinophils from entering the inflamed tissue. Connexons are gap junctions in endothelial cells and on immune cells such as macrophages that are composed of connexins and have been shown to alter ELAM expression. In this study, we investigated whether connexin 43 expression can be modulated by saturated or unsaturated fatty acids. We found that the saturated fatty acids, palmitic and lauric acid, increased expression of connexin 43 in bEnd.3 endothelial cells over a 24-hour period at concentrations ranging from 100μM to 400μM. Conversely, linoleic acid, an unsaturated fatty acid, decreased the expression of connexin 43 over a 24-hour period at concentrations from 400μM to 800μM. Connexins 37 and 40 were also examined but were not detected under the conditions used. These results suggest that the concentration and type of fatty acids in the blood can significantly impact connexin expression by vascular endothelial cells and the subsequent regulation of adhesion molecule expression necessary for eosinophil migration into inflamed tissues.

Table of Contents

Abstract iii

List of Tables vi

List of Figures vii

Manuscript

I. Introduction 1

II. Methods 4

III. Results 8

IV. Discussion 17

V. References 21

Extended Literature Review

I. Diet-Induced Obesity and Type 2 Diabetes Mellitus

Introduction 24
Toll-Like Receptor 4 and its Associated Ligands 25
Saturated Fatty Acid Levels 25
The Insulin Response 26

II. The Macrophage

M1 vs. M2 Macrophages and the Inflammatory Response 28
The M2 Macrophage Response 30

III. Eosinophils & Endothelial Cells

The Eosinophil, Eosinophil Migration and Connexins 33
Eosinophil Transmigration 34
Influences on Integrin Expression or Binding 40
Modifiers of Eosinophil Transmigration 41
Gap Junction Proteins 42
Endothelial Cells and PRRs 47
Endothelial Cell Response to Fatty Acids and Connexin Expression 49
Poly-Unsaturated Fatty Acids: Omega-3 vs. Omega-6 50

IV. Conclusion 51
V. References 53

List of Tables

Manuscript

I. Results

Table 1. Western blot band quantitation adjusted for differences in background intensities 14

List of Figures

Manuscript

I. Results

Figure 1. LPS Increases Cx43 expression in bEnd.3 endothelial cells 9

Figure 2. Low concentration (100μM) of palmitic acid increases Cx43 expression by 4 hours and decreases expression by 24 hours in bEnd.3 endothelial cells 10

Figure 3. High concentrations (400μM) but not low concentrations (100μM & 200μM) of palmitic acid increase Cx43 expression in bEnd.3 endothelial cells within a 24-hour period 11

Figure 4. Increasing concentrations (100-400μM) of lauric acid increase Cx43 expression in bEnd.3 endothelial cells within a 24-hour period 12

Figure 5a. High concentration (400μM) but not low concentrations (100μM & 200μM) of linoleic acid decrease Cx43 expression in bEnd.3 endothelial cells within a 24-hour period 13

Figure 5b. Increasing concentrations (400μM to 800μM) of linoleic acid decrease Cx43 expression in bEnd.3 endothelial cells within a 24-hour period 14

Figure 6. A) 7 day butyric acid differentiated HL-60 Clone 15 cell into an eosinophil, H&E Stain. B) Differentiated, Luxol-Fast-Blue stain C) Non-differentiated HL-60 Clone 15 cell, Luxol-Fast-Blue stain 16

Extended Literature Review

I. Diet-Induced Obesity and Type 2 Diabetes Mellitus

Figure 1. Intracellular mechanisms of inflammatory insulin resistance 28

II. The Macrophage

Figure 2. The M1 macrophage is activated by Pathogen Associated Molecular Patterns (PAMPs) such as LPS and SFA and by IFN-γ 33

III. Eosinophils & Endothelial Cells

Figure 3. Activation of β2 integrin receptors like LFA-1 and MAC-1 on eosinophils leads to the activation of two different intracellular pathways 37

Figure 4. Eosinophil recruitment and transmigration across the endothelial barrier 39

Figure 5. The effects of the anti-inflammatory and pro-inflammatory states on the expression of CD73 and Cx37 on endothelial cells and macrophages resulting in enhanced or reduced adhesion and thus migration across the endothelial barrier 46

Introduction

The chronic low-grade inflammation that defines obesity (1) results from a dominance of M1 macrophages resident in the adipose tissue. These macrophages are stimulated to produce inflammatory cytokines through the binding of lipopolysaccharide or saturated fatty acids (SFA) to the TLR-4 receptors which they express. Thus, the high fat diet of the obese individual continuously favors the differentiation of these tissue macrophages to the pro-inflammatory M1 phenotype (1-9). Inflammatory cytokines and diet-induced obesity both result in increased eotaxin secretion (4) by adipocytes and immune cells to attract eosinophils to the adipose tissue. The eosinophils, in turn, would produce IL-4 and IL-13 to shift the macrophage differentiation to the anti-inflammatory M2 phenotype in order to regain homeostasis. It has been observed, however, that despite the increased eotaxin production in the obese individual, the eosinophil numbers are reduced in adipose tissue when high levels of SFA and inflammatory cytokines are present. This suggests that the reduced eosinophil numbers in adipose tissue (10, 11) may be due to the cells' inability to migrate across the endothelial barrier into the tissue, quite possibly due to alterations in the adhesion molecules expressed in the presence of the inflammatory cytokines and SFA (10-14).

Connexins are gap junction proteins expressed in the vascular endothelium that are able to regulate the expression of endothelial cell adhesion molecules. While connexins have received considerable attention because of their role in intercellular communication and cardiovascular health and disease, more recent studies have revealed

their importance in tissue inflammation and repair as well. There are over 20 members in the connexin family of proteins. These proteins are defined by their ability to form hemi-channels in the plasma membrane to dock with their counterparts on neighboring cells, forming gap junctions for the propagation of electrical signals and the diffusion of ions and second messengers. (12-14). Three connexin family members are relevant to the expression of adhesion molecules that are used for leukocyte migration. Connexins 40 and 43 are capable of directly altering surface adhesion molecule expression, whereas Cx37 is located on smooth muscle cells and on macrophages and indirectly influences adhesion molecules expression through the release of ATP onto the endothelial cell surface.

Because vascular endothelial cells express receptors for inflammatory cytokines and TLR-4 receptors specific for LPS and SFA, it is possible that the cells respond to these ligands by altering connexin expression which in turn modifies adhesion molecule expression. The selectins as well as the integrins, vascular cell adhesion molecule 1 and intracellular adhesion molecule 1(VCAM-1 and ICAM-1), are responsive to changes in connexins and these adhesion molecules would impact the efficiency of eosinophil transmigration across the endothelial barrier (12-14). Previous studies have shown that Cx40 increases VCAM-1 and P-selectin expression and, while these molecules are necessary for recruitment and initial attachment of the eosinophils to the endothelial surface, strong binding would prevent the eosinophils from releasing VCAM-1 at the luminal surface for subsequent binding to ICAM-1; ICAM-1 is located on the membrane between adjacent cells and is critical for adhesion during transmigration into the inflamed adipose tissue.

The aim of this study is to determine whether saturated or unsaturated fatty acids can alter the expression of connexins on endothelial cells. We would predict that SFA binding to the TLR-4 receptor would increase expression of Cx43 while decreasing Cx40 expression on endothelial cells. This would induce strong expression of P-selectin and VCAM-1 on the luminal surface of the endothelial layer and impede the eosinophil transmigration. Conversely, UFA would increase Cx40 expression while decreasing Cx43 expression on the endothelial cells. This would be expected to reduce the levels of VCAM-1 expression such that eosinophil transmigration across the endothelial barrier would be enhanced. A second goal of this study is to determine whether eosinophils express Cx37 or Cx43, as do macrophages and neutrophils. Based on its function in macrophages, Cx37 expression by eosinophils would be expected to negatively influence surface adhesion molecule expression on the endothelial barrier and thus prevent their migration into the inflamed adipose tissue (13, 14).

Methods

Materials

Pre-stained, low molecular weight markers were purchased from BioRad (Hercules, CA). Primary antibodies were purchased as follows: Connexin 43 from Sigma Aldrich (St. Louis, MO), Connexin 40 from Chemicon (Billerica, MA) and Connexin 37 from Alpha Diagnostics International Inc. (San Antonio, Texas). Hematoxylin, glass slides and cover slips as well as materials for H&E staining were kindly provided by the Dermatology Associates of West Michigan.

Cell Culture

Endothelial cells: bEnd.3 endothelial cells were kindly provided by Dr. David Kurjiaka (Grand Valley State University, Allendale, MI). Cells were grown at 37°C with 10% CO_2 in high glucose DMEM (HyClone) containing 10% FBS, penicillin and streptomycin (700μg/ml penicillin and 700μg/ml streptomycin) in 100mm plates. The media was renewed every 2-5 days until the cells reached 80% confluence or greater.

Eosinophils: The HL-60 Clone 15-cell line was purchased from ATCC (American Tissue Culture Collection, Manassas, VA). The cells were maintained in RPMI 1640 with 10% FBS (ATCC) and 50μg/ml gentamycin (Sigma). The cells were induced to differentiate into eosinophils by seeding them at $2x10^5$ cells/mL in medium containing 0.5mM butyric acid (Sigma). Cell culture was continued for 7 days without changing the medium. Cell viability was assessed by 0.2% trypan blue dye exclusion. To assess eosinophil differentiation, cells were stained with Luxol-Fast-Blue stain (Sigma), a dye that specifically stains mouse eosinophil granules (15, 16). An H&E stain was also

performed to verify the differentiation of the cells into eosinophils (courtesy of Dermatology Associates of West Michigan). Pictures were taken to demonstrate cell differentiation into eosinophils. (See Figure 6).

Protein Sample Preparation

Endothelial Cells: 100mm plate cultures of confluent bEnd.3 endothelial cells were placed on ice and washed 3 times with 5mL of cold phosphate buffered saline solution (PBS). The cells were then scraped into a 1mL volume of PBS (Corning Costar 3008). The scraped contents were then transferred to a microcentrifuge tube and centrifuged at 7000xg for 5 minutes at 4°C. The supernatant (1X PBS) was removed and the cell pellet (whole cells) was resuspended in 50-100µL of 1X Laemmli sample buffer on ice. The mixture was sonicated twice at intensity 10 (Microson XL2000) for 15 seconds and then placed back on ice. Next, the samples were spun down for 10 minutes at 14,000xg in a 4°C centrifuge. The supernatant was transferred to a microcentrifuge tube and stored at -20°C until used.

Eosinophils: HL-60 Clone 15 cells, either differentiated or undifferentiated, were centrifuged at 1200 RPM (about 800xg) for 8 minutes at room temperature. The supernatant was removed and the cells were washed three times in 5mL of cold PBS. After the final wash, the protein samples were prepared as described above for the bEnd.3 cells.

