Endothelial Dysfunction, an Important Component of Insulin Resistance

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The Endothelium: a dynamic organ and a target tissue

The endothelium is a dynamic autocrine/paracine organ which regulates vascular tone and the interaction of the vessel wall with circulating substances and blood cells. The endothelium produces vasodilators and vasoconstrictors (Table 1), which under normal physiologic conditions are in balance. One of the major vasodilators is nitric oxide (NO), which has multiple vascular protective actions. These include inhibition of vascular smooth muscle cell (VSMC) growth and migration, platelet aggregation and thrombosis, monocyte adhesion, inflammation, and oxidation.[1] In contrast, vasoconstrictors such as angiotensin II (Ang II) promote vascular damage. Ang II is pro-inflammatory. In endothelial cells and vascular smooth muscle cells (VSMC), it stimulates 1) the expression of adhesion molecules, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM), which enhance the adhesion of circulating monocytes to the endothelial surface,[2] 2) expression of monocyte chemoattractant protein-1 (MCP-1) which promotes movement of monocytes into the vessal wall,[3] and 3) stimulates movement of moncytes.[4] Ang II also stimulates platelet aggregation and thrombosis, the letter by transactional activation of plasminogen activtor inhibitor-1 (PAI-1).[5]gene expression Ang II also promotes migration and growth of the VSMC, as well as increased expression of transforming growth factor -β (TGF-β) and is, thus, a major regulator of vascular remodeling.[6] Importantly, Ang II is also an oxidant, as it stimulates NAD(P)H oxidase, which enhances the conversion of NO to superoxide radicals. The health of the vasculature critically depends on normal functioning of the endothelium. Endothelial dysfunction is defined as an imbalance in which the effects of vasocontrictors outweighs the effects of vasodilators. This imbalance generally results from decreased NO bioactivity, which is measured as decreased vasodilatory capacity, but which also implies loss of vascular protection.

Well known risk factors for coronary artery disease including hypercholesterolemia, low high density lipoprotein cholesterol (HDLC), smoking, hypertension, diabetes, and hyperhomocystinemia are associated with endothelial dysfunction.[7-12] Both brachial artery and coronary artery responses to acetylcholine, which stimulates endothelial NO production, have been demonstrated to be abnormal in the presence of these risk factors. Since endothelial dysfunction occurs even in the absence of angiographically defined disease, endothelial damage has been implicated as preceding and probably contributing to the development of atherosclerosis.[13] In general, endothelial dysfunction predicts cardiovascular events. For example, in one prospective study of 150 subjects receiving coronary artery angiography, those in the lowest tertile of coronary artery vasodilatory responses to acetycholine had significantly greater events in the following two years compared to subjects in the middle and upper tertile of responses.[14] Endothelial dysfunction increases with age, above 40 years in men and 55 years in women.[15] Environmental factors including ingestion of a high–fat meal and physical inactivity are also associated with endothelial dysfunction.[16, 17] In normal people the effects of a high fat meal are transient. Infusion of Intralipid, containing a mixture of free fatty acids, can induce endothelial dysfunction, suggesting that free fatty acids may be damaging to the vascular wall.[18, 19]

Endothelial Dysfunction in Insulin Resistance and Diabetes

Several studies have demonstrated that NO-mediated vasodilation is abnormal in patients with type 2 diabetes.[20] Brachial artery responses were found to be abnormal to both endogenous and exogenous NO donors, suggesting that there was increased inactivation of NO, possibly caused by enhanced metabolism of NO or abnormal VSMC responses to NO due to alterations in signal transduction in the guanylate cyclase pathway.[20] Obese patients without frank type 2 diabetes

have also been demonstrated to have abnormal endothelial function.[21, 22] Many of these patients had impaired glucose tolerance (IGT) and, likely, multiple components of the insulin resistance syndrome. Both of these states of carbohydrate intolerance are associated with increased rates of coronary heart disease mortality, three to four fold in type 2 diabetes and two to three fold in IGT.[23]

