MECHANISMS OF DIABETIC COMPLICATIONS

Josephine M. Forbes and Mark E. Cooper

Diabetes Division, Baker IDI Heart and Diabetes Institute, Melbourne, Australia; Department of Medicine and Immunology, Monash University, Melbourne, Australia; and Mater Medical Research Institute, South Brisbane, Australia

Forbes JM, Cooper ME. Mechanisms of Diabetic Complications. Physiol Rev 93: 137-188, 2013; doi:10.1152/physrev.00045.2011.—It is increasingly apparent that not only is a cure for the current worldwide diabetes epidemic required, but also for its major complications, affecting both small and large blood vessels. These complications occur in the majority of individuals with both type 1 and type 2 diabetes. Among the most prevalent microvascular complications are kidney disease, blindness, and amputations, with current therapies only slowing disease progression. Impaired kidney function, exhibited as a reduced glomerular filtration rate, is also a major risk factor for macrovascular complications, such as heart attacks and strokes. There have been a large number of new therapies tested in clinical trials for diabetic complications, with, in general, rather disappointing results. Indeed, it remains to be fully defined as to which pathways in diabetic complications are essentially protective rather than pathological, in terms of their effects on the underlying disease process. Furthermore, seemingly independent pathways are also showing significant interactions with each other to exacerbate pathology. Interestingly, some of these pathways may not only play key roles in complications but also in the development of diabetes per se. This review aims to comprehensively discuss the well validated, as well as putative mechanisms involved in the development of diabetic complications. In addition, new fields of research, which warrant further investigation as potential therapeutic targets of the future, will be highlighted.

I. CLINICAL OVERVIEW OF THE DISEASE BURDEN

Diabetes, correctly termed diabetes mellitus, is a major epidemic of this century (540), which has increased in incidence by 50% over the past 10 years (129). This modern epidemic in some ways is rather surprising given that diabetes is one of the world’s oldest diseases, described in historical records of civilizations such as those found in ancient Egypt, Persia, and India (15, 154, 167). The World Health Organization states that ~347 million people worldwide were suffering from diabetes in 2008, which equates to 9.5% of the adult population (129). The incidence of diabetes is rapidly increasing with estimations suggesting that this number will almost double by 2030. Diabetes mellitus occurs throughout the world but is more common in developed countries. The greatest increase in prevalence in the near future, however, is expected to occur in Asia, the Middle East (4), and Africa, where it is likely that there will be an ~50% increase in diabetes in these parts of the world by 2030 (540).

A. Type 1 Diabetes

In type 1 diabetes, hyperglycemia occurs as a result of a complex disease process where genetic and environmental factors lead to an autoimmune response that remains to be fully elucidated (131). During this process, the pancreatic β-cells within the islets of Langerhans are destroyed, resulting in individuals with this condition relying essentially on exogenous insulin administration for survival, although a subgroup has significant residual C-peptide production (295). Type 1 diabetes is considered as a “disease of wealth” given that rates in westernized societies are increasing (234, 582). Type 1 diabetes comprises 10–15% of the diabetic population in countries such as Australia, but contributes in certain countries up to 40% of the total cost of diabetes, given its early onset, generally before the age of 30 years (http://www.jdrf.org.au/about-jdrf-australia/mediaroom). The genetic basis of this disease is not yet fully understood. Indeed, a number of major genetic determinants of type 1 diabetes such as alleles of the major histo-
compatibility locus (HLA) at the HLA-DRB1 and DQB1 loci (421) and more recently the HLA-B*39 locus (259) only account for some 40–50% of the familial clustering of this disorder. This suggests that there are other genetic loci involved in susceptibility to type 1 diabetes. Furthermore, there is an ~6% annual increase in the risk of developing T1D in developed nations (234, 582), which remains unexplained, but it is postulated to occur as a result of environmental triggers. This rising incidence may also be influenced by insulin resistance, which has been reported as a risk factor for type 1 diabetes (187). Although type 1 diabetes is an insulin-deficient state, features of insulin resistance are increasingly common, with the high prevalence of obesity in Westernized populations. In addition, this insulin resistance may be exacerbated by the high doses of exogenous insulin administered subcutaneously to type 1 diabetic subjects.

B. Type 2 Diabetes

Type 2 diabetes is the majority of the diabetes burden, comprising some 85% of cases. In this form of the disease, peripheral insulin resistance and compensatory hypersecretion of insulin from the pancreatic islets may precede the decline in islet secretory function. The tissues that most prominently demonstrate reduced insulin sensitivity include skeletal muscle, liver, and adipose tissue due to the particular requirements for glucose uptake and metabolism at these sites. However, it is increasingly considered that in most subjects the relative diminution in insulin secretion is the final event leading to hyperglycemia (278). Indeed, insulin secretory defects appear to be critical for the ultimate transition to overt type 2 diabetes, although residual insulin secretion from β-cells can persist for prolonged periods despite considerable disease progression. The increase in incidence of type 2 diabetes, especially in developing countries, follows the trend of urbanization and lifestyle changes, perhaps most importantly a “Western-style” diet with associated obesity. This suggests that environmental influences are also important contributors to this disease, which has a strong genetic component. It remains unlikely that genetic factors or ageing per se alone can explain this dramatic increase in the prevalence of type 2 diabetes. It remains to be fully determined as to how increased caloric and dietary fat intake in the context of reduced exercise with an associated increase in body weight ultimately lead to type 2 diabetes.

C. Complications of Diabetes

Diabetes is associated with a number of complications. Acute metabolic complications associated with mortality include diabetic ketoacidosis from exceptionally high blood glucose concentrations (hyperglycemia) and coma as the result of low blood glucose (hypoglycemia). This review will focus on arguably the most devastating consequence of diabetes, its long-term vascular complications. These complications are wide ranging and are due at least in part to chronic elevation of blood glucose levels, which leads to damage of blood vessels (angiopathy; FIGURE 1). In diabe-
tes, the resulting complications are grouped under “microvascular disease” (due to damage to small blood vessels) and “macrovascular disease” (due to damage to the arteries). Microvascular complications include eye disease or “retinopathy,” kidney disease termed “nephropathy,” and neural damage or “neuropathy,” which are each discussed in detail later within this review. The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes. Although the underlying etiology remains controversial, there is also myocardial dysfunction associated with diabetes which appears at least in part to be independent of atherosclerosis. Other chronic complications of diabetes include depression, dementia, sexual dysfunction, which are not discussed further within this review.

With conventional clinical management, the risk of the major chronic complications in type 1 diabetes based on the Diabetes Control and Complications Cohort (DCCT) and its followup study Epidemiology of Diabetes Interventions and Complications (EDIC) (415) are 47% for retinopathy, 17% for nephropathy, and 14% for cardiovascular disease. For type 2 diabetes, there are more limited data, with significant differences in the relative proportions of the various complications between Asian and Caucasian populations. For example, it appears that Asians tend to have a higher prevalence of nephropathy but a lower incidence of cardiovascular disease than Caucasians (84).

1. Nephropathy

Diabetic nephropathy represents the major cause of endstage renal failure in Western societies (206). Clinically, it is characterized by the development of proteinuria with a subsequent decline in glomerular filtration rate, which progresses over a long period of time, often over 10–20 years. If left untreated, the resulting uremia is fatal (400). Importantly, kidney disease is also a major risk factor for the development of macrovascular complications such as heart attacks and strokes (2). Hypertension (6) and poor glycemic control (612) frequently precede overt diabetic nephropathy, although a subset of patients develop nephropathy despite good glycemic control (148) and normal blood pressure. Once nephropathy is established, blood pressure is often seen to rise, but paradoxically in the short term, there can be improvements in glycemic control as a result of reduced renal insulin clearance by the kidney (23).

The early diabetic kidney also undergoes significant hypertrophy. This is characterized by enlargement of the kidney via a combination of both hyperplasia and hypertrophy, which is surprisingly often observed at the time of diabetes diagnosis (486). Hypertrophy is seen within the glomeruli, which is accompanied by mesangial expansion and thickening of the glomerular basement membrane. However, the proximal tubule, which constitutes greater than 90% of the cortical mass in the kidney, accounts for the greatest change in growth in diabetes (157, 532). As the tubule grows, more of the glomerular (urinary) filtrate is reabsorbed, which increases the glomerular filtration rate (GFR) via a feedback loop from the tubules (614). As a consequence of hyperfiltration and the diabetic milieu, the kidney filters increased amounts of glucose, fatty acids, proteins and amino acids, growth factors, and cytokines which are free to trigger a number of pathological pathways such as energetic imbalances, redox abnormalities, fibrosis, and inflammation (FIGURE 1). Ultimately, the deposition of extracellular matrix in the tubular component of the kidney (tubulointerstitial fibrosis) is postulated to be the major determinant of the progression of renal disease in diabetes (379).

Changes in hemodynamics, associated with blood pressure changes both systemically and within the kidney, have been reported to occur early in diabetes and are characterized by glomerular hyperfiltration. Glomerular hyperfiltration was initially postulated to be a major contributor to damage of the filtration component of the kidney, the glomerulus, as well as to preglomerular vessels (433). However, this role of hyperfiltration promoting general damage remains controversial, with some recent data suggesting that diabetic individuals who maintain normal glomerular filtration or hyperfiltration are actually protected against the progression to end-stage kidney disease (220). These hemodynamic changes are considered to occur as a result of changes in the metabolic milieu, release of vasoactive factors, alterations in signal transduction (FIGURE 1), as well as intrinsic defects in glomerular arterioles including electromechanical coupling. Proteinuria, which includes the protein albumin as a major component, often reflects changes in renal hemodynamics and is linked to changes in the glomerular filtration barrier, in particular changes within glomerular epithelial cells, termed podocytes.

Currently utilized therapies to treat diabetic renal disease largely target systemic blood pressure and/or intraglomerular hypertension. Applied the most widely are interventions
which alter the renin-angiotensin system (RAS) which includes angiotensin converting enzyme (ACE) inhibitors (6, 333) and angiotensin II (ANG II) receptor antagonists (59), which are considered first line therapies for diabetic nephropathy. Indeed, this strategy is an important component of most national and international treatment guidelines, along with strict glycemic control. It is important to note that early renal disease is a major risk factor for cardiovascular disease in individuals with diabetes (220). This suggests that more attention should be paid to the development of nephropathy in the early stages of the disease. However, the role of specific interruption of the RAAS in the prevention and management of early diabetic nephropathy remains controversial, with recent relatively disappointing results in this context (46, 377).

2. Retinopathy

Diabetic retinopathy is characterized by a spectrum of lesions within the retina and is the leading cause of blindness among adults aged 20–74 years (189, 245). These include changes in vascular permeability, capillary microaneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization). The neural retina is also dysfunctional with death of some cells, which alters retinal electrophysiology and results in an inability to discriminate between colors. Clinically, diabetic retinopathy is separated into nonproliferative and proliferative disease stages. In the early stages, hyperglycemia can lead to intramural pericyte death and thickening of the basement membrane, which contribute to changes in the integrity of blood vessels within the retina, altering the blood-retinal barrier and vascular permeability (189). In this initial stage of nonproliferative diabetic retinopathy (NPDR), most people do not notice any visual impairment.

Degeneration or occlusion of retinal capillaries are strongly associated with worsening prognosis (60), which is most likely the result of ischemia followed by subsequent release of angiogenic factors including those related to hypoxia. This progresses the disease into the proliferative phase where neovascularization and accumulation of fluid within the retina, termed macula edema, contribute to visual impairment. In more severe cases, there can be bleeding with associated distorting of the retinal architecture including development of a fibrovascular membrane which can subsequently lead to retinal detachment (189).

Diabetic retinopathy develops over many years, and almost all patients with type 1 diabetes (245, 506), and most having type 2 diabetes (297), exhibit some retinal lesions after 20 years of disease. Furthermore, whereas in type 1 diabetes the major vision threatening retinal disorder appears to be proliferative retinopathy (303), in type 2 diabetes there is a higher incidence of macula edema. Nevertheless, only a minority of such patients will have progression resulting in impaired vision.

In addition to maintenance of blood pressure and glycemic control, there are a number of treatments for diabetic retinopathy that have efficacy in reducing vision loss. These three treatments include laser photocoagulation, injection of the steroid triamcinolone, and more recently vascular endothelial growth factor (VEGF) antagonists into the eye, and vitrectomy, to remove the vitreous. However, there is no agreed medical approach to slow disease progression before the use of these rather invasive treatments.

3. Neuropathy

More than half of all individuals with diabetes eventually develop neuropathy (7), with a lifetime risk of one or more lower extremity amputations estimated in some populations to be up to 15%. Diabetic neuropathy is a syndrome which encompasses both the somatic and autonomic divisions of the peripheral nervous system. There is, however, a growing appreciation that damage to the spinal cord (530) and the higher central nervous system (641) can also occur and that neuropathy is a major factor in the impaired wound healing, erectile dysfunction, and cardiovascular dysfunction seen in diabetes. Disease progression in neuropathy was traditionally clinically characterized by the development of vascular abnormalities, such as capillary basement membrane thickening and endothelial hyperplasia with subsequent diminishment in oxygen tension and hypoxia. Inhibitors of the renin-angiotensin system and α1-antagonists improve nerve conduction velocities in the clinical context, which is postulated to be a result of increases in neuronal blood flow. Advanced neuropathy due to nerve fiber deterioration in diabetes is characterized by altered sensitivities to vibrations and thermal thresholds, which progress to loss of sensory perception. Hyperalgesia, paresthesias, and allodynia also occur in a proportion of patients, with pain evident in 40–50% of those with diabetic neuropathy. Pain is also seen in some diabetic individuals without clinical evidence of neuropathy (~10–20%), which can seriously impede quality of life (434).

Recently, however, there has been some controversy as to the inclusion of neuropathy as a “microvascular” complication, given that changes in neuronal blood vessels are considered by some investigators to be a secondary effect of an underlying neuronal and glial disorder associated with neuropathy rather than the vasculopathy being implicated as the cause of this group of complications. Indeed, recently there is some evidence suggesting that diabetic neuropathy selectively targets sensory and autonomic neurons over motor neurons, with little vascular involvement. In particular, the loss of epidermal (469, 546) and corneal innervation (364) has been noted. Indeed, nerve degeneration and loss of neuronal fibers within the cornea can be assessed and quantified noninvasively in patients with diabetes, using techniques such as corneal confocal microscopy (364, 478). Nerve degeneration at these sites has shown significant correlations with thermal thresholds and various measures of...
pain and pressure and neurological disability (364), suggesting that corneal confocal microscopy is a useful clinical tool for evaluating neural damage as a consequence of diabetes.

The size of neurons is also important. It appears that in diabetes, longer nerve fibers show an earlier loss of nerve conduction velocity with loss of their nerve terminals. This is the reason why tingling and loss of sensation and reflexes are often first observed in the feet and then ascend to affect other areas, in particular the hands. This syndrome is commonly termed a “glove and stocking” distribution, which includes numbness, dysesthesia (pins and needles), sensory loss, and nighttime pain. Spatial awareness of limb location is also affected early in the disease progression. This includes a loss of sensation in response to injury leading to callouses and other common foot injuries which places patients with diabetic neuropathy at high risk of developing foot and leg ulcers, which can ultimately result in amputation. Some diabetic individuals also incur multiple fractures and develop a Charcot joint, a degenerative condition seen in weight-bearing joints, characterized by bone destruction and eventually deformity. Progressive motor dysfunction is also common in diabetic neuropathy, which can lead to dorsiﬂexion of the digits of the hands and toes.

In addition to motor neuron dysfunction, the autonomic nervous system is also inﬂuenced by diabetes. One common abnormality in autonomic function seen in individuals with diabetes is orthostatic hypotension, due to an inability to adjust heart rate and vascular tone to maintain blood ﬂow to the brain. The autonomic nerves innervating the gastrointestinal tract are also affected leading to gastroparesis, nausea, bloating, and diarrhea, which can also alter the efﬁcacy of oral medications. In particular, delayed gastric emptying can dramatically affect glycemic control by delaying the absorption of key nutrients, as well as antidiabetic agents leading to imbalances in glucose homeostasis.

The wide variety of clinical manifestations seen with neuropathy, in addition to impaired wound healing, erectile dysfunction, and cardiovascular disease, can severely impede quality of life. Indeed, autonomic markers can predict which diabetic individuals have the poorest prognosis following myocardial infarction (36). Consistent with other complications, the duration of diabetes and lack of glycemic control are the major risk factors for neuropathy in both major forms of diabetes (148, 612). Other than optimization of glycemic control and management of neuropathic pain, there are no major therapies approved in either Europe or the United States for the treatment of diabetic neuropathy. In addition, as is seen with other complications, the mechanisms leading to diabetic neuropathy are poorly understood. At present, treatment generally focuses on alleviation of pain, but the process is generally progressive.

