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Role of the Endothelium in the Metabolic Syndrome: IIB or Not IIB

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The metabolic syndrome represents the commonly observed link between obesity, cardiovascular disease and metabolic disease, with the primary constituents being obesity, hypertension, insulin resistance and dyslipidemia. An example of the link between obesity and hypertension is a study in adolescents published in 2008. In this work, more than 300 subjects aged 12 to 18 had their intra-abdominal fat evaluated by magnetic resonance imaging, and metabolic syndrome was defined as having 3 or more abnormal parameters for blood pressure, body mass index, serum glucose-relative-to-insulin, triglycerides or high-density lipoprotein cholesterol level. In an evaluation of systolic blood pressure in these individuals, those who had high intra-abdominal fat measurements had consistently higher systolic blood pressure than those who had low intra-abdominal fat measures.¹ An example of the consequences that can arise from the metabolic syndrome is a study in the journal *Atherosclerosis* in 2009 that involved almost 100 healthy Latino children who were approximately 11 years old. They were assessed for the metabolic syndrome on 4 annual visits. At those times, they also had carotid intima-media thickness (cIMT) measured by ultrasound. They were characterized as either having no, intermittent, or persistent metabolic syndrome across all 4 annual visits. There was a marked increase in cIMT in the children who had persistent metabolic syndrome during the 4-year period. The increase in cIMT observed with persistent metabolic syndrome was consistent with that seen with approximately 10 years of atherosclerosis progression.² As a pediatrician quite concerned with the long-term health of children, it is quite clear that obesity does indeed elevate the risk of hypertension and preclinical vascular disease.

Let us briefly remind ourselves about the endothelium, its function in vascular health, and the critical enzyme endothelial nitric oxide synthase (eNOS). eNOS is found in the vascular endothelial cells, where it produces the signaling molecule nitric oxide (NO) to serve diverse functions. NO is anti-inflammatory and antithrombotic, and it promotes relaxation of the underlying smooth muscle and blunts smooth muscle growth and migration. In addition, the NO produced by eNOS is pro-angiogenic, and it promotes endothelial repair.³

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For several years now, our laboratory has been studying eNOS and its regulation, particularly as it relates to its localization on the plasma membrane. There are unique organelles on the plasma membrane where eNOS is targeted called caveolae, which are a subset of lipid rafts. The caveolae are small invaginations that were first appreciated by electron microscopists. In the early 1990s, we discovered that eNOS is localized to caveolae. We used many approaches to study the localization, one of which was to perform immunoelectron microscopy. We observed that antibodies to the structural protein caveolin directed gold particles to the caveolae, confirming the identity of the microdomains, and that caveolae were also the site where antibodies to eNOS directed gold particles.⁴

In our early studies of the regulation of eNOS in caveolae, we came to recognize that eNOS is involved in a complex signaling module. There are a number of ligand-receptor pairs functional in caveolae, many of which are G-protein-coupled receptors that activate kinases, in particular, phosphatidylinositol-4,5-bisphosphate 3-kinase and Akt kinase. These kinases alter the phosphorylation of particular residues on the eNOS protein to govern the level of activity of the enzyme. In the caveolae, eNOS interacts with the structural protein caveolin, and it is additionally regulated by calmodulin, calcium and HSP90.³

I will now discuss findings that we have made in preclinical models regarding eNOS regulation that we believe provide insights into the role of the endothelium in at least certain features of the metabolic syndrome. Let me introduce you to the inflammatory molecule C-reactive protein (CRP). This is a member of the pentraxin family of proteins. It is an acute phase reactant that can be elevated several hundred fold within 24 to 48 hours of an inflammatory response. It is synthesized and secreted primarily but not exclusively by the liver in response to cytokines, particularly IL6. CRP is used clinically as a marker of inflammation. For reasons that will be of interest in a moment, it serves the equivalent function of IgG in the horseshoe crab. Mice produce a homologous protein to CRP known as serum amyloid P component that also functions as an acute phase reactant. Several studies in humans have demonstrated that CRP levels are chronically elevated in obesity and other inflammatory conditions. Important to our discussion today, a modest elevation in CRP in the circulation is a recognized risk factor for hypertension and type II diabetes, which are 2 key components in the metabolic syndrome.

