Chitosan derivatives and the HUVEC inflammatory response

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Chitosan derivatives and the inflammatory response of HUVEC

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15 ECTS thesis submitted in partial fulfillment of a
Baccalaureus Scientiarum degree in Biochemistry and Molecular Biology

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Abstract

Chitin is a linear polysaccharide homopolymer composed of β-(1–4)-linked 2-deoxy-2-amino-D-glucopyranose. By partially removing the acetic groups of the acetyl glucosamine units of chitin, chitosan is produced. Chitosan is different to chitin as it doesn't have a free amino acid group, which makes it more soluble. Despite great interest in its bioactivity, few studies have examined the effects that chitin and its derivatives have on the immune response of endothelial cells. Increased inflammatory gene expression of genes such as intracellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1) by lipopolysaccharide (LPS) via the NF-κB transcription factor, is reduced in endothelial cells when treated with ChOS, according to Du *et al.* 2010. The aim of this project was to determine if chitosan derivatives affect the expression of the adhesion molecules ICAM-1 and VCAM-1, which are important molecules in the inflammation and immune response, with and without the presence of lipopolysaccharide of two different concentrations. The experiments were conducted on human umbilical vein endothelial cells (HUVEC) from three separate donors. This study showed no significant difference in ICAM-1 and VCAM-1 expression.

Útdráttur

Kítín er línuleg fjölsykra sem byggð er úr β-(1–4)-tengdum 2-díoxíð-2-amínó-D-glúkósapyranósa. Með því að fjarlægja sýruhóp amínósýrunnar af acetyl glúkósamín hluta kítíns er hægt að framleiða kítósan. Kítósan hefur þann kost fram yfir kítin að vera leysanlegt í súrum vatnslausnum vegna færri vetnistengja. Þrátt fyrir mikinn áhuga vísindamanna á lífvirkni kítósans hafa fáar rannsóknir einblínt á áhrif kítósan og kítósan afleiða á bólgusvörun í æðaþelsfrumum. Rannsóknir hafa sýnt að þegar meðferðum með kítósan afleiðum er beitt minnkar aukin tjáning vegna LPS á NF-κB umritunarþætti og tjáning ICAM-1 og VCAM-1 minnkar eins og sýnt er fram á í DU *et al.* 2010. Markmið þessarar rannsóknar var að athuga áhrif kítósan afleiða á tjáningu ICAM-1 og VCAM-1, sem eru mikilvægar sameindir í bólgu og ónæmissvari, með og án LPS ónæmisvaka í tveimur styrkjum. Rannsóknin var framkvæmd á æðaþelsfrumum úr naflastreng manna (HUVEC) úr þremur mismunandi gjöfum. Rannsóknin sýndi þó engan marktækan mun.

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Abbreviations

APS: Ammonium persulfate

ChOS: Chitosan oligosaccharides

FBS: Fetal bovine serum

HUVEC: Human umbilical vein endothelial cells

LPS: Lipopolysaccharide

ICAM-1: Intracellular adhesion molecule

IL-6: Interleukin 6

IL-8: Interleukin 8

PBS: Phosphate buffered saline

RIPA: Radioimmunoprecipitation buffer

SDS: Sodium dodecyl sulfate

TBS: Tris buffered saline

TBST: Tris buffered saline with tween

TChOS: Therapeutic ChOS

VCAM-1: Vascular adhesion molecule

Acknowledgements

First of all I want to offer my thanks to Dr. Pétur Henry Petersen for allowing me this opportunity to work on this project at his lab in Læknagarður, where all of the work was performed. I would also like to thank Steinunn Guðmundsdóttir for her instructions, supervision and overall guidance on the project. I also want to thank all the people at the laboratory at Læknagarður, where special thanks go to Egill E. Hákonarson. At last I would like to thank Dr. Bjarni Ásgeirsson and Dr. Haraldur Halldórsson for their help and involvement in the project.

1 Introduction

The immune-system's main response to tissue damage and invading microorganisms is to cause inflammation. Inflammation is a surviving mechanism, leading effector cells to battle with infectious agents. The innate immune cells (effector cells) do that by secreting cytokines and chemokines. Effector cells often have to cross endothelial cell linings, such as in arteries. When these molecules enter the bloodstream, capillaries expand and blood flow slows down. In addition adhesion molecules, such as the intracellular adhesion molecule (ICAM-1) and the vascular adhesion molecule (VCAM-1), are expressed on the cell surface. These adhesion molecules help leukocytes to merge with the endothelial cell wall. Cytokines at the same time signal the endothelium, which helps to make the capillary wall more permeable. This gives the plasma proteins and cells of the immune system the opportunity to enter the tissue[1,2].

When induced by lipopolysaccharide (LPS), an endotoxin found on the outer side of gram-negative bacteria, endothelial cells initiate a mixture of inflammatory responses, as described above. When an inflammatory response fails to resolve, it leads to chronic inflammation, and that may in turn lead to the development of cardiovascular disease. Therapeutic solutions to chronic inflammation are therefore of great interest. Chitin and chitin derivatives have in recent years shown promise in the fight against chronic inflammation[3,4,5].

1.1 Chitin and its derivatives

Chitin is a linear polysaccharide homopolymer composed of β-(1–4)-linked 2-deoxy-2-amino-D-glucopyranose[6]. It is very abundant in nature and is one of the fundamental component of the outer exoskeleton and tendons of crustaceans as well as most other invertebrates[7]. Chitin is also important in the cell wall of fungi. Due to its abundance, biocompatibility and low immunogenicity, chitin has been extensively studied for a broad range of applications such as a drug delivery system, in tissue engineering and as a antitumor and anti-fungal agent [8,9].

Numerous studies on chitins biomedical applications have been conducted and it has e.g. shown promise as a material in nanotechnology. Chitin has also interested researchers

by its possibilities in a wider range of applications in fields such as biotechnology and clinical science[8]. However, because of the intermolecular hydrogen bonds of chitin, it is insoluble in aqueous solutions[8]. This has largely prevented its usage [7].

