The Effect of Vytorin on Intracellular Lipid and Inflammation in Obese Subjects

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Abstract

Following the first demonstration by our group that macronutrient (glucose, cream and a high fat high carbohydrate meal) intake results in increased ROS generation and oxidative stress at the cellular and molecular level, we have now shown in our preliminary data that cream intake induces comprehensive inflammation as reflected in increased intranuclear NFκB binding, decreased IκBα expression, increased expression of IL-1β, IL-12, TNFα and other pro-inflammatory mediators. While carrying out these experiments, we asked whether cream intake was associated with an uptake of lipid by peripheral blood mononuclear cells (MNC). Indeed, there was a significant increase in intracellular lipid which was visualized as intracellular lipid droplets. The increase in intracellular lipid droplets was associated with an increase in intracellular superoxide generation; the expression of CD68, a marker for macrophages; and PECAM, the adhesion molecule which mediates trans-endothelial transfer of leucocytes. We also found that the lipid fractions to increase were cholesterol ester, triglyceride and fatty acids. In view of the tantalizing observation that the lipid droplet laden MNC appeared to be monocytes, looked like foam cells and the fact that CD68 expression had increased, there is a possibility that foam cells may be formed in peripheral circulation by monocytes after a lipid rich meal. This simple model of foam cell formation also lends itself for the study of the effect of various lipid lowering drugs. Our investigation will be the first to study this novel paradigm. We plan to study the effect of a cholesterol lowering agent, Vytorin (simvastatin and ezetimibe), on intracellular lipid in MNC, expression of CD68 and PECAM, ROS generation and inflammation in obese subjects. This investigation may provide an additional mechanism of action by which these drugs may reduce atherosclerosis.

SPECIFIC AIMS

Our recent work shows that following the intake of cream there is an increase in intracytoplasmic lipid and evidence of cellular inflammation in mononuclear cells (MNC) and monocytes. The main objective of this research is to investigate the effect of Vytorin (simvastatin and ezetimibe) on intracellular lipid accumulation and inflammatory mechanisms at the cellular and molecular level in the monocytes of humans, *in vivo*.

Hypothesis 1 (primary objective): Vytorin (simvastatin and ezetimibe) can decrease the accumulation of lipids in the circulating monocytes of obese subjects.

Aim 1.1: To study the effect of dairy cream (33 g) intake on the accumulation of cholesterol esters, cholesterol, triglycerides, fatty acids and phospholipids in the monocytes of obese subjects with LDL ≥100mg/dl before after 6 weeks of therapy with Vytorin or placebo.

Aim 1.2: To investigate the effect on cream intake on monocyte activation markers including CD68 and PECAM before and after 6 weeks of above mentioned drugs.

Hypothesis 2 (secondary objective): Dairy cream intake causes an acute oxidative and inflammatory response at the cellular and molecular level. This inflammatory response can be decreased after therapy with Vytorin .

Aim 2.1: To measure the activity and the expression of pro-inflammatory transcription factor NF-κB and its activating proteins, IκB Kinase β (IKKβ); and the expression of pro-inflammatory NF-κB-regulated genes and proteins including IL-1β, IL-12, TNFα in normal subjects after the intake of dairy cream before and after Vytorin or placebo for 6 weeks on obese subjects.

Aim 2.2: To measure the oxidative stress in MNCs induced by cream challenge before and after 6 weeks of Vytorin.

Hypothesis 3 (secondary objective): The intake of cream induces an increase in the expression of toll like receptor and the mediators of TLR signal transduction, and suppressor of cytokine signaling (SOCS)-3 in MNC and monocytes. This response can be decreased after therapy with above mentioned drugs.

Aim 3.1: To measure the expression of TLR-2, TLR-4, MyD88, SOCS-3 and TRIF in MNC and monocytes after cream intake in obese subjects before and after Vytorin or placebo for 6 weeks.

Background

We have previously demonstrated that the intake of macronutrients including glucose, cream and a high fat high carbohydrate (HFHC) fast food meal induces oxidative stress as reflected in an increase in ROS generation and an increase in plasma TBARS concentration(1). Furthermore, there is an increase in intranuclear NFκB binding and a decrease in IκBα, with an increase in IKKα and IKKβ(2). Clearly, therefore, these macronutrients induce post prandial oxidative stress and inflammation. These observations have potential implications in terms of the relationships between macronutrient intake and atherogenesis since post prandial glycemia and lipemia have been implicated in this process. In this respect, it is also important that the oxidative stress and inflammation induced by a HFHC meal is of a greater magnitude and lasts for longer in the obese than in normal subjects(2). In addition, our work has also shown that the obese start with a higher level of basal (fasting) oxidative and inflammatory stress than normal subjects. Since T2D patients have higher basal levels of oxidative and inflammatory stress, it is likely they will respond to macronutrient challenge with greater levels of oxidative and inflammatory stress. In this respect it is also relevant that equicaloric meals composed of fruit and fiber do not induce oxidative and inflammatory stress. Similarly, orange juice and alcohol do not induce either oxidative or inflammatory stress(3).

Our experiments with cream have shown that its intake induces not only an increase in ROS generation but also lipid peroxidation products in plasma (TBARS). Our recent data show that cream intake induces an increase in NFκB binding, IKKα and IKKβ activity **(Figure 1)** and an increase in the expression of several pro-inflammatory cytokines including IL-1β, IL-12 and IL-18 **(Figure 2)**. It also induces an increase in another pro-inflammatory transcription factor, Egr-1, and c-fos **(Figure 3)**. c-fos is a component of the transcription factor, AP-1 which regulates the expression of matrix metallo-proteinases (MMPs). Indeed, MMP-9 increases after cream intake and a HFHC meal (2). The pro-inflammatory effect of cream lasts for more than 3h.

