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Possible Role for Protein Kinase C in the Pathogenesis of Inborn Errors of Metabolism

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Abstract Protein kinase C (PKC) is a ubiquitous enzyme family implicated in the regulation of a large number of short- and long-term intracellular processes. It is hypothesized that modulation of PKC activity may represent, at least in part, a functional link between mutations (genotype) that lead to the pathological accumulation of naturally occurring compounds that affect PKC activity and perturbation of PKC-mediated substrate phosphorylation and cellular function in the corresponding diseases (phenotype). This model provides a unifying putative mechanism by which the phenotypic expression of some inborn errors of metabolism may be explained.

Recent studies in a cell-free system of human skin fibroblasts support the hypothesis that alteration of PKC activity may represent the functional link between accumulation of sphingolipids and fatty acyl-CoA esters, and perturbation of cell function in sphingolipidoses and fatty acid oxidation defects, respectively. Further studies will elucidate the effects of these alterations on PKC-mediated short- and long-term cellular functions in these diseases, as well as the possible role of PKC in the pathogensis of other diseases.

Key words: Protein kinase C (PKC), inborn errors of metabolism, sphingolipidoses, fatty acid oxidation, signal transduction, protein phosphorylation

Protein kinase C (PKC) is a ubiquitous enzyme family of at least 11 isozymes implicated in the regulation of a large number of short- and long-term intracellular processes, including transport processes, metabolic regulation, gene expression, and cell growth and differentiation [Nishizuka, 1986, 1988, 1989, 1992]. PKC subspecies exhibit subtly different enzymological properties, differential tissue expression and specific intracellular localization [Nishizuka, 1988, 1992].

PKC was originally shown to be tightly linked with the phosphatidylinositol and calcium signal transduction pathway [Nishizuka, 1986, 1992]. Diacylglycerol (DAG), the second messenger of this pathway, activates PKC by increasing its affinity to calcium, the other second messenger of this signal transduction pathway [Nishizuka, 1986]. More recently, evidence for a pivotal role for PKC in several signal transduction pathways has been presented [Nishizuka, 1989, 1992; Liscovitch, 1992]. More specifically, PKC seems to participate in a positive feedback mechanism of receptor-mediated activation of the phospholipases D and A_2 and in a negative feedback mechanism of phosphatidylinositol phospholipase C [Liscovitch, 1992]. These processes are probably propagated through both a series of short- and long-term PKC activation by DAG and by PKC-mediated activation or inactivation of other phospholipases, kinases, and phosphatases involved in the signal transduction complex [Liscovitch, 1992].

It is widely accepted that the final common step of transmembrane signaling is phosphorylation–dephosphorylation of proteins such as enzymes, transporters, and channels. PKC has been shown to phosphorylate serine and threonine residues, having a broad substrate specificity [Nishizuka, 1986]. PKC may also phosphorylate some nuclear proteins, thereby stimulating gene expression.

A role for PKC in mediating disease processes has been steadily emerging in recent years. A growing number of studies indicate that PKC may be linked to abnormal cell growth and differentiation and to cancerous processes. However, a role for this enzyme in the phenotypic expression of inborn errors of metabolism has not been delineated.

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HYPOTHESIS

The following cascade of cellular events is surmised: A particular inborn error of metabolism leads to the accumulation of a naturally occurring metabolite that inadvertently modulates PKC activity. Modulation of PKC activity leads to perturbation of phosphorylation of endogenous substrates and possibly of signal transduction and intracellular calcium homeostasis. These alterations may lead to the derangement of various short- and long-term cellular functions mediated by PKC as well as other calcium-dependent kinases, phosphatases, and proteases (Fig. 1). Thus, modulation of PKC activity may represent, at least in part, the missing functional link between a mutation (genotype) and perturbation of cellular function in the corresponding disease (phenotype).

This paper presents two opposing examples of this cascade. Both processes, although opposing one another, lead to alterations in PKC activity and thus may lead to the perturbation of the normal cascade of PKC-mediated intracellular events.

POSSIBLE ROLE IN SPHINGOLIPIDOSES

Sphingolipidoses are a group of lysosomal storage diseases in which sphingosine and its derivatives, specific for each disease, accumulate in tissues in which the metabolic turnover of these metabolites is maximal (e.g., spleen, lymph nodes, and brain). Sphingolipidoses arise as a consequence of deficiencies of particular lysosomal enzymes, involved in the degradation of specific sphingolipids. These complex compounds are normal cellular lipid constituents and are found in the plasma membrane of all eukaryotic cells. They are most abundant in brain and nerve tissue. Sphingolipids have been implicated in the regulation of diverse cellular functions such as maintenance of cell membrane and lipoprotein structure, cell–cell communication, modulation of cell-surface receptors, cellular growth, differentiation, and neoplastic transformation [Hannun and Bell, 1989; Hakomori, 1990; Merrill, 1991]. Of all sphingolipids, gangliosides are of particular interest because of their abundance in neural tissue and their role in cell proliferation and differentiation. Their precise mechanism of action is not known, but it is generally accepted that they may be involved in cellular signal transduction pathways [Hakomori, 1990].