BCA assay of protein concentration

Protein concentration was measured using a standard BCA assay kit (Pierce), sample buffer (1X Laemmli sample buffer), and 1X PBS. 10µL of each sample was used to

determine the concentration of protein per sample. Bovine serum albumin was used to generate a standard curve.

Gel Electrophoresis

A 10% SDS PAGE gel was prepared and 30-40μg of protein was loaded per lane Gels were run overnight at 6-8mA per gel. Proteins were then transferred to PVDF membranes and stored at 4°C until used for Western blotting.

Western blot

Membranes were blocked in 5% non-fat dry milk (Carnation) in Tris-buffered saline-Tween (TBST) for 30 minutes. A calculated dilution (1:2000) of primary antibody was added directly into the milk solution and the membrane allowed to rock for 2 hours at room temperature or overnight at 4°C. The membranes were then washed with TBST 5 times, rocking each wash for 5 minutes each. Next, a goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) was diluted 1:10,000 in 5% milk-TBST and incubated on the blot for 90 minutes at room temperature. Membranes were then washed again with TBST as described. After the final wash, the chemiluminescent substrate (Pierce SuperSignal West Pico) was added to each membrane and rocked for approximately one minute to ensure that it covered the entire membrane. Membranes were then wrapped in Saran Wrap and exposed to X-ray film (Midwest Scientific) for both 10-15 minutes and overnight.

Cell stimulation

LPS was used at 10μg/ml to stimulate the cells for the time indicated. 10% BSA was created by dissolving 1 gram of low endotoxin BSA into 10mL of 1X PBS. The mixture was gently warmed and vortexed until the BSA completely dissolved into solution. The

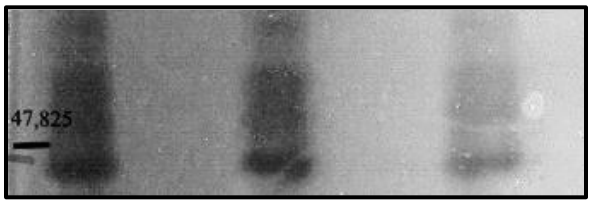
final mixture was then sterile-filtered and stored in the refrigerator until used. Saturated fatty acids (SFA): 30mM stock solutions of SFA (palmitic, lauric and/or linoleic acid) in ethanol were prepared and stored at -20°C. Stock solutions were diluted to 5mM-30mM working solutions by adding them in a 1:7 volume ratio to 10% fatty acid-free BSA in PBS. The solutions were incubated for 1-2 hours at 37°C until all precipitations were completely dissolved, then sterile-filtered and stored in the refrigerator. The vehicle control used a mixture of ethanol and BSA alone in place of SFA. On the day of the experiment, a final SFA concentration of 100-800μM was used to stimulate the cells for 0, 4 or 24 hours, based on the reported rat physiological range for serum non-esterified fatty acid levels from 100-200μM under non-fasting conditions to 500-600μM in fasting conditions (17). Equal volumes of control solution were placed into the other plates to ensure a similar environment for cell growth. This was done for the endothelial cells only.

Results

The bEnd.3 endothelial cell line is derived from the cerebral cortex tissue of mouse brain. According to the ATCC, these cells should express the following adhesion molecules: intracellular adhesion molecule 1(ICAM-1), vascular adhesion molecule 1 (VCAM-1) & mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1). E & P-selectin can be induced by TNF-α, LPS or interleukin-1. These cytokines will also increase expression of ICAM-1 on the cells. Based upon these references from ATCC, as well as other studies, we assumed that the endothelial cells contained the necessary receptors (such as TLR-4) to respond with appropriate connexin expression (18, 19). All experiments used bEnd.3 cells that were under passage 30.

Time-dependent effects of LPS on Cx43 expression

Because LPS and SFA are able to both act through the TLR-4 receptor to induce inflammation, we wanted to test this to see if they produced a similar profile of connexin expression. Hypothetically, LPS binding to the TLR-4 on the endothelial cells should up regulate Cx43 expression on endothelial cells. Therefore, we stimulated confluent bEnd.3 endothelial cells with 10 μg/ml of LPS for 4 or 24 hours for comparison with SFA stimulation. We found that over a 24-hour time frame (after adjusting for the background), LPS increased Cx43 expression within 4 hours which then returned to control levels by 24 hours (Table 1). This was 57% higher than the vehicle control (Table 1). Because the background intensity in the Western blots was not uniform throughout, Table 1 provides a quantitation of band intensity, with the background subtracted, for ease of comparison.

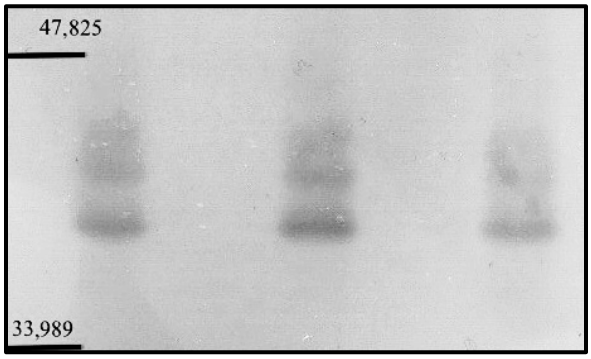


1 2 3

Figure 1: LPS Increases Cx43 expression in bEnd.3 endothelial cells. Confluent bEnd.3 endothelial cells were stimulated with 10μg/ml for 4 hours (lane 2) or 24 hours (lane 3) and the expression of Cx43 was analyzed by Western blot. The control is shown in lane 1.

Time-dependent effects of palmitic acid on Cx43 expression

Palmitic acid (PA), a C16:0 SFA has been shown by others to bind to and activate endothelial cells through the TLR-4 receptor and to influence connexin expression. This in turn regulates expression of endothelial leukocyte adhesion molecules (ELAMs) such as selectins and vascular cell adhesion molecule-1 (VCAM-1). PA has been reported to increase connexin 43 expression as well as ELAMs and its influence is proportionate with incubation time (20, 21). To confirm these reports in the bEnd.3 endothelial cells, a low concentration (100μM) of palmitic acid was used to stimulated the cells for 4 and 24 hours. Connexin 43 expression was then compared between the treated and control cells. As shown in Figure 2, there appeared to be small changes in band intensity during the exposure to the palmitic acid. The 4-hour stimulation has a darker intensity than the control indicating a transient increase in expression followed by a decrease in expression by 24 hours (Figure 2). The data indicates a 34% increase in Cx43 expression after 4 hours of exposure, which decreased to 14% at 24 hours (Table 1). This would suggest that a low concentration of palmitic acid is able to increase the expression of Cx43 by bEnd.3 endothelial cells within a 4-hour period in a manner similar to that seen with LPS.



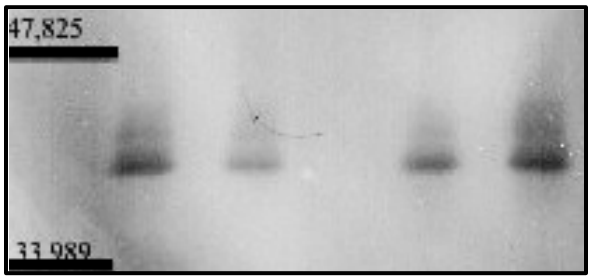
1 2 3

Figure 2: Low concentration (100µM) of palmitic acid increases Cx43 expression by 4 hours and decreases expression by 24 hours in bEnd.3 endothelial cells. Confluent bEnd.3 endothelial cells were stimulated with 100µM of palmitic acid for 4 hours (lane 2) or 24 hours (lane 3) and the expression of Cx43 was analyzed by Western blot. The control is shown in lane 1.

Concentration dependent effects of palmitic acid on Cx43 expression

As mentioned above, palmitic acid has been shown to increase connexin 43 expression as well as ELAMs and its influence is proportionate with incubation time (20, 21). Since we did not see a sustained increase in Cx43 expression using 100µM, we tested the effects of higher concentrations of PA (100 - 400µM) on Cx43 expression in bEnd.3 endothelial cells at 24-hours. The 100µM stimulation for 24 hours had similar Cx43 expression as compared to the control group, consistent with the results in Figure 2. As concentration began to increase from 100µM to 400µM however, the intensity of the band greatly increased (Figure 3). According to the data provided in Table 1, while 100µM and 200µM concentrations decreased Cx43 expression by 40% and 32% respectively compared to the control, the 400µM concentration increased expression by 25% (Table 1). This suggests that a concentration of 400µM of PA for 24 hours is sufficient to elevate Cx43 expression in bEnd.3 endothelial cells. Also noteworthy is that

the phosphorylated forms of Cx43 become stronger than the control after 24 hours of treatment with 400µM PA.



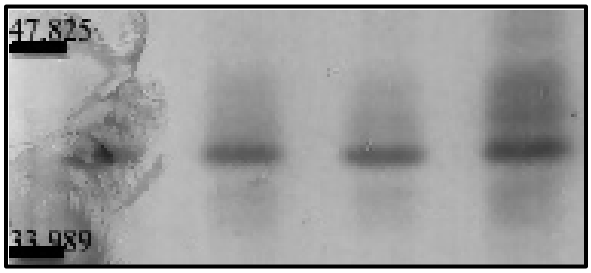
1 2 3 4

Figure 3: High concentrations (400µM) but not low concentrations (100µM & 200µM) of palmitic acid increase Cx43 expression in bEnd.3 endothelial cells within a 24-hour period. Confluent bEnd.3 endothelial cells were stimulated with 100µM (lane 2), 200µM (lane 3) or 400µM (lane 4) of palmitic acid for 24 hours and the expression of Cx43 was analyzed by Western blot. The control is shown in lane 1.

Concentration dependent effects of lauric acid on Cx43 expression

Lauric acid (LA), a C12:0 SFA, has not been investigated in terms of its effects on connexin or ELAM expression, but it has been shown to be a potent activator of TLR-4 receptors in dendritic cells (22). In order to investigate the effect of chain length on the activity of SFA, lauric acid was used to treat the endothelial cell for comparison with palmitic acid. We hypothesized that because lauric acid is a SFA, it should produce similar results to that of palmitic acid if they both engage the TLR-4 receptor. With this in mind, we evaluated the effects of 100 - 400µM lauric acid on Cx43 expression in bEnd.3 endothelial cells for 24-hours. The results indicate that as the concentration of LA increases, the expression of Cx43 steadily increases as well (Figure 4, Table 1). At 400µM LA the expression of Cx43 in the bEnd.3 endothelial cells was 75% higher than the control (Figure 4,Table 1) after 24 hours of treatment. With this information we can

conclude that SFA increases Cx43 expression in endothelial cells regardless of chain length. In both cases, Cx43 was increased after 24 hours of treatment, although there is some suggestion that the shorter chain LA may have more potent effects.