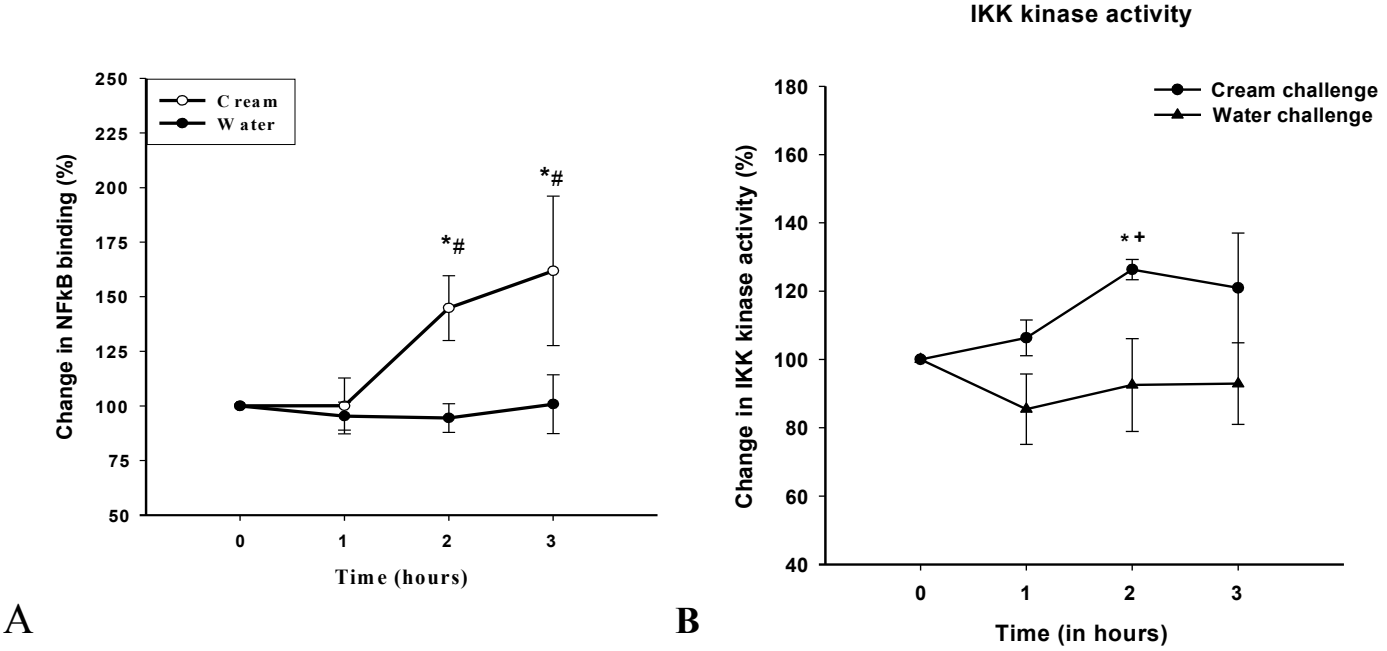

Figure 1: Percent change in NFκB (A) and IKKβ (B) activities in MNC following cream intake. N=12, * $P<0.05$ by One Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus the baseline, # $P<0.05$ by Two Way Repeated Measures ANOVA for comparison versus control group.