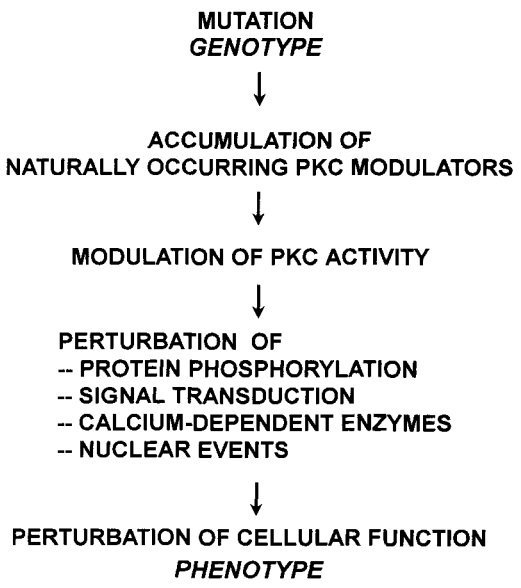

Fig. 1. Possible cascade of PKC involvement in the pathogenesis of inborn errors of metabolism.

The most prevalent backbone of the sphingolipids is sphingosine, a naturally occurring long-chain amino base. Sphingosine may be synthesized de novo by serine palmitoyltransferase, but its main source is the breakdown of cellular sphingolipids by ceramidases. The underlying mechanism linking the intracellular accumulation of sphingolipids in sphingolipidoses, the histopathology, and the perturbation of cell function, has not been fully elucidated.

PKC is an attractive candidate for playing a pivotal role in the pathogenesis of sphingolipidoses [Hannun and Bell, 1987]. It is well established that sphingosine [Hannun et al., 1986] and its derivatives, lysosphingolipids [Hannun and Bell, 1987] and gangliosides [Kreutter et al., 1987], are potent and reversible inhibitors of PKC in vitro in partially purified PKC preparations [Hannun et al., 1986], platelets [Hannun and Bell, 1987; Hannun et al., 1986], neutrophils, and HL-60 cells [Hakomori, 1990; Merrill, 1991]. These metabolites exert their effect on PKC activity either by competitive inhibition with DAG [Hannun et al., 1986; Hannun and Bell, 1987] or by direct interaction with the regulatory domain of the kinase [Kreutter et al., 1987]. Physiologically, sphingosine and lysosphingolipids are believed to function in one of two ways [Merrill, 1991]. Low levels of these compounds may tonically inhibit PKC activity and prevent its activation by basal levels of DAG; thus, PKC will be activated only by a

physiological generation of DAG, following activation of the cell. Alternatively, sphingosine and lysosphingolipids may function as second messengers [Liscovitch, 1992]. According to this model, lysosphingolipids are generated from the corresponding sphingolipids in response to certain cell agonists, analogous to the generation of DAG from membrane phospholipids. These lysosphingolipids may alter cell function by inhibition of PKC or other targets that are involved in signal transduction such as phospholipase C [Merrill, 1991]. Signal transduction mechanisms involving gangliosides may lead to alterations in PKC activity, ganglioside-stimulated protein kinase, and ganglioside-inhibited protein kinase [Chan, 1988].

There is evidence to suggest that sphingosine may function as a second messenger through its metabolite sphingosine-1-phosphate, which has been shown to stimulate the release of calcium from intracellular stores and to increase phosphatidic acid levels by activating phospholipase D [Desai et al., 1992]. In addition to its inhibition of DAG, sphingosine has been shown to block the effects of the calcium–calmodulin complex and its dependent enzymes, thus inhibiting both branches of the phosphatidylinositol signaling pathway [Jefferson and Schulman, 1988]. Taken together, these findings provide a putative unifying mechanism by which accumulation of sphingolipids may alter a large variety of cellular functions in sphingolipidoses, both by a direct effect on PKC and by interfering with the sphingomyelin-mediated signal transduction pathway.

In a preliminary study in this laboratory, PKC activity was shown to be inhibited in a cell-free system of skin fibroblasts derived from patients with sphingolipidoses [Boneh, 1990]. Inhibition of PKC activity was specific in that it was demonstrated only in the particulate fraction (composed of membranous organelles), but not in the cytosolic fraction. Moreover, PKC activity was inhibited in the particulate fraction of cells in which sphingolipids accumulate (e.g., Niemann-Pick type A, mucolipidosis type 4), whereas it was normal in those cells in which sphingolipids do not accumulate (e.g., metachromatic leukodystrophy, Niemann-Pick type C) [Boneh, 1990].

