

**A NUTRIENT MIXTURE CONTAINING ASCORBIC ACID, LYSINE, PROLINE, ARGININE,
CYSTEINE, AND GREEN TEA EXTRACT SUPPRESSES AUTOCRINE INFLAMMATORY
RESPONSE IN CULTURED HUMAN AORTIC SMOOTH MUSCLE CELL**

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Abstract

Introduction:

Recognition of the involvement of inflammatory processes in atherosclerotic lesion initiation and development of pathological consequences initiated a search for an effective inhibitor. Naturally occurring compounds demonstrate a wider spectrum of biological activity and fewer side effects than synthetic drugs. Mixtures of natural compounds often produce synergistically enhanced therapeutic action.

Objective:

This prompted us to investigate whether a unique nutrient mixture (NS), containing ascorbic acid, lysine, proline, arginine, N-acetyl cysteine and tea phenolics, could reduce an autocrine response of human aortic smooth muscle cell (SMC) to inflammatory stimuli. Cultured SMC were challenged with tumor necrosis factor-alpha (TNF α) or lipopolysaccharide (LPS) in the presence or absence of NS. Expression of leading mediators of inflammatory reaction was assayed with ELISA (R&D Systems).

Results:

2.5-fold induction of interleukin-1 α (IL-1 α) content in cellular media was completely reversed in the presence of 20 μ g/ml NS (containing 20 μ M ascorbic acid). Secretion of pro-interleukin-1 β (pro-IL-1 β) and of its activator, caspase-1, was inhibited by 46% and 67%, respectively. This resulted in significant reduction of IL-1 β formation. Secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) was also dramatically reduced. Moreover, addition of NS significantly inhibited expression of cell adhesion molecules: sP-selectin and monocyte chemoattractant protein-1 (42% and 65% inhibition, respectively). Anti-inflammatory effects of NS exceeded the sum of actions of its individual components.

Conclusion:

From these data we conclude that the mixture of ascorbic acid, tea phenolics, and selected amino acids tested has a strong potential against involvement of vascular cells into inflammatory response to pathogens.

1. Introduction:

Atherosclerosis and its associated vascular complications are the principal causes of cardiovascular and cerebrovascular diseases leading to myocardial infarction and stroke, respectively. Every year over 12 million people worldwide die of the results of atherosclerosis, heart infarctions, and strokes. According to the American Heart Association's 2004 Heart and Stroke Statistical Update, over 64 million people worldwide suffer from CVD, which has been the leading cause of death in the US for decades [(American Heart Association (2004)].

Certain drastic behavioral modifications by the arterial wall smooth muscle cell (SMC) have been considered key steps in the formation of atherosclerotic lesions. These include: massive migration of SMC from the media to the intima layer of the vessel, dedifferentiation of SMC to proliferating phenotype, and increased secretion of inflammatory cytokines. These events trigger vessel wall thickening and monocyte recruitment from blood, leading to progressive development of atherosclerotic plaques. Cellular adhesion molecules on the surface of endothelial cells cause monocyte adherence to the endothelium and subsequent migration into the arterial wall. Production of these adhesion molecules is directly stimulated by inflammatory cytokines. Several studies suggest that adhesion molecules may be an important target for the prevention and treatment of atherosclerosis and CVD as formation of atherosclerotic lesions was found to be significantly decreased in mutant mice that don't express macrophage colony stimulating factors and monocyte chemoattractant protein-1 [Qiao, Tripathi et al. (1997), Gu, Okada et al. (1998)].

Originally reported by Russell Ross, atherosclerosis is now recognized as an inflammatory disease [Ross (1999)]. Regardless of the amount of obstruction due to a given atherosclerotic lesion, the increased risk of atherosclerotic events, such as unstable angina and myocardial infarction, appears to be related to chronic sub-clinical inflammation. Inflammation of the vascular wall constitutes a major factor in the development of atherosclerosis, atheroma instability and plaque disruption, and leads to

local thrombosis and the clinical presentation of acute coronary syndromes. Endothelial-cell injury is the main stimulus for atherosclerotic plaque development. The well-known “risk factors”, such as dyslipemia, diabetes, hypertension, obesity, immunity, infection, hyperhomocysteinemia, and smoking, are morbidities that stimulate or enhance the underlying inflammatory process. In his review of the chain of local arterial endothelial cell reactions to injury, the behavior of inflammation markers, and the effects of specific drugs that possess additional anti-inflammatory effects, Altman proposes the concept of athero-inflammation as the meeting point of the different morbidities mentioned above [Altman (2003)].

