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COMPREHENSIVE INVITED REVIEW

Redox Control of Protein Kinase C: Cell- and Disease-Specific Aspects

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Abstract

Hormones, growth factors, electrical stimulation, and cell–cell interactions regulate numerous cellular processes by altering the levels of second messengers, thus influencing biochemical reactions inside the cells. The Protein Kinase C family (PKCs) is a group of serine/threonine kinases that are dependent on calcium (Ca^{2+}), diacylglycerol, and phospholipids. Signaling pathways that induce variations on the levels of PKC activators have been implicated in the regulation of diverse cellular functions and, in turn, PKCs are key regulators of a plethora of cellular processes, including proliferation, differentiation, and tumorigenesis. Importantly, PKCs contain regions, both in the N-terminal regulatory domain and in the C-terminal catalytic domain, that are susceptible to redox modifications. In several pathophysiological conditions when the balance between oxidants, antioxidants, and alkylants is compromised, cells undergo redox stress. PKCs are cell-signaling proteins that are particularly sensitive to redox stress because modification of their redox-sensitive regions interferes with their activity and, thus, with their biological effects. In this review, we summarize the involvement of PKCs in health and disease and the importance of redox signaling in the regulation of this family of kinases. *Antioxid. Redox Signal.* 13, 1051–1085.

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I. Introduction

Protein phosphorylation is widely recognized as an important event in transmembrane signal transduction in eukaryotic cells. It is therefore not surprising that a large number of protein phosphorylating enzymes exist. In general, they are divided into serine/threonine kinases and tyrosine kinases on the basis of the phosphate-accepting amino acid in the substrate protein. The term PKC defines a large family of serine/threonine kinases that are highly conserved in evolutionary terms. When activated, PKCs can be translocated from one intracellular compartment to another, thus being able to affect a wide variety of cellular processes.

The intracellular redox state is routinely subjected to modifications during cell life. Changes in the redox state are due to the action of reactive oxygen species (ROS) and alkylating agents that are produced during normal cell life (70, 148). In several pathophysiological conditions, cells undergo redox stress, showing an altered balance between oxidants, antioxidants, and alkylants. In general, proteins are important targets since changes in their redox state can have consequences on their activity. PKCs are particularly important as they are part of the cell signaling machinery and contain regions that are susceptible to redox modifications, both in the N-terminal regulatory domain and in the C-terminal catalytic domain (90).

A. The PKC family: Members, structure, and activators

PKC was originally discovered in 1977 as a histone protein kinase activated by Ca^{2+} and diacylglycerol (DAG), phospholipids, and/or phorbol esters (254). It is now known to consist of a family of isoenzymes that differ in their structure, cofactor requirement, and substrate specificity. The various PKC isoforms have been subdivided into three classes (Fig. 1): a) the classical, or conventional, PKCs (cPKC: α, βI, βII, and γ) are activated by Ca^{2+} and DAG; b) the novel PKCs (nPKC: δ, ε, η, and θ) are activated by DAG, but are Ca^{2+}-independent;

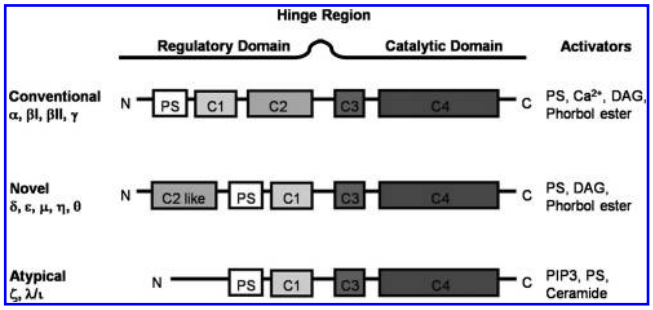

FIG. 1. Classification and structure of the different PKC isoforms. All PKC family members are constituted by four conserved domains (C1–C4) separated by a hinge region. The pseudosubstrate site (PS) keeps the protein in its inactive form. Activators are indicated on the *right side of the picture*.

and c) the atypical PKCs (aPKC: λ, ι, and ζ) are insensitive to both Ca^{2+} and DAG (164, 184, 261). Finally, an additional class is represented by PKCμ, also called PKD. This kinase, like aPKCs, is insensitive to Ca^{2+} and DAG, but apparently it still requires a pseudosubstrate site (PS) as a cofactor (261). Since PKCμ does not fit into any of the major PKC subfamilies, it will not be discussed further in this review.

The existence of PKC heterogeneity poses the question as to why so many isoforms exist and what the functional relevance of each of them could be. The high degree of conservation of the individual PKC isoforms across various mammalian species indicates that maintaining the heterogeneity is somehow "worthwhile," and suggests that each could have a specific relevance for the organism. Marked differences in tissue distribution exist among the PKC isoforms. Some, such as PKCα, δ, and ζ, are expressed widely, whereas others, such as PKCγ, η, and θ, are restricted to only one tissue type, or a few (109). Moreover, most cell types show only a specific subset of PKC isoforms. Differences in subcellular localization of the various PKC isoforms have also been reported. Compartmentalization of PKC isoforms appears to be dynamic and can change in a manner that is dependent on the activation state of the cell.

Intracellular PKC receptors (RACKs; receptors for activated C kinases) have been described that presumably contribute to the subcellular localization of PKCs (169). This fundamental interaction of PKCs with RACKs is mediated through the regulatory domain of PKC (241). RACKs can thus be responsible not only for the unique subcellular localization of different activated PKC isozymes, but also for the different intracellular distribution of the same isoform in different cell types. Indeed, if the RACK expression changes in different cell types, the PKC localization will also be affected. Localization of activated PKC isozymes to different subcellular sites confers specific substrate phosphorylation and different cellular responses. By binding to its respective RACK, each PKC isozyme is positioned next to a subset of protein substrates and away from others, and is thus bestowed with different functional specificies (241). Since the multiple PKC isoforms are discrete functional entities with distinct cellular and subcellular localizations, it is likely that they possess unique patterns of sensitivity to input signals. Thus, the heterogeneity of the PKC family appears to be a means of allowing the cell to diversify and finely tune messages coming in from the outside.

The various PKCs clearly mediate unique functions; even the 50 amino acid difference in the alternatively spliced forms of PKCβ (βI and βII) appears to be responsible for the unique role of each PKCβ isoform (31). Generally, the PKC polypeptide consists of a C-terminal catalytic domain and an N-terminal regulatory domain that are separated by a flexible hinge region (247). cPKCs contain four homologous domains (C1, C2, C3, and C4) interspaced by isozyme-unique variable domains (V1, V2, V3, V4, and V5). The C1 region is a putative membrane-binding domain. The C2 region appears to be related to the Ca^{2+} sensitivity of the enzyme. The C3 region

contains the catalytic site and the ATP-binding site. The C4 region seems to be necessary for recognition of the substrate to be phosphorylated. As for nPKCs, they lack the C2 homologous domain (and thus do not require Ca^{2+} for activation). Finally, aPKCs lack both the C2 and one-half of the C1 homologous domain (and thus are insensitive to DAG and Ca^{2+}) (184).

In the absence of activating cofactors (Ca^{2+}, DAG, phospholipids, etc), the regulatory domain inhibits the kinase activity of the catalytic domain. This inhibitory effect is exerted by a sequence motif termed pseudosubstrate site (PS). The PS is a moderately conserved amino acid sequence that resembles a consensus phosphorylation site in PKCs substrates where, instead of the serine/threonine residue, an alanine residue is present (91, 128). A PS is present in the regulatory domain of all PKCs. The PS appears to interact with the substrate-binding pocket in the catalytic domain, thereby suppressing kinase activity. Binding of cofactors reduces the interaction between PS and catalytic site. Thus, activation of PKC by its cofactors can be considered as a shift in the equilibrium between two states of the catalytic domain (*i.e.*, PS-bound and PS-unbound). Ca^{2+} and DAG signals sequentially activate the cPKC isoforms (Fig. 2). Ca^{2+} acts rapidly, but DAG binding to PKC is initially prevented by a pseudosubstrate clamp which keeps the DAG-binding site inaccessible and delays both Ca^{2+}- and DAG-mediated kinase activation. After the end of Ca^{2+} signals, bound DAG prolongs the kinase activity (187). DAG binding to the PKC regulatory domain increases its affinity for membrane lipids and consequently stabilizes its association. Other lipids such as arachidonic acid, phosphatidylinositol (PI) 4,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (PIP3) can also stimulate PKC activity. Phorbol esters mimic DAG activation of PKCs by binding to the same sites within the regulatory domain, thus inducing similar conformational changes. Prolonged treatment with phorbol esters selectively downregulates the expressed levels of PKC. This invariably results in decreased responsiveness of cells to further stimulation with either phorbol ester or a physiologically relevant ligand. On the contrary, nPKC isoforms are activated directly by DAG without Ca^{2+} priming since, in this case, the DAG-dependent domain binds membranes with sufficiently high affinity to recruit nPKCs in the absence of any other targeting mechanism (61). Finally, aPKCs, which are insensitive to both Ca^{2+} and DAG, are also activated by lipid components, such as PI, phosphatidic acid, arachidonic acid, and ceramide. Among these lipids, phosphatidylinositol-3,4,5-trisphosphate (PIP3) has been proposed to play a critical role in the regulation of aPKCs in cooperation with PI-dependent protein kinase 1 (PDK1). Indeed, when PIP3 concentration is increased in response to cell stimulation, PDK1 binds to PIP3, via its pleckstrin homology domain, and becomes activated. Then, PDK1 interacts with aPKCs and phosphorylates its kinase domain, promoting autophosphorylation of the kinase. aPKCs simultaneously and directly interact with PIP3 which releases PS-dependent autoinhibition. Both contributions of PIP3 and PDK1 are necessary for the complete and stable activation of aPKCs (105).

Selective oxidative modification at the N-terminal regulatory domain induces PKC activation (84), while alterations at the C-terminal catalytic domain result in complete inactivation of the kinase (83). Two pairs of zinc fingers are located in the regulatory domain, and the high concentration of cysteine residues in this region makes this site an attractive target for redox regulation (90). Oxidant treatment of PKC produces a form that does not bind phorbol esters and is catalytically active in the absence of Ca^{2+} and phospholipids. PKC catalytic domains are inactivated by loss of free sulfhydryls required for its function, thus making PKC a potential target for anticancer agents as well as tumor promoters. Indeed, some chemopreventive compounds can inactivate PKC by oxidizing the thiols present within the catalytic domain (90). Also, nitric oxide (NO) inactivates PKC by reacting as NO(x) with cysteine sulfhydryls (85). The balance between reduced and oxidized GSH, the major cellular antioxidant, is an important mechanism for counteracting the effects of ROS and alkylating agents (259). PKC is adequately positioned to react with lipophilic agents and peroxides but not with water-soluble GSH. Both regulatory and catalytic domains of PKC can easily undergo oxidative modifications by hydrogen peroxide. This, as well as additional regulation by tyrosine phosphorylation, can lead to the activation of the mitogen-activated protein kinase (MAPK) pathway, which in turn

