

The pathogenesis of the chronic complications of diabetes mellitus

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Diabetes mellitus is currently defined as blood glucose concentrations that are elevated above normal. The specific values chosen – originally by the National Diabetes Data Group in 1979,¹ and recently modified for fasting values² – were based on epidemiological data correlating fasting and post-oral glucose load blood glucose concentrations with the occurrence of microvascular complications. Thus, the group of diseases we call diabetes is defined by hyperglycemia and its attendant risk for complications.

The association between microvascular complications (ie, nephropathy, retinopathy, and neuropathy) and hyperglycemia has been appreciated for many years. However, it is only since the DCCT (Diabetes Control and Complications Trial) carried out in subjects with Type 1 diabetes (T1DM),³ and the UKPDS (United Kingdom Prospective Diabetes Study) in subjects with T2DM⁴ that a cause and effect relationship between elevated glucose and these complications in humans was universally accepted. In contrast to microvascular disease, the role of hyperglycemia in the pathogenesis of macrovascular disease (ie, the accelerated atherosclerosis associated with diabetes) has been more difficult to document. This is due, in part, to the multiple, additional, powerful, risk factors for atherosclerosis that commonly cluster in individuals with T2DM. However, in both the DCCT and UKPDS, there was a tendency towards a reduction in macrovascular complications in subjects with better glycemic control that did not reach statistical significance.

The mechanisms by which elevated levels of glucose cause microvascular complications are not completely understood. However, a number of cellular and molecular changes triggered by abnormal activation of various glucose metabolic pathways has been proposed to account for these effects.⁵ Four of these mechanisms have been studied over a number of years, and a fifth is the subject of more recent investigations by several laboratories, including our own (Table 1). This issue of *Endocrinology Rounds* will review these mechanisms.

The aldose reductase (polyol) pathway

It has long been understood that excess glucose entering cells results in an osmotic force that promotes the net accumulation of intracellular water, leading to cell swelling.⁶ This scenario is most prominent in the lens and accounts for blurred vision in the setting of hyperglycemia associated with poorly controlled diabetes. Blurred vision is reversible over several weeks with restoration of normal glucose levels. Although glucose itself is freely permeable across membranes by virtue of the bi-directional facilitated diffusion that is mediated by glucose transporters, its conversion to



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Table 1: Mechanisms proposed to contribute to the microvascular complications of diabetes

- Aldose reductase (polyol) pathway
- Formation of advanced glycation endproducts (AGEs)
- Oxidative/carbonyl stress
- Activation of protein kinase C (PKC)
- Increased flux through the hexosamine biosynthesis pathway (HBP)

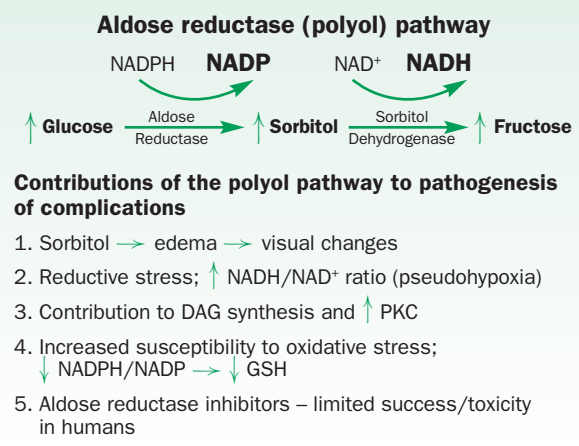
sorbitol by aldose reductase, and subsequently to fructose by sorbitol dehydrogenase (Figure 1), results in the accumulation of nonpermeable sugars. Although this osmotic effect causes cellular edema, it does not appear to account for chronic microvascular disease. Indeed, despite success in moderating the microvascular complication equivalents in animal models of diabetes,⁷ inhibitors of the aldose reductase pathway have had limited success in human studies.⁸⁻¹⁰

The best results have been achieved in diabetic neuropathy when minimal improvements and/or inhibition of the deterioration of nerve conduction velocity are demonstrated.⁸⁻¹⁰ One explanation for the lack of efficacy is that inadequate concentrations of inhibitor have reached nerve tissue sites, since dosing is limited by adverse effects. However, it is conceivable that the polyol pathway plays an indirect role in the pathogenesis of complications, and for this reason, inhibiting only this pathway will have little or no effect. The indirect contributions of glucose metabolism via the polyol pathway are discussed below.