Several clinical studies investigated the relationship between the levels of cytokines and stable acute coronary syndromes. Recent studies have found increased levels of other "immune system" substances (macrophage colony stimulating factor (MCSF), IL-1_β, IL-6 and C-reactive protein (CRP) in patients with coronary atherosclerosis when contrasted with levels in healthy subjects. [Tashiro, Shimakawa et al. (1997), Ikonomidis, Andreotti et al. (1999)]. Furthermore, the level of both MCSF and IL-1_β in the bloodstream correlates closely with the extent of coronary artery disease.

Thus, there is sufficient evidence to suggest that inflammatory cytokines and growth factors play a significant role in the development of atherosclerosis and its complications. Treatments aimed at blocking the inflammatory process may be a promising area to look for new, more effective treatments or methods of prevention. Furthermore, naturally occurring compounds demonstrate a wider spectrum of biological activity and fewer side effects than synthetic drugs and a mixture of natural compounds often produces synergistically enhanced therapeutic actions. This reasoning prompted us to investigate the effect of a mixture of nutrients, including ascorbic acid, lysine, proline, arginine, N-acetyl cysteine and epigallocatechin gallate, on the autocrine response of human aortic SMC challenged with pathogenic stimuli.

2. Methods and Materials:

2.1. Cell Culture

Human aortic smooth muscle cells (obtained from Clonetics) were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% fetal bovine serum, penicillin (100 µg/ml) and streptomycin (100 µg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37⁰C in a tissue culture incubator equilibrated with 95% air and 5% CO₂ and used for experiments at passages 5th-8th. Cell media components were obtained from GIBCO.

2.2. Cytokine Expression Studies

SMC were plated into 24-well plastic plates at 50,000 cells per well and grown to confluence. Cell culture medium was replaced with 0.5 ml serum-free DMEM supplemented with 0.1% bovine serum protein and indicated amounts of nutrient mixture. After incubation for 24 hours, media were replaced with fresh DMEM/BSA media containing the same amounts of nutrient mixture and a stimulator: 10 ng/ml of tumor necrosis factor alpha (TNF- α), 0.1 mg/ml bacterial lipopolysaccharide (LPS), or no stimulator as control. Following 24 hours incubation, conditioned media were collected and frozen at -80⁰C individually for cytokine assay. Cell protein was measured by BCA protein micromethod (Pearce) after cell layer washing with phosphate buffered saline (PBS) and dissolving in 0.1N NaOH for 2 hour at 37⁰C. Test samples of cell protein content per well did not differ significantly from control (un-supplemented) samples, indicating unimpaired cell viability. The cytokine level in cell-conditioned media was assayed with ELISA kits (Quantikine, R&D Systems) according to manufacturer's protocol. All experiments were performed at least twice in triplicates.

2.3. Composition of the Nutrient Mixture (NS)

Stock solution of the nutrient mixture (total weight 4.4 Gm) is composed of the following nutrients: vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1mg.

2.4. Statistical Analysis

The results for each representative study are expressed as mean cytokine concentrations \pm SD for the groups. Data was analyzed by independent sample “t” test.

3. Results

3.1. SMC secretion of Interleukin – 1 alpha:

TNF- α (10 ng/ml) induced SMC secretion of IL-1 α to 132% of that in the control. However, in the presence of 20 μ g/ml NS, IL-1 α SMC expression was not only reversed, but also reduced to 60% ($p=0.0066$) of the control LPS-induced SMC secretion of interleukin-1alpha (IL-1 α) increased 250% over that in the control cell culture and was completely reversed ($p>0.0001$) in the presence of 20 μ g/ml NS (containing 20 μ M ascorbic acid). (Figure 1).