4. Cardiovascular disease

There is increased risk of cardiovascular disease (CVD) in diabetes, such that an individual with diabetes has a risk of myocardial infarction equivalent to that of nondiabetic individuals who have previously had a myocardial infarction (228). CVD accounts for more than half of the mortality seen in the diabetic population (228, 319), and diabetes equates to an approximately threefold increased risk of myocardial infarction compared with the general population (155). In type 1 diabetes, it is not common to see progression to CVD without an impairment in kidney function (220, 475). In type 2 diabetes, kidney disease remains a major risk factor for premature CVD, in addition to dyslipidemia, poor glycemic control, and persistent elevations in blood pressure (161) (FIGURE 1).

Cardiovascular disorders in diabetes include premature atherosclerosis, manifest as myocardial infarction and stroke as well as impaired cardiac function, predominantly diastolic dysfunction. Diabetic individuals at risk of CVD are treated with intensive regimens including strict glycemic control, administration of blood pressure-lowering agents such as those targeting the renin angiotensin system, lipid-lowering therapy with statins and/or fibrates and antiplatelet agents, such as aspirin.

Atherosclerosis is a complex process involving numerous cell types and important cell-to-cell interactions that ultimately lead to progression from the “fatty streak” to formation of more complex atherosclerotic plaques. These complex atherosclerotic plaques may then destabilize and rupture, resulting in myocardial infarction, unstable angina, or strokes. The precise initiating event is unknown; however, dysfunction within the endothelium is thought to be an important early contributor. The endothelium is crucial for maintenance of vascular homeostasis, ensuring that a balance remains between vasoactive factors controlling its permeability, adhesiveness, and integrity such as ANG II and nitric oxide, but this balance appears compromised by diabetes (441). Localized abnormalities drive atherogenesis, where immune cells including macrophages and T cells can bind to the vessel wall (432). This initiates movement of low-density lipoprotein into the subendothelial space leading to foam cell and fatty streak formation (209) which are commonly seen at sites of turbulent flow such as bifurcations, branches, and curves (501). Ultimately, proliferation of smooth muscle cells and matrix deposition, often with associated necrosis, result in the formation of a complex atherosclerotic plaque, which may occlude the blood vessel at the site of formation such as in the coronary or femoral circulation or become an embolus occluding blood vessels at distant sites, commonly originating from within carotid vessels and reaching the cerebral circulation.

Damage to the myocardium in the absence of hypertension and coronary artery disease may also occur in diabetes, and
this has been termed diabetic cardiomyopathy (53, 509). Cardiomyopathy is characterized by diastolic dysfunction (288). Diastolic dysfunction is an inability of the heart to relax and undergo filling during the diastolic part of the cardiac cycle. It is frequently subclinical and requires a high degree of suspicion for diagnosis which involves the use of sophisticated echocardiography techniques (482). With clinical progression, diastolic dysfunction may result in diastolic heart failure, which is best described as the presence of clinical signs and symptoms of heart failure in the presence of near-normal systolic function. Diastolic dysfunction is observed in up to 40–60% of subjects with heart failure (32, 689, 690), with diabetic individuals overrepresented (348).

The major clinical consequence of diastolic dysfunction is exertional dyspnea, which impedes the capacity of diabetic individuals to perform exercise, an important aspect of diabetes management, particularly in the context of obesity. Diastolic dysfunction is thought to arise as the result of a number of pathological processes. These include stiffening of the myocardium due to cross-linking and extracellular matrix deposition, hypertrophy, and neuronal abnormalities.

Overall, atheroma and myocardial damage are likely to occur at least in part as consequences of hypertension, altered vascular permeability, and ischemia. Importantly however, long-term glycemic control, biochemically defined using measures of HbA1C, remains the best predictor of CVD risk in both type 1 (52) and type 2 diabetic individuals (79). Gene expression within the vasculature is markedly altered by oxidative stress and chronic inflammation (178), which each tip the balance from an anti-inflammatory and antithrombotic vessel towards a pathogenic pro-inflammatory and thrombogenic state. There is also a failure of vascular repair in diabetes (586), with a reduction in endothelial progenitor cells (145, 586), further enhancing complications in multiple organs as described above, thereby imparting the significant morbidity and early mortality seen in both major forms of diabetes.

II. ANIMAL MODELS OF DIABETES COMPLICATIONS

A. Models of Microvascular Complications

1. Nephropathy

Animal models are important in preclinical testing given the interactions of the kidney with many other systems within the body and the role of the kidneys of the ultimate excretion of many therapeutic agents.

The most widely utilized animal model of diabetes involves the low-dose injection of the beta cell toxin streptozotocin, which displays a range of early functional and structural changes reminiscent of human diabetic nephropathy (22, 61). These include kidney hypertrophy, elevations in glomerular filtration, progressive leaching of albumin (albuminuria) and protein into the urine, and ultrastructural changes such as glomerular basement membrane thickening and mesangial expansion. These models, however, do not progress to more advanced renal disease seen in humans which is characterized by a loss of glomerular filtration, overt proteinuria, and advanced structural lesions (22, 61).

New models reminiscent of type 1 diabetes, such as the Akita (224), CD-1 mice with low-dose streptozotocin (626), OVE26 mice (684), and eNOS-deficient mice (681), have been recently developed by a number of investigators including the animal models of diabetes complications consortium in the United States (http://www.diacomp.org/). Mice with endothelial nitric oxide synthase (eNOS) deficiency and OVE26 mice currently appear to be the best murine models of advanced renal disease, showing a decline in glomerular filtration in the context of advanced structural lesions (22). It remains to be determined if newer mouse strains will ultimately prove to be more useful preclinical models for diabetic renal disease given that the severity and nature of the renal lesions vary depending on the genetic background (C57 vs $129$) in the Akita mouse (224) and eNOS-deficient mice do not generally respond as clearly to certain therapies used in humans, such as inhibition of the renin-angiotensin system (309).

Lepr(+/+)C57BL/KsJ (db/db) mice, which have a leptin receptor mutation, develop type 2 diabetes, characterized by comorbidities commonly seen in humans including elevated systolic blood pressure, obesity, and hyperlipidemia. The db/db mouse on this background overeats and progresses to type 2 diabetes in three stages. First, up to ~8 wk of life, these mice produce excessive amounts of insulin but do not have diabetes. They next develop type 2 diabetes, characterized by high plasma levels of insulin and glucose (weeks 9–10). These mice then develop more advanced type 2 diabetes by week 14, with high blood glucose concentrations and inadequate insulin secretion, requiring exogenous insulin administration for survival. At this stage, these mice develop declining GFR and overt proteinuria and have advanced kidney structural damage by week 20 (588). The recently described BTBR ob/ob mouse develops even more advanced renal lesions in the context of declining GFR (22). Therefore, these two models may prove to be more useful models of nephropathy resulting from type 2 diabetes.

All of these rodent models have limitations, and therefore, it is often considered preferable to look for changes that are common to more than one model and, most importantly, to relate these changes, where possible, to the temporal development of human diabetic nephropathy.
2. Retinopathy

There have been a large number of species studied as models of diabetic retinopathy, including monkeys, dogs, rats, and mice (170). In general, mammalian models develop the early stages of retinopathy, which includes degeneration of retinal capillaries. Unfortunately, periretinal (intravitreal) neovascularization is not seen in any animal model of diabetes, most likely due to the shorter lifespan of rodents. This has resulted in the emergence of a number of nondiabetic models of retinal disease where neovascularization is secondary to other factors such as relative hypoxia such as seen in oxygen-induced retinopathy (359), or with overexpression of growth factors such as VEGF (438) or insulin-like growth factor I (IGF-I) (508) in the eye. Although only very early stage changes are seen in animals models of diabetes, retinal neurons do degenerate in diabetic rats (34) and mice (33). Despite this, animal models do not develop measurable visual impairment or blindness due to diabetes.

These animal models have been able to assist us in understanding the retinal neovascularization in diabetes, although we are not able to fully describe the contribution of neurodegeneration to vision loss as seen in diabetic patients. Multiple intraretinal or extraretinal cell types are thought to contribute to retinopathy, which adds to the complexity of this complication. It is anticipated that the development of models, which develop more severe retinal lesions, may offer new insights into the pathogenesis of diabetic retinopathy and aid in the search for new targets for therapy.

3. Neuropathy

As with other models of diabetic microvascular complications, rodents generally lack advanced clinical characteristics of microvascular complications, which in neuropathy include segmental demyelination and axon and fiber loss (536). While these changes may be useful in defining the role of certain pathogenic pathways in early diabetic neuropathy, these models may not provide clues for the mechanisms responsible for axon loss and neurodegeneration in diabetes, in particular the selectivity for sensory and autonomic neurons. A longer duration of diabetes can precipitate discernible nerve pathology in some diabetic rodent models (68, 283), with some loss of skin sensory fiber terminals (42, 93), in addition to sensory loss. In particular, this can be seen in the Ins2 Akita mouse which has neuritic dystrophy and neuropathic of the sympathetic ganglia and is therefore very useful as a model of autonomic neuropathy in diabetes (526).

As for other complications, the lack of overt degenerative neuropathy in diabetic rodent models is likely a consequence of the relatively short life span of rodents or could be a manifestation of the physically shorter axons, and this has prompted studies in larger mammals. In support of this, diabetic dogs and non-human primates develop nerve con-

duction slowing and corneal hyposensitivity after years of hyperglycemia (171) and epidermal fiber loss (448), but degenerative neuropathy is minimal even in these larger animals. Surprisingly, diabetic domestic cats (397) have demonstrated functional and degenerative neuropathy indistinguishable from that seen in humans. Thus the potential of this feline model as a tool to investigate therapies for degenerative neuropathy is currently under evaluation.

B. Models of Macrovascular Complications

There are a number of animal models that have used to examine the pathological mechanisms which contribute to the macrovascular complications seen as a result of diabetes. The classic model of streptozotocin injection in rodents is not particularly useful for studying atherosclerosis given that although diabetes enhances vascular permeability (628) and enhances early cardiomyopathy (175), advanced atherosclerosis is not present. This is most likely a consequence of the generally anti-atherogenic profile of rodents, with high-density lipoprotein (HDL) rather than low-density lipoprotein (LDL) being the major lipoprotein present in the systemic circulation and the highly effective lipid-clearance mechanisms found in rodents (196). Furthermore, the db/db mouse model does not develop atherosclerotic lesions unless crossed with an apolipoprotein E-deficient (ApoE KO) mouse despite obesity, hyperlipidemia, advanced kidney disease, and cardiomyopathy (638). Indeed, mice are relatively resistant to the development of atherosclerosis unless they are bred onto vulnerable genetic backgrounds, such as is seen in the apolipoprotein E-deficient mouse (discussed below).

Therefore, models where genetic or dietary manipulations resulting in hyperlipidemia are combined with hyperglycemia have been developed. The first and currently most widely used of these is the apolipoprotein E-deficient mouse, which develops accelerated atherosclerosis following the induction of diabetes with streptozotocin (76, 451). However, it is difficult to determine in this model the individual contributions made by lipids and glucose to atherosclerosis, given that both hyperglycemia and more pronounced dyslipidemia are commonly seen with diabetes in this model (451). In addition, the relevance of this model may be limited, given that exogenous insulin administration is not required and the characteristic hyperlipidemia seen in these mice is different from humans with type 1 diabetes where hyperlipidemia does not generally manifest until after the development of renal disease.

More recently, mice that are deficient in the LDL receptor (LDL-R) have been bred. When the mice were made diabetic and fed a cholesterol-free diet, there was accelerated atherosclerosis when compared with nondiabetic animals, enabling investigators to postulate that hyperglycemia rather than dyslipidemia was responsible for the
accelerated atherosclerosis (491). These studies have also revealed that diabetes-associated dyslipidemia also accelerated lesion progression above that seen with hyperglycemia alone, confirming that glucose and lipids appear to be independent risk factors for atherosclerosis in diabetes. Furthermore, overexpression of the enzyme aldose reductase in these mice produces atherosclerotic changes more akin to those seen in diabetic humans (621).

Rodent models, such as streptozotocin-induced diabetes and the db/db mouse, can also be useful in studying cardiomyopathy as a result of diabetes, in the absence of coronary artery disease. However, there appear to be some specific cardiac differences between the models of type 1 and type 2 diabetes, and this highlights the likelihood that the pathogenesis of cardiomyopathy, particularly in its early stages, may not be identical in these two forms of diabetes (69).

Mouse models of macrovascular complications have a number of significant limitations. For instance, despite developing advanced atherosclerotic lesions and intraplaque hemorrhage, they do not reliably display evidence of thrombosis and plaque rupture. There are some models, however, including diabetic non-human primates which develop advanced cardiovascular disease similar to that seen in human subjects, which may be vulnerable to thrombotic events (109, 202). As is seen for microvascular disease, most models have significant limitations given that in humans, a diabetes duration of many years and often decades is required for the development of macrovascular disease.

Irrespective of these caveats, studies in animal models of diabetes ranging from rodents to swine models (151, 571) to non-human primates (251) have provided insight into fundamental mechanisms that may accelerate atherosclerosis, ultimately resulting in end organ infarction, which represents the major cause of mortality in individuals with diabetes. Hence, for preclinical testing, it may be rational to use small animal models to elucidate the utility of targets that have been identified and to perform early high-throughput compound screening in such models. For final lead compounds, these findings could then be confirmed in non-human primate models to validate therapeutic targets of interest for subsequent translation into the human context.

III. OVERVIEW OF COMMON MECHANISMS OF DAMAGE

A. Glucose: The Master Switch

1. Controlling blood glucose

The chronic elevation in blood sugar, termed “hyperglycemia,” is the major diagnostic biochemical parameter that is seen in the two major forms of diabetes (Figure 1). Ultimately, the most effective way to reduce the risk for vascular complications in both type 1 and type 2 diabetes is to achieve optimal glycemic control with the goal of reaching normoglycemia as early as possible in the course of the disease (148, 612). Type 1 diabetes is associated with an absolute insulin dependency, and in type 2 diabetes, as many as 50% of individuals ultimately require insulin to control hyperglycemia. The importance of insulin as an approach to reduce the burden of diabetic complications has been best studied in the DCCT/EDIC trial (148). In these type 1 diabetic subjects, intensification of insulin therapy either by increasing the daily frequency of injections or a continuous insulin infusion approach via a pump, led to improvements in micro- and macrovascular complications. These benefits on the outlook for individuals with type 1 diabetes have been considered the result of an improvement in overall glycemic control by various organs (Figure 2) rather than a specific independent effect of insulin.

In experimental models of diabetes complications, exogenous insulin therapy has been shown to protect against the development and progression of both micro- and macrovascular diabetic complications (575, 653). Interestingly, intranasal insulin (188) and pancreatic islet transplants (219) have also shown superior protection when compared with exogenous insulin in insulin-dependent rodents, suggesting that there may be other factors related to insulin secretion and trafficking that are important in the prevention of complications such as a potential beneficial effect of C-peptide (275) and the immune system (discussed below). C-peptide is a portion of the proinsulin molecule that is ultimately cleaved before insulin signaling occurs and is therefore a byproduct of endogenously produced but not exogenously administered insulin.

In type 2 diabetes, the situation remains more complex, as outlined below, since there are a range of pharmacological strategies used to control hyperglycemia in this condition. Although there is convincing evidence that optimizing glycemic control by targeting a number of sites (Figure 2) is the most effective therapeutic strategy in the clinical management of microvascular complications of diabetes (204, 531, 560), the recent ADVANCE (454) and ACCORD (203) studies have shown that more intensive glycemic control does not necessarily reduce the risk of cardiovascular disease.

There are numerous agents used to control hyperglycemia in type 2 diabetes. These include insulin-sensitizing agents such as thiazolidinediones (330) and metformin (273), whose primary role is to improve insulin resistance and glucose uptake into peripheral tissues. Interventions that stimulate insulin secretion from the pancreas,
namely, sulfonylureas (612) and the glucagon-like peptide-1 (GLP-1) agonists (225) and dipeptidyl peptidase-IV (DPPIV) inhibitors (472), are also widely used in clinical practice to address the relative insulin deficiency seen in the context of concomitant insulin resistance.

Some of these antihyperglycemic agents have direct effects on the development and progression of diabetic complications. For example, thiazolidinediones, which are peroxisome proliferator activated receptors (PPAR \( \gamma \)) agonists (14), have shown beneficial effects on complications, which are independent of their glucose lowering action (449). Indeed, the protective effects of PPAR \( \gamma \) agonists in the diabetic kidney appear to be modulated via prevention of activation of proximal tubular cells (580) and reduced secretion of profibrotic cytokines such as hepatocyte growth factor (HGF; Ref. 338) and other factors. The utility of thiazolidinediones has been overshadowed, however, by the increased incidence of cardiovascualar events including myocardial infarction seen with the PPAR \( \gamma \) agonist rosiglitazone (426).

