World Congress on the Insulin Resistance Syndrome, 2009

Cellular mechanisms of insulin resistance

This is the first of four articles summarizing presentations at the Seventh World Congress on the Insulin Resistance Syndrome, held in San Francisco, California, on 5–7 November 2009, pertaining to cellular mechanisms of insulin resistance.

Circadian rhythms and insulin resistance

Peter Grant (Leeds, U.K.) introduced the topic of circadian rhythms and their relationship to insulin resistance. The thrifty genotype hypothesis (1) suggests a survival advantage of insulin resistance, presumably during periods of famine. Insulin resistance should then be considered a physiologic process, with duration the critical issue, so that prolonged insulin resistance leads to disease—rather than what might be considered beneficial insulin resistance after increased nutrient ingestion in preparation for famine. Animals that prepare themselves for hypernation have adipocyte hypertrophy, hyperphagia, inflammatory changes, hyperinsulinemai, hyperglycemia, and reduced energy expenditure—all short- to medium-term processes. Seasonal animals have marked insulin resistance just prior to hibernation (2), with seasonal cycling related to the fat phenotype; a secondary period of fat accumulation occurs during springtime. There is an endogenous clock that sets up oscillating systems in virtually every tissue, synchronized by a central clock regulatory mechanism. "You disrupt the clock at your own peril," Grant noted, with sleep duration a risk factor for development of diabetes. Cardiovascular physiology is under circadian variation, affecting blood pressure, endothelial function, vascular tone, lipid metabolism, platelet and leukocyte function, and thrombosis. Effects of shift working in man include gastrointestinal, reproductive, and metabolic disturbances; insulin resistance; fasting and postprandial hypertriglyceridema; and increased risk of cardiovascular disease (CVD), "so this is not a minor change." One could hypothesize that seasonal insulin resistance benefits the hibernating animal, but "man seems," Grant said, "to have completely lost contact with 'the animal,' . . . [putting] on weight year after year."

Joseph Bass (Chicago, IL) reviewed information about the molecular clock and its linkage to metabolic control. There are hypothalamic areas regulating weight gain and weight loss. Genetics studies of rodents with the ob mutation and of flies with mutation of clock have contributed to understanding these areas, with positional cloning furthering understanding of the clock genes in mammals and improving our understanding both of circadian rhythms and of appetite regulation. The cloning of *clock* and its dimeric partner *Bmal* shows alternating patterns of stimulation and suppression. A number of transcription factors such as *PPARα*, *PPARγ*, and oxysterol ligands "are co-wired with the clock," linking metabolism to diurnal rhythms and leading Bass to raise the interesting question of whether drugs affecting these nuclear factors might alter circadian rhythms.

Obesity represents imbalance between energy intake and expenditure, with *clock* mutants having increased intake accounting for weight gain in a fashion parallel to human obesity, which also may involve disruption in circadian rhythms. The normal mouse eats mostly in the dark period (since mice are nocturnal), but *clock* mutant mice are what Bass termed "people-ized," with increased light and decreased dark food intake, leading to increased adipose tissue and liver fat, perhaps relevant to these features of the metabolic syndrome. *clock* is expressed in fat and liver and also in the immune system, cardiovascular system, kidney and gut, with disruption of circadian rhythms then affecting metabolic processes in all these tissues. Conversely, Bass said, a high-fat diet disrupts the circadian rhythm, suggesting that the environment alters the regulatory role of the central clock mechanism and implying a multiplicity of factors synchronizing the organism's circadian clock. Life span control is regulated by calorie availability, with roles of sirtuins via nuclear stores of the metabolic switch factor nicotinamide adenine dinucleotide (NAD), as sirtuins deacetylate histones and other factors involved in gene transcription. Nuclear NAD does not reflect the redox state of the cell, as does mitochondrial NAD, but itself varies across the circadian cycle rather than simply being a constant "housekeeping molecule" and, hence, can be thought of as both detecting metabolic fluxes and coupling these to changing circadian gene regulatory cellular programs. Normal metabolism requires entrainment of the organism to its environment, corresponding to periods of feeding and hormonal patterns, while overfeeding induces circadian asynchrony, which misaligns hormonal and metabolic cycles.