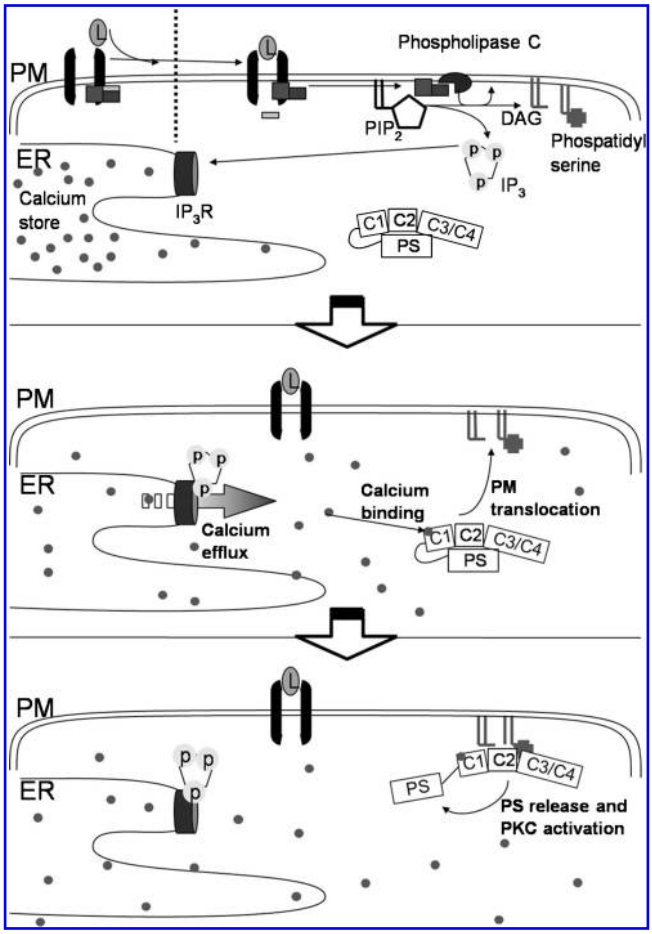

FIG. 2. Schematic activation of conventional PKC. The activation of PKC is preceded by the binding of external stimulus that activates a G protein-coupled receptor, which activates a stimulating G protein. The G-protein activation promotes the generation of DAG and IP3 by activation of phospholipase C. The Ca^{2+} release from ER into the cytoplasm, induced by IP3, promotes PKC activation, which translocates to the membrane, anchoring to DAG and PS.

promotes transcriptional activity and changes in gene expression patterns (90).

B. ROS regulating pathways in the cell and relationship with PKC and disease

All cells produce free radicals in the normal course of metabolism. The main sites for free radical generation are: i) mitochondria (the major source of ROS in most cells), ii) peroxisomal fatty acid metabolism, and iii) microsomal P450 enzymes.

i) Mitochondria may generate more than 85% of the total ROS. Most of the oxygen consumed by aerobic organisms is reduced to water by the enzyme cytochrome *c* oxidase in the terminal reaction of the mitochondrial respiratory chain. However, partial reduction of the oxygen molecule can occur at the same time, and reactive free radicals are constantly generated. Univalent reduction of the oxygen molecule leads to the production of the superoxide radical ($O_2^{\cdot -}$) by complexes I and III. This exists in equilibrium with its conjugated acid, the hydroperoxyl radical (HOO·), which dismutates to hydrogen peroxide (H_2O_2). In order to prevent damage, H_2O_2 must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to catalyze the decomposition (dismutation) of H_2O_2 into oxygen and water molecules. This enzyme is not essential for survival, since mice genetically engineered to lack catalase show a normal phenotype. The hydroxyl radical (OH·) can also be formed from H_2O_2 under specific conditions (95).
ii) Peroxisomal metabolism of fatty acids can lead to free radical formation. Many of the intermediates in prostaglandin and leukotriene biosynthesis and metabolism are free radical species, which could contribute to the generation of oxidative damage (95).
iii) Numerous xenobiotics are metabolised to free radical species by normal cellular detoxification mechanisms such as the cytochrome P450 system. This may be a relatively minor component of the cellular sources of ROS in normal situations, but in specific subjects (*e.g.*, smokers) the system can be an important source of oxidants in the body (95).

Several other enzyme systems also generate ROS. NAD(P)H oxidases located in the plasma membrane of neutrophils and various other cell types are responsible for the generation of radical species as part of the cellular response to invading organisms. Many tissues also contain a xanthine dehydrogenase/xanthine oxidase enzyme system that generates superoxide when the cells are stressed.

NO is a key vertebrate biological messenger, playing a role in a variety of biological processes. It is biosynthesized endogenously from arginine and oxygen by various NO synthase (NOS) enzymes and also by reduction of inorganic nitrate. There are several mechanisms by which NO has been demonstrated to affect the biology of living cells. These include oxidation of iron-containing proteins such as ribonucleotide reductase and aconitase, activation of the soluble guanylate cyclase, ADP ribosylation of proteins, protein sulfhydryl group nitrosylation, and iron regulatory factor activation. NO is also a radical but it is neither a strong oxidizing nor a reducing agent. However, when produced in excess, or in addition to ROS, NO is capable of a variety of reactions, which can produce much more reactive and potentially damaging free radicals. NO reacts with superoxide to form a very potent oxidizing agent, peroxynitrite ($ONOO^-$) (95).

Lipid peroxidation refers to the oxidative degradation of lipids. This process involves free radicals that interact with lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene groups that possess especially reactive hydrogens. Three major steps characterize the reaction: initiation, propagation, and termination. In the initiation step, a fatty acid radical is produced. The initiators in living cells are most notably ROS, such as OH ·, which combines with a hydrogen atom to make water and a fatty acid radical. Fatty acid radicals are not very stable molecules and they react readily with molecular oxygen, creating a peroxyl-fatty acid radical. This, too, is an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide (or a cyclic peroxide if reacting with itself). This mechanism continues as the new fatty acid radical can react in the same way. The chain reaction stops when two radicals react and produce a nonradical species. Living organisms have evolved different molecules that speed up termination by catching free radicals and therefore protecting the cell membrane (95).

Under conditions of oxidative stress, superoxide dismutase (SOD) acts as an endogenous cellular defence system that degrades O_2^- into oxygen and hydrogen peroxide, with the latter being further detoxified by glutathione peroxidase or catalase. There are two forms of SOD: the Mn enzyme in mitochondria (SOD2) and Cu/Zn enzyme present in the cytosol (SOD1) or extracellular surfaces (SOD3) (236).

The thiol-containing compound glutathione (GSH) acts as the primary cellular homeostatic "redox buffer." It is present in cells at concentrations varying from 5 to 10 mmol/l (172). Under normal conditions, approximately 1% of the total cellular GSH is in the oxidized form (GSSG). Thus, oxidation of only a small proportion of the GSH in a cell can shift significantly the GSH:GSSG ratio, with consequent implications for cell signaling. Indeed, total cell GSH levels may act as a signal transducer or modulator. When GSH levels are low, the cell environment will be oxidizing and the function of enzymes, particularly those with thiol groups, will be altered (50).

ROS can act through several different pathways of signal transduction, making use of signaling molecules such as Ca^{2+}, protein kinases, phosphatases and phospholipases. In this review we will describe the ROS-dependent PKC activity.

Many different agents can act on PKC behavior and may contribute to the regulation of diseases. Benzoyl peroxide, for example, induces two different—but complementary—mechanisms in tumor promotion: one involves the copper-mediated free radical formation and mutagenic DNA oxidation; the second mechanism involves metal ion-independent direct interaction with redox-cycling thiols of membrane proteins, such as PKC, that trigger cell signaling (2). Fatty acid hydroperoxides have been shown to induce membrane translocation of PKC. Sodium m-periodate acts as a tumor promoter and also as a mitogen in some cell types. Moreover, the cigarette smoke-induced effects on PKC are likely to be

involved not only in promoting tumor cell growth, but also in the development of invasive disease. In support of this idea, hydroquinone, catechol, or whole cigarette smoke condensate (*i.e.,* cigarette smoke tumor promoters) induce PKC translocation (and activation) from the cytosol to the plasma membrane in lung cells (86) and produce biological changes that are similar to those evoked by phorbol esters (276). All these negative effects are inhibited by a variety of PKC inhibitors and an antioxidant such as vitamin E succinate, at low concentrations (1–25 μM), inactivates the PKC reducing side effects (90).

Other important players in the redox regulation of PKC are represented by NO and the thioredoxin system.

NO, a free radical generated by NOS, is an important regulator of numerous processes in the nervous, immune, and cardiovascular systems, including smooth muscle relaxation, thus resulting in vasodilation of arteries and increasing blood flow. Besides mediating normal functions, sustained levels of NO production result in direct tissue toxicity that contributes to different pathophysiological states. In particular, endogenous NO production by lung endothelial cells has been shown to selectively reduce pulmonary hypertension; however, excessive NO production can be toxic to a number of cells, including vascular endothelial cells through sulfhydryl oxidation of PKC (121). Indeed, exposure to high levels of NO results in loss of the catalytic activity of conventional, novel, and atypical PKC. On the other hand, as redox-sensitive biological targets, PKCs are regulated also by oxidoreductases, including thioredoxin reductase, a thiol-disulfide selenoprotein that operates in the cellular defense against oxidative stress. Interestingly, the existence of a thioredoxin–PKC complex has been demonstrated (271) and this close proximity is critical for restoring the catalytic activity of PKC. Moreover, excessive NO exposure causes oxidation of endogenous thioredoxin, leading to a decrease of redox regulatory activity, required to maintain PKC catalytic activity. Therefore, thioredoxin levels are critical for maintaining the function of PKC. These data emphasize that PKC is a logical candidate for redox modification by oxidants and antioxidants that may in part determine its cancer-promoting and anticancer activities.

C. The intracellular distribution of PKC is sensitive to redox changes

In its inactive state, PKC is mainly located in the cytosol. Translocation from one intracellular compartment to another (typically the plasma membrane) is, for all PKCs, a key step in the activation process. Oxidative stress induces PKC translocation that is specific for different isoforms and different cell types. For example, in mouse embryonic fibroblasts (MEFs), oxidative stress triggers translocation of PKCα, β, δ, and ε isoforms from the cytosol to the plasma membrane. Under the same conditions, PKCζ translocates to the nucleus (Fig. 3). This behavior is not general for all cell types; for example, in HeLa cells, PKCβ does not translocate to the plasma membrane after H_2O_2 treatment (227). The intracellular redox state controls the selectivity of PKC isoform activation and thus changes the sensitivity of the different isoforms to cell stimulation, as observed in the case of PKCα during agonist stimulation after H_2O_2 pre-treatment. Indeed, the treatment with H_2O_2 renders PKCα insensitive to histamine stimulation, an agonist coupled to the generation of DAG and inositol 1,4,5-trisphosphate (IP3) (and thus the release of Ca^{2+} from intracellular stores with consequent increase of cytosolic Ca^{2+} concentration, $[Ca^{2+}]_c$). On the contrary, in untreated cells, as expected for a cPKC, histamine induces PKCα oscillations (*i.e.*, different translocation to the plasma membrane and re-translocation to the cytosol). This inhibition is specific for PKCα and not for all other cPKCs; for example, H_2O_2 pretreatment does not modify the sensitivity of PKCβ to histamine (227). These data could thus explain why stimuli that in principle could activate a broad number of PKC isoforms become selective for a specific isoform by delivering either a reductive or an oxidative stress.

Signaling pathways that induce variations in the level of PKC activators have been implicated in the regulation of diverse cellular functions. As such, PKCs are key regulators of a plethora of cellular processes, including proliferation, tumorigenesis, cell death, neurodegeneration, differentiation, preconditioning, and aging. The following paragraphs will focus on some aspects of PKC signaling that are related to these cellular processes. Particular emphasis will be placed on the role of oxidative stress in the modulation of PKC activity.

