Reviews

Protein kinase C activation: isozyme-specific effects on metabolism and cardiovascular complications in diabetes

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Abstract Protein kinase C (PKC) is a family of multi-functional isoenzymes, activated by diacylglycerols (DAGs), which play a central role in signal transduction and intracellular crosstalk by phosphorylating at serine/threonine residues an array of substrates, including cell-surface receptors, enzymes, contractile proteins, transcription factors and other kinases. Individual isozymes vary in their pattern of tissue and subcellular distribution, function and Ca^{2+} /phospholipid cofactor requirements, and in diabetes there is widespread activation of the DAG-PKC pathway in metabolic, cardiovascular and renal tissues. In liver, muscle and adipose tissue, PKC isozymes have been implicated both as mediators and inhibitors of insulin action. Activation of DAG-sensitive PKC isoforms, such as PKC- θ and PKC- ε , down-regulates insulin receptor signalling and could be an important biochemical mechanism linking dysregulated lipid metabolism and insulin resistance in muscle. On the other hand, atypical PKC isozymes, such as PKC- ζ and PKC- λ , have been identified as downstream targets of PI-3-kinase involved in insulin-stimulated glucose uptake, especially in adipocytes.

Glucose-induced de novo synthesis of (palmitate-rich) DAG and sustained isozyme-selective PKC ac-

tivation (especially but not exclusively PKC- β) has been strongly implicated in the pathogenesis of diabetic microangiopathy and macroangiopathy through a host of undesirable effects on endothelial function, VSM contractility and growth, angiogenesis, gene transcription (in part by MAP-kinase activation) and vascular permeability. Interventions that increase DAG metabolism (e.g. vitamin E) and/or inhibit PKC isozymes (e.g. the β -selective inhibitor LY333531) ameliorate the biochemical and functional consequences of DAG-PKC activation in experimental diabetes, for example improving retinal blood flow and albuminuria in parallel with reductions in membrane-associated PKC isozyme activities. Thus, a greater understanding of the functional diversity and pathophysiological regulation of PKC isozymes is likely to have important clinical and therapeutic benefits. [Diabetologia (2001) 44: 659–673]

Keywords Protein kinase C, PKC isoenzymes, insulin resistance, diacylglycerol, vascular complications, endothelial dysfunction, vascular permeability, PKC- β PKC

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Abbreviations: Akt, also called protein kinase B; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ET-1, endothelin-1; ECM, extracellular matrix; GS, glycogen synthase; GSK-3 β , glycogen syn-

thase kinase 3-beta; IRS-1, insulin receptor substrate-1; MAP-kinase, mitogen-activated protein kinase; NO, nitric oxide; cNOS & iNOS, constitutive & inducible nitric oxide synthase; PA, phosphatidic acid; PC, phosphatidylcholine; PI-3-kinase, phosphoinositide-3-kinase; PKC, protein kinase C (c, conventional; n, novel; a, atypical groups); PLA₂, phospholipase A₂; PS, phosphatidylserine; TG, triglyceride; TGF β , transforming growth factor-beta; TNF- α , tumour necrosis factor-alpha; VPF (or VEGF), vascular permeability factor.

Fig. 1. The catalytic and regulatory domain structures of conventional, novel and atypical PKC isozymes with conserved regions (C1–C4) and variable regions (V1–V5), and binding sites for Calcium, phosphatidylserine (PS), DAG and ATP

Protein kinase C (PKC): a family of multifunctional isoenzymes

Adding and removing phosphate groups is an important physiological mechanism for regulating intracellular proteins, including enzymes, receptors and second messengers. Thus, a variety of receptor-mediated responses and metabolic pathways are activated and deactivated by intracellular kinases (enzymes that add phosphate groups) and phosphatases (enzymes that remove phosphate groups) which in turn are themselves regulated by extrinsic biochemical signals such as hormones and growth factors. Cellular kinases are broadly divided into two types: those that phosphorylate proteins at tyrosine residues (tyrosine kinases) and those that phosphorylate serine and threonine sites (serine/threonine kinases). There are three major serine/threonine kinases widely distributed in all tissues: cyclic AMP-dependant protein kinase (also known as protein kinase A, PKA), Akt (also known as protein kinase B, PKB) and the calcium-phospholipid activated kinase, protein kinase C (PKC).

PKC was first identified over 20 years ago as a single proteolytically-activated kinase in rat brain [1] but in fact PKC is a family of structurally and functionally related proteins derived from multiple genes (at least 3) and from alternative splicing of single mRNA transcripts (Fig.1) [2, 3]. Activation and translocation of PKC from the cytosol to the plasma membrane occurs in response to a transient increase

in diacylglycerols (DAG) or exposure to exogenous tumour-promoting agents known as phorbol esters. The primary structure of PKC, a single polypeptide chain, can be divided into 4 conserved domains (C1–C4) separated by 5 variable regions (V1–V5) (Fig.1). The NH₂-terminal half of the polypeptide (i.e. C1, C2, V1, V2 and part of V3) constitutes the regulatory domain, while the CO₂H-terminal half (i.e. C3, C4, V4 and V5) forms the catalytic domain [4]. The functional domains are located in the conserved regions. Thus, the C1-region, which contains two cysteine-rich zinc finger-like regions, is responsible for DAG or phorbol ester binding. The C2-region is responsible for Ca²⁺-binding while the C3-region contains the ATP binding site and the C4-region contains the main catalytic site (Fig.1).

