NITRIC OXIDE AND ITS ROLE IN HEALTH AND DIABETES

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The Nobel Prize was awarded to three Americans in 1998 for their work on discovering Nitric Oxide (NO) and clarifying its role in health. Their most important contributions lay in describing the effect of NO on the circulation that, as everyone knows, is disturbed to one degree or another in diabetic patients. The question then becomes is NO metabolism or action deranged in diabetic patients and could NO have a role in preventing some of the consequences in diabetes? We certainly believe so and will develop this theme in this and succeeding articles.

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Summary Overview

Nitric Oxide

Nitric oxide (NO), is a free radical gas that is a powerful regulator of circulation (it is an endogenous vasodilator) and a neurotransmitter (it helps in the processing of nerve signals as they cross synapses). L-arginine, one of 20 amino acids that make up proteins, is the only amino acid that generates significant amounts of NO.

The enzymes that produce NO from L-arginine

Nitric Oxide Synthase (NOS) is the enzyme that generates NO from L-arginine. This enzyme exists in three different forms (called isoforms), NOS 1, NOS 2 and NOS 3. Each isoform synthesizes NO but does so under different conditions. Often all three isoforms will be found in the same cell but occasionally one cell will contain only one of the isoforms. This is important because many see or hear the term ***nitric oxide*** and assume that it refers to all cells under all conditions. Each of the three isoforms is described below.

1.NOS1 is the neural (or brain) isoform, sometimes referred to as bNOS. It helps in synaptic transmission, the processing of nervous information from nerve to nerve across gaps between

the nerves called synapses and from peripheral nerves to the brain.

2. NOS2 is called inducible or iNOS. This enzyme generates extraordinarily high concentrations of NO, in part to kill bacteria. NOS2 (iNOS) takes several hours to be mobilized and the response is due to an injury or infectious process. NOS2 produced by macrophages is responsible, in part, for their effects to repair injury and to ward off infections. Extraordinarily high concentrations of NO (100 to 1000 times normal) are produced very locally by this isoform. Unlike NOS1, which is part of normal neurotransmission, there must be something very abnormal (a wound, tissue damage, hypoxia, bacterial infection, etc.) to induce this enzyme.

3. The third isoform is ecNOS (or NOS3) which stands for "endothelial cell" NOS. This isoform is active at all times (it doesn't need to be induced as does iNOS) and is found in endothelial cells which are the cells that line the inner surface of all blood vessels and lymph ducts. EcNOS is activated by the pulsatile flow of blood through vessels (the stretching and relaxation of the blood vessel wall in response to each beat of the heart). This leads to a "shear stress" on the membrane of the endothelial cells as the column of blood in the vessel moves forward and then stops. This NO, produced by ecNOS, maintains the diameter of blood vessel so that perfusion of tissues (skin, muscle, nerves, and bone) is maintained at optimal levels. In addition, ecNOS mediated NO causes angiogenesis, which is the growth of new blood vessels. This is especially important in healing an ulcer or wound on the skin.

NO initiates and maintains Vasodilation

NO initiates and maintains vasodilation through a cascade of biological events that culminate in the relaxation of smooth muscle cells that line arteries, veins, lymphatics. While somewhat complex, the sequence of biological events that are triggered by NO is described below:

Step 1. NO gas released from nitrosothiols in hemoglobin or from endothelial cells diffuses into smooth muscle cells that line small blood vessels.

Step 2.Once inside the smooth muscle cell, NO binds to an enzyme, called guanylate cyclase (GC) and this binding results in GC activation.

Step 3. Activated GC is able to cleave two phosphate groups from another compound called guanosine triphosphate (GTP). This results in the formation of cyclic guanosine monophosphate (cGMP) that is used to phosphorylate (Phosphorylation is the addition of a phosphate group) proteins, including the smooth muscle contractile protein called myosin.

Step 4. Once phosphorylated, smooth muscle cell myosin relaxes, resulting in dilation of the vessel that was originally exposed to NO.

This vasodilation continues until a phosphatase enzyme dissociates the phosphate from myosin (which may be delayed by Viagra). Since vasodilation

through NO only occurs when there is GC able to bind NO, additional NO, is sequestered for future use as a nitrosothiol, including those found in hemoglobin.

NO influences neurotransmission and mediates pain

NO has both a direct and indirect influence on neurotransmission. NO, by affecting cGMP, allows phosphorylation of ion channels, especially potassium channels necessary for normal transmission of nerve signals. NO also increases blood flow. This allows sufficient oxygen and glucose to be transported to nerve cells, positively affecting ATP production and, in turn, potassium/sodium homeostasis essential for neurotransmission. Increases in blood flow may also allow the oxygen dependent isoform, bNOS, to produce more NO.

In addition to improving neurotransmission, NO functions to reduce pain. No reduces pain directly by increasing cGMP (the mechanism by which opioids work), and *indirectly* by increasing circulation to restore normal membrane potential and reduce pressure on nerves due to localized edema.

NO is important in the process wound healing and tissue repair

Nitric Oxide (NO) and its interrelationship with essential growth factors is critically involved in the entire continuum of events associated with wound repair. NO is a powerful stimulator of cell division (proliferation) and maturation, particularly formation of appropriate cell receptors (differentiation). NO is a necessary mediator of neovascularization, i.e., the formation of new and eventually mature blood vessels (angiogenesis) and lymph ducts to nourish the healing tissues. NO increases the number of fibroblasts (fibroblastic proliferation) and thereby enhances collagen formation for the healing wound. Lastly, L-arginine and NO are necessary for the proper cross-linking of collagen fibers to one another, via proline, to minimize scarring and maximize the tensile strength of healed tissue.

NO is often impaired in people with diabetes

Both Type I and Type II diabetic patients have a reduced ability to generate NO from L-arginine, reflected in part by direct measurements of plasma nitrate and nitrite levels. Several factors influence nitric oxide production and metabolism.

As part of normal metabolism of L-arginine small amounts of a natural inhibitor of NOS are formed (asymmetrical dimethyl arginine (ADMA). Normally, ADMA does not accumulate in the blood because it is rapidly eliminated in the urine through normal kidney function. Reduced kidney function as part of aging (more than 20% of all Americans over 65 have Type 2 diabetes) or due to kidney dysfunction, which is accelerated by diabetes, may prevent the elimination of the major NOS inhibitor, ADMA, thereby limiting the production of NO.

Nitric oxide synthase (NOS) from which NO is derived, is a pH dependent enzyme. It is active at slightly alkaline (basic) conditions but is suppressed by acidotic conditions. In diabetes, glycolysis and ketoacidosis force pH toward acid conditions and this may account, in part, for the reduced production of NO.

Adequate Oxygen is necessary for the activity of NOS and therefore NO. Circulation is notoriously impaired in diabetic patients, which limits available NOS and NO.

Lastly, people with diabetes often experience elevated glucose levels. Some of this glucose becomes incorporated into hemoglobin and is measured as glycosylated hemoglobin (Hgb) or HgbA1C. Glycosylated hemoglobin binds NO in the form of nitrosothiols very tightly so that any NO that is formed cannot be easily released from RBC to help maintain blood flow through smooth muscle cell relaxation.

In summary, acidosis, low oxygen, and/or accumulation of ADMA are responsible for the decreased production of NO. What NO is available is tightly bound to glycosylated hemoglobin limiting its release and smooth muscle cells where NO affects essential cellular functions.

NO deficiency may impair the health of people with diabetes

Reduced production and higher than normal binding, may be partly responsible for the poor circulation in diabetic patients and would be one of the reasons for their high propensity to develop an ulcer. Additionally, poor nitric oxide metabolism is thought by some researchers to be the cause of peripheral neuropathy, the nerve damage often observed in people with diabetes.