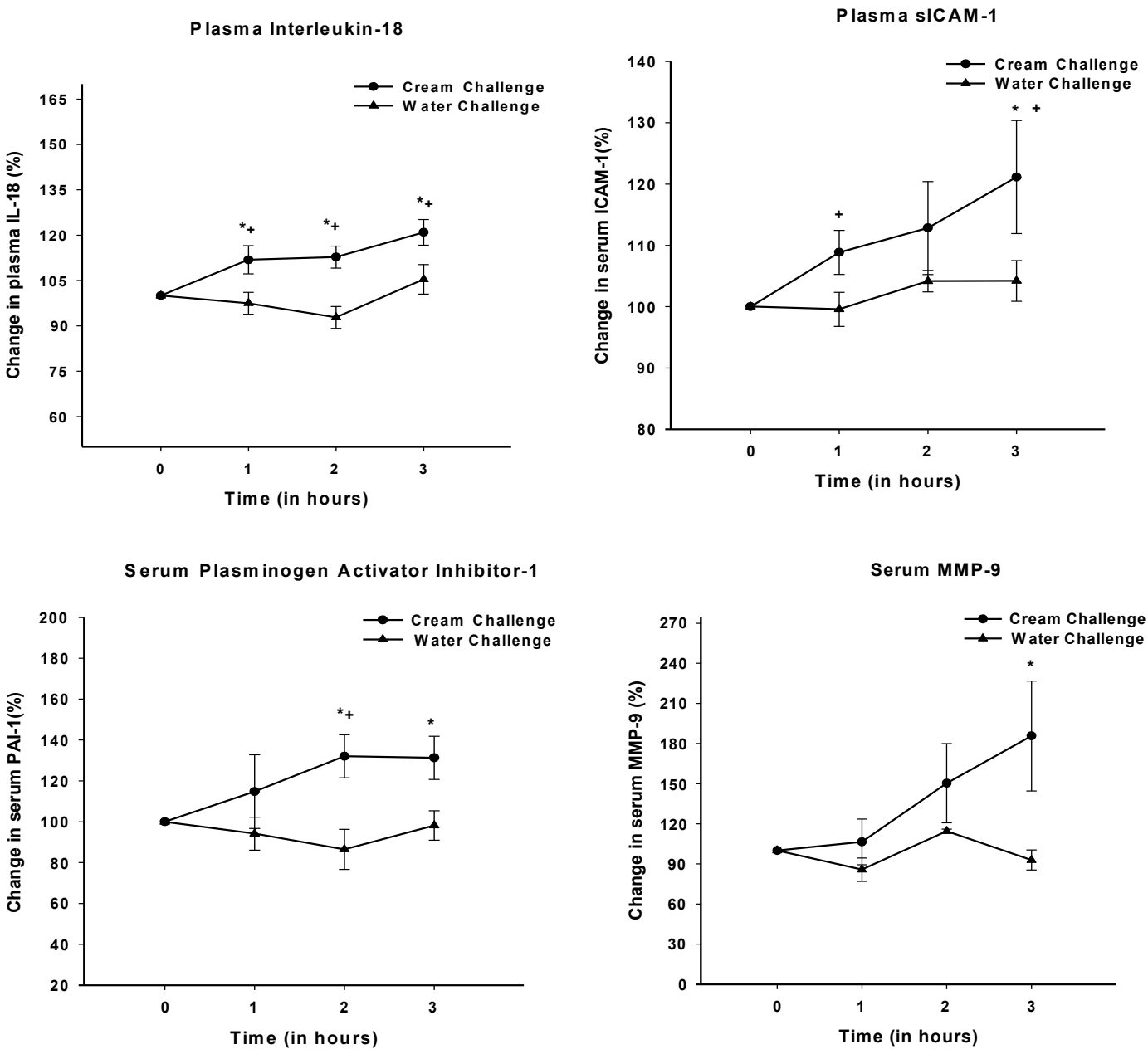

Figure. 2: Percent change in plasma/Serum levels of various mediators: Graphical representation of values obtained by ELISA for IL-18 (A), ICAM-1 (B), PAI-1 (C) and MMP-9 (D). All values are presented as mean±SE. * $P<0.05$ by One Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus the baseline, $^{+}$ $P<0.05$ by Two Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus control group.

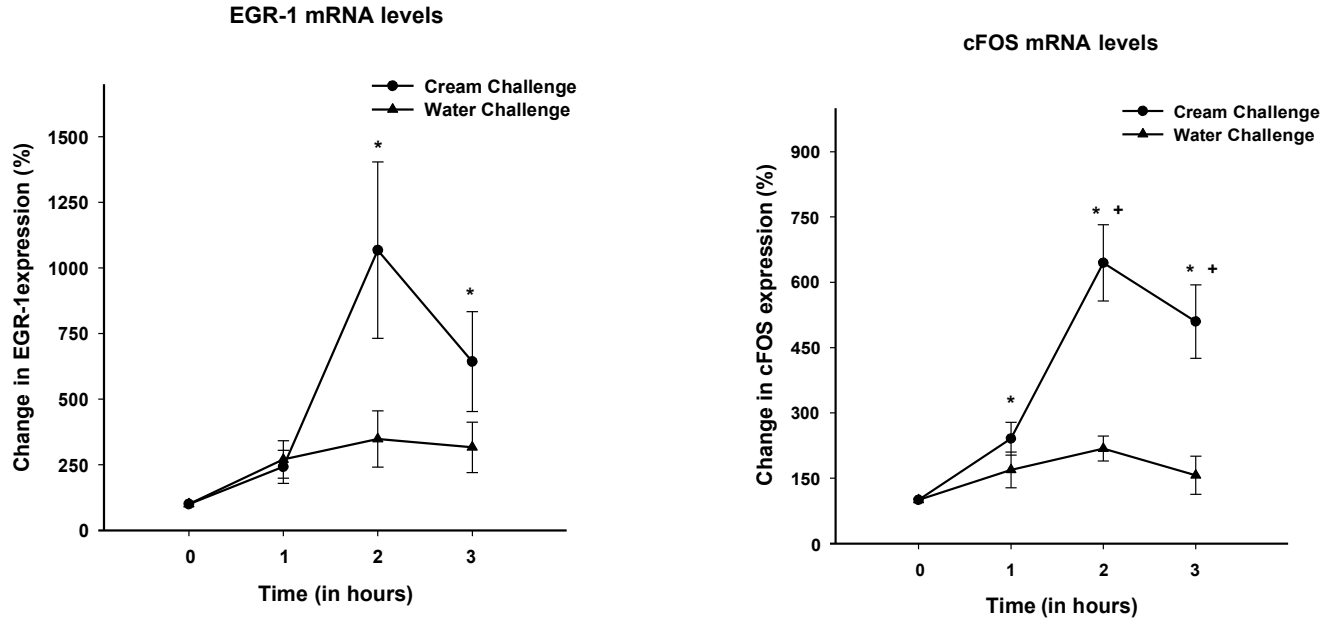

Figure 3ercent change in mRNA levels of Egr-1 and c-fos by RT-PCR: All values are presented as mean±SE. n=12. * $P<0.05$ by One Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus the baseline, $^{+}$ $P<0.05$ by Two Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus control group.

In our most recent experiments, we have also shown that following the intake of cream the expression of TLR-4 **(Figure 4A)** is increased both at mRNA and protein level. This increase is observed at 1h and is sustained for at least 3h. Similarly, there is an increase in the expression of SOCS-3 following cream intake both at mRNA and protein levels **(Figure 1B)**. This combination of effects is important since it has implications not only in terms of post prandial inflammation but also for a potential role in the pathogenesis of insulin resistance. The selective deletion of each of these two proteins leads to a protection from diet induced obesity related insulin resistance.