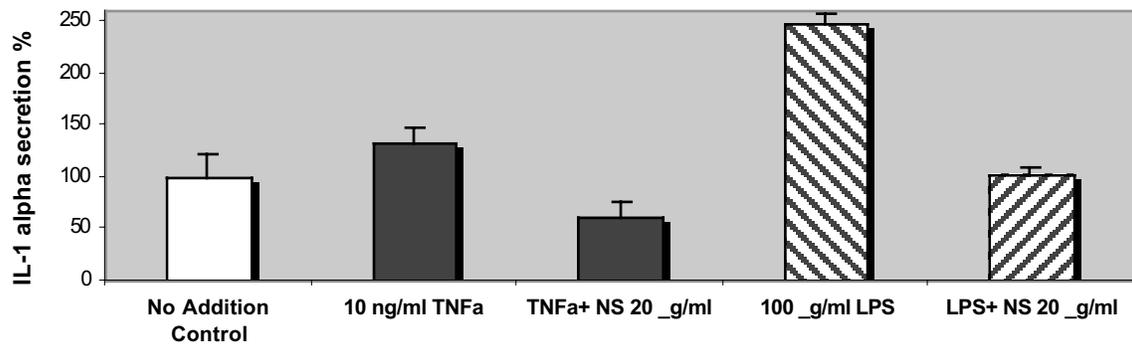


Figure 1 – Human aortic SMC-secretion of interleukin-1 alpha induced by TNF-alpha or LPS in the presence of the nutrient mixture (NS). Mean control value is 0.1 pg/ml.

3.2. SMC secretion of pro-interleukin – 1 beta:

Secretion of pro IL-1 β by SMC challenged with TNF α (10 ng/ml) increased to 137% of the control and was reversed and dramatically inhibited (to 16% of the control; $p=0.0004$) in the presence of 20 μ g/ml NS, showing enhanced inhibition from synergistic effect of low levels of ascorbic acid (20 μ M) and EGCG (3 μ M)) (Figure 2). Significant inhibition (to 35% of control; $p=0.002$) was seen in the presence of EGCG 10 μ g/ml (30 μ M) and some inhibition (to 119% of control) was seen with ascorbic acid (25 μ g/ml)

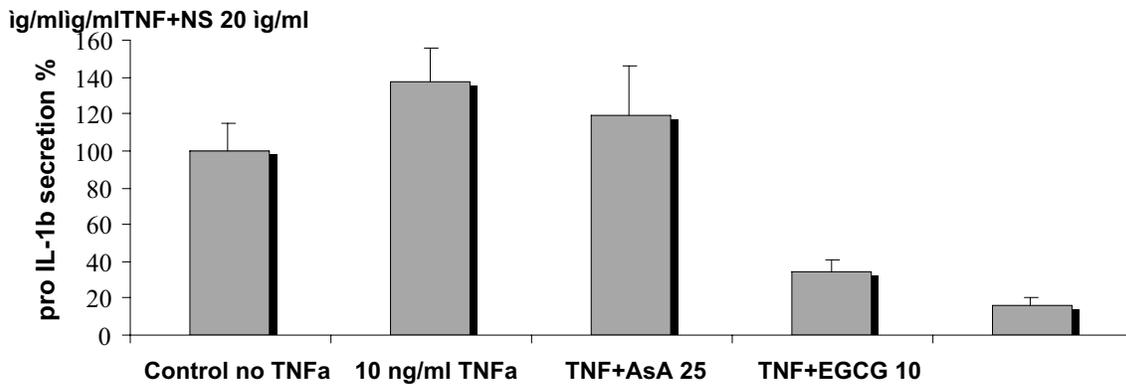


Figure 2 – Human aortic SMC secretion of pro-interleukin-1 beta induced by TNF-alpha in the presence of various nutrients. Mean control value is 74.6 ng/ml.

3.3. SMC secretion of caspase-1:

SMC secretion of caspase-1 (activator of pro-interleukin-1 β) was increased to 231.1% of the control with LPS challenge and completely reversed (to 86.1%; p=0.0001) in the presence of the nutrient mixture. See Figure 3.