A total of 12 isozymes of PKC have so far been cloned and characterised [5]. Brain and liver contain most PKCs but each isoform shows a different pattern of tissue distribution, substrate specificity and cofactor requirements (Table 1). The Group A (classical) PKC isoforms (cPKC- α , - β_1 , - β_{II} and - γ) are calcium- and phospholipid-dependent and possess the full primary amino acid sequence (C1–C4 and V1–V5). They are activated by phosphatidylserine (PS), Ca²⁺ and DAG (or phorbol ester). Group B (novel) PKCs (nPKC- δ , - ϵ , - η , - θ and - μ) lack the C2-region and are therefore calcium-independent but phospholipid-dependent; they require PS and DAG for activation. The Group C (atypical) PKC isoforms (aPKC- ι , - λ and - ζ) also lack a C2-region, as well as one of the zinc finger-like cysteine-rich motifs in the C1 region, and are calcium and phospholipid independent. Both PKC- λ and PKC- ι are species homologues. Although the atypical PKCs are dependent on the presence of PS for their catalytic activity, these isoforms are not activated by Ca²⁺, DAG or

Table 1. Protein Kinase C isoforms

	Isoform	Tissue Distribution	Co-factor requirements		
			Ca ²⁺	DAG	PS
Conventional cPKCs	α	widespread	✓	✓	✓
	β_1	widespread	✓	✓	✓
	β_{II}	widespread	✓	✓	✓
	γ	brain	✓	✓	✓
Novel nPKCs	δ	widespread	–	✓	✓
	ε	brain, heart	–	✓	✓
	η	heart, skin, lung	–	✓	✓
	θ	muscle, brain, blood cells	–	✓	✓
	μ	lung epithelial cells	–	✓	✓
Atypical aPKCs	ζ	widespread	–	–	✓
	ι/λ *	kidney, brain, pancreas	–	–	✓

phorbol esters. In addition to the allosteric effectors, post-translational phosphorylation of PKC also appears to constitute an important mechanism for regulating PKC translocation and isozyme activity [5, 6].

Activation of one or more isoforms of PKC leads to a variety of biological responses, including changes in cell proliferation and differentiation, transmembrane ion transport, glucose and lipid metabolism, smooth muscle contraction and gene expression [7–9]. Very little is known about the functional specificity of different PKC isoforms and experimental work has been hampered by the lack of isoform-specific substrates for use in radioenzymatic assays. Pathophysiological studies have implicated PKC- β and PKC- δ in hyperglycaemia-induced vascular dysfunction [10]. They have implicated PKC- ζ in insulin signalling [11–13] and abnormalities of cell growth and differentiation, including hyperplasia and hypertrophy [14], and PKC- ε and PKC- θ in muscle insulin resistance [15–17].

DAG-mediated PKC activation in diabetes. Intracellular release of DAG is the primary step leading to activation and translocation of PKC and various species of DAG (varying in fatty acid composition) are generated from 4 principal sources (Fig.2). These sources are, firstly, classical receptor-mediated, phospholipase C-catalysed hydrolysis of inositol phospholipids; secondly, the release of DAG from phospholipase D-mediated hydrolysis of phosphatidylcholine (PC); thirdly, through release of non-esterified fatty acids (NEFAs) from precursor lipids by the action of phospholipase A₂; and, finally, de novo synthesis of DAG from phosphatidic acid (PA). This latter pathway is mainly responsible for hyperglycaemia-induced DAG formation in a range of metabolic and cardiovascular tissues in diabetes [18] but high glucose levels also increase the turnover of PC [19]. Thus, the fatty acid composition of excess DAGs that accumulate in diabetic tissues are consistent with a release of DAG from phospholipase D-mediated

hydrolysis of phosphatidylcholine and de novo synthesis of DAG from phosphatidic acid [20].

In addition, DAG-mediated activation of PKC is augmented by specific NEFAs of varying chain lengths. For example, non-esterified fatty acids and their CoA esters (especially arachidonic, oleic, linoleic and linolenic acids) appear to activate PKC synergistically with DAG [21, 22], and it has been suggested that *cis*-unsaturated fatty acids act as enhancer molecules [23]. Thus, in diabetes increased NEFA concentrations could enhance hyperglycaemia-induced PKC activation, independently of de novo synthesis of DAG [24].

Fig.2. Major reactions of animal glycerolipid metabolism. DAG is a central intermediate in the synthesis and turnover of all major glycerolipids. As such, DAGs are derived from multiple sources, including (1) phospholipase-C (PLC) mediated hydrolysis of phosphoinositides, (2) phospholipase-D (PLD) mediated hydrolysis of phosphatidylcholine (PC) by phosphatidic acid, (3) release of free fatty acids from precursor lipids through phospholipase A₂ (PLA₂) mediated, and (4) de novo synthesis of DAG from a glycolytic intermediate, glyceraldehyde-3-phosphate (G-3-P), under conditions of hyperglycaemia

Protein Kinase C isozymes – inhibitors & mediators of insulin action

Numerous lines of investigation suggest that the pathological accumulation of naturally occurring lipid metabolites could affect PKC activity and thus lead to dysregulated substrate phosphorylation and cellular function [25]. The PKC has been directly implicated in insulin signalling [26] and significantly increased PKC activity has been reported in liver [27] and muscle [28] tissues from diabetic humans and rats (Fig. 3). There is evidence that short-term exposure of various cell types to insulin leads to rapid formation of DAG [29], followed by activation and translocation of PKC [30], but increased membrane-associated PKC has been reported in humans (Fig. 3) and animals with diabetes characterised by hyperinsulinaemia [27, 28] and hypoinsulinaemia [31, 32], presumably reflecting tissue-selective and isoform-selective differences in the insulin regulation of PKC.