1 2 3 4

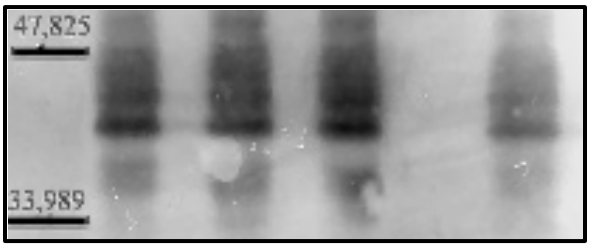
Figure 4: Increasing concentrations (100-400µM) of lauric acid increase Cx43 expression in bEnd.3 endothelial cells within a 24-hour period. Confluent bEnd.3 endothelial cells were stimulated with 100µM (lane 2), 200µM (lane 3) or 400µM (lane 4) of lauric acid for 24 hours and the expression of Cx43 was analyzed by Western blot. The control is shown in lane 1.

Concentration dependent effects of linoleic acid on Cx43 expression

Unsaturated fatty acids are known to bind to the PPARγ nuclear receptor to reduce inflammation by interfering with the de-repression of inflammatory cytokine gene transcription. Since saturated and unsaturated fatty acids are expected to have opposite effects on connexin 40 and 43 expression, we evaluated the effects of various concentrations of linoleic acid (LnA) on Cx43 expression in bEnd.3 endothelial cells. Because LnA is an UFA, we hypothesized that it would act like other UFAs such as the omega-3 alpha-linolenic acid and reduce Cx43 expression in a non-time dependent manner (20, 23, 24).

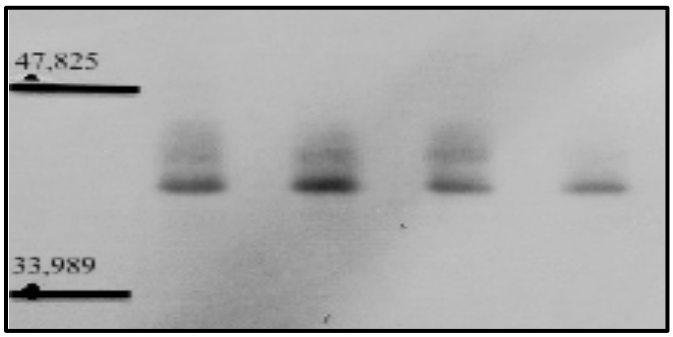
According to Figure 5a and Table 1 combined, it can be seen that the 100µM stimulation over 24 hours resulted in an increase in Cx43 expression (30%). Similarly,

the 200µM stimulation resulted in a darker, more intense band while the 400µM stimulation was actually less intense than the control and lower concentrations of LnA (Figure 5a). After adjusting the data for any background differences, we found that at 200µM of LnA that Cx43 expression began to decrease as compared to the 100µM stimulation (17%) but was still 13% higher than the control (Table 1). In contrast, the 400µM stimulation decreased expression of Cx43 by 33% (Table 1). This fairly significant decrease in Cx43 expression at the higher concentration of LnA led us to further explore even higher concentrations of LnA on bEnd.3 endothelial expression of connexins. Figure 5b and Table 1 indicate that the effects of 400µM LnA on Cx43 expression were negligible in this trial; however, 800µM of LnA resulted in a 36% decrease in Cx43 expression as compared to the control. While there is still some variability or inconsistency in the trials conducted, we can generally conclude that stimulation using high concentrations of LnA for 24 hours decreases Cx43 expression in bEnd.3 endothelial cells. This is of interest as the effects of LnA on connexin expression have been controversial (20, 23, 24)



1 2 3 4

Figure 5a: High concentration (400µM) but not low concentrations (100µM & 200µM) of linoleic acid decrease Cx43 expression in bEnd.3 endothelial cells within a 24-hour period. Confluent bEnd.3 endothelial cells were stimulated with 100µM (lane 2), 200µM (lane 3) or 400µM (lane 4) linoleic acid for 24 hours and the expression of Cx43 was analyzed by Western blot. The control is shown in lane 1.



1 2 3 4

Figure 5b: Increasing concentrations (400µM to 800µM) of linoleic acid decrease Cx43 expression in bEnd.3 endothelial cells within a 24-hour period. Confluent bEnd.3 endothelial cells were stimulated with 400µM (lane 3) or 800µM (lane 4) of linoleic acid for 24 hours and the expression of Cx43 was analyzed by Western blot. The control with regular media is shown in lane 1 and control with ETOH-BSA is shown in lane 2.

	Lane	Time (hrs)	Concentration	Total Intensity	Ratio to Control	Result
	1	Control	0	19723		
A	2	4	LPS 10 µg/ml	31055	1.57	**Increase**
	3	24	LPS 10 µg/ml	18434	0.93	**No change**
	1	Control	0	15495		
B	2	4	100 µM PA	20750	1.34	**Increase**
	3	24	100 µM PA	13264	0.86	**Decrease**
	1	Control	0	15287		
C	2	24	100 µM PA	9366	0.61	**Decrease**
	3	24	200 µM PA	11882	0.78	**Decrease**
	4	24	400 µM PA	19052	1.25	**Increase**
	1	Control	0	10656		
D	2	24	100 µM LA	15797	1.48	**Increase**
	3	24	200 µM LA	16292	1.53	**Increase**
	4	24	400 µM LA	18636	1.75	**Increase**
	1	Control	0	15287		
E	2	24	100 µM LnA	19842	1.30	**Increase**
	3	24	200 µM LnA	17208	1.13	**Increase**
	4	24	400 µM LnA	11845	0.77	**Decrease**
	1	Control	0	11793		
F	2	Vehicle	ETOH BSA	12918	1.10	**No change**
	3	24	400 µM LnA	11320	0.96	**No change**
	4	24	800 µM LnA	7505	0.64	**Decrease**

Table 1: Western blot band quantitation adjusted for differences in background intensities. A-F corresponds with the sequence in which the results are presented above. (Abbreviations: PA = palmitic acid, LA = lauric acid, LnA = linoleic acid)

Expression of connexins on eosinophils

Immune cells such as macrophages have been shown to express connexins, including Cx37 (12, 14). The expression of this specific connexin in an inflammatory environment allows the macrophage to release ATP, which has downstream effects of up-regulating ELAM expression on endothelial cells (12, 14). Because eosinophils have some similarities to macrophages in terms of migration, it is possible that eosinophils also express connexins to modify endothelial expression of ELAMs. This might explain why eosinophils are unable to migrate into inflamed adipose tissues.

HL-60 Clone 15 leukemic cells have been shown in the literature to serve as an *in vitro* model of eosinophils. These cells can be differentiated to eosinophils by treatment for seven days with butyric acid (15). Using this protocol, the differentiated cells were analyzed by Western blot for the expression of Cx37 and Cx43, the two connexins known to be involved with release of ATP onto the endothelial surface. The control, which consisted of a non-differentiated HL-60 Clone 15 leukemic cell produced the same results. This led us to conclude that eosinophils differentiated from the HL-60 Clone 15 cell line do not express connexins. Confirmation that the cell line differentiated into eosinophils was determined by staining with Luxol-Fast-Blue stain and by H&E staining. (Figure 6).

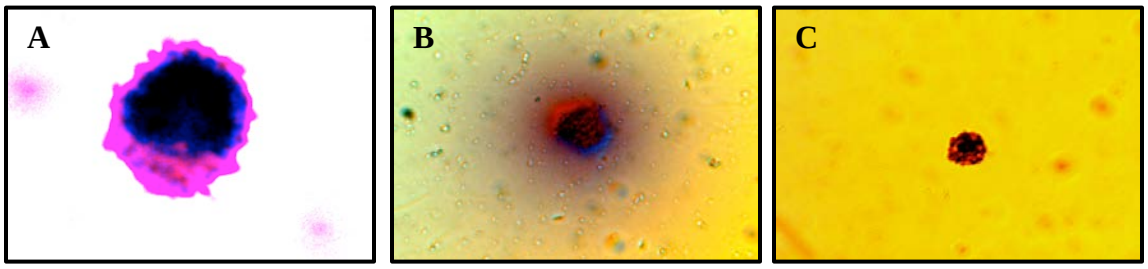


Figure 6: A) 7 day butyric acid differentiated HL-60 Clone 15 cell into an eosinophil, H&E Stain. B) Differentiated, Luxol-Fast-Blue stain C) Non-differentiated HL-60 Clone 15 cell, Luxol-Fast-Blue stain

Expression of Cx40 and Cx37 by bEnd.3 endothelial cells

The expression of connexins 40 and 37 were investigated in bEnd.3 endothelial cells in a similar fashion to Cx43. Unfortunately, we were unable to identify any expression of either connexin with unstimulated cells. Cx40 expression was continually evaluated in each experiment along with Cx43 but we failed to find any indication of Cx40 expression.

Discussion

In this study the effects of SFA and UFA on the expression of endothelial connexin 43 were investigated as a potential means to alter eosinophil transendothelial migration. We also looked at Cx37 and 40 expression but were unable to detect these proteins. We predicted that LPS as well as any chain length of SFA would increase Cx43 expression and thereby promote eosinophil transmigration since these are all ligands for TLR-4. Conversely, we expected linoleic acid, an UFA, to decrease Cx43 expression and therefore prevent eosinophil migration across the endothelial barrier.

The results support the hypothesis in that both long (PA) and short chain (LA) SFA were able to increase the expression of Cx43 in bEnd.3 endothelial cells. Increasing LA concentrations resulted in increasing Cx43 expression while PA only increased expression at higher concentrations (400μM) (Table 1). This concentration of palmitic acid represents the high end of the physiological range for rodents (100μM to 600μM) and is much higher than the normal, nontoxic blood concentration of free fatty acids in humans (10-30μM) (17, 20). However, any concentration of PA below 400μM resulted in a decrease in expression of Cx43 (Table 1). We also found that higher concentrations (400-800μM) of a polyunsaturated fatty acid (linoleic acid) led to a decrease in Cx43 expression in bEnd.3 endothelial cells. Some other findings included an increase in Cx43 expression in response to LPS and also that differentiated HL-60 Clone 15 eosinophils do not express connexins 37 or 43.

This data provides evidence to suggest that fatty acids might be involved in modulating the expression of adhesion molecules on the endothelial surface in diet-induced obese individuals. Excess SFA in the diet-induced obese individual could

increase connexin 43, leading to endothelial leukocyte adhesion molecule (ELAM) expression (VCAM-1 and selectins) that would prevent eosinophils from migrating into the inflamed tissue. A simple change in diet, incorporating more UFA such as omega 3 and omega 6 fatty acids, could reverse this change by decreasing connexin 43 expression. Eosinophils would then attach to the endothelium and extravasate due to a higher expression of ICAM-1 than VCAM-1. Also, the fact that eosinophils do not appear to express connexins supports the idea that fatty acids might directly modulate ELAMs on the endothelium without contribution by the eosinophils themselves.