In understanding the ways that NO can reduce pain, it is easy to realize its significance in people with diabetes. Impaired circulation is a typical consequence of this disease. Disturbed membrane potential would be anticipated thus decreasing the stimuli necessary for nerve firing and perception of pain. Additionally, this impaired circulation often leads to swelling in the extremities, exerting pressure on the nerves, which also causes pain. Lastly, NO mediated increases in cGMP may be impaired limiting its ability to directly reduce neuropathic pain.

Part 1. <u>Discovery of NO, Nobel Prize, relevance in vasodilation</u>

This is the first in a series of articles that relate specifically to nitric oxide (NO), a free radical gas that is a powerful regulator of circulation (it is an endogenous vasodilator) and a neurotransmitter (it helps in the processing of nerve signals as they cross synapses). L-arginine, one of 20 amino acids that make up proteins, is the only amino acid that generates significant amounts of NO. Both circulation and neural function are impaired in diabetic patients, more so if tight glucose control is not maintained.

The Nobel Prize was awarded to three Americans in 1998 for their work on discovering NO and clarifying its role in health. Their most important contributions lay in describing the effect of NO on the circulation that, as everyone knows, is disturbed to one degree or another in diabetic patients. The question then becomes is NO metabolism or action deranged in diabetic patients and could NO have a role in

preventing some of the consequences in diabetes? We certainly believe so and will develop this theme in this and succeeding articles.

The blood flow and nerve responses are rapid. Small increases in NO lead to both vasodilation and to better sensory perception. We will discuss the enzymes that generate NO in later articles in this series. Suffice it to say that NO metabolism is necessary for normal circulation (venous, arterial, and lymph flows) and for the ability to sense pain, temperature, and pressure. Diabetic patients have deficits in circulation, which often lead to blindness, kidney dysfunction, heart disease, and ulcers in the lower legs. Clearly circulation is impaired in diabetic patients. In addition, peripheral neuropathy (the inability to sense pressure or temperature in the feet) is a consequence of diabetes in many patients. Diabetic peripheral neuropathy (DPN) is a primary cause of ulcers and eventual amputation of digits or even whole limbs.

Is there evidence that NO metabolism is impaired in diabetic patients? The answer is yes and that too will be discussed in a later article.

L-arginine, the source of NO is released from proteins and small peptides in the small intestine and is then absorbed, along with other amino acids into the circulation from which it is delivered to every cell in the body. Some L-arginine is metabolized for NO synthesis and some is used for protein synthesis. In endothelial cells, the small cells that make up capillaries and line every blood vessel and lymph duct in the body, L-arginine can be converted to NO. This occurs only if the enzyme that makes NO and it's co-factors are available in adequate amounts. In diabetic patients, athrsoclerotic disease often occludes a portion of a vessel so that the endothelial cells are not able to properly absorb NO. If the endothelial cell can't take up L-arginine, then NO synthesis will be impaired. Moreover, if athrosclerotic disease is present, oxygen delivery to all cells is impaired and molecular oxygen is one of the cofactors needed by the enzyme to generate NO from L-arginine. The NO diffuses into the smooth muscle cells that surround the endothelial lining of blood vessels cells causing a biologic chain of events that lead to smooth muscle cell relaxation. This results in more blood flow to the tissues. Tissues that are hypoxic (deprived of good, normal circulation) can not produce as much NO as do normal, well oxygenated tissues. Thus an initial period of hypoxia leads to declines in NO production and less and less blood flow over time, a viscous cycle to say the least. It is no wonder that diabetes is a progressive disease with wounds, kidney, heart, and eye disease becoming worse and worse over time.

The next article in this series will examine the enzyme that generates NO from L-arginine. This is an important topic since there are three forms of the enzyme and each exerts slightly different effects on the amounts and timing of NO production.

Part 2. <u>Isoforms of Nitric Oxide Synthase</u>

Nitric Oxide Synthase (NOS) is the enzyme that generates NO from L-arginine as described in Part 1 of this series. However, the enzyme exists in three different

forms called isoforms. Each isoform synthesizes NO but does so under different conditions. Often all three isoforms will be found in the same cell but occasionally one cell will contain only one of the isoforms. This is important because many see or hear the term ***nitric oxide*** and assume that it refers to all cells under all conditions. This is not the case as outlined below.

NOS1 is the neural (or brain) isoform, sometimes referred to as bNOS. It helps in synaptic transmission, the processing of nervous information from nerve to nerve, across gaps between the nerves called synapses, and from peripheral nerves to the brain.

NOS2 is called inducible or iNOS. This enzyme generates extraordinarily high concentrations of NO, in part to kill bacteria. NOS2 (iNOS) takes several hours to be mobilized and the response is due to an injury or infectious process. NOS2 produced by macrophages is responsible, in part, for their effects to repair injury and to ward off infections. In other words, when the body mounts an inflammatory response to injury, macrophages are attracted to the site of injury where they produce large amounts of NO. Extraordinarily high concentrations of NO (100 to 1000 times normal) are produced very locally by this isoform. In fact, reports suggest that wound (ulcer) fluid may contain levels of NO that are very high and can only be attributed to iNOS. Unlike NOS1, which is part of normal neurotransmission, there must be something very abnormal (a wound, tissue damage, hypoxia, bacterial infection, etc.) to induce this enzyme. For the wound community that event is usually anything that threatens integrity of the skin.

The third isoform is ecNOS (or NOS3) which stands for "endothelial cell" NOS. This isoform is active at all times (it doesn't need to be induced as does iNOS) and is found in endothelial cells which are the cells that line the inner surface of all blood vessels and lymph ducts. ecNOS is activated by the pulsatile flow of blood through vessels. What does pulsatile mean? It is the stretching and relaxation of the blood vessel wall in response to each beat of the heart. Each time the heart beats it leads to an acute increase in the diameter of the blood vessel, followed by an equally acute return to a normal diameter. This leads to a "shear stress" on the membrane of the endothelial cells as thew column of blood, in the vessel moves forward and then stops. This NO, produced by ecNOS, maintains the diameter of blood vessel so that perfusion of tissues (skin, muscle, nerves, and bone) is maintained at optimal levels. In addition, ecNOS mediated NO causes angiogenesis, which is the growth of new blood vessels. This is especially important in healing an ulcer or wound on the skin.

One interesting interplay of iNOS and ecNOS is in tissue repair. Initially,NO is generated from iNOS in order to ward off infection and to destroy and remove the irreversibly damaged, necrotic tissue. This is often referred to as the inflammatory stage of wound repair. This phase lasts only a short time (a few days with an acute wound) and then ecNOS is (or should be) mobilized to cause vasodilation and angiogenesis to induce the healing response. NO will relax smooth muscle cells and thus dilate veins, arteries, and lymphatics. This increases blood supply both to the repairing tissues and from the damaged region. The latter removes metabolic waste products, reduces edema, and prevents swelling that would otherwise compress capillaries. In the absence of adequate blood supply tissue will remain hypoxic and heal only slowly, if at all. Moreover, since iNOS is produced in large part by white

blood cells (WBC), vasodilation permits delivery of additional WBC to the area that needs to be defended from infection. There are wounds that do become infected and often only marginal reduction of the infection is seen even with high dose and high potency antibiotics. By now most should realize that if the vascular bed (arteries, veins, and lymphatics) were dilated, more of the antibiotic would get to site of infection. Thus it is essential that ecNOS be activated to produce NO. Clearly both ecNOS and iNOS play a role in wound healing; neither alone is sufficient to achieve full recovery. In diabetic patients, however, ecNOS activity is often well below normal so these patients cannot produce NO at normal levels.