In a discussion of nuclear receptors as integrators of circadian rhythms and metabolism (and, hence, as potential pharmacologic targets), Bart Staels (Lille, France) reviewed the multiplicity of nuclear receptors, particularly those activated by dietary lipids: peroxisome proliferator–activated receptor (PPAR)α, -δ, and -γ; liver X receptor (LXR)-α and -β, farnesoid X receptor (FXR), retinoid X receptor (RXR), Receptor Tyrosine Kinase-like Orphan Receptors (RORα), and Rev-erbα, of which the latter two are linked to circadian rhythms. Nuclear receptors are linked to *clock* (3,4), with integration of this system with nuclear receptors. This suggests transcriptional control of metabolic pathways by circadian oscillators, with the light/daytime effect on the suprachiasmatic nucleus of the

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hypothalamus and the effect of food acting initially on peripheral tissues, also setting in motion circadian pathways altering metabolic transcription factors.

In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial, the effects of fenofibrate on microvascular and peripheral vascular disease were independent of the degree of glycemic control and of blood pressure and medications (5). How, Staels asked, would activation of PPARα influence microvascular disease? He pointed out the effect of fenofibrate in combination with the antioxidant CoQ10 on endothelial function (6), with evidence that the combination also decreases blood pressure, particularly lowering nocturnal levels (7), which would not have been observed in the data collected in the FIELD study. Evaluation of physiologic parameters throughout the diurnal cycle may be important in better understanding metabolic illness and the effects of treatments.

Rev-erbα shows tremendous circadian variation in liver, muscle, adipocyte, macrophages, and endothelium. Its circadian rhythmicity in liver is maintained in isolated hepatocytes (8). The related nuclear receptor RORα binds to the same site but with activating effects, while Rev-erbα is inhibitory, although it also inhibits its own gene expression, further affecting the cyclicity, whereas PPARα-RXR enhances Rev-erbα expression. RORα is bound by hydroxyl-cholesterol, while Rev-erbα is bound by heme, suggesting the complexity of modulators of circadian rhythmicity. Transcriptional control of adipogenesis by PPARγ is enhanced by Rev-erbα overexpression. RORα4 is expressed in visceral and subcutaneous adipose tissues and decreases adipocyte fatty acid and glucose uptake, and its overexpression impairs adipocyte differentiation. In a mouse not expressing RORα, preadipocyte differentiation is particularly enhanced when exposed to PPARγ and other activators of adipocyte differentiation, while introduction of RORα with a viral vector reverses this effect. The RORα-deficient mice have increased atherosclerosis, with increased inflammation, although triglyceride levels are decreased in RORα-deficient mice while increased in Rev-erbα–deficient mice, further suggesting an interrelationship of circadian and metabolic processes. Bile acid metabolism has long been recognized to have strong circadian rhythmicity, with mice not expressing Rev-erbα having decreased bile acid synthesis as a result of Rev-erbα negatively regulating *cyp7A1*, the gene encoding cytochrome P450 7A1. Furthermore, Rev-erbα, the signal of which is increased by PPARα and -γ, LXR, FXR and RORα, inhibits LXR-activated pathways in a variety of tissues in the macrophage affecting the ABCA1 reverse cholesterol transport and TLR4 inflammatory processes.

Fred Turek (Chicago, IL) reviewed sleep and circadian dysregulation and related disorders, particularly of metabolic rhythms, referring to an article by Staels entitled "When the Clock Stops Ticking Metabolic Syndrome Explodes" (9). Although synchronized in the suprachiasmatic nucleus, clocks genes are present in all metabolic tissues and control at least 10% of all metabolic processes. There is an association of shift work with metabolic dysregulation (10), based on a mismatch of circadian rhythms leading to metabolic dysregulation. Circadian timing of food intake contributes to weight gain, with daytime feeding of mice leading to weight gain (11), suggesting that humans, too, by eating at the wrong times have greater weight gain. Inappropriate time of feeding is additive to the weight gain seen in *Clock* mutant animals.

