AuPS/ASB Meeting - Adelaide 2010

Symposium: Lipid Metabolism and disease: new insights from the lab to the clinic

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Chair: Matthew Watt

Circulating ceramide, inflammation and insulin resistance

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Obesity is associated with an increased risk of developing insulin resistance, a condition that precedes the development of type 2 diabetes. Although this relationship is well recognised, the mechanisms linking obesity to insulin resistance remain unresolved. One explanation is that obesity is associated with an 'oversupply' of lipids, which leads to the storage of lipids in tissues such as the liver and skeletal muscle that in turn cause insulin resistance. Another is that obesity is accompanied by low-grade inflammation that negatively impacts on insulin signal transduction. Ceramide accumulation in skeletal muscle is associated with the development of insulin resistance and pharmacological blockade of ceramide ameliorates lipid-induced insulin resistance in obesity (Holland *et al.*, 2007). Ceramides are also known to circulate in plasma, even though they are insoluble in aqueous environments. While plasma ceramides are increased in type 2 diabetes patients (Haus *et al.*, 2009), the mode of ceramide transport *in vivo* and the role of circulating ceramide in the development of insulin resistance and inflammation remains unresolved.

We first assessed ceramide levels in human plasma which was fractionated by fast performance liquid chromatography. The eluted fractions containing lipoproteins were identified by analysis of the ultraviolet absorption spectrum and confirmed by measuring cholesterol in each fraction. Ceramide was transported exclusively by very low density lipoproteins (VLDL, ~10%), low density lipoproteins (LDL, ~50%) and high density lipoproteins (HDL, ~40%). Total plasma ceramide content was increased in obese, type 2 diabetes (T2DM) patients compared with lean, insulin sensitive aged-matched individuals (Lean: 13.8 ± 1.1 *vs* T2DM: 17.4 ± 1.3 μM). Ceramides were higher in LDL (55%, $P = 0.006$) of T2DM, but not in VLDL ($P = 0.76$) or HDL ($P = 0.16$). LDL-ceramide was positively associated with insulin resistance (HOMA-IR), fasting insulin and glucose but not body mass index (a marker of adiposity), triglyceride or cholesterol. Thus, LDL-ceramide is associated with insulin resistance in humans.

To assess circulating ceramide function in cell culture, a novel approach was used to replicate the *in vivo* environment by creating LDL containing ceramides. This method depletes the LDL core of neutral lipids which allows for the introduction of ceramide into an intact LDL. LDL-ceramide (either C16:0 or C24:0 ceramide) mildly decreased insulin-stimulated glucose uptake in L6-GLUT4myc myotubes after 24 h treatment (~30%), whereas no effect was observed at 6 h. The reduction in insulin responsiveness was not associated with an impairment in Akt phosphorylation, suggesting no direct effect on distal insulin signalling. Interestingly, intracellular ceramide was increased with LDL-ceramide, although we are unable to provide definitive evidence for receptor-mediated uptake. LDL-ceramide activated pro-inflammatory signalling in RAW 264.7 macrophages, resulting in c-Jun terminal kinase activation and increased TNF-α secretion, but surprisingly, not IL-6 secretion . The conditioned media from LDL-ceramide treated macrophages decreased insulin-stimulated glucose uptake in L6-GLUT4myc myotubes, whereas no effect was observed with LDL conditioned media.

Overall, these results show that ceramides are elevated in the plasma of obese, type 2 diabetes patients and that this is related to insulin resistance, and not adiposity or generalised dyslipidemia. Furthermore, LDL-ceramide can cause insulin resistance in skeletal myotubes, albeit mildly, and activate pro-inflammatory signalling in macrophages, that in turn creates a milieu that decreases insulin sensitivity.

Haus JM, Kashyap SR, Kasumov T, Zhang R, Kelly KR, Defronzo RA & Kirwan JP. (2009). Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. *Diabetes* **58**, 337-343.

Holland WL, Brozinick JT, Wang LP, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts TA, Siesky A, Nelson DH, Karathanasis SK, Fontenot GK, Birnbaum MJ & Summers SA. (2007). Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metabolism* **5**, 167-179.