Finally, NO generated at physiologic levels, via ecNOS, will suppress the activity of the enzyme iNOS. This is why there is usually only a transient increase in iNOS activity in the normal response to wounds or tissue damage. In diabetic patients, with low production of NO from ecNOS, iNOS may not be inhibited and iNOS mediated NO production may remain high well beyond its intended time. This could contribute to continuous and uncontrolled tissue destruction, thereby slowing the healing process.

We have not fully explored bNOS or brain (neural) NOS activity in this discussion However, as we develop the theme of reversal of diabetic peripheral neuropathy later in this series of articles, one should remember that all three isoforms of NOS including bNOS, require molecular oxygen in order to function appropriately. Clearly, neural transmission (sensation of pain, pressure, and temperature) will be impaired if circulation (delivery of oxygen) is impaired. Thus, synaptic transmission and the proper processing of nervous stimuli need oxygen (controlled in part by ecNOS) in order for bNOS to carry out its NO mediated activity.

In the next part of this series we will discuss how NO formation and/or availability, especially from ecNOS, is altered in the diabetic patient.

Part 3. NO (NITRIC OXIDE) METABOLISM IN DIABETIC PATIENTS

To understand NO metabolism in diabetic patients we must first discuss the normal process of NO formation. NO is a gas that is also a short-lived, unstable free radical and, within seconds of production, must become stabilized. To do so, it reacts with one or more elements or biologic compounds, as described below.

First, NO may diffuse into smooth muscle cells and bind to an enzyme called guanylate cyclase (GC). As discussed in more detail in the next article in this series, this binding to GC initiates the process of vasodilation.

Secondly, NO can interact with oxygen to form the stable nitrates and nitrites that are measurable in serum, urine, or saliva. Clinically, nitrates and nitrites reflect the status of a particular patient in producing NO at the time the measurement is made. Higher concentrations of nitrate and nitrite are suggestive that high amounts of NO have recently been produced.

Lastly, NO may bind to sulfur (S) elements that are found as a part of certain amino acids, such as cysteine, and in other biologic compounds, such as glutathione, a well-recognized anti-oxidant. This binding to S molecules results in the formation of nitrosothiols. (A nitrosothiol is another name for a compound in which NO is attached to sulfur). Later, the NO can be released from the nitrosothiols to cause biologic responses such as smooth muscle cell relaxation and vasodilation.

Hemoglobin is a protein within RBC and is made up of two alpha chains and two beta chains. Although hemoglobin is well known for its ability to carry oxygen to tissues, a less well-appreciated fact is that on the beta chain are cysteine amino acids (which contain S) that bind NO as nitrosothiols. Thus hemoglobin carries both NO, which may be subsequently released, and oxygen.

Both Type I and Type II diabetic patients have a reduced ability to generate NO from L-arginine, reflected in part by direct measurements of plasma nitrate and nitrite levels. Several factors influence nitric oxide production and metabolism. Because NO is derived from the amino acid L-Arginine, one of the amino acids that make up proteins, it is clear that adequate protein intake is essential for NO production. However, simply adding more L-arginine to the diet of diabetic patients may not solve the problem of low NO production.

First, as part of normal metabolism of L-arginine small amounts of a natural inhibitor of NOS are formed. These inhibitors do not accumulate in the blood because they are rapidly eliminated in the urine provided kidney function is normal. The major inhibitor is named asymmetrical dimethyl arginine (ADMA). ADMA does, however, accumulate as kidney function declines and many diabetic patients lose kidney function as part of the disease process. Therefore, increasing dietary L-arginine, in an attempt to increase NO production, may be counterproductive in diabetic patients with decreased kidney function. Reduced kidney function is a part of aging (more than 24% of all Americans over 65 have Type 2 diabetes) and kidney dysfunction, which is accelerated by diabetes, may prevent the elimination of the major NOS inhibitor, ADMA. In this case, the production of NO would be low because NOS activity was inhibited by ADMA.

From our last article, you will recall that NO is produced from L-arginine due to the enzymatic activity of nitric oxide synthase (NOS). NOS is a pH (acid/base measurement) dependent enzyme; it is active at slightly alkaline (basic) conditions but is suppressed by acidotic conditions. In diabetes, glycolysis and ketoacidosis force pH toward acid conditions and this may account, in part, for the reduced production of NO since a slightly basic pH is ideal for NOS enzymatic activity.

Oxygen is a cofactor for the activity of NOS and therefore adequate oxygen is necessary for NO production. In the absence of sufficient oxygen there is less NO produced because the enzyme NOS will not function as well as normal. Circulation (in other words blood flow that brings oxygen to a particular site) is notoriously impaired in diabetic patients. One can appreciate the magnitude of the reduced blood flow, and the concomitant reduction in oxygen delivery, with non-invasive modalities such as scanning laser Doppler, TcPO2, or ABI measurements.

Plasma nitrate and nitrite concentrations are often lower in both Type I and Type II diabetic patients than in normal subjects thus indicating lower levels of NO production, irrespective of whether kidney function is below normal. JV Boykin, Jr., M.D. recently made an interesting observation. He noted that diabetic patients who didn't heal with growth factor therapy (to be discussed in a later article in this series) had very low levels of nitrates and nitrites in their urine, whereas those that did heal had higher, near normal concentrations of urinary nitrates and nitrites. The interpretation was that failure to heal a diabetic ulcer might be related to low rates of NO production. It is not clear yet whether, in diabetic patients, low L-arginine intake, acidosis, low oxygen, or accumulation of ADMA, or all of these are responsible for the ***decreased production of NO***, reflected by low urinary nitrate and nitrites. Most likely, all these events are occurring simultaneously.

In diabetes, glucose levels are elevated. Some of this glucose becomes incorporated into hemoglobin and is measured as glycosylated hemoglobin (Hgb) or HgbA1C. Glycosylated hemoglobin binds NO in the form of nitrosothiols very tightly so that ***any NO that is formed cannot be easily released*** from RBC to help maintain blood flow through smooth muscle cell relaxation.

To summarize, it is clear in diabetic patients that acidosis, low oxygen, and/or accumulation of ADMA are responsible for the decreased production of NO. Tighter binding to glycosylated hemoglobin may also limit release of NO to the plasma and smooth muscle cells. Most likely, all these events are occurring simultaneously and they account for the low plasma and urine levels of nitrates and nitrites in diabetic patients. Reduced production and higher than normal binding, may be partly responsible for the poor circulation in diabetic patients and would be one of the reasons for their high propensity to develop an ulcer. In the next article, we will discuss the mechanism by which NO causes vasodilation.

Part 4. <u>How Nitric Oxide (NO) causes vasodilation</u>

NO initiates and maintains vasodilation through a cascade of biological events that culminate in the relaxation of smooth muscle cells that line arteries, veins, lymphatics. While somewhat complex, the sequence of biological events that are triggered by NO is described below:

Step 1. NO gas released from nitrosothiols in hemoglobin or from endothelial cells, diffuses into smooth muscle cells that line small blood vessels.

Step 2.Once inside the smooth muscle cell, NO binds to an enzyme, called guanylate cyclase (GC) and this binding results in GC activation.

Step 3. Activated GC is able to cleave two phosphate groups from another compound called guanosine triphosphate (GTP). This results in the formation of cyclic guanosine monophosphate (cGMP) that is used to phosphorylate (Phosphorylation is the addition of a phosphate group) proteins, including the smooth muscle contractile protein called myosin.