Our results are in agreement with the work of others. For example, it has been found that SFA are associated with increased ELAM expression on endothelial cells as well as with increased connexin 43. While lauric acid has not been previously studied, our results with palmitic acid are in agreement with other studies (20, 21, 26). This data indicates that SFA of different chain lengths is able to act on endothelial cells through the appropriate receptor and modulate their state of activation by increasing expression of Cx43 (Figures 3 & 4). This would be associated with either a loss of inhibition or a promotion of expression of endothelial leukocyte adhesion molecules such as VCAM-1 or various selectins (21). Hypothetically, eosinophils would still be able to attach to the activated endothelium but expression of VCAM-1 might override that of ICAM-1, thus, preventing the eosinophil from detaching to migrate across the endothelial barrier (21, 27, 28). This is because VCAM-1 is required for initial attachment of the eosinophil to the endothelium and for its activation. However, a subsequent conformational change and decrease in VCAM-1 affinity is necessary while simultaneously increasing affinity for ICAM-1. The increased binding of eosinophils to ICAM-1 allows the cells to undergo

diapedesis and cross the endothelial barrier. Without being able to cross the endothelium, the eosinophils would not be available to secrete the cytokines needed to maintain the M2 macrophage population, thus favoring the default activation of monocytes and macrophages towards the M1 macrophage state due to the molecular environment within the adipose tissue (6, 7).

The effects of UFA, specifically omega 6 fatty acids such as linoleic acid or arachidonic acid, on ELAMs such as VCAM-1 and ICAM-1 are controversial. There are some reports that have shown no effect while others have shown an increase or decrease in adhesion molecule expression (14, 20, 21, 23). Our data indicates that linoleic acid decreases Cx43 expression in bEnd.3 endothelial cells (Figure 5). A decrease in Cx43 expression would result in a subsequent decrease in expression of VCAM-1 and endothelial-expressed selectins, with ICAM-1 expression remaining relatively constant (21, 27, 28). Although VCAM-1 would be decreased, it is possible that there would still be enough expression for eosinophils to attach to the endothelium. With a higher ratio of ICAM-1 to VCAM-1, the eosinophils would be able to easily release VCAM-1 and bind to ICAM-1, allowing them to migrate across the endothelial barrier and enter the adipose tissue.

If the eosinophil is able to migrate into adipose tissue then it should be able to produce the necessary cytokines to maintain the M2 population within the specific tissue (28). Maintenance of M2 macrophages would result in a healthy balance of anti-inflammatory molecules and prevent complications such as insulin resistance and adipocyte death from occurring (6, 7).

Areas of improvement for this study would include the use of a positive control for Cx43 expression to further validate the Cx43 Western blots. Additional experiments to directly detect the cellular adhesion molecules expressed in response to SFA and UFA stimulations would confirm our assumption that modulation of connexin affects ELAM expression. Other avenues of interest to strengthen the study would be to investigate the effects of an omega-3 fatty acid on connexin expression as these are proven to be the most beneficial fatty acids and have anti-inflammatory properties as compared to omega-6 fatty acids (20, 23, 24). Finally, combinations of SFA with LPS, UFA with LPS and SFA with UFA might contribute additional useful information, as would the use of additional concentrations of fatty acids and longer stimulations.

Although the HL-60 Clone 15 cell line has been shown by others to differentiate into eosinophils using the treatments used in this study, our efforts to confirm this differentiation were disappointing. This causes us to proceed rather cautiously with any conclusions about connexin expression and function in eosinophils. It would be desirable to repeat the experiments with primary human eosinophils or with a human eosinophil cell line.

To conclude, connexin expression can in fact be altered by fatty acids. SFA such as palmitic and lauric acid serve to increase connexin 43 expression while UFA like linoleic acid appear to decrease Cx43 expression at higher concentrations similar to those of a diet-induced obese individual. With further investigation, manipulation of connexin expression may be a key strategy for altering endothelial cell activation and leukocyte adhesion to combat pathologies associated with diet-induced obesity.

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I. Diet-Induced Obesity and Type 2 Diabetes Mellitus

Introduction

At least 2.8 million adults worldwide die each year as a result of being overweight or obese. In addition, obesity contributes to 44% of those with diabetes, 23% of those with ischemic heart disease and 7% to 41% those with cancer (1). With the doubling of the rates of obesity worldwide within 3 decades (1), the overall health problems associated with obesity can be viewed as life threatening. For example, obesity is a known risk factor for the development of diabetes. In fact, obesity is strongly associated with type-2 diabetes (2). This is of interest because diabetes, in turn, can also have serious health implications aside from obesity. Examples of health problems related to diabetes are increased risk of blindness, heart attacks, strokes and kidney failure. Diabetics also have twice the risk of death as compared to non-diabetics. Thus, diabetes poses many threats to one's health.

The link between type-2 diabetes and obesity is reflected in the updated definition of obesity as a state of chronic low-grade inflammation (3). This inflammation is a result of the difference in diets between individuals: a diet rich in saturated fatty acids (SFA) enhances inflammation whereas a diet rich in unsaturated fatty acids (UFA) reduces inflammation (3, 4, 5).

Toll-Like Receptor 4 and its Associated Ligands

When an individual consumes a diet rich in saturated fat, the result is an increase in circulating free SFAs. These free SFA activate Toll-like Receptor 4 (TLR-4) receptors on macrophages (6, 7, 8) similar to the lipopolysaccharides (LPS) that are released by invading bacteria. SFAs are structurally similar to LPS and therefore are both bound by the pattern recognition receptor (7) TLR-4. The binding of either ligand to the TLR-4 receptor causes the secretion of inflammatory cytokines (6, 7).

Saturated Fatty Acid Levels

There are three reasons for the increase in free SFA concentration when consuming a high fat diet. The first is simply the naturally associated increase in levels of saturated fat within the body when consistently consuming a diet high in saturated fat. For example, normal, nontoxic, physiological blood concentrations of free fatty acids average from 10-30 μM; therefore, concentrations greatly exceeding this value would be considered unhealthy (9). When these concentrations of SFA are high in a cell, it can enhance their release in 2 ways: due to endoplasmic reticulum stress response and adipose tissue hyperplasia (7).

Excess glucose and fatty acids (FA) are usually stored as triacylglycerides for later use, leading to their accumulation in adipose tissue. When an individual continues to consume excess calories, the “storage capacity” for triacylglycerides within adipocytes can be exceeded. The accumulation of fatty acid within cells increases their binding to fatty acid-binding protein (FABP/AP2). This FABP/AP2 alters the endoplasmic reticulum (the endoplasmic reticular stress) initiating an Unfolded Protein Response

(UPR). The UPR can also be stimulated by excess nutrients and microhypoxia that results when nutrient and oxygen supply become insufficient for the hyperplastic tissue (6, 7, 10). Initiating a UPR activates the transcription factor nuclear factor –kappa B (NF-kB), which increases the production of inflammatory cytokines by the adipocytes (6, 7, 10). At the same time, the insulin-signaling pathway is inhibited through the serine phosphorylation of the Insulin Receptor Substrate 1 (IRS-1: discussed later). Further, activation of the UPR will cause the adipocyte to undergo apoptosis (programmed cell death). However, prior to apoptosis, the adipocyte will attract macrophages through the release cytokines like monocyte chemotactic protein 1 (MCP-1) due to both the activation of the UPR as well as nutrient overload that exceeds the rate at which the adipocyte can dispose of the fatty acids and results in accumulation of fatty acids within the tissue (11, 12).

In addition to the ER stress response, the accumulation of adipose tissue begins to exceed the capacity of the vascular supply to provide oxygenation. When the adipose tissue becomes hypoxic, necrosis (cell death) of the adipose tissue results, leading to outcomes similar to those associated with the ER stress response (6, 7, 10).

The Insulin Response

In a lean individual, the insulin response is initiated by insulin binding to its receptor, a tyrosine kinase, on the surface of the adipocyte, or any other cell/tissue, which activates intracellular substrates, referred to as insulin receptor substrate-1 and 2 and the mitogen-activated protein kinase (MAPK) cascades. These substrates are phosphorylated and activate phosphatidylinositol 3-kinase (PI3K). PI3K activation leads to the phosphorylation and activation of the protein kinase B (PKB)/Akt which translocate the

glucose transporter 4 (GLUT4) to the plasma membrane of the adipocyte. This allows for the uptake of glucose by the adipocyte and of its conversion to glycogen and fatty acids within the adipocyte (13, 14, 15).

For an individual consuming a diet high in SFAs, the risk of developing a resistance to insulin that defines type 2 diabetes is elevated. This is due to the release of inflammatory cytokines tumor necrosis alpha, interleukin 1 beta and interleukin 6 (TNF-α, IL-1β and IL-6) by M1 macrophages in response to activation of the TLR-4 receptor by LPS or SFA. Once released within adipose tissue, these inflammatory cytokines can interfere with the insulin-signaling cascade. This causes insulin resistance within the resident tissue. The current finding for the development of insulin resistance is that these cytokines, as well as SFA and other inflammatory related molecules, stimulate the phosphorylation of c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor kappa beta kinase (IKKβ: 22). Phosphorylation of these transcription factors contributes to insulin resistance in 2 ways.

First, activation of IKKβ and JNK by inflammatory mediators inhibits the activity of IRS-1: serine phosphorylation of IRS-1 dampens the normal insulin signaling pathway. This prevents activation of PI3K and thereby reduces the resulting translocation of GLUT4 to the plasma membrane of the adipose cell. Second, the production of inflammatory cytokines is elevated (25) (Figure 1). These two outcomes both contribute to insulin resistance and poor glucose metabolism because GLUT4 is not inserted into the membrane and this negatively affects glycogen synthesis.

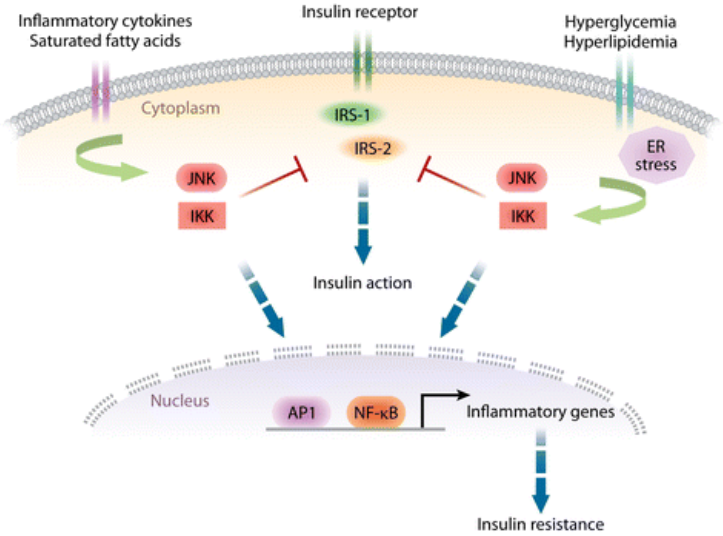

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Fig. 1: Intracellular mechanisms of inflammatory insulin resistance. Insulin action is transduced from the cell surface to cytoplasmic and nuclear responses via tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and -2. However, serine phosphorylation of these same substrates by Jun N-terminal kinase 1 (JNK1) and inhibitor of nuclear factor κB (NF-κB)–kinase β (IKKβ), the central mediators of stress and inflammatory responses, potently inhibits insulin action, thereby directly linking these responses to insulin resistance. In addition, the transcriptional activation of inflammatory genes by JNK1 and IKKβ induces insulin resistance in an autocrine and paracrine manner in tissues. Moreover, in states of obesity, JNK1 and IKKβ signaling pathways are activated by an increased influx of free fatty acids and glucose. Abbreviations: AP1, activator protein 1; ER, endoplasmic reticulum (7).