II. Involvement of PKCs in Neoplastic Transformation

The function of PKC in cancer is complex, primarily because much of the data indicate that the isozymes subtly regulate many pathways involved in cellular transformation. Overall, increased PKC levels have been associated with malignant transformation in several cell lines, including breast (185), lung, and gastric carcinomas (242). These aspects establish a clear role of PKC in tumorigenesis and, at the same time, spawn questions about the pivotal contribution of individual isozymes. How is this family of serine/threonine kinases involved in tumor promotion? How do PKC isozymes regulate the diverse cellular processes, and what are their roles in carcinogenesis? This section will focus on the role of the different PKC isoforms in cell cycle regulation and tumor progression, with particular interest in the contribution of the redox signal. Recent studies imply that ROS can play an essential role in cell biology and physiology. As a result of the dynamic interplay between ROS generation and antioxidant action, high concentrations of ROS are pathogenic, while moderate amounts of oxidant species take part in cell regulation, acting as mediators and signal transduction molecules (104). The dosage of the redox signal is important in the modulation of cell function: indeed, the cell cycle is arrested when cells are exposed to moderate levels of oxidative stress, whereas very low doses of ROS stimulate cell proliferation. The mitogenic signals, mediated through the generation of ROS, activate redox-sensitive transcription factors, influencing molecular and biochemical processes responsible for changes observed during cell differentiation, senescence, and transformation (251). As mentioned before, PKCs are subjected to a complicated cellular redox regulation. Studies *in vivo* and *in vitro* confirm the biphasic behavior of PKC in response to different oxidative injuries. High doses of prooxidant compounds (carbon tetrachloride and ethanol) cause hepatic PKC inactivation and proteolytic degradation, while low doses induce stimulation of kinase activity (56). Indeed, Ward *et al.* have suggested that depletion of GSH during oxidative stress removes a mechanism for negative regulation of PKC and, consequently, provides a permissive environment

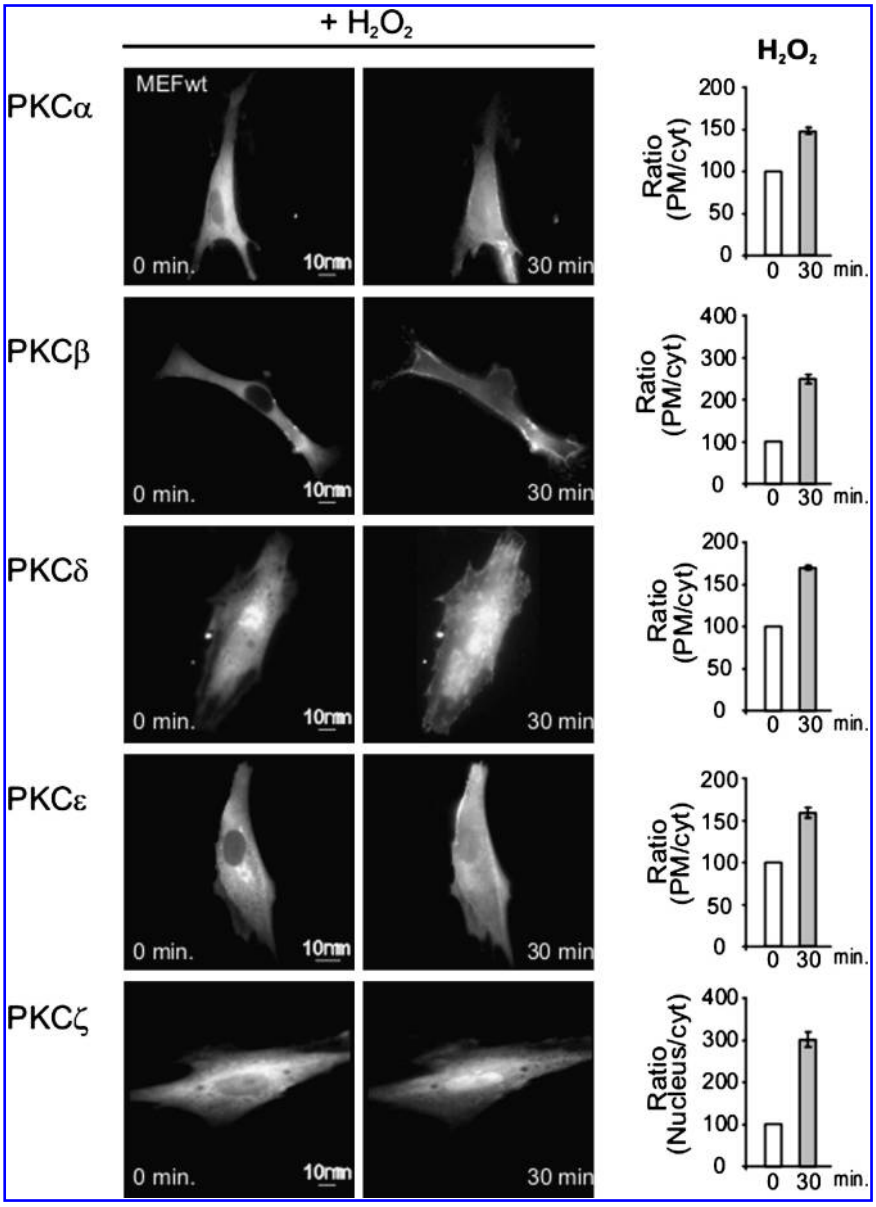

FIG. 3. Intracellular distribution of PKC. Fluorescence imaging of PKCαGFP, PKCβGFP, PKCδGFP, PKCεGFP, and PKCζGFP chimeras (transduced into wild-type MEFs via an adenoviral vector) upon an oxidative challenge (H_2O_2 1 m*M*,30 min). All the chimeras show predominantly cytosolic distribution. After the addition of H_2O_2, a clear plasma membrane staining (PKCαGFP, PKCβGFP, PKCδGFP, and PKCεGFP) or nuclear localization (PKCζGFP) was detected. The graphs indicate the increase in fluorescence in respect to time zero, calculated as the fluorescence ratio of plasma membrane (PM) or nucleus to the average cytosolic (cyt) fluorescence.

for PKC activity and tumor promotion (269). It has been confirmed that a highly reactive Cys residue expressed in the catalytic domain of each PKC isoenzyme is subject to S-thiolation, inactivating the enzyme (269). In particular, this group has shown that GSH and *N*-acetylcysteine (a precursor of cellular GSH) irreversibly inactivate purified PKC isoenzymes, reporting that brief treatment of cultured rat fibroblasts or human cancer cells with these results in a decline in the level of Ca^{2+}- and PS-dependent PKC activity. As reported by Ward and Gundimeda (93), it is clear that PKC can play an important role in oxidant-mediated tumor progression.

In support at this theory, β-carotene and retinoids function as anticarcinogenic agents, antagonizing the biological effects of pro-oxidants on PKC (30). The exact role of these antioxidants in carcinogenesis and cancer progression remains very controversial. More evidence has demonstrated that some antioxidants (α-tocopherol or β-carotene) are associated with a lower incidence of specific types of cancers (lung, prostate, and breast) (3). On the contrary, no evidence was found in gastrointestinal cancer prevention after antioxidant supplements (20).

A. Altered PKC expression in cancer

There is a limited number of cases in which PKC mutations in humans are linked to a cancer phenotype. However, altered levels of PKC isoforms can be found in many types of human cancers; often, altered expression of PKC can also be linked to the disease progression.

A single point mutation in PKCα (D294G) was found in a subpopulation of highly invasive pituitary tumors (6) and in thyroid follicular adenomas and carcinomas (212). This mutation alters an amino acid sequence (GDE motif) present in the V3 hinge region that links the regulatory and catalytic domains of PKCα. It confers a defect in translocation to the plasma membrane and consequently impairs substrate phosphorylation. The GDE motif might also be involved in the

interaction with anchoring/scaffolding proteins required for PKC membrane association.

The levels of PKCβII play an important role in colonic epithelial cell proliferation and progression to colon carcinogenesis. A dramatic increase in the amount of PKCβII was observed in both aberrant crypt foci (ACF; preneoplastic lesions of the colon) and colon tumors, as compared to normal colonic epithelium. Of particular interest, PKCε has been shown to be upregulated in various types of cancer (270), whereas PKCα and PKCδ are downregulated (110, 193). In some cancers, there is a striking correlation between changes in PKC expression and disease progression. However, it is currently not clear whether changes in PKC expression involve genetic and/or epigenetic effects, especially because the mechanisms that control PKC gene expression are largely unknown.

B. PKC and cell-cycle regulation

The role of PKC in modulating cellular proliferation is well documented, but the role of specific PKC isozymes in controlling cell-cycle events is still controversial (21, 80). In fact, different PKC isozymes can confer distinct and contrasting effects on proliferation (167).

The orderly transition between phases of the cell cycle is carried out by a family of cyclins, which bind to and activate specific cyclin-dependent kinases (Cdks) (103). Under normal circumstances, the cell cycle proceeds without interruptions. However, if cycling cells receive damage, they usually have the capacity to pause temporarily in G_1, S, or G_2 phase, repair the damage (if possible), and re-engage the cell cycle. The checkpoint pause often involves a suppression of cyclin/Cdk activity.

Control modes of Cdk activity appear to be modulated by PKC. In fact, several lines of evidence point to the involvement of PKC in cell cycle progression during the G1 and the G2/M phases (Fig. 4).

Activation of PKC during the G1 phase, in some cell systems, may exert positive or negative growth regulatory effects, depending on the time of PKC activation (283). Treatment of human fibroblasts or vascular smooth muscle cells with phorbol 12-myristate 13-acetate (PMA) inhibits DNA synthesis triggered by growth factors (18) blocking cell cycle in G1 phase. On the contrary, PMA stimulates DNA synthesis in some cells such as Swiss 3T3 and lymphocytes (123, 231). The reason for these different effects of PMA is unclear, although it is likely to be the result of the expression of different sets of PKC in these cells. Indeed, PKCα has been shown to have antiproliferative effects in various cell types, including intestinal and pancreatic cells (54). In the case of intestinal cells, PMA treatment causes cell-cycle arrest in G1 in a PKCα-dependent manner. In another study, it was shown that the ectopic expression of the novel PKCη isoform in NIH 3T3 cells delayed the G1/S transition; upon growth stimulation, synchronized NIH 3T3 cells overexpressing PKCη entered the S phase at a slower rate, exhibiting a longer G1 phase (152).

Amongst the nPKC isoforms, the activation of PKCδ causes proliferation defects both in G1 and G2 phases of the cell cycle. Recent studies showed that phorbol-ester treatment arrests lung adenocarcinoma cells in G1 through PKCδ activation (179). The transition from G2 to M is also regulated by the PKC pathway. The cell cycle can be interrupted in the G2

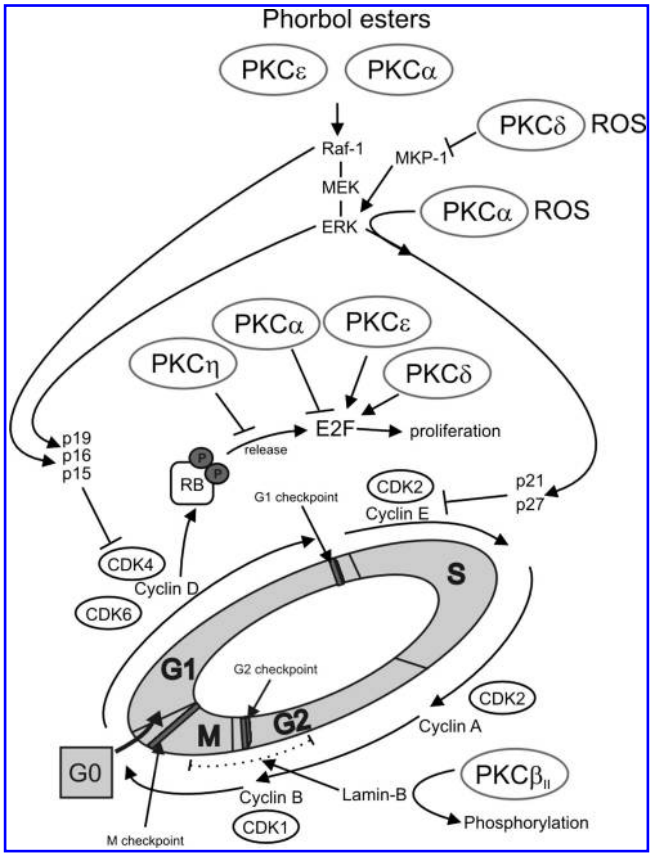

FIG. 4. Involvement of different PKC isoforms in cell cycle regulation. The figure shows some molecular targets and PKC-dependent pathways crucial for the induction of growth arrest and tumor progression.

phase by PMA, indicating that the G2/M transition can be negatively regulated by PKC.