The notion that PKC activation might phosphorylate and down-regulate one or more steps involved in glucose transport and metabolism emerged in the mid-1990s [33], supported by preliminary evidence that PKC phosphorylates the insulin receptor [34] and glycogen synthase [35]. In subsequent studies, changes in DAG-PKC signalling in liver, muscle and adipose tissues have been associated with insulin resistance due to ageing [36, 37], obesity [38], high-fructose and high-fat feeding [15, 16, 39] as well as muscle denervation [40]. In addition, PKC has been implicated in the phenomenon of 'glucose toxicity', whereby sustained hyperglycaemia both in Type I (insulin-dependent) and Type II (non-insulin-dependent) diabetes mellitus exacerbates insulin resistance [41]. As well as glucose-induced formation of DAG enhancing phosphorylation and down regulation of the IR, increased glucose flux through the hexosamine biosynthesis pathway during hyperglycaemia has been shown to inhibit glucose transport through the activation of PKC [42]. The PKC has also been directly implicated in lipolysis and adipose tissue metabolism [43, 44] and transfected hepatocytes that over-express PKC become insulin resistant [45].

There has been considerable interest in the interrelationships between dysregulated lipid metabolism, fatty acid composition of membrane phospholipids and insulin resistance in skeletal muscle [46]. In both obesity and Type II diabetes, there are close inverse relation between muscle triglyceride (TG) content and in vivo insulin sensitivity [47, 48]. Higher concentrations of DAG (an intermediate in the synthesis of TG and phospholipids) have been proposed as the key biochemical link, by PKC activation, between dysregulated lipid metabolism and greater muscle insulin resistance [25, 46]. Thus, accumulation of lipids and their metabolites could inadvertently modulate PKC activity. Two novel PKCs, PKC- θ and PKC- ε ,

Fig. 3. Immunodetection of membrane-associated PKC- α , - ϵ and - ζ in liver tissue from lean, obese and obese diabetic patients. Reproduced from reference 27

have been particularly implicated in this hypothesis. Both high-fructose and high-fat feeding are associated with increases in circulating and intramuscular TG content in rodents, and recent studies have shown isoform-selective increases in muscle PKC signalling in these dietary-induced models of insulin resistance [15, 16, 39] (Fig. 4). Moreover, treatment of high-fat fed rats with an antilipolytic drug, BRL49653, which reduced muscle DAG and non-esterified fatty acid mass, improved insulin sensitivity and reversed the diet-induced changes in nPKC expression [49]. Similarly, ex vivo experiments using human tissues showed that insulin is less capable of stimulating insu-

Fig. 4 (A–D). High-fat fed rats develop insulin resistance and increased DAG and triglyceride content of red gastrocnemius muscle relative to starch-fed controls (**A, B**). Correspondingly, the high-fat fed rats (●) have increased PKC- θ (and PKC- ϵ) in the membrane fraction (relative to cytosol) of red gastrocnemius muscle compared with starch-fed controls (○) (**C, D**). Reproduced with permission from reference 16

lin receptor substrate-1 (IRS-1) tyrosine phosphorylation, phosphoinositide-3-kinase (PI-3-kinase) activation and glucose transport in the skeletal muscle from obese subjects than from lean subjects [50]. Furthermore, PKC- θ has been particularly implicated in the inhibitory effects of fatty acids on insulin-induced PI-3-kinase activation [17].

PKC- θ is one of the more recently identified PKC isoforms [51] and has a unique pattern of tissue distribution [52]. PKC- θ is expressed predominantly (and almost exclusively) in skeletal muscle, haematopoietic tissues, testis and platelets, and it is the major isoenzymic form of PKC in skeletal muscle [51]. A study has shown that expression of PKC- θ is threefold higher in tensor fascia latae (TFL, an insulin-resistant white muscle) than in the soleus muscle (an insulin-sensitive red muscle) in the hindlimb of normal rats [15]. In addition, selective activation of muscle PKC- θ and PKC- ϵ has been associated with dietary-induced models of insulin resistance in normal rats [15, 16, 39] and DAG-PKC activation after food intake in the muscles of obese Zucker rats [38], whereas in other experimental conditions associated with muscle insulin resistance (e.g. rodent models of hypertension and ageing) no changes in

nPKC expression or subcellular distribution were observed [37,53]. Thus, specific changes in PKC- θ and PKC- ϵ seem to be associated with feeding and diet-related changes in muscle lipid content and insulin sensitivity.

Phosphorylation events seem to be particularly important in the mechanism of tumour necrosis factor- α (TNF- α) induced insulin resistance, especially in adipocytes [54]. Recent evidence suggests that PKC mediates the inhibitory effect of TNF- α on insulin signalling by its effects on discrete PKC isoforms, e.g. PKC- ϵ [55] and/or PKC- β and PKC- ζ [56, 57]. Similarly, increases in NEFA concentrations lead to a reduction in insulin-stimulated PI-3-kinase activity by the selective activation of PKC- θ [17].