Recent evidence suggest that endothelial dysfunction can be detected earlier in the spectrum of insulin resistance, prior to the presence of any distinct carbohydrate intolerance. Quinones, et al,[24] studied endothelial function in Mexican American subjects with only modest changes associated with the insulin resistance syndrome. They employed positron emission tomography (PET) to non-invasively assess coronary artery endothelial responses to cold-pressor testing (CPT). Dipyridamole was also used to access coronary artery vascular smooth muscle vasodilatory capacity. Insulin resistant subjects had half of the coronary artery vasodilatory responses to CPT compared to insulin sensitive subjects, while the vascular smooth muscular cell responses were similar in both groups.[24] In fact, the endothelial responses to CPT in the insulin resistant group, were similar to that previously seen in a group of smokers.[25] Thus, in relatively young individuals who are obese, even in the absence of major components of insulin resistance syndrome, coronary artery endothelial dysfunction is detectable. In another study evaluating microvascular endothelial-dependent vasoreactivity, Balletshofer, *et al*,[26] found abnormal vasoreactivities in non-diabetic relatives (siblings and children) of patients with type 2 diabetes. The relatives of patients with type 2 diabetes who were insulin resistant displayed abnormal vasomotion.. Thus, insulin resistance itself appears to be associated with endothelial dysfunction. This observation has multiple implications which relate to alterations in insulin signaling in the

vasculature, endothelial dysfunction as a *cause* of insulin resistance, and insulin, itself, contributing to accelerated vascular damage.

Insulin, is a vasodilator and stimulates endothelial NO production.[27, 28] In target tissues, insulin stimulates two major pathways, the phosphatidylinositol 3-kinase (PI3-K) pathway and the mitogen-activated protein kinase (MAPK) pathway (Figure 1). Following binding to its receptors activation of PI3K is critical for insulin–mediated glucose uptake into insulin-dependent target tissues, such as skeletal muscle, heart and fat.[29] This pathway has also been shown to regulate insulin-dependent endothelial NO production.[30] Thus, in the presence of a defect in insulin-mediated glucose uptake, Baron and his colleagues,[31] demonstrated that there is also a defect in insulin-stimulated endothelial vasodilation. Thus, a systemic defect in the PI3 kinase pathway, which likely "defines" insulin resistance, leads to a combined defect in insulin-mediated glucose transport and in insulin-stimulated endothelial vasodilation. Insulin is also a well known growth factor; the MAPK pathway mediates this action of insulin.[32] In the vasculature this pathway not only mediates cellular growth, but also mediates the ability of endothelial cells, VSMC and monocytes to migrate. It also appears to mediate expression of the prothrombotic, profibrotic factor, PAI-1, by a variety of stimuli.[33] Thus, by mediating cell growth and migration, proinflammatory and prothrombotic responses, the MAPK pathway may be proatherogenic. An important question is whether this pathway is also attenuated in states of insulin resistance. In the obese, insulin resistant Zucker rat, although activation of the vascular PI3K pathway by insulin is blunted, the MAPK pathway is activated normally in response to insulin.[34] Similar responses have been shown in humans. Mandarino and colleagues,[35] measured PI3K and MAPK activity in gluteal biopsies taken before and after insulin infusion in normal subjects, obese non-diabetic subjects and patients with type 2 diabetes. Activation of the

PI3K pathway was blunted in both the obese non-diabetic subjects and patients with type 2 diabetes compared to lean controls, while all three groups had similar activation of the MAPK pathway in response to insulin infusion.[35] This data clearly demonstrates that insulin resistance and the accompanying defects associated with the insulin resistance syndrome are dependent on a defect in a specific insulin signaling pathway, the PI3K pathway, while functions mediated by the MAPK pathway operate normally. In fact, there is also data to suggest that the impaired activation of the PI3K pathway by insulin is associated with enhanced activation of the MAPK pathway in vascular cells.[36] In the presence of insulin resistance, it is possible that hyperinsulinimia may be proatherogenic.

As insulin resistance may contribute to endothelial dysfunction, defects in NO-mediated vasodilation may contribute to insulin resistance. An infusion of N^G-monomethl-l-arginine (L-NMMA), which inhibits NO synthase, not only impairs endothelial-dependent vasodilation, but also has been reported to impair insulin-mediated glucose uptake.[37] L-NMMA inhibited leg muscle glucose uptake by 21%.[37] Another group were unable to demonstrate that infusion of L-NMMA could alter whole-body glucose uptake, despite its effects to reduce forearm blood flow and increase blood pressure.[28] Nevertheless, the idea that endothelial function could regulate insulin-mediated glucose uptake is intriging. This relationship might help to explain the observation in two clinical trials that, two different agents which improve endothelial function, an angiotensin converting enzyme inhibitor (ACEI) and a statin not only slowed the progression of coronary artery disease and cardiovascular death, but prevented the onset of type 2 diabetes in high risk patients, both by approximately 30%-35%.[38, 39] Thus, these observations suggest that as the progression to cardiovascular disease is attenuated, the progression to diabetes is also

attenuated. Elucidation of common mechanisms mediating these events will be important to understanding their intimate relationship.