Watanabe *et al.* generated several CHO cell lines overexpressing PKC isoforms α, βII, δ, and ζ and showed that in these cells, only PKCδ have a direct role in the G2/M transition (270). Some years later, it has been demonstrated that also the PKCβII isoform is required for the G2/M transition (260).

In this scenario, the interplay between ROS and PKC in cell cycle regulation is not so clear. Indeed, while the damaging effects of ROS on DNA have been extensively studied, the effects of ROS on checkpoint responses have not. As for PMA, it is known that high levels of ROS may play a key role in establishing growth arrest and senescence (253) through PKCδ activation and cycle arrest in M phase. Similarly, it has been reported that ROS mediate G_1-S arrest also by PKCα activation, important for trigger cell migration and metastasis (277).

In conclusion, a considerable amount of data establish the pivotal role of PKC in regulation of the cell cycle. Unfortunately, it appears to be a rather ambiguous scenario, complicating the approaches towards efficient pathologies therapy strategies.

III. PKC Family as Key Player in the Apoptotic Process

Protein kinases have been implicated both in the upstream induction phase of apoptosis and in the downstream

execution stage, as direct targets of caspases (226). In this section, we will focus on the role of PKC, given that its role in programmed cell death has been intensively investigated in recent years. Other serine/threonine protein kinases also play a role in apoptosis, for example, the MAPK family, cyclic AMP-dependent protein kinase (PKA), and protein kinase B (PKB). However, their involvement has not been fully characterized and the reader is referred to recent reviews on this topic for more details (47).

PKCs can have a dual and contradictory role in apoptosis (Fig. 5): the activation of specific PKC isoforms may protect cells or induce cell death. Among the classical PKCs, 12-0-tetradecanoylphorbol-13-acetate (TPA) induces cytochrome *c* release (and thus cell death) in U-937 leukemia cells through a PKCβ-dependent mechanism (196). In gastric cancer cells, indomethacin-induced apoptosis is in part mediated by differential regulation of PKC isoforms expression; enhanced expression of exogenous PKCβ protects against this form of apoptosis (286). Moreover, as described below (see Fig. 8), when PKCβ is activated by oxidative challenges, it causes the phosphorylation and import of p66shc into mitochondria, where it acts as an oxidoreductase. This initiates a feed-forward cycle of ROS production, leading to the opening of the mitochondrial permeability transition pore (mPTP), the release of caspase cofactors, and ultimately triggers apoptotic cell death.

Powell and co-workers demonstrated that in a cell line derived from a human prostate cancer, the presence on the cell membrane of PKCα correlates with spontaneous apoptosis, while its absence is associated with resistance to TPA-induced apoptosis (211).

Among the novel isoforms, most evidence supports a key role of PKCδ. For example, Lynch and co-workers showed that basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by reducing [Ca^{2+}] changes through a PKCδ-dependent pathway (155). PKCδ appears to have a role also in the release of cytochrome *c* from mitochondria. Indeed, TPA induces translocation of PKCδ from the cytoplasm to mitochondria and this translocation results in the release of cytochrome *c* and the activation of caspase-3 (225). Mitochondrial localization is not unique to the PKCδ isoform. Also PKCα has been shown, in the same cell types, to be present in mitochondria, where it may cause Bcl-2 phosphorylation and suppression of apoptosis (233).

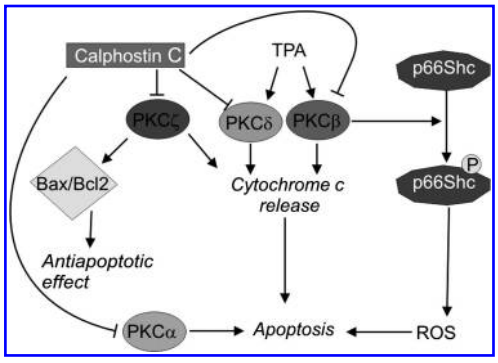

FIG. 5. Schematic representation of how apoptosis is triggered. TPA activates PKCδ and β that induce cell death through cytochrome c release from mitochondria. PKCβ also acts on p66Shc, phosphorylating it, and allowing ROS formation. Activation of PKCα directly promotes apoptosis. PKCζ can induce cytochrome c release, leading to apoptosis, but it can modify also the status of Bax/Bcl2 proteins, exhibiting an anti-apoptotic effect. The same can be obtained using calphostin c that inhibits all PKC isoforms. 234×162 mm (96×96 DPI).

Most of the examples described above concern apoptosis induced *in vitro*, under selected experimental conditions. A particularly interesting phenomenon, of major physiological relevance (that involves both Ca^{2+} and PKC) is that related to the termination of the immune response. To maintain T cell homeostasis, once the Ag has been cleared, activated lymphocytes are removed by apoptosis (232). This form of apoptosis involves the TCR-induced expression of the CD95 ligand (CD95L) on the surface of T cells (27, 178). Once CD95L is expressed on the T cell surface, it induces T cell apoptosis through activation of CD95 (9). It has been shown that PKCθ in cooperation with calcineurin plays an essential role in regulating CD95 expression and activation-induced cell death (266).

Atypical PKC isoforms also appear to play a role in apoptosis. PKCζ has been reported to be anti-apoptotic (14). PKCζ modulates the apoptotic machinery at the mitochondrial level by modifying the Bax/Bcl-2 ratio, but also by modulating cytochrome *c*-mediated caspase activation (69). It should be noted, however, that PKCζ augments mitochondrial Ca^{2+} uptake, which is a signaling change that should sensitize the cell to apoptotic challenges (204). A plausible explanation for this apparent discrepancy is that the anti-apoptotic effect of PKCζ is mostly at the transcriptional level; indeed, PKCζ was reported to control the expression levels of Bcl-2 family members and other apoptotic regulators (69).

Direct support for a role of PKC in apoptosis comes from the demonstration that the antibiotic calphostin C (a potent PKC inhibitor) induces rapid apoptosis in human acute lymphoblastic leukemia (ALL) by two additive effects: the inhibition of PKC through an irreversible oxidative inactivation and the modulation of a cytosolic Ca^{2+} rise. Calphostin C was shown to induce rapid Ca^{2+} mobilization from intracellular stores of ALL cell lines, and its cytotoxicity correlates well with the magnitude of this Ca^{2+} signal. In fact, calphostin C-induced death is suppressed by loading the cells with the Ca^{2+} chelator BAPTA (285). Similar to calphostin C, hypericin (a naturally occurring substance found in the common St. John's Wort; Hypericum species) also induces apoptosis by oxidatively inactivating PKC (86).

Daunorubicin (DNR) and 1-β-D-arabinofuranosylcytosine (ara-C), the most active anti-leukemic agents, activate the sphingomyelin-ceramide (SM-CER) cycle, leading to apoptosis by stimulating—through a redox-dependent mechanism—the c-Jun N-terminal kinase module, and AP-1 DNA binding affinity (17, 159, 160). Such an apoptotic pathway has also been described for vincristine, ionizing radiation, Fas agonist, and TNF (144). Lyn, a src family member, plays a critical role in drug-induced N-SMase stimulation. Indeed, ara-C activates Lyn through ROS production and the activated Lyn interacts with N-SMase. The SM-CER apoptotic pathway is controlled by potent regulators that can operate either upstream or downstream of CER production. Among them, PKC activity seems to play a crucial role. Indeed, PKC stimulation induced by phorbol esters or DAG not only inhibits CER-induced apoptosis (118), but also limits DNR-induced

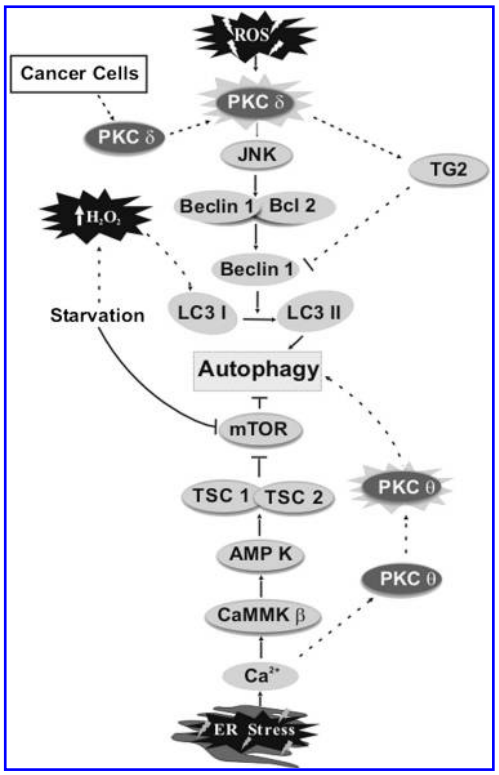

FIG. 6. A proposed model for redox/PKC regulation of autophagy. Production of ROS induces JNK activation in a PKCδ-dependent manner. Activated JNK determinates phosphorylation of Bcl-2, releasing Beclin-1 to induce autophagy. Starvation promotes autophagy activation via mTOR inhibition and simultaneously triggers accumulation of ROS (probably H_2O_2), which is also necessary for autophagosome formation. Promotion of the autophagy process is caused by disruption of [Ca^{2+}] ER homeostasis, which leads the activation of CaMKK-b, AMPK, and TSC-1/2, that in turn results in inhibition of mTOR. The increase in Ca^{2+} induces phosphorylation of PKCθ that leads to the autophagic process.

N-SMase stimulation, CER generation, and apoptosis (158). The role of PKC in regulating N-SMase is also supported by another study that showed that PKC inhibition by calphostin C resulted in N-SMase stimulation (39). In an elegant set of experiments, Benzombes and colleagues demonstrated how PKCζ overexpression results in a reduction of drug-induced ROS production, Lyn activation, N-SMase stimulation, CER production, as well as in apoptosis inhibition and drug resistance (16). Moreover, PKCζ inhibition promotes apoptosis in leukemia cells exposed to etoposide and TNF-α, as well as sensitized tumor cells grown in nude mice to etoposide (69). These results thus suggest that PKCζ reduces the sensitivity of cancer cells to chemotherapeutic agents and indicate that this PKC isoform may be a useful target for tumor cell chemosensitization.

However, as discussed, the available literature concerning PKC involvement in apoptosis is contradictory. Indeed, the different activities of the individual isoforms remain to be fully characterized. The essential role of this kinase family in both cell survival and apoptosis suggests that specific isoforms may function as molecular sensors, promoting cell survival or cell death depending on environmental cues.