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Dual, but opposing, roles for the double stranded RNA-dependent protein kinase in metabolic homeostasis

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Chronic inflammation is a hallmark of obesity and contributes to the development of numerous diseases, including insulin resistance, Type 2 diabetes, atherosclerosis and cancer. Metabolic and immune signalling pathways are intimately linked and understanding how nutrient excess promotes cellular inflammation is of considerable importance in understanding the pathogenesis of many chronic metabolic diseases. A recent study in *Cell* identified double stranded RNA-dependent protein kinase (PKR) as a core component of a "metabolic inflammasome" linking stress signalling to metabolic disease (Nakamura *et al.*, 2010). Using murine embryonic fibroblasts and macrophages from PKR-deficient mice on an inbred C57BL/6 background our results confirm that PKR is required for the induction of pro-inflammatory responses triggered by nutrient excess. However, and remarkably, PKR-deficient cells had 2 to 3-fold increases in the levels of numerous intracellular lipid types, including diacylglycerol and ceramide, lipid species linked to inflammation and insulin resistance, following treatment with fatty acids.

To examine the *in vivo* consequences of PKR deletion, we placed PKR-deficient and wild type (WT) mice on a high fat diet. PKR knockout (KO) mice had increased total body fat mass, higher leptin levels and increased lipid accumulation in skeletal muscle and liver. Furthermore, high fat fed PKR KO mice were hyperinsulinaemic, glucose intolerant and insulin resistant, compared to WT mice. Gene expression analysis of PKR-deficient and wild type macrophages, skeletal muscle and liver identified marked increases in the levels of the fatty acid transporter (FAT) CD36 and several fatty acid binding proteins (FABP). While we observed significantly greater recruitment of macrophages into the white adipose tissue of PKR KO mice, this was associated with similar levels of the pro-inflammatory genes Tnf and Il6 but higher levels of the anti-inflammatory gene Il10. We conclude that while PKR deletion may confer protection from nutrient excess-driven inflammation, it also promotes lipid accumulation, most likely *via* an increase in the expression of FAT/CD36 and specific FABP. Importantly, *in vivo* excess lipid accumulation appears to be the predominating effect of PKR deletion, leading to exacerbated glucose intolerance and insulin resistance.

Nakamura, T., Furuhashi, M., Li, P., Cao, H., Tuncman, G., Sonenberg, N., Gorgun, C.Z., and Hotamisligil, G.S. (2010). Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. *Cell* **140:** 338-348.

The emerging role of HDL in glucose metabolism

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The association of low plasma levels of high-density lipoprotein (HDL) with states of impaired glucose metabolism and type 2 diabetes mellitus is well established, but the mechanistic links remain to be fully elucidated. Recent data from our laboratory (Drew *et al.*, 2009) and others suggests that HDL directly influences glucose metabolism through multiple mechanisms. This presentation will discuss the emerging evidence and mechanisms by which HDL modulates glucose metabolism in the context of the well established actions of HDL.

Low HDL has been traditionally considered an atherosclerotic risk factor on the basis of convincing epidemiology, demonstrating an association with negative cardiovascular outcomes, even at very low levels of LDL. The process of cholesterol removal from peripheral cells such as macrophages, for transport to the liver and subsequent excretion is termed reverse cholesterol transport, and is generally viewed as the most important anti-atherosclerotic action of HDL. However, in addition to reverse cholesterol transport, HDL is now known to convey an impressive spectrum of protective properties including inhibition of inflammation, oxidation and thrombosis, as well as vasodilatation *via* nitric oxide.

Recent experimental and clinical developments linking HDL to glucose metabolism suggest yet another beneficial action of HDL that may have relevance to diabetes. We have shown that HDL elicits reductions in blood glucose in patients with type 2 diabetes (Drew *et al.*, 2009) which likely occurs through multiple actions including stimulation of pancreatic β-cell insulin secretion (Drew *et al.*, 2009; Fryirs *et al.*, 2010) and increased glucose uptake into skeletal muscle *via* activation of the AMP-activated protein kinase (AMPK) signaling pathway (Drew *et al.*, 2009). In addition, given the established role of lipid accumulation and inflammation in the pathogenesis of type 2 diabetes, it is highly likely that the reverse cholesterol transport and anti-inflammatory actions of HDL in metabolic tissues contribute to improved insulin sensitivity and thus glucose homeostasis. We therefore hypothesized that HDL may improve insulin sensitivity *via* lipid removal and anti-inflammatory actions in macrophages associated with excess adiposity/ectopic lipid deposition. A variety of macrophage cell models including RAW 264.7 (mouse), THP-1 (human) and primary human macrophages from healthy participants were incubated separately with an acetylated LDL lipid challenge and then co-treated with either HDL (50μg/mL) or vehicle for 18 hours. Fresh conditioned media from macrophage cultures was applied (1:10) to primary human skeletal muscle cell cultures derived from 5 unmedicated patients with type 2 diabetes for 24 hours and insulin-mediated glucose uptake (2-deoxyglucose) measured. In all models, acetylated LDL treatment reduced insulin-mediated glucose uptake to basal levels and co-treatment with HDL restored insulin mediated glucose uptake to control levels. These data suggest that macrophage inflammation associated with excess/ectopic adiposity is reduced by HDL and these effects may contribute to improved insulin sensitivity and glucose homeostasis.