II. The Macrophage

M1 vs. M2 Macrophages and the Inflammatory Response

When monocytes leave the blood and enter the tissues, they differentiate into a macrophage. When this differentiation occurs, the cytokine environment within the designated tissue will dictate whether the monocyte becomes an M1 or M2 macrophage. This is important as it determines the cytokines the macrophage will secrete upon activation.

For example, when there is a rise in SFA levels, individuals consuming a diet rich in SFA will exhibit two associated changes that occur with the macrophage. The first occurs when the macrophage is activated through the binding of LPS or SFA with the TLR-4 receptor to produce and secrete several inflammatory cytokines. This leads to the polarization of the macrophages to become M1 macrophages that will secrete pro-inflammatory cytokines like TNF- α, IL-6 and IL-1β that are responsible for the development of insulin resistance (2, 3, 7, 16, 17). Therefore, the M1 macrophage can be characterized as a pro-inflammatory macrophage whose abundance correlates with free SFA levels and contributes to insulin resistance (6) (Figure 2).

The second associated outcome is the down regulation of the peroxisome proliferator-activated receptor (PPAR). PPAR is a nuclear membrane receptor expressed by the M2 macrophage whose ligands are UFAs (6, 7). When activated by UFAs they lead to the production of anti-inflammatory cytokines such as IL-10. Recently IL-10 was shown to decrease inflammation and (more importantly) increase insulin sensitivity, although the mechanism of action is unclear (13, 16). PPAR consists of a gamma, alpha and beta/delta form (PPARγ, PPARα, & PPARβ/δ) and these different forms are up-regulated on the macrophage nuclear membrane surface in response to Interleukins-4 and 13 (IL-4 and IL-13) binding to their receptors on the macrophage. These two cytokines are produced by several types of immune cells including eosinophils (6, 16). Without these cytokines, PPAR expression would not increase within the macrophage (7).

In light of the discussion above, UFA and SFA are best viewed as competitors. When concentrations of SFA are higher than UFA, there will be more activated M1 than M2 macrophages within adipose tissue due to the down regulation of the PPARγ receptor

and pro-inflammatory cytokines secretion. This increases the inflammatory response within adipose tissue and ultimately leads to the development of insulin resistance (6, 16). Conversely, if concentrations of UFA are greater than that of SFA, as with lean individuals, there will be more activated M2 than M1 macrophages in adipose tissue due to the binding of UFA to PPARγ in macrophages. This will lead to the down regulation of the inflammatory cytokine genes and promote the production of anti-inflammatory cytokines (Figure 2) (6, 7, 16).

The M2 Macrophage Response

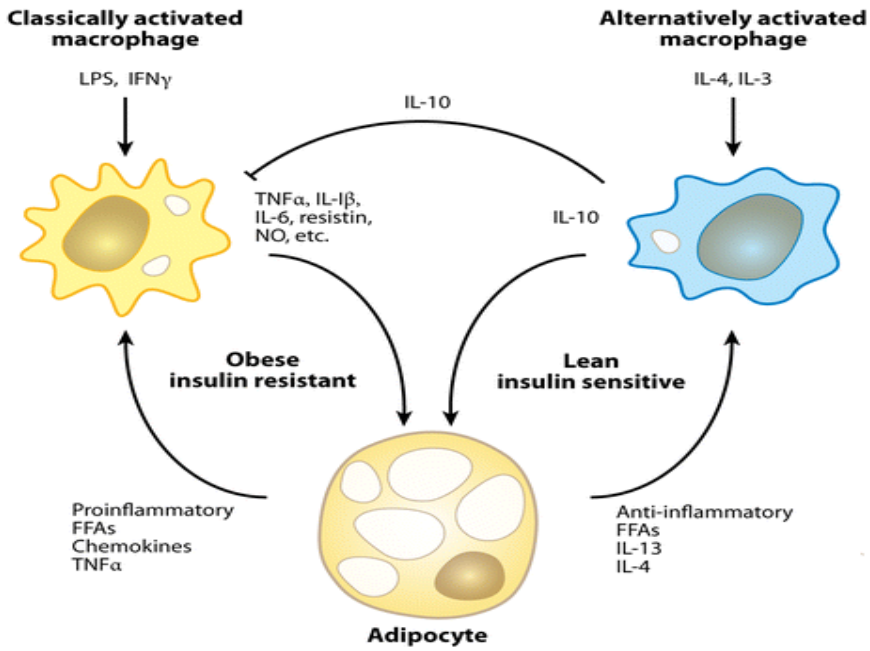
The energy substrate utilized by macrophages is affected by the presence prevailing fatty acids. When the UFA binds to the PPARγ receptor, it redirects the energy metabolism of the M1 macrophage towards a more glycolytic pathway. The M1 macrophage relies on a glycolytic program to rapidly provide the energy and reducing equivalents necessary for intense, short-lived bactericidal activity. However, when UFA engages with the PPARγ receptor, the macrophage's energy program shifts to the more efficient and sustainable fatty acid oxidation and oxidative glucose metabolism program associated with the M2 macrophage. This enhances the oxidative metabolism of the M2 macrophage, facilitating the break down of fat by phagocytosis of free fatty acid and reduction of fatty acid levels within the adipose tissue (6). Therefore, a lean individual consuming a diet rich in UFA will have less fat due to the response created by the ligand-receptor relationship between UFA and PPARγ which would in turn reduce SFA levels in adipose tissue.

The binding of UFA to PPARγ within M2 macrophages in adipose tissue also negatively regulates components of the inflammatory pathway such as NF-kB to reduce the production of inflammatory cytokines. This involves two inflammatory gene suppressors Nuclear Receptor Corepressor (NCoR) and Silencing Mediator of Retinoic acid and Thyroid hormone receptors (SMRT). These suppressors are normally bound to inflammatory cytokine transcription activators and are removed in response to activation of the TLR-4 receptor. When PPARγ is activated by UFAs, a small intracellular portion of the receptor covalently couples with SUMO1, which is a small ubiquitin-like modifier. This leads to the "SUMOylation" of PPARγ which independently associates with the inflammatory gene, hindering the removal of NCoR and SMRT. Because the NCoR/SMRT complexes are not removed from inflammatory cytokine transcription activators, the expression of inflammatory cytokines cannot occur. This would favor the M2 phenotype over M1 macrophages within adipose tissue and allow for an increased number of M2 macrophages due to the higher probability of their activation. Once again, this would lead to the break down of more fat, the production of the insulin-sensitizing interleukin-10 and a decrease in the inflammatory cytokine levels that contribute to insulin resistance (Figure 2) (6).

When UFA concentrations rise in individuals who have a healthier diet, it is important that M2 macrophages express the corresponding PPARγ receptor in order to respond. This PPARγ expression is dependent upon IL-4 and IL-13, which are produced by immune cells such as eosinophils in adipose tissues. These cytokines bind to the corresponding receptors on the macrophage to induce expression of PPARγ. By increasing the presence of PPARγ in the macrophage these cytokines increase the

availability of PPARγ receptor and directing the macrophage to the M2 phenotype. By increasing the number of M2 macrophages in adipose tissue, IL-4 and IL-13 decreased SFAs and inflammatory cytokine levels within adipose tissue while also enhancing insulin sensitivity (6, 7).

In summary, the consistent presence of SFA associated with a high fat diet will activate M1 macrophages in adipose tissue through TLR-4 leading to the secretion of inflammatory cytokines TNF- α, IL-6 and IL-1β. This contributes to insulin resistance. Simultaneously, PPARγ receptor expression is decreased and thus the presence of the M2 macrophages in adipose tissue will decline as they become converted to M1 macrophages and inflammation and insulin resistance become dominant (6, 7, 16).



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Annu. Rev. Physiol. 72:219–46

Fig. 2: The M1 macrophage is activated by Pathogen Associated Molecular Patterns (PAMPs) such as LPS and SFA and by IFN-γ. The M2 macrophage is activated by IL-4 and IL-13 which are produced by Eosinophils, Basophils and NK cells. This leads to continued M2 production and associated secretion of IL-10 (6).

III. Eosinophils & Endothelial Cells

The Eosinophil, Eosinophil Migration and Connexins

Given this information, the transformation from M1 to M2 macrophages appears to be critical for reducing inflammation resulting from diet-induced obesity. In theory, this re-polarization could be accomplished by exposure to IL-4 and IL-13. These cytokines would induce PPARγ expression to convert macrophages to the M2 phenotype, promoting insulin sensitivity and combating the insulin resistance associated with type-2 diabetes. However, the source of these cytokines within healthy (UFA dominant) adipose tissue has been unclear, as has the reason for their reduced production in the obese individual. Recent work suggests that the source of the cytokines is eosinophils (18).

Eosinophils play a major role in sustaining M2 macrophage numbers in adipose tissue by secreting a majority of the IL-4 present in perigonadal adipose tissue mice (18, 19). The reason for the reduced secretion of IL-4 and IL-13 in the adipose tissue of obese individuals has yet to be resolved but may involve the eosinophil. The eosinophils present in adipose tissue may be stimulated by SFAs or inflammatory cytokines to produce less IL-4 and IL-13. They might also be stimulated to secrete inflammatory by SFA binding to the TLR-4 receptors (20). Alternatively, the eosinophils might be dying off within the adipose tissue, as TNF- α can stimulate eosinophil apoptosis (16). Further, as the eosinophil must cross the endothelium to reach the adipocyte, a decrease in eosinophils / endothelial cells interaction would reduce eosinophil migration into adipose tissue. This study will focus on the effects of free SFA on the migration of eosinophils.

Eosinophil Transmigration

Eosinophils migrate in a similar manner to other leukocytes, which involves a three-step process. First, selectins expressed on the eosinophil and endothelial cell loosely tether these cells to reduce eosinophil velocity in the blood. This slow, rolling eosinophil begins to expresses integrins that bind to cellular adhesion proteins on the endothelium to prevent further rolling (firmly adhere to endothelium). This interaction of the integrins with the endothelial adhesion proteins is further strengthened by the chemokines that attracted the eosinophil to the inflamed tissue. Finally, adhesion ligands in combination with chemokines secreted by cells in the surrounding tissue activate the eosinophil and attract the eosinophil into specific tissues (21, 22, 23, 24).