IV. Role of PKC in the Autophagy Process

Protein turnover is essential for removing defective proteins and for contributing to the pool of amino acids required for continued protein synthesis, particularly in times of limited nutrient availability. The ubiquitin–proteasome and autophagy–lysosomal pathways are the two major routes for protein and organelle clearance in eukaryotic cells (189). Autophagy—from the Greek "auto" (self) and "phagy" (to eat)—refers to any cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome. Autophagy is a dynamic process of protein degradation, typically observed during nutrient deprivation, and occurs when cells need to "self-cannibalize" or degrade their constituents (48). Recently, autophagy has been the object of renewed interest from oncologists, since different types of cancer cells undergo autophagy after various anticancer therapies (Fig. 6). At least three forms have been identified: chaperone-mediated autophagy, micro- and macroautophagy, that differ with respect to their physiological functions and the mode of cargo delivery to the lysosome (48). In this review, we will focus on macroautophagy (herein referred to simply as autophagy), the major regulated catabolic mechanism that eukaryotic cells use to degrade long-lived proteins and organelles.

Autophagy occurs constitutively at low levels in all eukaryotic cells to perform housekeeping functions such as the destruction of dysfunctional organelles (124). Dramatic upregulation of cytoplasmic and organelle turnover, for example, occurs in the presence of external stressors (starvation, hormonal imbalance, oxidation, extreme temperature, and infection), and internal needs (generation of source materials for architectural remodeling, removal of protein aggregates) (163). Signaling pathways that regulate autophagy are extremely complex, since numerous feed-forward and feedback loops and cross-talks with many other signaling networks are involved.

Recently, several studies have proposed that redox stress, in particular hypoxic stress, may be involved in the induction of autophagy (200). In fact, various defense mechanisms have been developed to protect the cell against oxidative stress, such as upregulation of antioxidants, removal of specific proteins by the ubiquitin–proteasome system, and removal of damaged proteins and organelles by autophagy. Although autophagy has been characterized in many contexts, the signaling pathways that activate it in response to hypoxic stress have not been studied extensively yet.

Specifically, autophagy is highly regulated through the coordinated action of various kinases, phosphatases, and guanosine triphosphatases.

One of the key regulators of autophagy is the serine/threonine protein kinase mTOR (mammalian target of rapamycin, homolog of the yeast Atg8), but recent studies have shown that PKCs play a critical role in the regulation of autophagy (Fig. 6), even if the exact mechanism remains ambiguous.

Recent observations showed that the PKC–JNK axis is involved in hypoxia-induced autophagy (36). It has been demonstrated that acute hypoxic stress induces autophagy through a process involving PKCδ. Upon stress, a rapid activation of PKCδ occurs, with a consequent JNK activation, that in turn releases Beclin-1 from its inhibitor Bcl-2, leading to autophagy induction during the early phase of hypoxia. These

effects were attenuated by dominant negative PKCδ or in MEF/PKCδ-null cells.

Chen and co-workers in 2008 demonstrated that this hypoxic stress utilizes a rapid recruitment and activation of PKCδ in a pathway BNIP3-independent (37). BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) is a member of the Bcl-2 family reported to play a critical role in hypoxia-induced autophagy and regulated by the hypoxia-induced transcription factor HIF-1α (263).

Taken together, these results lead to the conclusion that a PKCδ/JNK1-dependent pathway is required for autophagy in the early response to hypoxic stress, whereas a BNIP3-dependent pathway is required for autophagy in response to prolonged hypoxia, possibly related to the accumulation of ROS during chronic exposure to hypoxia (282).

Recently, another PKC isoenzyme, PKCθ, a member of the nPKC family, has been demonstrated to mediate autophagy during stress conditions (234). Pharmacological inhibition of PKCθ or siRNA-mediated knockdown of PKCθ prevented the autophagic response to ER stress. Treatment with the ER stressors thapsigargin or tunicamycin induced PKCθ phosphorylation and activated autophagy. Sakaki and co-workers have hypothesized that PKCθ activation and autophagy in response to ER stress is independent from the mTOR kinase signal transduction pathway (235). This observation conflicts with recent findings by Hoyer-Hansen *et al.* (108) who concluded that autophagy in response to thapsigargin treatment occurs through inactivation of mTOR kinase. It is possible that a PKCθ-dependent pathway is required for autophagy in response to acute severe ER stress, whereas an mTOR-dependent pathway is required for autophagy in response to chronic ER stress, possibly related to secondary effects of ER stress on nutrient metabolism, but the exact mechanism by which PKCθ is activated in response to this ER stress condition remains unknown.

V. PKC Isoforms as Aging Actors

The first correlation between oxidative stress and aging was proposed by Harman in 1956, based on the evidence that all organisms live in an environment with ROS (101): mitochondrial respiration, the mechanism for energy production in eukaryotic cells, spawns ROS from the electron transport chain. The universal nature of ROS was emphasized by the identification, a decade later, of SOD (71), an enzyme whose sole function seemed to be the removal of $O_2^{\cdot-}$. This provided a mechanistic support for Harman's hypothesis. The fact that mitochondria consume the greater part of intracellular oxygen in order to produce ATP, led to the general hypothesis that to a higher metabolic rate corresponds a shorter lifespan. This is not always true, especially for primates and birds, which tend to live longer than would be predicted by their metabolic rates. However, a careful analysis of mitochondria of these species showed a lower production of ROS despite their metabolic rate (141). These data strongly suggest the importance of ROS production in the aging process, rather than the metabolic rate. As a matter of fact, oxidative stress and altered mitochondrial function have consistently been proposed to parallel organism deterioration, and overexpression of antioxidants have shown to extend the lifespan of transgenic animals (245). In this framework, the study of genes involved in ROS production and oxidative stress is currently of great interest for the scientific community. Caloric restriction is the only nongenetic mechanism known to extend the lifespan in mice, and this is strictly correlated with a lower production of ROS.

The relationship between PKC and oxidative stress has been the subject of intense research (90). Several unique structural aspects of PKC make it a highly susceptible direct target for oxidants and chemopreventive antioxidants. As already mentioned, both the regulatory and catalytic domains of PKC contain cysteine-rich regions that are targets for redox regulation (87). When oxidized, the autoinhibitory function of the regulatory domain is compromised and, consequently, cellular PKC activity is stimulated. Treatment with H_2O_2 causes phosphorylation and subsequent activation of some PKC isoforms, such as PKCβ and PKCδ (135).

PKCδ has an established role in activating, through phosphorylation, $p47^{phox}$, an essential component of NADPH oxidase, thus increasing ROS production (256). The levels of active PKCδ were shown to be increased during replicative senescence in human diploid fibroblasts (273). Moreover, it has been demonstrated that PKCδ acts also as a critical downstream mediator of the ROS signaling pathway (256). Based on this evidence, Takahashi *et al.* described an interesting cooperation between PKCδ, ROS production, and $p16^{INK4a}$, a senescence-related gene (253) (Fig. 7). Soon after the initial discovery of $p16^{INK4a}$, it was observed that its level increased progressively with the proliferative history of cells in culture (4,99). Strategies such as caloric restriction, which extend lifespan, also seem to reverse the age-dependent increase in $p16^{INK4a}$ transcription (140), suggesting that this gene is the real effector of aging. The $p16^{INK4a}$ signaling pathway, in conjunction with mitogenic signals, induces elevated intracellular ROS and a consequent activation of PKCδ, establishing a positive feedback loop that sustains ROS production. Moreover, the activation of ROS–PKCδ signaling blocks cytokinesis in an irreversible manner, partially reducing the levels of WARTS (a kinase involved in cytokinesis), and promotes cell-cycle arrest in human senescent cells (253).

Like PKCδ, also the β-isoform is activated by oxidative stress, such as H_2O_2 (206). Its activation is required for the phosphorylation of p66Shc, a lifespan determining protein (208). This 66 kDa protein is a genetic determinant of lifespan in mammals, regulating ROS metabolism and apoptosis (82). Downregulation of p66Shc causes an increase of lifespan in mammals with no known pathological consequences (166), and MEFs knock-out for this gene are resistant to apoptotic death induced by oxidative stress (206). In about 20% of fibroblasts of higher organisms, p66Shc is localized to mitochondria and oxidative stress promotes a translocation of part of the cytosolic pool of p66Shc to this organelle (191). Within mitochondria, p66Shc has a redox activity (when bound to cytochrome *c*, it promotes electron transfer from cytochrome *c* to molecular oxygen) (82), with a consequent increment of ROS production and aging. In wild-type MEFs, the application of oxidative stress causes a drastic reduction in mitochondrial Ca^{2+} spike evoked by agonist stimulation, an early consequence of mitochondrial damage. It has been shown that p66Shc has to be phosphorylated at serine 36 to be active (166). This phosphorylation can be mediated by several protein kinases and is indispensable for life-span regulation by p66Shc. Our group has shown that inhibition (with Hispidin, a specific PKCβ inhibitor) or silencing of PKCβ protects cells

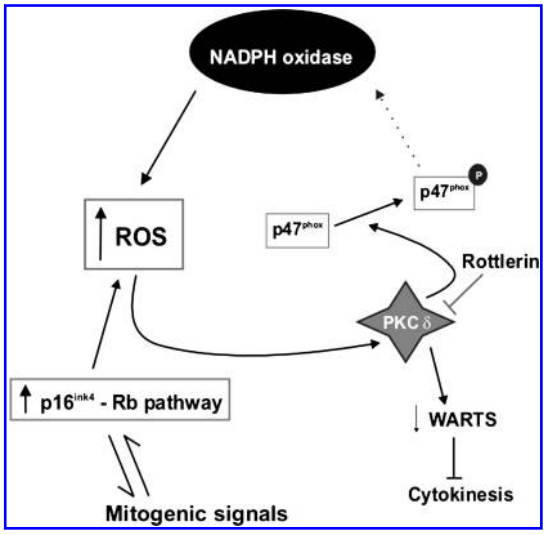

FIG. 7. Cooperation between PKCδ, ROS, and p16INK4a. The p16INK4a /RB1 signaling pathway, in cooperation with mitogenic signals, induces elevated intracellular ROS and a consequent activation of PKCδ, establishing a positive feedback loop that sustains ROS production. PKCδ phosphorylates p47phox an essential component of NADPH oxidase that increases ROS production. The activation of ROS–PKCδ signaling blocks, in an irreversible manner, cytokinesis (partially reducing the levels of WARTS) and promotes cell-cycle arrest in human senescent cells.