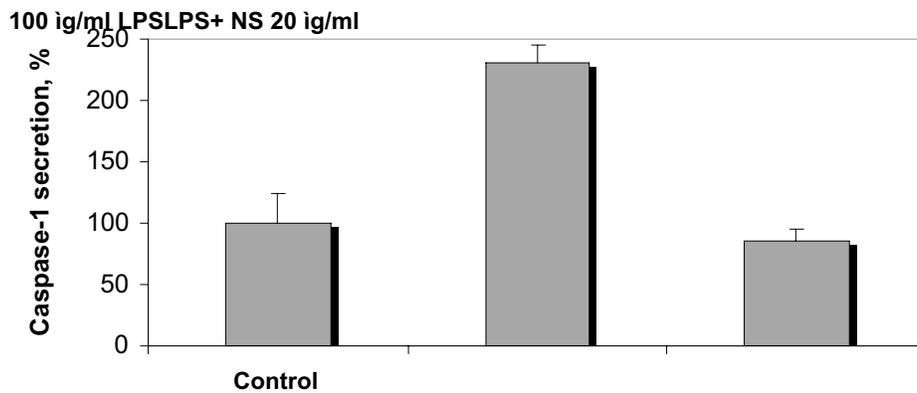


Figure 3 – Human aortic SMC secretion of caspase-1 induced by LPS in the presence of the nutrient mixture (NS). Mean control value is 0.9 pg/ml.

3.4. SMC secretion of IL-1 beta

Induction of interleukin 1beta (IL-1 β) secretion by SMC challenged with TNF- α or LPS was completely reversed in the presence of NS (Figure 4). TNF- α -induced SMC secretion of IL-1 β increased to 349.2% of the control, which was dramatically decreased to 46.7% (p<0.0001) of the control in the presence of 20 μ g/ml of the nutrient mixture.

LPS induced SMC secretion of IL-1 β to 485.5% of the control, which also was significantly reduced to 76.5% ($p < 0.0001$) of the control in the presence of NS.

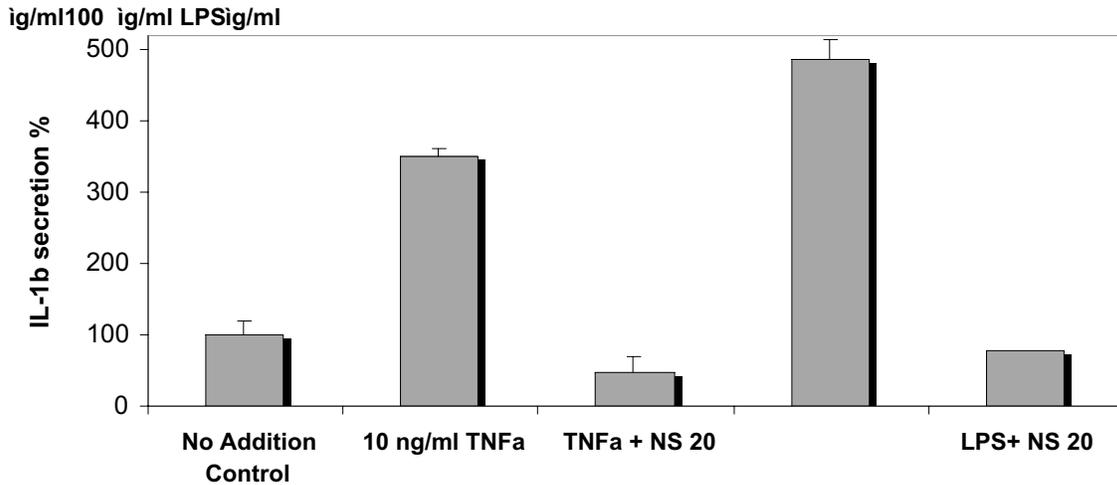


Figure 4 – Human aortic SMC secretion of interleukin-1 beta induced by TNF- α or LPS in the presence of the nutrient mixture (NS). Mean control value is 0.1 pg/ml.

3.5. SMC secretion of IL-6

TNF- α induced SMC secretion of interleukin –6 (IL-6) increased to 181.9% of control. IL-6 secretion was completely reversed and further inhibited to 53.2% ($p < 0.0001$) of control in the presence of 100 μ g/ml NS (Figure 5).