1.1.1 Chitosan and chitosan oligosaccharides (ChOS)

By partially removing the acetic groups of the acetyl glucosamine units of chitin, chitosan can be produced (*figure 1*). In chitosan, between 50-90% of the acetyl groups have been reduced to amine groups[8].

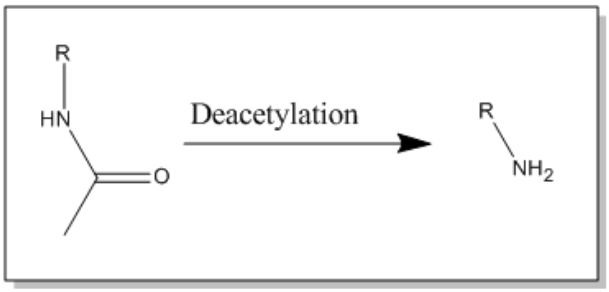

Figure 1: Deacetylation of carboxyl acid to amine.

Chitosan is different to chitin as it is soluble in acidic solutions due to fewer intramolecular hydrogen bonds [10]. The fact that chitosan is soluble makes it much easier to use as a substrate for various medical research [8]. Chitosan can be manipulated into different shapes and forms, depending on its usage. This can be convenient for various applications, such as drug carrying, coating for implants as well as a substitute for bone and skin [11].

Smaller polymers can be produced from chitosan via hydrolysis or enzymatic reaction. These small polymers are called chitosan oligosaccharides (ChOS) (*figure 2*). ChOS are more soluble in water than chitosan and have been extensively studied regarding biological activities, absorption and biocompatibility. Recent publications suggest that ChOS may have anti-inflammatory abilities in endothelial cells. ChOS have even been recommended as food additives in recent years in some parts of Asia, due to its properties [9,10].

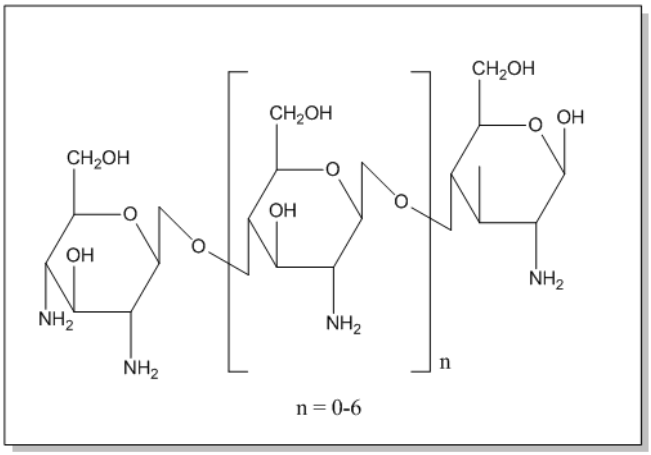

Figure 2: Chitosan oligosaccharide polymer.

1.2 Human endothelium derived from umbilical veins

The endothelial cells form a thin layer on the interior of vesicles, called endothelium. The endothelium is what separates the lumen and the rest of the cell wall from circulating blood. It plays important roles, e.g. as a semi-selective barrier, assisting with the process of blood clotting and the inflammation process[1, 2].

In human research where the aim is to mimic in vivo conditions of the endothelium, primary endothelial cells from umbilical veins (HUVECs) are the most commonly used [12]. They are, like other endothelial cells, very sensitive to stimulation from pathological invasion and upon aggravation cause a variety of inflammation responses[13]. Because of that, because they are easy to cultivate, and because new donor cells can easily be obtained, HUVECs are a prime tool to investigate pathogenesis on vascular inflammation and the molecular mechanisms that trigger inflammation[9].

Cultured endothelial cells grow as a homogenous monolayer were the cells are large, morphologically similar, polygonal and often with poorly defined borders. *Figure 3* is an example of dense monolayer HUVECs. The cell doubling time is around 92 hours [14].

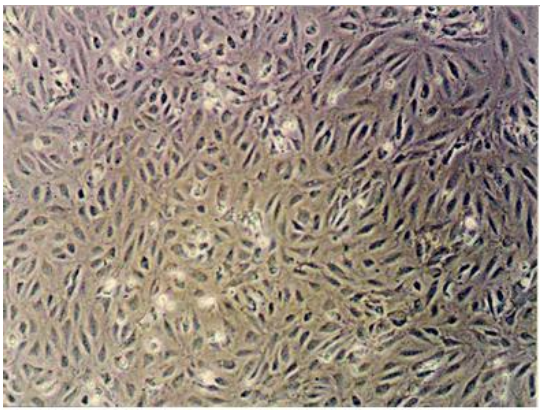

Figure 3: Phase contrast image of HUVEC culture in a petri dish (Shore et al. 2004)

1.3 ICAM-1 and VCAM-1 adhesion molecules

Intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecules-1 (VCAM-1) are parts of the immunoglobulin superfamily and play an important role in inflammatory response[15]. The *NF-κB* pathway regulates expression of these proteins. After transcription, they are relocated to the cell membrane, were they play an important role in the inflammatory response of the endothelium. The pathway does that by assisting monocytes and neutrophils to merge into the endothelium. Upregulation of ICAM-1 and VCAM-1 occurs at an early stage of vascular inflammation. This makes leukocytes mediate to "active" cells and it can lead to endothelial dysfunction. That is a systemic pathological state of the endothelium, defined by imbalance between dialing and constricting substances produced by the vascular endothelium[12].