Step 4. Once phosphorylated, smooth muscle cell myosin relaxes, resulting in dilation of the vessel that was originally exposed to NO.

As one can imagine, only a limited number of GC enzymes are present in any one smooth muscle cell and once all the GC enzymes have been activated, additional NO will not initiate any further vasodilation. Any "extra" NO is simply sequestered as a nitrosothiols bound to hemoglobin in RBC for future use.

Eventually the phosphate groups bound to myosin in smooth muscle cells must be removed to return the blood vessels to their normal diameter. This removal, or de-phosphorylation, is accomplished by another enzyme, a phosphatase. If the phosphatase enzyme is inhibited, then NO/GC/cGMP mediated vasodilation will be sustained for a longer period of time. This, in fact, is the basis of the erectile dysfunction drug Viagra™; which inactivates the phosphatase.

What does this mean for people with diabetes and for their physicians? Clearly, normal vasodilation cannot occur in patients whose NO ***production or release*** is depressed, as we pointed out in previous articles. Without vasodilation, healing of ulcers will be slow, development of nerve damage will accelerate, and circulation to organs such as eyes, kidney, heart, and intestine will remain below normal.

Some may ask whether it isn't "too much" vasoconstriction rather than "too little" vasodilation that characterizes poor perfusion in people with diabetes? In normal subjects, the control of perfusion involves several vasoconstrictor hormones and activation of sympathetic nerves, which together cause vasoconstriction. To induce vasodilation, the body must reduce these biologic responses or counter them with vasodilators such as NO (or a prostaglandin called prostacyclin). Therefore, in the absence of normal concentrations of NO, even normal levels of vasoconstrictive hormones or nervous activity results in abnormally low blood flow (vasoconstriction and its effect to reduce tissue perfusion). One does not need to implicate "too much" vasoconstrictive activity (via hormones or nerves) as a cause of perfusion problems in people with diabetes, although this certainly can be a contributing factor in some instances.

In summary, NO causes vasodilation by initiating a cascade of biological events that relax smooth muscle cells lining blood vessels. This vasodilation continues until a phosphatase enzyme dissociates the phosphate from myosin (which may be delayed by Viagra). Since vasodilation through NO only occurs when there is GC able to bind NO, additional NO, is sequestered for future use as a nitrosothiol, including those found in hemoglobin. NO is the most important of the body's countermeasures against normal vasoconstriction and, if production or release of NO is impaired, as in the case of people with diabetes, poor circulation, and all the consequences thereof, ensues.

Many people with diabetes exhibit loss of sensation, a phenomena that is linked directly to an abnormality in nerve function. The loss of nerve structure and function has been attributed to a decreased circulation induced, in part, by decreased production of NO. The relationship between NO, vasodilation, blood flow, and nerve function will be discussed in the next article.

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Part 5. NO and neurotransmission

Diabetic patients are particularly at risk for damage to sensory and motor nerves in the feet or to dysfunction of the autonomic nervous system that innervates internal organs, for example, the intestine. The clinical diagnosis of the latter condition is gastroparesis. NO is an important signaling molecule conveying information from one nerve to another, including non-cholinergic, non-adrenergic (NCNA) nerves. NCNA nerves control smooth muscle cells, which regulate gastric emptying and intestinal motility. Reduced availability of NO in diabetic patients may be one cause of gastroparesis.

Nerves communicate with one another across synapses and several biochemical compounds diffuse from one nerve to the second nerve. NO is one of these biochemical "neurotransmitter" molecules and is produced by both brain tissue and peripheral nerves.

NO has both a direct and indirect effect on neurotransmission. The direct effect relates to permeability of nerve membranes regulating ion transport that is important for nerve signal transmission. Indirectly, NO enables nerves to properly function by causing increases in blood flow (vasodilation) allowing essential oxygen and nutrients to be transported to nerve cells.

Direct: Dispersal of ions across the nerve cell membrane is dependent, in part, on transporter proteins that act as channels for ion transport. These channels regulate the permeability of the cell membrane. As was the case for the smooth muscle cell protein myosin, the contractile protein described in part 4, phosphorylation of these channels is essential in controlling ion permeability of the membrane of the nerve. Physiologic changes in ion permeability determine the transmission of impulse along the nerve. In nerve cells, NO generates cGMP (as described in Part 4), which results in phosphorylation of a nerve cell ion channel that is permeable to potassium ions. Thus, NO must be present in order to regulate membrane permeability to potassium ions, which is necessary for nerve signal transmission. Normalization of the inadequate NO levels in diabetic patients can ***directly*** impact nerve function by improving nerve membrane permeability to potassium ions.

Indirect: Poor circulation to feet and the lower leg, possibly a result of impaired NO-mediated vasodilation, results in swelling (edema), tissue damage, and ulcers. The lack of oxygen and nutrients also adversely affects nerves that also rely on oxygen and glucose to generate the energy source, adenosine triphosphate (ATP). ATP maintains ions such as potassium and sodium at normal physiologic concentrations inside and outside nerve cells. If oxygen and glucose delivery to nerves is impaired, then normal levels of ATP will not be generated. This event adversely affects

potassium/sodium homeostasis across the membrane. The nerves will not receive and process information (touch, temperature) when the potassium and sodium ions become chronically disturbed due to the lack of sufficient oxygen and nutrients. In addition, bNOS found in some peripheral nerves is, like ecNOS, is an oxygen dependent enzyme. The lack of oxygen available to the nerve itself would impair formation of NO and compromise neurotransmission. Nerves, like other tissues are supplied with oxygen through blood vessels. By inducing vasodilation and improving circulation, NO can improve nerve function by increasing available oxygen and glucose, thereby allowing ATP production to establish normal ion concentrations across the nerve cell membrane. Specifically, increased oxygen availability to the bNOS enzyme will improve impaired formation of neural NO and thus neurotransmission.

In summary, NO has both a direct and indirect influence on neurotransmission. NO, by affecting cGMP, allows phosphorylation of ion channels, especially potassium channels necessary for normal transmission of nerve signals. NO also increases blood flow. This allows sufficient oxygen and glucose to be transported to nerve cells, positively affecting ATP production and, in turn, potassium/sodium homeostasis essential for neurotransmission. Increases in blood flow may also allow the oxygen dependent isoform, bNOS, to produce more NO.

NO is also a powerful regulator of cell division and proliferation necessary for tissue repair In the next article, we will discuss the involvement of NO in tissue repair and wound healing, including the regeneration of nerves.

Part 6. Nitric Oxide's effects on Proliferation/differentiation: fibroblasts, blood vessels (angiogenesis), and skin

Nitric Oxide (NO) and its interrelationship with essential growth factors is critically involved in the entire continuum of events associated with wound repair, including cell division, maturation, neovascularization, and collagen synthesis including proper cross-linking of collagen fibers.

NO is a powerful stimulator of cell division. This is called proliferation, one cell into two, two into four, four into eight, and so on. For wounds to heal, new tissue is formed through induced division of existing cells. Several of the 10 to 20 known growth factors are necessary to induce cell division required in tissue repair. Of these, epidermal growth factor and/or keratinocyte growth factor, which are important for re-epithelialization and wound closure, cannot perform their biological function without their common chemical mediator, NO. Additionally, NO is important in duplicating some of the components of the cell so that each new cell is identical to its parent.

Proliferating cells must then differentiate into mature cells capable of responding to external signals. NO also stimulates the process of differentiation, in part, by regulating the formation of other proteins within the cell. One critical protein is the cytoskeleton, a complex network of proteins that form the internal structure of the

cell. These cytoskeletal proteins exert many functions, one of which is the insertion of receptors into the cell membrane. One end of some of these proteins is exposed to the external environment (the interstitial fluid) and the other end of the protein communicates with the cell interior (the cytoplasm). In the absence of NO, cytoskeletal protein development does not occur. Thus, without NO, a cell cannot form proteins that recognize, process and transmit information from outside of the cell to the cell interior.