Research in this area is in a preliminary phase, with the potential for HDL elevation to provide metabolic protection yet to be proven in a chronic context. However, findings to date provide fertile ground for mechanistic speculation regarding links between HDL and glucose metabolism in the context of diabetes (where HDL is low and fasting plasma glucose is poorly controlled) and aerobic conditioning (where HDL is high and fasting plasma glucose is tightly controlled). These findings highlight the possibility that HDL-raising therapies already in advanced clinical development for vascular disease may also have efficacy in the prevention and management of type 2 diabetes.

Drew BG, Duffy SJ, Formosa MF, Natoli AK, Henstridge DC, Penfold SA, Thomas WG, Mukhamedova N, de Courten B, Forbes JM, Yap FY, Kaye DM, van Hall G, Febbraio MA, Kemp BE, Sviridov D, Steinberg GR & Kingwell BA. (2009). High-density lipoprotein modulates glucose metabolism in patients with type 2 diabetes mellitus. *Circulation* **119,** 2103-2111.

Fryirs MA, Barter PJ, Appavaroo M, Tuch BE, Tabet F, Heather AK & Rye KA. (2010). Effects of high-density lipoproteins on pancreatic β-cell insulin secretion. *Arteriosclerosis, Thrombosis and Vascular Biology* **30:** 1497 - 1499.

Calorie restriction versus exercise: which produces the best health outcomes?

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The prevalence of obesity is rapidly escalating worldwide and obesity is closely linked to the development of insulin resistance, metabolic syndrome and type 2 diabetes. Negative energy balance is key in reversing the metabolic defects associated with obesity and produces an array of health benefits that cannot be matched by any one drug that is currently on the market. These include improved lipid profiles and insulin sensitivity, reduced ectopic lipid deposition, blood pressure and reduced inflammatory cytokine production. However, the optimal method to achieve negative energy balance is debated. Here I compare studies of reduced energy intake to increased energy expenditure, and in particular focus on the differential effects of these interventions on lean mass preservation, energy metabolism and insulin action.

In free living studies, moderate calorie restriction (CR) nearly always produces greater weight loss, but there is evidence to suggest that aerobic exercise may provide equal or greater health benefits and is better to maintain weight loss following CR. Here, I report findings from the CALERIE studies, where we compared 25% CR versus 12.5% CR plus 12.5% increase in energy expenditure by aerobic exercise training under stringent laboratory conditions for 6-months in healthy overweight individuals. In this study, energy requirements were carefully assessed, all foods were provided for 3.5 months whilst individuals attended weekly training sessions to learn how to accurately count calories, and all of the exercise sessions were conducted under supervision. Under these conditions, CR and CR+EX produced equal energy deficits; and thus equal losses in body weight, subcutaneous fat cell size and subcutaneous, visceral and liver fat stores by MRI. No changes were observed in intramyocellular lipid, but CR+EX led to slightly greater preservation of lean body mass at 3-months, and greater improvements in fitness and insulin sensitivity at 3- and 6-months. Similar results were produced from the CALERIE study conducted at Washington University where participants were randomised to either 20% CR or a 20% increase in physical activity alone.

We also observed that energy expenditure in the 24-hour chamber was reduced more than predicted based on the loss of mass in both CR and CR+EX indicating metabolic adaptation, but there was no change in spontaneous physical activity. Total daily energy expenditure was reduced only in CR, and was greater than was accounted for based on the decrease in sedentary energy expenditure. This suggests that the CR group also reduced daily activity. This result is found following prolonged CR in monkeys, although CR rodents that are given access to a running wheel exercise more than *ad-libitum* fed animals. We also observed that CR and CR+EX produced similar increases in mitochondrial biogenesis and SIRT1 expression in muscle and equivalent reductions in DNA damage, although functional changes in mitochondria were not observed. Interestingly, a recently completed study that we have conducted of high fat overfeeding also produced an increase in mitochondrial biogenesis, despite induction of insulin resistance, lending support to the growing body of evidence that suggests that mitochondrial dysfunction may be a consequence rather than a cause of insulin resistance.

So, should we promote dieting or exercise? This probably depends on whether the goal is weight loss or maximal improvement in health. Without close dietary and exercise support, exercise alone is unlikely to produce much weight loss. However, dieting alone reduces sedentary energy expenditure and physical activity which will promote weight regain over time. We have shown that the combination of CR+EX prevents reductions in total energy expenditure, and may also provide slightly greater health benefits than dieting alone.