1.4 Effects of chitin, chitosan and ChOS on the inflammatory response

Despite great interest in its bioactivity, only a few studies have explored the anti-inflammatory ability and the effects that chitin and its derivatives have on the immune system in endothelial cells such as HUVECs[7,4]. One of the most important factors in chronic inflammation is the nuclear factor-kappa B (NF-κB). NF-κB is present in a dimer consisting of two subunits, p65 and p50. These subunits are located in the cytoplasm of cells and are bound to an inhibitor called I-κB. When cells are treated with an inflammatory agent such as LPS, an I-κB kinase gets activated, phosphorylating the I-κB

which starts I-κB-α ubiquitination and then degradation of I-κB by proteasomes[16], thereby rendering it useless to inhibit. This enables the NF-κB transcription factor to translocate to the nucleus. When relocated to the nucleus NF-κB activates target genes which translate into adhesion molecules such as ICAM-1 and VCAM-1 (*figure 4*) [9,7].

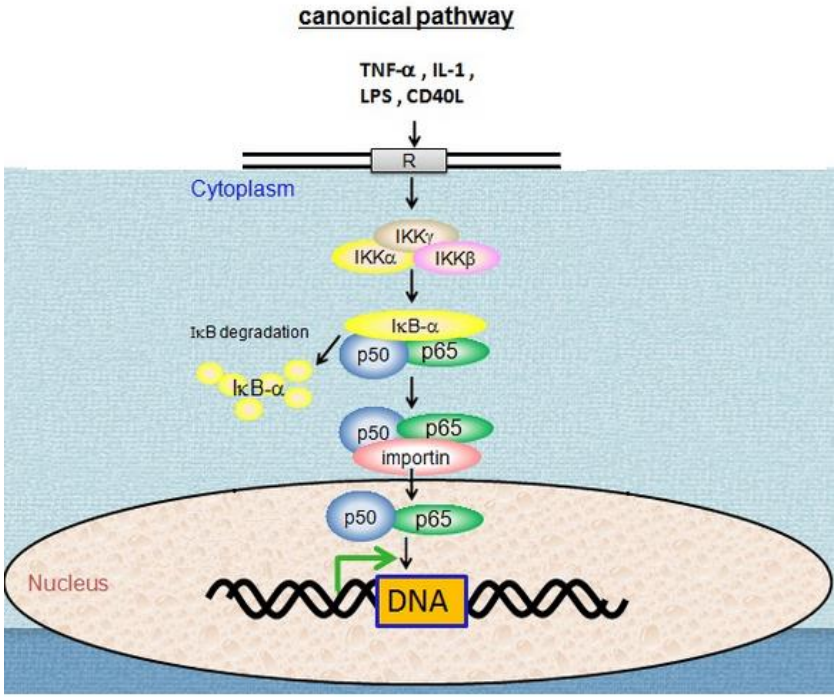

Figure 4: The NF-κB pathway. When stimulated with infectious agents, I-κB is degraded and NF- κB is activated and relocated in to the nucleus. This starts translation on genes for adhesion molecules such as ICAM-1 and VCAM-1.

1.5 Previous studies on the subject

Inflammatory gene expression induced by LPS is reduced in ChOS pretreated endothelial cells[5,3,9]. The molecular mechanism behind this reduction of inflammation in ChOS treated endothelial cells has not been thoroughly described[4]. The current hypothesis is that ChOS induces anti-inflammatory effects with post-translational modulation of the NF-κB transcription factor by disrupting LPS-induced O-GlcNAc modification of NF-B/p65. This would otherwise result in activation of the NF-κB pathway as described above and in Xu *et al.* 2014[9]. This could be accomplished by inhibition of the mitogen activated protein

kinase (MAP kinase), signaling and down-regulation in expression of, for example, E-selectin and ICAM-1, as described in Du *et al.* 2013[4]. Other studies support these claims, such as Du *et al.* 2010[5] and Du *et al.* 2011[13] . In both of those studies, the expression of IL-6 and IL-8 were inhibited in LPS-induced HUVEC with ChOS. This was also suggested in Yu *et al.* 2011[3] where ChOS was shown to inhibit IL-6 as well as TNF-α expression through the MAP kinase pathway and the PI3K/Akt signaling pathway (*figure 5*).

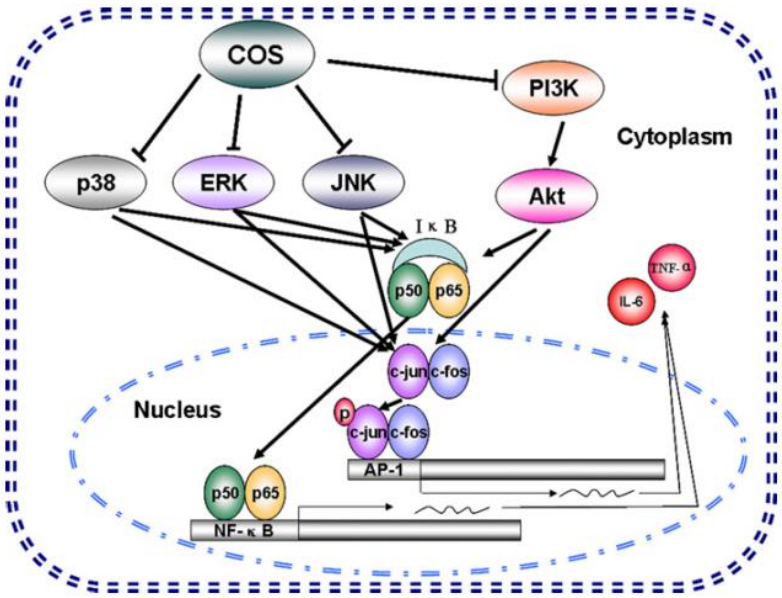

Figure 5: Effects of chitosan oligosaccharides on the NF-κB pathway as suggested in Yu et al. 2011[3]. ChOS (here written as COS) is believed to inhibit various pathways that would otherwise lead to gene translation of adhesion molecules such as ICAM-1 and VCAM-1.