Stated another way, without cell division and receptor formation, mediated in part by NO, wound healing will not occur.

Formation of new blood vessels, called angiogenesis, is essential for wound healing otherwise newly formed tissue will eventually deteriorate again due to lack of oxygen and nutrients. Growth factors, including vascular endothelial growth factor (VEGF) determine the extent of revascularization of damaged tissues. All growth factors bind to receptors on the cell surface and generate NO-mediated cGMP. Therefore, NO is a powerful and necessary mediator of angiogenesis.

JV Boykin first suggested that NO-mediated wound vascularization was an important mechanism for impressive wound healing obtained through the use of hyperbaric oxygen (HBO) therapy. Importantly, Boykin recognized that the enhanced wound healing could not be explained by HBO's effect on hyperoxia alone and he suggested that the additional oxygen helped to stimulate NOS activity and NO formation. We urge investigators to explore the extent to which HBO, and other maneuvers, activates ecNOS so that effective treatment strategies might be developed to enhance the activity of growth factor based products that may be dependent upon restoring normal NO for maximum efficacy.

Fibroblasts are cells that also respond to growth factors. NO increases the number of fibroblasts (fibroblastic proliferation) and thereby enhances collagen formation for the healing wound. Fibroblast growth factor exists in several isoforms but each causes local increases in NO production by fibroblasts. Furthermore, L-arginine availability ensures that the collagen that is formed is structurally similar to native collagen, i.e., that which was present prior to the injury to the skin. L-arginine is absolutely necessary for the proper cross-linking of collagen fibers to one to another, via proline, one of metabolites of L-arginine metabolism. Without L-arginine and thus NO and proline, collagen cross-linking is disrupted and the collagen that is formed is structurally abnormal. Scars or poor tendon/ligament integrity are principally manifestations of inadequate amounts of both L-arginine and NO early in the healing process brought about by fibroblasts.

In summary, NO is critical in many of the cellular processes involved with wound healing. NO is a powerful stimulator of cell division (proliferation) and maturation, particularly formation of appropriate cell receptors (differentiation). NO is a powerful and necessary mediator of neovascularization, i.e., the formation of new and eventually mature blood vessels (angiogenesis) and lymph ducts to nourish the healing tissues. NO increases the number of fibroblasts (fibroblastic proliferation) and thereby enhances collagen formation for the healing wound. Lastly, L-arginine and NO are necessary for the proper cross-linking of collagen fibers to one another,

via proline, to minimize scarring and maximize the tensile strength of healed tissue. Nerves must also "regrow" in healing tissues.

Part 7. Nitric Oxide (NO) and Relief of Pain

NO offers pain relief in a number of ways. In fact, NO is the mediator of the analgesic effect of opioids such as morphine. In this article we describe how NO affects pain responses and, in particular, certain pain responses in people with diabetes.

Nerves can only function if they are structurally intact. Nerves must have a normal membrane potential maintained by ion (potassium and sodium) pumps that derive energy from the synthesis of ATP. However, compromised circulation, which is often the case in people with diabetes, causes nerves to malfunction, due in part to the absence of normal amounts of oxygen and nutrients (such as glucose), which together synthesize ATP. Lack of adequate oxygen and nutrients, and the lower synthesis of ATP, adversely affects normal membrane potential. Under normal conditions, nerves operate at -70 mV (millivolts) and fire (signaling pain) at -20 mV. Due to lack oxygen and nutrients, the membrane potential more closely approximates -20 mV and in those circumstances it takes little stimuli to reach the firing threshold. Poor circulation to the nerves prevents them from sending the appropriate signals (for pressure and temperature) to the brain and often the poor circulation is first perceived as pain. NO mediated vasodilation will increase delivery of oxygen and nutrients to poorly perfused nerves to re-establish their normal membrane potential. Patients with diabetes are often given strong painkillers in an attempt to modulate the pain. These drugs do nothing to restore normal nerve function.

Reduced perfusion as a result of acute injury or chronic circulatory disorders causes swelling or edema, and this added fluid accumulation exerts pressure on the nerves, which can cause pain. Swelling also compresses capillaries that provide oxygen to the nerves (and other tissues as well). Consider the pain that occurs when, as a child, you put a rubber band around your finger. The finger turned blue, it swelled, and it eventually became so painful that you had to remove the rubber band. NO, which increases arterial flow to nerves and venous drainage away from nerves, counters the "rubber band" like effect of impaired diabetic circulation and in doing so removes the edema and swelling.

Medical researchers have often missed the significance of research conducted nearly a decade ago. NO was shown in the early 1990's to be the mediator of the analgesic effect of opioids such as morphine (SH Ferreira, 1991). Other studies, by this same group, showed that this beneficial effect was due, in part, to a morphine-mediated increase in NO and then in cGMP. Thus, it now appears that morphine binds to a nerve cell receptor, initiates a release of NO and there is a subsequent diminution in pain, mediated by cGMP. Therefore, raising local levels of NO can mitigate pain.

In understanding the ways that NO can reduce pain, it is easy to realize its significance in people with diabetes. Impaired circulation is a typical consequence of this disease. Disturbed membrane potential would be anticipated thus decreasing the stimuli necessary for nerve firing and perception of pain. Additionally, this impaired circulation often leads to swelling in the extremities, exerting pressure on the nerves, which also causes pain. Lastly, diffuse extremity pain is often associated with peripheral neuropathy. NO mediated increases in cGMP may directly reduce this neuropathic pain.

In summary, NO may reduce pain associated with diabetes *directly* by increasing cGMP (the mechanism by which opioids work), and *indirectly* by increasing circulation to restore normal membrane potential and reduce pressure on nerves due to localized edema.

The next article will discuss diabetic peripheral neuropathy (DPN), the leading cause of ulcers and amputations among people with diabetes. This is especially timely because on October 17, 2001, CMS published a Decision Memorandum recognizing DPN with loss of protective sensation as a localized illness of the feet.

Part 8. Nitric Oxide(NO) and diabetic peripheral neuropathy (DPN)

Diabetic Peripheral Neuropathy (DPN; peripheral nerve damage) is a common complication of diabetes. Almost 70% of people with diabetes develop DPN within 5 years and after 5 years the incidence rate increases to almost 100%. DPN most often begins as a tingling feeling and insidiously progresses to loss of sensation to hot and cold and to pressure. Additionally, DPN sometimes manifests itself as diffuse pain in the extremities. DPN is uncomfortable, may lead to poor balance and higher risks of falls, and is dangerous to those who have it. We have all come in contact with people with insensate feet who have developed ulcers because they did not sense poorly fitting shoes or unexpected foreign objects.

Recently, the Centers for Medicare and Medicaid Services (formerly HCFA) in Decision Memorandum CAG 00059 characterized DPN with loss of protective sensation (LOPS) as a localized illness of the foot and ***the most important factor*** leading to amputation in people with diabetes. Diagnostically, this Decision Memorandum states that DPN with LOPS is determined by insensitivity to a Semmes Weinstein 5.07 Monofilament at 2 sites on the bottom of the foot.

Just how does DPN occur in people with diabetes? The causation is debated among researchers and clinicians in the field. One theory is that progressive loss of circulation to the peripheral nerves is the cause of DPN. Another theory is that DPN is due to nerve dysfunction possibly due to accumulation of sorbitol on peripheral nerves. According to the NIDDK (Peripheral Neuropathy: The Nerve Damage of Diabetes), other researchers believe that lack of nitric oxide or poor nitric oxide metabolism may be the culprit. The debate and research goes on and hopefully one day we will have the answer.