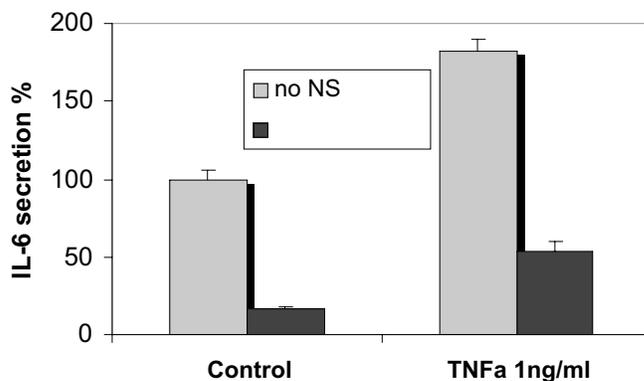


Figure 5 – Human aortic SMC secretion of interleukin-6 induced by TNF- α in the presence of the nutrient mixture (NS). Mean control value is 22.2 ng/ml.

3.6. SMC secretion of MCP-1

Induced smooth muscle cell secretion of monocyte chemoattractant protein (MCP-1), which leads to migration of monocytes into the atherosclerotic plaque, was almost completely reversed in the presence of 100 μ g/ml of the nutrient mixture (Figure 6).

TNF- α -induced MCP-1 expression increased to 218.5%. In the presence of NS, SMC MCP-1 expression was inhibited to 125.5% ($p < 0.0001$).

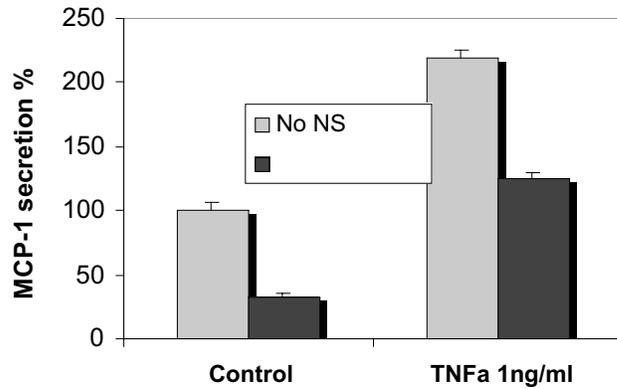


Figure 6 – Human aortic SMC secretion of MCP-1 induced by TNF- α in the presence of the nutrient mixture (NS). Mean control value is 12.7 ng/ml.

3.7. SMC secretion of sP-selectin

Secretion of sP-selectin (monocyte adhesive mediator) by smooth muscle cells challenged with TNF- α (10 ng/ml) and LPS (100 μ g/ml), was inhibited by 40% ($p = 0.001$) and 30% ($p = 0.038$) respectively in the presence of NS (Figure 7).

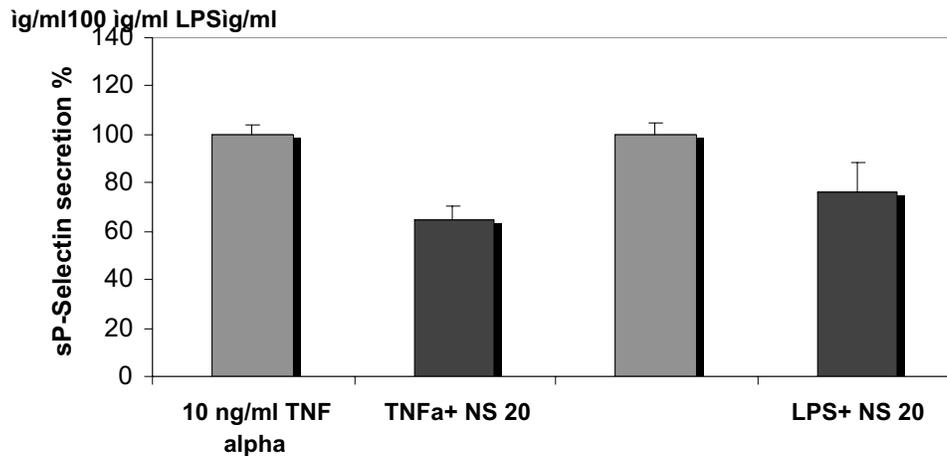


Figure 7 – Human aortic SMC secretion of sP-selectin induced by TNF- α or LPA in the presence of the nutrient mixture (NS). Mean control value is 0.3 pg/ml.