The presence of endothelial dysfunction early in the spectrum of insulin resistance strongly suggests that vascular damage, potentially associated with oxidation, inflammation and thrombosis is also present. Therefore, early recognition of the presence of insulin resistance treatment of insulin resistance, at least with lifestyle modification and aggressive management of risk factors, is critical in the prevention of atherosclerosis and potentially in the prevention of diabetes. Whether those approaches that improve endothelial function in insulin resistance also decrease cardiovascular risk remain to determined.

Mechanisms of Endothelial Dysfunction in Insulin Resistance

Multiple, interrelated mechanisms contribute to endothelial cell dysfunction in insulin resistance. Some components of the insulin resistance syndrome such as low HDLC, and hypertension, are, themselves, independent risk factors for atherosclerosis and are known to be associated with abnormal endothelial function.[40] Two other components of the insulin resistant syndrome, increased small, dense LDLC (the moiety of LDLC that is highly susceptible to oxidation) and increased uric acid, closely associate with coronary artery events and likely also contribute to alterations in endothelial function.[41, 42] The exact mechanism by which dyslipidemia contributes to endothelial dysfunction is unknown. ENOS is known to associate into calveolae, which are characteristic flask-shaped invaginations of the plasma membrane of a variety of cell types including endothelial cells and VSMC.[43] Calveolae are cholesterol rich and also contain a variety of signaling proteins. Animals who lack a calveolae associated protein, calveolin 1, have flaccid arteries with decreased vasoconstrictive responses to AngII and endothelin and constituitive eNOS activity.[44] Addition of oxidized LDL (ox LDL) to cultured endothelial cells

caused calveolin and eNOS to translocate from calveolae to an intracellular membrane compartment, which was associated with a reduction in acetycholine responsiveness and decreased cholesterol content in the calveolae.[45] Thus, disruption of the calveolae complex has been thought to be associated with decreased eNOS activity and endothelial dysfunction. HDL prevented the oxLDL-mediated decrease in cholesterol in calveolae, prevented the translocation of eNOS and calveolin from calveolae, and prevented the decrease in responsiveness to acetylcholine.[46] These effects of HDL occurred because HDL donated cholesterol to the calveolae complex.[46] These cellular events are consistent with the proatherogenic effects of LDLC and oxLDLC and the protective effects of HDLC.

The presence of hypertension and other atherosclerotic risk factors is associated with increased vascular AngII generation and activity.[47] AngII is known to stimulate the MAPK pathway to potentially enhance the action of insulin to promote cell growth and movement and increase vascular PAI-1 expression. Since Ang II and insulin activate a common signaling pathway, increased sensitivity to AngII may occur in the hyperinsulinemic, insulin resistant state.[48] In addition, AngII stimulates ICAM-1 and MCP-1 through the MAPK pathway in endothelial cells and VSMC, (Figure 1).[2, 3, 49] Many of the components of the insulin resistance syndrome likely have direct effects to alter endothelial vasoreactivity; however, many of these factors likely decrease NO activity through oxidation pathways (Figure 2).

Oxygen-derived free radicals impair endothelium-dependent relaxation. NO formed in the endothelium is inactivated by superoxide anion radical O_2 to form a stable peroxynitrite anion, $ONOO^-$.[50] Superoxide anions depress endothelium-dependent arterial relaxation induced by acetylcholine by inactivation of NO.[51] AngII, oxLDLC, cyclin stretch and other agents which damage the vasculature stimulate NADH/NADPH oxidase and other enzymes which catalyze the

production superoxide anions, reactive oxygen species (ROS) metabolize NO, (Figure 2).[52] ROS generation is enhanced in vessels of hypertensive animal models and in atherosclerotic lesions of humans and animals.[53-55] This process appears to not only promote vascular damage, but to be involved in the maintenance of hypertension.

Tetrahydrobiopterin (BH_4), the essential cofactor for the catalytic activity of eNOS, results in eNOS "uncoupling" favoring oxygen free radical generation if proper cofactor concentrations are not maintained.[56, 57] Critical BH_4 levels are normally met by *de novo* synthesis, regulated by GTP-cyclohydroxylase-1, and dihydrobiopterin (BH_2)-recycling mediated by dihydropterin reductase (DHPR).[58] BH_4 is a potent natural reducing agent and is depleted during high states of oxidative due to excessive oxidation. Uncoupling eNOS results in decreased NO production and favors the generation of superoxide anion and increased oxidative stress.[59] Treatment with BH_4 has been shown to improve endothelial function in experimental diabetes and smoking while intra-arterial infusion of BH_4 significantly improves function in hypercholesterolemic patients. [60, 61] [62] In addition, insulin resistance has been shown to diminish DHPR activity in human coronary arteries with resultant BH_4 depletion, from decreased BH_2 recycling.[63] This lead to increased oxidative stress levels and endothelial dysfunction as measured by intracoronary acetylcholine stimulation.[63]

