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WHITE PAPER:

“The Efficacy of Dietary Supplementation
for Enhanced Nitric Oxide Synthesis:
The Scientific Evidence”

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Introduction

Approximately twenty years ago, it was discovered that a particular factor produced within endothelial tissue specifically relaxes the adjacent smooth muscle of blood vessels thereby allowing vasodilation and increased blood flow to various tissues including skeletal muscle. This factor was initially referred to as endothelium-derived relaxing factor but is now known as nitric oxide. While nitric oxide serves as an important signaling molecule involved in numerous physiological functions (such as improved glucose transport and antioxidant properties) the role of nitric oxide and control of skeletal blood flow is the primary issue generally addressed with various proposed nutritional supplements. The marketing of these products is generally based on the assumptions that increased blood flow to exercising muscles will enable 1) increased levels of work output, and/or 2) increased resistance to fatigue with submaximal levels of work, and/or 3) increased recovery following exercise training.

Nitric oxide ‘stimulators’ comprise one of the primary categories of sports nutritional supplements along with ‘sports drinks/energy drinks’, whey protein powders, and creatine monohydrate products. Unfortunately, most of the information shared with the public is based on unsubstantiated manufacturer claims and not on scientifically based evidence. Prior to discussing commercial products currently available in this category, a review of the various mechanisms involved in control of blood flow during exercise is presented.

Redistribution of Blood Flow During Exercise

The demand for increased blood flow during exercise is met by increased cardiac output and redistribution of blood flow from inactive tissues to the muscles undergoing exercise stress. Cardiac output is the total amount of blood ejected by the heart per minute; increased with elevations in heart rate with some contribution by increased stroke volume, depending on the type of exercise activity. Cardiac output increases two -to- three fold during intense exercise. However, increased blood flow to active musculature is predominately accomplished through redistribution of blood flow.

At rest, approximately 15% to 20% of the total cardiac output is directed to the skeletal muscles. In contrast, the increased metabolic demand during maximal exercise requires over 80% of the total cardiac output to supply the active muscles. This redistribution of blood flow, or shunting, is accomplished by

adjustments in systemic and localized vasomotor tone. The walls of arterioles contain a layer of smooth muscle which is responsive to both extrinsic neural control and a process of auto regulation. This system of auto regulation is an intrinsic process which provides regulation of local blood flow in response to alterations in perfusion pressure and is generally considered to occur without influence of extrinsic neural or hormonal factors.

The arterioles are the site of major resistance to blood flow and therefore, peripheral resistance is primarily determined by the status (current radius) of the arterioles. Extrinsic stimulation by sympathetic nerves influences the tone or tension of muscles lining the walls of vascular vessels. Increased sympathetic nerve activity tends to produce vasoconstriction in these resistance blood vessels, via the neurotransmitter noradrenalin; thereby reducing cross-sectional area and blood flow to those tissues. In this way, blood flow can be reduced in non-active musculature during exercise.

During the early stages of exercise, blood flow is increased within the exercising muscles to some extent by reductions in sympathetic activity, but primarily due to locally mediated factors which produce smooth muscle relaxation and dilation of the local arterioles and precapillary sphincters. The combination of extrinsically controlled vasoconstriction, reduced blood flow in inactive muscle, and auto regulated vasodilation in the active muscles effectively establish redistribution or shunting of blood during exercise to meet the increased metabolic demands.

Vasodilation – Opening the Doorway to Muscle

Metabolic demands of exercising muscles produce acute localized responses which in turn serve as signals influencing vasodilation. In this way, blood flow to muscle during heavy exercise can be increased to 25 – 50 times those of resting levels (17).

As previously mentioned, the arterioles are the primary point of peripheral resistance and therefore are often considered the principal gatekeeper of blood flow to skeletal muscle. However, it is vital to consider the role of precapillary sphincters in this process. The capillary bed is the only means by which the exchange of nutrients and waste products can be made between muscle tissue and the blood stream. Precapillary sphincters control blood flow into the capillary bed and therefore also into muscle. If these sphincters are constricted, then blood flow is directed through metaarterioles, primarily bypassing most capillaries. The process of

vasodilation also involves relaxation of the precapillary sphincters thereby allowing blood flow into the capillary bed and providing a complete perfusion of the muscle tissue supplied by the bed. Thus, blood flow to skeletal muscle is determined by the vascular tone of both the feed arterioles and the precapillary sphincters.