Three selectins are expressed on the endothelium, and some leukocytes, that are involved in transendothelial migration of eosinophils: selectins L, E and P. L-selectin is expressed on eosinophils and P and E-selectins are expressed on endothelial cells (21). Eosinophil rolling is dependent on P-selectin. The expression of this integral membrane protein is stimulated by cytokines IL-4 and IL-13. This is important as an environment saturated with UFA and a large M2 macrophage population has higher levels of these two cytokines thus leading to increased eosinophil migration to the specific tissue. Also, this selectin is longer than the other two selectins, which allows the endothelium to capture more leukocytes (21). L-selectin plays a role in the initial recruitment and attachment of the eosinophil (21, 23, 24).

The primary ligand for P-selectin is P-selectin glycoprotein ligand 1 (PSGL-1); CD24 expressed on eosinophils is also a receptor for this selectin. The eosinophils favor binding to P-selectin due to the higher levels of PSGL-1 on their surface as compared to sialyl Lewis x (E-selectin or L-selectin ligand). In addition, the inflammatory environment with TNF-α present would enhance the role of P-selectin in initial attachment of eosinophils to the endothelium (16, 21, 25, 26, 27).

Another eosinophilic protein involved in tethering to the endothelium is very late antigen-4 (VLA-4 or alpha4 beta1 integrin). This proteins binds to the vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells. This interaction is key to eosinophil recruitment as blocking VLA-4/VCAM-1 interaction reduces eosinophil migration across endothelial monolayers within minutes but has no effect over longer periods of time (21, 23, 24). This interaction is also able to mediate eosinophil migration across the endothelial barrier (23, 24). Aside from VLA-4, eosinophils also express an

α4β7 integrin receptor on their surface that binds to its principle ligand MAdCAM-1, primarily expressed in mucosal tissues (e.g., gastrointestinal tract) and contributes to diseases of altered eosinophil recruitment (21, 25).

Although VCAM-1 is important in eosinophil migration, binding to the endothelium can also be independent of VCAM-1, relying solely on P-selectin and intracellular adhesion molecule 1 (ICAM-1: 23, 24, 28). ICAM-1, an adhesion molecule/β_2 integrin ligand, is expressed both on the luminal surface of the endothelial cell as well as between adjacent cells. The abundance of ICAM-1 on the surface of the endothelial allows eosinophils migration across the endothelial barrier without the use of VCAM-1 as VCAM-1 is expressed only on the luminal surface (23, 24). Blocking the interaction between endothelial ICAM-1 and β_2 integrins on eosinophils prevents the eosinophil from migrating across the endothelial barrier. Therefore, the VLA-4/VCAM-1 interaction is necessary for recruitment and initial attachment of the eosinophil but ICAM-1 is required for eosinophil arrest and migration across the endothelium (23, 24).

Once the eosinophils are arrested by the endothelium, they must then be activated (i.e., change cytoskeletal conformation and expression of adhesion molecules) in order to create the firm endothelial adhesion that allows transmigration. This involves the binding of VLA-4 to VCAM-1 which activates several intracellular pathways within eosinophils through phosphorylation of Cbl, a protein product of the c-*cbl* proto-oncogene, and Phospholipase C (PLC) (See Figure 3). Cbl activates ZAP-70 and PLC-γ, which can play a role in adhesion-dependent functions of eosinophils. Activation of the eosinophil through VLA-4 and VCAM-1 interaction also enhances the generation of superoxide

anions by the eosinophil resulting in activation of the eosinophil's VLA-4 receptor (23, 24, 29, 30).

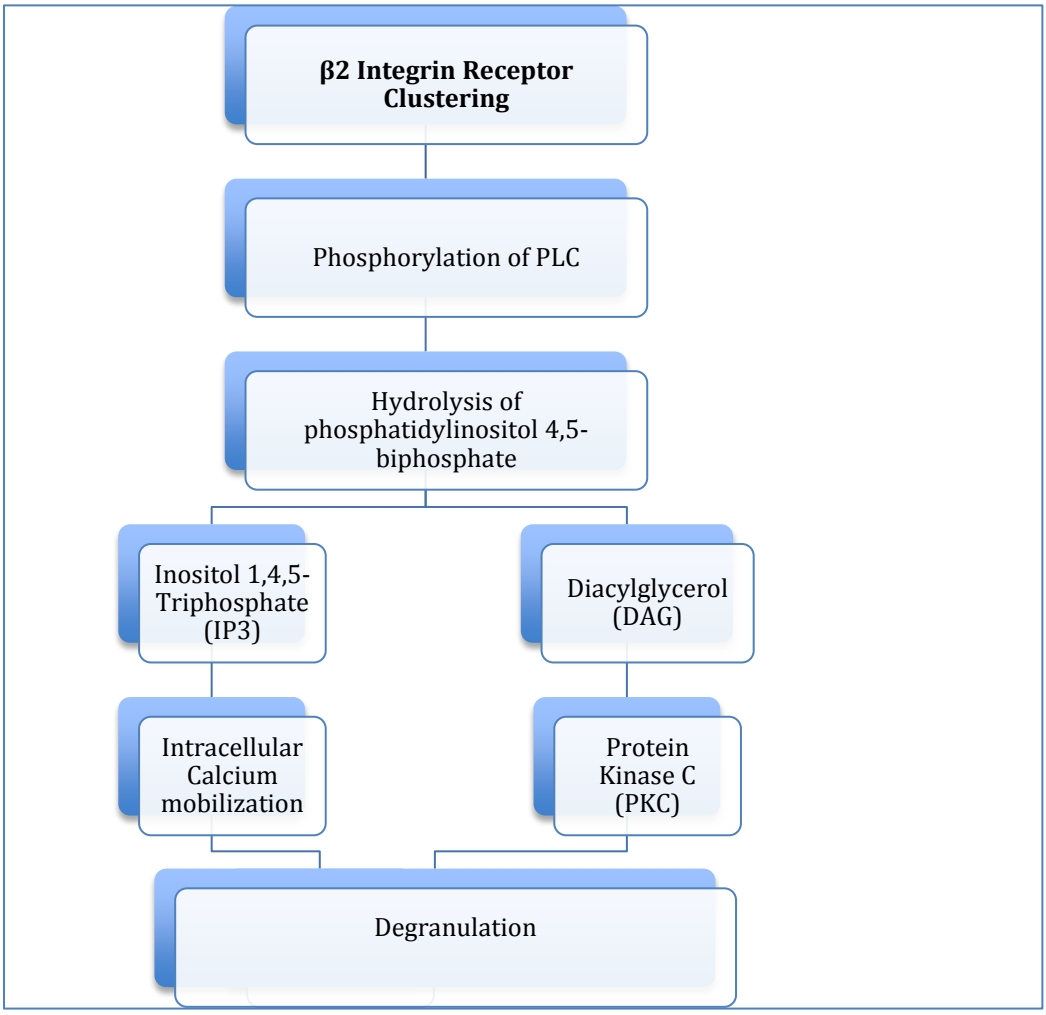

Fig. 3: Activation of β2 integrin receptors like LFA-1 and MAC-1 on eosinophils leads to the activation of two different intracellular pathways. Activation of both the IP3 and PKC pathways lead to eosinophil activation and subsequent degranulation.

The activation of VLA-4 increases eosinophil binding to VCAM-1 through enhanced affinity for the VCAM-1 on the endothelium creating a positive feedback mechanism. After this process of firm adhesion begins, the eosinophil undergoes a

conformational change via activation through surface adhesion proteins such as the α_4 and β2 integrins MAC-1 and LFA-1 (CD11b/18 and CD11a/18) and ICAM-1 on the endothelial cells. These interactions mediate eosinophil adhesion to the endothelium by supporting the activation of the β2 integrins, through the conformational change, on the eosinophil further enhancing the interaction with the abundant ICAM-1 on the endothelium. This activation signal can result from selectin binding, the interaction of the eosinophil with VCAM-1 or soluble factors like chemokines (Platelet Activating Factor and RANTES) or cytokines (TNF-α) (21, 23, 24). Also noteworthy is that ICAM-1 can be expressed by eosinophils and interact with similar receptors on the endothelium, thus the interaction can go either way (31). This process is summarized in figure 4.

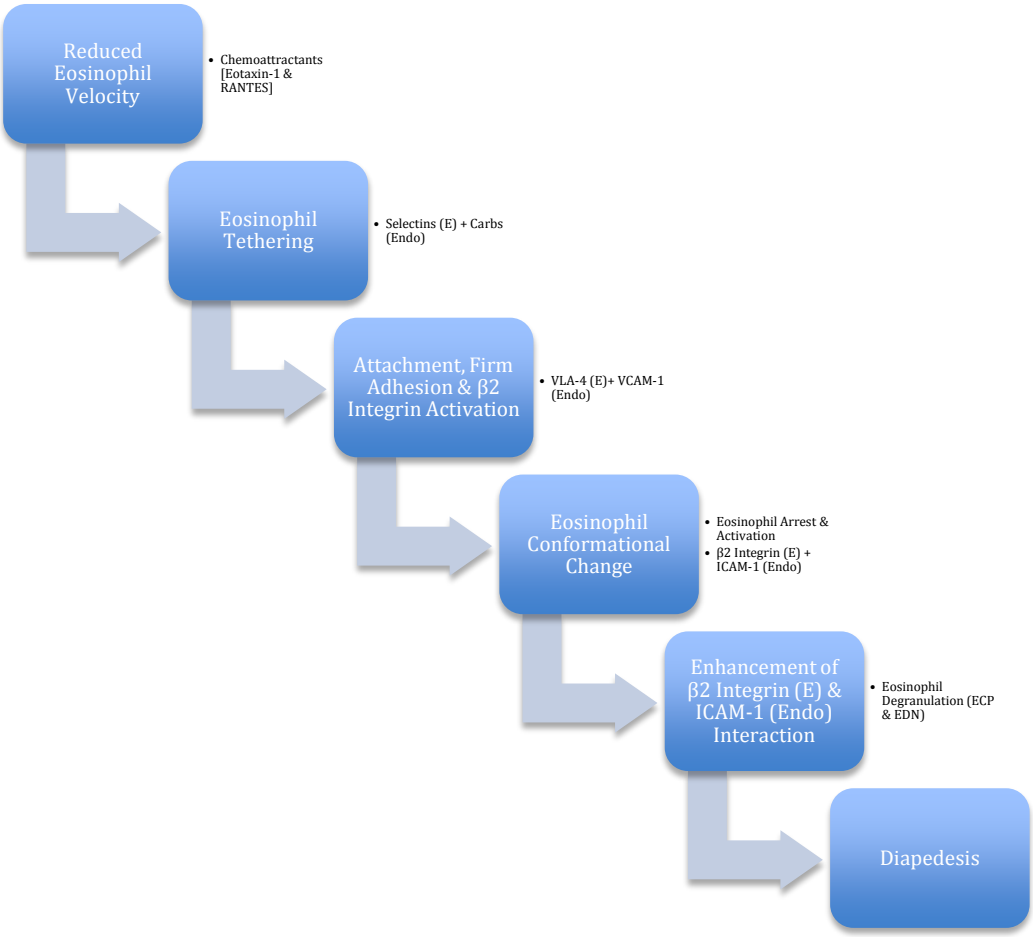

Fig. 4: Eosinophil recruitment and transmigration across the endothelial barrier. The eosinophil is first recruited to the specific tissue by chemokines resulting in a decrease in eosinophil velocity within the blood and allows the eosinophil to roll along the endothelial lining. Selectins on the eosinophil further reduce eosinophil velocity by grabbing, not binding, onto carbohydrates on the luminal surface of the endothelium. Upon tethering, the interaction between VLA-4 on the eosinophil and VCAM-1 on the endothelium occurs and begins the process of firm adhesion while simultaneously activating the eosinophil. The activation of the eosinophil results in a conformational change in the eosinophil's cytoskeleton promoting β_2 integrin interaction with ICAM-1 on the endothelium. This final interaction results in the degranulation of the eosinophil leading to enhanced interaction between β_2 integrin and ICAM-1 and finally results in diapedesis of the eosinophil and complete migration across the endothelial barrier (75, 76). (E = eosinophil, Endo = endothelium, ECP = eosinophil cationic protein, EDN = eosinophil-derived neurotoxin)