The formation of advanced glycation endproducts (AGEs)

Glucose undergoes oxidation-reduction chemical changes at very slow rates that are accelerated in the presence of metal ions such as Fe^{++} and Co^+ . The more oxidized “keto” structure facilitates a nonenzymatic reaction with protein- NH_2 (amino) groups.¹¹ This time-, temperature-, and glucose concentration-dependent reaction has been utilized to monitor glycemic control by the development of assays for $\text{HbA}_{1\text{C}}$ (glycated hemoglobin) and fructosamine (glycated albumin). The $\text{HbA}_{1\text{C}}$ assay represents a major advance in our ability to assess metabolic control and institute therapy as required.¹² The close correlation of $\text{HbA}_{1\text{C}}$ with the development of microvascular complications has led to its use in guidelines for diabetes treatment.²

Figure 1: The aldose reductase pathway and its contribution to diabetes complications



The reaction of glucose with protein in the pathogenesis of microvascular disease is more complex than that described above for the glycation of hemoglobin. It appears that the formation of dicarbonyl compounds intracellularly, rapidly generates even greater amounts of reactive intermediates, both 6-carbon derivatives of glucose such as 3-deoxyglucosone, and 3-carbon fragmentation products of the glycolytic intermediate, glyceraldehyde-3-phosphate, called glyoxal and methylglyoxal.¹³ These reactive species form covalent linkages with the amino groups of proteins both intra- and extracellularly that result in altered protein structure and function. The mechanism by which such AGEs cause significant dysfunction is not clear. Apart from the direct protein modification alluded to above that has been demonstrated for some extracellular matrix proteins such as collagen and laminin,¹⁴ circulating AGEs have been found to bind to receptors for AGEs, called RAGE, on various cells (endothelial cells, macrophages and mesangial cells).¹⁵ Binding to these receptors appears to generate reactive oxygen species (ROS) that are involved in stimulating the expression of cytokines such as $\text{TNF}\alpha$ (tumour necrosis factor- α) and IL-1 (interleukin-1),¹⁶ permeability factors such as VEGF (vascular endothelial growth factor),¹⁷ and even pro-coagulant molecules, eg, PAI-1 (plasminogen activator inhibitor-1).¹⁸

Further evidence for the role of AGEs in causing complications is derived from experiments in which AGE formation was inhibited. Aminoguanidine, an agent with multiple free- NH_2 groups, was discovered to interfere with the formation of AGEs by binding to the reactive intermediates. In rodent models of

diabetes, administration of aminoguanidine was quite effective in decreasing the development of diabetes complications.¹⁹ While some effectiveness was demonstrated in early human studies, significant toxicity may limit the clinical application of this agent. Recently, the effects of the AGEs have been blocked using a new technique that inhibits their interaction with the receptor, RAGE. In one interesting study, a soluble form (extracellular portion) of this receptor was infused into an atherosclerosis-prone (ApoE^{-/-}) mouse that was rendered diabetic. The acceleration of the atherosclerosis with diabetes was blocked by the soluble protein that bound the AGEs in the circulation, thus preventing their interaction with RAGE.²⁰

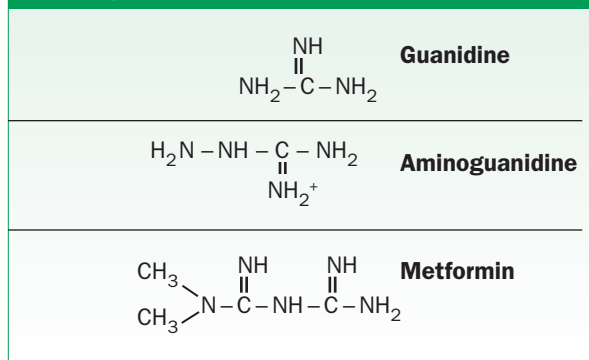
Humans possess several mechanisms to clear AGEs from their circulation, as well as to metabolize the reactive intermediates (eg, glyoxal).²¹ It is possible that some individuals are more sensitive to high glucose because of a reduced ability to metabolize these intermediates and/or clear AGEs. Taken together, there is good evidence that advanced glycation does contribute to diabetes complications – perhaps significantly to macrovascular disease – and that further investigation of methods to interrupt this process is worthy of attention.