Vasodilation is increased by auto regulatory mechanisms initiated by:

- **Decreased tissue oxygen concentration (pO₂)** – Either oxygen supply is reduced or oxygen demand is increased
- **Increases in blood flow** – produced with muscular work
- **Temperature** – increased with exercise
- **Increased concentrations of Carbon Dioxide** – produced during periods of increased oxidative metabolism
- **Decrease in pH** (increase in acidity)
- **Increases in Adenosine** – Adenosine is formed during periods of hypoxia
- **Increased Potassium Concentrations** - K⁺ is produced by skeletal muscle contractions faster than Na⁺ / K⁺ - ATPase pump activity

In general, each of the factors listed above increases with ongoing and intensifying exercise stress and progressively contributes to the vasodilation process and control of local blood flow to exercising muscles. Limitations in vasodilation will result in reduced blood flow and restrictions in exercise intensity and/or duration.

Vasodilation during exercise is dramatically influenced by increases in nitric oxide.

Nitric Oxide

Nitric oxide is synthesized in the endothelium from its precursor L-arginine via enzymatic action of endothelium nitric oxide synthase (eNOS), in a process that also requires oxygen and reduces inorganic nitrate. Nitric oxide is a very reactive gas, having a half-life of only a few seconds, but easily diffuses across membranes and into various tissues. This molecule easily crosses from the endothelium into the adjacent smooth muscle of the arterioles; where it binds to the enzyme guanylyl cyclase which in turn converts guanosine-5'-triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). Cyclic GMP serves as an important second messenger for many physiological functions including the relaxation of smooth vascular muscle. The smooth muscle relaxation is produced when cGMP signals different mechanisms including inhibition of calcium into the smooth muscle and activation of

potassium channels. Cyclic GMP also stimulates the protein kinase which activates enzymatic activity which dephosphorylates myosin light chains again producing relaxation of the smooth vascular muscle. Interestingly, prescription drugs such as Sildenafil (trade name Viagra) inhibit the breakdown of cGMP in order to augment the vasodilatory effects of nitric oxide in the treatment of male erectile disorder.

Nitric oxide relaxes the smooth muscle of the arterioles and produces vasodilation via both direct and indirect mechanisms. The indirect mechanisms inhibit the influences of the previously mentioned sympathetic vasoconstrictor influences. The direct vasodilatory mechanisms of nitric oxide are classified as either flow dependent or receptor mediated. The former mechanism is produced when increased levels of blood flow establish shearing forces which stimulate the endothelium to release calcium which then activates the production of nitric oxide. Receptor mediated vasodilation is initiated when various receptors positioned within the endothelial tissue are activated, again releasing calcium and activating the production of nitric oxide. Specific receptors included in this process include those sensitive to acetylcholine, bradykinin, substance-P, and adenosine.

Nitric oxide provides other vascular effects including anti-thrombotic effects (inhibits adhesion of blood platelets to the endothelium), anti-inflammatory effects (inhibits adhesion of leukocytes to the endothelium), and anti-proliferative effects (inhibits hyperplasia of smooth muscle). Limited production or bioavailability of nitric oxide has been associated with increased levels of vasoconstriction resulting in increased vascular resistance and hypertension. In time, limitations in arteriole compliance may produce vascular hypertrophy and stenosis. Limited levels of nitric oxide have also been associated with thrombosis and inflammation related to platelet and leukocyte adhesion to the endothelium, respectively.

Recent evidence indicates that nitric oxide also regulates glucose metabolism as well as glucose and fatty acid oxidation in cardiac and skeletal muscle as well as in adipose tissue. (13) Nitric oxide has also been shown to enhance lipolysis in adipocytes. Increased levels of nitric oxide in insulin-sensitive tissues promotes substrate uptake and removal as a result of increased blood flow through those tissues.