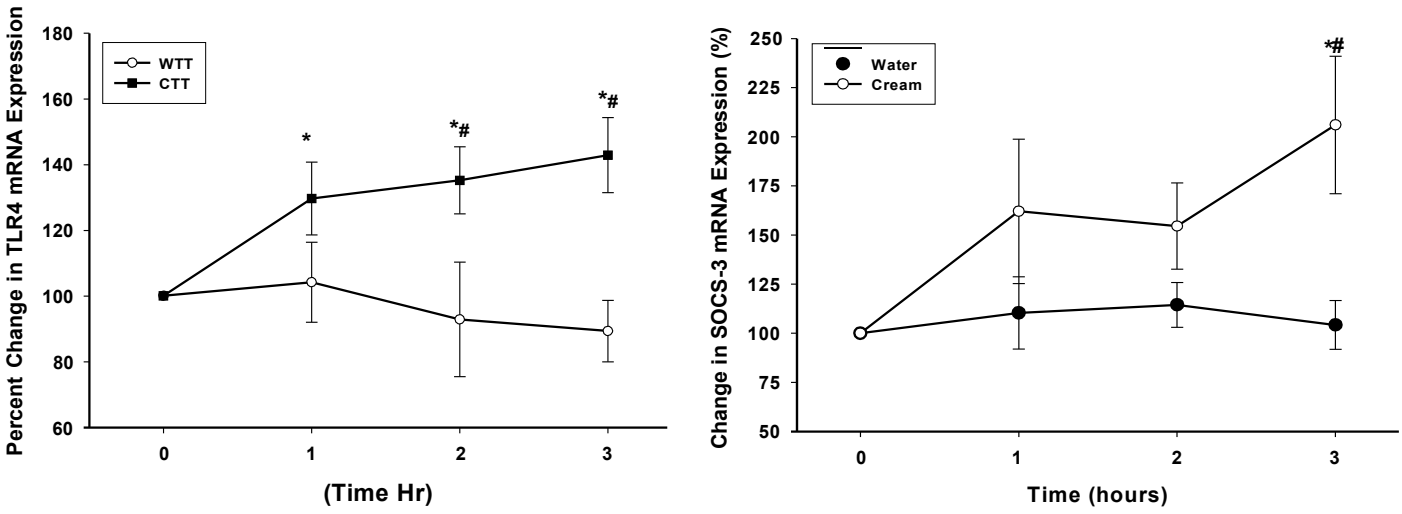

Figure 4: Percent change in mRNA levels of TLR4 and SOCS-3 by RT-PCR: All values are presented as mean±SE. n=12. * $P<0.05$ by One Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus the baseline, $^{\#}$ $P<0.05$ by Two Way Repeated Measures ANOVA followed by Holm-Sidak method for comparison versus control group.

During our experiments with cream exploring its effects on oxidative and inflammatory stress, we asked whether its intake would increase intracellular lipid in MNC. Indeed, we were able to demonstrate an increase in intracellular lipid in MNC both chemically and microscopically **(Figure 5-6)**. These dramatic changes raised important issues. Firstly, it was important to define whether this increase was in monocytes, the cells which infiltrate the arterial intima to form foam cells by taking up lipid, and thus forming the fatty streak. The second was to define the type of lipid contained in these cells. We also asked whether these cells expressed more CD68, the characteristic marker for macrophages and whether they expressed more of PECAM, the adhesion molecule which is cardinal to the process of trans-endothelial transfer of leucocytes. Indeed, there was an increase in the expression of CD36, CD68 and PECAM. These findings were indicative of an increase in the ability of these cells to take up lipid, to transform to the macrophage phenotype and to develop the key adhesion molecule which would potentially facilitate their trans-endothelial transfer of these cells into arterial wall.

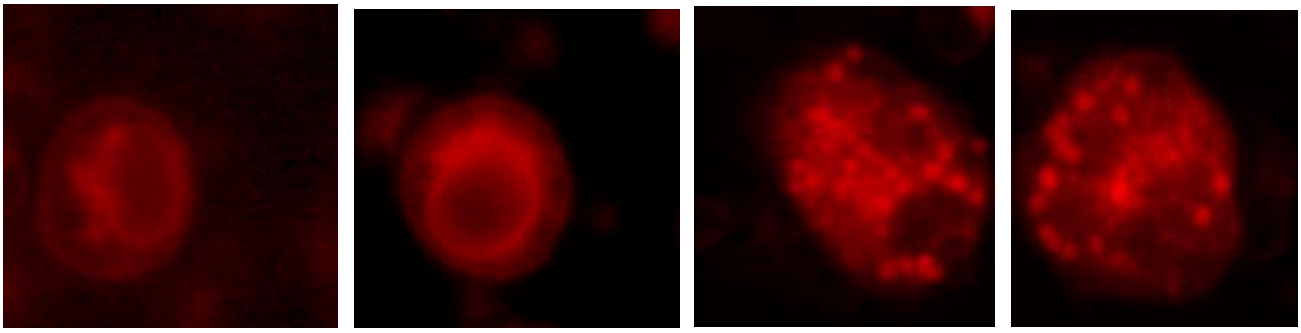

Figure 5: Lipid droplet accumulation in MNC after cream intake in normal subjects. Nile Red stain of MNC is shown before (0 hour) and after (1, 2 and 3 hours) lipid intake. At the indicated times, cells were fixed and stained for lipid. This experiment was performed 6 times. Peak lipid accumulation was at 2h. Results from one experiment are shown.