In the following study, we showed that inhibition of PKC activity in the particulate fraction of fibroblasts from mucolipidosis type 4 patients was remarkably similar and differed from that in Niemann-Pick type A, both in its nonreversibility by elevated concentrations of the activating lipids and in its reversibility following partial purification of the enzyme [Boneh and Bach, 1993]. These data may suggest that inhibition of PKC activity in the particulate fraction of sphingolipidoses is dependent on the type of metabolite(s) accumulating, in agreement with the findings of differential Ki for sphingosine derivatives and PKC [Hannun and Bell, 1987].

We have also provided evidence for a differential pattern of PKC-mediated phosphorylation of endogenous substrates in a cell-free system of mucolipidosis type 4 and control fibroblasts [Boneh and Bach, 1993].

Members of the PKC isoenzyme family are found in high concentrations in various areas of the mammalian brain [Nishizuka, 1986, 1988, 1989, 1992]. However, it is not at all clear whether all subspecies of PKC are inhibited in sphingolipidoses and what the relative inhibitory effect of each accumulating metabolite might be. It is possible that particular PKC subspecies are inhibited by particular sphingolipids but not by others. In view of the tissue specificity of PKC subspecies (including that of specific brain cells), an answer to this question may be relevant in linking the clinical expression of the mutation with its biological expression. Alternatively, it is possible that the neurological dysfunction, observed in some of the sphingolipidoses, may reflect the overall effect of an apparently perturbed PKC activity on myelination and neural induction. This implies a possible generalized effect of PKC inhibition in the developing brain, regardless of the subspecies of the kinase.

Taken together, several unanswered questions await further studies using intact cells in order to further characterize the role of PKC in the pathogenesis of this group of diseases.

POSSIBLE ROLE IN FATTY ACID OXIDATION DEFECTS

Mitochondrial fatty acid oxidation plays a major role in energy production during prolonged fasting, when carbohydrate stores are depleted and during aerobic exercise. At least 15 distinct defects in mitochondrial fatty acid oxidation have been described, comprising carnitine deficiency as well as defects in many of the enzymes in the β-oxidation pathway. In addition to the mitochondrial diseases. Fatty acid β-oxidation defects are found in several peroxisomal disorders such as Zellweger syndrome and pseudo-Zell-

weger, infantile Refsum's disease, and neonatal adrenoleukodystrophy (autosomal recessive). In X-linked adrenoleukodystrophy, where there is no deficiency in peroxisomal β-oxidation enzymes, there probably is an impaired ability of peroxisomes to activate very-long-chain fatty acids.

The variable clinical findings in fatty acid oxidation disorders are believed to be due to intracellular accumulation of fatty acids, acyl-CoA, and acyl-carnitine esters. Corkey and Deeney [1990] showed abnormal intracellular profiles of fatty acyl-CoA esters in liver and muscle cells, suggesting their accumulation in mitochondrial fatty acid oxidation defects. These investigators also found a direct relationship between the chain length of the fatty acid and the extent to which intracellular calcium concentration was reduced in cultured glioma cells, and concluded that the accumulating acyl-CoA esters may perturb signal transduction and cellular metabolic pathways. Likewise, Wilson and Sargent [1993] provided evidence for the accumulation of very-long-chain fatty acids in the brain white matter of adrenoleukodystrophy patients. These workers also found alterations in the biochemical structure of sphingomyelin in these patients.

Support for the hypothesis that PKC may play a role in linking between these findings and the perturbations in cellular processes in fatty acid oxidation defects is derived from studies in which activation of PKC by fatty acids of various chain lengths, and their CoA esters, has been shown using a purified enzyme preparation [Murakami et al., 1986; Shinomura et al., 1991; Khan et al., 1992], crude cellular extracts [McPhail et al., 1984; Majumdar et al., 1991], intact platelets [El Touny et al., 1990; Yoshida et al., 1992], T-cell lymphocytes [Szamel et al., 1989], leukemic megakarioblasts [Kitagawa et al., 1991], and isolated hepatocytes [Diaz-Guerra et al., 1991].