Arginine and Nitric Oxide

Arginine is the precursor for nitric oxide leading to the supposition that oral supplementation can serve as a potent nitric oxide stimulator. This position is also based on the findings that significant increases of blood serum arginine levels induce significant levels of vasodilation in healthy persons when fasting (12). However, such blood levels of arginine require direct infusion as high oral doses generally are not tolerated. In fact, oral dosages as low as 10 grams per day have been associated with significant gastric distress (16).

A substantial body of research dating back to the 1990's, demonstrates that oral feeding of arginine is ineffective for increasing nitric oxide production, as compared to intravenous infusion, which is largely impractical. In one study performed by Adams, (1) young healthy men were supplemented with arginine in powder form for 3 days (7 grams three times daily). Plasma arginine levels were variable among the subjects resulting in inconsistent and non-significant vascular results; such that oral supplementation did not increase endothelium-dependent vasodilation. In a 1996 study, healthy males received 20 g per day of oral L-arginine for 28 days (7). Oral supplementation did not have a significant effect on endothelial function in healthy persons and results further indicated that orally ingested arginine dissipated to other pathways as indicated by a change in total amino acid profile but not in the L-arginine plasma concentration.

The application of oral L-arginine has been examined in regards to nitric oxide production in various clinical populations. Patients with severe heart failure received 20 g of L-arginine supplementation per day for 28 days in a study performed by Chin-Dusting and associates (8). This relatively high dosage over a four-week period was not successful in restoring function of endothelial nitric oxide synthesis in these patients. Neither were there changes in nitric oxide levels in older adults (65 yrs and older) after supplementation of 8.5 g of L-arginine over a four-week period (19).

Bode-Boger et al. (4) designed a comprehensive study to examine the different dynamics of intravenous versus oral supplementation. Subjects received a single dose of L-arginine intravenous infusion of 30 g, 6 g, and placebo. On separate days, participants also received 6 g of L-arginine and placebo orally. First and foremost, oral supplementation of L-arginine at 6 g was found to be 70% bioavailable, but of which 50% may be converted into ornithine (6).

Moreover, peak plasma concentrations were considerably lower for oral as compared to intravenous infusion of L-arginine. Total peripheral resistance was not significantly affected by either oral ingestion or intravenous infusion at 6 g, but a significant decrease in resistance and increase in cardiac output was apparent for 30 g infusion. This study did demonstrate that a single intravenous dose of L-arginine, greater than 28 g, positively affected hemodynamic conditions in healthy persons. However, while these results are significant, they remain largely impractical especially for marketing oral L-arginine supplementation to the general public with the express purpose of developing strength and mass via increases in vasodilation and nutrient delivery.

For reasons beyond gastric distress, dosage, and bioavailability, the theory of arginine supplementation is flawed. While it was well established that arginine is a precursor for nitric oxide, it is not automatic that arginine concentration directly limits the rate of nitric oxide production. Kurz and Harrison (15) found arginine levels in healthy persons to be greater than what is reportedly sufficient to activate eNOS and therefore produce nitric oxide. They coined the term 'arginine paradox' to refer to this apparent physiological contradiction. Other studies have examined the effects of arginine-free diets on nitric oxide levels. Castillo et al (6) found no changes in nitric oxide synthesis over a six-day period without ingestion of any arginine (6). Another research group examined the plasma concentrations of L-arginine and nitric oxide compounds during 24 hours of an L-arginine and nitrate/ nitrite free diet with and without supplementation of L-arginine (3.8 g/d) (21). While the plasma L-arginine levels were greater with the supplement, there were no variations between conditions in plasma nitric oxide compound concentrations. Thus, the research shows that while arginine is the precursor of nitric oxide, it is not rate limiting.

Dietary supplementation of arginine has also been examined in conjunction with different modes of exercise. Robinson and associates (16) applied 10 g of arginine with 70 g of simple carbohydrates to examine the effects on glucose levels, forearm blood flow, and blood pressure following either a session of resistance training, after a cycling endurance session, or after a rest period. None of the outcome measures were significantly affected by the arginine and carbohydrate ingestion.