During the early 2000s, we learned a lot about CRP, not solely as a marker of inflammation but as a mediator of adverse effects of inflammation on the vascular endothelium. What we discovered is that CRP potently inhibits the transcription of the eNOS gene, thereby decreasing the abundance of the enzyme protein. In addition to that effect, CRP acutely inhibits the activation of eNOS. Those cumulative effects both on transcription and on acute regulation of the enzyme lead to CRP causing marked decreases in NO production by the endothelium.⁵⁻⁷

Because of this, CRP very potently can be pro-inflammatory and, for example, promotes monocyte adhesion to the endothelium. We performed additional studies in which we evaluated the basis for CRP signaling in the endothelium. The example I am going to provide is that insulin signaling in the endothelium is disrupted by CRP. Let us first consider normal insulin signaling. Insulin very potently activates eNOS by first binding to the insulin

receptor. This leads to the phosphorylation of insulin receptor substrate 1, leading to sequential activating phosphorylation of PI3 kinase, Akt kinase and eNOS. What we observed is that with CRP exposure, insulin no longer activates eNOS. In particular, the critical phosphorylation event on eNOS does not occur in response to insulin. The immediate upstream activating phosphorylation of Akt in response to insulin also does not occur. This suggests that CRP is perturbing signaling between PI3 kinase and Akt kinase.

Using different reconstitution strategies, we determined that CRP is inhibiting signaling in response to insulin by binding to a receptor on endothelium, that is, a member of the IgG family of receptors known as Fc receptors (FcR). The specific receptor involved is Fc γ RIIB. Further studies showed that the CRP bound to Fc γ RIIB inhibits insulin signaling through a particular domain on the FcR called the ITIM domain. That domain is involved in recruiting and activating the phosphatase SHIP-1. Therefore, the basis for CRP signaling in the endothelium is through binding to Fc γ RIIB. This ligand/receptor interaction activates the phosphatase SHIP-1, and SHIP-1 inhibits signaling downstream of PI3 kinase. As a result of those effects, there is blunted production of NO in response to agonists such as insulin.⁸

With this information about how CRP signals in endothelial cells in mind, let us discuss how we determined whether CRP influences blood pressure and insulin sensitivity. We evaluated blood pressure by radio telemetry in mice that are transgenic for CRP (TG-CRP) and in control wild-type mice on identical CF1 background. In the TG-CRP mice, the relative amount of CRP in the circulation is manipulated by altering dietary carbohydrate intake, as the transgene is regulated by the phosphoenolpyruvate carboxykinase (PEPCK) promoter. The first studies were performed on a standard diet. Under those circumstances, TG-CRP mice displayed marked systolic hypertension compared with control mice. The mice were then placed on a high carbohydrate diet, turning off the CRP transgene and lowering serum CRP, and whereas systolic blood pressure fell in TG-CRP, there was no change in BP in control mice. The mice were then switched to a low carbohydrate diet, activating the CRP transgene, and BP rose in TG-CRP mice and was unaffected in control mice.⁹ These studies demonstrated that, at least in the mouse, CRP promotes hypertension, and that CRP-induced hypertension is reversible. Thus, if one can lower CRP, the blood pressure will fall in suit.

In more recent studies that have just been completed, we determined whether the effects of CRP on blood pressure in the mouse involve Fc γ RIIB. By crossing TG-CRP mice with mice null for Fc γ RIIB, we determined that the IgG receptor is required for CRP to induce hypertension in mice. Whether CRP and Fc γ RIIB contribute to the hypertension characteristic of the metabolic syndrome in humans is yet to be determined.

Let us now turn to what we have been able to learn about CRP and Fc γ RIIB and metabolic health. These were studies performed in the TG-CRP mice. First, we observed that TG-CRP mice have fasting hyperglycemia and fasting hyperinsulinemia. We then performed glucose tolerance tests (GTTs) and found that the GTTs are abnormal in TG-CRP mice. The increase in serum glucose that occurs in response to a bolus dose of glucose was exaggerated in TG-CRP mice. We also performed insulin tolerance tests (ITT), which evaluate the fall in glucose after an insulin dose, and found that the TG-CRP mice have a blunted response to insulin and therefore an abnormal ITT. The most rigorous test of insulin sensitivity is to

perform euglycemic-hyperinsulinemic clamp studies, in which mice are given a stable infusion of a set dose of insulin to evaluate the amount of glucose that must be infused at the same time to achieve a very finite range of serum glucose. We found that to achieve the goal range of serum glucose, TG-CRP mice require half as much glucose infusion as control mice. This indicates that TG-CRP mice are extremely insulin resistant. We then went on to determine the role of Fc γ RIIB in CRP-induced insulin resistance by looking at mice derived from crossing the TG-CRP mice with the Fc γ RIIB null mice. Mice with elevated CRP but lacking the receptor had normal fasting glucose and insulin, and normal GTT and ITT, indicating that Fc γ RIIB mediates CRP-induced insulin resistance. This then certainly raised many questions about how Fc γ RIIB, a receptor for IgG and for CRP, causes insulin resistance. We performed a series of immunohistochemical studies to determine which insulin target cell types and tissues express Fc γ RIIB. We did not find Fc γ RIIB in skeletal muscle myocytes, hepatocytes or adipocytes. However, Fc γ RIIB was quite abundant in micro-vascular endothelium, particularly in skeletal muscle microvascular endothelium. This was demonstrated by both immunohistochemical studies and by immunofluorescence, including negative studies in Fc γ RIIB null mice. This suggested that perhaps CRP and the FcR are working in concert to alter glucose regulation events involving the skeletal muscle and its microvasculature.