1.6 Aim

The purpose of this project was to determine if chitosan derivatives (ChOS) affect the expression of the adhesion molecules ICAM-1 and VCAM-1 in HUVEC, with and without the presence of LPS of two different concentrations. This would confirm if chitosan derivatives have any effect on the inflammatory response of human endothelial cells.

2 Material and methods

2.1 HUVECs

Human umbilical vein endothelial cells (HUVECs) were obtained from Dr. Haraldur Halldórsson. They had been isolated from human umbilical cords with collagenase treatment. Cells were grown with Vasculife EnGS (Lifeline Cell Technology) medium supplemented with 0.2% EnGS, 5 ng/ml recombinant human EGF, 50 µg/mL ascorbic acid, 10 mM L-glutamine, 1 µg/mL hydrocortisone hemisuccinate, 0.74 U/mL heparin sulfate, 2% fetal bovine serum (FBS), 20 U/mL penicillin and 20 µg/mL, streptomycin and kept at 37°C in 95% humidified 5% CO_2 atmosphere.

The HUVECs were split when reaching confluence (3-5 days) by treating them with 0.05 M trypsin (Gibco) in EDTA for 1-3 minutes or until they got loose from the surface. They were diluted in media and seeded in a 1/5 dilution. Cells were used in early passage, which is the number of times the cells have reached confluence and been split (no older than passage three) by treating them with test substrates when they had reached confluence so in vivo conditions would be simulated in the best way possible.

HUVECs were then grown in twelve well plates, 3.60 cm^2, until they had formed a dense confluent monolayer on the plastic surface. After confluence was gained test substrates were used to stimulate the cells [15].

2.2 Chitosan and ChOS

Therapeutic chitosan oligosaccharide (TChOS) was obtained from Genís ehf. A solution of de-acetylated chitosan (Sigma) were made by dissolving in 30 mL 1 M NaOH solution and kept at 90°C for one hour to destroy endotoxins[17]. The solution was centrifuged, washed once with ddH_2O and then five times with sterile PBS. “Small” chitosan (<30 µm) was made from the original chitosan solution by sonicating the solution at full power on ice for 15 minutes. It was then filtered with a 30 µm filter; estimated material loss was about 66%. Chitosan oligosaccharide lactate (ChOS lactate (Sigma)) was dissolved in endotoxin-free

PBS, vortexed and sterile filtered (0.45 μm). Substrate strength administered in HUVEC treatment was 100 μg/ml [15].

2.3 Western blotting

2.3.1 Protein extraction

Cold PBS was used to rinse the cells and they were then lysed with RIPA lysis buffer (50 μl for each well on a 12 well plate). Proteins were extracted from cells with RIPA lysis buffer, which included a protease cocktail (Sigma) as well as $NAVO_3$, PMSF and NaF. The incubation period was ten minutes on ice while the plates were also tilted occasionally. Wells were scraped with pipette tip and cell lysate was collected in an eppendorf tube. Next the lysates were sonicated for two minutes on medium setting in Diagenode Bioruptor (three cycles). Lysate was then kept on ice for ten minutes and centrifuged at 12,000 g for 20 minutes at 4°C. Samples were then stored at -20°C. Protein concentration was determined using Bradford reagent with bovine serum albumin as standard (BSA). Quantification showed 0.075-0.250 μg/ml [15].

2.3.2 Electrophoresis and transfer

A 16 μl of the sample was mixed with 4 μl of 5x SDS loading buffer (4% w/v SDS, 20% v/v glycerol, 120 mM Tris, 5% v/v β-mercaptoethanol (β-ME)) and incubated at 90°C for 10 minutes. 15 μl of the sample were loaded on a 10-12.5% SDS-polyacrylamide gel and electrophorized at 110 V for 60-90 minutes in running buffer (200 mM glycine, 0.1% w/v SDS, 25 mM Tris). The gels were then transferred to a nitrocellulose membrane (Macherey-Nagel) at 400 mA for 60 minutes in transfer buffer (25 mM Tris, 200 mM glycine, 20% v/v MeOH). The membrane was then dried [15].

2.3.3 Immunoblotting

After the membranes had been dried, they were blocked in TBS (Tris buffered saline, 20mM Tris, 137mM NaCl) with 5% w/v skimmed milk powder for one hour. Primary incubation lasted three hours at room temperature or overnight at 4°C with a primary antibody solution consisting of blocking solution 0.1% v/v Tween (Sigma) and anti-I-CAM-1, anti-VCAM-1 (1:500, Santa Cruz) and anti-β-actin (1:10,000, Millipore).

The membrane was washed four times for five minutes in TBST (TBS w/ 0.1% v/v Tween), then incubated for three hours at room temperature and incubated with fluorescent

secondary antibody (1:20,000) for 1 hour at room temperature. After incubation, the membrane was washed four times for 5 minutes in TBST and scanned and analyzed in the Odyssey imaging system (Li-cor)[15].

2.3.4 Statistical analysis

All quantification of western band strength and western image processing was performed in Image Studio 2.0. All statistical analysis and statistical imaging was performed in PRISM Graphpad 6.0.

3 Results

3.1 HUVEC stimulation

To determine if chitin derivatives have an effect on the expression of inflammatory pathways, HUVECs were stimulated with an inflammatory agent for two hours before the substrates were applied. Cells were primed with LPS in two different strengths: 1 µg/ml and 100 ng/ml. The chitosan substrates administered were; chitosan, small chitosan, chitosan lactate and TChOS. This was done in three replicates on cells from three separate donors. ICAM-1 and VCAM-1 levels were measured via western blot to decide if stimulation with the substrate had an effect on respectable protein levels. *Figure 6* shows measured sample concentration which was determined with Bradford reagent method.