Arginine-AKG

Many, if not most, of the current commercial nutritional supplements that claim to enhance nitric oxide levels utilize

arginine alpha-ketoglutarate (AKG) as the primary 'active ingredient'. Alpha-ketoglutarate is an important intermediate in the Krebs's cycle, following isocitrate and prior to succinyl CoA. While AKG provides important metabolic functions, there is no scientific evidence that arginine AKG has any effect on nitric oxide production. (5). In fact, there have been no studies to actually examine nitric oxide levels or vasodilation with supplementation of arginine AKG.

Most popular products that claim to enhance nitric oxide levels are actually a combination of multiple agents, generally in a 'proprietary blend'. Generally, the ingredients of a proprietary blend are not listed with the actual concentrations. Thus, the actual ingredient contents are not known by the general public. The common claim is that there is a special combination of agents involved which in combination or through a synergistic effect produce dramatic increases in nitric oxide levels and subsequent vasodilation. Interestingly, two of the most common items included in these combination products are creatine and sugar. Again, there is absolutely no scientific evidence that any of these commercial oral arginine AKG combination products actually has any direct effect on the production of nitric oxide.

Research has identified facts related to L-arginine supplementation and nitric oxide: 1) L-arginine is not rate-limiting to nitric oxide production, 2) L-arginine is approximately 70% bioavailable but reflects an altered amino acid profile without substantial increases in serum arginine levels (4), and 3) as an oral supplement the amino acid is ineffective for increasing endothelium-dependent vasodilation (7). Having this knowledge, then why is L-arginine so widely touted and promoted as a supplement for enhancing blood flow and nutrient delivery? A more sound theory should be considered involving the hormone insulin.

Insulin, Nitric Oxide and Vasodilation

Dating back to 1994, it was shown that insulin itself acts as a regulator for vasodilation and blood flow by modulating nitric oxide synthesis/release but not the actions of nitric oxide (20). Cleland and associates (9) demonstrated that local insulin mediated vasodilation is dependent on insulin sensitivity. This concept was extended by Ueda et al. (22) who found that the vasodilatory effects of insulin are actually dependent on local uptake of glucose by insulin sensitive tissues. Furthermore, the modulation of nitric oxide by insulin may be diminished in conditions of (the) insulin resistance thereby increasing the incidence of hypertension.

In 1995, Beaumier and associates (2) showed a 25% increase in plasma insulin levels from an arginine-supplemented diet as compared to a normal diet; even when total dietary nitrogen level and carbohydrate consumption were accounted for. Giugliano et al. (11) demonstrated L-arginine infusion had a positive effect on hemodynamics resulting in a reduction of blood pressure, increase in blood flow in the femoral artery, inhibition of platelet aggregation, significant rise in heart rate, and plasma catecholamine levels. These authors related the vasodilatory effect with endogenous insulin secretion, a subsequent effect of the L-arginine infusion.

It is apparent that while L-arginine is the precursor for nitric oxide, the amino acid itself is not responsible for increases in nitric oxide production and thus vasodilation. In fact, the corrected theory presents itself in that L-arginine supplementation may augment the role of insulin in mediating the action of nitric oxide; thus, leading to greater vasodilatation and delivery of nutrients in healthy persons.

The vasodilatory effects of insulin certainly suggest that agents (other than arginine products) would be effective in enhancing blood flow during exercise. Certainly, the most commonly used agent is simple sugar. The most popular 'sports drinks' are composed primarily of sugar and electrolytes. Many of the popular nitric oxide products also contain high levels of sugar. Certainly, those products do result in an insulin response and some magnitude of vascular vasodilation, which may provide benefits during exercise. However, one must question the use of such high levels of sugar during regular training sessions. While the relationship of such products to the alarming rate of diabetes is not known at this time, the reliance on high doses of simple sugars on a daily basis certainly gives cause for consideration.

Effective and safe means to enhance vasodilation via nutritional supplementation as support of the nitric oxide mechanisms would provide dramatic advantages over the limited options currently available.