We then looked at the effect of CRP on skeletal muscle blood flow responses to insulin. This involves measuring blood flow in the hind limb, particularly to the muscle, during a continuous insulin infusion. In normal mice, skeletal muscle blood flow increases in response to insulin, and this is critical to the enhancement in skeletal muscle glucose disposal. In contrast, we found that there was absolutely no change in blood flow to the skeletal muscle in response to insulin in the TG-CRP mice. Paralleling this impairment in blood flow response to insulin, there was an almost 50% fall in glucose uptake in the skeletal muscle in the TG-CRP mice. Therefore, CRP has a major negative impact on skeletal muscle glucose disposal, and this underlies CRP-induced insulin resistance.

We then looked at the role of Fc γ RIIB in CRP impairment in skeletal muscle glucose uptake. We found that mice with elevated CRP lacking Fc γ RIIB have perfectly normal skeletal muscle glucose uptake. To confirm the observations made in mice expressing the CRP transgene, we turned to experiments in wild-type mice administered either vehicle or CRP every other day for 2 weeks. In vehicle-treated mice over the 2-week period, there was not a change in fasting glucose or insulin. In contrast, with CRP treatment, there was a marked increase in fasting glucose and insulin. These studies with exogenous CRP confirmed the observations in the TG-CRP mice. In addition, in parallel, the CRP administration to wild-type mice blunted skeletal muscle glucose uptake.¹⁰

To evaluate whether the impairment in skeletal muscle glucose uptake caused by CRP is indeed related to its effects on eNOS within the skeletal muscle microvasculature, we turned to studies in a knock-in mouse in which the critical serine residue that is phosphorylated to activate eNOS (Ser 1176) in the mouse has been mutated to aspartic acid (Asp 1176). The carboxyl side group in aspartic acid mimics the negatively charged phosphate group. Thus, eNOS with Asp 1176 is a phosphomimetic form of eNOS, and the knock-in mice have a mutant form of eNOS that cannot be inhibited by CRP. So knowing that, we evaluated the

effect of CRP injection on skeletal muscle glucose uptake in wild-type versus eNOS-Asp 1176 knock-in mice. Although in wild-type mice CRP treatment blunted skeletal muscle glucose uptake, there was no inhibition of uptake in the eNOS-Asp 1176 knock-in mice. We were then able to cross the TG-CRP mice with the eNOS knock-in mice, and we were excited to find that in the TG-CRP mice that have the phosphomimetic eNOS, their normal fasting glucose and insulin were preserved. More importantly, the GTT and ITT returned to normal. This finding demonstrates that the ability of CRP and Fc γ RIIB to cause the type II diabetes phenotype in mice is because of the inhibition of eNOS activation in the endothelium and resulting attenuation of skeletal muscle glucose disposal.¹⁰

I conclude by summarizing the new observations made in mice regarding mechanisms of inflammation associated with obesity and hypertension and insulin resistance (Figure 1). With obesity, the evidence in mice indicates that there is likely a change in one of the ligands for Fc γ RIIB, and this ligand may well be CRP generated by the liver. In partnership with Fc γ RIIB, the ligand potently antagonizes eNOS, and in resistance blood vessels, this leads to the hypertension phenotype. In the skeletal muscle microvasculature, the ligand:Fc γ RIIB inhibition of eNOS blunts skeletal muscle glucose disposal, thereby causing insulin resistance. Thus, the endothelium may well be the common site for processes that cause both hypertension and insulin resistance. Future studies can focus on determining whether comparable mechanisms occur in humans, and strategies can then potentially be developed to manipulate the response to the Fc γ RIIB ligand or its production, or to manipulate the function of Fc γ RIIB and perhaps prevent these key aspects of the metabolic syndrome, namely the hypertension and insulin resistance.

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FIGURE 1.

Mechanisms underlying CRP-induced hypertension and insulin resistance. In the setting of obesity and other chronic inflammatory conditions, there is a persistent modest elevation in CRP production, most likely primarily by the liver. In partnership with FcγRIIB, CRP blunts eNOS activation. In resistance blood vessels, this results in hypertension, and in skeletal muscle microvasculature this causes impairment in skeletal muscle glucose disposal leading to insulin resistance. CRP, C-reactive protein.