One potentially important recent observation in this regard was in the UKPDS. In this study, the group of patients receiving metformin had significantly fewer complications than the other groups over the 10-year follow-up.⁴ This was independent of glycemic control, suggesting an additional effect. The structure of metformin contains a number of free-NH₂ groups (Figure 2) and recent studies suggest that they have direct interactions with reactive glucose derivatives such as glyoxal.²² Further work will determine if metformin can significantly interfere with AGE formation and will provide an explanation for this clinical observation.

Oxidative stress

Oxidative stress is defined as the generation of reactive oxygen species (ROS) in excess of their removal. This may be due to increased ROS formation, decreased antioxidant defense mechanisms, or both. Although it has been documented that increased generation of ROS is stimulated by high glucose, the precise mechanisms, and their role in pathogenesis, have not been defined. Early studies show that glucose autoxidation occurs in the presence of metal ions

Figure 2: Guanidine is the basic structure of both aminoguanidine and metformin. The amino groups can interact with glucose and its reactive metabolites, to prevent AGE formation



generating O₂^{•-} (superoxide) and H₂O₂ (hydrogen peroxide) and, if superoxide dismutase (SOD) and/or catalase are impaired, this may result in the formation of hydroxyl radicals (OH[•]) that react rapidly with, and damage, protein and DNA.²³

The importance of this phenomenon has been questioned and the concept of “carbonyl stress” has been proposed.¹³ This concept relates to the generation of reactive carbonyl groups (discussed above) with AGE formation and the fact that some formative pathways of AGE linkages may result in the generation of ROS. This suggests that increased ROS may be an epiphenomenon and may explain why antioxidant therapies have thus far been disappointing in clinical trials. However, ROS may be important participants in pathogenesis in two other ways.

- First, as discussed above, interactions with RAGE induce ROS.²⁴ These ROS may serve as intracellular signaling molecules, which are known intermediates for signalling by TNF α and angiotensin.²⁵ There are many redox-sensitive enzymes and transcription factors, providing a plausible mechanism by which ROS could alter cell structure and function.²⁶
- A second, very provocative, recently proposed mechanism for the contribution of ROS to diabetes complications is their enhanced production in the mitochondria and the subsequent inhibition of glycolysis. This is explained in more detail below.

Apart from an increase in the formation of ROS, it has been noted that skin fibroblasts cultured from subjects with diabetes complications have poorer antioxidant defense mechanisms than those derived from subjects without complications, further supporting

a pathogenic role and suggesting that endogenous antioxidant defense systems may be genetically determined risk factors.²⁷ In animal models, very large doses of combinations of multiple antioxidants have shown some effect in protecting against the cellular manifestations of diabetes complications.²⁸ Whether this is the case in human subjects requires the development and testing of more potent and long-lasting targeted agents. These are currently under development.

Activation of protein kinase C (PKC)

The PKC family of enzymes includes the serine/threonine kinases that consist of at least 11 isoforms, divided into 3 groups:²⁹

- the conventional, cPKCs, are activated by DAG (diacylglycerol) and Ca⁺⁺
- the novel, nPKCs are activated by DAG
- the atypical, aPKCs, respond to phospholipids such as PIP₃ (phosphatidylinositol 3,4,5-triphosphate).

PKCs function as cell-signaling enzymes, playing a role in a diverse set of functions such as cell growth, differentiation, apoptosis, cytoskeletal rearrangement, protein trafficking, and cell polarity. As such, they are generally stimulated by ligand receptor interactions. Thus the cPKCs are activated with the acute generation of DAG that is formed by the breakdown of membrane PIP₂ (phosphatidylinositol 4, 5 bisphosphate) by activation of PLC (phospholipase C). However, a number of years ago, it was demonstrated that cPKCs, and more recently the nPKCs, may be activated by DAG, synthesized *de novo* in the presence of hyperglycemia.³⁰ Increased metabolism of glucose to the glycolytic intermediate, glyceraldehyde-3-phosphate, and its conversion to dihydroxyacetone phosphate (DHAP) results in increased substrate concentrations that drive *de novo* DAG formation. This process is promoted by increased NADH, a by-product of the elevated flux through the polyol pathway (see above).