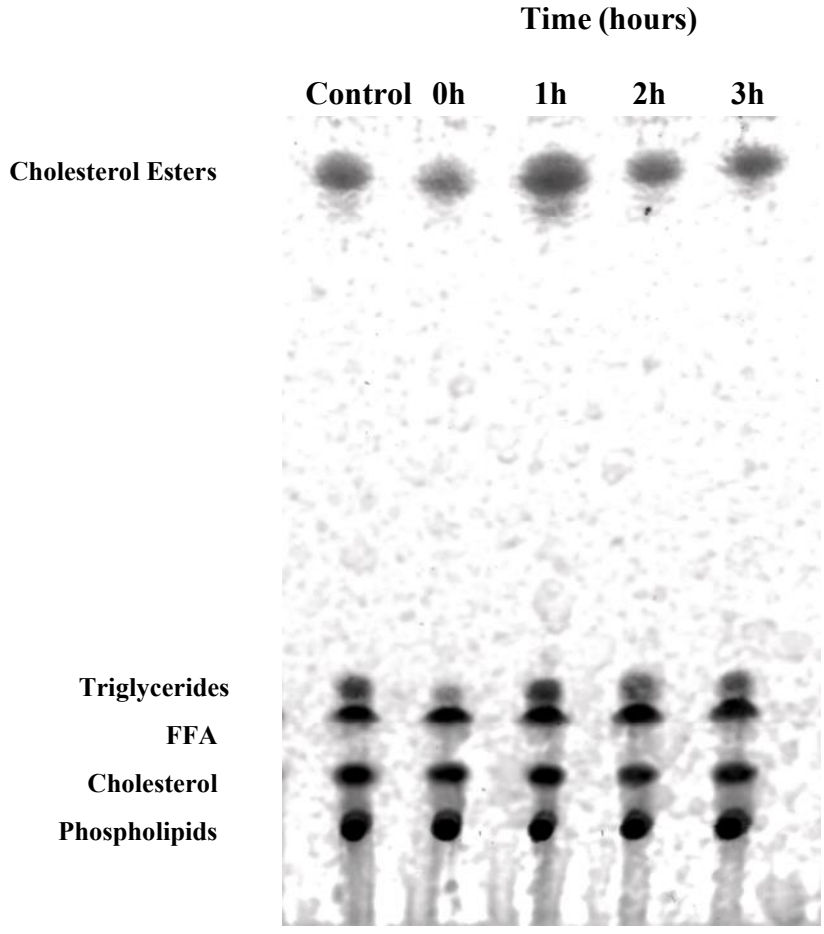

Figure. 6: Intracellular accumulation of lipids in the MNC analyzed by Thin Layer Chromatography (TLC): A representative TLC of lipids extracted from the MNC at the baseline and following cream challenge in one of the subjects. Lane 1 is the pooled control sample, lane 2 represents intracellular lipids at the baseline and lanes 3, 4 and 5 represent intracellular lipids at 1, 2 and 3 hours following the cream intake.

Our most recent experiments have also shown that following cream intake, monocytes separated from the MNC by magnetic beads coated with CD14 antibody show lipid loading, inflammation and an increase in the expression of CD68 and PECAM **(Figure 7)**. The non-monocytic fraction of MNC does not demonstrate significant lipid intake. Furthermore, the lipid laden monocytes have the typical appearance of the foam cell. We are not certain of the fate of this cell in circulation and whether one important destination for this cell is the atherosclerotic plaque (MCP-1 and CCR-2). This needs to be investigated further. However, in the mean time, the possibility that this 'foam cell' formed after the intake of cream and possibly other fatty meals, is a potential resident of the atherosclerotic plaque has to be entertained. After all, the concept that the uptake of lipid by monocyte/macrophages occurs only in the arterial wall after the arrival of both the cell and LDL-C in the arterial intima, is a product of a series of experiments conducted, in vitro, followed by a series of hypotheses to define a potential mechanism. Our data do not exclude the possibility that lipid may be taken up in the atherosclerotic plaque. However, they seriously raise the possibility that the foam cell may be formed post prandially in the circulation.

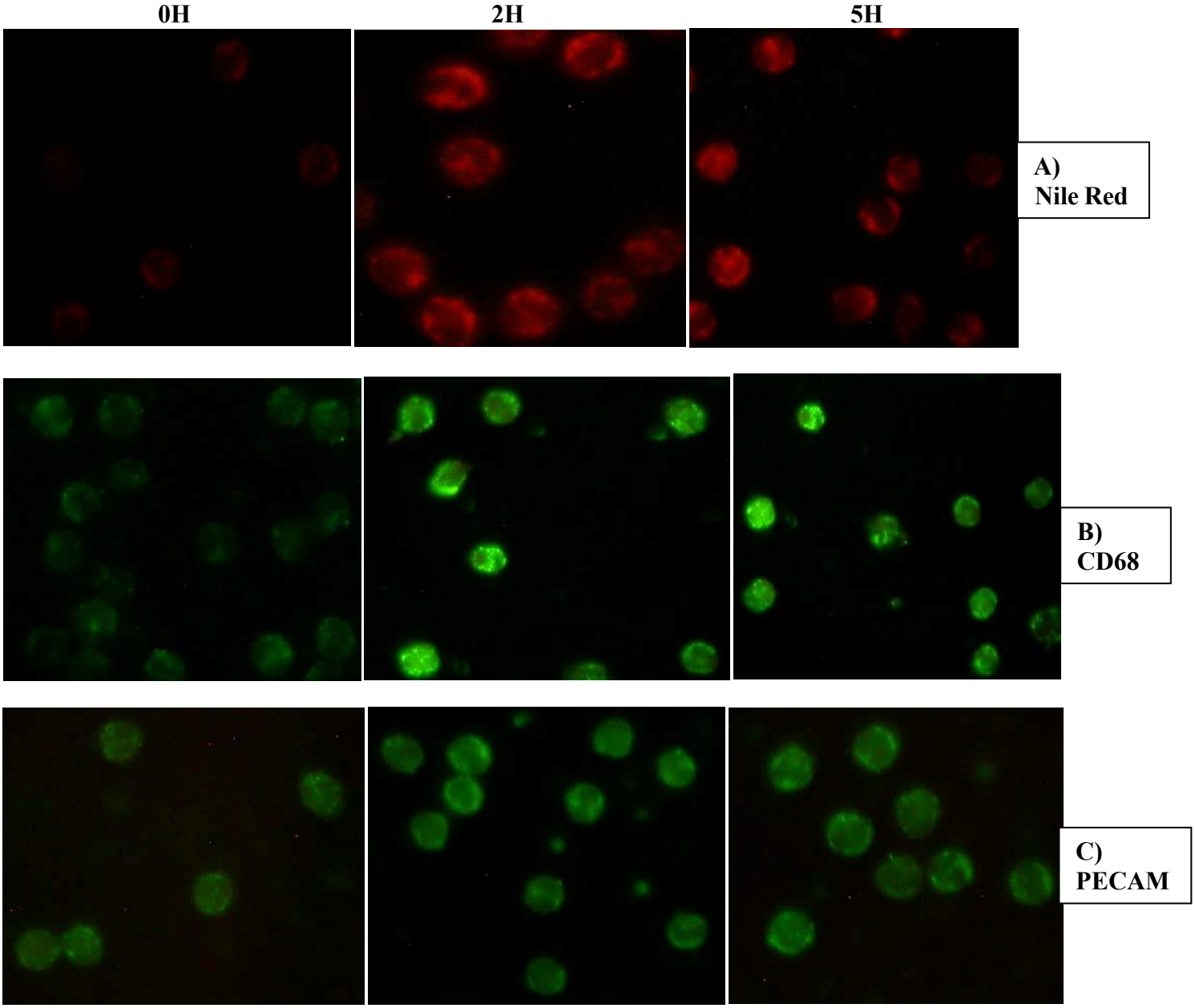

Figure 7: Lipid accumulation and activation of Monocytes before and at 2H and 5H following cream intake in normal subjects. A) Nile Red stain of monocytes, B) immunohistochemistry staining of CD68 and C) immunohistochemistry staining for PECAM. Data representative of N=8.