Pharmacological studies of phorbol ester-induced PKC activation and the effects of non-specific PKC inhibitors (e.g. staurosporine-like compounds) have also provided important information. For example, several studies have shown that phorbol esters antagonise the effects of insulin [58] and inactivate the insulin receptor tyrosine kinase [59], whereas PKC inhibitors seem to enhance glucose-induced activation of hepatic glycogen synthase [60] and promote glucose uptake in isolated adipocytes [44]. Similarly, okadaic acid (a protein phosphatase inhibitor) promotes an insulin resistant state in adipocytes through a sustained serine/threonine phosphorylation of IRS-1 [61, 62]. In a PKC- β knockout mouse model, Standaert et al showed modest increases in glucose uptake in specific tissues (i.e. 25% in soleus muscle and 50–100% in adipocytes), consistent with a small reduction in the inhibitory effect of one PKC isozyme on glucose homeostasis [63].

Previous review articles found some difficulty reconciling apparently contradictory research showing roles for PKC both as a mediator and inhibitor of insulin action [26] but it now seems clear that distinct PKC isoforms serve widely different functions and do so in a tissue-dependent manner. Thus, whereas the Ca^{2+} -independent isozymes, PKC- θ and PKC- ε , exert negative regulatory effects on insulin action, especially in skeletal muscle, the atypical PKCs such as PKC- ζ which are not activated by DAG, appear to be stimulated by polyphosphoinositides derived from PI-3-kinase activation by insulin, for example in adipose tissues [12, 13, 64, 65]. Insulin could also affect mRNA expression of individual PKC isozymes [66]. It is therefore likely that some PKC isoforms (e.g. PKC- ζ) function as mediators of insulin action downstream of PI-3-kinase, while other PKCs (e.g. PKC- θ and PKC- ε) have negative regulatory effects on insulin signalling [12, 13, 65].

PKC-mediated phosphorylation and insulin receptor function. The IR tyrosine kinase on the β -subunit is an important target for PKC phosphorylation [55, 67] and recent work has shown that PKC- β_1 and - β_{II} exert strong inhibitory effects on the IR tyrosine kinase – autophosphorylation step [68]. A study has also shown that glucose and phorbol ester-induced activation of PKC- α , - ε and - ζ causes a rapid inhibition of IR tyrosine kinase activity [69], whereas PKC- α , - δ and - θ require IRS-1 in order to inhibit the IR tyrosine kinase in HEK 293 cells [70]. The PKC- θ has an especially strong inhibitory effect on insulin receptor signalling by the phosphorylation of IRS-1 [70] and recent work has shown that serine residues 994 and 1023/25 on the IR are particularly important for PKC- θ and PKC- β_{II} mediated inhibition of IR autophosphorylation [71].

The PKC-mediated phosphorylation is clearly an important mechanism of hyperglycaemia-induced IR inhibition [41, 69, 72] and a PKC inhibitor prevents glucose-related down-regulation of the IR tyrosine kinase [73]. A recent clinical study examining PKC pathways in skeletal muscle concluded that the decreased tyrosine kinase activity of the IR in obese subjects was due to hyperphosphorylation on serine/threonine sites mediated by PKC, especially PKC- β [74]. PKC activation also increases IR degradation [75].

In the immediate down stream signalling events following IR stimulation, PKC phosphorylates and modulates the IRS proteins [62, 72], particularly at the serine 612 position [76], and inhibits insulin-stimulated Akt1 and Akt3 activity [77]. Although some PKC isoforms seem to exert a negative regulatory effect on insulin signalling, others, including PKC- ζ and PKC- λ , are among several serine/threonine kinases (including Akt/PKB) that have been identified as down stream targets of PI-3-kinase [12, 13]. On the

other hand, PKC- θ is selectively activated by a rise in circulating NEFA concentrations and leads to a reduction in insulin-stimulated PI-3-kinase activity [17].

PKC and glucose transport. Although GLUT transporter proteins are not directly regulated by phosphorylation, there is evidence that PKC-related phosphorylation events, perhaps involving intermediate substrates, indirectly influences glucose uptake. For example, pharmacological studies have shown increased glucose transport (secondary to increased GLUT1 and GLUT4 expression) in parallel with decreased PKC activity [78], and GLUT4 trafficking pathways could be regulated by PKC [79]. On the other hand, early studies suggested that PKC activation, whether by DAG or phorbol esters, increases glucose uptake [44, 80], even though more recent work by the same group has shown that only atypical PKC isozymes (e.g. PKC- ζ) are involved in mediating insulin-stimulated glucose transport [12, 65]. Thus, initial, apparently contradictory findings can be explained on the basis that certain PKC isoforms, such as PKC- ζ and PKC- λ , have been identified as down stream targets of PI-3-kinase [12, 13], while DAG-sensitive PKCs (i.e. certain cPKCs and nPKCs) have negative regulatory effects on insulin signalling [65]. Thus, different isoenzymes of PKC appear to function separately as mediators or inhibitors of insulin action.

PKC and glycogen synthase activity. Glycogen synthase (GS) is the rate-limiting enzyme involved in non-oxidative glucose metabolism, which is the major pathway of glucose disposal in resting skeletal muscle. GS exists in two interconvertible forms: an inactive phosphorylated form and an active dephosphorylated form. PKC is one of several kinases that phosphorylate GS directly [35], and individual PKC isoforms could reduce GS activity in muscle [28], but in the liver PKC- α , - β and - γ phosphorylate glycogen synthase kinase-3 β (GSK-3 β , the most potent kinase regulating hepatic GS activity), which in turn leads to a reduction in glycogen synthesis [81].

