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## Laboratory of Metabolic Control

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### Control of Cellular Redox States

*Veech*

Central to energy homeostasis is the control of cellular redox states and the linked phosphorylation potential. The redox state is a measure of the ability of metabolic reactions to donate or accept electrons. Within the cell there are many redox couples with varying potentials. For example the free cytosolic NAD couple readily accepts electrons from the glycolytic pathway and has a potential of about -0.19 V. In contrast, the free cytosolic NADP couple, used for the synthesis of lipids and other reactions has a much more negative and therefore powerful redox potential of -0.42 V, allowing synthetic reductions to be driven to completion. Intermediate between these cytosolic co-factor redox couples is the mitochondrial NAD couple whose potential is usually about -0.28 V. This intermediate potential allows the mitochondria to synthesize ATP in the combined reactions of electron transport and ATP synthesis.

Linked to and determined by the NAD redox couples of cytoplasm and mitochondria is the phosphorylation potential, the ATP/ADP $\times$ Pi ratio, or the energy of the ATP bond. Depending upon the redox energies available between the mitochondrial NAD couple and the terminal electron acceptor, molecular O<sub>2</sub>, the energy of cellular ATP can vary particularly in disease states. In non pathological states, the energy of ATP hydrolysis is tightly regulated, varying only about 10%, between -53 to -60 kJ/ mole of ATP hydrolyzed.

The cell possesses many other redox couples wherein common co-factors link and integrate multiple reactions sharing the common co-factors. Examples of such co-factor determined redox states are the mitochondrial co-enzyme Q/ co-enzyme QH<sub>2</sub> couple and the folate couples, to name only a few. The former mitochondrial couple is of particular importance for two reasons. Firstly, the distance between the redox states of the mitochondrial NAD and Q couples determines the energy of ATP synthesized by the mitochondria. Secondly, the major source of cellular free radical production is the spontaneous reaction of the intermediate Q semiquinone with oxygen. Oxidation of the Q couple therefore decreases the production of free radicals, an important etiological factor in many disease states.

ATP hydrolysis has been called “the energy currency” of the cell. Many disease states are characterized by a lowering the energy of ATP hydrolysis. One the most important links between the energy of ATP hydrolysis and other cellular reactions is the extent of inorganic ion

gradients between the extra and intracellular phase of the cell. These gradients between  $\text{Na}^+$  and  $\text{Ca}^{2+}$  between the phases are determined by the energy of ATP hydrolysis as are the gradients of the 9 major inorganic ions. In turn the gradients of inorganic ions determines the distribution of water between intra and extracellular phases accounting for the universal phenomena of the loss of intracellular  $\text{K}^+$  and the gain of intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$  during injury of the cell of any type. Cellular redox and phosphorylation states can vary with the type of substrate being metabolized. This variability in energy available from the metabolism of substrates is inherent in the difference between the heats of combustion of the various metabolic substrates being combusted to  $\text{H}_2\text{O}$  and  $\text{CO}_2$  in the citric acid cycle and the mitochondrial processes of oxidative phosphorylation. Redox and phosphorylation states can also be changed by variance in hormonal or disease status of the organism. Recognition that change of substrate being used for the generation of metabolic energy production can alter these central controlling cellular co-factor couples present new and unexpected therapeutic opportunities in a variety of diverse disease states <sup>1</sup>.

### **Metabolomics**

*Pawlosky, King, Crutchfield, Kashiwaya, Veech*

The determination of cellular redox and phosphorylation states cannot be determined by direct measurement of the nucleotides involved such as NAD, NADH, ADP, AMP and the like for reasons reviewed the above reference. Instead, various intermediary metabolites of enzymatic reactions of high capacity relative to flux must be measured to determine the redox or phosphorylation state in the compartment of interest. This can be accomplished by established methods of enzymatic analysis. However these methods are slow and labor intensive. Accordingly, we have developed new methods for the analysis of new samples of tissue using capillary electrophoresis combined with mass spectrometric which allows the quantitative determination of the products and reactants of the enzymes comprising a metabolic pathway of interest as well as the determination of the flux through that pathway using stable isotopes. Such measurements, when combined with knowledge or determination of the kinetic constants for the enzymes involved and the equilibrium constant for each reaction under conditions appropriate to the in vivo situation allow for the determination of the degree of control of flux exerted by each enzyme within the pathway. It is the alteration of flux through metabolic pathways that define the disease phenotype. Knowledge of the control strength of each step suggests new approaches where the disease phenotype may be returned to normal <sup>2</sup>.

Of particular interest to the programs of NIAAA are the metabolic effects of acetate, the primary metabolite of ethanol. Ethanol is almost totally converted to acetate in the liver. The acetate formed then leaves liver where it is metabolized in extra hepatic tissues, including brain. Relatively little attention has been paid to the effects of acetate metabolism upon brain. Using techniques which are unique to this laboratory, namely brain blowing, which eliminates post mortal changes in energy metabolites and a combination of enzymatic analysis with CE and GC MS we have now completed a metabolomic survey of the changes in brain metabolism resulting from the metabolism of acetate. The most striking finding is that the metabolism of acetate by brain leads to a lowering of  $\Delta G'$  of ATP hydrolysis.