Cardiovascular disease is currently the leading cause of death in the developed countries (4). Atherosclerosis is the most important cause of cardiovascular disease. It is a chronic inflammation of the arterial wall. Vascular disease, including in the heart and brain, is associated with monocyte activation and differentiation to macrophages which infiltrate into the intima of arteries. Activated monocytes/macrophages are capable of uptaking native and modified lipids from the surrounding matrix and transform to foam cells (lipid-laden macrophages). Aggregates of foam cells form fatty streaks which evolve to a more complex atherosclerotic lesion which eventually leads to clinical manifestations. This process involves the participation of many cell types including leucocytes, endothelial cells and smooth muscle cells secreting many compounds and enzymes that participate in the progression of the atheroma.

The current screening tests for vascular disease are not sensitive enough to detect vascular disease in its early stages. Most of the current technology including EKG stress test, thallium stress test, ultrasound, angiography, CT scans and MRI are not capable of detecting micro-atherosclerotic lesions or evaluate foam cell formation, the crucial step in atherogenesis. Thus, an early and direct screening test is needed to identify subjects with risk of developing vascular disease years earlier than other technologies so the proper prevention/treatment programs can be initiated. Plasma lipid levels are one of the risk markers for vascular disease since higher plasma lipids, especially low-density lipoprotein cholesterol (LDL-C), lead to increased lipid infiltration to the intima where it will be uptaken by activated monocytes/macrophages and begins the transformation to foam cells. Although low-density lipoprotein cholesterol (LDL-C) remains the primary target for vascular disease prevention, many individuals who have vascular disease do not have substantially elevated LDL-C. This indicates that plasma lipids alone do not necessary reflect increased foam cell formation in the intima and therefore, atherosclerosis. Thus, there is a need for other biomarkers that can reflect the events occurring in the intima and is crucial for early detection and prevention of vascular disease.

Postprandial lipemia, a state characterized by increased triglycerides and triglyceride rich lipoproteins (TRLP) is related to an increased risk of atherosclerosis and cardiovascular disease (5). The mechanisms underlying this association are not clearly understood. Part of this association can be explained by the effect of TRLP on the vasculature. Vogel et.al. found that intake of a single fatty meal containing 50 g fat and 900 kcal impairs flow-mediated vasodilatation (6). A single high fat meal containing 50 g fat and 760 kcal stimulated endothelial expression of adhesion molecules in normal healthy subjects(7). Furthermore, hypertriglyceridemia also seems to affect haemostatic variables. TRLP induce the production of antifibrinolytic, PAI-1 from cultured human umbilical vein endothelial cells (8). The activity of coagulation factor VII increases in response to TRLP remnants (9). A few studies have examined the effect of a fatty meal or lipid particles on the leukocytes. A challenge with 33 g of dairy cream has been shown to induce an increase in reactive oxygen species (ROS) generation by MNC, similar to an equicaloric amount of glucose (1). The incubation of macrophages with chylomicrons and VLDL from 15 hypertrigylceridemic subjects resulted in the conversion to cells similar to foam cells seen in the atherosclerotic plaque (10). But the acute effect of lipids intake on the circulating MNC or monocytes, in particular on intracellular lipid accumulation and inflammation is not known.

Inflammation at the cellular level can be described as an increase in the intranuclear binding of the proinflammatory transcription factor NFκB to promoters of pro-inflammatory genes like TNFα. Many different stimuli have been shown to activate NFκB, such as cytokines, oxidants, viruses, immune stimuli, and lipopolysaccharides (11). Activation of NFκB has also been implicated in atherosclerosis (12). The genes regulated by NFκB include cytokines (e.g. IL-1β, IL-18), chemokines (e.g. MCP-1, MIF), adhesion molecules (e.g. ICAM, VCAM), metalloproteinases (e.g. MMP-1, MMP-9). Other transcription factors which are involved in inflammation include Egr-1 and AP-1. Egr-1 is a rapidly and transiently induced by a variety of extracellular stimuli related to hypoxia and vascular injury, growth factors, cytokines, and physical damage to the blood vessels (13-16). It induces the transcription of TNF α, ICAM-1, PAI-1, TF, TGFβ, and MMPs. Egr-1 also regulates tissue factor (TF) expression, which is a cell surface receptor of coagulation factor VII and initiates the extrinsic pathway of coagulation (15). AP-1 modulates transcription of various inflammatory genes including MMP-9 (17). MMP-9 hydrolyses extracellular matrix components and allows the spread of inflammation. MMP-9 has also been implicated in atherosclerotic plaque destabilization (18).

Toll-like receptors (TLRs) activate intracellular signaling pathways that lead to the induction of inflammatory cytokine genes such as TNF alpha, IL-6, IL1 beta and IL-12 in response to binding of microbial and foreign products including lipopeptides and peptidoglycans from Gram positive bacteria and endotoxin

from gram negative bacteria. TLRs activation stimulates the recruitment of a set of intracellular TIR-domain-containing adaptors, including MyD88, TRIF. Recruitment of MyD88 leads to activation of MAP kinases and transcription factor NFκB to control the expression of inflammatory genes. It has been shown that high fat diet induces insulin resistant in mice in a TLR4 dependent fashion. We would like to investigate the effect of lipid lowering drugs on cream intake induced increase in TLRs and downstream signaling.