Although several isoforms are reported to be activated in different tissues subject to complications, the two major ones are PKC-β and -δ.³⁰ The attractiveness and strength of the PKC hypothesis as a mediator of complications derives from two observations: first, that activation of PKC results in many of the abnormalities found in the retina and kidney exposed to high glucose (Table 2), and second, that recent studies with a PKC-β-specific inhibitor have demonstrated success in preventing these complications in rodent models of diabetes.³¹ If this approach will be successful in

Table 2: Activation of PKC modulates enzyme activity and gene expression

A. Enzyme activity

- Inhibits eNOS → promotes endothelial dysfunction
- Activates NADPH oxidases → increases ROS
- Activates PLA₂ → inhibits Na⁺, K⁺, ATPase

B. Gene expression

- Increases VEGF → increases vascular permeability
- Increases PAI-1 → decreases fibrinolysis
- Increases TGFβ₁, ECM proteins → increases mesangial expansion

The above are selected examples of the consequences of PKC activation in various tissues that are relevant to the pathogenesis of microvascular disease. eNOS, endothelial nitric oxide synthase; PLA₂, phospholipase A2; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; PAI-1, plasminogen activator inhibitor-1; TGFβ₁, transforming growth factor β₁; ECM, extracellular matrix.

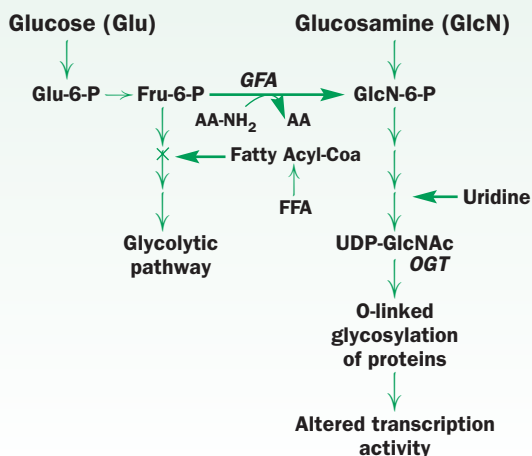
human subjects with diabetes is a question currently being investigated in large multicentre trials.

The hexosamine biosynthesis pathway (HBP)

The most recently recognized pathway of glucose metabolism that appears to contribute to the pathogenesis of complications is the HBP. This pathway consists of the conversion of fructose-6-phosphate (F6P) to glucosamine-6-phosphate (Glc-6-P) by the rate-limiting enzyme GFA (glutamine F6P amidotransferase). The Glc-6-P is then utilized to generate UDP-N-acetylglucosamine (UDP-GlcNAc). This end-product is utilized for protein glycosylation.³² One particular form of glycosylation, created by the addition of a single GlcNAc onto *ser* residues (O-GlcNAcylation), is a post-translational covalent modification that, much like phosphorylation, can occur on nuclear and cytosolic proteins altering their functional properties. This modification is regulated, at least in part, by the availability of UDP-GlcNAc and has been found to occur frequently on proteins involved in transcriptional regulation, (ie, specific transcription factors and their interacting proteins).³³ In this way, flux through the HBP has the potential to regulate gene expression. Indeed, it has been demonstrated that enhanced flux via the HBP stimulates the expression of leptin, the satiety hormone, in the peripheral insulin target tissues, fat and muscle.³⁴ In these tissues, both excess glucose and lipid (free fatty acids) can increase HBP flux (Figure 3) and leptin expression; therefore, it has been proposed that the HBP has a physiological role as a nutrient-sensing pathway. In fact, the HBP was first proposed to contribute to hyperglycemia- and free fatty acid-induced insulin resistance.^{35,36}

Figure 3: The hexosamine biosynthesis pathway

Glucose is metabolized via fructose-6-phosphate to glucosamine-6-phosphate by the rate-limiting enzyme glutamine fructose-6-P amidotransferase (GFA). This results in an increase of endproducts such as UDP-GlcNAc, used for O-linked glycosylation of proteins. The HBP flux is increased by high glucose, by a block in the glycolytic pathway mediated by increased free fatty acids (FFA), by administration of glucosamine or uridine, and potentially by alteration of GFA activity.