## **Ketosis and Neurological Disease**

*Kashiwaya, King, Crutchfield, Bergman, Pawlosky, Osei-Hyaman, Srivastiva, Burns, Clarke Veech*

A ketogenic diet has been used for over 100 years in the treatment of refractory epilepsy. However such high fat, low carbohydrate diets are not suitable for treatment of patients over 17 years of age because of poor patient tolerance and the atherogenic potential and other abnormalities resulting from elevation of blood cholesterol, triglyceride and free fatty acids. Accordingly, in collaboration with the Department of Defense and Oxford University we have been developing an oral form of ketone bodies suitable for human use. Because the metabolism of the ketone body D-•-hydroxybutyrate leads to elevation of the energy of ATP hydrolysis above that resulting from the metabolism of glucose such a diet has potential application in many disease phenotypes beyond the treatment of epilepsy. An important group of this class of disease phenotypes is those of neurodegeneration, among which is Parkinson's disease.

Several years ago we demonstrated that administration of the ketone body, D-•-hydroxybutyrate preserved mesencephalic neurons from death caused by MPTP, a neurotoxin resulting in immediate Parkinsonism in humans. It has subsequently been shown that treatment of C57 black rats with a ketogenic diet prevented mesencephalic damage from administered MPTP. More recently, a ketogenic diet has been shown to reduce tremor and rigidity in a small study of human patients by about 60% while improving mentation.

More recently we have shown that partial inhibition of NADH dehydrogenase (Complex I) by rotenone in cultured dopaminergic neuroblastoma cells could be overcome by D-•-hydroxybutyrate. Abnormalities in complex I have long been reported in Parkinsonism and recent <sup>31</sup>P-MRS studies of occipital cortex shows a lowered PCr/ATP ratio or energy of ATP hydrolysis in this area not generally thought to be involved in Parkinsonism. These observations suggest that elevation of ketones deserve a wider therapeutic test in this disease <sup>3,4</sup>.

In attempts to by pass a number of the complications of inducing ketosis without feeding ketogenic diet, we have developed ketone esters suitable for oral administration. We have completed animal toxicity of newly synthesized ketone esters. In collaboration with DARPA and Oxford University, we have taken steps to obtain FDA approval to feed this new food to human subjects. Subsequently we have received approval from the DoD human ethics board to feed these esters to human subjects. We intend to commence the first in human toxicity tests of these materials, early in 2009.

## **Control of Sirt1**

*Srivastiva, Kashiwaya, Pawlosky, King, Veech*

Sirt1 is an NAD dependent histone deacetylase the activity of which is thought to be related to the life extending properties associated with caloric restriction. The factors controlling the amounts of Sirt1 are not well understood. Neither are those factors controlling the activity of this enzyme. Many studies have attempted to relate the activity of Sirt1 to measurements of total NAD. For reasons stated above, measurement of total NAD bear little relationship to the amount of total free NAD in cytoplasm or nucleus, which are the compartments of interest for understanding of the control of Sirt1, largely located in nucleus.

In collaboration with Dr Vittorio Sartorelli we showed that the activity of Sirt1 in myoblasts was related to changes in the free cytoplasmic  $\text{NAD}^+/\text{NADH}$  ratio, which is the same as that in nucleus. We are continuing our studies of the factors controlling the amount and activity of this enzyme<sup>5</sup>.

More recently, it has been reported that feeding ethanol alters the expression of SIRT 1<sup>6</sup> an enzyme reputed to be important in regulating life span in a number of species.