Metformin has shown conflicting results with respect to the complications of diabetes. In diabetic humans, metformin therapy has been associated with a worsening of peripheral neuropathy, which appears to be related to its effects on vitamin B\( \text{_{12}} \) (645). Interestingly, modulation of vitamin B has also shown detrimental effects in diabetic nephropathy in humans (250). Conversely, there appear to be beneficial effects of metformin on macrovascular complications including in atherosclerosis and atherothrombosis (504) and myocardial infarction (3) in type 2 diabetes patients, best investigated in the UKPDS. Benefits on diabetic renal disease have also been shown following the administration of metformin in humans (204) and in animal models (576). These favorable effects of metformin on diabetic complications have been attributed to improvements in dyslipidemia, a reduction in proinflammatory profiles, decreased ox-

**FIGURE 2.** Interactions among glucose homeostatic pathways and target cells susceptible to diabetes complications. Target cells include endothelial cells, podocytes, proximal tubular cells, Müller cells, cardiomyocytes, and neuronal cells; GLP-1, glucagon-like peptide; IGF-1, insulin-like growth factor; FFA, free fatty acid.
idative and carbonyl stress, and restoration of endothelial function within the vasculature.

The newest class of oral antihyperglycemic drug for type 2 diabetes, which are currently in an advanced stage of clinical development, are the inhibitors of the sodium-dependent glucose transporter 2 (SGLT2), which exploit the role of the kidney in maintaining glucose homeostasis. Every day, some 180 g of glucose are filtered by the kidneys in a healthy normoglycemic subject, equivalent to approximately one-third of the total energy consumed by the human body. Almost all of this glucose requires reabsorption following filtration, primarily in the proximal tubule of the kidney, such that urine is almost free of glucose. This is different in diabetes, where the filtered glucose exceeds the transport capacity of the kidney tubular system and thus glucose appears in the urine (glycosuria) (372). Therefore, this therapeutic approach, namely, the selective pharmacological inhibition of the kidney SGLT2, which inhibits renal reabsorption of glucose, increases urinary excretion of glucose, which leads to a reduction in plasma glucose levels (356, 615). This is an insulin-independent approach to reduce plasma glucose levels currently being extensively evaluated in type 2 diabetes (31, 416). However, since SGLT2 inhibition does not rely on insulin levels or action, one cannot exclude a potential role for this new class of antidiabetic agent in type 1 diabetes. Indeed, in this setting there may be a use for these agents first by improving glycemic control and second by lowering the dose of exogenous insulin administration.

Hyperglycemia, however, is not the only major factor effecting complications. There is evidence that low blood glucose, hypoglycemia (695), may also be of import given its severe consequences, such as seizures, accidents, coma, and death. Indeed clinically, it is now appreciated that the greatest benefits on the complications of diabetes may be seen following minimization of plasma glucose and insulin “excursions” including low blood glucose concentrations, thereby providing better overall glycemic control. Whether “less excursions” reflecting glycemic control in individuals impact directly upon the complication rates remains to be determined. Although glucose variability is still considered by certain investigators to play a role in susceptibility and progression of diabetic complications (80, 401), it is possible that hypoglycemia per se, often seen in diabetic subjects with increased glucose excursions, plays a key role in explaining some of the deleterious outcomes seen in poorly controlled diabetic subjects. Indeed, in the recent ADVANCE study, an association between hypoglycemia and major macrovascular events such as myocardial infarction was observed, with the authors postulating that hypoglycemia could be a marker of a patient who is more vulnerable to adverse clinical outcomes (695).

2. Losing control of energy production

Cells within tissues that are prone to diabetic complications, such as endothelial cells, are not able to modulate glucose transport rates to prevent excessive accumulation of intracellular glucose (237). Hence, energy production in these cells becomes uncontrolled in the context of diabetes and eventually is impaired. Glucose-derived molecules, which enter cells, are usually fed into the first energy production pathway, glycolysis (FIGURE 3). Glycolysis, which requires no oxygen, is referred to as anaerobic metabolism and is known to be abnormal in diabetes (66). However, glycolysis is not efficient, with only four ATP molecules made from one molecule of glucose. Hence, eukaryotic cells shuttle the pyruvate produced from glycolysis to mitochondria, where it is oxidized and used for oxidative phosphorylation. Oxidative phosphorylation is a highly efficient pathway that uses energy released from nutrients via the Kreb’s cycle and from fatty acid and amino acid oxidation to produce 15 times more ATP (20). Glucose-derived intermediates are the most efficient facilitators of ATP generation and use less oxygen than substances such as free fatty acids (FIGURE 3). Energy released from nutrients as electrons flows through the respiratory chain, which facilitates the transport of protons across the inner mitochondrial membrane. The resulting electrochemical proton gradient is used to generate chemical energy in the form of ATP. This group of reactions can be uncoupled to produce heat or to terminate ATP production by collapsing the mitochondrial membrane potential. Simplistically, these interactive step-wise energy production reactions could be termed as a “controlled burn” of carbohydrates and fats. Hence, the highly regulated energy production in these cells is likely to become uncontrolled in the context of diabetes as a result of excess substrate availability, in particular glucose. Eventually, it is possible that glucose transport be slowed in order for cellular “self-preservation” or as a result of impaired insulin signaling. This perceived “lack” of intracellular glucose for metabolism may ultimately shift the substrates for energy production from glucose intermediates to those derived from free fatty acids. In the diabetic heart, where free fatty acids are the predominant source of energy (some 60%), there is likely to be increased uptake of free fatty acids to compensate for a loss of glucose-mediated ATP production and in response to an increased free fatty acid gradient due to hyperlipidemia.

Abnormalities in energy production are thought to be major contributors to the development of diabetic complications. Indeed, these changes are common manifestations seen in both microvascular (9, 18, 122, 499) and macrovascular (70, 180) disease. These include abnormalities in delivery of substrates, switching the ratios of cell specific fuel sources among glucose intermediates, fatty acids and amino acids, changes in respiratory chain protein function, and uncoupling of the respiratory chain (182). Uncoupling of the respiratory chain can occur to terminate ATP production by dissipating the mitochondrial membrane potential leading to the liberation of heat (FIGURE 3). Dysregulation of the family of proteins which regulate this process, the uncoupling proteins, has been previously reported at sites of dia-
betic complications (240, 462, 510, 624). The ultimate loss of ATP content within complication-prone tissues is considered to occur relatively late in the development of the disease. This suggests that although mitochondrial abnormalities may be early manifestations of the disease, the ultimate loss of ATP generation is likely to contribute to end-organ decline and cell death in the later stages of disease progression (64, 578). Indeed, it is likely that changes which enhance energy production from excesses in substrates such as glucose and free fatty acids are more likely pathological events, which occur early in the development of complications. In addition to more conventional benefits, restriction of energetic imbalances is probably another reason as to why glycemic control appears so effective in modulating the incidence of vascular complications by decreasing the delivery of glucose into tissues to prevent the switching to other fuel sources such as free fatty acids under inappropriate conditions (659). Conversely, the apparent lack of effect of glycemic control in preventing the progression of CVD, seen in studies such as ADVANCE, VADT (165), and ACCORD (203, 454), may also be related to lower cellular glucose uptake in the context of a high utilization of free fatty acids, although this remains to be clearly demonstrated.

3. Hexosamine biosynthesis

Glucokinase/hexokinase is an important enzyme involved in the transport of glucose into cells. The expression of this enzyme is controlled by the enzyme glucose-6-phosphate dehydrogenase (G6PDH), which forms part of the pentose phosphate pathway (66). Changes in this rate-limiting enzyme have been observed at sites of diabetes complications (447, 679). The pentose phosphate pathway provides ribose for the production of NAD(P)H, glutathione (GSH) reductase, and aldose reductase.

Once glucose is transported inside the cell, most of it is metabolized via glycolysis, through steps involving the conversion of glucose-6-phosphate to fructose-6-phosphate. When intracellular glucose concentrations are high, however, glycolysis can divert fructose-6-phosphate stepwise to UDP N-acetylglucosamine. This is important given that N-acetylglucosamine is used for posttranslational modifica-

**FIGURE 3.** Overview of fuel production within the mitochondria. NAD/H, nicotinamide adenine dinucleotide (reduced); FADH₂, flavin adenine dinucleotide (reduced); I, complex I (NADH dehydrogenase); II, complex II (succinate dehydrogenase); III, complex III (cytochrome c reductase); IV, complex IV (cytochrome-c oxidase); mPT, mitochondrial pore; CoA, coenzyme A.
tion of proteins within the cytosol and nucleus by single O-linked N-acetylglucosamine (O-GlcNAc) glycosylation (see below in protein modifications). These resulting sugar residues can compete with phosphate groups altering gene expression in diabetic tissues (162, 306, 520).

4. Aldose reductase

Increased flux through the sorbitol/polyl pathway was documented more than 40 years ago in the hyperglycemic setting (166). In this pathway, NAD[P]H delivered from the pentose phosphate pathway under high glucose conditions catalyzes the conversion of glucose to sorbitol using NAD(P)H derived from the pentose phosphate pathway. This is facilitated by the enzyme aldose reductase, which has a physiological role in detoxification of aldehydes into inert alcohols. During hyperglycemia, consumption of NAD(P)H by aldose reductase could inhibit antioxidant capacity by depletion of reduced glutathione and ultimately glutathione peroxidase activity. Elevations in intracellular sorbitol also provide the mitochondrial electron transport chain with excess NADH, which is a substrate for complex I. Intracellular accumulation of sorbitol can also result in osmotic stress which damages proteins via oxidation reactions. There is some evidence that diuretics which decrease osmotic stress can protect from cell death in cells exposed to hyperglycemia (466).

Overall, mice generally display relatively low levels of aldose reductase, but overexpression enhances vulnerability to diabetes-induced atherosclerosis and ischemia/reperfusion injury (258, 621). In addition, aldose reductase inhibitors (ARIs) and animal models with a genetic deletion of this enzyme each provide protection against the development of both microvascular (378, 570) and macrovascular (258, 621) complications. The translation of aldose reductase inhibition to the clinical context has proven disappointing, with decades of investigation not ultimately leading to a widely used treatment (380, 433). The only potential role for these ARIs is likely diabetic nephropathy, but even in this context, the results have not been particularly impressive.

5. Insulin resistance

Insulin resistance is broadly defined as the loss of cellular signaling in response to the hormone insulin. The tissues most affected by reductions in insulin sensitivity are skeletal muscle, liver, and adipose tissue, although there are many cells which depend on insulin-mediated glucose uptake. Commonly, skeletal muscle accounts for 75% of insulin-dependent uptake of circulating glucose, which is either immediately utilized or stored as glycogen. In the liver, insulin-mediated uptake of glucose leads to storage as glycogen and a reduced hepatic output of glucose by restricting gluconeogenesis. In addition, insulin signals a reduced need for lipid metabolism within the liver, resulting in the mobilization of free fatty acids for storage in adipose tissue. The mechanisms thought to be important for the development of insulin resistance are not further discussed within this review; however, they have been elegantly reviewed previously (43, 141).

The incidence of insulin resistance is increasing worldwide, largely in parallel with the rise in obesity rates. However, there is evidence that insulin resistance is also present in children with type 1 diabetes, where obesity is traditionally rarely seen (187). As our population does increase its body fat mass however, it is possible that insulin resistance may play a more important role in the development and progression of type 1 diabetes (128).

Impaired glucose uptake as a result of impaired insulin signaling has become increasingly apparent in tissues not generally considered as “insulin resistant” such as at sites of diabetes complications (604, 637). Indeed, a recent study has demonstrated that a deficiency in insulin receptor signaling in podocytes of the kidney can induce a disease state reminiscent of diabetic nephropathy even in the setting of normoglycemia (637). Paradoxically, reduced postprandial glucose uptake resulting from impaired insulin signaling is perceived by the liver as “low glucose availability,” which mobilizes fatty acids from adipose tissue as an alternate energy source, upregulating glucose-generating pathways (gluconeogenesis and glycogenolysis). Consequently, there is an accumulation of free fatty acids and hyperglycemia, which change the localization and expression of glucose transporters such as GLUTs and SGLTs at sites of diabetic complications. Indeed, there has been some protection afforded against the development of diabetic complications previously with selective targeting of glucose transporters in microvascular complications such as SGLT2 (363, 443) and GLUT-1 in nephropathy (635, 676). Diabetic cardiomyopathy is also improved by targeting GLUT-4 transporters (173).

Deficient insulin signaling is often identified experimentally by detecting a reduction in serine-473 Akt phosphorylation in insulin-target tissues, as one of the many steps involved in the insulin signaling pathway (600). Indeed, a loss of serine-473 Akt phosphorylation has been identified at sites of diabetic complications (538, 637) particularly in models of type 2 diabetes. Conversely, Akt activity is increased in some tissues and vascular beds affected by complications in type 1 diabetes (674, 675). While increases in Akt activity are reversible by tight metabolic control, the combination of hyperglycemia and insulin treatment results in enhancement of mTOR activity (discussed below). An adequate explanation for this paradox at sites of diabetes complications remains to be established but may be resolved by evaluation of therapeutic benefits of pharmacological modulators of Akt activity.
B. Obesity

1. Nutrient overload

Obesity is a particularly common comorbidity seen in individuals with type 2 diabetes. In type 1 diabetes, obesity may be present in some patients and is often a result of exogenous insulin administration rather than excess caloric intake. Indeed, obesity per se is known to exacerbate the development of diabetic complications (135, 666). This is likely due to the concomitant abnormalities seen in nutrient and calorie overload, insulin sensitivity, and secretion, in addition to a lack of physical activity, which are all likely contributors to vascular complications.

High-calorie diets commonly include a high content of saturated fat. There is certainly accumulating evidence to suggest that consumption of high-fat diets can exacerbate the development of diabetic complications (150, 550). It is also thought that obesity-induced neuropathy can be improved by dietary restriction of fat intake (435). Perhaps the most compelling evidence of the role of dyslipidemia, which could occur as the result of a high dietary fat consumption in diabetic complications, comes from studies using statins, which are discussed in detail elsewhere in this review. Conversely, increasing the consumption of beneficial fats such as through intake of fatty fish (138) or using polyunsaturated fatty acid supplements such as fish oil (535) have shown benefits in diabetic individuals, particularly on endothelial function and cardiovascular outcomes.

The American Diabetes Association suggests that there is no real consensus on whether restriction of dietary protein or carbohydrate has any long-term benefits on the development of diabetic complications (5). There are, however, a few studies which contain evidence that a reduction in dietary protein intake may be of benefit for diabetic nephropathy (233, 496). Similarly, although glycemic index has shown no association with the development of vascular complications, there is some research showing that a diet which contains increased content of whole grains compared with refined grains in humans may be protective (194, 388). Overload of, or a deficiency in, certain micronutrients including vitamins and minerals may also exacerbate the complications of diabetes, but this is not discussed further here.

Rodent models of diabetic nephropathy also show considerable improvement following either caloric restriction (412) or alternate day feeding patterns (603). Caloric restriction can also delay cardiovascular disease in diabetic rodents (394), primates (114), and individuals with type 2 diabetes (231). However, long-term compliance to such a regimen is unlikely, and therefore, mimetics of caloric restriction are currently being tested in aging populations (266). Nevertheless, the effects of these mimetics on diabetic complications remain unknown. Ultimately, long-term weight loss using very-low-calorie diets may be more achievable in diabetic individuals given a recent study which provides a new understanding of the reasons behind the high rate of weight regain after diet-induced weight loss (562). Bariatric surgery to restrict caloric intake, leading to substantial weight loss in morbidly obese individuals, has also shown benefits on chronic kidney disease (417) and on risk factors for cardiovascular disease (249, 547), but is more effective in concert with increases in physical activity (212). Bariatric surgery is increasingly being considered in adolescents with type 2 diabetes, with marked benefits on glycemic control. It is likely therefore that we will observe the benefits of this intervention on diabetic complications in the future.

Physical activity has also been shown to have effects on complications in animal models (51), via a reduction in circulating concentrations of a number of factors including advanced glycation end products (AGEs), insulin, and cytokines. As seen in nondiabetic individuals, regular moderate exercise can improve a number of factors relevant to the development of microvascular complications and may be an effective nonpharmacological approach if compliance issues could be overcome (51).

2. Adipokines

Adipose tissue is highly secretory, releasing a number of factors that are modulated in response to hyperglycemia. These are thought to induce a number of effects both systemically and likely on surrounding tissues (374), which may be important to the development of diabetic complications. Initially, this was postulated to be through effects on insulin sensitivity, which is not discussed further in this review; however, more recently adipokines have been shown to confer direct effects on organs susceptible to diabetic complications, and these are outlined below (FIGURE 2). These adipokines include adiponectin, a 30-kDa circulating plasma protein. Adiponectin modulates a number of metabolic processes, in particular those associated with glucose homeostasis and fatty acid catabolism, and is found in relatively high concentrations within the circulation. Adiponectin circulates in multimeric forms and binds to two adiponectin receptors (AdipoR1 and AdipoR2) inducing signaling via stimulation of 5’-adenosine monophosphate activated protein (AMPK) and likely other intracellular pathways (658). Some of the protective effects of adiponectin appear to be via improvements in oxidative stress (581). It has been reported that a reduction in circulating adiponectin is predictive of progressive kidney (514), retinal (665), and cardiovascular complications in diabetes. In contrast, however, increases in circulating adiponectin concentrations have also been shown to correlate with vascular complications in type 1 diabetic individuals (192, 227). In rodent models, elevating adiponectin concentrations attenuates, while prevention of the interaction of adiponectin with its receptors worsens kidney disease in diabetes (538).
Leptin is a 16-kDa hormone that plays a key role in regulating energy intake and expenditure. It achieves these effects via ligation to leptin receptors in the hypothalamus where it inhibits appetite. The absence of leptin (or its receptor) leads to uncontrolled food intake (hyperphagia) resulting in obesity. Indeed, a leptin receptor mutation results in the db/db mouse described above as an excellent animal model of diabetic complications. Leptin has an important role in the development of type 2 diabetes per se, but recently, it has been implicated in the development of complications. Specifically, intravitreal leptin concentrations are increased in individuals with proliferative diabetic retinopathy (197, 358), and leptin has been shown to stimulate ischemia-induced retinal neovascularization (561). Deletions of the leptin receptor in mice also result in autonomic neuropathy (211). Systemically, elevations in leptin concentrations are associated with renal disease (647), and infusion of exogenous leptin leads to renal disease in various experimental models (647). This could be partly because leptin has been reported to be proinflammatory, albeit in other contexts (195, 349).