Influences on Integrin Expression or Binding

After the interaction between eosinophil β2 integrins and endothelial ICAM-1, activation of a tyrosine kinase and phosphoinositide metabolism in the eosinophil causes their degranulation and release Eosinophil Cationic Protein (ECP) and eosinophil-derived neurotoxin. These products enhance the expression of endothelial ICAM-1, further strengthening the interaction between endothelium and eosinophil.

Once tightly bound to the endothelium, eosinophils transmigrate, which requires β2 integrins on the surface of the eosinophil. Specifically, this process requires the interaction of LFA-1 and Mac-1 integrins with their endothelial receptors ICAM-1 and ICAM-2. The interaction between VLA-4 and VCAM-1 (a β1 integrin) partially inhibits eosinophil transmigration: as eosinophils are activated, their cellular adhesion molecules switch their preference, through a cytoskeletal conformation changes their affinity from VCAM-1 to ICAM-1 which allows transendothelial migration (21, 23, 24).

The eosinophil must also be primed in order to transmigrate. This priming occurs through the binding of cytokines such as IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) to the eosinophil. These cytokines promote chemoattractant-dependent eosinophil migration across the endothelial monolayer. Platelet activating factor (PAF) released by endothelial cells also promote eosinophil transmigration by increasing binding to ICAM-1 and decreasing expression of endothelial PSGL-1. Eosinophils also require the shearing forces of the flowing blood to maximize transmigration. Shear stress activates endothelial cells, which enhances their cytokine-stimulated secretion of chemokines (32). With these conditions comes the requirement of the soluble/adhesion molecules already mentioned (21, 25, 26, 27, 32, 33).

Modifiers of Eosinophil Transmigration

Taking a step back, eosinophil recruitment by specific tissues is mediated by several chemokines such as eotaxin and RANTES as well as cytokines such as IL-4, IL-5, and TNF-α. The source of these chemokines ranges from leukocytes such as macrophages and Th2 cells to tissue cells such as adipocytes (16, 17, 25, 26, 27, 31, 33, 34, 35). Eotaxin is the most potent chemoattractant signal for eosinophils and is involved in trafficking of eosinophils into specific tissues such as the mucosal tissues of the gastrointestinal tract and the peritoneal cavity (25, 26, 33, 34).

The chemokine eotaxin-1, also known as CCL11, is a member of the CC chemokine family (CC refers to adjacent cysteine residues: 12, 20, 21, 22, 56). Eotaxin is a non-glycosylated polypeptide secreted by epithelial cells, fibroblasts, macrophages and eosinophils (35). There are 2 additional isoforms of eotaxin [eotaxin-2 (CCL24) and eotaxin-3 (CCL26)] all of which are ligands for the chemokine receptor CCR3 located on eosinophils (16, 26, 27, 25, 32, 34).

Eotaxin-1, -2 or -3 can be secreted by epithelial cells, leukocytes and adipocytes (17, 35). Both differentiated adipocytes and pre-adipocytes secrete eotaxin, with the latter secreting more (35). In response to specific stimuli, adipocytes secrete high levels of eotaxin. For example, TNF-α elevates eotaxin secretion by 40-fold in 3T3-L1 pre-adipocytes/adipocytes (35). Also, IL-4 and IL-5 secretion by eosinophils can increase eotaxin secretion by adipocytes as well, with IL-4 enhancing secretion greater than IL-5 (35). Eotaxin secreted by other cells such as macrophages or eosinophils increase eotaxin secretion by adipocytes (35). Therefore, eosinophils could create a positive feedback loop

whereby eotaxin (or IL-4) secretion within adipose tissue would recruit more eosinophils and thereby promote the M2 macrophage (anti-inflammatory) response in lean individuals.

Another interesting aspect of eotaxin secretion by adipocytes is that it is impact by diet: a high fat diet elevates adipocytes eotaxin secretion. Switching to a low fat diet reduces eotaxin secretion as does maintaining a low fat diet (17, 35). This is consistent with the idea that TNF-α can elevate eotaxin secretion by adipocytes because obesity is an inflammatory state and TNF-α is an inflammatory cytokine.

From the evidence presented above, the number of eosinophils within adipose tissue should be maintained or increased by elevating eotaxin. However, eosinophil numbers have been reported to decrease under these conditions. It is possible that the obese state impedes the migration of eosinophils into the peritoneal cavity as well as other areas of adipose tissue. This could involve alterations in either endothelial cell or eosinophil that reduce transmigration. Interesting, eotaxins have been shown to alter the expression of endothelial cell gap junction proteins (connexins). These connexins play an important role in the regulation of endothelial cell function in the vasculature and may be involve in the eosinophil paradox (37, 38, 39, 40).

Gap Junction Proteins

Connexins have received considerable interest for their ability to moderate inflammatory responses (38, 40). To form a gap junction between cells, 6 connexins associate into hemi-channels (connexon) that dock with a connexon on a neighboring cell. Once formed, these gap junctions gate to the open state allowing the diffusion of

electrical and chemical signals (< 1kDa) between cells (gap junctional intercellular communication: GJIC) (38-40). Hemichannels are fluid in the plasma membrane moving laterally to dock with their counterparts in the neighboring cells to form gap junctions.

Because tissues depend on connexons for homeostasis and expression of the 22 isoforms of the connexin protein is tissue specific, any dysregulation or mutation in a gene coding for a connexin can result in disease such as cataractogenesis, deafness, multiple skin disorders and heart disease (39). Relevant to the current study, the expression and/or the connectivity of connexins in tissues can be altered both in vitro and in vivo under inflammatory conditions such as obesity. Inflammatory mediators such as LPS and TNF-α can inhibit GJIC by phosphorylation of intracellular portions of connexons to alter the gating of the channel as well as its permeability (38, 39). Inflammation decreases the expression of endothelial cell Cx40, while increasing endothelial cell Cx37 expression. Interestingly, Cx37 is also expressed by leukocytes. Thus, inflammation would alter the endothelial layer and could thereby contribute to its pathogenesis in the affected tissues (38, 39, 40).

In the current study, we are particularly interested in endothelial connexins that alter expression of adhesion molecules like VCAM-1 which would affect leukocyte adhesion in states of inflammation such as those induced by diet. Thus, Cx37, Cx40, and Cx43 are the focus of this study due to their role in inflammatory diseases such as atherosclerosis and asthma (37, 38, 39, 40).

Cx40 is expressed in endothelial cells of healthy individuals and some smooth muscle cells (SMCs). It prevents macrophage and leukocyte adhesion indirectly by preventing the surface expression of VCAM-1 (38, 39, 40). The coexpression of CD73

and Cx40 on the surface of endothelial cells down regulates VCAM-1 expression and thus decreases leukocyte or macrophage adhesion (mechanism will be discussed shortly). If Cx40 is absent or impaired due to inflammation, there will be increased VCAM-1 expression and an increase in macrophages adhesion to the endothelial surface. Also, Cx40 is part of an anti-inflammatory signaling pathway within the pulmonary system that prevents the adhesion of neutrophils (38, 39, 40).

Like Cx40, Cx43 can also be expressed by both SMCs and endothelial cells. In terms of atherosclerosis, this connexin is considered “atherogenic” because its presence is related to increased macrophage infiltration within the vasculature, aiding in the progression of atherosclerotic plaque development. This connexin is also expressed on the surface of both macrophages and neutrophils, which could allow for intercellular communication between these cells respectively, as well as with other cell types (38, 39, 40). Cx43 expression on SMCs and leukocytes can be up regulated in the presence of IFN-γ along with either LPS or TNF-α, thus showing its importance in the development of inflammatory diseases. Aside from atherosclerosis, Cx43 has been linked to a pro-inflammatory state within the lungs involving the propagation of calcium waves between endothelial cells. These calcium waves promote the expression of P-selectin on the surface of the vasculature that facilitates leukocyte rolling on the vascular surface and thus leads to increased adhesion of leukocytes (mechanism promoting P-selectin expression is unknown) (38, 39, 40).

Cx37 is located on endothelial cells, some smooth muscle cells and macrophages and is protective against inflammatory diseases. Although this connexin plays an important role in both early and late atherosclerosis (38, 40), it also impacts macrophage

function. As shown in figure 5, Cx37 hemichannels on macrophages release ATP into the extracellular matrix. ATP is degraded by ectoenzymes CD39 and successively by CD73 into AMP and then to nucleosides and inosine. In the anti-inflammatory state, CD73 degrades AMP to adenosine (Ado), which binds to an $A_{2B}R$ receptor on the endothelial cells to reduce VCAM-1 expression to very low levels. This would decrease the number of macrophages that cross the endothelial barrier. Simultaneously, Cx40 expression is enhanced within endothelial cells (40). Conversely, in a pro-inflammatory state CD73 is down-regulated on the endothelial surface due to a decrease in Cx40 expression and a increase in Cx43 expression caused by an inflammatory stimulus. In a parallel response, the Cx37 hemichannel is either blocked or expression on the macrophage is reduced to prevent the release of ATP into the extracellular environment. In the event that expression was reduced, small amounts of released ATP are able to bind to the purinergic receptor P_2Y triggering Ca^{2+}-dependent signaling that propagates between endothelial cells via Cx43 and increases the expression of endothelial leukocyte adhesion molecules (40). This allows for enhanced inflammatory cell adhesion and migration across the endothelial barrier in the tissue of interest (38, 40).