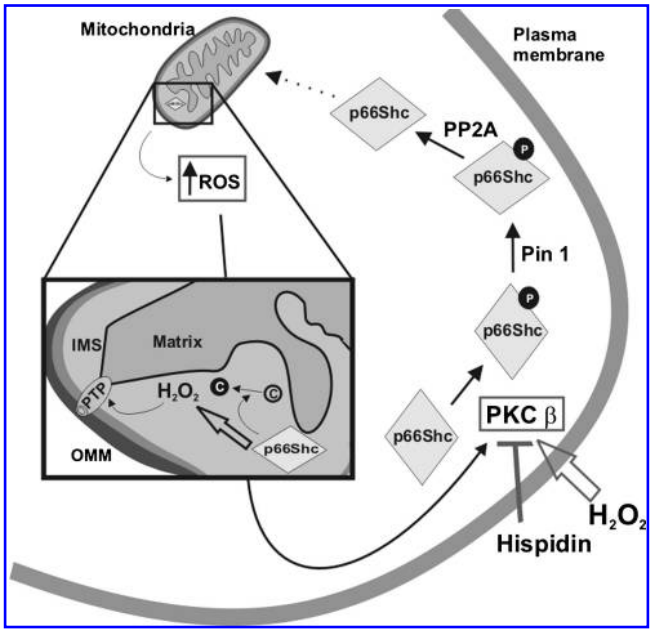

FIG. 8. PKCβ, p66Shc, and ROS. PKCβ is activated by H_2O_2 (ROS) and induces p66Shc phosphorylation, thus allowing p66Shc to be recognized by Pin1, isomerized, dephosphorylated by PP2A, and imported into mitochondria. Here, active p66Shc oxidizes reduced cytochrome c (*from white to black*) and catalyzes the reduction of O_2 to H_2O_2. PTP opening by H_2O_2 then leads to swelling and apoptosis. The increment of ROS production feeds this "vicious circle" with a consequent senescence progression. IMS, intermembrane space; OMM, outer mitochondrial membrane.

against H_2O_2 challenge. Furthermore, overexpression of PKCβ reproduces the Ca^{2+} signaling defect only in cells expressing p66Shc. Thus, there is a strong dependence between PKCβ and p66Shc activity at the mitochondrial level, explained by alterations in mitochondrial Ca^{2+} homeostasis (206). Activation of PKCβ by oxidative stress leads not only to phosphorylation of p66Shc, but also promotes binding of p66Shc to Pin1, a peptidyl-prolyl isomerase that induces cis-trans isomerization of phosphorylated Ser-Pro bonds, causing mitochondrial translocation of p66Shc (Fig. 8). The discovery of this interaction between p66Shc and Pin1 solved an apparent contradiction: p66Shc, although phosphorylated in the cytosol, is found nonphosphorylated inside mitochondria. Indeed, the phosphorylation sites, once isomerized by Pin1, become targets for dephosphorylation by PP2A (a type 2 serine/threonine phosphatase) (278). The current understanding of the signal transduction pathway of p66Shc involves an initial phosphorylation of this protein by PKCβ (activated by oxidative stress) and, after a conformational change induced by Pin1 (and subsequent dephosphorylation), translocation into mitochondria for exerting its oxidoreductase activity (206).

Despite the importance of oxidative stress in aging process is undoubted, evidences about a beneficial role of antioxidant treatments are still scarce and equivocal (76). A perfect example of this unclear scenario is represented by melatonin, a potent free radical scavenger, especially towards highly toxic hydroxyl radicals, which seems to have a potential capacity to extend life span (7) and to stimulate a number of antioxidative enzymes (223). On the other hand, melatonin, as for many other antioxidants, can act as a pro-oxidant under certain conditions (7, 45,192).

VI. PKC as a Rheostat in Neurosurvival and Death

Neurodegeneration is a phenomenon where neurotransmission becomes progressively compromised, characterized by axonal degeneration and other anatomical losses within the nervous system. This condition directly affects neurons and other neuron-related cells (*i.e.,* oligodendrocytes in the case of multiple sclerosis). Gradually, a very large and heterogeneous group of cells become involved, leading ultimately to the death of the patient. In the enormous variety of signal transduction pathways and molecular targets investigated in neurological disorders, a lowest common denominator could be represented by mitochondria, a key regulator of cell death or survival. In recent years, oxidative damage has acquired an important role in the etiopathology of neurodegenerative diseases. Studies on human frontal cortex samples show an increased expression of genes that regulate stress response and oxidant scavenging (154). It has been widely demonstrated that ROS are capable of triggering apoptotic/necrotic neuronal death, leading to neurodegeneration and related diseases. At the same time, mitochondria are the most important source of ROS within the cell; they are produced in high doses by the respiratory chain and scavenged by mitochondrial antioxidants (195). Because PKCs are redox sensors, as previously mentioned, it is logical to propose that these kinases act as possible decoders of oxidative stress in neuronal physiopathology (Fig. 9).

The first elegant work demonstrating PKC neuroprotective activity was carried out by Pamela Maher in 2001 (156). This

work revealed that PKCs mediate neurosurvival—after cell death is induced by oxidative stress—by activating ERK 1/2 and JNK kinase. It has also been shown that this protection, experimentally induced by TPA, increases levels of PKCα and ε (already associated with cell death protection) (248, 274) while, on the contrary, it reduces expression of PKCδ (involved in positive modulation of programmed cell death together with the β-isoform) (168).

Another study looked at PKC as a survival factor in brain tissue by activating the same pathways as insulin, involving MAPK, especially ERK1 and ERK2 (181). Both these kinases, components of the neurotrophin receptor pathway, participate in neurological development increasing the density of dendritic spines following brain-derived neurotrophic factor exposure (5) and dendritic growth through the glial derived-neurotrophic factor (281). Insulin-induced activation of PKC does not seem to require Akt participation, as noted in the scarce literature related to this feature, probably due to differential pathways activated by insulin receptors (181).

All these events are relevant in synaptogenesis, underlining the role of PKC during learning and brain remodelling. Synaptogenesis occurring in the hippocampus during learning requires an increase in PKC activity. In addition, the PKC activator bryostatin enhances memory formation and synaptogenesis in rat. Remodelling of synapses is also a reparation event during cerebral ischemia. The activity and expression of PKCs during this kind of damage varies, depending on the cerebral subregion or animal model. The role of the γ isozyme is not defined precisely, but seems to be quite important, especially after studies in mice lacking this isoform showed that these appear to be protected from a hypoxic condition, but not from reperfusion injury. On the other hand, PKCε appears to be fundamental in preconditioning mediated by NMDA (40). At the same time, bryostatin, a potent PKC activator, can enhance memory and learning by blocking dendritic spine loss (250). The neurosurvival and regeneration could occur as a result of damages such as ischemia, hypoxia, or inflammatory response, causing oxidative stress and activating the prosurvival activity of the PKCs. In PC12 cells, treatment with cobalt or xantine/xantine oxidase, as mediator of oxidative stress, induce neuritogenesis by activating PKC (89). The oxidative condition causes the activation of PKC and its migration to the plasma membrane resulting in the phosphorylation of GAP 43, a neurotrophic factor already associated with neuroregeneration, rearrangment of the cytoskeleton, and neurite growth. Moreover, an excessive oxidant condition causes the inactivation of PKC, degeneration of the neurite, and cell death.

The modulation of PKC by oxidative stress, generated during ischemic stroke, could represent a physiological system of protection and a molecular rheostat for accelerated neuronal repair. The protective role of PKCs in neurosurvival could go even further than synaptogenesis mediated by ERK1 and 2, participating in the development of one of the most representative neurodegenerative conditions, Alzheimer's disease. Indeed, PKCs are involved in the processing of amyloid precursor protein (APP) in multiple ways. APP is the precursor of the β-amyloid peptide, responsible for the creation of insoluble aggregates within brain tissues of Alzheimer patients, ultimately leading to cell death. This precursor is physiologically present as a transmembrane protein that can undergo different modifications by secretase isozyme cleavage. The combined activity of β-secretase and γ-secretase is required to obtain the mature β-amyloid fragment, while activity of α-secretase produces an extracellular soluble fragment that is not amyloidogenic. Thus, the first pathway is responsible for the progression towards neurodegeneration while, on the contrary, α-processing has been proposed to be protective by reducing the pool of APP available for β-processing (96).

Recently, it has been found that phorbol esters, strong PKC activators, could enhance α-processing so much that it is now the most used method for reducing APP formation. The α, β, and ε PKC isozymes are all involved in APP processing with different roles (216). PKCα is directly involved in α-APP formation and, in parallel, in reduced β-processing, probably by promoting APP movement to the plasma membrane through the TGN (49). Moreover, bryostatin, a novel PKCα activator,

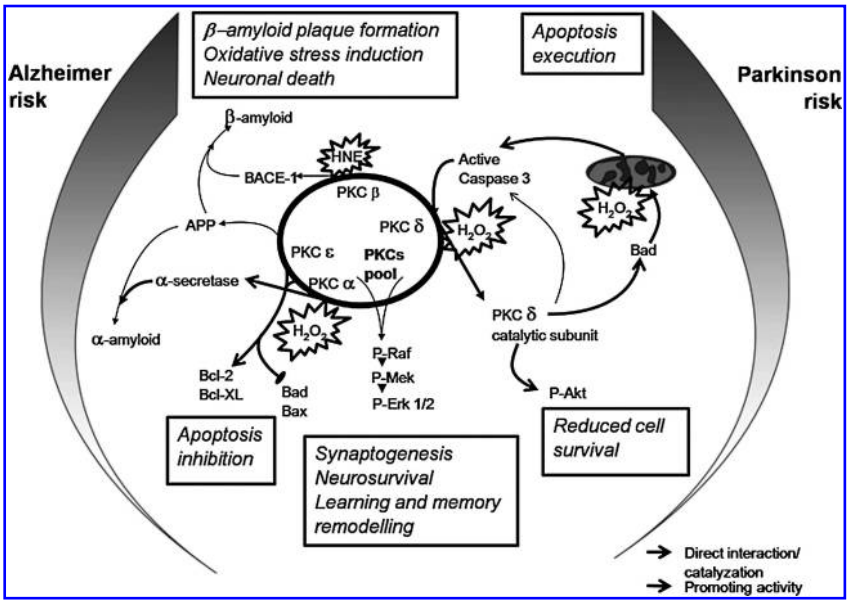

FIG. 9. Schematic representation of the main PKC pathways addressed in this review concerning Alzheimer's and Parkinson diseases. PKCs are commonly targeted to the mitogenic signal transduction pathway Raf/Mek/Erk. During oxidative conditions ("*spiky balloons*") different isozymes directly catalyze (*filled arrows*) or promote (*empty arrows*) effects modulating Alzheimer or Parkinson risk. 129×92 mm (150×150 DPI).

has been demonstrated to be able to induce PKCα translocation to the membrane in a murine model of Alzheimer's disease. In this model, bryostatin not only activated PKC, but also reduced mortality and Aβ formation (65). The molecular model for PKCα involvement is not completely defined. It has been proposed that it could be mediated by direct phosphorylation of Ser^{655} in the APP cytoplasmic tail, in the perimembrane space (115), without affecting APP expression itself, suggesting that PKCα regulates APP cleavage activity or other post-translational modifications (49). Differently, the PKCε isozyme was shown to modulate APP expression (49). Interestingly, APP overexpression induces PKCε downregulation without affecting the levels of other isozymes (149), suggesting a negative feedback mechanism between APP expression and PKCε regulating activity. This evidence, focusing attention on PKC regulation of APP processing, was gathered after PKC activation by drugs or protein overexpression. It would be interesting to reproduce the same observation following PKC activation by oxidative stress. In agreement with this concept, is the development of new compounds that, activating PKC, promote neurosurvival in a cortical neuron oxidative toxicity model. Kozikowski and co-workers (138) developed a series of benzolactam derivatives, some of which promote strong neurosurvival during GSH deprivation. At the same time, these compounds promote PKC activation and α-amyloid synthesis, damaging β-amyloid production. This work provides evidence for a role of oxidative activation, and the relevance of PKC as a target for the treatment of Alzheimer.

As indicated before, regarding synaptogenesis by PKC, it has been shown that PKC activators for specific isoforms could promote memory formation and, at the same time, reduce the accumulation of β-amyloid and tau protein in brain, hinting at a double protective role in the inhibition of Alzheimer progression (249). A different approach—and unexpected results—were published by Paola and co-workers in NT2N differentiated cells. The authors demonstrated that an oxidised product typical of brain tissues damaged by oxidative stress, 4-hydroxynonenal, could activate PKCβII, resulting in increased β-processing of APP (197). Similarly, it was demonstrated that the PKCβI isoform promotes the formation of β-amyloid by APP (230). Recently, new hints have arisen regarding oxidative stress and β-amyloid accumulation. It has been shown that 4-hydroxynonenal could cause the overexpression of BACE1, the β site APP-cleaving enzyme. This modulation requires the JNK/p38 MAPK pathway (257) that has been shown to be regulated by PKCs.