Ob/ob mice show altered sleep regulation, with increased sleep time but also greater sleep fragmentation; leptin treatment rapidly normalizes sleep patterns far earlier than the improvement in body size. Sleep appears to be of great importance in the development of obesity, diabetes, and CVD. There are ~4,000 sleep clinics in the U.S., but Turek would term what is needed "circadian clinics." Sleep deprivation causes metabolic abnormalities; short sleep causes insulin resistance and, possibly, overweight (12,13) Turek predicted that coming research will show relationships of sleep abnormalities to CVD, cancer, gastrointestinal illness, attention deficit, and depression, and he showed his study of weekly reversal of the light-dark cycle increasing mortality in a CVD model (14).

Garet FitzGerald (Philadelphia, PA) further discussed the molecular clock from the point of view of its relationship to CVD (15), reviewing its highly conserved characteristics from plants to animals to man and its multiple negative feedback as well as positive feed-forward regulators. Experimental models of reduced expression of all of the clock genes do not show eliminated rhythmicity, reflecting the high degree of redundancy of the system (16). There are multiple biological networks in various tissues, with the connections between these networks often involving the clock system, regulating physiologic processes; the ability to recover from insulin-induced hypoglycemia, for example, is decreased in animals not expressing either of the circadian genes *bmal1* or *clock* (17). There are a number of oscillating subsets of genes expressed in vasculature, including those important in vascular integrity and those related to carbohydrate and lipid metabolism and to adipocyte differentiation (which make use of the clock systems), and specific coactivaors and corepressors such as *sirt1* affect clock systems (18). The relationship of metabolism to the clock system is suggested by the circadian patterns of plasma metabolites (19). Mice not expressing the clock system component gene *Bmal1* have accelerated atherosclerosis in a high fat–induced atherogenesis model, and adipocyte tissue–specific *Bmal1* suppression leads to obesity with standard as well as with high-fat diets, with increased food intake during the light period of normally reduced nutrient ingestion and with muscle insulin resistance but increased adipocyte glucose uptake. Circadian rhythms in blood pressure are related to the diurnal variation in myocardial infarction and stroke frequency (20), although this does not prove whether the epidemiologic finding is related to *clock* or is a time-dependent effect of environmental stressors. When this question was addressed in a vascular injury model, thrombus formation showed diurnal variation, which was abolished by endothelium-specific inactivating *clock* mutations (21). *Bmal1* and *clock* are critical to the maintenance of circadian rhythms in blood pressure (22). FitzGerald concluded that the three major clock genes, *bmal1, clock,* and *cry* likely have distinct roles in maintaining circadian variation in blood pressure and pulse and possibly regulating catecholamine levels, with catecholamine o-methyl transferase expression under tissue-specific circadian control. *Bmal1* modulates the stress response, with the hypertensive response of mice to immobilization abolished in animals not expressing *bmal1* but the steroid response conserved suggesting other mediators. The system is robust and highly conserved and regulated, with core elements of the clock influencing thrombogenesis, blood pressure, and lipid and glucose metabolism, and having impact on a number of regulatory systems relevant to cardio-

vascular function, leading FitzGerald to speculate that communications must occur between peripheral clocks and those in the central nervous system.