PKC and insulin secretion

An increase in intracellular Ca^{2+} in the beta cell in response to insulin secretagogues, including glucose, directly triggers exocytosis of the insulin granules. Second messengers, such as cAMP and DAG, increase insulin release through protein phosphorylation events mediated by PKA and PKC, respectively. Apart from increasing intracellular Ca^{2+} levels, these phosphorylation reactions enhance insulin release by heightening the Ca^{2+} sensitivity of the secretory apparatus [82]. Recent work has shown that PKA and

PKC act synergistically on different steps upstream of exocytosis in the insulin secretory cascade [83].

Conventional and atypical isoforms of PKC are expressed in pancreatic β -cells, including the most recently cloned isoenzyme, PKC- ι [84]. The PKC- ζ is particularly important in carbachol-stimulated insulin release [85], and there is increasing evidence that PKC activation regulates the second-phase insulin secretory response [86]. (The first phase insulin response is primarily cAMP and Ca^{2+} -dependent). Extrinsic factors such as endothelin-1 could augment insulin secretion through PKC-dependent pathways [87].

Hyperglycaemia-induced PKC activation: a central pathway in the development and progression of diabetic vascular complications

DAG-PKC signalling in vascular tissues exposed to high glucose. Several biochemical mechanisms have been proposed to explain the structural and functional abnormalities associated with prolonged exposure of cardiovascular tissues to hyperglycaemia (Table 2), but this review will focus on the clinical, pathophysiological and therapeutic significance of glucose-induced DAG-PKC activation.

Glucose is transported into vascular cells by GLUT1 transporters and then metabolised mainly through glycolysis (< 5% of intracellular glucose is metabolised by the aldose reductase pathway, even under conditions of hyperglycaemia). The GLUT1 is up-regulated by high extracellular glucose concentrations and hypoxia [88], and the increased glycolytic flux results in increased de novo synthesis of DAG (Fig. 2). Increases in total DAG, and palmitate-rich DAG, have been reported in various cells and tissues from diabetic animals and humans, including the retina, aorta, heart and glomeruli [10, 89, 90] (Table 3). A study has recently shown that fatty acids play an important role in modulating PKC activity in vascular cells, independently of de novo DAG synthesis, and that increased formation of DAG, by itself, is insufficient to activate PKC [24]. The NEFAs also increase the affinity of cPKCs for Ca^{2+} such that PKC activation occurs even at basal Ca^{2+} concentrations [91].

Thus, hyperglycaemia, even acutely [92], leads to increased DAG-PKC signalling in a variety of cardiovascular and renal tissues (Table 3). Investigators at the Joslin Diabetes Centre in Boston first made the important observation of differential isoform and tissue-dependent PKC responses to hyperglycaemia [10]. They showed that hyperglycaemia-induced DAG is rich in palmitate and speculated that different species of DAG preferentially activate one or more PKC isoenzymes in different tissues, for example, PKC- β_{II} in the heart and aorta, PKC- α , - β_{II} and

Table 2. Major pathways implicated in the pathogenesis of hyperglycaemia-induced vascular injury

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- Non-enzymatic glycation: formation of advanced glycation end-products (AGEs)
 - Polyol pathway : aldose reductase-mediated changes in sorbitol and myoinositol
 - Protein Kinase C activation via hyperglycaemia-induced DAG accumulation
 - Redox potential alterations : changes in free radicals and oxidation state
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- ϵ in the retina, and PKC- α , - β_{I} and - δ in glomerular cells [93]. It is interesting that PKC- β_{II} is the dominant isoform response in macrovascular tissue, including smooth muscle and endothelial cells, and the retina [10, 94], whereas in glomerular cells PKC- β_{I} is predominantly overexpressed in response to high glucose [95]. Although a wide range of tissue and cell types show increased DAG-PKC signal transduction under experimental conditions of diabetes (Table 3), these changes are by no means universal. For example, PKC activity in the brain and peripheral nerve tissue is not changed [96].

Accumulation of DAG in vascular tissues in hyperglycaemic states is ameliorated by D- α -tocopherol (vitamin E), which activates DAG kinase and facilitates the conversion of DAG to PA [97, 98]. Several studies have shown that glucose-induced PKC activation is attenuated by vitamin E [94, 99] and that the functional consequences of PKC activation are correspondingly reversed, for example in the kidney [95] and retina [100].

Activation of the DAG-PKC pathway, by the phosphorylation of many different PKC substrates, has a number of undesirable effects on pathophysiological processes which contribute to the long-term structural and functional abnormalities associated with microvascular and macrovascular complications (Fig. 5).

Endothelial dysfunction & monocyte adhesion. The vascular endothelium plays a crucial role in regulating haemostasis (coagulation and fibrinolysis), blood vessel tone, vascular permeability and local platelet activation. Endothelial dysfunction and endothelial cell activation, involving many of these processes, are early features of diabetes which precede the development of overt microangiopathy and macroangiopathy [101, 102]. Apart from nitric oxide (NO), a huge array of substances is released from endothelial cells in response to biochemical and physical stimulation, for example angiotensin II, endothelin-1 (ET-1), TGF β , vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1, prostaglandins and von Willibrand factor (vWf) protein. The vWf has been used as a surrogate marker of endothelial cell activation which correlates with macrovascular death rates in diabetes [103].