L-Carnitine and Carnitine Esters

L-Carnitine is an important nutrient that is directly involved in the transfer of long-chain fatty acids such as triglycerides into the mitochondria ('cell's powerhouse'). This process involves the cleaving of fatty acids from triglycerides for release into the bloodstream. Therefore, L-carnitine controls to some extent the

conversion of fatty acids into energy available to fuel both cardiac and skeletal muscular contractions. Increased energy supply via triglycerides reduces reliance on the oxidation energy system and spares glycogen stores. Greater transport of fatty acids to the mitochondria should spare glycogen, enhance endurance, and stamina. While supplementation of L-carnitine is generally believed to increase long-duration aerobic performance, research has demonstrated mixed results with not all outcomes showing a benefit. Carnitine can be bound to either acetic or propionic acid as acetyl L-carnitine or propionyl- L-carnitine, respectively.

Acetyl-L-Carnitine is the more commonly known ester form of L-carnitine. This form is known to differ from L-carnitine in its much greater capacity to cross the blood-brain barrier. This activated form of carnitine has been shown to share with L-carnitine the ability to transfer fatty acids into the mitochondria while also producing neurological effects. Scientific evidence currently supports claims of enhanced mental clarity and focus. Acetyl-L-carnitine has been applied successfully in the treatment of patients with Alzheimer's disease and other neurological disorders.

Propionyl-L-Carnitine is a naturally produced form of carnitine that has a high affinity for both cardiac and skeletal muscle. This affinity is related to its interaction with carnitine transferase which enhances the cellular concentration of carnitine. Thus, propionyl-L-carnitine significantly increases the rate of fatty acid metabolism. Additionally, this form of carnitine also provides an energy substrate, propionate, which is converted via the anaplerotic pathway into succinate. Additional supply of succinate fuels the Krebs cycle even during periods of hypoxia. Controlled research trials have demonstrated that oral treatment with propionyl-L-carnitine enhances exercise duration and maximal oxygen uptake in patients with heart failure. Other studies have shown this agent to be particularly effective in increasing exercise capacity and blood flow velocity in persons with peripheral vascular disease. Propionyl-L-carnitine is available in Europe as a prescription medication and is currently undergoing Phase III trials in the United States.

Glycine Propionyl-L-Carnitine Hydrochloride, USP

During 2005, glycine propionyl- L-carnitine HCl, USP, (GlycoCarn®, GPLC) became available for use in food supplement products for the first time. This molecular compound is composed of the propionyl ester of carnitine in addition to a glycine component.

Glycine is considered as a glucogenic amino acid in that it helps to regulate blood sugar levels. This amino acid is also very important in the formation of creatine. Glycine propionyl- L -carnitine is available in over 80 different commercial products primarily in the health promotion and life-extension markets and is manufactured as GlycoCarn® by Sigma-Tau Industries, S.p.A. (Rome, Italy). As a member of the AminoCarnitines®, more information regarding this and the other next generation dietary L-carnitines is available on their website: www.aminocarnitines.com

The effects of glycine propionyl- L-carnitine supplementation have been recently investigated by Dr. Richard Bloomer of the University of Memphis in two different controlled studies. Dr. Bloomer's first study examined the effects of eight weeks of glycine propionyl-L-carnitine supplementation in conjunction with a cycling endurance training program (18). The study subjects, 42 untrained men and women, were randomly assigned to either a placebo group, or to one of two groups that received either 1.5 or 4.5 grams per day of the supplement.

All research subjects participated in testing prior to and after an 8-week training period with assessments including exercise performance, markers of oxidative stress, and of cardiovascular health. Alondialdehyde, a marker of oxidative stress, was significantly reduced in both supplement groups compared to placebo. Wingate power testing indicated similar training gains among the three study groups. Similarly, there wasn't a significant difference between groups in pre- and post-training values of maximal oxygen consumption as measured during a graded incremental treadmill test. However, there was a trend ($p = 0.090$) for greater increases in anaerobic threshold with training in the two supplemented groups (10%, 9%) compared with the placebo group (3%).

Perhaps the most startling of the findings of this study are in regards to the measurement of nitrates/nitrites as an indication of nitric oxide. The results indicated that the relative change in those concentrations in the study group supplemented with 4.5 grams per day (+55%) was significantly greater than the changes displayed by the 1.5 gram per day group (+13%) and the placebo group (+8%).