Adipocytes and insulin resistance

A number of different views of the adipocyte and its relationship to insulin resistance were presented at the Congress. Sonia Caprio (New Haven, CT) reviewed the role of ectopic fat and the relationships between adipocyte size and adipogenic potential of subcutaneous tissue, inflammation, and insulin resistance in obese adolescents. There is a great deal of heterogeneity in insulin resistance among obese adolescents, with studies of insulin-resistant obese, insulin-sensitive obese, and lean adolescents showing muscle and visceral fat composition to predict the degree of insulin resistance (23,24). Fat storage may take place in subcutaneous, pericardial, perivascular, or visceral adipose tissue in or insulin-sensitive tissues such as liver, skeletal muscle, and pancreas. Focusing on ectopic fat in liver and muscle to identify factors that cause some but not other obese children to develop abnormal glucose metabolism, Caprio asked whether the degree or the distribution of obesity is of greatest importance. Limited or inefficient fat storage in the subcutaneous depot might be related to liver, muscle, and visceral fat accumulation. In a study of 118 obese adolescents, stratified by visceral fat, there was an inverse relationship between visceral and subcutaneous fat, while visceral fat correlated with hepatic fat, with the degree of insulin resistance when fasting insulin–based measures were used, as well as with prevalence of metabolic syndrome (25). Liver fat was associated with the likelihoods of impaired glucose tolerance, impaired fasting glucose, and type 2 diabetes. Subcutaneous fat biopsy was performed in 15 study participants with low visceral/high subcutaneous fat and in 18 with high visceral/low subcutaneous fat, revealing an expanded population of small adipose cells and impaired adipose cell differentiation in the latter group in association with increased visceral and hepatic fat and with insulin resistance; subcutaneous fat characteristics did not correlate with muscle fat. PPARγ2 expression appeared to reflect the abnormality of impaired adipocyte differentiation, and a trend to greater degrees of adipose tissue macrophage infiltration was seen in the biopsy specimens from children with insulin resistance.

Sam Cushman (Bethesda, MD) further discussed fat cell size distribution studies. Adipocytes, he said, consist of a "huge lipid droplet" with cellular elements contained in an outer rim of the cell. Adipocytes have secretory as well as storage functions, with adipocyte-specific deletion of the glucose transporter (GLUT)4 causing muscle and whole-body insulin resistance, presumably related to secreted factors or to ectopic fat deposition. In analysis of fat cell size of 11 insulin-resistant and 15 insulin-sensitive individuals, both groups had large but similar numbers of small adipocytes and (contrary to the hypothesis) the size of the large adipocytes was roughly similar between the groups.

Adipocytes maturation proceeds, with undetermined controlling factors, from stem cells to preadipocytes, with subsequent adipocyte formation controlled by activation of the nuclear receptor PPARγ. The methodology used does not give specific information about very small cells but allows quantitation of cells varying from 20 to 200 microns in size. Measurement of mean cell size (to assess hypertrophy) and total cell number (to assess hyperplasia) allows ascertainment of recruitment, growth/shrinkage, fluctuation, and death of adipocytes. On a high-fat diet with progressive weight gain, large adipocyte numbers decreased but size increased, suggesting more rapid growth but with greater susceptibility to cell death; all the fat depots studied had similar behavior. After the high-fat diet was stopped, the number of small cells decreased, suggesting death of precursor cells, with reduction in size of the large adipocytes. Over time there were cycles of cell growth. With administration of rosiglitazone there was rapid recruitment of adipocytes with increase in cell number over the initial 10 days and a subsequent shift from smaller to larger cells, although Cushman pointed out that thiazolidinediones may not actually stimulate differentiation of stem cells, which would explain why the initial "burst" is followed by increase in adipocyte size rather than number. He hypothesized that adipogenesis is triggered when available adipose cells cannot absorb the energy flux.