3.8. SMC secretion of siCAM-1

Secretion of siCAM-1 by SMC challenged with TNF- α (10 ng/ml) was reduced by 24% ($p = 0.029$) in the presence of NS (Figure 8).

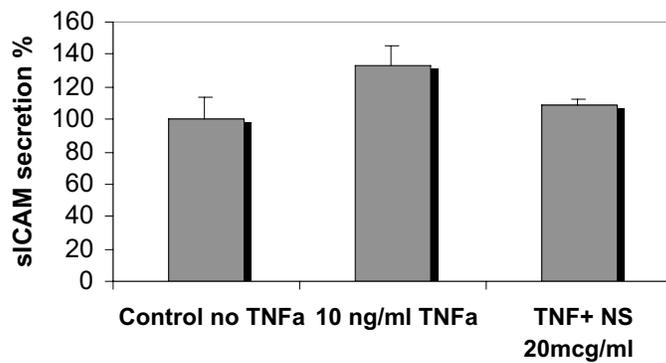


Figure 8 – Human aortic secretion of siCAM-1 induced by TNF- α in the presence of the nutrient mixture (NS). Mean control value is 1.3 pg/ml.

4. Discussion:

In this study we investigated the effects of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine and epigallocatechin gallate on the enhanced expression of inflammatory mediators by cultured human vascular smooth muscle cells challenged with TNF- α or LPS. Human aortic SMC expression of each inflammatory mediator studied was enhanced by TNF- α or LPS and significantly and dramatically inhibited by the presence of the nutrient mixture.

As shown in Figure 9, infection, oxidative stress, mechanical stress, nutrient imbalance cause artery wall damage and subsequent release of inflammatory mediators by SMCs. During this process, signaling molecules called cytokines are produced that accelerate inflammation. The nutrient mixture was seen to inhibit the expression of each of these cytokines along the chain of events leading to monocyte adhesion and progression of the atherosclerotic plaque. In addition, secretion of MCP-1, sP-selectin and siCAM-1 lead to attraction and adherence of monocytes to the endothelium, followed by monocyte migration across the endothelium and into the arterial wall. The nutrient mixture also acted to inhibit SMC secretion of these inflammatory mediators as well. Once trapped in the arterial wall, monocytes engorge oxidized cholesterol and are converted into fat-laden foam cells. Formation and aggregation of foam cells is the first manifestation of atherosclerosis, leading to the arterial narrowing and, eventually, to full-blown CVD.