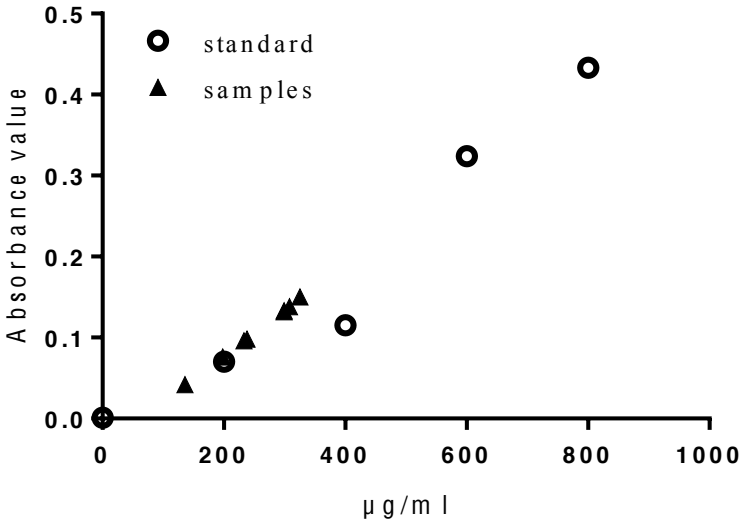

Figure 6: Bradford reagent protein quantification. Samples proved to be between 0.075-0.300 µg/ml.

After confirming the protein concentration (*figure 6)*, the protein was prepared for western blotting. Each sample was run through 12.5% SDS-PAGE gel to separate proteins according to size. Next the proteins were transferred to a membrane and immunoblotted with the according antibodies.

3.1.1 HUVEC induced with chitosan and ChOS without LPS stimulation

HUVECs were administered with chitosan and chitosan derivatives, as described. However, ICAM-1 and VCAM-1 signals were too low to detect (*figure 7*).

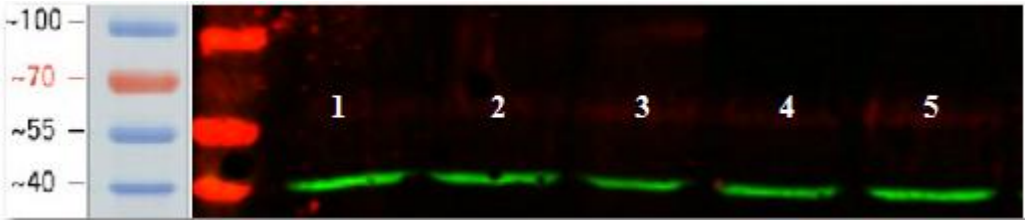

Figure 7: Immunoblot with antibody for ICAM-1 (110 kd) and β-actin (40 kd). HUVEC were not primed with LPS in this experiment. Samples put on wells are: 1 media only, 2 chitosan lactate, 3 small chitosan, 4 chitosan, 5 TChOS. ICAM-1 is not visible due to low signal strength.

3.1.2 HUVEC stimulated with LPS 100 ng/ml

After stimulating the cells with LPS, western blot was performed on samples where ICAM-1 was examined and normalized against β-actin (*figure 8*).

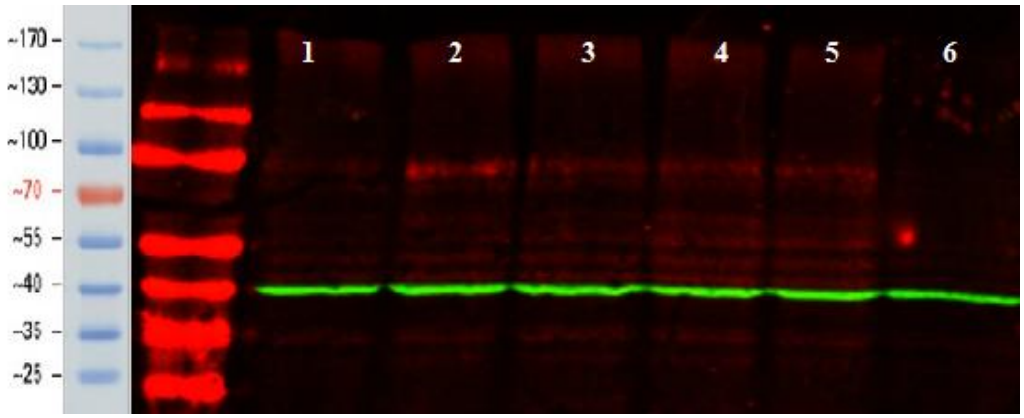

Figure 8: Immunoblot with antibody for ICAM-1 (110kd) and β-actin (40kd). HUVEC were primed with LPS (100 ng/ml). Samples put on wells are: 1 no substrate, 2 chitosan lactate, 3 small chitosan, 4 chitosan, 5 TChOS, 6 blank.

After ICAM-1 signals of samples had been normalized to β-actin with Li-Cor image studio 2.0, all samples were organized by donor (*figure 9*).

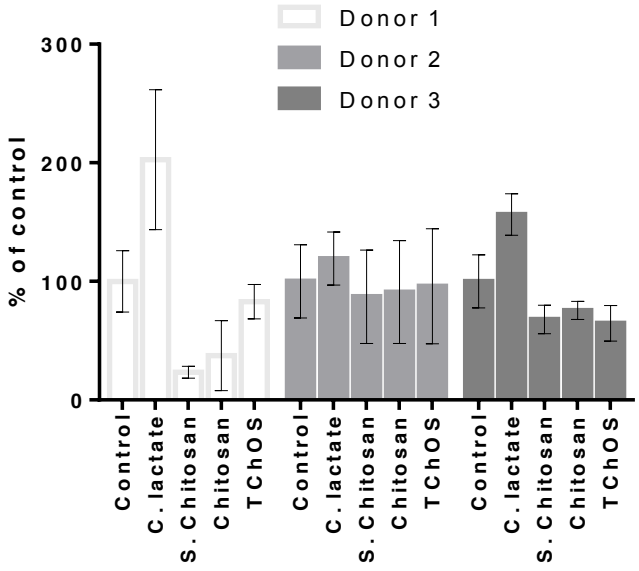

Figure 9: Effects of LPS stimulation (100 ng/ml) on ICAM-1 expressed in HUVEC from three donors. ICAM-1 signal is normalized to β-actin and then fixed to a percentage with control group used as standard. Bars represent standard error of the means. (N=3).