C. Lipids

1. Dyslipidemia

Dyslipidemia, as assessed by standard measures, includes raised plasma triglycerides and LDL-cholesterol, in the context of decreased HDL-cholesterol. However, the measurement of these “classical” lipids alone does not adequately represent the complex dyslipidemia and abnormal lipid metabolism that is associated with both forms of diabetes. In type 2 diabetes, there is often an associated dyslipidemia, the major abnormalities being elevated triglycerides and low HDL cholesterol. Abnormalities in lipid handling are not traditionally associated with type 1 diabetes, except in some subjects as an elevation in HDL cholesterol. Indeed, dyslipidemia is thought to develop later in the course of type 1 diabetes, often concomitantly with the development of renal disease and in particular macroproteinuric. There has, however, been one large study which has suggested specific lipid abnormalities in the early stages of type 1 diabetes (442). In that study, individuals who developed type 1 diabetes had reduced serum levels of succinric acid and phosphatidylcholine (PC) at birth.

Broadly, hyperlipidemia can result in increased uptake of free fatty acids by cells, both by passive diffusion and through protein-mediated pathways. The most common proteins that mediate fatty acid uptake into tissues are CD36 and members of the fatty acid binding protein (FABP) family. Changes in the expression of CD36 within the diabetic kidney have been previously reported (569). In addition, circulating soluble CD36 (sCD36) concentrations (40) and monocyte expression of sCD36 are higher in diabetic patients (513). Studies have also inferred that serum A-FABP and E-FABP concentrations may be biomarkers for evaluating progressive nephropathy and associated cardiovascular risk in individuals with type 2 diabetes (664). However, most of the evidence of a pathological role for these proteins in disorders such as atherosclerosis and cardiomyopathy has been reported in the non-diabetic context (150, 317).

2. Lipid lowering

Perhaps the most prominent effects of lipid lowering in diabetic individuals are seen on their cardiovascular risk profile (208, 293). The two major classes of compounds studied to date target either 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (statins) or PPARα, using fibrates including fenofibrate and gemfibrozil. While statins are thought to have broad-ranging benefits in diabetic individuals, fibrates have shown inconsistent results. Fenofibrate has many pleiotropic effects that include anti-thrombotic and anti-inflammatory effects, in addition to improving flow-mediated dilatation. It is likely that the superiority of protection afforded by statin therapy compared with fibrates lies in its specific ability to potently lower LDL-cholesterol, a major risk factor for cardiovascular disease, not specific to the diabetic setting.

Statins inhibit HMG-CoA reductase, which is involved in the synthesis of sterols, isoprenoids, and other lipids through the mevalonate pathway. HMG-CoA reductase is the rate-limiting step in cholesterol synthesis in humans. Indeed, many individuals with diabetes will be prescribed statins as part of an overall treatment strategy to prevent or slow the progression of vascular complications, in particular, cardiovascular disease. The utility of targeting HMG-CoA reductase with statins has expanded beyond direct limitation of cholesterol synthesis. This is due to the discovery that statins have pleiotrophic cardiovascular health benefits that appear to be independent of improvements in plasma cholesterol (27). Statins have been shown to have anti-inflammatory effects (552), which is postulated as the result of their capacity to limit production of key downstream isoprenoids that are required for various aspects of the inflammatory response. Recently, it has been suggested that statin therapy may in certain populations promote the development of diabetes, although historically, pravastatin had been reported to indeed reduce the risk of diabetes (474). This issue remains an ongoing controversy (473, 518), but it appears that effects of statin therapy on the development of diabetes, either positive or negative, are modest at best.

Dyslipidemia has been reported to be particularly important for the development of neuropathy (134). In type 1 diabetes, dyslipidemia develops later in the disease and is often seen to coincide with the delayed onset of diabetic neuropathy (623). In type 2 diabetes, a high number of cases of peripheral neuropathy (as many as 10–20% of
patients) present at the time of diagnosis of diabetes. This is likely exacerbated by serum lipids and increases in body mass index which are independently associated with the risk of developing diabetic neuropathy. Indeed, in some studies, only persistent elevation in plasma triglycerides correlated with rapid progression of diabetic neuropathy, as assessed in sural nerves. Fenofibrate therapy has also been associated with a lower risk of amputations, particularly in the absence of macrovascular disease, probably as the result of its pleiotropic effects. This suggests that fenofibrate may have clinical utility for the prevention of diabetes-related lower-limb amputations. It remains unclear, however, as to the exact nature of the relationship between dyslipidemia and neuropathy.

Dyslipidemia is also thought to be a comorbidity influencing the progression of diabetic kidney disease. Statins have specific renoprotective actions and have been shown to reduce albuminuria and to prevent a decline in GFRs in both experimental and clinical diabetic renal disease. It is likely that at least some of these benefits may be due to lipid lowering. These benefits include anti-inflammatory effects, attenuation of oxidative stress, and improvements in other thrombotic markers. Statins can also affect the posttranslational prenylation of proteins within cells. This may also influence the progression of diabetic renal disease.

Studies have also shown a rationale for the use of fenofibrate in diabetic nephropathy. In experimental models, a genetic deficiency in PPARα worsened diabetic renal disease, while treatment of db/db with fenofibrate improves renal disease. There is also evidence from the FIELD study, which has demonstrated the renoprotective effects in diabetic nephropathy.

There is more convincing evidence for the use of fenofibrate in diabetic retinopathy. In the FIELD study, fenofibrate reduced laser treatment for macula edema and proliferative diabetic retinopathy. In the ophthalmology sub-study of FIELD, both macular edema and necessity for laser treatment was significantly reduced by fenofibrate. Conversely, a large-scale clinical trial of statins has shown no reduction in laser treatment or benefits on progressive retinopathy, despite a significant reduction in cardiovascular events. The ACCORD-EYE study has demonstrated that the combination of fenofibrate and simvastatin can lower the progression to diabetic retinopathy by as much as 40% compared with simvastatin alone. Thus both the FIELD and ACCORD-EYE studies have confirmed renoprotective effects of fenofibrate. Current experimental studies are in progress to determine if PPARα agonism is directly influencing key pathways including VEGF in the diabetic retina. It remains to be fully elucidated as to whether lipid-lowering drugs have downstream effects on macular edema and the prevention of vision loss.

D. Blood Pressure and Hemodynamics

1. Introduction

In addition to metabolic factors, hemodynamic factors are known to contribute to the development and, in particular, the progression of diabetic complications. These hemodynamic factors include systemic and tissue-derived components of the renin-angiotensin-aldosterone system (RAAS). The importance of hemodynamic factors is clearly emphasized in clinical studies where systemic hypertension is commonly associated with accelerated vascular complications including macrovascular disease, nephropathy, and retinopathy. Although hypertension is often considered a manifestation of diabetic renal disease, it is also an important systemic factor in exacerbating or promoting diabetic vascular complications.

2. The RAAS

The hormonal cascade that is the RAAS is thought to be a master controller of blood pressure and fluid balance within the body. Organs prone to diabetic complications appear to have their own functional tissue RAAS. Over the last decade, it has become increasingly appreciated that the RAAS is an extremely complex pathway. Indeed, some of the new discoveries with respect to this hormonal cascade such as the identification and characterization of more recently discovered components such as the putative pro-renin receptor and the enzyme angiotensin converting enzyme-2 (ACE2) may be particularly relevant to the diabetic setting.

A) Effector Molecules of the RAAS. The major arm of the RAAS is that which generates the vasoconstrictor ANG II, which also influences extracellular volume and is a key regulator of mean arterial blood pressure. More recently, however, the actions of angiotensins produced in the vasodilator arm of the RAAS such as angiotensin 1–7, may also play pivotal roles in the development of diabetes complications.

The liver synthesizes angiotensinogen, which is the major source of circulating angiotensin I (ANG I) in mammals. Angiotensinogen can be secreted in response to a number of stimuli including tissue injury and bacterial infection. Hepatic release can also occur as part of a feedback loop modulated via ANG II. However, the liver is not the only source of circulating angiotensinogen given that the angiotensinogen gene is expressed at many sites of diabetic complications including the kidney and the heart. There is also evidence in rodent models that overexpression of angiotensinogen causes tubular necrosis in the kidney. Superoxide has been shown to be an effector molecule for tissue damage in mice with transgenic expression of human renin and angiotensinogen.
The hormone renin is synthesized and stored as an inactive precursor, preprorenin, within the juxtaglomerular (JG) apparatus of the kidney cortex (217, 226). Prorenin is secreted through fenestrated capillaries from the JG after activation producing the majority of circulating renin. Renin is the hormone responsible for the cleavage of angiotensinogen to ANG I, and this reaction occurs primarily in the systemic circulation. There are a number of pathways that are thought to influence the secretion of prorenin. These include renal pressure sensors (baroreceptors) (411), endocrine pathways, and intracellular mechanisms and are often independent of systemic blood pressure.

Novel inhibitors of renin are currently being used in chronic kidney disease and hypertension. It appears that aliskiren in this context provides equivalent renoprotection to that seen with angiotensin receptor blockade (643). Its exact mechanism of action remains to be elucidated; however, there is evidence that its beneficial effects are the result of binding to the prorenin receptor complex (FIGURE 4) (467). The (pro)renin receptor is widely expressed at sites of diabetic complications including the kidney (422) where blockade has shown renoprotection in animal models (260).

Perhaps the most well-recognized enzyme of the RAAS is ACE-1 (commonly known as ACE; FIGURE 4). ACE is a promiscuous protein which cleaves a number of peptides including ANG I into ANG II, substance P, luteinizing hormone, and bradykinin. ACE-1 is localized at a number of sites of diabetic complications and may be regulated renally and hepatically via midkine (247).

Neutral endopeptidase (NEP) can hydrolyze ANG I to yield angiotensin 1–7 and smaller peptides such as angiotensin 1–4. Angiotensin 1–7 was originally postulated to be a vasodilatory peptide with benefits on kidney function (37). However, more recent evidence suggests that angiotensin 1–7 per se may be detrimental in certain contexts (172), and chronic administration of angiotensin 1–7 accelerates kidney disease in diabetic animal models (534).

Aldosterone is released from the adrenal glands in response to various angiotensins including ANG II and III as well as changes in serum potassium concentrations. Within the kidney, aldosterone acts as a hormone to increase the reabsorption of sodium ions and water in addition to the release of potassium ions into the urine for excretion. Aldosterone promotes water and salt retention by the distal tubule leading to increases in blood volume, which ultimately result in elevations in systemic blood pressure (63). A decline in blood pressure leads to the release of aldosterone from the adrenal gland increasing sodium reabsorption in the kidney and gastrointestinal tract. An increase in sodium alters extracellular osmolarity, which produces a complimentary rise in systemic blood pressure. Aldosterone elicits the majority of its effects via ligation to the mineralocorticoid receptor.

FIGURE 4. The renin-angiotensin cascade. ACE, angiotensin converting enzyme; NEP, natriuretic peptide; Ang, angiotensin.
**B) ANGIOTENSIN RECEPTORS.** The biological effects of ANG II are mediated via at least two specific receptor subtypes, the angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors (136), which are each widely expressed, including at sites of diabetic complications (50, 74, 159). The pressor actions of ANG II appear to be mediated via ligation with the AT1 receptor including vasoconstriction and activation of the sympathetic nervous system. Indeed, AT1 KO mice tend to have lower blood pressure, and when diabetes is induced in these mice, there is less renal injury consistent with the AT1 receptor playing a pivotal role in mediating diabetes-related renal injury (644). These findings build on a large body of evidence using AT1 receptor antagonists, which have demonstrated end organ protection in diabetic complications (59). These agents appear to block a large number of effects of ANG II including production of fibrotic cytokines and extracellular matrix accumulation as well as improve renal albumin permeability probably via effects on cytokines such as VEGF and on podocyte structure and function via effects on nephrin, a protein localized to the slit pore of the podocyte that is implicated in various proteinuric disorders.

Despite only sharing 34% sequence homology with the AT1 receptor, the AT2 has similar binding affinity for ANG II (136). It adult tissues, the AT2 receptor is not highly expressed (445), but is abundant in fetal tissues, where it is believed to play an important role in tissue development including nephrogenesis most prominently seen in AT2 receptor-deficient mice (339).

The role of the AT2 receptor in diabetic complications remains to be fully defined. Whereas in most contexts the AT2 receptor appears to act as a functional antagonist to the AT1 receptor via protection against ROS-mediated damage (558), there are increasing data to show that in the diabetic context it may have actions similar to that of the AT1 receptor. These include induction of cytokines such as VEGF, which may be of particular importance in diabetic retinopathy as well as promoting macrophage infiltration, presumably via NFκB-dependent proteins such as RANTES and MCP-1 implicated in the cellular changes which promote atherosclerosis. Indeed, recent studies using both an AT2 receptor antagonist and AT2 KO mice suggest that in diabetes, suppression of the AT2 receptor leads to reduced macrovascular disease (305), although in the absence of diabetes, AT2 KO mice have been reported by some groups to have greater susceptibility to vascular injury (57, 271).

**C) THERAPEUTIC TARGETING OF THE RAAS.** Clinically, the most widely applied RAAS blockers interrupt the conversion of ANG I to ANG II, namely, angiotensin converting enzyme-1 inhibitors (ACEI), agents which compete with ANG II for binding to the AT1 receptor, AT1 antagonists (ARB), or drugs which inhibit binding of aldosterone to the mineralocorticoid receptor. There have been many-scale clinical trials performed with these agents, and these have highlighted that therapeutic targeting of the RAAS has beneficial effects that go beyond blood pressure reduction. Given that ACE is a promiscuous enzyme which cleaves a number of substrates, it is possible that at least some of its success is due to its diversity of substrates, including substances such as bradykinin. There is, however, little evidence in humans that ACE inhibitors are superior to other therapeutics targeting this axis such as ARBs (673).

Not surprisingly, almost all clinical practice guidelines include drugs that inhibit the RAAS as first line therapies for individuals with diabetic complications. This group of agents has minimal side effects and preserves residual renal function, thereby maximizing protection against cardiovascular disease. In addition, RAAS blockade also has arguable benefits on most other microvascular complications of diabetes. A significant number of prospective, randomized, controlled studies have confirmed the protective effects of ACEIs (6, 333) or ARBs (59) in diabetic renal and cardiovascular disease.

It has become recently apparent that more complete blood pressure lowering can be achieved by combining agents targeting prorenin/renin receptor signaling with RAS blockade (AVOID; Ref. 452). As with other RAS blockers, direct renin inhibitors also show many beneficial nonhemodynamic effects in diabetic complications.

Compounds that are considered as aldosterone antagonists interfere with ligation to the mineralocorticoid receptor and are therefore antihypertensive and often diuretic. Inhibition of the actions of aldosterone using inhibitors such as spironolactone exhibits direct renoprotective effects (574). In addition, beneficial effects on retinopathy in rodents (646) via inhibition of the RAAS in a model of retinopathy have been reported. Benefits in the treatment of heart failure have also been shown (577). Taken together, these findings suggest that targeting the mineralocorticoid receptor may provide added benefits beyond those seen with other targets within the RAAS for the treatment of diabetic complications. However, the common side effect of hyperkalemia is a major limitation of widespread use of this class of agents in diabetic subjects with nephropathy.

**D) THERAPEUTIC TARGETING OF NON-RAAS PATHWAYS FOR HYPERTENSION.** β-Adrenergic receptor blockers afford their hypotensive effects through modulation of the sympathetic nervous system. Therapeutic interventions targeting this pathway attenuate sympathetic stimulation by competitive inhibition of catecholamine binding to β-adrenergic receptors (490). There are three major receptors, the most widely applied being those that compete for binding with β1-adrenergic receptors. Within the kidney, β-adrenergic receptors influence the secretion of renin, in addition to modulating vasoconstriction within kidney blood vessels. There
is some controversy as to the safety of this approach in individuals with diabetes due to the capacity of adrenergic receptors to influence peripheral vascular compliance and to inhibit hypoglycemic awareness, in addition to influencing glycemic control and lipid metabolism (248, 573). Recently, successful targeting of the sympathetic nervous system as a hypotensive strategy by bilateral renal denervation has been examined (315, 521). Renal denervation also appears to have metabolic effects on glucose homeostasis in nondiabetic individuals (360). Targeting of the sympathetic nervous system as an approach to combat diabetic complications may warrant future investigation.