Fig. 5. Increased DAG-PKC activation in vascular cells and tissues exerts a variety of undesirable effects which contribute to the functional and structural abnormalities characteristic of diabetic microangiopathy and macroangiopathy. Some of these effects can be ameliorated by vitamin E, a DAG kinase activator, or isozyme-specific PKC inhibitors such as LY333531

There is increasing evidence that PKC activation is an important biochemical mechanism in hyperglycaemia-related and diabetes-related endothelial dysfunction. For example, impaired (NO-mediated) vasodilation and increased release of ET-1 in the retina involve PKC-mediated inhibition of constitutive nitric oxide synthase (cNOS) [104] and induction of ET-1 gene expression [105, 106]. This is clinically important because retinal blood flow decreases before overt retinopathy develops [107] and PKC inhibition restores retinal microvascular haemodynamics [89]. In contrast, in the kidney vasculature, PKC activation could increase (by iNOS induction) or decrease NO production according to cell type, tissue location and duration of diabetes [108, 109]. Individual isoforms of PKC regulate iNOS expression in both a positive and negative manner [110]. Both PKC activation and increased NO production also contribute to the hyperpermeability response to increased glucose [111].

Activated normal platelets produce local vasodilation by the release of platelet-derived ADP, which in turn stimulates endothelial release of NO, but in diabetic patients this pathway is impaired. Recent work has shown that impaired platelet-mediated vasodilation due to hyperglycaemia is caused by increased DAG levels and PKC-mediated stimulation of phospholipase A₂ (PLA₂) activity [112]. In addition, the reduced Na⁺-K⁺-ATPase activity in diabetes is explained by PKC-dependent increases in cytosolic PLA₂ and prostaglandin E₂ concentrations [113, 114].

Endothelins are particularly important in the pathogenesis of diabetes complications [115], especially in the retina, and PKC activation increases ET-1 gene expression [105, 106]. A study has shown that overexpression of PKC-β_{II} and PKC-δ (but not PKC-ζ) enhanced, in parallel, PKC activities, and

basal and glucose-induced ET-1 mRNA expression by at least twofold [105]. Similarly, phorbol esters increased ET-1 release and PKC inhibition decreased ET-1 release from aortic and pulmonary endothelial cells [116]. The effects of ET-1 on the heart, including calcium homeostasis, myocyte contractility and the growth-promoting effects of ET-1, seem to be mediated by PKC-ε [117].

Monocyte binding to endothelial cells is an important early event in atheroma formation and one that is enhanced in diabetes [118]. A recent clinical study has shown that membrane-associated PKC activity is increased in monocytes from diabetic patients and that total PKC activity and expression of the glucose-sensitive PKC-β_{II} isoform decreased by 40% under euglycaemic conditions [92]. Because increased PKC activity in monocytes enhances their adhesion to the vascular wall, increases fibrinogen binding [119] and promotes differentiation into macrophages [120], augmentation of this signal transduction pathway could contribute to the accelerated progression of atheroma in patients with diabetes. The adhesion molecules most involved in monocyte-endothelial cell interactions are E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). High glucose up-regulates ICAM-1 protein and mRNA expression, but not VCAM-1, by a PKC-NFκB-dependent pathway [121].

Vascular smooth muscle contractility, growth & differentiation. The PKC plays a pivotal role in the signal transduction of vascular smooth muscle (VSM) cell contraction [122], and the increased contractile responsiveness of VSM observed in diabetes has been attributed, in part, to sustained hyperglycaemia-induced PKC activation [123]. Increased PKC activity in VSM cells occurs within minutes of exposure to high extracellular glucose levels (15–20 mmol/l), and the PKC response quickly reverses if glucose levels fall [124]. A study has shown that, in contrast to the transient PKC response to pressor hormone stimulation, glucose-induced PKC activation in VSM is much more sustained [124]. Increases in PKC activity in VSM have been implicated in the regulation of cell-surface hormone receptor density, intracellular signalling responses to pressor hormones, ion channel activity, phosphorylation of myofibrillar proteins, expression of the actin-myosin regulatory protein caldesman and intracellular pH [122, 125]. Overexpression of PKC-β_{II} in a transgenic heart model led to phosphorylation of troponin-I and decreased myofilament responsiveness to Ca²⁺ [126]. Short-term exposure of VSM cells to high glucose also increases vascular permeability factor (VPF) mRNA by a PKC-β-dependent pathway [127]. The VPF is essentially an endothelial cell-selective mitogen involved in angiogenesis and vascular permeability.

Table 3. Diabetes-related activation of the DAG-PKC pathway in vascular cells and tissues

	Species	DAG content	PKC Activity	Isoforms activated	Reference
Cells in Culture					
Aortic Endothelial	Rat, bovine	↑	↑	β	10, 141
Aortic VSM	Rat, human	↑	↑	$-\beta$	98, 124, 150
Retinal Endothelial	Bovine	↑	↑	$-\beta_{II}$, $-\delta$	89, 105, 141
Retinal Pericytes	Bovine	NM	↑	$-\beta_{II}$, $-\delta$	105
Renal mesangial	Rat	↑	↑	$-\alpha$, β_I	95, 145
Renal glomerular	Rat	↑	↑	$-\alpha$, β_I	95
Tissues					
Heart	Rat, human	↑	↑	$-\beta$, $-\epsilon$	10, 129, 125
Retina	Rat, canine	↑	↑	$-\beta_{II}$, $-\delta$	89, 18
Aorta	Rat, canine	↑	↑	$-\beta$	10, 94
Glomeruli	Rat, mouse	↑	↑	$-\delta$, $-\beta_I$, $-\alpha$	90, 158, 153
Monocytes	Human	NM	↑	$-\beta_{II}$	92