Figure 9 shows visible difference between different stimulations where it is clear that C. lactate increases ICAM-1 signal. Some variance can also be seen between donors. However, some western bands needed to be omitted due to low quality as it might affect results (*figure 10*).

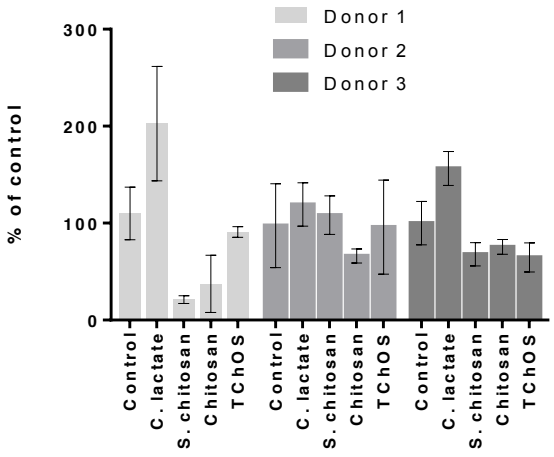

Figure 10: Effects of LPS stimulation (100ng/ml) on ICAM-1 expressed in HUVEC from three donors. ICAM-1 signal is normalized to β-actin and then fixed to a percentage with control group used as standard. Due to low quality of five western bands those samples were removed Bars represent standard error of the means. (N=3).

Figure 10 shows that by removing low quality samples, standard deviation decreases and significance thereby increases. *Figure 11* shows all samples measurements for the same substrate pooled together to look at the total difference.

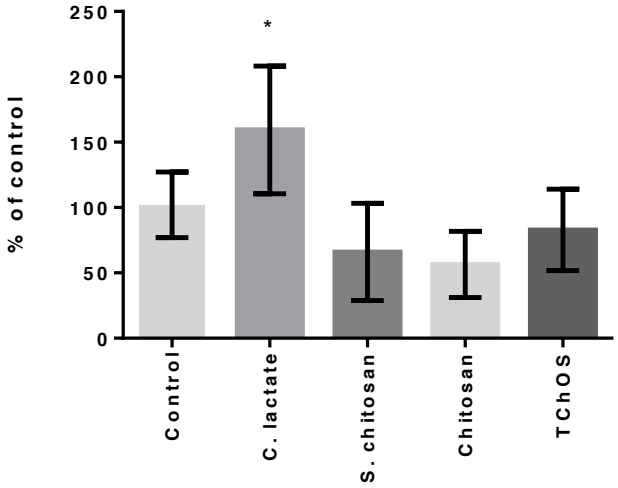

*Figure 11: Effects of LPS stimulation (LPS 100 ng/ml) on ICAM-1 expressed in HUVEC from three donors. All donors pooled, low quality samples omitted. Bars represent the standard error of the mean. * represents significant difference from control acquired by one way anova ($p<0.05$).*

Figure 11 shows notable difference in ICAM-1 signal strength depending on substrate used. C. lactate is the only substrate that shows significant difference from control ($p<0.05$).

Western blotting was also performed where VCAM-1 was examined. However due to little time and low initial total sample size, not all samples were blotted to look at V-CAM-1. *Figure 12* shows western blot on VCAM-1 and β-actin.

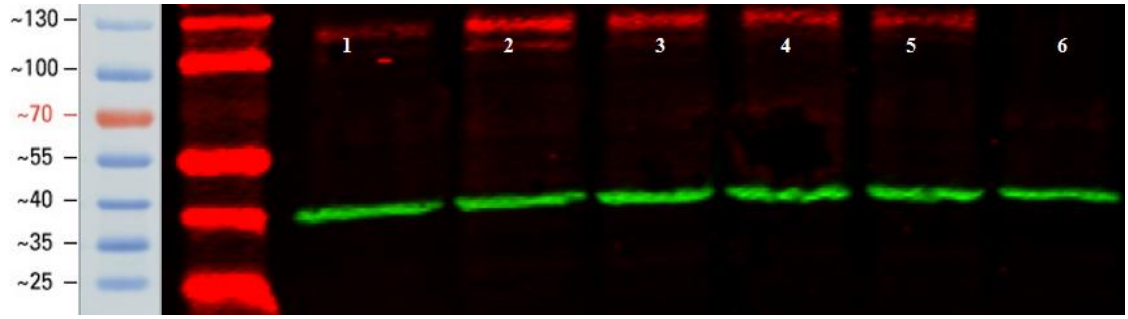

Figure 12: Immunoblot with antibody for VCAM-1 (110kd) and β-actin (40kd). HUVEC were primed with LPS (100 ng/ml). Samples put on wells are: 1 no substrate, 2 chitosan lactate, 3 small chitosan, 4 chitosan, 5 TChOS, 6 blank.

3.1.3 HUVEC stimulated with LPS 1 µg/ml

Figure 13 shows where western blotting is performed on samples from the same three donors, where LPS concentration was 1 µg/ml. *Figure 14* shows where ICAM-1 signals have been organized by donor as in chapter 3.1.2.