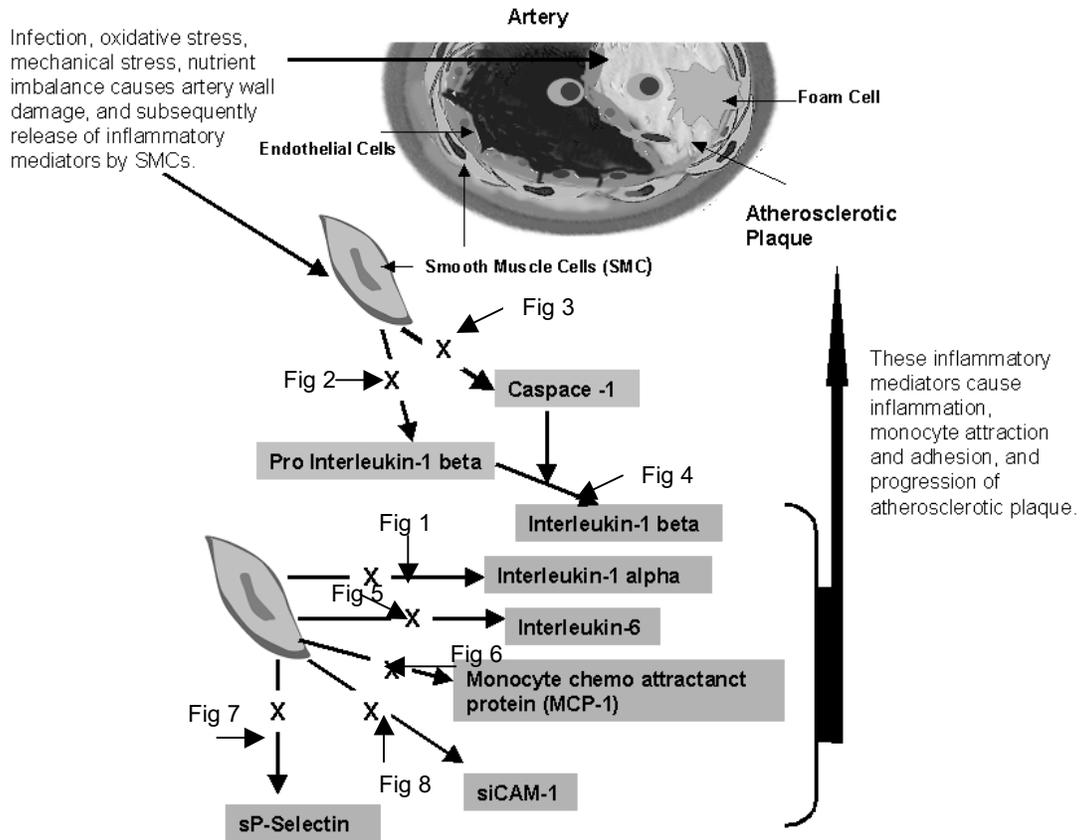


Figure 9 – Summary graphic showing inhibitory action of NS on specific inflammatory mediators, resulting in interruption in development of atherosclerotic plaque.

The results of this study are important since multiple clinical studies support the significance of these inflammatory mediators in the pathogenesis of coronary atherosclerosis and unstable cardiac syndromes. For example, one clinical study, measured plasma levels of IL-1_β, TNF-_α, and IL-6 in 97 patients: 67 with stable angina, 24 with unstable angina and 15 healthy controls. Mean levels of IL-1_β were significantly higher in patients with unstable angina when compared to patients with stable angina and IL-6 levels were found to be elevated in patients with angina [Simon, Yazdani et al. (2000)]. In a study of 131 Japanese subjects (79 with known atherosclerosis (40 with unstable angina and 39 with stable atherosclerotic disease) and 52 control), concentrations of hsCRP and IL-6 were highest in the unstable angina

group and lowest in the control group. This study makes an association between the presence and degree of inflammation and the presence of atherosclerosis. It further makes an association between the degree of inflammation and the instability of that atherosclerosis by noting an increase in inflammatory cytokines in the unstable angina group compared with the stable atherosclerosis group [Yamashita, Shimada et al. (2003)].

Furthermore, clinical studies have shown that among patients admitted for unstable angina, those who experienced complicated in-hospital course had significantly higher levels of cytokines, specifically IL-1_β and IL-6, than those who had an uneventful course [Biasucci, Liuzzo et al. (1999)].

The synergistic anti-atherogenic effects of nutrient supplementation with a mixture of vitamins, minerals, amino acids, coenzymes and other nutrients were demonstrated in a pilot study of the effect of nutrient supplementation on progression of early coronary atherosclerosis. In this study, the extent of coronary calcification in 55 patients diagnosed with early coronary atherosclerosis was measured prior to nutrient supplementation and after one year of intervention, using an Imatron C-100 Ultrafast CT scanner. Progression of coronary calcification, as determined by the CAS score, decreased significantly (from 0.49 mm² to 0.28mm² monthly growth) after one year of nutritional intervention [Rath and Niedzwiecki (1996)].

Furthermore, large studies in patients with heart disease have found that those with high blood levels of other "immune system" substances -- CRP, leukocytes and fibrinogen -- are more likely to suffer progressively worse symptoms, including heart attack [Dabesh, Collins et al. (1998), Andreotti, Burzotta et al. (1999)]. The presence of these substances suggests that inflammatory processes interacting with or, perhaps, originating from atherosclerotic blood vessels may somehow cause or promote thrombosis, or blockage of the artery by a blood clot.

5. Conclusion:

From these data, we conclude that the nutrient mixture of ascorbic acid, tea phenolics, and selected amino acids, has a strong inhibitory potential against vascular cell inflammatory responses to pathogenic stimuli. In view of these results and the experimental and clinical studies demonstrating the important role played by inflammatory mediators in the development of atherosclerosis and coronary complications, nutrient synergy is a promising therapeutic agent for atherosclerosis and its associated complications.

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