↑ = increased; NM = not measured

PKC also plays an important role in VSM and cardiomyocyte growth and differentiation, partly by activating the mitogen-activated protein (MAP) kinases which in turn phosphorylate transcription factors and alter the balance of gene expression [128]. This pathway can affect cell phenotype, promote cell division or increase the production of extracellular matrix material. In the diabetic heart, PKC activation seems to be particularly important in remodelling [129]: PKC- α and $-\beta$ have been implicated in cardiac hypertrophy [130] and PKC- ϵ seems to have a protective effect in myocardial preconditioning [131]. Overexpression of PK- β_{II} in a transgenic mouse model led to increased left ventricular (LV) hypertrophy and fibrosis, with reduced LV function, accompanied by up regulation of mRNA expression for fetal myosin heavy chain, *c-fos*, TGF β and collagen types IV and VI [130]. Activation of PKC- ζ has been shown to be an essential step in mitogenic signal transduction [132], for example in mediating the growth-promoting effects of angiotensin II on VSM [133] and pathological conditions such as hypoxia selectively activate PKC isoforms in cardiomyocytes [134].

A variety of polypeptide growth factors have been shown to be important in the physiological regulation of VSM contraction, migration and growth, including competence growth factors such as platelet-derived growth factor (PDGF) and progression factors such as insulinlike-growth factor-1 (IGF-1). Changes in intracellular Ca²⁺ and pH are important modulators of growth factor-induced mitogenesis and contraction but the underlying pathways are linked to PKC activation [135]. The VSM cell apoptosis induced by reactive oxygen species is also PKC-dependent [136].

Vascular permeability and angiogenesis. The loss of endothelial barrier function is an early pathophysiological feature of diabetic angiopathy. Various stimuli, including high glucose, activate second messenger systems in endothelial cells, particularly PKC, which initiate intracellular contractile events, shape change

(‘rounding up’ of endothelial cells) and leakage of macromolecules. The PKC-mediated phosphorylation and relaxation of cytoskeletal and adhesion proteins, such as caldesmon and vimentin, as well as up regulation of NOS, account for the increase in vascular permeability in response to high glucose [111, 137] and PKC- β_I overexpression augments phorbol ester-induced hyperpermeability [138]. The PKC- α has been implicated in the permeability response to high glucose, an effect that was abolished using a specific antisense oligonucleotide [139].

The cytokine VPF (also known as vascular endothelial growth factor, VEGF, or vasculotropin) is a homodimeric glycoprotein secreted by VSM cells that exerts potent effects on vascular permeability and angiogenesis. The VPF gene expression is up-regulated in VSM cells exposed to high glucose through a PKC-dependent pathway [127] and increased VPF levels have been detected in ocular fluid of patients with diabetic retinopathy [140]. The VPF is the principal mediator of hypoxia-stimulated angiogenesis and neovascularisation in the eye but also plays a key role in fetal development, wound healing, collateral vessel formation and tumourgenesis. The VPF binds to one of two cell-surface receptors: KDR/flk-1, expressed only on endothelial cells, especially in the eye, and flt-1, expressed on endothelial and non-endothelial cell types. The VPF binding to either receptor triggers phosphoinositol hydrolysis, with the release of DAG and activation of PKC- α , $-\beta$ and $-\delta$ [141], and VPF-induced cell proliferation is blocked by non-specific and β -selective PKC inhibitors but not by an antisense oligonucleotide to PKC- α [141]. Intra-vitreous administration of VPF increases membrane translocation of PKC- α , β_{II} and $-\delta$, and the VPF-induced permeability and cell growth responses are abolished by selective PKC- β inhibition [142].

There is a synergistic interaction between the angiogenic effects of VPF and basic fibroblast growth factor (bFGF) which is at least partly due to up regulation of KDR/flk-1 expression by bFGF through a

PKC and MAP kinase-dependent pathway not involving PKC- β [143]. Thus, PKC signalling appears to be central to several pathways involved in the regulation of angiogenesis and new vessel formation [144].

Glomerulopathy and extracellular matrix deposition. Expansion of the glomerular mesangium is an early feature of diabetic nephropathy, and hyperglycaemia promotes increased synthesis and reduced degradation of extracellular matrix (ECM) components. Glucose-induced formation of fibronectin and type IV collagen by mesangial cells is PKC-dependent [145] and PKC- β_1 seems to be particularly up-regulated in glomerular cells exposed to high glucose [10]. Isoform-specific translocation of PKC has been confirmed in glomerular cells from diabetic rats, and there is now good evidence of PKC-mediated serine-23 phosphorylation (accompanied by reduced activity) of the glomerular Na⁺-K⁺-ATPase [146]. This in turn affects cellular adhesion, vascular permeability and Na-H transport. In addition, recent work has shown that high glucose stimulates mesangial cell proliferation through the PKC-NF κ B pathway [121].

Growth factors also play a key role in basement membrane thickening and increased ECM deposition, in particular TGF β and connective tissue growth factor (CTGF) which increase collagen, fibronectin and laminin synthesis. Hyperglycaemia-induced expression of CTGF in cultured mesangial cells was blocked by a PKC inhibitor [147, 148], and up regulation of TGF β by glucose involves PKC-dependent stimulation of AP-1 binding sites on the transcription factors *c-fos* and *c-jun*.

Interestingly, two therapeutic interventions that reduce proteinuria and slow the progression of diabetic nephropathy, aminoguanidine and the angiotensin converting enzyme inhibitor ramipril, both prevent diabetes-related increases in glomerular PKC activity [149], suggesting that this signal transduction pathway plays a central role in disease progression.