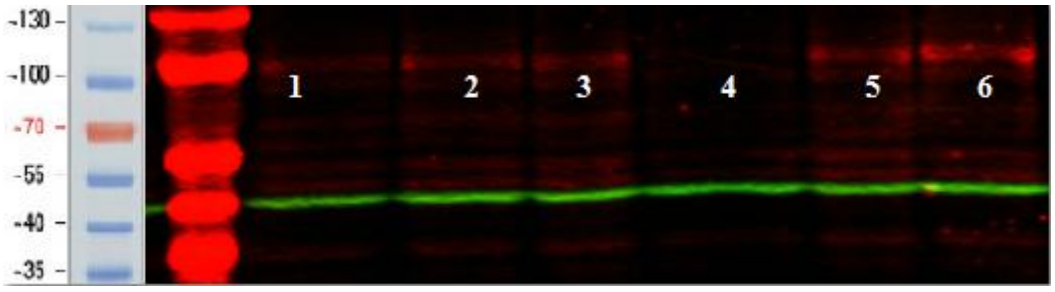

Figure 13: Immunoblot with antibody for ICAM-1 (110kd) and β-actin (40kd). HUVEC were primed with LPS (1 µg/ml). Samples put on wells are: 1 no substrate, 2 chitosan lactate, 3 small chitosan, 4 chitosan, 5 TChOS, 6 blank.

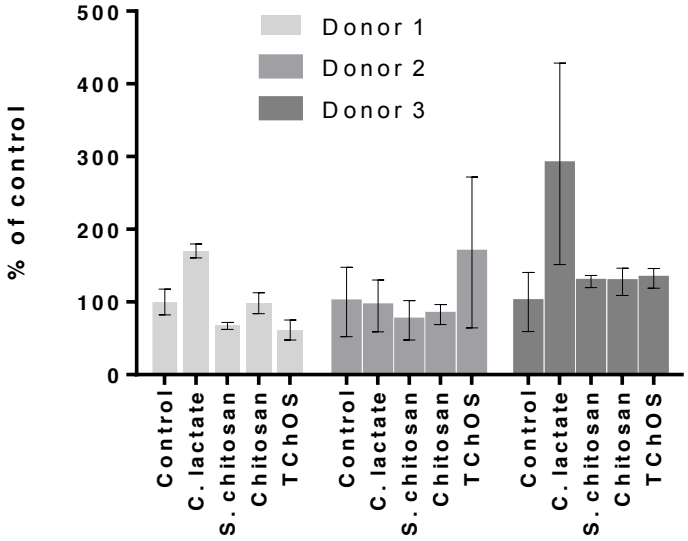

Figure 14: Effects of LPS stimulation (LPS 1µg/ml) on ICAM-1 expressed in HUVEC from three donors. ICAM-1 signal is normalized to β-actin and then fixed to a percentage with control group used as standard. Bars represent standard error of the means. (N=3).

Figure 14 shows a greater variance between donors than *figure 9* does. Less variance is detectable between different treatments. Some western bands needed to be omitted here as well due to low quality as it might affect results (*figure 15*).

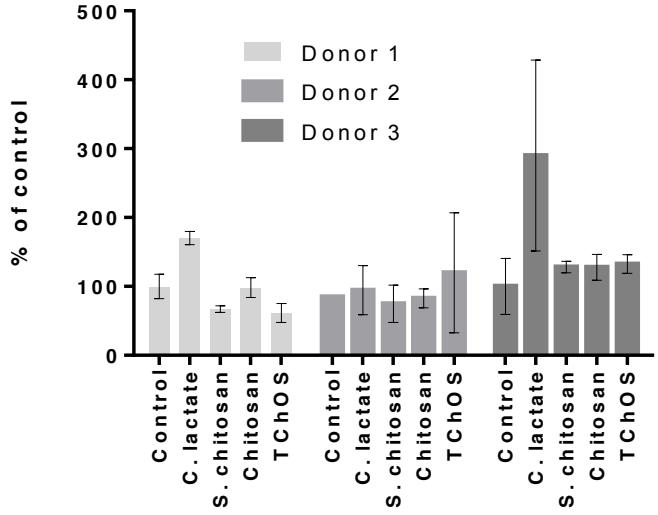

Figure 15: Effects of LPS stimulation (LPS 1µg/ml) on ICAM-1 expressed in HUVEC from three donors. ICAM-1 signal is normalized to β-actin and then fixed to a percentage with control group used as standard. Due to low quality western bands 4 samples were removed. Bars represent standard error of the means. (N=3).

By removing lower quality samples as done in *figure 15*, standard deviation of samples deceases. This leads to higher significance of measurements. *Figure 16* shows all samples measurements for the same substrate pooled together to look at the total difference.

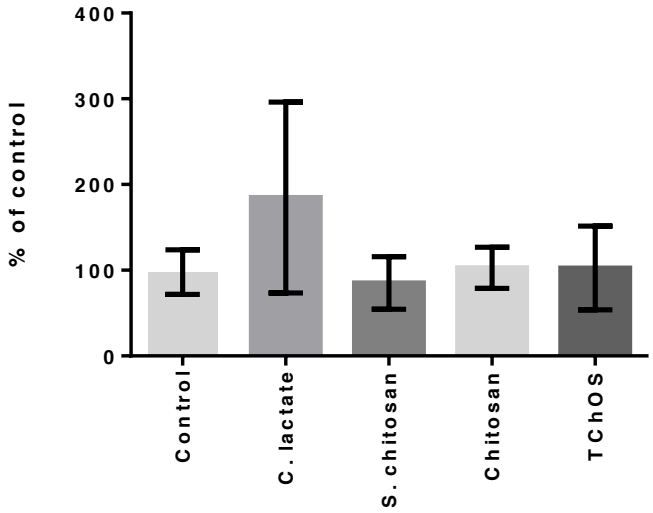

Figure 16: Effects of LPS stimulation (LPS 1µg/ml) on ICAM-1 expressed in HUVEC from three donors. All donors pooled, low quality samples omitted. Bars represent the standard error of the mean. One way anova did not show difference between substrates that can be interpreted as statistically significant ($p<0.05$).

Figure 16 shows limited difference between stimulations except for increase in C. lactate. This difference is however not statistically significant.