### **Metabolite Control of Transcription**

*Shireesh Srivastava, Yoshihiro Kashiwaya*

Summary: It is becoming increasingly clear that transcription of many of the proteins encoded in the genome is controlled by small metabolites whose concentrations vary depending upon environmental conditions. Thus consuming a diet rich in carbohydrate leads to the transcription of the genes encoding the major enzymes of glycolysis required to form the precursor acetyl CoA as well as the enzymes of the hexose monophosphate pathway producing the NADPH required for fat synthesis. The transcription factor is called ChREBP and responds to dietary carbohydrate. Combined with the effects of SREBP, the sterol responsive binding protein described by Brown and Goldstein, and responsive to insulin, these two transcription factors are of major importance in obesity, type II diabetes and vascular diseases. This elegant co-ordinate control of ChREBP is exerted by the simple hexose monophosphate pathway metabolite, xylulose 5-P [1]. Changes in the redox state of the pyridine nucleotides are the hallmark of changes in metabolic status and are known to be profoundly altered by alcohol ingestion. A number of transcription factors are now known to be controlled by the redox state of the pyridine nucleotides. These include: NPAS2, the so-called clock gene responsible for circadian rhythm (Rutter, J. et al, Science 293: 510-514, 2003); CtBP, the transcriptional co-repressor playing a role in development and transformation (Fjeld, C. et al, PNAS 100: 9202-9207, 2003); Oct-1, the transcription factor regulating expression of nuclear histones, H2B (McKnight, S. Cell 114:150-152, 2003); and Sir2, the gene silencer thought to play a central role in the life extending properties of caloric restriction in yeast, *C. elegans*, and in mammals as well. In recent work, done in collaboration with other groups as NIH, we have shown that the activity of SIR2 in myoblasts is controlled by changes in the free cytosolic  $[\text{NAD}^+]/[\text{NADH}]$  [2]. Currently it is thought that the activity of this enzyme is controlled by inhibition by nicotinamide with a  $K_i$  of over 150 micromolar. We are continuing the examination of changes induced in the amount of Sirt1 present in different dietary conditions. It is not now clear how control is related to change in the  $[\text{NAD}^+]/[\text{NADH}]$  ratio. It goes without saying that changes in  $[\text{NAD}^+]/[\text{NADH}]$  are characteristic of both alcohol ingestion and ketosis and would therefore be expected to alter the activity of the above listed transcription factors.

Reducing insulin/IGF signaling allows for organismal survival during periods of inhospitable conditions by regulating the diapause state, whereby the organism stockpiles lipids, reduces fertility, increases stress resistance, and has an increased lifespan. The Target of Rapamycin (TOR) responds to changes in growth factors, amino acids, oxygen tension, and energy status; however, it is unclear how TOR contributes to physiological homeostasis and disease conditions. We have shown<sup>7</sup> that reducing the function of *Drosophila* TOR results in decreased lipid stores and glucose levels. Importantly, this reduction of dTOR activity blocks the insulin resistance and metabolic syndrome phenotypes associated with increased activity of the insulin responsive transcription factor, dFOXO. Reduction in dTOR function also protects against age-dependent decline in heart function and increases longevity. Thus, the regulation of dTOR activity may be

an ancient "systems biological" means of regulating metabolism and senescence that has important evolutionary, physiological, and clinical implications.

Changes in energy demand have been reported to induce an increase in mitochondrial mass and in a number of mitochondrial enzymes. The effects upon mitochondrial mass of feeding a ketone ester diet are now being investigated.

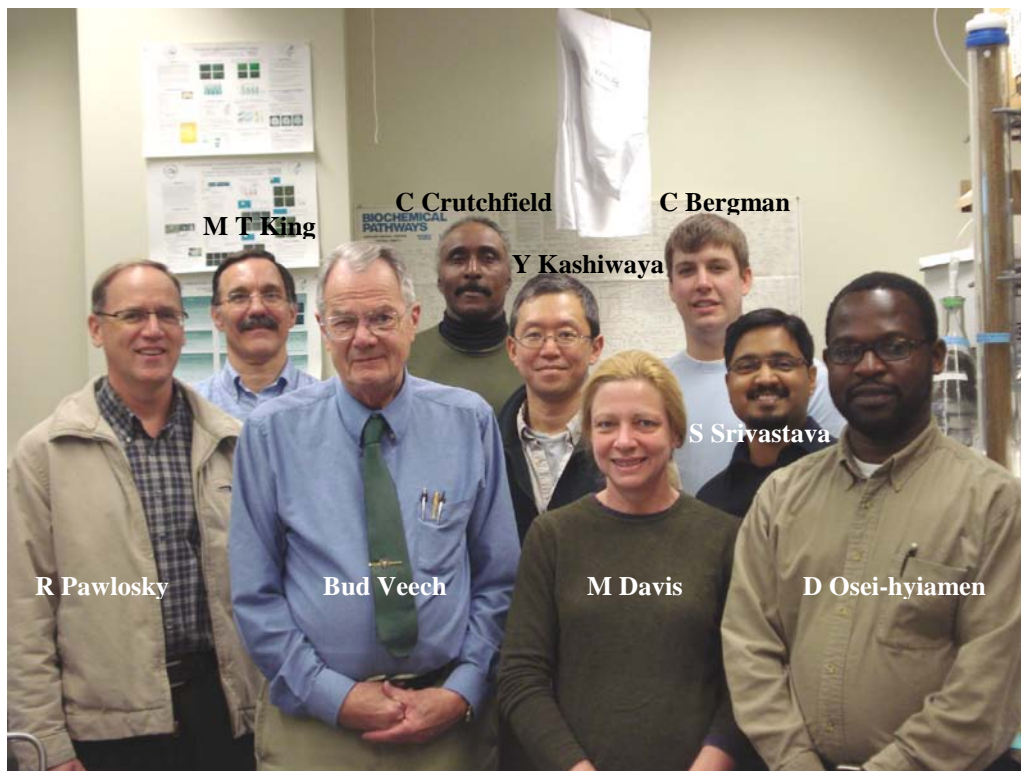
### **Collaborators**

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**Team Ketone**