Calcium channel blockers are another class of antihypertensive agents widely used in clinical practice, particularly for cardiovascular disorders (56). Pharmacologically, these agents reduce the cellular uptake of calcium or its mobilization from intracellular stores. As a class, calcium channel blockers are thought to combat hypertension by lowering peripheral vascular resistance, decreasing the responsiveness of the vasculature to ANG II, and facilitating diuresis (25, 559). The use of calcium channel blockers in individuals with diabetes as first line therapy remains controversial given their modest effects on renal and cardiovascular outcomes when compared with conventional blockade of the renin-angiotensin system (39, 334, 619). However, increasingly these agents are being used as part of combination regimens with blockers of the RAAS and have been shown in that setting to be useful in terms of blood pressure lowering and reduced adverse cardiovascular outcomes, as seen in the diabetic cohorts from the ACCOMPLISH (19) and ASCOT trials (444).

E. Protein Modifications and Turnover

1. Protein folding

One of the most complex processes that occurs within cells is the folding of the translated linear strands of amino acids into a fully functional three-dimensional protein. This involves an assembly line with strict regulation by a number of factors that guide nascent proteins to select the correct shape from an almost infinite array of possibilities (153). There is, however, some consolation, with the amino acid sequence dictating the biologically active conformation of a protein. Indeed, stoichiometry leads the way, where the chain of amino acids fluctuates through many conformations to identify a structure that is the most energetically efficient (153).

Misfolded proteins can occur as a consequence of a number of different processes outlined below including genetic mutations and interruption of posttranslational modifications. Indeed, it is important that there were no errors in transcription and translation of the gene that will change the amino acid sequence. An incorrect amino acid chain sequence often yields a misfolded protein. The second layer of this is posttranslational modifications, some of which are discussed below.

Posttranslational modifications alter the stoichiometry of the amino acid chain and thus have profound effects on the energy signature and the ultimate conformation of the folded protein. Some posttranslational modifications discussed below that are relevant to diabetes include advanced glycation, glycosylation, and phosphorylation. Indeed, changes in the functional properties of the protein are major contributors to chronic diseases including diabetic complications, which share the pathological feature of aggregated misfolded protein deposits and many excessively posttranslationally modified proteins including those modified by advanced glycation. These dysfunctional proteins are often unable to perform their normal intracellular functions and may not be able to be secreted from cells to complete their extracellular functions (529). This suggests the exciting possibility that “protein misfolding” may present a common target for therapeutic intervention in diabetic complications (111).

2. Autophagy

Autophagy is a cellular process that is responsible for the breakdown of proteins into their constituent amino acids during times of need, including starvation and metabolic disorders such as diabetes (487). These amino acids are primarily fed into the mitochondria for oxidation to produce ATP via oxidative phosphorylation. Therefore, changes in autophagy could facilitate the use of inappropriate fuels for energy production, which is a common phenomenon seen at sites effected by diabetic complications. During autophagy, part of the plasma membrane forms an autophagosome that then fuses with lysosomes and other cell structures to obtain the hydrolytic enzymes required for protein hydrolysis. This process is facilitated by a number of autophagy-related proteins (398). This includes the protein beclin, which is also known as autophagy related gene 6 (Atg-6; Ref. 340). Beclin is thought to be a critical factor involved in autophagy in mammals by facilitating the formation of autophagosomes (340).

Insulin is a known inhibitor of autophagy where it acts to limit the formation of autophagosomes (110, 365). This suggests that fluctuations in plasma insulin concentrations could have profound effects at sites of diabetic complications via effects on cellular breakdown and processing of spent or damaged proteins. It has been shown that in insulin-sensitive cells, including B cells, autophagy is increased (110, 385). Conversely in obesity, a common comorbidity for individuals with type 2 diabetes, hyperinsulinemia, as a result of nutrient overload, has been shown to decrease autophagosome formation. However, later in type 2 diabetes, autophagy appears to be increased in accordance with lower insulin concentrations, which may be an adaptive
response to protect against cell death (511). This penultimate effort to preserve cellular autophagy to facilitate the removal of damaged cellular structures and maintain energy production is thought to be one reason for the increase in life span seen with caloric restriction (124, 385). Indeed, caloric restriction also appears to be of benefit in experimental diabetic nephropathy (394, 412, 603).

In the vascular complications of diabetes, the study of autophagy is a relatively recent area of research. The growth factor HGF has been shown to play a role in the amelioration of diabetic vascular complications, at least in part, via autophagic clearance of proteins modified by advanced glycation (437). With respect to diabetic neuropathy, there is one study which shows that exposure of a neuronal cell line to sera from individuals with type 2 diabetes and neuropathy leads to the formation of autophagosomes and the expression of beclin-1 (606). In cardiomyocytes exposed to high glucose conditions, cell death is also associated with the expression of the autophagy marker beclin-1 (670). In addition, there is evidence that the serine threonine kinase target of rapamycin (mTOR) can regulate autophagy in renal cells (671). Furthermore, recent studies have shown that either deletion or upregulation of components of the mTOR pathway, including the mTOR complexes mTORC1 and mTOR2 (210, 267), contribute to the development of diabetic nephropathy. These studies suggest that autophagy is a tightly regulated cellular process where either chronic overactivity or inactivity may contribute to structural and functional decline at sites of diabetic complications. This exciting area of research warrants further investigation.

3. Posttranslational modifications

A) N-linked glycosylation. Glycosylation is a form of enzymatic posttranslational modification resulting in the addition of glycans onto proteins, lipids, and other organic molecules. This is a site-specific and targeted process in which the donor is usually an activated nucleotide sugar. There are five major types of glycosylation occurring intracellularly, which result in the addition of glycans onto molecules; however, this review is limited to the discussion of N- and O-linked glycosylation (FIGURE 5). N-linked glycosylation, commonly within the endoplasmic reticulum (ER), is important for the folding of a number of eukaryotic proteins which affects their trafficking and secretion. N-glycans which are imparted during N-glycosylation affect protein
folding, quality control, ER-associated degradation, ER-to-Golgi trafficking, and retention of glycoproteins in the apical membrane such as is seen for receptors.

 Interruption of N-glycosylation has been shown to result in the intracellular accumulation of misfolded proteins and the inability of glycoproteins to be trafficked to their correct cellular compartments, including to the plasma membrane for secretion. This phenomenon, termed ER stress, has been shown as a common pathological feature of many chronic diseases including diabetic complications. In the late 1980s, investigators characterized the unfolded protein response (313), where cells are thought to activate three major signaling cascades. The first of these is protein kinase RNA (PKR)-like ER kinase (PERK), the second is the inositol-requiring protein-1 (IRE1α), and the third is the transcription factor-6 (ATF6) pathway. The PERK pathway has a role in slowing protein translation, whereas the ATF6 and the IRE1α cascades are postulated as transcriptional regulators of ER chaperone genes which ultimately restore correct protein folding and ER-associated degradation of damaged proteins. In concert, these three pathways relieve the accumulation of misfolded ER proteins by targeting damaged proteins for recycling via autophagy. It remains controversial as to whether all three arms need to be activated in order for a true ER stress response to be initiated intracellularly to restore cellular homeostasis in protein folding.

In diabetes however, it is thought that these ER stress protective pathways are overwhelmed, initiating proapoptotic pathways (302, 654). Indeed, at sites of diabetic complications, ER stress is thought to be a common phenomenon (126) initiated by a number of important pathological pathways such as advanced glycation (264) and ANG II (564). There is some discordance, however, as to whether oxidative stress specifically is able to initiate ER stress, independent of glucose (403, 541).

It has been previously documented that activation of the ER stress response occurs within the diabetic kidney (404, 476, 651) or in isolated renal cells under high glucose conditions (483). Furthermore, microarray studies using human renal biopsies from individuals with diabetes have demonstrated higher expression of specific ER stress associated proteins including BiP (HSPA5), calnexin, and XBP-1 (344).

Within the retina, overexpression of 58-kDa inhibitor of protein kinase [P58(IPK)] by intravitreal injection of a purified recombinant adeno-associated virus vector (rAAV2)-P58(IPK) protects against diabetic retinopathy via improvements in ER stress (671). Furthermore, inhibition of ER stress ameliorated inflammation in the retinas of diabetic and oxygen-induced retinopathy mouse models (336). These findings suggest that ER stress is potentially an important mediator of retinal inflammation in diabetes.

There is also some evidence in diabetic macrovascular disease to suggest that ER stress is an important pathological pathway. Inhibition of the renin-angiotensin system using the AT receptor blocker valsartan can ameliorate ER stress, thereby preventing cardiomyocyte apoptosis (652). Induction of ER stress using excess administration of glucosamine that is seen in diabetic vessels induces accelerated atherosclerosis reminiscent of that seen in diabetes (640). Indeed, further studies using streptozotocin-induced diabetic apoipoprotein E-deficient mice suggest that accumulation of intracellular glucosamine as a result of hyperglycemia results in endothelial ER stress that precedes the onset of atherosclerosis (300).

B) O-LINKED GLYCOSYLATION. O-linked glycosylation is a late posttranslational process occurring within the Golgi apparatus (229), although single O-linked N-acetylgalactosamine (O-GlcNac) sugar residues can occur within the nucleus and cytosol (as discussed in hexosamine biosynthesis). O-glycosylation is the enzymatic addition of galactosamine to serine or threonine residues, which is rapidly followed by other carbohydrates (such as galactose or sialic acid). This form of posttranslational modification process is particularly important for many extracellular matrix proteins such as collagens and proteoglycans facilitating their role as “connective tissues.” For example, O-glycosylation of proteoglycans, which adds glycosaminoglycan chains to a proteoglycan core protein, produces properties which assist in cell-cell adherence. This also could be an important process in the development and progression of complications but is not discussed further within this review.

Another important role of O-GlcNAc modification of proteins is to mediate the actions of the hexosamine synthesis pathway during times of glucose flux, although this is thought to be a pathological rather than a homeostatic process (see above). In both micro- and macrovascular disease in diabetes, there is evidence of excessive O-GlcNAc modification of proteins (66). In cardiomyocytes and rodent models of diabetes, increases in O-GlcNAcylation have been shown to impair calcium cycling (108), modulate cardiac hypertrophy (368), and inhibit functional phosphorylation of proteins such as phospholamban, an important regulator of the activity of the key calcium-dependent protein SERCA-2 (667). Angiogenesis is also affected by excesses in protein O-GlcNAc modification which is thought to inhibit Akt signaling (355) and alter the transcription of the important angiogenic protein angiopoietin-2 (663). An elevation in O-GlcNAc-modified proteins has also been detected in human kidney biopsy specimens (143).

C) ADVANCED GLYCATION. Advanced glycation of free amino groups on proteins and amino acids is a nonenzymatic posttranslational modification, which begins with covalent attachment of heterogeneous sugar moieties (FIGURE 6). This reaction, first discovered 100 years ago and termed the
“Maillard reaction” (361), is influenced by many factors including intracellular glucose concentrations, pH, and time. Physiologically, advanced glycation is postulated as an evolutionary pathway for labeling of senescent cellular amino acids for their recognition and ultimate turnover, but it is likely that this is an oversimplistic view of these complex modifications. Indeed, recent evidence has shown that advanced glycation may modulate insulin secretion (123) and signaling (78, 488), although the ultimate influence of this on diabetic complications is yet to be determined. In addition, advanced glycation is viewed to stabilize extracellular matrix proteins via cross-linking. It is likely that these posttranslational modifications have other as yet undiscovered physiological roles.

Persistent hyperglycemia and oxidative stress accelerate the formation of AGEs (193). In diabetes, not only do long-lived proteins become more heavily modified, but short-lived proteins are also altered by advanced glycation. In addition, glycolytic metabolites of glucose such as glyoxal and products of the Kreb’s citric acid cycle are much more efficient initiators of intracellular advanced glycation than glucose per se. AGE pathways are as heterogeneous as their products and occur as the result of complex biochemical reactions involving the formation of Amadori products, the pentose phosphate pathway glyceraldehyde-3-phosphate, and formation of the reactive carbonyl methylglyoxal (596). As a consequence of AGE formation, there is often concomitant liberation of reactive oxygen species (193).

The consequences of modification of proteins by advanced glycation are numerous. First, extracellular generation of AGEs has effects on matrix-matrix, cell-cell, or matrix-cell interactions. This has been shown under pathological conditions to excessively cross-link the matrix resulting in stiffening (86, 120, 290). This may occur as a consequence of intracellular AGE modification of extracellular matrix proteins, altering their secretory properties and folding. In particular, modification of collagens including type IV collagen, a basement membrane glycoprotein, has been shown to alter cell adhesion, thereby changing physiological protein interactions (281, 406, 544).

Second, intracellular posttranslational modification of proteins by advanced glycation can directly alter trafficking, protein function, and turnover since AGEs are likely to be generated much more rapidly within cells. Despite the fact that excess uptake of glucose is the major reason for increases seen in the formation of intracellular AGEs, it is likely that situations where there is altered production of
glycolytic or Kreb’s cycle intermediates or reactive oxygen species would also lead to modification of proteins by advanced glycation (230).

The third pathway by which AGEs may exert their pathological effects is via interaction with cellular receptors. There are many AGE receptors (353, 555, 566, 661), but the most widely scrutinized in diabetic complications is the receptor for advanced glycation end products (RAGE). RAGE is a pattern recognition receptor that binds to multiple ligands such as AGE modified proteins, HMGB1 (427), S100 calgranulins (238), and β-amyloid (238). Its physiological role is thought to be an amplification of immune and inflammatory responses, given that RAGE is highly expressed on mucous membranes (45, 523). The ligand of AGEs to RAGE also results in NAD(P)H oxidase (639) and mitochondrial (122) dependent ROS generation. Although RAGE has a number of ligands other than AGE-modified proteins, these are not extensively discussed in this review but may contribute to the pathogenesis of complications in diabetes.

Advanced glycation, most likely via RAGE, can activate common downstream pathways which contribute to fibrosis via excess accumulation of extracellular matrix proteins, most likely induced via RAGE (118, 556). Specifically relevant to diabetic complications, AGEs can induce the production chemokines such as of monocyte chemoattractant protein (MCP-1) (263, 269, 656), profibrotic cytokines and growth factors including transforming growth factor-β1 (TGF-β1) (337, 607, 655) and connective tissue growth factor (CTGF) (611), and the angiogenic growth factor VEGF (544).

Transcription and translation of the RAGE gene produces a number of protein splice variants (252, 280, 668), the most commonly generated being the membrane-bound RAGE and circulating RAGE, known as endogenous secretory RAGE (esRAGE) (668). Circulating soluble RAGE can also be produced via cleavage of membrane-bound RAGE by proteases such as ADAM-1 (677). The capacity of soluble RAGE, a so-called decoy receptor, to compete for ligands appears to play an important role in the development and progression of diabetic complications. In diabetic individuals with complications, studies now conclusively show that increases in soluble RAGE are predictive of both cardiovascular events (112, 253, 423, 593) and all-cause mortality (424, 593). Early in disease, however, there may be a decrease in the levels of circulating soluble RAGE (185, 221). Furthermore, in neuropathy, there appears to be no association between circulating soluble RAGE and either peripheral or autonomic neuropathy in diabetes (254).

Possibly some of the most convincing data implicating advanced glycation in the development and progression of diabetic complications come from studying the predictive value of the intermediate AGE hemoglobin A1C. Studies in both type 1 (EDIC/DCCT) and type 2 diabetes (UKPDS/ACCORD/ADVANCE) conclusively show that elevation in HbA1C is one of the most useful, prognostic indicators for CVD risk in individuals with diabetes. Therefore, it is not totally surprising that elevations in circulating concentrations of RAGE ligands including AGEs (565) and HMGB1 (662) are predictive of macrovascular complications in diabetes. Moreover, we have identified that these RAGE ligands may form complexes, which facilitate more extensive binding and signal transduction via the RAGE receptor (456). Furthermore, one of the most consistent predictors of vascular complications in individuals with type 1 diabetes of long (200) or extremely long duration (i.e., greater than 50 years) are tissue levels of AGEs (565). In addition, there may be some utility for urinary AGE concentrations as biomarkers of diabetic kidney disease given that the ultimate fate of most AGE-modified proteins and peptides from within the body is excretion via the kidney (121, 190, 396).

Overall, evidence to suggest a pathological role for advanced glycation in diabetic complications primarily comes from rodent studies, which have clearly shown the efficacy of AGE lowering therapies such as pyridoxamine (72, 86, 127, 142), thiamine (29), alagebrium chloride (75, 184, 186), and OPB-9195 (410) as well as lowering AGE dietary intake (682) in averting and retarding experimental diabetic nephropathy. The role of dietary AGE intake remains controversial with our group having not shown benefits of reducing dietary AGE intake in experimental diabetic nephropathy (578). The use of thiamine and benfotiamine, however, in human clinical trials has been generally modest or disappointing (21, 480), with vitamin B supplementation actually worsening renal disease in diabetes (250).