4 Discussion

The purpose of this project was to determine if chitosan oligosaccharides affected the expression of inflammatory proteins such as ICAM-1 and VCAM-1 in endothelial cells. LPS in two concentrations was used to induce inflammation.

4.1 Effects of chitosan and ChOS on HUVECs stimulated with LPS

The first experiment shows that the chitin derivatives did not induce ICAM-1 or VCAM-1 expression (*figure 6*). Positive control would have helped in determination of band strength. Previous studies [4 5 9 13] have shown that ChOS can have anti-inflammatory effects on HUVECs previously stimulated with LPS. The aim of this study was to look at the effect that ChOS have on ICAM-1 and VCAM-1 adhesion molecules in LPS primed cells. Some difference in ICAM-1 levels can be detected between ChOS substrates when all donors were pooled together at low LPS (100 ng/ml) stimulation (*figure 11*) with a one way anova test ($p<0.05$). Because of an overall lack in band signal, these results may carry low significance. No significant difference could be seen in ICAM-1 signal strength where LPS stimulation concentration was 1 µg/ml.

It may also be noted, that due to the effort put into examining ICAM-1, not enough samples were left to run statistical analysis of VCAM-1 as was planned in the beginning. Samples that were western blotted to examine VCAM-1 showed the same pattern as ICAM-1 (*figure 12*). These results may be interpreted as showing that these experiments did not show an anti-inflammatory effect.

By western blotting and using the Li-cor imaging system, ICAM-1 and VCAM-1 were successfully isolated and imaged. Results showed low signal and great variance in the adhesion molecule strength. This is interesting because the Bradford reagent measurement of protein concentration showed sufficient protein concentration in the samples. Furthermore, previous experiments done by Steinunn Guðmundsdóttir showed a much

stronger signal in western blotting analysis using the exact same protocols.

Determining the cause of the problems behind low protein signals in western blots took up a great deal of research time and samples. Protein extraction was examined and is a plausible explanation for weak ICAM-1 and VCAM-1 bands, although this is unlikely due to relatively strong actin bands, as well as high protein concentration in Bradford reagent tests. All samples were sonicated evenly and all samples had an equal amount of protease inhibitors according to the protocol and were exactly the same as those used to prepare samples by S. Guðmundsdóttir.

The preparation of the western itself is also a possible explanation to low strength in ICAM-1 and VCAM-1 bands. It is for example possible that the SDS loading buffer that was used did not work with larger proteins but better with smaller proteins as that would explain good actin bands and bad ICAM-1 and VCAM-1 which have more than double the molecular weight of the actin. This might also explain weaker bands from samples generated in this study next to strong bands from a previous study, as the old samples had been prepared with another SDS loading buffer. Other precautions were taken to try to determine the cause of low quality signals such as exchanging all solutions involved in the western blot protocol.

Samples from these experiments were compared to older similar experiment with known strong ICAM-1 signal prepared by S. Guðmundsdóttir. This was done to determine if something in later stages of the western blot was the cause of bad signals.

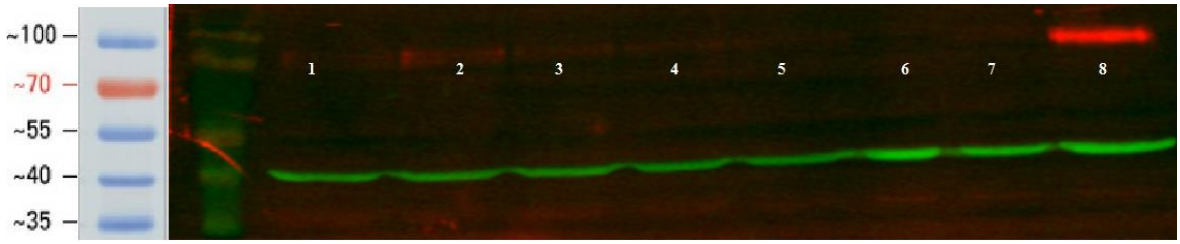

Figure 17: Immunostaining of a western blot for ICAM-1 and β-actin. Samples from this study in wells 1-7. Sample from a previous study done by S. Guðmundsdóttir in well 8. There is a clear difference between signal strengths.

As can be clearly seen in *figure 17*, the cause of the low signal is not due to any stages of the western blotting technique (with the exception of SDS loading buffer).

A plausible cause for low quality signals of adhesion molecules might be that cells used might have been insensitive and the inflammatory pathway might for some reason not have transcribed the adhesion molecules as highly as other donors before. That possibility could have been prevented if the HUVECs of many donors had been pooled together before being used for experiments. Another possibility is that the LPS used didn't have the same effects as it had previously, and because the samples were stimulated early in the process, it was not possible to retry and see if new LPS would have increased the signal of ICAM-1 and V-CAM-1. LPS calculations might also have been done wrong, but it is a highly unlikely cause as samples without LPS showed no sign of ICAM-1 and VCAM-1 as they were expected to do under normal circumstances.

4.2 Conclusion

Chitosan derivatives did not induce any changes in ICAM or VCAM on their own. Change in ICAM-1 expression was detected in HUVEC cells with added chitosan lactate, previously stimulated with 100 ng/ml LPS as shown in figure 10. Some difference was detected between other substrates and control groups at LPS strength of 100 ng/ml where small chitosan, chitosan and TChOS showed lower ICAM strength than the control group. However, this was not statistically significant. The experiments encountered some problems with signal strength and that might affect these results.

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Figure references

Figure 3: http://pubs.rsc.org/en/content/articlelanding/2004/cp/b312189e#!divAbstract [Obtained 5 May]

Figure 4: (original figure edited) http://eoncosurg.com/inhibition-of-canonical-nf-kb-and-uppression-of-inflammation-and-cancer-growth-by-designed-inhibitor-dhmeq [Obtained 3 May]