Our preliminary data demonstrate that monocyte transformation to foam cell begins in the circulation in response to increased inflammation and oxidative stress that activates lipid uptake and/or de novo synthesis. These activated-lipid laden monocytes will then migrate to the subendothelium and uptake more lipids and progress to form the atherosclerotic lesion. This observation represents an early stage in atherogenesis and can be used, therefore, as an early screening test for vascular disease. Other leucocytes, including T-cells and B-cells, are also recruited to the injury site and participate in the process of plaque formation. Determination of intracellular levels monocytes and lymphocytes, therefore, represents a direct and early marker of monocyte/macrophage activation level and the process of foam cell formation and therefore, the risk for atherosclerosis and vascular disease. The current proposal provides a method for the determination of post cream challenge levels of intracellular lipids in monocytes as a mechanistic model for vascular disease.

Significance of the Study

1. Clinical relevance. Since the formation of the foam cell is potentially important in atherogenesis, it would be important to determine whether drugs that lower LDL cholesterol and atherogenic risk decrease formation of these foam cells.
2. Mechanistic and biological/pathological significance. The confirmation of our preliminary data in a larger study is important since these data provide the first evidence that the formation of the foam cell may occur in circulation and that the monocyte may get loaded with lipid after every fatty meal. This may potentially contribute to atherogenesis. This study may provide a model to study novel mechanisms of action of lipid lowering drugs.

Study Sample:

This will be a double blind, prospective study conducted in 20 qualifying volunteers. Subjects will be recruited from the hospital and university. All subjects will sign a written informed consent approved by the institutional review board of SUNY at Buffalo. Women and members of minority groups will be encouraged to participate in this study and efforts will be made to recruit subjects from these groups.

Subject Assignment:

Experiments will be carried out in obese subjects. There will be 2 groups in the study:-
Group 1 will receive Simvastatin 40 mg and Ezetimibe 10 mg daily combination pill (Vytorin) for 6 weeks
Group 2 will receive a matching placebo pill for 6 weeks

Randomization:

Subjects will be randomized to 2 groups with 10 patients in each using a computer generated randomization chart.

Group 1 will be getting vytorin 40/10 mg daily for 6 weeks
Group 2 will receive placebo daily for 6 weeks.

Inclusion Criteria for all groups:

- Age: 18 to 65 years of age.
- Obese (BMI $\geq$30 kg/m^2)
- LDL cholesterol of $\geq$100 mg/dl
- Subject will be available for duration of the study and willing to comply with all study requirements
- Written and informed consent signed and dated
- Not taking any vitamins or antioxidants

Exclusion Criteria*:*

- Currently using anti-hyperlipidemic therapies
- Triglycerides >500 mg/dl.
- Myocardial infarction, angioplasty/stent placement or coronary artery bypass surgery in the past 6 months
- Patient on chronic use of non-steroidal anti-inflammatory drugs or steroids
- Hepatic disease
- Renal impairment
- History of drug or alcohol abuse
- Participation in any other concurrent clinical trial
- Use of an investigational agent or therapeutic regimen within 30 days of study.
- Smoker
- Pregnancy
- Premenopausal women who are not on birth control pills and have not had a hysterectomy or tubal ligation
- Anemia with hemoglobin <12 g/dl

RECRUITMENT OF SUBJECTS:

The recruitment into the study will be carried out by the following methods:

1. Patients for the Diabetes-Endocrinology Center of WNY Clinic
2. Diabetes-Endocrinology Center of WNY - Research Division - Recruitment Database

Accrual Rate: On an average, we plan to recruit 1 patient per week.

All subjects will have completed the following procedures prior to participating in the study.
1) Medical History;
2) Physical Exam
3) Informed consent
4) Baseline lab draws to measure CBC, SMA and lipid profile. All labs will be drawn in the fasting state in the morning before 10am.

Informed Consent

Study process and required visits will be explained to patient in lay terms. All subjects will be asked to sign a consent form approved by IRB and a copy given to all subjects.

STUDY PROCEDURES

Subjects will be randomized to receive Vytorin or matching placebo for 6 weeks.

Visit 1 at week 0: Subjects will arrive after having fasted (10 hour) overnight. A fasting blood sample will be taken, followed by cream challenge and blood sample will be drawn 1, 2, 3 and 5 hours after the cream challenge. **Cream challenge**: Subjects will be given 100 mL of dairy cream (gourmet heavy whipping cream, Land 'O' Lakes Inc., Arden Hills, MN) to be consumed over 10 minutes. This preparation of dairy cream has 33 g fat (300 Calories) per 100 mL.

Subjects will then be given a supply of their drug to be taken once a day at bedtime.

Visit 2 at week 3: Subject will come fasting for a short visit for a blood draw at 4 weeks.

Visit 3 at Week 6: A fasting blood sample will be taken, followed by cream challenge same as the 1st visit and blood sample will be drawn at 1, 2, 3 and 5 hours after the challenge. Subjects will then be discharged from the study.

COMPENSATION FOR PARTICIPATION:

To help defray the costs of your participation, you will receive $150.00 for completing the entire study. In the event that you do not complete the entire study, you will receive $60.00 at Visit 1 and Visit 3 each, and $30 for Visit 2. You will not be paid for the screening visit. . Please be aware that occasionally additional visits are

required, for safety evaluations or to repeat blood test, and that these visits are not a part of the 3 required visits. There will be no additional compensation for extra visits.