Alzheimer's disease is characterized early on by abnormal levels of oxidative stress (107) and in mouse the β-processing of APP is preceded by oxidative stress (222). It is now possible to draw a plausible scenario—even if hypothetical—for the different PKC isozymes; indeed, the oxidative stress induced by the progression of Alzheimer's disease affects the equilibrium of different PKC isoforms, thus regulating APP expression or α- and β-processing. During Alzheimer's progression, an imbalance is created either by β-processing mediated by altered isozyme expression within brain tissues, or by abnormal oxidative stress production.

Interestingly, a significant amyloidogenic inhibition has been shown by derivatives of rasagiline, a propargylamine already used for the treatment of Parkinson disease (19). These anti-Parkinson drugs could modulate activation of α-secretase and stimulate the α-processing of APP through the MAPK/PKC pathway (64). Rasagiline is able to neuroprotect against a broad variety of neuronal insults and Parkinson-stimulating drugs (*e.g.*, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), 6-hydroxydopamine) by inhibiting the mitochondria-initiated apoptotic pathway (161). It has been demonstrated that, following rasagiline exposure, MAPK is activated, PKCα and ε are translocated to the plasma membrane, and Bcl-2 family members are modulated. This results in the upregulation of Bcl-2 and Bcl-XL, with a parallel downregulation of Bad and Bax, with the corresponding effects on cellular survival. The double effect on cell physiology (on the MAPK pathway and Bcl-2 family) is probably not due to a double affinity of rasagiline for different proteins, but rather to the ability of the PKC family to regulate Bcl-2. PKCα, upon pharmacological stimulation, translocates to mitochondria and promotes phosphorylation of Bcl-2 (233).

The accumulated data on rasagiline have prompted interest in the potential role of the PKC family on Parkinson progression. This disease, like other neurodegenerative disorders, has become increasingly associated with ROS production. Mitochondrial ROS production has been implicated in Parkinson, with many patients showing a deficiency in complex I of the respiratory chain and depletion of GSH in substantia nigra neurons (240). Accordingly, MPTP induces apoptosis in dopaminergic neurons by inhibiting complex I and inducing ROS production. Recently, oxidative stress has been proposed as an activator of PKCδ in dopaminergic neuronal cells. The δ isozyme undergoes phosphorylation of the regulatory domain (53), and is translocated to the membrane (227). It does not appear to be neuroprotective (as described by Maher) and has been proposed as an inducer of neuronal reperfusion injury and apoptosis after ischemia (40). In this model, PKCδ could activate neutrophil adhesion and, after an increase in DAG, promote phosphorylation of Akt (leading to inactivation) and translocation of Bad to mitochondria (facilitating caspase-3 release). In dopaminergic neurons, the phosphorylation of PKCδ (induced by H_2O_2) has been demonstrated to promote its cleavage by caspase-3 (126), leading to the loss of the regulatory domain and causing a constitutive activation of the kinase. Moreover, this situation promotes the activation of caspase-3 (127), thus creating a positive catastrophic feedback loop that leads to progressive neuronal death.

Morover, Kaul *et al.* (126) later showed that PKCδ proteolytic activation during oxidative stress is mediated by phosphorylation of Tyr311. Within the wide group of phosphorylation that could modify PKC activity, Tyr311 appears to be a critical step in this process (136), probably due to its close proximity to the caspase-3 cleavage site. Although the authors did not completely clarify the molecular mechanisms, strong evidence was provided for the involvement of $p60^{src}$ as a catalytic mediator for the phosphorylation at Tyr311 upon oxidative stress.

It is not clear why PKCδ participates in the cell death of such a specific subset of neurons in Parkinson disease, while it affects a broad neuronal pattern in the case of Alzheimer's disease. The reasons for this variability are probably connected to the molecular actors in each specific oxidative stress situation (for a more elucidating review, see (147)). The aim of this section was to indicate that, independently of the exact mechanism of action, the balance between different PKC isoforms is fundamental in neuronal pathophysiology.

Moreover, it is now clear that selective inhibitors or activators are real tools for the development of efficient therapeutic strategies against a very large group of neuronal dysfunctions.

VII. PKCε and δ: Role in Ischemic Preconditioning and Ischemia/Reperfusion Injury

As discussed in other parts of this work, the PKC family of serine/threonine kinases are involved in many different signaling processes. Within the nPKC subfamily, PKCε and δ show a high degree of homology and similar substrate specificity (190), suggesting that they may have similar targets in signal transduction pathways. This part focuses on these two Ca^{2+}-insensitive PKC isoforms, particularly on the opposing roles played in ischemic preconditioning and ischemia/reperfusion injury.

Myocardial ischemia occurs when blood flow to the heart is impaired, such as during acute myocardial infarction or cardiac surgery; there are two distinct components of damage: (i) *ischemic injury*, which results from the initial loss of blood flow and (ii) *reperfusion injury*, which occurs upon restoration of oxygenated blood flow. Reperfusion is a prerequisite for cellular survival but, paradoxically, it can exacerbate the damage that occurs during the ischemic period (the severity of which depends on the duration of the preceding ischemia and the efficiency of blood flow at reperfusion). This reperfusion injury results in necrotic and apoptotic death of cells that were only moderately injured during the preceding ischemic insult. Increases in cellular [Ca^{2+}] and ROS, initiated in ischemia and then amplified upon reperfusion, are thought to be the main causes of reperfusion injury.

The most potent known mechanism of protecting the heart from ischemia/reperfusion is by 'preconditioning' the heart with brief periods of intermittent ischemia and reperfusion prior to the prolonged ischemic event (262). This protocol was first shown to offer strong protection against reperfusion injury in the dog heart by Murry *et al.* in 1986 (176) and has since been confirmed in all species investigated, including humans (134). Ischemic preconditioning (IPC) protects the heart from ischemia and reperfusion-induced damage by inducing intracellular signaling pathways, which increase cellular resistance to ischemia/reperfusion, thus reducing infarct size. Several triggers released from the heart during the preconditioning ischemia have been proposed for IPC, including adenosine (as a result of metabolic breakdown of ATP), bradykinin, and an endogenous opioid (62) (Fig. 10, upper part). These three ligands occupy their respective G protein-coupled receptors (GPCRs), which ultimately work in parallel to activate PKC through different pathways. While adenosine couples directly to PKC through the phospholipases, bradykinin and opioids do it through a complex pathway that includes, in order: PI3-kinase, Akt, endothelial NOS, guanylyl cyclase, PKG, opening of mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP} channels) (see below), and activation of PKC by redox signaling. There is accumulating evidence supporting a role for the modest increase in ROS (produced by mitochondria during the IPC protocol) in the activation of PKC (129). ROS are known to activate PKC in the isolated heart and IPC can be prevented if free radical scavengers are present during the preconditioning phase (130). ROS mediate signal transduction in the early phase of IPC acting as second messengers to trigger PKC through redox signaling: oxidation of critical cysteine residues on PKC isoforms is known to cause their activation and thus provides a mechanism by which ROS could turn on PKC.

As discussed above, initial reports suggested that the activation of PKC is cardioprotective and may mediate ischemic preconditioning-induced protection (280). However, the exact role of PKC in the survival of cardiac cells has remained controversial. Indeed, different (and sometimes opposing) roles in the process have been reported for specific PKC isozymes (75, 268). Ping *et al.* demonstrated that all 11 isoforms of PKC are present in rabbit myocardium and that IPC activates the ε and η isoforms (203). Subsequent studies demonstrated that the lack of PKC isozyme-selective tools made it difficult to discern the role of PKC in preconditioning and ischemia/reperfusion injury. The use of isozyme-specific PKC knockout and overexpression in transgenic mice, as well as isozyme-specific inhibitors and activators [recently developed by Mochly–Rosen's group (169)], have helped to address this question. Isozyme-specific PKC activators are short peptides that induce translocation of a specific PKC isozyme by mimicking the action of the respective isozyme-selective anchoring protein, RACK, on the isozyme; these peptides are called pseudo-RACKs. Isozyme-specific inhibitors are peptides derived from the RACK-binding site on a PKC isozyme (6–8 amino acids long). These peptides prevent the binding of each PKC isozyme to its RACK [reviewed in (246)]. Using this approach, it has been found that selective activation of PKCε confers cardiac protection, whereas selective inhibition of PKCε abolishes protection induced by IPC (58). Additional indication for the critical role of PKCε in cardiac protection was obtained from genetically modified mice. Mice overexpressing PKCε in their hearts are protected from ischemic damage, and PKCε knockout mice are unresponsive to both ischemic and pharmacological preconditioning, further confirming the essential role of PKCε in this process (119, 239). In contrast to the cardiac protective role of PKCε, selective activation of PKCδ (with the PKCδ-selective activator peptide) caused increased damage from ischemic insults both in neonatal cardiac myocytes and in adult isolated rat cardiac myocytes (*ex vivo* and in isolated hearts infused with the PKCδ-selective activator prior to ischemia). Conversely, inhibition of PKCδ with the selective inhibitory peptide resulted in protection (38). Based on these studies, it is now evident that PKCε and PKCδ have distinct temporal and opposing roles in regulating myocardial damage induced by ischemia/reperfusion. Activation of PKCε before ischemia protects the heart by mimicking preconditioning, whereas inhibition of PKCδ during reperfusion protects the heart from reperfusion-induced damage [reviewed in (28)]. How can two highly homologous PKC isoenzymes play such opposing roles? Although the signaling mechanisms that link PKC to ischemic preconditioning and ischemia/reperfusion injury are not completely characterized, several pathways have been proposed. The following section will analyze separately the mechanisms by which PKCε and δ exert their opposing effects.

As stated, PKCε plays a critical role in signaling pathways that protect the heart from ischemia/reperfusion. Upon activation, PKCε translocates to multiple subcellular sites. Previous studies suggest that mitochondrial translocation of PKCε is required for cardioprotection. Emerging evidence also suggests that the cardioprotective target of PKCε resides