Our laboratory at the Banting and Best Diabetes Centre at the University of Toronto and others have postulated that the HBP may also be activated in non-insulin target tissues, specifically those manifesting complications. It has been demonstrated that exposure of glomerular mesangial cells to glucosamine, which bypasses the rate-limiting enzyme GFA to markedly increase UDP-GlcNAc, could mimic the effects of high glucose and stimulate the expression of genes and the secretion of proteins such as TGF β ₁, the extracellular matrix (ECM) proteins (fibronectin, collagen,³⁷ and laminin), and the pro-atherosclerotic, procoagulant, PAI-1 (plasminogen activator inhibitor-1).³⁸ These proteins are known to play a role in mesangial ECM expansion that leads to severe diabetic nephropathy.³⁹ Furthermore, Nerlich showed that the GFA enzyme is upregulated in the kidneys of human subjects with diabetic nephropathy.⁴⁰

More detailed studies at our laboratory have revealed that the expression of PAI-1 by high glucose was dependent on its flux through the HBP and that this required activation of a specific transcription factor called Sp1.³⁸ Sp1 is known to be heavily glycosylated and high glucose caused increased glycosylation and a reciprocal decrease in phosphorylation of this transcription factor.⁴¹ A competition between

glycosylation and phosphorylation is an attractive hypothesis to explain the differential regulation of such proteins.³³ It must be kept in mind, however, that associations do not prove a cause-effect relationship and so, this observation may not completely explain the mechanism of regulation of gene expression. Furthermore, our ability to inhibit the HBP *in vivo* is limited and this inhibition is required to determine the extent of the contribution of the HBP to diabetes complications.

Potential mechanisms of altered glucose metabolism in the setting of hyperglycemia

From the above discussion, the pathogenesis of the complications of diabetes share a common point: the increased flux of glucose via metabolic pathways, which under normal physiological conditions, account for only a minor portion of glucose metabolism. Excess flux results in the generation of “toxic” products, altered cell signaling, and gene expression, and these together induce tissue damage. Provided there are adequate numbers of glucose transporters in the cell surface plasma membrane so that glucose can freely move down its concentration gradient, the presence of high concentrations of circulating glucose will drive all of the pathways. It was recently proposed that glucose flux down these minor pathways was proportionately further enhanced by hyperglycemia because of a block in the glycolytic pathway at the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Excess glucose metabolism results in increased mitochondrial glucose oxidation during which ROS, superoxide (O₂⁻), formation occurs during the electron transport process.⁴² GAPDH is very sensitive to cellular redox state, and both the reflection in the cytosol of increased ROS produced in the mitochondria, as well as the relative increase in NADH/NAD⁺ ratio caused by the polyol pathway activation (see above), result in a 66% reduction in activity.⁴³ In this way, all the above pathways branching off the major glucose-glycolytic metabolic pathway at, or above, the glyceraldehyde-3-phosphate intermediate, are activated (Figure 4).

In cells in culture, dissipation of excess ROS with the antioxidant enzyme MnSOD (manganese-dependent superoxide dismutase) or of the mitochondrial proton gradient with UCP-1 (uncoupling

Figure 4: Multiple alternate metabolic pathways of glucose metabolism are enhanced in the setting of hyperglycemia by increased glucose entry into cells as well as by a reactive oxygen species (ROS)-mediated inhibition of glycolysis distal to glyceraldehyde 3-P

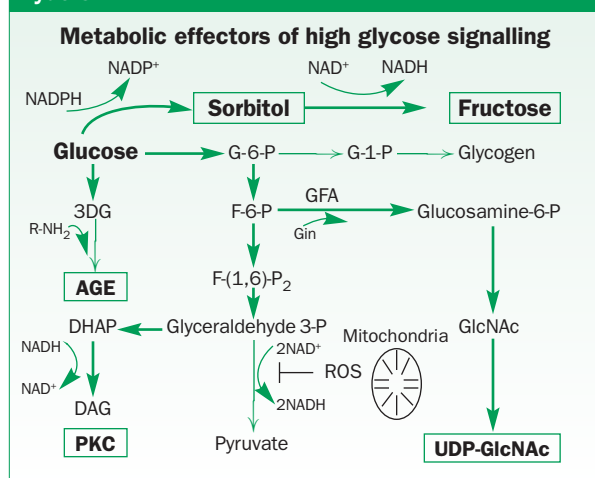


Table 3: Therapeutic approaches to prevent the complications of diabetes

A. Current

- Optimize glycemic control
- Screen for retinopathy (laser photocoagulation)
- Screen for microalbuminuria (ACE inhibitor or AT₁ receptor blocker)
- Foot care
- Detect and treat hypertension
- Detect and treat hyperlipidemia/dyslipidemia

B. Future

- Aldose reductase inhibitors
- Blockers of AGE formation
- PKC inhibitors
- Antioxidants
- OGT (O-linked N-acetylglucosamine transferase) inhibitors

protein-1), was able to normalize glucose flux through all of these pathways.⁴² These results point to the possibility of developing novel therapies directed at these fundamental mechanisms.