This study appears to be the first report of an oral food supplement product producing increased blood concentrations of nitric oxide, as measured by the surrogate marker of plasma nitrate/nitrite. However, these findings should be considered specifically in

regards to the study population. That is, these study findings, indicate a response to glycine propionyl- L-carnitine in the non-trained population while undergoing a program of cycling endurance training.

Dr. Bloomer's second study with glycine propionyl-L-carnitine (3) expanded on the findings of his earlier work in which supplementation increased the nitric oxide levels of untrained subjects. He realized that this agent might possibly provide the type responses sought by the consumers of the current nitric oxide products in the sports nutrition field. Thus, the second study examined the effects of glycine-propionyl-L-carnitine in a group of 15 previously resistance trained men. Each subject was tested following a month of supplementation and after a month of placebo treatment.

The supplement (4.5 grams per day) was consumed for 4 weeks during a 10-week study period in a cross-over design with a 2-week washout period. Subjects underwent assessment following each of the two 4-week supplement periods. The testing procedure involved six minutes of upper arm occlusion that end with one minute of isometrically grasping a handgrip dynamometer. This occlusion testing applies a standard blood pressure cuff inflated to 200mmHg and is commonly used in clinical studies of blood flow through the brachial artery. In clinical settings, Doppler ultrasound technology is commonly used to directly measure the cross-sectional area and flow through the artery.

In this study, the research question regarded nitric oxide levels. Dr. Bloomer and his associates took blood samples prior to, immediately following, and three and ten minutes after the occlusion period. The research team used the standard procedures to assay the blood plasma for nitrate/nitrite as the indication of nitric oxide. (As previously discussed, nitric oxide is a very reactive gas with very limited half-life thereby making direct measurement difficult).

The results of this controlled trial demonstrated a significant general main effect ($p = 0.0008$) for increased nitrite levels across all time points of testing. With supplementation of glycine propionyl- L-carnitine, there was a greater than 30% increase in nitrate/nitrite levels across the testing period.

The nitrate/nitrite values following occlusion were significantly greater with supplementation compared with (the) placebo. With supplementation those levels were increased by 16% over resting levels at three minutes and increased by 17% at ten minutes post-

occlusion. Conversely, the placebo condition produced only increases of 4% and 6% after three and ten minutes, respectively.

Dr. Bloomer and his research associates are to be commended for their fine work in this area. These two studies are the first to document the effectiveness of an oral food supplement in the enhancement of nitric oxide production.

The discovery of nitric oxide has enticed many in the quest to optimize cardiovascular function in both diseased states and athletic settings. Unfortunately, many nutritional products widely marketed as nitric oxide enhancers are without scientific evidence of any direct effects on the compound.

The findings of Dr. Bloomer's initial two studies indicate that glycine-propionyl-L-carnitine may provide a safe and effective means to enhance nitric oxide synthesis. The findings that supplementation of this nutrient can increase the plasma nitrate/nitrite levels in resistance trained men in response to blood flow occlusion are particularly dramatic. Based on the assumption of the established effects of nitric oxide on vasomotor tone, it may be presumed that glycine-propionyl-L-carnitine Hcl will produce significant elevation of blood flow to exercising muscle.

While the general functions of nitric oxide have been well described, those functions were observed without the ability to modify them. It is unknown what the direct effects of altered levels of nitric oxide would be on cardiovascular function during exercise. The lack of an effective means to alter nitric oxide production has limited such thoughtful inquiries to little more than wistful conjecture.

Dr. Bloomer's studies suggest that glycine-propionyl-L-carnitine may provide an effective means to enhance vasodilation and blood flow in exercising muscles. However, this presumed logical assumption must be complemented with the appropriate scientific investigations to directly examine blood dynamics during exercise. Similarly, controlled studies must be performed to examine the performance effects of this nutrient. However, based on the results of the existing literature, glycine-propionyl-L-carnitine appears to be a most promising prospect for application in the sports nutrition arena.

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