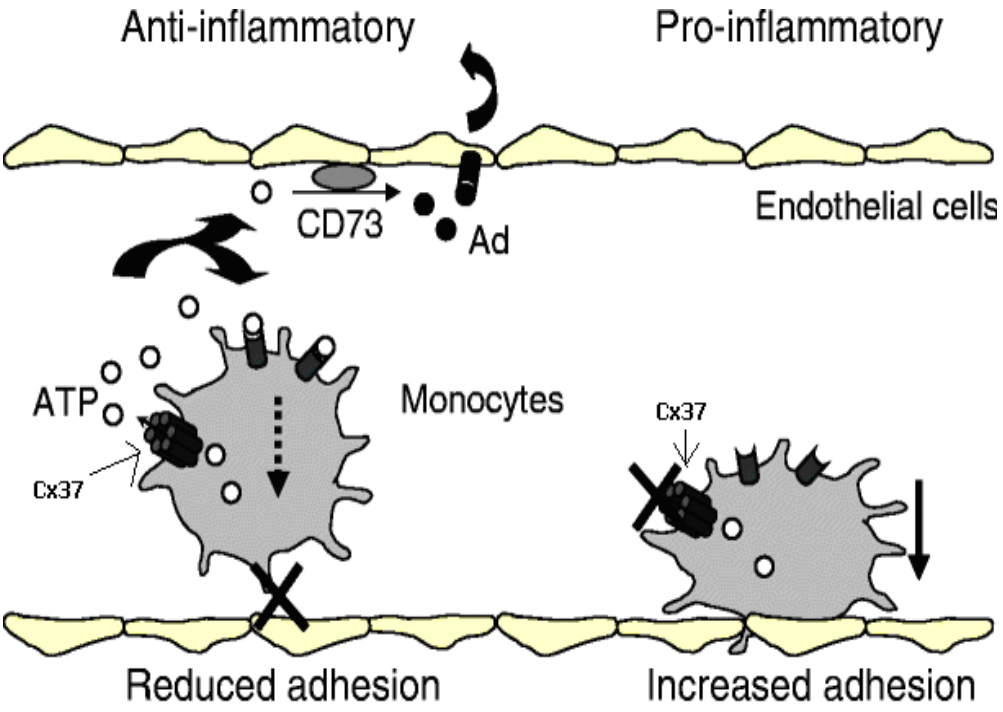

Figure 5: The effects of the anti-inflammatory and pro-inflammatory states on the expression of CD73 and Cx37 on endothelial cells and macrophages resulting in enhanced or reduced adhesion and thus migration across the endothelial barrier (38).

Based upon the data presented above, we will address the reason(s) underlying the decrease in eosinophil levels within obese individuals on a high fat diet that occurs despite elevated levels of eotaxin. Though the relationship between connexins and eosinophils remains largely unknown, Cx43 appear to influence eosinophil migration. Decreasing Cx43 expression within subepithelial fibroblasts in nasal polyps increased eosinophil infiltration (28). Thus, the expression of Cx43 by eosinophils could be the key to understanding the regulation of eosinophil migration. Specifically, connexins on eosinophils or on the endothelial cells lining the blood vessels of the peritoneal cavity may reduce eosinophil population in adipose tissue by altering proteins responsible for

endothelial-leukocyte adhesion. Therefore, this proposal will investigate the impact of free SFA on connexin expression in eosinophils and endothelial cells. If a cellular environment high in saturated fat can alter the expression of connexins on either cell type then this could explain the reduction in eosinophil migration.

Endothelial Cells and PRRs

Endothelial cells are unique in that they are one of the most abundant cells in the body and together they form the largest interconnected organ in the human body (41). Their abundance and function as a barrier to all body cavities and blood vessels render them an important part of the body's defense system, more specifically the innate immune system. Endothelial cells contain many surface receptors such as pattern recognition receptors (PRR) and adhesion molecules such as selectins (41). These surface molecules allow the endothelial layer to react to specific soluble stimuli such as LPS, chemokines, and cytokines and respond with regulation of leukocyte migration from the blood stream into various tissues (41, 42, 43).

Of the surface molecules expressed on endothelial cells, TLR-4 appears to be most important in the innate immune response. As mentioned before, TLR-4 is a pattern recognition receptor that is located on many cells from leukocytes like macrophages and eosinophils to non-bone marrow-derived parenchymal cells like endothelial cells (41, 42, 43). Expression of TLR-4 on the endothelial cells makes them susceptible to activation by LPS and the subsequent alterations in adhesion molecules expression as well as cytokines and chemokines secretion.

Similar to the monocytes, the interaction between LPS and TLR-4 on the surface of endothelial cells results in the activation of the transcription factor NF- kB by MyD88, IL-1 receptor-associated kinase (IRAK), IRAK-2 and TNF receptor-associated factor-6 (42, 43). Activated endothelial cells secrete inflammatory cytokines and chemokines and subsequent alteration of selectin and integrin expression. However, unlike the monocyte endothelial cell activation is dependent upon the soluble CD14 rather than a membrane bound CD14 (42).

Upon the activation of the endothelial cell by TLR-4, the cell will begin to change expression of adhesion molecules to favor leukocyte rolling and transmigration across the endothelial barrier and into tissues. This occurs through an increase in P-selectin expression to tether cells like neutrophils, eosinophils and macrophages, allowing them to tightly bind to the endothelium and initiating the process that ultimately leads to transmigration. This is important in the rapid clearance of a bacterial infection (41). TLR-4 activation can also alter endothelial connexin expression which could affect expression of surface adhesion molecule (i.e. Cx43 could increase P-selectin expression: 39, 40).

Along with expression of PRRs such as TLR-4, endothelial cells also express PPARγ that is activated by UFAs (43). Similar to macrophages, the activation of this subunit on endothelial cells dampens the inflammatory response by interfering with NF-kB activation. Therefore, PPARγ activation by UFA would resolve an inflammation response (43).

Endothelial Cell Response to Fatty Acids and Connexin Expression

Endothelial cells expression of TLR-4 and PPARγ receptors allows them to interact with both saturated and polyunsaturated fatty acids. This means that fats impact not only their internal function, in terms of cell stress, but also their expression of surface molecules and regulation of cell migration and innate immune function.

For example, SFA such as palmitic acid (C16:0) and lauric acid (C12:0) act through TLR-4 on endothelial cells to induce translocation of Cx43 to the mitochondria membrane which can depress mitochondrial membrane potential in cells (marker of endothelial cell stress and dysfunction. It alters membrane potential by acting as a channel, similar to a potassium channel regulated by ATP, which is thought to have cytoprotective effects on the cell by enhancing the mitochondrial function within the cell (9).

Mitochondrial membrane potential and that membranes permeability maintains the function of the electron transport chain. When a cell is undergoing the ER stress response, hypoxia or is being stimulated by LPS or TNF-α, the mitochondrial membrane potential decreases due to substrates like free FA or toxic free radicals. These substrates cause a dysfunction in the respiratory chain thereby increasing the reactive oxidative species (ROS) that can lead to cell death through apoptotic or necrotic mechanisms. Therefore, the ideal situation would be to increase mitochondrial membrane potential and decrease Cx43 expression within the mitochondria. UFAs like arachidonic acid (AA; n-6 UFA) and eicosapentanoic acid (EPA; n-3 UFA) or their precursors (linoleic and α-linolenic acids respectively) act through PPARγ to elevate inner mitochondrial membrane potential in endothelial cells to combat cell stress, prevent apoptosis and in some cases

increase Cx43 translocation to the mitochondrial membrane (9). Therefore, these UFAs or their precursors appear as ideal targets for combating the effects associated with an inflammatory environment (i.e. cell death, inflammatory cell migration, etc.) (9, 44, 45).

Poly-Unsaturated Fatty Acids: Omega-3 vs. Omega-6

With UFAs becoming the center of focus to combat problems associated with diet-induced obesity, research has led to the grouping of UFAs into beneficial and non-beneficial. EPA, or its precursor α-linolenic acid, is an omega-3 fatty acids derived from fish oils that is the most beneficial of the UFAs. This source of fatty acid has been shown to not only be anti-inflammatory, but also decreases monocyte adhesion and reverses insulin resistance. Alternatively, AA and linoleic acid, are omega-6 fatty acids that stimulate inflammation. In fact, they are one of the breakdown products of apoptotic adipocytes present in diet-induced obese individuals that contributes to the inflammatory environment associated with medical complications (9, 44, 45).

Arachidonic Acid increases monocyte adhesion to endothelial cells regardless of endothelial cell activation due to inflammatory cytokines. This occurs through the increased endothelial expression of integrins and selectins like ICAM-1, VCAM-1 and E-selectin (44). However, the effect of AA on connexin expression in endothelial cells remains questionable, as it has also been shown to have similar results as EPA. This could be due to the fact that both UFAs act through PPARγ and down regulate inflammatory transcription factors (9, 44, 45, 46). Regardless of previous research, more research must be done to determine the exact benefits and differences between the two fatty acids until a viable explanation is reached.

IV. **Conclusion**

The fact that endothelial cells express both TLR-4 and PPARγ receptors opens a realm of possibilities when questioning the impact of SFA and UFA on migration of eosinophils across the endothelial barrier. Specifically, it gives two possibilities in regards to why there is a lack of eosinophils migrating into the peritoneal cavity in diet-induced obese individuals; there is a change in endothelial cells or in the eosinophil. As mentioned before with the eosinophil, the three subcategory problems were as follows: 1) Eosinophils can transmigrate but die in the tissue due to the cellular environment, 2) eosinophils migrate to the cavity but are unable to cross the endothelial barrier, or 3) the eosinophils are transmigrating into the tissue but have shifted to secretion of pro-inflammatory cytokines and thus contribute to the problem.

On the other hand, endothelial cells could be the root of the problem for why eosinophils are not transmigrating into the affected tissue, resulting in the unfavorable polarization to M1 macrophages within the specific tissue. Focusing on endothelial cells and incorporating the idea of connexin expression then the problem could be simple, which is that SFAs are binding to TLR-4 receptors on the endothelial cells resulting in increased expression of adhesion molecules that facilitate eosinophil migration into adipose tissues. If eosinophils were entering the tissue in this state of inflammation then it would be possible that they are compromising their secretion of cytokines IL-4 and IL-13 and instead secreting pro-inflammatory cytokines that could amplify the M1 response leading to insulin resistance.

If SFAs such as palmitic acid or lauric acid are the root of the problem then the answer could be as simple as naturally consuming, taking supplemental or injected forms of Poly unsaturated fatty acids such as alpha-linolenic acid, an Omega-3 fatty acid, or linoleic acid, an Omega-6 fatty acid (9, 44, 45). This would increase expression of cardio-protective connexins such as Connexin40 and Cx37, while decreasing CX43 expression to dampen inflammatory cell migration and maintaining the appropriate balance of M2 to M1 macrophages and eosinophils within the adipose tissue to prevent insulin resistance.

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