Approaches to Improve Endothelial Functions in Insulin Resistance

Lowering known atherosclerotic risk factors such as high LDLC with statins or hypertension with ACEI or increasing HDLC with fibrates improve endothelial function.[64-67] These treatments have also been demonstrated in clinical trials to reduce atherosclerotic risk.[38, 68, 69] Thus, it is clearly important to improve HDLC and aggressively control LDLC and blood pressure in all patients with insulin resistance, even before frank type 2 diabetes develops. The

current American Diabetes Association recommendation for LDLC is less than 100 mg/dl, HDLC greater than 45 mg/dl and for blood pressure (in agreement with the National Kidney Foundation, NKF) less than 130/80 mmHg.

Fibrates have dual effects to increase HDLC as well as activate peroxisome-proliferator-activated receptor (PPARα) in the vessel wall.[70] PPARα activation decreases vascular inflammation, growth and oxidation and appears to be vascular protective.[71] In contrast, other approaches have been shown to improve endothelial function, but not to improve cardiovascular endpoints (Table 2). Estrogen replacement therapy improves endothelial function in postmenopausal women, but did not improve cardiovascular morbidity and mortality.[72-75] Vitamin E administration has also been shown to improve endothelial function; however, several clinical trials have not demonstrated an effect on reducing atherosclerosis risk.[76-78] In the HERS TRIAL, hormone replacement therapy increased cardiovascular events during the first year in high risk women; these events were largely prothrombotic.[73] Estrogen is known to stimulate PAI 1, platelet aggregation and other thrombotic mechanisms, so that these factors appear to out weigh the impact of estrogen on endothelial function.[79] The cause of the discrepant results between vitamin E and endothelial cell function and its impact on cardiovascular endpoints is unknown; however, these observations suggest that in addition to the improvement in endothelial function, therapies aimed at decreasing cardiovascular events must be assessed in clinical trials.

There are other promising approaches which improve endothelial function in insulin resistance and diabetes, but which need testing by clinical trials with cardiovascular endpoints. Thiazolidinediones, ligands to the nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ), improve insulin-mediated glucose uptake and reverse nearly all of the components of the insulin resistance syndrome.[80] These ligands enhance expression of genes

involved in the insulin signaling cascade, primarily in the PI3K pathway, which likely involves direct effects in skeletal muscle, as well as modulating the complicated cross talk between adipose tissue and skeletal muscle.[81] In contrast, these agents inhibit several nuclear events activated by the MAPK pathway and, thus, decrease cell growth and movement in the vasculature and decrease circulating PAI-1 levels.[81] Ligands to PPARγ appear to restore the balance between the two major cell signaling pathways that are activated by insulin. Troglitazone (removed from the United States market in 2000) and rosiglitazone have also been shown to improve endothelial function when measured in peripheral arteries[82, 83]. Rosiglitazone has recently been shown to improve coronary artery endothelial cell function in insulin resistant subjects when measured by PET.[84] Several trials are currently underway to address the impact of rosiglitazone and pioglitazone on cardiovascular endpoints.

Hyperglycemia contributes to endothelial dysfunction. When normal subjects are made hyperglycemic during hyperglycemic clamp, endothelial dysfunction can be induced.[85] Hyperglycemia elevates diacylglycerol concentrations which translocates and activates protein kinase C (PKCβ) in endothelial cells.[86] Glucose also increases superoxide production which activates PKCβ.[87] PKCβ also increases NAD(P)H oxidase which augments ROS production decreasing bioavailability of NO. These effects of hyperglycemia can be reversed by a PKCβ inhibitor, LY333531.[88] In humans, LY333531 improves endothelial dysfunction induced by hyperglycemia in normal subjects and in patients with diabetes.[88]

Vitamin C improves endothelial-dependent vasodilation in type 2 diabetes and other states of endothelial dysfunction.[51, 89-91] It is possible that vitamin C in combination with vitamin E or that high dose vitamin C itself may attenuate cardiovascular risk. Vitamin C increases the bioavailability of NO and may even increase NO production.[92] Vitamin C also improves

hyperglycemia-induced endothelial dysfunction, suggesting hyperglycemia contributes to vascular superoxide anion formation.[93] L-arginine improves endothelial function; however, there are no cardiovascular endpoint studies with this amino acid approach.[94]

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