Tracy McLaughlin (Stanford, CA) discussed the relationship of insulin resistance to obesity. The correlation coefficient of steady-state plasma glucose (SSPG) with BMI in a large study was 0.58, suggesting "a large amount of variability at any given BMI." In the most insulin-resistant tertile, as BMI increases the prevalence of insulin resistance increases, but even with severe obesity some individuals are in the mid-tertile of insulin resistance. "This relationship is very likely to explain the clinical morbidities associated with obesity," she said, because in every BMI group, those in the more insulin-resistant tertile have higher blood pressure, triglyceride, and fasting and 2-h glucose. Individuals with insulin resistance who maintain weight loss show stability or even further improvement in SSPG, although those who are insulin sensitive initially do not have further improvement (26). Lipids, inflammatory markers, and asymmetric dimethylarginine improved in the insulin resistant group, while those who were insulin sensitive at baseline did not show change in these parameters with weight loss (27). McLaughlin posed a number of questions that have not been fully answered regarding the interrelationships of obesity and insulin resistance. Why do thiazolidinediones, acting at adipocyte nuclear receptors, improve insulin action in skeletal muscle? Does visceral fat promote insulin resistance or is impaired subcutaneous fat storage the actual abnormality, with the subcutaneous adipocyte having a protective role? Are individuals with greater capacity for adipocyte differentiation and proliferation inherently less likely to develop ectopic fat and, hence, insulin resistance? McLaughlin pointed out the association of insulin resistance with intramyocellular fat (28) in support of this notion. She reviewed the conflicting data regarding the relationship of adipocyte size with insulin sensitivity, noting that there are increased proportions of small adipocytes in insulin resistance but that thiazolidinediones further increase the small adipocyte number, although it may be that these small cells would subsequently increase in size and so promote greater insulin sensitivity. The peak adipocyte diameter increases with weight for individuals with BMI 25–29.9 kg/m^2, but from BMI levels of 30 to >50 kg/m^2 there is no further increase in adipocyte peak diameter. Does obesity involve stimulation of differentiation of new cells?

Phillip Scherer (Dallas, TX) discussed relationships between adipose tissue dysfunction and insulin resistance. He asked: what are the major issues encountered during fat expansion leading (in dysfunctional adipose tissue) to inflammation? He showed histologic evidence of in-

creased extracellular matrix in adipose tissue of obese animals, which can be shown in measurement of a variety of collagen molecules, with PPARγ agonist treatment downregulating this profibrotic signal. Scherer compared lean and *ob/ob* mice and *ob/ob* mice not expressing collagen VI; fat cell size further increased in the latter group, but glucose tolerance improved, suggesting that adipose tissue fibrosis prevents further expansion, causing necrosis and leading to insulin resistance (29). Another issue is adipose tissue hypoxia. Expanding fat has similarity to expanding malignant tissue in its potential for an inadequate vascular supply. The vascular density of fat in the *ob/ob* mouse is decreased, with attenuated postprandial increase in fat pad blood flow. On a high-fat diet, then, fat cells may rapidly expand, causing local hypoxia and triggering signals such as hypoxia-inducible factor 1α, which stimulates fibrosis and oxidative processes, leading to necrosis and then to local followed by systemic inflammation. The presence in adipose tissue of macrophages and in circulation of inflammatory markers may then be late occurrences. PPARγ agonists are antifibrotic, stimulate angiogenesis, and inhibit macrophages, preventing this process.

Adiponectin is unique among adipocyte cytokines in that its plasma levels correlate inversely with fat mass. It correlates with greater insulin sensitivity, is suppressed by inflammatory processes, and is increased by PPARγ agonists. It is a complicated molecule circulating in trimer, hexamer, and multimer forms. Females have higher total and high-molecular weight adiponectin levels due to levels of the high–molecular weight form, which correlates better than total levels with insulin sensitivity (30). It circulates at high levels but turns over rapidly, and its role has not yet been determined (31). Another relevant concept is endoplasmic reticulum (ER) stress, which is associated with obesity and decreases in obese individuals after weight loss (32). ER stress and hypoxia appear to suppress adiponectin secretion so that it may be seen as an integrated marker of adipose tissue health, with anti-inflammatory, antisteatotic, insulin-sensitizing (particularly in liver [33]), cardioprotective, antifibrotic, and anti-apoptotic effect, although a unifying mechanism has not been described. Scherer noted the possibility that, as with PPARγ agonists, its angiogenic effect could promote tumor growth. He suggested that adiponectin appears to be involved in the "metabolic flexibility" sustaining insulin sensitivity. "When you hear 'dysfunctional fat,'" he concluded, "think hypoxia, fibrosis, and also the stress response, the unfolded protein response." These in turn cause systemic inflammation.