METHODS:

1) **MNC and PMN isolation by Ficoll-hypaque method:** Blood samples will be collected in Na-EDTA as an anticoagulant. Three and a half mL of anticoagulated blood sample are carefully layered over 3.5 mL of PMN medium (Cedarlane Laboratories, Hornby, ON). Samples are centrifuged and at the end of the centrifugation, two bands separate out at the top of the RBC pellet. The top band consists of MNC, while the bottom consists of PMN. The MNC band is harvested and washed twice with Hank's balanced salt solution (HBSS). This method provides yields greater than 95% pure PMN and MNC suspensions.
2) **Monocytes isolation by magnetic beads.** Monocytes will be isolated from MNC fraction by magnetic beads coated with CD14 antibodies (Maltyni Biotec, Auburn, CA) .
3) **NF-κB activation:** Nuclear NF-κB DNA binding activity will be measured by EMSA. DNA-binding protein extracts will be prepared from monocytes by high salt extraction as described by Andrews et al (19). NF-κB gel retardation assay will be performed using double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NF-κB binding site is radiolabeled with γ-P^{32} by T4 kinase. Then, 5
   μg of the nuclear extract will be mixed with the incubation buffer and the mixture is pre-incubated at 4° C
   for 15 minutes. Labeled oligonucleotide (60,000 cpm) is added and the mixture is incubated at room temperature for 20 minutes. Samples will be then applied to wells of 6% non-denaturing polyacrylamide gel. The gel is dried under vacuum and exposed to X-ray film. The specificity of the band is established by super shift EMSA using antibodies against p65 and p50 proteins. Negative and positive controls will be also used to confirm the specificity of the bands.
4) **Western blotting:** MNC total cell lysates are prepared as and electrophoresis and immunoblotting will be carried as described before (20). Polyclonal or monoclonal antibodies against TLR4 (Abcam, Cambridge, MA), CD68, (Transduction labs, CA), PECAM, IKKβ and actin (Santa Cruz Biotechnology, CA) are used and the membranes are developed using super signal, chemiluminescence reagent (Pierce Chemical, IL). Densitometry is performed using molecular analyst software (Biorad, CA) and all values are corrected for loading with actin.
5) **ROS generation measurement by chemiluminescence as an index of NADPH oxidase activation:** Five hundred μL of PMN or MNC (2×10^5 cells) will be delivered into a Chronolog LumiAggregometer cuvette. Luminol is then added, followed by 1.0 μL of 10 mM formylmethionyl leucinyl phenylalanine (fMLP). Chemiluminescence is recorded for 15 minutes. In this assay system, the release of superoxide radical as measured by chemiluminescence, has been shown to be linearly correlated with that measured by the ferricytochrome C method. The interassay coefficient of variation of this assay is 8 %.
6) **Nile Red staining of intracellular lipids:** Freshly isolated monocytes are layered on slides treated with poly lysine solution for 20 minutes. Fix cells with freshly prepared 3.7% formalin solution followed by 2 PBS washes, 3 minutes each. Cover cells with freshly prepared working solution of Nile red stain (1:100 of stock solution in PBS) for 5 minutes. Fluorescence microscope will be used to examine lipid stain at excitation maximum =549nm and emission maximum = 628nm. **Intracellular Lipids Scoring:** 100 monocytes will be examined from each sample for their stain intensity and will be given a score (0-4) according to the following system:
   0 No positive stain in cytoplasm
   1 up to 25% of cell cytoplasm stains positive
   2 25% - 50% 0f cell cytoplasm stains positive
   3 50% - 75% of cell cytoplasm stains positive
   4 all/total cell cytoplasm stains positive
7) **Circulating pro-inflammatory mediators:** Plasma MMP-9, IL-1β and IL-12 will be assayed with ELISA Kits from R&D systems (Minneapolis, MN).
8) **Quantification of Pro-inflammatory gene mRNA expression in MNC and monocytes by Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR):** Total RNA is isolated using commercially available RNAqueous®-4PCR Kit (Ambion, Austin,TX). The quality and quantity of the

isolated RNA is determined before using the RNA. One μg of total RNA is reverse transcribed using Advantage RT-for-PCR Kit (Clontech, CA). Real Time RT-PCR is performed using Cepheid Smart Cycler (Sunnyvale, CA) in which 2 μL cDNA, 10 μL Sybergreen Master mix (Qiagen, CA) along with 0.5 μL of 20 μM gene specific primers for IL-1β and IL-12 (Life Technologies, MD) are used. The specificity and the size of the PCR products are tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel. All values are normalized to actin, ubiquitin C and cyclophilin A expression.

9) DATA ANALYSIS:

Statistical analysis will be carried out using SPSS and SigmaStat software (Jandel Scientific, CA). All data will be expressed as mean ± S.E of arbitrary units and percent change is calculated from the means. To evaluate similarity between the study groups, baseline values for subject's demographics will be compared using appropriate parametric and non-parametric tests based upon the nature of the data. To account for the anticipated wide inter-patient variability, baseline measures will be standardized to 100%. All subsequent values will be reported as the percent change from the baseline value. Comparisons for each endpoint will be made using repeated measures ANOVA.

The primary endpoint of the study is to evaluate the change in intracellular lipids in MNCs following cream challenge before and after Vytorin therapy for 6 weeks. Our preliminary studies have shown an increase of 70±24% in intracellular lipids staining following cream intake in normal subjects. Assuming approximately a 25% decrease in the cream induced intracellular lipid accumulation, a sample size of 20 subjects per group should provide adequate power ($\beta = 0.8$) to detect a statistically significant difference ($\alpha = 0.05$), provided the standard deviation of the residuals is not equal to or greater than the mean difference.

Secondary endpoints: We will measure markers of monocyte activation including CD68 and PECAM expression as well as the activity and the expression of pro-inflammatory transcription factor NF-κB and its activating protein, IKKβ, the expression of pro-inflammatory NF-κB-regulated genes, TLR-4, and SOCS-3 and oxidative stress in monocytes following cream challenge before and after Vytorin (simvastatin and ezetimibe). The decrease in intracellular lipids before and after combination of the Simvastatin and Ezetimibe will also be analyzed.

Data Safety Monitoring Plan:

A data and safety monitoring board is not required for this study. The Investigator will be responsible for the proper conduct, data collection and safety monitoring. Subjects will be given an adverse event diary and instructed to call the Investigator for any problems or changes in their physical condition or medications. Any serious adverse events will be reported immediately to the University IRB. Recruitment and accrual will be reported to the IRB quarterly. Annual reports regarding patient recruitment follow up, adverse events, dropout rates, and findings of the study will also be sent to the University IRB.

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