While the pain associated with DPN may respond to drug therapy (for instance, Neurontin) or to topicals (lidocaine or capsaicin), clinicians have had little in their armamentarium to reverse the progressive loss of sensation observed with DPN. Clinical trials for a class of drugs known as aldose reductase inhibitors have been unsuccessful. However, Dellon has reported success with a surgical intervention similar to that employed in carpal tunnel release. Additionally, alpha lipoic acid is under investigation in Europe as having potential to increase sensation in cases of DPN.

The American Diabetes Association, based on the 10-year study of 1,441 patients with IDDM, recommends, that tight glucose control is one of the best ways to delay the onset or progression of DPN. Tight glucose control may delay the onset of LOPS in DPN, by decreasing the accumulation of sugar molecules called sorbitol within the nerves themselves. Reducing serum glucose levels also lowers the concentration of glycosylated hemoglobin (HbA1c). We have already mentioned in an earlier article that people with diabetes produce lower than normal levels of nitric oxide that may account for decreases in blood flow and a decreased capacity of blood vessels to dilate. Now we know that even the low amounts of NO produced are tightly bound to glycosylated hemoglobin. Not surprisingly, when HbA1c is elevated, the NO that is present in red blood cells is not easily released to promote vasodilation. This may account, in part, for very low blood flow to the nerves of the feet and, thus to the symptoms of DPN.

In view of the risks associated with DPN, either slowing its progression or, hopefully, reversing its course, is a worthwhile clinical goal. A promising new approach involves the use of an FDA cleared, non-invasive, medical device which may increase local levels of NO, thereby restoring circulation to the feet of people with DPN. It is hypothesized that that this device may promote a NO-mediated increase in circulation to help to regrow nerves that were lost to DPN or it may reestablish normal function in those nerves that still remain in the feet, or both. The first report (A Kochman, et. al., JAPMA 2002, in press) of the effect of this drug-free technology on patients with DPN is very encouraging. After only one month of treatment (3X/week for 30 min/day), 42/42 patients who were insensate to a Semmes Weinstein 5.07 Monofilament when they entered the study were able to feel this 5.07 Monofilament at the conclusion of the study. In other words, ***loss of protective sensation was no longer clinically present.***

The next article will detail why this device, the Anodyne ® Therapy System, may be able to alter local levels of nitric oxide so as to restore sensation lost with diabetes.

Part 9. How Light (Photo Energy) May Increase Local NO and Vasodilation

Light mediated vasodilation was first described by R F Furchgott, in his nitric oxide research that lead to his receipt of a Nobel Prize in 1998. Later studies conducted by other researchers confirm and extend Furchgott's early work and demonstrate the ability of light or photo energy to influence the localized production or release of NO and stimulate vasodilation through NO's effect on cGMP (as discussed in detail in Part

4). This finding suggests that properly designed illumination devices may be effective, noninvasive therapeutic agents for patients who would benefit from increased localized NO availability.

At first blush, some might question that something as simple as light can have such a profound biological effect. However, the biological importance of light has been recognized for quite some time. Various wavelengths of light are absorbed by chemical compounds, which then lead to biologic responses. Sunlight absorbed by chloroplasts in plant cells permits formation of starch. Sunlight absorbed by human skin generates vitamin D. Blue light applied to the back of the knee will alter human circadian rhythm. Some wavelengths of light, including near infrared and ultraviolet (UV) light cannot be seen with the human eye, and yet UV causes biologic effects, especially in the skin. Near infrared photo energy also exerts biologic effects

All light, visible or invisible, consists of photons. The size or mass of the photons is dependent on the specific wavelength of the light. Considerable research has been conducted about light (photo energy). This research shows that the target tissues must first absorb light in order to have a biological effect. Additionally, absorption is best achieved when the light is 1) directed perpendicular to the skin, and 2) placed in direct contact with the skin. Moreover, photo energy emitted from a source that produces of a homogenous wavelength is often more effective therapeutically than light composed of several wavelengths (for example white light)

Recent research supports the hypothesis that some wavelengths of photo energy are absorbed by hemoglobin and that intense illumination can release the NO from hemoglobin (specifically from the nitrosothiols in the beta chain of the hemoglobin molecule) in red blood cells (RBCs). This finding provides the basis of a potentially profound noninvasive therapeutic device for patients who would benefit from increased localized NO availability. Since RBCs are continuously delivered to the area of treatment, there is a natural supply of NO that can be released from each new RBC that passes under the light source and is exposed to the appropriate wavelength of photo energy. Since the half life of the NO released under the area of illumination is only 2 to 3 seconds, NO release is very local, preventing the effect of increased NO from being manifested in other portions of the body. What's more, dosage is taken care of the body itself. As you will recall, vasodilation from NO is based its effect on the enzyme Guanylate Cyclase (GC), which forms cGMP to phosphorylate myosin and relax smooth muscle cells in the vascular system. Once available levels of GC are saturated with NO, or once maximum levels of cGMP are achieved, further vasodilation through illumination will not occur until these biologic compounds return to their pre-illumination status.

One device that employs illumination to apparently increase the localized levels of NO is The Anodyne Therapy System. This FDA cleared medical device delivers near infrared (890 nm) photo energy from 60 super luminous diodes mounted on flexible pads that can be placed in direct skin contact; in addition, the flexible pads assure that the photo energy is delivered perpendicular to the skin to maximize absorption. Tests conducted with a scanning laser Doppler (Moor Instruments) demonstrate that the near infrared photo thermal energy delivered by the Anodyne Therapy system can increase localized microcirculation by as much as 3200% after just 30 minutes.

Further tests show that increased microcirculation achieved by the Anodyne Therapy System on neuropathic feet is 10 times more than that achieved with warmth alone.

In summary, intense illumination of the skin may non-invasively increase the localized release of NO from hemoglobin. The effectiveness of the illumination is dependent upon absorption by the targeted tissues, which is based on wavelength, skin contact, and perpendicular delivery. The potential net effects of skin contact illumination are those we have previously discussed in relation to NO, i.e., better blood flow via stimulation of GC, acute delivery of growth factors and white blood cells, fibroblastic differentiation and proliferation, angiogenesis, reduced edema, and mediation of pain.

The next article will be a summary of the biology of NO, as outlined in previous parts of this series; in addition, we will discuss how this very important molecule regulates so many other important biologic functions which are important for the health of diabetic patients.

Part 10. Nitric Oxide (NO) and Its Role in Wound Prevention and Wound Healing

In previous articles we have alluded to the positive effects of NO on wound healing. In this article we address the overall implication of NO in wound prevention and wound healing.

Vasodilation:

By now, most readers will appreciate that the risk of developing a lower extremity ulcer in people with diabetes may be greatly reduced if loss of sensation due to peripheral neuropathy is either prevented or can be reversed. To do so, requires an improved blood flow. NO is a powerful regulator of acute vasodilation, both for arteries, veins, and lymphatics. The increase in blood flow fills capillaries that were underperfused bringing oxygen and nutrients to the peripheral nerves and tissue. In addition, the enhanced venous drainage as well as the increase in lymphatic motility helps to remove edematous fluid that accumulates in the wound area. The latter effects of NO allow more oxygen and nutrient delivery to the wound site and speeds the removal of metabolic waste products from the area. Simply put, hypoxia and ischemia are reversed.