Amelioration of hyperglycaemia-induced DAG-PKC activation in vascular tissues

Vitamin E. Several in-vitro studies have shown that α -tocopherol (vitamin E) prevents the rise in DAG and subsequent PKC activation in vascular cells exposed to high glucose [94]. Following i.p administration in diabetic rats, vitamin E ameliorated the increase in membrane PKC activity in the aorta [150], improved retinal blood flow [100] and reduced proteinuria [95]. The mechanism of this effect involves the stimulation of DAG kinase and the increased conversion of DAG to phosphatidic acid [97–99]; vitamin E has no effect on already-activated PKC

[100]. Impaired retinal microvascular blood flow correlates with DAG-PKC activation [89], and the therapeutic potential of high-dose vitamin E has recently been confirmed in a preliminary clinical study showing modest improvements in retinal perfusion [151].

Pharmacological inhibitors of PKC. Given that PKC is of such fundamental importance in cell signalling, it seemed highly unlikely only a few years ago that PKC inhibition would be a viable target for clinical drug development. First and second generation PKC inhibitors, such as the staurosporine-like compounds and isoquinolinesulphonamides (e.g. GF109203X), block the catalytic domain of PKC which carries a high degree of sequence homology with other kinases and are therefore non-specific for PKC. More recently, however, the recognition that PKC is a family of isoenzymes with significant biochemical and functional differences, coupled with mounting experimental evidence linking specific isozymes (e.g. PKC- β) with key pathological processes, has led to renewed interest in isoform-selective blockade of PKC activation using antisense oligonucleotides [133] or macrocyclic bis-indolylmaleimide compounds [152]. It has been shown that PKC- β_{II} and PKC- β_1 are preferentially activated in cardiovascular and renal tissues, respectively, in diabetes, and the chemical characterisation and in vivo profile of a highly-selective and orally active PKC- β inhibitor, LY333531 have been reported [153]. This compound showed considerable selectivity and reversibility for PKC- β_1 and - β_{II} with half-maximal inhibitory constants (IC₅₀ values) of 4.7 and 5.9 mmol/l, respectively, compared with IC₅₀ values of 52 mmol/l for PKC- η and 300 mmol/l or more for all other PKC isoforms [153]. In diabetic rats, treatment with LY333531 had no effect on glucose-induced accumulation of DAG in the retina and glomeruli, but tissue PKC activities were significantly reduced in parallel with functional improvements in glomerular filtration rate, albuminuria and retinal blood flow. Low-dose therapy (0.1 mg/kg) reduced retinal PKC activity in diabetic rats but higher doses (1 and 10 mg/kg) were required to achieve the same effect in the kidney. The drug had no effect on retinal and renal total PKC activity in non-diabetic animals, confirming that in normal conditions there is no preferential activation of PKC- β [153].

The therapeutic profile of LY333531 has been evaluated in a range of experimental models related to diabetic vascular complications. In the eye, reductions in PKC activity were accompanied by improved blood flow [154] and the normalisation of diabetes-related reductions in Na⁺-K⁺-ATPase and Ca²⁺-ATPase activities [155]. The role of PKC in mediating the ocular effects of VPF has also been confirmed: LY333531 reduces VPF-induced retinal permeability [142] and neovascularisation [156]. Preliminary clini-

cal studies have shown favourable effects on retinal blood flow [157], and larger multicentre clinical trials to evaluate the effects of LY333531 on progression of diabetic retinopathy, especially maculopathy, are currently in progress.

In human mesangial cells cultured in high glucose medium, LY333531 attenuates PKC activation and normalises arachidonic acid release, PGE₂ production and Na⁺-K⁺-ATPase activity [95]. Oral treatment in diabetic rats reduced the excess production of ECM proteins [95], and in *db/db* mice LY333531 10 mg/kg for 4 months reduced albumin excretion rate and mesangial expansion [158]. There is also interest in the effects of this compound on nerve conduction [159] and erectile dysfunction.

Conclusions

This family of multifunctional PKC isoenzymes plays a fundamental role in signal transduction and intracellular 'crosstalk' in many different tissues by phosphorylation and modification (up or down regulation) of enzymes, receptors, transcription factors and other kinases. In liver, skeletal muscle and adipose tissues, there is increasing evidence that PKC activation represents an important biochemical link between dysregulated lipid metabolism and insulin resistance. Different PKC isozymes could function as mediators (e.g. PKC- ζ) or inhibitors (e.g. PKC- θ and - ε) of insulin action. In muscle, most of the available evidence suggests that the accumulation of DAGs leads to PKC-mediated down regulation of the IR and downstream signalling events, including the IRS proteins. Glucose-induced de novo synthesis of DAG and PKC activation is likely to be an important mechanism of 'glucose toxicity', both in peripheral and pancreatic tissues.

Activation of the DAG-PKC pathway in cardiovascular and renal tissues is now recognised as a key biochemical event in the pathogenesis of diabetic complications, and hyperglycaemia-induced formation of palmitate-rich DAG seems to preferentially activate PKC- β in retinal, endothelial, VSM and glomerular cells. PKC activation adversely affects gene transcription (e.g. ECM protein synthesis), ion transport, vascular tone and endothelial permeability. The search for isozyme-selective PKC inhibitors is clearly feasible, as illustrated by the PKC- β inhibitor LY333531, and offers exciting opportunities to block pathways involved in the initiation and progression of microangiopathy and macroangiopathy.

Literature Search: A search of the literature was undertaken using PubMed (Medline) using the following keywords PKC, insulin, complications, PKC isozymes, diabetes, heart, diacylglycerol, PKC inhibitor, insulin resistance, vascular.

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