Ulf Smith (Gothenburg, Sweden) discussed aspects of impaired differentiation of human preadipocytes in abdominal obesity and insulin resistance. He suggested that "waist circumference is a better marker than BMI" of insulin resistance, noting that 25–30% of obese individuals are not insulin resistant while 25% of nonobese individuals are insulin resistant. Irrespective of BMI, he said, enlarged fat cells are associated with insulin resistance (using a different methodology from that described by Cushman and McLaughlin). He then termed insulin resistance obesity "hypertrophic obesity," which he contrasted with hypercellular obesity not related to insulin resistance. Obese mice overexpressing adiponectin, for example, have normal insulin sensitivity despite massive obesity, further supporting this notion. He noted that subcutaneous fat comprises most adipose tissue and suggested that visceral fat, like epicardial and liver fat, be considered "an ectopic depot" (34).

Enlarged adipocytes, he said, recruit inflammatory cells, including T-lymphocytes expressing CD3 (involved in acquired immune system activation) and CD8 (involved in macrophage recruitment), mast cells (35), and plasma cells. This contributes to insulin resistance by producing a variety of proinflammatory cytokines and by showing increased nuclear factor-κB and c-Jun kinase pathway activity, leading macrophages to assume a proinflammatory phenotype (36). Tumor necrosis factor-α is produced by adipocytes in an inactive form, requiring local inflammatory cells for activation, and acts to prevent differentiation of preadipocytes into fully differentiated adipocytes, which further produce inflammatory cells and display a macrophage-like phenotype rather than accumulating lipids, reducing recruitment of new adipocytes (37).

Smith asked, "Why don't the cells differentiate?" The Wnt signaling pathway, first described in *Drosophila*, is involved in growth and development, keeping stem cells in an undifferentiated state, with abnormalities in this pathway related to oncogenesis. Wnt ligands bind to membrane receptors including *TCF7L2*, polymorphisms of which are the most well recognized risk genes for type 2 diabetes. Wnt activation increases intracellular concentrations of β-catenin, which in turn induces a variety of genes. When Wnt is not present during differentiation, β-catenin is degraded. Smith suggested that tumor necrosis factor-α acts via Wnt activation. Wnt signaling leads in bone marrow to formation of osteoblasts rather than adipocytes from precursors, and it appears that a mechanism of thiazolidinediones reducing bone mineral density involves PPARγ induction of an inhibitor of Wnt, favoring differentiation into preadipocytes, while cytokines increase Wnt signaling. WISP-2, a marker of Wnt signaling, is associated with obesity, intra-abominal fat and high waist-to-hip ratio, and risk factors for type 2 diabetes. WISP-2 is elevated in newly diagnosed type 2 diabetes.

Michael Jensen (Rochester, MN) discussed the role of dysregulated adipose tissue lipolysis in the metabolic complications of obesity, pointing out that lower-body obesity is associated with normal free fatty acid (FFA) and upper-body (and visceral) obesity with high FFA. The portal fat hypothesis is that lipolysis by visceral fat cells is increased, increasing FFA flux to the liver (38). Jensen pointed out that insulin resistance is associated with high FFA and that experimental approaches increasing FFA reduce muscle insulin sensitivity, increased insulin secretion, and increase hepatic glucose output, suggesting FFA to be a central player. Adipocyte FFA release is tightly linked to metabolism, which is increased in the fasting state or with exercise, when lipolysis may increase fivefold, whereas after feeding—when FFA production is not required—insulin rapidly inhibits lipolysis by >90%; the range from lowest to highest levels of FFA release is 50-fold. FFA release is greater with upper-body obesity, which is associated with less suppression during a hyperinsulinemic clamp (39) or in the postprandial state (40). Jensen pointed to evidence that upper-body subcutaneous fat FFA release is less well inhibited than that from the splanchnic bed or leg postprandially (41), suggesting that visceral fat is not the source of increased circulating FFA and likely contributes to FFA delivered to the liver rather than that entering the systemic circulation (42). Upper-body rather than visceral obesity, then, may be associated with elevation in circulating FFA,

leading to abnormal islet function (as the pancreas is perfused by the systemic circulation) and, perhaps by its effect on the vasculature or on intramyocellular fat, to insulin resistance.

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