The mechanism(s) by which fatty acids activate PKC are not fully understood. One possible explanation for this activation is that fatty acids and DAG activate PKC by different mechanisms [Murakami et al., 1986]. El Touny et al. [1990] showed that oleic acid preferentially activated PKC in the soluble fraction of platelets, having no effect on the membrane-bound enzyme. Another interesting observation in that study was that sphingosine was more potent in inhibiting PKC activation by phosphatidylserine/DAG than by oleate. Moreover, oleate did not alter phorbol dibutyrate binding to platelet membranes, nor did it enhance the autophosphorylation of PKC. It was suggested that soluble PKC would be a target for *cis*-unsaturated fatty acids, while membrane-bound PKC would be targeted by phosphatidylserine/DAG [El Touny et al., 1990]. Another possible explanation is that the accumulating fatty acid acyl-CoA esters are diverted into synthesis of diacyl (and triacyl) glycerols by phosphatidate phosphohydrolase, thereby activating PKC [Brindley, 1984]. It should be noted that although the acyl groups of most physiological DAGS consist of stearate and arachidonate, modifications in the fatty acyl substituents of these DAGs have been shown to contribute to alterations in the activity of PKC [Lapetina et al., 1985; Strawn et al., 1989].

By contrast to some of the studies cited above, several investigators have shown that unesterified fatty acids (arachidonic, oleic, linoleic, and linolenic) activated PKC synergistically with DAG [McPhail et al., 1984; El Touny et al., 1990; Yoshida et al., 1992]. Thus, it was suggested that *cis*-unsaturated fatty acids act as enhancer molecules, rather than second messengers, since DAG was absolutely required for the fatty acid action [Yoshida et al., 1992].

We have shown that the long-chain fatty acids esters palmitoyl-CoA and oleoyl-CoA (saturated and monounsaturated, respectively) had a more pronounced regulatory effect on PKC activity than the saturated medium-chain octanoyl-CoA in the particulate fraction of human skin fibroblasts [Nesher and Boneh, 1994]. These findings are in agreement with previous reports. Furthermore, these results may accord with the demonstration of a direct correlation between the chain length of the acyl-CoA accumulating and the decrease in intracellular calcium concentration [Corkey and Deeney, 1990].

In the absence of DAG, the natural activator of PKC, palmitoyl- and oleoyl-CoA, and, to a lesser extent, octanoyl-CoA, enhanced PKC activity in both cytosolic and particulate fractions. We concluded that abnormal intracellular accumulation of fatty-acyl CoA esters may cause an inadvertent activation of PKC activity in cells that are not stimulated by an external stimulus and, hence, in which DAG concentration is not high.

The phosphorylation pattern of endogenous cytosolic and particulate proteins was altered by palmitoyl-CoA and oleoyl-CoA. However, our data indicated that the effects of palmitoyl-CoA

and oleoyl-CoA on the phosphorylation of endogenous substrates cannot be assigned solely to PKC activation, but rather to activation (or inactivation) of several kinases and phosphatases. It is not clear whether activation of other kinases and phosphatases precedes, coincides with, or is the result of PKC activation by acyl-CoA esters. It is hoped that studies in intact cells will lead to better understanding of this question.

FUTURE PERSPECTIVES

The results of these studies raise further questions: (1) What is the relevance of the findings in a cell-free system to intact cells? (2) Are specific PKC subspecies inhibited or activated in each disease, respectively (i.e., inhibition by particular sphingolipids; activation by particular chain-length and saturability of fatty acids)? (3) What is the target protein(s) affected by the perturbation of PKC activity? (4) How do these alterations modulate agonist-induced signal transduction in the cells? (5) What is the effect of the alteration of PKC activity on nuclear events and gene expression? (6) Is it possible to revert the perturbation of PKC activity and endogenous substrate phosphorylation by correcting the enzymatic defect? Answers to these questions may be of relevance in linking the clinical expression of the mutation to its biochemical expression.

In addition to these "vertical" studies, the proposed hypothesis has to be tested by "horizontal" studies. These should include diseases in which other natural compounds that directly modulate PKC activity accumulate. One possible candidate is bilirubin [Amit and Boneh, 1993]. Further studies will elucidate the indirect role of PKC in the pathogenesis of inborn errors of metabolism, i.e., in conjunction with the wider scope of signal transduction mechanisms [Sassone-Corsi and Borrelli, 1992]. For example, it has been shown that PKC activity is increased in the membranous fraction of renal tubuli of the *Hyp* mouse, a murine counterpart of the human X-linked hypophosphatemic rickets [Boneh and Tenenhouse, 1990]. It would be of interest to examine whether this alteration in PKC activity is the consequence of the effect of decreased 1α,25-dihydroxyvitamin-D_3 on the sphingomyelin cycle [Hannun, 1994]. Another example is ceroid lipofuscinosis, in which abnormalities in the phospholipid constituents of the cellular membrane, as well as modulation of signal transduction and PKC activity have been shown [Bennett et al., 1993].

In summary, the role of PKC in the pathogenesis of several inborn errors of metabolism is being characterized and provides new areas of research. This putative pathogenetic mechanism offers a unifying explanation for diverse biological consequences of presumably single mutations.

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