Growth factors:

Increased circulation through NO also provides an increased delivery of platelets, the source of platelet-derived growth factor. Addtionally, other growth factors and the cells that produce them will also have greater access to the wound area. Each of these growth factors is necessary for complete tissue remodeling in a healing wound. However, as Dr. Boykin pointed out, growth factors such as becaplermin, fail to achieve an acceleration of the wound healing process if the patient is deficient in NO.

Elevation the concentration of NO locally, in addition to simple vasodilation, facilitates the action of all growth factors in speeding cell division to rapidly replace damaged tissue. Thus, local increase in NO near the wound site will cause initial cell proliferation and then differentiation. These cellular activities relate to all tissues involved including blood vessels (angiogenesis), lymph ducts (lymphogenesis), muscles, epithelial cells, and nerves.

Inflammation:

NO will down regulate the activity of iNOS, which produces large amount of peroxynitrite (ONOO). INOS activity is important to destroy injured cells, in order to prepare the site of injury for new cell growth. However, uncontrolled activity of iNOS continues the inflammatory process and tissue destruction. Reducing iNOS activity with small, local amounts of NO will reduce shorten the inflammatory stage of wound healing and speed the repair process.

Immune Response:

It has been reported that dietary L-arginine will increase the concentration and activity of T-lymphocytes. This effect is likely mediated by NO itself rather than by L-arginine and thus NO is considered to be a powerful mediator of immune defenses. Therefore, in addition to NO mediated vasodilation that aids in the recruitment of white blood cells which defend against bacterial infections in non-healing ulcers, NO apparently strengthens the immune system, especially T-cells.

Skin flaps:

Wounds are often covered by grafts from other areas of the body. To survive, this viable tissue must be nourished with a good blood supply. We suspect that local elevations of NO for several days before as well as after surgery, in diabetic or other patients with reduced concentrations of NO in their circulation, would enhance the viability of these grafts. In fact, enhanced viability of skin grafts due to NO has been reported by Suzuki in Plastic Reconstructive Surgery (1998).

Cardiovascular integrity:

Diabetes is accompanied by serious cardiovascular disease. NO reduces platelet adhesion so in theory, there should be fewer atherosclerotic events. The ability of NO to grow new blood vessels reduces ischemia locally and removes edematous fluid in areas of low perfusion. Thus, the threat of clot formation, hypoxic or ischemic injury, and swelling of tissues are all minimized by elevations of NO toward normal.

Cumulative effect:

Continued elevation of local NO availability builds on the physiologic and biochemical effects, which were begun, with the first dose of NO. It is similar to starting up a staircase, where the first elevation of NO (first step) exerts positive effects on wound healing. Subsequent NO is important since the wound is never again as poor biochemically and physiologically as it was prior to the first increase in local NO. The

first NO exposure stimulates acute angiogenesis, perhaps only one or two new capillaries. However, using the staircase analogy, subsequent NO delivery to the wound site will result in the progressive development of many new blood vessels. What starts as a modest acute vasodilation eventually results in a well perfused, well healed tissue bed, one in which a subsequent ulcer is very unlike to occur. This is not to say that an ulcer won't occur in another area of the body based on the underlying disease state.

Approaches to elevating NO:

There are caveats to the treatment of wounds with a source of NO. First, systemic administration of dietary supplements such as L-arginine must be able to reach the wound and if swelling, edema, and tissue damage impinge on the local blood supply, then dietary supplements will have little value. Furthermore, acidosis and low oxygen availability in the immediate wound area, compromise the ability of the enzyme NOS to make NO from L-arginine.

Adverse drug interactions:

Many diabetic patients receive diuretics for kidney or cardiovascular disease. Diuretics may cause problems with potassium and magnesium metabolism. Magnesium is a regulator of intracellular calcium which itself is a co-factor for NOS activity. Magnesium also helps regulate intracellular potassium content and the excretion of potassium by the kidney. Addressing these possible co-morbid factors in a diabetic ulcer, may speed the healing of an otherwise slow healing wound.

Importantly, potassium imbalance also affects transmembrane potential in nerves. Clearly, the sensation of pressure, temperature, balance, and pain can be adversely affected unless the possible effects of diuretic usage are considered by the healthcare professional in the overall approach to diabetic ulcer management.

In summary NO, by stimulating vasodilation and normal membrane potential, may reduce the likelihood of peripheral neuropathy and thereby the major risk factor for diabetic ulcers. NO also positively influences wound healing by increasing vasodilation, promoting cell division and proliferation, angiogenesis, collagen formation and collagen cross-linking. Since people with diabetes are often low in NO, localized increases in NO availability may speed the healing of refractory diabetic ulcers.

The next article will discuss our experience with the Anodyne Therapy System as an adjunctive modality in wound care.

Part 11. Wound Care with Nitric Oxide Therapy— The Basis for The Anodyne System

Use of the Anodyne Therapy System (ATS) appears to elevate NO locally so that blood flow can be increased directly at the site of application. This increase in blood

flow is the basis of the therapeutic benefits of the ATS on pain and neuropathy, as we discussed earlier. Recall that arterial AND venous dilation occur in the presence of NO; throughput at the wound site is increased. Wounds such as diabetic ulcers, venous stasis ulcers, and pressure ulcers fail to heal without adequate blood flow to and from the site of injury. The ATS, which certainly increases circulation, might be expected to assist in the healing of wounds for reasons mentioned in Part 10, last week.

The first study to document an ATS-mediated improvement in wound healing was published in the peer reviewed journal, Advances In Skin and Wound Care 12:35, 1999. The study described some of the results from an IRB approved, double blind study on VA patients with venous stasis ulcers of long duration (6-40 years!). To enter the study the patients exhibited a current wound(s), which had to be present for at least a year, AND demonstrating non-healing with other treatments. Under the protocol, patients treated themselves at home for 30 min. per day with the ATS using only a wet to moist covering held in place with a stocking. Half of the patients were treated with placebo ATS devices offering warmth alone and the other half received active treatment. Within 3 weeks all the patients on active ATS showed remarkable acceleration of healing while those on placebo ATS did not. The physicians, for ethical reasons, began treating all patients in the study with active ATS. Additionally, patients with non-healing diabetic foot ulcers were treated with the ATS at another hospital. These studies were not placebo controlled, but the patients had failed to improve when treated with most, if not all, conventional wound care products. These patients were treated for 1-3 times per week as outpatients! These wounds also healed quickly.

The authors waited for over a year to publish these results to determine the quality and duration of healing. Subsequent evaluation (including capillary refill time, tissue integrity, and minimal scarring) demonstrated excellent tissue remodeling and, as a result, no subsequent breakdown in the healed wounds was observed. As might be expected, punch biopsies, for collagen analysis were not performed because patients and physicians were reluctant to "reinjure" the wound that had finally healed after so many years. Further follow up at four years on a number of patients has shown no reoccurrence of the wounds. Interestingly, we used a Scanning Laser Doppler (Moor Instruments) to measure the micro perfusion in the healed wound sites and observed that, four years after treatment, the blood flow in the healed area was 3 times better than blood flow in the surrounding area where no wound had ever occurred. These findings of excellent, very local tissue remodeling and presumed angiogenesis at the healed wound site would be expected with NO mediated increases in local blood flow and NO mediated enhancement of vascular and epithelial growth.

The results of this study have encouraged numerous wound centers to evaluate ATS for the treatment of refractory wounds. Dr. Mark Melin of Minneapolis recently reported success in more than 75% of patients during an interview aired on the Ivanhoe Network. Evaluators in Wisconsin have reported improved wound healing in refractory venous ulcers and improved viability of skin flaps using ATS. This center has also noted increases in TcPO2 values after use of ATS. Increases in TcPO2 cannot be attributed to anything else but an increase in oxygen availability at the site, which is believed to result from the vasodilation induced by NO. Lastly, a well-known rehabilitation hospital in Colorado has reported high levels of success in

treating previously unresponsive pressure ulcers. Other researchers are evaluating the speed of healing of diabetic ulcers using ATS.