Aminoguanidine, essentially the first AGE inhibitor to be extensively investigated (67), is another AGE-lowering therapy that can also inhibit the actions of nitric oxide synthase. This AG inhibitor was efficient in animal models (67, 402, 553) and did show potential benefits in human clinical trials (26). Unfortunately, the binding of this agent to AGE intermediates, which prevents AGE modifications in humans, produced new molecules previously unseen by the immune system, resulting in the deposition of unique circulating immune complexes in some individuals due to its mechanism of action, actually worsening renal impairment in these type 2 diabetic subjects. Although reduction in AGEs remains an extremely promising approach for therapeutic intervention, more careful pharmacological targeting of this pathway is required.

Manipulation of the enzyme glyoxalase-1 which is responsible for the removal of the AGE precursor methylglyoxal also lowers the tissue accumulation of AGEs (65, 545). This has been shown to translate into functional and structural
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benefits for diabetic neuropathy (44) and retinopathy (41, 392). Therefore, approaches which increase the activity of glyoxalase-1 or decrease the accumulation of methylglyoxal warrant further investigation as therapeutic targets in diabetic complications.

Administration of soluble RAGE or RAGE-neutralizing antibodies (181) in rodent models of diabetes have also shown protection against complications, which is also seen in RAGE-deficient mice (122, 407, 551, 578, 639). Not surprisingly, transgenic overexpression of RAGE worsens kidney disease in both nondiabetic and diabetic mice (657). When one examines these studies in totality, they suggest that the accumulation of AGE-modified proteins and their interaction with RAGE do contribute to both the development and progression of diabetic nephropathy. This is most likely due to RAGE modulation being upstream of many important pathological pathways relevant to diabetic complications, including ROS generation (122, 636), activation of the immune system (30, 90, 92), release of cytokines (191), and a newly discovered role in glycemic control (78, 123, 183). Therefore, it is not difficult to see why several major programs have been established to identify novel RAGE antagonists or probably even more important molecules that can mimic the action of soluble RAGE. However, the intrinsic role of RAGE in innate and adaptive immunity (366, 405, 599) must be considered carefully during the design of potential pharmacological agents to treat complications in the already potentially immunocompromised environment of diabetes. For example, it is very well known that hyperglycemia per se can influence neutrophil function, thereby reducing the resistance to certain infections (455).

D) PHOSPHORYLATION. One of the pathways implicated in the development of diabetic complications involves activation of the key intracellular second messenger protein kinase C (PKC). This family of enzymes includes at least 11 isoforms, which have been classified into 3 groups: the conventional group which includes PKC-α, β1, β2, and γ; the novel group; and the atypical group. After initial studies showing that glucose can directly activate certain isoforms, subsequent studies revealed that other stimuli characteristic of the diabetic milieu such as AGEs (591) and ANG II (507) can also promote PKC activation. Seminal studies by the Joslin group (201, 327, 328) and others (382) have emphasized the central role of PKC-β in particular PKC-β1 and -βII, in various diabetic complications including nephropathy, retinopathy, and cardiac dysfunction (384, 572, 627). This led to an active drug discovery program ultimately resulting in the development and clinical evaluation of a relatively selective PKC-β inhibitor, ruboxistaurin (268), which inhibits both the PKC-βI and -βII isoforms. This agent, initially known as LY333531, was shown 15 years ago to have renal and retinal benefits (16, 312). Further studies confirmed renoprotection in other animal models as well as defining key molecular events in the diabetic kidney that appeared to be PKC-β dependent such as enhanced renal TGF-β expression. Subsequently, various clinical trials were performed which revealed modest effects of this agent on diabetic retinopathy, nephropathy, and some effects on urinary albumin excretion, of doubtful clinical significance (17, 54, 97, 132). Currently, this drug has not progressed to clinical use but remains under ongoing active investigation, with its long-term future as a potential treatment in diabetes remaining precarious.

To further examine the role of the various PKC isoforms in diabetic complications, a series of mice with deletions of the individual isoforms have been generated. The PKC-β KO mouse after induction of diabetes did not develop renal hypertrophy (384). This occurred in association with attenuation of diabetes-associated upregulation of proteins which compose the extracellular matrix including collagen and fibronectin as a result of reduced expression of the key prosclerotic growth factors TGF-β and CTGF. Consistent with the major effect of PKC-β being via a TGF-β-dependent pathway, no decrease in urinary albumin excretion was observed, a phenomenon similar to that seen in diabetic rodents treated with a TGF-β1-neutralizing antibody (91). Another group also studied PKC-β KO mice and demonstrated a reduction in diabetes-associated increases in certain markers of oxidative stress, in the context of no major effects on expression of various subunits of the enzyme NADPH oxidase (439). As reported by the other group, attenuation of expression of prosclerotic cytokines and extracellular matrix proteins was also observed.

Additional experiments have now been performed examining another PKC isoform, PKC-α. This isoform is also up-regulated at sites of diabetic complications including the kidney. In contrast to the PKC-β KO mice, the PKC-α KO mice in response to diabetes have more prominent effects on urinary albumin excretion (387). Indeed, diabetic PKC-α KO mice not only have attenuation of albuminuria, but this is associated with a decline in renal VEGF expression, a growth factor that has been implicated in enhanced albumin permeability including across the kidney as well as restoration within the glomerulus of the podocyte specific protein nephrin (605). This is a highly relevant finding, since nephrin is strongly implicated in the pathogenesis of proteinuria including in the diabetic setting with nephrin gene deletion, nephrin gene mutations, and acquired nephrin deficiency as seen in diabetes all associated with increased proteinuria. Other studies have included experiments in PKC-ε KO mice, which develop albuminuria, mesangial expansion, and tubulointerstitial fibrosis even without diabetes (383). Further evaluation of these mice indicated that PKC-ε regulates TGF-β1, although the importance of this interaction has not been fully defined in the diabetic context. Indeed, with the increase in PKC-ε that has been seen in the diabetic kidney, it is possible that the up-
regulation of this particular PKC isoform represents a protective response to renal injury.

A family of mitogen-activated protein kinases (MAPK) including p38 initiate a cascade of intracellular events in response to stimuli such as cytokines, and are thought to be integral mediators of cell differentiation, apoptosis, and likely the development of diabetic complications (286). Indeed, a range of MAPK have been examined in the diabetic setting including p38 MAPK (261, 308, 492, 642). This work was stimulated by in vitro studies demonstrating that mechanical stretch in mesangial cells led to p38 MAPK activation ultimately resulting in enhanced TGFβ-1 and fibronectin expression. This was followed by studies which identified increased gene expression and activity of certain enzymes from the MAPK family (286). Subsequently, it was shown that reactive intermediates such as methylglyoxal that are increased in diabetes could activate p38 MAPK (345). Furthermore, certain effects of AGEs also appeared to involve this signaling pathway (87). With respect to human diabetic nephropathy, there is increasing evidence demonstrating enhanced expression of phosho ERK and p38 MAPK in the diabetic kidney. This phenomenon is seen in a range of renal cell populations including mesangial cells, podocytes, endothelial cells, proximal tubular cells, and mononuclear cells within the interstitium. To further explore the role of this isoform, p38 inhibitors have been administered to diabetic rats and were demonstrated to have effects on intrarenal hemodynamics and blood pressure (308). Furthermore, a p38 inhibitor, SB203850, has been shown in vitro to attenuate glucose-induced tubular cell apoptosis (484). It remains to be ascertained how effective targeting p38 MAPK will be on long-term renal functional and structural manifestations of diabetic nephropathy.

This pathway has not been as extensively assessed in the diabetic retina, but it has been shown in vitro that certain glucose-mediated effects on retinal pigmented epithelial cells are mediated by p38 MAPK and ERK (672). In addition, in models of experimental diabetes, inhibition of p38 MAPK improves retinopathy and sensory nerve function (163). Furthermore, diabetic mice with a deficiency in M KK3 which is an upstream kinase of p38 MAPK do not develop nephropathy (343).

c-Jun NH2-terminal kinases (JNKs) consist of 10 isoforms derived from the genes JNK1 to -3. They also belong to the MAPK family and as such are responsive to stress stimuli and facilitate apoptosis and T-cell differentiation. There have been several reports suggesting JNK signaling is elevated in both human and experimental diabetic complications, in particular nephropathy. To further explore the role of JNK, studies have used either inhibitors of JNK or mice with genetic deficiencies in JNK1 or JNK2 (262, 342). Surprisingly, each of these approaches has exacerbated urinary albumin excretion and worsened the integrity of the glomerular filtration barrier. These data contrast with data from other models of progressive renal disease (357). Interestingly, blockade of JNK signaling has been shown to be antiatherogenic (633) in addition to attenuating retinal neovascularization in a model of retinopathy of prematurity (223). However, the role of JNK in diabetic retinopathy remains to be fully defined.

F. Redox Imbalances

1. The mitochondria

Superoxide (O2•-) generation by dysfunctional mitochondria in diabetes has been postulated as the primary initiating event in the development of diabetic complications (425). Within mitochondria, over 90% of oxygen in humans is metabolized during oxidative phosphorylation where glucose metabolites and other fuels donate electrons to reduce molecular oxygen, resulting ATP generation. Despite this being a highly regulated process, some 1% of oxygen is only partially reduced to O2•- instead of fully to water by resident antioxidant enzymes under physiological conditions. There are two major sites where electron leakage can occur to produce superoxide within the mitochondria (FIGURE 3, namely, NADH dehydrogenase (complex I) and at the interface between coenzyme Q (CoQ) and complex III (609). Therefore, based on in vitro studies (425), it has been hypothesized that excess production of O2•- is via the premature collapse of the mitochondrial membrane potential so that electron leak to form O2•- and then H2O2 rather than ATP production. This, however, has yet to be satisfactorily substantiated in vivo in models of diabetes. Nevertheless, there are a number of studies that have shown mitochondrial functional abnormalities at sites of diabetic complications, and this remains an area of active research interest (70, 105, 122, 505, 542, 590, 685).

Therefore, one could rationalize that antioxidants that target mitochondrial superoxide production may be of benefit in diabetic complications. One such agent is idebenone, which has preferential mitochondrial uptake by organs such as neurons, kidney, and cardiac tissues. Indeed, this compound is used in human respiratory chain diseases such as Friedreich ataxia where mitochondrial generation of ATP appears to be preserved (236), particularly in cardiac tissues. Indeed, administration of exogenous coenzyme Q has shown therapeutic benefits in animal models of diabetic complications (256, 557).

MitoQ, an agent under investigation for the treatment of Alzheimer’s disease in humans, is selectively taken into mitochondria as the result of a lipophilic triphenylphosphonium cation (http://www.antipodeanpharma.com) (215). Studies determining the therapeutic potential of MitoQ to decrease vascular complications in experimental models of
type 1 diabetes (82) have shown some promise, and this is an area of research warranting further attention.

2. NAD(P)H oxidase

NAD(P)H oxidase was originally discovered in neutrophils where it produces vast quantities of $O_2^-$ by electron transport to augment host-pathogen defenses. The enzyme complex is usually composed of membranous and cytosolic components and a GTPase, rac1 or rac2. Nox-4 is a unique subunit originally identified in renal tissues, in that it does not require the other subunits to generate $O_2^-$. Although originally discovered in the cytosol, Nox-4 has been recently discovered in the mitochondria (48). Another homolog of gp91phox is Nox-5.

Numerous nonphagocytic cell types at sites of diabetes complications express NAD(P)H (216), but the capability for production of $O_2^-$ is significantly less than in immune cells such as neutrophils. This is likely due to their differential physiological roles in that white blood cells use NAD(P)H oxidase as a killing mechanism while in nonphagocytic cells the ROS generated are postulated to act as second messengers. However, binding of several cytokines and hormones such as ANG II and AGEs to their receptors rapidly activates NAD(P)H oxidase (28, 592). This is also seen in diabetic mice with deletions of the specific NAD(P)H oxidase subunits (584) or following treatment with anti-sense oligonucleotides (213) in the context of improvements in end-organ function. Although NAD(P)H oxidase as a potential pathogenic mediator of hyperglycemia induced ROS production, there are some major challenges to be overcome when targeting this pathway including redundancy among Nox isoforms (160) and the capacity of pharmacological agents not to interfere with intracellular $O_2^-$ generation for use as either second messengers or in host-pathogen defense.

3. Nitric oxide synthase

Nitric oxide is a common free radical with a role in cellular signaling which is produced by numerous cell populations in mammals. Nitric oxide synthase (NOS) has a number of isoforms, namely, inducible (iNOS), neuronal (nNOS), and endothelial (eNOS), which produce NO from NADPH, $\tau$-arginine, and oxygen often in the presence of cofactors such as bihydrobiopterin (BH$_4$) and flavin adenine dinucleotide (FAD). One of the major roles of NO is vascular dilatation following its release from endothelial cells. Indeed, NO is one of the most powerful vasodilators and is generally thought to be vasoprotective in the context of diabetes (367).

In diabetes, there is previous evidence that uncoupling of NOS due to restriction of $\tau$-arginine availability is a major source of superoxide at sites of diabetic complications (517), which is produced in preference to NO in that context. Indeed, administration of $\tau$-arginine to db/db mice prevents cardiac fibrosis (298). There is, however, some controversy as to the contribution of NOS uncoupling to diabetic complications. Early in disease development, NO production within tissues is thought to increase (307) as a result of changes in NOS activity (517), and therefore, it has been postulated that therapeutic blockade of this pathway could be beneficial at this time (100). Indeed, a deficiency in iNOS (618) or pharmacological inhibition of NOS (332, 692) improves nerve conduction velocity in animal models of diabetic neuropathy.

In contrast, the majority of studies performed later in the progression of diabetes suggest that functional decline in complication-prone organs is seen in concert with a state of progressive NO deficiency (471). These changes in NO production are attributed to multiple mechanisms such as glucose and AGE quenching as well as inhibition and/or post-translational modification of NOS. Indeed, several studies support this view, with chronic NO inhibition having been identified to have no effects (554) or detrimental outcomes for renal disease as a consequence of diabetes (282). This is also the case for blockade of nNOS in experimental diabetic neuropathy, where nNOS-deficient mice are not protected against diabetes-induced loss of sensory perception and intraepidermal nerve fiber loss (692).

Asymmetric dimethylarginine (ADMA) is a naturally occurring amino acid that is a natural inhibitor of NO production (116, 140). There is evidence to suggest that circulating ADMA concentrations are increased in individuals with both type 1 and type 2 diabetes and correlate with enhanced risk for cardiovascular disease (583). ADMA concentrations are also postulated to be influenced by elevations in circulating levels of LDL cholesterol (49). It is likely, therefore, that high LDL concentrations leading to increases in ADMA could compound deficiencies in NO production seen late in diabetes. ADMA is eliminated through metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) and subsequent urinary excretion. Therefore, reduced glomerular filtration by the kidney as is seen in chronic kidney disease including diabetic nephropathy could also elevate circulating levels of ADMA via decreases in renal clearance.

The amino acid homocysteine can regulate the activity of DDAH, thus influencing NO production. Indeed, an elevation in homocysteine is considered a risk factor for the development of both microvascular (99, 199, 519) and macrovascular complications in diabetes (320, 352). The clinical utility of decreasing homocysteine concentrations is currently under scrutiny (597). This is due to the results of a clinical study showing that supplementation of vitamins B$_6$, B$_9$, and B$_{12}$ to decrease homocysteine concentrations exacerbated the decline in renal function and increased the
risk of vascular disease in patients with diabetic nephropathy (250). This, however, is not the first disappointing result in this field. The Heart Outcomes Prevention Evaluation (HOPE-2) study found no effect of high-dose B₆, B₉, and B₁₂ cosupplementation on death from cardiovascular disease, while the risk of unstable angina was actually increased (351). Furthermore, the Homocysteine in Kidney and End Stage Renal Disease (HOST) study of patients with advanced kidney disease demonstrated no effect of high-dose vitamin B on risk of cardiovascular disease or death (272). The cardiovascular morbidity and mortality in the Atherosclerosis and Folic Acid Supplementation Trial (ASFAST) also showed that there was no slowing of atheroma progression or improvement in cardiovascular morbidity or mortality in individuals with chronic renal failure despite lowering of homocysteine concentrations (694).

These complex temporal changes in NO production seen during the evolution of diabetic complications make it difficult to determine the clinical applicability of approaches which inhibit NOS activity given that a deficiency in NO production seems to be an equally important pathological contributor to this group of diseases.

4. Antioxidants

Mammals have highly conserved antioxidant systems to combat tissue ROS generation. The first of these is superoxide dismutase (SOD), of which there are three major isoforms: copper zinc superoxide dismutase (CuZnSOD, SOD1), manganese SOD (MnSOD, SOD2), and extracellular SOD (SOD3). SOD catalyzes the reduction of superoxide to hydrogen peroxide. The second process in the stepwise reduction of superoxide to water involves the antioxidant glutathione peroxidase (GPx), which converts hydrogen peroxide to water. There are many other important cellular antioxidants, such as glutathione and numerous vitamins, but these are not discussed here due to space constraints (218, 446, 595).