Current approaches and future therapy

The current approach to the prevention of diabetes complications includes first and foremost an attempt to maintain optimal glycemic control. This may be achieved with multiple daily injections of insulin in subjects with either Type 1 or Type 2 DM, and with various combinations of diet, exercise, and oral hypoglycemic agents in T2DM. (These will not be reviewed here as it is beyond the scope of this article). In recent studies, prevention or delay of T2DM has been successful with diet and exercise (lifestyle intervention), and to a lesser extent, with metformin⁴⁴ or acarbose.⁴⁵ Clearly, prevention of the onset of hyperglycemia is a powerful way to stop microvascular complications.

In addition to attention to glycemia, it has been found that ACE (angiotension-converting enzyme) inhibitors and recently, AT₁ (angiotensin type 1) receptor blockers will inhibit the progression of nephropathy,^{46,47} retinopathy,⁴⁸ and cardiovascular disease.⁴⁹ The aggressive treatment of hypertension, commonly associated with T2DM, has also been demonstrated to reduce complications. Finally, treatment of hyperlipidemia, both elevated LDL cholesterol and high triglycerides/low HDL dyslipidemia, improves cardiovascular risk. In combination, these

measures dramatically improve the prognosis of subjects with diabetes. It is also important to screen for the presence of complications since laser treatment of retinopathy can prevent blindness and protection of feet can prevent infection and amputation.

Future treatment modalities based on our understanding of pathogenesis as discussed above, such as PKC inhibitors, aldose reductase inhibitors, blockers of AGE formation, and newer antioxidants are under development and/or investigation by the pharmaceutical industry (Table 3). It is certain that within the next 10 years, there will be new therapeutics to inhibit the devastating effects of chronic hyperglycemia and improve the quality of life of those with diabetes.

Questions

Why do aldose reductase inhibitors seem to improve neuropathy more than the other microvascular complications such as retinopathy?

This appears to relate to tissue differences in the activity of these pathways. For example, in equivalent hyperglycemia, glucose flux through the polyol pathway in the lens may be 33% of total, but it is only 11% in the erythrocytes. The clinical implication is that multiple drugs are needed to prevent all of the complications of diabetes since each of the above mechanisms likely contributes to a variable extent in different tissues. This is similar to the approach to treatment of hypertension, which often requires 3, or even 4, agents.

You discussed the stimulation of PAI-1 by the HBP. Does the HBP contribute to macrovascular disease or atherosclerosis?

This remains to be definitively demonstrated, but there is increasing supportive evidence. Recently, Du et al showed that increased glucose flux via the HBP results in glycosylation of *ser* 1177 of the enzyme eNOS (endothelial nitric oxide synthase).⁵⁰ Insulin activates eNOS to cause vasodilation, and abnormalities of NO generation and endothelial function are early manifestations of atherosclerosis observed in diabetes. The phosphorylation of this same *ser* residue of eNOS is stimulated by insulin, resulting in its activation. Thus, modification by GlcNAc will inhibit enzyme activity and may promote macrovascular disease.

Many of my patients are taking oral glucosamine for arthritis. What should I tell them if they have diabetes?

We do not yet know whether oral glucosamine sulphate, as prescribed for arthritis, is absorbed in amounts that will raise serum concentrations high enough to enhance tissue uptake. Furthermore, glucosamine is taken up into cells by glucose transporters, and in the presence of high glucose, even less will enter cells. However, the evidence in favour of glucosamine markedly promoting flux down the HBP and altering gene expression should make us cautious. As a rule, patients with diabetes should probably avoid glucosamine and use alternative medications for arthritis.

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14-18 June, 2002

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Tel: 703 549-1500 Ext. 2134

E-mail: meetings@diabetes.org

19-22 June, 2002

Endocrine Society's 84th Annual Meeting

San Francisco, California

CONTACT: Beverley Glover

E-mail: Bglover@endo-society.org

Website: www.endo-society.org

2-5 October 2002

Canadian Diabetes Association and the Canadian Society of Endocrinology Metabolism Professional Conference and Annual Meetings

Vancouver, British Columbia

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