Clinical reports indicate that ATS is being successfully used adjunctively along with numerous dressings including Fibracol and Panifil. Since ATS treatments are of short duration (30 minutes per day), non-invasive, and can be self administered by the patient adjunctively along with most wound dressings, the ATS appears to be a promising new innovation in wound care. Addtionally, by reversing diabetic peripheral neuropathy and restoring protective sensation, ATS may be able to reduce the incidence of diabetic foot ulcers and amputations in the first instance.

In summary, the Anodyne Therapy System is the first non-invasive medical device that has been shown to locally affect NO levels. In view of the essential physiological role NO plays in wound healing and pain reduction, the ATS (by affecting NO) may be of substantial clinical benefit to many patients. The ATS may provide its most meaningful clinical benefits to people with diabetes. These individuals exhibit impaired NO metabolism, reduced blood flow to nerves and tissue and consequent peripheral neuropathy, non-healing wounds, amputations and reduced life expectancy. Initial clinical research and reports show that ATS supports wound healing, reduces pain and reverses peripheral neuropathy in people with diabetes. Thus, it appears very likely that the ATS can improve the quality of life for people with diabetes and reduce direct and indirect cost associated with this chronic disease.

The next article will be the last in this series. It will summarize the role and importance of NO for patients, their physicians, and the healthcare profession in general.

Part 12. <u>The Current Science and Benefit of Nitric Oxide and Diabetes</u>

Diabetic patients, their physicians and health care providers, including certified diabetic educators, all recognize that **diabetes is a disease in which blood flow slowly and insidiously decreases over time**. The heart, kidneys, eyes, skin, and nerves all exhibit signs of reduced blood flow. One organ may show symptoms earlier than another, but all organs eventually demonstrate reduced function associated with the progressive decrease in blood flow. **The key to slowing this progressive deterioration of organ function is to delay the decrease in blood flow, or if possible, to restore it back toward normal levels.**

The body's natural vasodilator is nitric oxide (NO) but its production by diabetic patients is often 50% or more below normal levels. In addition, any NO that is formed is very tightly bound to hemoglobin within RBCs (and possibly to other heme proteins in other cells) so that it cannot be easily released to cause a needed increase in blood flow.

The past articles in this series have shown that there are several ways to increase the production or the local release of NO. Oxygen is critical to NO production but with poor circulation to sites such as nerves and skin, not enough oxygen is available to

fully activate the enzyme that generates NO from the amino acid L-arginine. In addition, as blood flow decreases, the generation of ATP (from oxygen and glucose) diminishes and high amounts of acid molecules are formed. Acidosis also diminishes the production of NO. Since production is diminished, the only readily useful source of NO is that already stored in cells, such as RBCs. The problem is how to release the stored NO so that local vasodilation can occur. The Anodyne Therapy System™ (ATS), mentioned several times in this series, turns out to be an easy to use, efficacious, non-invasive, drug free way to accomplish the local release of NO from RBC. The ATS uses near infrared light (NIR) that penetrates far deeper into tissues than do shorter wavelengths such as ultraviolet (UV).

Why is a wavelength of light so effective in releasing NO from RBC? To answer this question one must acknowledge the important contributions of physician/scientists who have studied NO for so many years. The 1998 Noble Prize in Medicine or Physiology was awarded to three Americans, one of whom, Dr. R.F. Furchgott, noted that NO could be made available acutely when he shined white light on tissues and that, as a result, blood flow increased. Since light is made up of several different wave lengths (or colors), subsequent research studies explored the beneficial effects of individual colors to determine which might be better at causing NO production or release and the accompanying vasodilation. Studies with visible colors were followed by experiments with single wavelengths (monochromatic) of non-visible light such as ultraviolet (UV) and near infrared (NIR).

Once NO is made available following the ATS's effect on RBC, and local blood flow (both arterial perfusion and venous return) is increased, skin ulcers heal faster and sensory and other nerve functions improve toward normal (All these events occur through the effect of nitric oxide to elevate cGMP and the phosphorylation events that follow).

Loss of sensation, especially in the feet of diabetic patients, has always been a progressive and irreversible side effect of diabetes; there was, until the ATS became available for clinical use in 1994, no known treatment for diabetic peripheral neuropathy. Since Loss Of Protective Sensation (LOPS) is the **leading cause of diabetic foot ulcers and amputations**, saving the foot has become a goal of all agencies (Federal, for profit, and not-for-profit) interested in diabetes foot care. One important recommendation to diabetic patients is to exercise. Exercise is much easier to engage in if blood flow is restored to near normal levels. In addition, exercise itself produces NO in blood vessels (by a process known as "shear stress") so that once circulation is restored toward normal levels, exercise helps to sustain the improvement in blood flow. However, it is quite difficult for a diabetic patient, with poor baseline blood flow and little or no sensation in their feet, to engage in this needed exercise.

Use of the ATS now appears to be a simple way to reverse LOPS (i.e., to restore protective sensation). Restoration of protective sensation is the leading way to dramatically decrease the incidence of ulceration and reduce the number of diabetes-related amputations performed each year. Associated costs of simply just treating diabetic ulcers, rather than healing them, should also decrease since the ATS also helps to heal (not just treat) diabetic ulcers. Importantly the ATS mediated increase in local NO, besides just increasing blood flow, oxygen, and nutrient delivery, has

other secondary effects. The ATS mediated increase in circulation restores the ability of cells such as fibroblasts to secrete growth factors, and NO enhances cell division so that new cells are formed faster than in the absence of NO. In addition, the increase in circulation delivers more white blood cells to the area so that healing and infection control can be achieved naturally. If needed, antibiotics are also delivered more effectively to the site of an infection when localized blood flow is increased. This is a remarkable series of outcomes, all attributed to increasing local NO at the treatment site.

It is highly unlikely that we would have been able to draft this series of articles on diabetic peripheral neuropathy and wound healing if Dr. Furchgott had not been curious about the effects of light in his laboratory experiments so many years ago. In fact, even he recognized the importance of NO to diabetic patients.

Dr. Furchgott wrote a lengthy article in 1998 entitled **"Nitric oxide: from basic research on isolated blood vessels to clinical relevance in diabetes"**, An R Acad Nac Med (Madrid) 115: 317-331. The Anodyne Therapy System capitalizes on these Nobel Prize winning laboratory experiments and appears to lead, non-invasively, to a biologic correction of diabetic circulatory problems that previously defied any consistent mechanical or pharmaceutical treatment.

Patients with serious diseases, and even their physicians, are often frustrated with the slow pace by which basic research is translated into treatments or cures. This series of articles highlights that such results do occur, and we encourage everyone to continue to support basic research conducted by the National Institutes for Health. One never knows where the next breakthrough will occur. We hope that the information on NO and diabetes, provided in this series of articles over the last 12 weeks for patients and their healthcare providers, will stimulate discussion about the importance of NO in ameliorating some of the symptoms of diabetes.

In conclusion, we recognize that research continues in the field of nitric oxide every day and that, in the future, targeted pharmaceutical drugs might be developed to locally increase nitric oxide. In the meantime, the Anodyne Therapy System™ is a **currently available, viable approach to locally stimulating NO release so as to increase circulation and reduce pain, two outcomes that may be of particular advantage to diabetic patients**.

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http://www.diabetesincontrol.com/burkearchive/