In organs affected by diabetic microvascular disease, there is consistent evidence that the expression and activity of antioxidant enzymes is altered (81, 144, 244, 393). Interestingly, GPx-1-deficient mice have increased tissue hydrogen peroxide concentrations in the context of an increase in the incidence of macrovascular (335), but not microvascular disease (137), most likely because of redundancy with respect to other renal GPx isoforms. Overexpression of catalase in experimental models of type 2 diabetic nephropathy also appears to be protective (62). However, the utility of modulating antioxidant activity as a potential therapy for diabetic complications remains to be determined in particular in light of the disappointing results obtained to date using agents such as α-tocopherol in diabetic humans. However, α-lipoic acid, although not affecting primary end points in clinical studies, has shown clinically meaningful effects on pain and muscle weakness in diabetic polyneuropathy (688).

G. Inflammation

1. Introduction

The acute inflammatory response is an integral part of innate immunity, which is triggered in response to a real or perceived threat to tissue homeostasis. While the innate immune response is relatively nonspecific, adaptive immunity allows the human body to recognize and remember pathogens. This results in the ability to mount an enhanced inflammatory response following reexposure to a particular pathogen. In brief, acute inflammation occurs with the primary aim being the removal of perceived pathogens and initiation of wound healing in the damaged tissue. Not surprisingly, inflammation is a finite process that resolves via apoptosis and subsequent clearance of activated inflammatory cells as soon as the threat of infection abates and sufficient repair to the tissue is finalized.

Inflammation is carefully orchestrated by a cascade of factors such as proinflammatory cytokines, chemokines, and adhesion molecules that initiate the interaction between leukocytes and the endothelium and guide directional leukocyte migration towards infected or injured tissue. Proinflammatory cytokines (for example, tumor necrosis factor (TNF-α) and interleukins) and chemokines (such as Chemokine C-C motif ligand-2 and 5; CCL2 and CCL5 and fractalkine CX3CL1) released from infected/injured tissue activate the endothelium to increase the expression of the adhesion molecules E-selectin, intercellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1).

While acute inflammation as part of innate and adaptive immunity is beneficial, excessive or uncontrolled inflammation can promote tissue injury. Indeed, chronic inflammation is thought to be a characteristic feature seen at sites of diabetic complications. In clinical studies, circulating inflammatory markers are increased in patients with type 1 and type 2 diabetes, and the levels of these markers appear to predict the onset and progression of diabetic complications. The use of nonsteroidal anti-inflammatory compounds such as cyclooxygenase-2 (COX-2) inhibitors (discussed below in detail) or high-dose aspirin given to diabetic individuals for other indications has also provided evidence of a role for inflammation in the development of complications such as retinopathy (304), nephropathy (47, 95), and macrovascular disease (500). However, not all studies have shown benefits following the use of these anti-inflammatory agents (304), and some of these agents cannot be considered as long-term treatment for diabetic nephropathy because nonsteroidal anti-inflammatory drugs often have nephrotoxic side effects (47, 95).
2. Adhesion molecules

At sites of diabetic complications, hyperglycemia, hypertension, and dyslipidemia induce activation of the endothelium resulting in inflammation via a variety of mechanisms, including oxidative stress, NF-kB activation, dysregulation of NOS, and formation of AGEs.

Activation of the endothelium is a common manifestation in diabetes (441), where ICAM-1, VCAM-1, and E-selectins are expressed. These adhesion molecules facilitate recruitment of leukocytes and their extravasation, enabling their infiltration into tissues at sites of diabetic complications (585). In diabetic nephropathy, deletion of ICAM-1 protects against the development of renal disease in both experimental type 1 (440) and type 2 mouse models (104). This is also seen in diabetic retinopathy where blockade of ICAM-1 is protective against blood-retinal barrier breakdown, capillary occlusion, and endothelial cell damage (246, 395). Furthermore, lowering the expression of adhesion molecules with a neutralizing antibody against VCAM-1 (437) improves atherosclerosis in rodent models.

Soluble isoforms of VCAM-1 and ICAM-1 can be released from activated endothelial cells and are regarded as markers of inflammation (331). These soluble factors can cause activation of leukocytes and their chemotaxis to damaged tissue sites. Increased circulating levels of sVCAM-, sICAM-1, and E-selectin are closely associated with both their increased surface expression on endothelial cells (329, 495) and with diabetic renal, retinal, and macrovascular complications in humans (174, 381, 386, 522). There also is some evidence linking the levels of sVCAM-1 and sICAM-1 to diabetic neuropathy (277). Along with another platelet specific adhesion molecule, P-selectin, the levels of sICAM-1 are significantly higher in patients with neuropathy, and this associates with markers such as impaired nerve conduction velocity and vibration perception threshold (158, 255).

3. Leukocyte infiltration

Phagocytic cells such as monocytes and macrophages are often the first infiltrating cells that arrive at sites of diabetic complications (318, 516) in response to chemotactic molecules, in particular CCL2, CX3CL1, and CCL5. Indeed, rodent studies have suggested a causal role for monocytes and macrophages in the development of diabetic complications (101). In addition, blockade of the production of chemotactic molecules such as CCL2 (102) is also beneficial in preventing diabetic complications in rodent models. In models of experimental diabetes, a deficiency in CCL2 impedes renal monocyte and macrophage accumulation and improves diabetic renal injury (102, 103). These effects need to be considered in the context of a putative metabolic effect of CCL2 on insulin sensitivity (283).

In experimental diabetes, excessive CCL-2 signaling and consequent reorganization of the actin cytoskeleton affects nephrin expression in glomerular podocytes, which alters podocyte structure and function leading to albuminuria. These changes in cytoskeletal rearrangement are likely to be important for other cell types affected by diabetes. Furthermore, administration of pharmacological antagonists of the CCL-2 receptor, CCR-2 in experimental models of diabetic nephropathy, reduced renal hypertrophy and macrophage infiltration within renal glomeruli (284, 287).

Elevations in urinary excretion of MCP-1 may be a valid diagnostic marker of vascular complications in individuals with diabetes (73). The chemokine fractalkine (CX3CL1) is also known to be elevated within the circulation with both microvascular (512, 669) and macrovascular disease (73). In diabetic retinopathy, elevated circulating inflammatory markers including CCL-2 and CCL-5 have been shown to correlate with the degree of retinal damage (386).

Upon arrival and activation within damaged tissues, monocytes and macrophages facilitate the chemotaxis of other leukocytes such as T cells via secretion of a number of factors including interleukin-1β (IL-1β) and CCL5. Despite the role of T cells in the development of diabetes complications being a relatively new area of investigation, there are some rodent studies showing that depletion of T-cell populations at sites of vascular injury is beneficial (214). Conversely, however, other studies have shown that depletion of both B- and T-cell populations using diabetic Rag1 KO mice does not influence the development and progression of diabetic nephropathy (341). Two functional polymorphisms in CCR5 (RANTES, the receptor for CCL5) that inhibit its expression on immunocompetent cells are associated with increased risk of diabetic nephropathy in type 1 diabetes, specifically in men (399). Polymorphism of the CCR5 gene is also associated with increased risk for diabetic nephropathy in individuals with type 2 diabetes (409).

4. Inflammatory cytokines

Cytokines are a complex group of molecules capable of triggering differential effects on cells depending on factors such as cell type, timing, and the context of their expression. These molecules are able to share receptors and act synergistically to amplify their effects, and therefore conceptually, it is likely that cytokines and their receptors could be difficult to target therapeutically given that their temporal expression may alter many times over the course of the development and progression of diabetes complications. Cytokines are usually classified broadly according to their pro- or anti-inflammatory actions.

IL-1 is a cytokine that is primarily released by immune cells but is also secreted by resident monocytes, macrophages, adipocytes, and other cells at sites of diabetic complications. One of the first roles discovered for this cytokine is
the recruitment and activation of other leukocytes by enhancing the expression of adhesion molecules (235). In addition, there is some suggestion that inhibition of IL-1β might represent a safer alternative than vascular endothelial growth factor (discussed below) in retinal degeneration (322). The release of IL-1 also has a number of other effects on cells, including secretion of prostaglandins that affect vascular permeability via changes in local hemodynamics (463, 464), which may also be relevant for cells at sites of diabetic complications. Indeed, in diabetic neuropathy, IL-1β has been postulated to contribute to nerve damage (115) and miscommunication between Schwann cells and axons during the early stages of diabetic neuropathy (548).

IL-6 is a proinflammatory cytokine and an important mediator of cell proliferation, endothelial cell permeability, and matrix overproduction (515). White adipose tissue is the source of large quantities of adipokines such as IL-6 and TNF-α in diabetic patients (503). Circulating concentrations of TNF-α are elevated in individuals with either type 1 or type 2 diabetes compared with healthy control subjects (323, 418). Both IL-6 and TNF-α have been shown to influence glial cell and neuron behavior, contributing to the pathological processes relevant to both diabetic neuropathy and retinopathy (158). Clinical studies inhibiting TNF-α signaling using the pharmacological agent pentoxifylline are currently further exploring the renoprotective effects of this agent (419). Given that the primary role of TNF-α is the regulation of immune cells, any therapy targeting this axis would need to be extensively tested to assiduously define any side effects.

C-reactive protein (CRP) is another circulating protein that is released by the liver (PMID 12813013) in response to inflammation. CRP is a pattern recognition receptor whose physiological role is to activate the complement system (594). CRP is thought to be a sensitive biomarker for cardiovascular disease (458), but it has also been suggested that this molecule could be selectively targeted as a therapy for CVD (458).

5. Growth factors

Insulin is arguably the major growth factor associated with tissue growth and survival. Hyperinsulinemia has been associated with organ and tissue hypertrophy. In this context, hypertrophy and hyperplasia are most commonly seen at the major sites of peripheral insulin signaling such as the liver, skeletal muscle, and adipose tissue. The complexity of these growth-related actions of insulin, however, are underpinned by the fact that during sustained insulin resistance, where insulin signaling is diminished, skeletal muscle atrophies, while in general white adipose tissue and the liver increase in size. It is therefore difficult to pinpoint all the sites at which insulin-related defects may influence the development of diabetic vascular complications. We have however suggested throughout this review that there are a range of insulin-related effects on the development and progression of diabetic complications.

IGF-I and -II also bind to the insulin receptor (198) and are primarily produced by the liver in response to changes in growth hormone. In addition, the IGFs bind to at least two other receptors, IGF receptors 1 and 2, as well as a number of regulatory binding proteins (164). Although IGF-I is a well-known growth factor, its most notable growth effects in the nondiabetic context are on the kidney (176), which are thought to occur as a result of systemic growth hormone changes leading to local elevations in IGF-I (222).

IGF-I is thought to be a major contributor to early changes in the diabetic kidney including hypertrophy and increases in GFR (527). Indeed, there is a large body of evidence linking the GH/IGF-I axis to renal structural and functional abnormalities in diabetes (4, 257, 321, 429), with this link being more extensively characterized in type 1 diabetes (528). In retinopathy, the role of IGF-I appears to be rather complex. On one hand, a chronic deficiency of both insulin and IGF-I within the diabetic retina may lead to degeneration of neurons and capillaries resulting in ischemia. However, on the other hand, excesses of insulin caused by acute abundance of exogenously administered insulin or hyperinsulinemia alter IGF-I concentrations and enhance VEGF expression during ischemia (88).

In diabetic neuropathy, there is also some evidence implicating members of the IGF-I axis as pathological mediators of peripheral neuropathy and hypoglycemia (106, 391, 687). There have also been studies performed that suggest that erythropoietin (EPO) has synergy with various IGF-I functions, including neuroprotection, and therefore may be a potential substitute for IGF-I in the treatment of autonomic neuropathy (525). Another study has also shown that EPO peptides have some beneficial effects on autonomic neurite degeneration in diabetes (524).

The IGF-I/IGF-II pathway is similarly implicated in the development and progression of macrovascular diabetic complications. For example, either systemic overexpression of IGF-I (279) or cardiac specific overexpression of the IGF-I receptor (257) prevent the onset of diabetic cardiomyopathy. There is also evidence in diabetic atherosclerosis of a pathogenic role for the IGF-I/IGF-II axis (533, 625, 629).

TGF-β is a superfamily with three mammalian isoforms. The major isoform, TGF-β1, is synthesized as an inactive or latent form, complexed with latency-associated protein and secreted into the extracellular matrix. This complex is then cleaved by proteolytic enzymes, leading to the generation of the active form. TGF-β1 ligation to the binds to the TGF-β type II receptor (TGF-βRII), which then combines with the TGF-β type I receptor (650). This results in a signaling cascade involving the phosphorylation of Smad proteins.
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(371). Studies have shown that a range of stimuli increase TGF-β1 expression including hyperglycemia, AGEs, ANG II, lipids, and various products of oxidative stress (184, 648, 649). TGF-β1 is arguably the most potent inducer of tissue fibrosis, and chronic administration of a neutralizing TGF-β1 antibody improves renal function and structure in models of type 1 (537) and type 2 diabetes (691). However, the utility of TGF-β1 as a target for therapeutic intervention is impeded by its essential role in inflammatory and immune processes. Hence, although this molecule is a central mediator for many fibrotic processes which occur at sites of diabetes complications, it may be preferable to modulate tissue TGF-β1 levels via an alternative approach.

Although less extensively studied, TGF-β2 has also been implicated in diabetic complications, in particular nephropathy (242, 243). The role of this isoform is increasing being investigated, with recent data linking its actions to downstream effects of some micro RNA species (629).

Another profibrotic cytokine, connective tissue growth factor (CTGF), is also being considered as a pathogenic mediator of diabetic complications (75, 493, 610). CTGF expression is mediated by a number of factors commonly expressed in diabetes including TGF-β1, hyperglycemia, or mechanical stretch (493). The accumulation of AGEs has been reported to specifically increase CTGF expression, initially in dermal fibroblasts (611) but subsequently in other kidney (610) and cardiac cells (75). Moreover, anti-inflammatory agents such as aspirin can prevent the diabetes-mediated increase in CTGF and mesangial expansion in experimental models of diabetic renal disease (362). A phase I study of FG-3019, using a humanized anti-CTGF antibody, has been completed in patients with diabetic nephropathy, which was well tolerated and improved microalbuminuria (11). Subsequent studies are planned in diabetic patients with microalbuminuria (http://www.fibrogen.com/trials).

The angiogenic growth factor VEGF was initially considered to be a major mediator of retinal neovascularization in diabetes (438, 616). Recent findings, however, have demonstrated the importance of VEGF at other sites of diabetic complications (494, 626). The VEGF family (VEGF A-D) stimulates cellular responses by binding to cell surface tyrosine kinase receptors, the most common of which is VEGFR-2 (KDR/Flik-1), which is known to mediate most of the known cellular responses to VEGF. VEGF is most commonly expressed by the vascular endothelium, although pleiotropic effects have been identified on a number of other relevant cell types (e.g., stimulation of monocyte/macrophage migration, neurons, and renal epithelial cells). We and others have previously shown both in vivo and in vitro increases in VEGF expression at sites of diabetes complications that can be modulated by a number of therapies including AGE inhibitors (591) and AT receptor blockade (494, 678). Despite VEGF being an important contributor to diabetic complications, there is some controversy surrounding the utility of VEGF as a therapeutic target. Indeed, some studies suggest that VEGF blockade is renoprotective (139) and that overexpression of VEGF-A in podocytes of adult mice causes glomerular disease (620). In contrast, other experimental studies suggest VEGF is a critical survival factor and that blockade of this pathway may in fact promote cellular damage (13). These differential effects are also seen with anti-VEGF antibodies (192, 193). Furthermore, therapeutic interventions targeting VEGF for diabetic retinopathy have resulted in development of proteinuria in humans, such as that seen with Avastin, a humanized VEGF antibody (497). More recently, targeting of VEGF receptor signaling via deletion of the FLT-1 receptor in podocytes (316) or using SU5416, a VEGFR tyrosine kinase inhibitor (567), have shown protection against diabetic renal disease. Studies in the retina have also defined VEGFR1 as an important target for the treatment of retinopathy (77).

6. Cyclooxygenase

Cyclooxygenase (COX) is a family of enzymes, COX-1 to -3, which facilitate the formation of prostanooids, such as prostaglandins, prostaoyclin, and thromboxane. While COX-1 is expressed almost ubiquitously, inflammation and mitogens can stimulate the expression of COX-2. Some specific reactions that COX enzymes are involved with are the conversion of arachidonic acid to prostaglandin H2 and the oxygenation of two other essential fatty acids, dihomoy-linolenic acid (omega-6) and eicosapentaenoic acid (omega-3). These omega fatty acids are competitive inhibitors within COX pathways, which is the rationale for the use of dietary forms of these fatty acids (e.g., fish oil) to reduce inflammation. Furthermore, nonsteroidal anti-inflammatory drugs, such as aspirin and ibuprofen, are thought to provide their beneficial effects via inhibition of COX enzymes.

In diabetes, COX-2 inhibitors protect against the development of nephropathy (96, 413, 479). In addition, overexpression of COX-2 within podocytes predisposes the kidney to diabetic glomerular injury, most likely via a (pro) renin-mediated mechanism (94).

COX-2 inhibition has also shown efficacy in diabetic neuropathy, where intrathecally administered COX-2 inhibitors attenuate mechanical hyperalgesia (375, 617) in rodents. Selective COX-2 inactivation also protects against sympathetic denervation and left ventricular dysfunction, which is thought to be the result of improved intramyocardial oxidative stress and inflammation and attenuation of myocardial fibrosis in diabetes (296). In retinopathy, where COX-2 is an important mediator of angiogenesis (296), COX-2 inhibitors have also shown efficacy in preventing neovascularization by normalizing retinal oxygenation.
The selective COX-2 inhibitor nimesulide also prevents endothelial dysfunction in the hindlimbs of diabetic rats (8).

7. NF-κB

NF-κB is a transcription factor that is thought to be an important modulator of diabetic complications. Commonly, this dimer is composed of p50 and p65 subunits (35), which are sequestered in the cytosol via binding to the inhibitor of NF-κB, I-κBa. Following phosphorylation by IκB kinase β (IKKβ), a fine-tuning controller of the nuclear factor NF-κB pathway, NF-κB dimers are translocated to the nucleus. The active p65 subunit in particular is thought to be central to the transcription of numerous genes including angiotensinogen, cytokines, and adhesion molecules in the diabetic environment (35, 45). Not surprisingly, hyperglycemia (465), excess ROS (425), iNOS activation (420), and AGEs stimulate the translocation of NF-κB to the nucleus to induce transcription of numerous target genes (660).

Recent studies in diabetic individuals using the compound Bardoxolone methyl have shown potential benefits of chronic kidney disease primarily via improvements in estimated GFR (460). This therapy activates the Kelch-like ECH-associated protein 1 (KEAP-1)-Nrf-2 pathway (324). KEAP-1 plays a role in NF-κB regulation by controlling the ubiquitination and therefore breakdown of IKKβ. Indeed, depletion of KEAP1 leads to the accumulation and stabilization of IKKβ and to the upregulation of NF-κB-derived factors. Therefore, this pathway should be further investigated in the future as a potential target for other diabetes complications given that KEAP-1-Nrf-2 pathways have also been shown to regulate ROS production in cardiac cells (579).

Pyrrrolidine dithiocarbamate (PDTC) is a NF-κB inhibitor that has been used in both diabetic (326) and nondiabetic animal models of renal disease where it is renoprotective (485), although the toxicity of this drug has inhibited its direct translation to the clinical setting. Indeed, our group has demonstrated that NF-κB plays a role in early renal macrophage recruitment and infiltration in the diabetic kidney (326, 347). Moreover, diabetes-induced increases in NF-κB activation are prevented by numerous therapeutics including metformin (270), aspirin (683), vitamin B derivatives (232), carnosine (436), and thiazolidinediones (370). It is possible that in diabetic vascular complications, NF-κB is a central node which controls the downstream pathogenic consequences of hemodynamic and glucose-dependent pathways. However, approaches to inhibit NF-κB have not been explored fully in diabetes in humans, most likely as a result of the intimate involvement of this transcription factor in a number of essential cellular processes including apoptosis and host pathogen defense (276, 314).

8. Toll-like receptors

Toll-like receptors (TLRs) are a family of pattern recognition receptors with a diverse number of ligands including LPS, HMGB1, and fragmented DNA from necrotic cells, which play a role in both innate and adaptive immunity (239). TLRs can also mediate responses to a number of host molecules including ROS, the RAGE ligand HMGB1, breakdown products of tissue matrix, and heat shock proteins (HSP). Thus TLR are thought to be central modulators of a number of pathological conditions, infectious diseases, autoimmune and neurodegenerative diseases, and cancer.

A role for TLRs in the development of diabetes complications comes from studies performed in rodent models where these receptors have been deleted. Mice deficient in the TLR2 do not develop chronic inflammation or incipient diabetic nephropathy (147). Knockout of TLR4 in mice also attenuates the proinflammatory state of diabetes (146). In experimental macrovascular disease, TLR4 is required for early-intimal foam cell generation at lesion-prone aortic sites in ApoE KO mice, as is TLR2. Intimal SMC surround and penetrate early lesions, where TLR4 signaling within these early plaques may influence lesion progression (241).

Given that TLRs have also been identified in the mammalian nervous system, on cells such as glia, neurons, and neural progenitor cells (498), it is likely that these receptors also contribute to neuropathy and retinopathy, with this area warranting detailed investigation.

H. Gene Regulation

1. Metabolic memory

The contribution of hyperglycemia per se to macrovascular disease needs to be reconsidered in the context of the recent disappointing findings from the ACCORD and ADVANCE clinical trials, which explored the effects of strict glycemic control in type 2 diabetic subjects with established cardiovascular disease (203, 454). It is possible that the lack of a beneficial effect on cardiovascular mortality in these large-scale clinical studies relates to the relatively short duration of the trials (<5 yr), but could also reflect the irreversible vascular changes as a result of pathways that were induced prior to hyperglycemia such as advanced glycation. Indeed, this was previously suggested for diabetic retinopathy more than 20 years ago (169), where elegant studies in dogs showed the progression of retinal disease despite good glycemic control. However, although disputed by the investigators of the ACCORD study, hypoglycemia as a result of intensive glycemic control has been suggested to be another explanation for the increased mortality that was seen in that study with intensive glycemic control. The ADVANCE study has also explored the potential deleterious impact of hypoglycemia and reported that severe hypoglycemia may...
have contributed to severe adverse outcomes but could equally just be a marker of vulnerability to major macrovascular events (695).

Another possibility is “hyperglycemic memory” or a legacy effect as a result of previous episodes of hyperglycemia (83), possibly via epigenetic mechanisms including glucose-induced effects on histone modifications leading to modulation of vascular gene expression (168, 622) which persists despite a return to normoglycemia. Metabolic memory describes the phenomenon that has been observed in a number of large clinical trials where early intensive glycemic control has a sustained impact on reducing the risk of subsequent diabetic complications. These effects are seen even after the study has been completed and long after patients had returned to more conventional glycemic control. In type 1 diabetes, this phenomenon was observed in the DCCT where two groups were followed, one with strict glycemic control and one with a more conventional treatment strategy (148). After the study completion, the glycemic control in these intensively treated subjects returned to levels similar to before the study, and these individuals were followed for another 10 years as part of the followup study to the DCCT, known as the EDIC study (72). Interestingly, the protective effects of strict glycemic control on diabetic microvascular (72) and macrovascular complications were maintained (414). In experimental models of diabetes, a similar metabolic memory phenomenon has been observed in cell culture (168) and in animal models where restoration of glycemic control at specific time points using islet transplantation was unable to prevent retinopathy (85, 169, 311).

It remains to be fully determined if this is the result of programming a reversible epigenetic (discussed below) memory effect on cells via good glycemic control. Indeed, this legacy effect is likely to be particularly relevant when considering the vulnerability of diabetic individuals to major macrovascular events such as myocardial infarction.

2. Histone modifications

Remodeling of chromatin, a complex of DNA and histone proteins, can occur via at least two major processes. The first are the posttranslational protein modifications of the histone tails by processes such as acetylation, methylation, advanced glycation, ubiquitylation, and phosphorylation. The second is via direct modification of DNA by the addition of methyl groups commonly at CpG sites. Each of these modifications alters the DNA structure exposing or concealing specific gene sequences enhancing or inhibiting gene transcription. These processes represent some of the many regulators of gene transcription, which occur independent of changes in the underlying DNA sequence, which are heritable and may persist given that they remain through many cell divisions. Recently, these posttranslational modifications, such as DNA and histone methylation, have been suggested as contributors to diabetic complications (119, 489) since they regulate many cellular processes including proliferation, differentiation, and apoptosis in disease states such as cancer (470, 539). Epigenetics could also help explain how reexposure to certain states such as postprandial hyperglycemia may determine cell memory and affect future cell responses to stimuli.

In diabetic complications, experimental models have revealed a similar metabolic memory phenomenon to that seen in humans, which supports the postulate that there is a central role for epigenetic pathways, including modifications of histones. The potential effects of high glucose on these various epigenetic pathways is summarized in FIGURE 7.

Histone acetyltransferases (HATs) are responsible for histone acetylation within chromatin, which usually results in gene activation via “opening” of the DNA to allow for transcription factor and RNA polymerase II binding. Conversely, histone deacetylases (HDACs) remove lysine acetylation and in general oppose the actions of HATs as components of repressor complexes (310, 502). It is important to appreciate that histone acetylation is a dynamic process that is likely to be influenced by changes in glucose concentrations (468).

In contrast, methylation of histones within chromatin has been considered to be more constant and long-lasting, although increasingly this is also thought to be a dynamic process. This process of histone methylation leads to modification of both lysine and arginine residues, which can affect both gene repression and activation. Protein arginine methyltransferases (PRMTs) are commonly involved in gene regulatory events via mono- or dimethylation of arginine residues (325). At lysine residues, methylation is often complex given that they can be mono-, di-, or trimethylated, but these events have only been identified at some sites (369). For example, histone H3 lysine 4 methylation (H3K4me) is typically associated with gene activation while histone H3 lysine 9 methylation (H3K9me), in general, represses gene transcription (369). There are also numerous lysine demethylases (HDMs) that have been discovered which can also alter gene expression, emphasizing the bidirectional nature of histone methylation (543, 608). In addition to the histone, genomic methylation can also influence gene regulation with DNA methylation considered more stable than the changes seen within the histone code. However, recently it has been clearly shown that DNA methylation changes in response to transient hyperglycemia (468).

A number of changes in expression and activity of HDAC, histone methyltransferases (HMTase), HATs, and histone demethylases (HDMs) have been identified at sites of diabetes complications. Most of these studies have been performed in endothelial cells, where changes in these enzymes have been associated with the regulation and transcription of specific genes including the NF-kB subunit p65 (55, 152, 686). Preclinical studies in leukocytes including monocytes from diabetic patients have exhibited epigenetic modifications includ-
ing changes in effects on histones such as H3K9me2 and H3K4me2, which are associated with immune and inflammatory pathways (389, 390). In addition, TGF-β1 treatment of renal mesangial cells increases the histone methyltransferase SET7/9, which is associated with the expression of profibrotic genes in these cells (563).

Reversal of epigenetic memory, or epigenetic therapy, has gained interest for the treatment of diabetic complications. Indeed, curcumin (a derivative of turmeric), which is an inhibitor of histone acetyl transferases, can lower the expression of a number of inflammatory genes and has shown promise in the treatment of diabetic nephropathy in both humans (299, 613) and in experimental models of diabetes (568, 601).

Other approaches targeting epigenetic regulation in experimental models of diabetes (12, 205, 428) have also shown promise. Furthermore, it is likely that these compounds may be useful for the treatment and prevention of atherosclerosis (179) and retinal neovascularization (301) given previous beneficial effects seen in the nondiabetic context with respect to these disorders.

3. Sirtuins

The sirtuin family of proteins also known as sir-2 are categorized as class III histone deacetylases that play complex and important roles in ageing-related pathological conditions such as cancer and the dysregulation of metabolism. There are seven members of this family in humans, divided into four classes, and these are evolutionarily highly conserved across most species. Sirtuins can affect gene transcription, apoptosis, and resistance to stress, as well as modulate energy efficiency during restricted calorie intake. Unlike other known protein deacetylases, sirtuin-mediated deacetylation is coupled to NAD hydrolysis, which is the reason for the cellular link between their enzymatic activity directly to the energy status of the cell compartments.

There are a number of experimental studies in diabetes which link sirtuins to the development of diabetic complications. Resveratrol protects against development of diabetic nephropathy via changes in phosphorylation of histone H3 and Sir-2 (329, 602). Another therapy, fidarestat, which targets aldose reductase, improves cardiac function in diabetic mice through a sirtuin-dependent mechanism (156). In addition, specific sirtuin isoforms are thought to regulate and protect mitochondrial function (459), which is known to be disturbed at sites of diabetic complications.

4. MicroRNA

MicroRNAs are short sequences of RNA that directly bind complementary mRNA, thereby arresting their translation into protein or targeting these mRNAs for degradation. The complementary sequences for miRNA binding within mRNAs are located within the 3′ untranslated region (3′-UTR). Mi-
croRNAs originate predominantly from the random formation of hairpin loops in “noncoding” introns of DNA, but have also evolved by duplication and modification of existing miRNAs (431). These sequences have added a whole new level of complexity to gene transcription and the ultimate production of proteins by cells, further complicated by their often biphasic actions on protein expression. miRNAs are involved in the normal functioning of all eukaryotic cells and so similarly their dysregulation is known to contribute to a number of disease processes (274) including diabetes complications (289). It remains, however, to be determined if selective in vivo targeting of these miRNAs is possible at sites of diabetic complications.

MicroRNAs, as regulators of renal changes in diabetes, are perhaps the most well studied (289). Most of this research has focussed on miRNAs that target molecules involved in renal fibrosis via effects elicited by TGF-β1. miR-192 targets zinc finger E-box-binding homeobox 2 (ZEB2), a protein involved in early growth and development including nephrogenesis. In experimental diabetes, expression of miR-192 is reported to be increased (291), resulting in repression of ZEB2 and consequently deposition of type 1 collagen, most likely via repression of Smad7 signaling (107). This has also been shown in other renal diseases (631, 632). However, the status of miR-192 within the diabetic kidney remains controversial with conflicting results by other groups (630). A growing number of other miRNAs are also implicated in the accumulation of extracellular matrix in organs affected by diabetes, including miR-21 (680), miR-29 (477), miRNA-216a (291, 292), miR-377 (634), and miRNA-93 via regulation of a number of TGF-β-stimulated molecules. In addition, protection against the accumulation of collagen I and fibronectin and decreases in VEGF expression have also been shown in the diabetic kidney and retina by the miRNA 200 family (350, 629).

**IV. SUMMARY/CONCLUSION: CURRENT CHALLENGES IN THE DESIGN OF NEW THERAPIES TO COMBAT DIABETES COMPLICATIONS**

As one can see from the scope of this review, the pathogenesis of the vascular complications of diabetes is incredibly “complicated” as depicted by the number of pathways implicated [Figure 8]! Therefore, it is not surprising that these disorders as a result of diabetes are named complications, given that the dictionary meaning of the word complicated...
is “something that is difficult to analyze or understand.” Indeed, we have a difficult task ahead to address the current and future disease burden of these predominantly vascular complications. Diligent control of glycemia and blood pressure (693) have stabilized the level of morbidity and mortality associated with diabetes in most developed nations. However, good glycemic control alone has been shown to be insufficient to prevent death from a cardiovascular event (203, 454) and may in fact increase the risk of adverse events (695). In particular, the concern is that a vast number of new cases of diabetes are now originating from developing nations (129), and hence, it is likely that less stringent management in these nations due to resource issues may result in a greater incidence of vascular complications worldwide, despite better management in developed nations.

Ultimately, our goal is to prevent or reverse the vascular complications seen in diabetic individuals. In particular, it is critical that we not only understand the mechanisms that lead to disease development and progression, but also how these changes occur in a temporal manner. We also need to consider the development and progression of complications in the context of abnormalities in glucose handling involving many different organs such as sites of peripheral insulin resistance and islet secretory abnormalities. Animal models are useful and often powerful tools to establish temporal patterns of progression and to implicate the involvement of particular molecules in end-organ protection or pathology. Indeed, studying the early development of complications in animal models may provide clues as to the initiators and early promoters of disease, rather than focusing on those changes that are consequences of progression. The results seen within animal models, however, must be interpreted with caution, remembering the limitations of these models with regular reference back to the human condition, which is critical for defining the relevance of these experimental findings.

In the interim, while we search for agents to better manage diabetic complications, earlier screening of patients for renal impairment seems a worthwhile strategy, since this is a major risk factor for cardiovascular disease (220) and all cause mortality (376). Of course, this should occur in concert with appropriate management of hyperglycemia, obesity, hyperlipidemia, and hypertension. This is particularly important given that a number of previous studies have shown reduced efficacy of the various interventions, once the disease has progressed beyond a certain point (169, 184, 461). Indeed, research should focus on understanding which particular events are involved in this transition from reversible or preventable disease to a point of no return, where the disease progresses despite our best efforts.

Finally, we must not forget that within the body, glucose abnormalities in concert with relative insulin deficiency are the key determinants of diabetic complications. Indeed, more research should be targeted toward elucidating the initial functional and structural patterns altered by the inevitable but common changes in glucose uptake and trafficking that occur at sites of diabetes complications. These events provide the scaffolding for the subsequent development of complications in individuals in the context of a particular genetic susceptibility to these disorders. Hence, identification of why certain persons with diabetes progress to complications whereas others remain remarkably resistant to developing these vascular disorders is of paramount importance. This puzzle is likely to be solved using the combined approaches of genetics, epidemiology, physiology, and biochemistry.

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Address for reprint requests and other correspondence: M. Cooper, Baker IDI Heart and Diabetes Institute, 75 Commercial Rd., Melbourne, Victoria, Australia (e-mail: Mark. Cooper@bakeridi.edu.au).

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