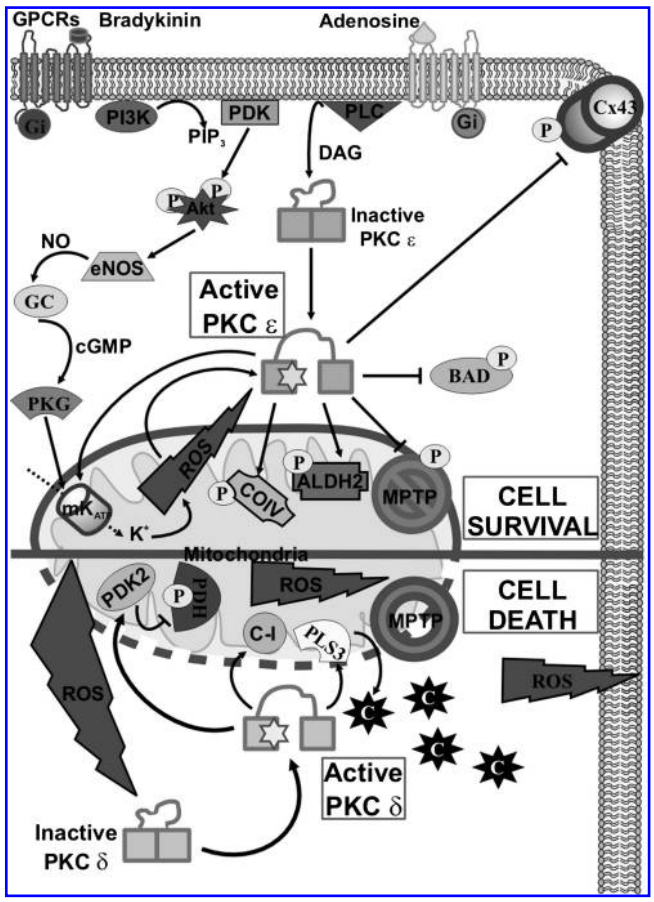

FIG. 10. Schematic representation of the molecular mechanisms by which PKCε and δ exert their opposing effects in ischemic preconditioning and ischemia/reperfusion injury. During ischemic preconditioning (*upper part*), several stimuli (including adenosine, bradykinin, endogenous opioids, and increase in intracellular ROS) induce PCKε activation and its translocation into mitochondria. This leads to: i) mitoKATP channels opening (thus preserving mitochondrial function and generating local ROS which can further activate PKCε in a positive feedback mechanism); ii) inhibition of mPTP opening; iii) activation of COIV; and iv) phosphorylation of ALDH2. PKCε activation also induces phosphorylation of Bad (which renders Bad unable to participate in apoptosis) and Cx43 (causing a reduction in gap junction permeability). This cardioprotective signaling pathway promotes cell survival and ultimately results in protection from ischemia/reperfusion injury. In contrast, the massive increase of intracellular ROS that occurs during ischemia/reperfusion damage (*lower part*), leads to PKCδ activation and its translocation to mitochondria. Here it regulates mitochondrial function and further ROS generation through: i) activation of PDK2, allowing phosphorylation and inactivation of PDH; ii) activation of NADPH oxidase-like activity of complex I (C-I); and iii) activation of PLS3, resulting in increased cardiolipin oxidation and cytochrome c (C) release. Moreover, PKCδ-mediated elevation in ROS can lead to mPTP opening, with resultant loss of membrane potential and cytochrome c release, triggering cell death.

in mitochondria. These organelles mediate diverse cellular functions including energy generation and play an important role in intracellular signaling through the generation of ROS and regulation of intracellular Ca^{2+} signaling (97). In addition, mitochondria are key regulators of cell viability and play a central role in both the necrotic and apoptotic cell death pathways. Proposed mitochondrial targets of PKCε include mitoK_{ATP} (46), components of the mPTP (11), ROS generation, and components of the electron transport chain (188). The mitoK_{ATP} channels, located in the inner mitochondrial membrane (IMM), have emerged as important components of the IPC signaling pathway (113). These channels are closed under normal conditions but, as ATP levels decline, open during periods of metabolic stress. Although the mechanism remains controversial, the opening of mitoK_{ATP} channels results in an increased mitochondrial K^+ flux (174) that is thought to confer cardioprotection by regulating either (i) mitochondrial Ca^{2+} uptake (106), (ii) mitochondrial matrix volume (59), or (iii) mitochondrial ROS production (186). A mechanistic link between mitoK_{ATP} and PKC was established when it was found that activation of PKC with PMA could potentiate the effects of diazoxide (a specific mitoK_{ATP} channel opener) in isolated cardiomyocytes (238). Other studies have demonstrated that mitoK_{ATP} opening may be the source of mitochondrial ROS, suggesting that it may serve as a trigger, rather than an end-effector of IPC (139). Recent studies have demonstrated that mitoK_{ATP} and PKCε directly interact in the IMM, that PKCε is required for the opening of mitoK_{ATP}, and that PKCε activators induce the opening of mitoK_{ATP}, while its inhibitors and a protein phosphatase reverse these effects. These studies suggest that mitochondrial PKCε may also contribute to cardioprotection by regulating mitoK_{ATP} channel opening. Identification of the molecular components of this channel is necessary to reveal the PKCε phosphorylation site. Another proposed mitochondrial target for PKCε is the mPTP. Under normal physiological conditions, the IMM is almost impermeable to metabolites and ions. However, on reperfusion, a large nonspecific pore known as the mPTP can open, permeabilizing the IMM (98). The consequences of sustained mPTP opening are mitochondrial swelling, release of mitochondrial proteins (such as cytochrome *c*) and uncoupling of oxidative phosphorylation, that ultimately causes the collapse of the mitochondrial membrane potential, resulting in cell death. The exact structure of mPTP is an area of current controversy and not all studies are in agreement (79). It is currently thought that the pore may be composed of ANT (adenine nucleotide translocase) in the IMM, VDAC (voltage-dependent anion channel) in the outer mitochondrial membrane (OMM) and cyclophilin D in the mitochondrial matrix (219). In isolated adult rat cardiomyocytes, preconditioning increased the time necessary to induce mPTP opening and was associated with translocation of PKCε to the mitochondria. Blockade of ROS with the scavenger *N*-acetyl-l-cysteine prevented PKCε translocation to the mitochondria and also the inhibition of mPTP. Also in the same report, it was shown how photoexcitation of a discrete cellular area induced local ROS production, which in turn was found to induce PKCε translocation (120). Thus, local ROS/redox signaling may stimulate mitochondrial translocation of PKCε, resulting in the inhibition of mPTP opening, thus reducing necrotic cell death on reperfusion. Moreover, PKCε has been demonstrated to interact with putative components of the mPTP in

heart mitochondria. Immunoprecipitation of mouse cardiac mitochondria revealed that PKCε physically associates with both ANT and VDAC and that PKCε can phosphorylate VDAC (11). It is therefore possible that PKCε phosphorylates mPTP during ischemia, preventing mPTP from opening at reperfusion. Other studies have identified COIV (cytochrome *c* oxidase subunit IV) as a substrate for PKCε. *In vitro* phosphorylation of COIV, induced by PMA, is blocked by the PKCε-selective inhibitory peptide (188). Furthermore, hypoxic preconditioning of neonatal cardiomyocytes induces coprecipitation of PKCε and COIV, resulting in phosphorylation of COIV and a 4-fold increase in cytochrome *c* oxidase activity; this activity was attenuated by the PKCε-selective inhibitory peptide (94). Taken as a whole, these findings suggest that PKCε interacts with COIV in cardiac mitochondria to enhance electron transport chain activity, modulating myocardial bioenergetics and increasing ATP synthesis, thus providing enhanced resistance to ischemic injury.

A further player with which PKCε is involved is connexin 43 (Cx43). Cx43 was immunoprecipitated with PKCε and co-localized at gap junctions. PKCε was demonstrated to phosphorylate Cx43 in rat cardiomyocytes (55) and in human heart (25), causing a reduction in gap junction permeability, which prevented the spread of injury to adjoining cells. Recent studies have reported that a fraction of Cx43 is located in mitochondria and that mitochondrial Cx43 levels increase following IPC (23). At present, however, a mechanistic role for mitochondrial Cx43 in IPC remains undefined.

Lastly, some studies have suggested that PKCε may also regulate the levels of the pro- and anti-apoptotic Bcl-2 family; this may be a way in which the damage from ischemia and reperfusion is propagated. For example, PKCε activation was found to induce indirectly the phosphorylation of Bad, thus preventing Bad from participating in apoptosis (15). In other systems, PKCε activity may also regulate the expression of anti-apoptotic Bcl-2 (92). This may help explain how PKCε is cardioprotective during preconditioning. As a final point, a recent and interesting report from Mochly–Rosen's group should be noted (169). This group found that aldehyde dehydrogenase 2 (ALDH2) is another mitochondrial target of PKCε. It was established that PKCε phosphorylates ALDH2, resulting in an increase of ALDH2 catalytic activity, which correlates with cardiac protection from ischemia. Further, co-immunoprecipitation experiments confirmed the association of ALDH2 and PKCε in the mitochondrial fraction. It is therefore likely that PKCε can enter mitochondria, phosphorylate ALDH2 directly and, in so doing, induces cardioprotection (32). All these data suggest that a concerted, integrated response, rather than phosphorylation of one exclusive target, confers PKCε-mediated cardioprotection.

Regarding PKCδ, its inhibition protects against reperfusion-induced cell damage by preventing both necrotic and apoptotic cell death decreasing infarct size by ~80% (112). PKCδ activation and translocation to mitochondria was shown to play a role in reperfusion-mediated damage: it negatively regulates mitochondrial function and induces apoptosis or necrosis (175). This is accomplished by a diminished ADP-linked mitochondrial respiration (43), decreased tricarboxylic acid cycle activity (42), lowering of the cellular pH and decline in the rate of ATP production (112), increased generation of ROS (43), and induction of apoptosis through the release of cytochrome *c* (175). During ischemia/reperfusion, mitochondria are a major source of ROS: in the early minutes of reperfusion, total myocardial H_2O_2 levels increase by ~600% (244). Enhanced oxidative stress activates PKCδ through oxidation of key redox-sensitive residues within the enzyme and/or activation of PKCδ-phosphorylating enzymes. Konishi *et al.* were the first to show that treatment of COS-7 cells with H_2O_2 results in phosphorylation and activation of PKCδ (135). Moreover, H_2O_2 treatment of perfused hearts as well as hearts subjected to cardiac reperfusion show significant translocation of PKCδ to mitochondria (43). In addition to ROS activating PKCδ, the generation of ROS is in turn controlled by PKCδ. To support this hypothesis, knockout mice lacking PKCδ exhibit a loss of ROS formation by the endothelium when subjected to cell stress agents such as UV and TNF-α, and were resistant to death by the ROS generator H_2O_2 (143). Mitochondrial ROS-generating targets for PKCδ have yet to be identified. However, several interesting possibilities exist. First, activation of pyruvate dehydrogenase kinase 2 (PDK2) leads to phosphorylation and inactivation of PDH (pyruvate dehydrogenase), causing enhanced fatty acid oxidation and anaerobic glycolysis, with reduced ATP regeneration (217). Second, direct activation of NADPH oxidase-like activity of complex I: PKCδ may regulate some NADPH oxidase-like function within cardiac mitochondria to increase ROS generation. A third possibility could be the activation of phospholipid scramblase-3 (PLS3, an enzyme responsible for flipping phospholipids from the IMM to the OMM), resulting in increased cardiolipin oxidation and cytochrome *c* release (150). In all three cases, cellular oxidative stress would be expected to increase, further activating PKCδ and resulting in a positive feedback loop. If the stimulus is strong enough, ROS levels would become so elevated that mPTP opening would occur, with resultant loss of membrane potential, further amplification of ROS production, ultimately leading to apoptotic or necrotic cell death. Propagation of the injury can occur as ROS diffuse to neighbouring cardiomyocytes (287). In this way, PKCδ could be defined as a "redox transducer" regulating a tunable system in which apoptosis will only be triggered if the initial stimulus is large enough to evoke a sustained response. Indeed, numerous antioxidants have been shown to provide partial cardioprotective benefit from ischemic reperfusion injury (100).

The central role of oxygen free radicals in the development of reperfusion injury led to a widespread interest in the use of antioxidant therapy to attenuate reperfusion injury (265). Antioxidants have been tested in several experimental and clinical models with mixed success (213). Despite positive observations in classic models of experimental ischemia and reperfusion, clinical experience with antioxidants has been disappointing. Indeed, therapy with human recombinant superoxide, designed to attenuate angioplasty-induced reperfusion injury, demonstrated no beneficial effects (72); although this may be related to cell impermeability, this study cast a shadow on the development of antioxidant strategies for reperfusion injury. Even the protective effects of vitamin E (α-tocopherol), the major lipid-soluble antioxidant, are controversial. Many investigations demonstrated the importance of vitamin E for protection against cardiac ischemia-reperfusion injury using vitamin E deficient animal models (13, 117, 133). Nevertheless it requires prolonged and very high levels of oral treatment to achieve cardiac concentrations that are protective from reperfusion injury (100).

Another proposed mechanism suggests that PKCδ translocation to the mitochondria facilitates the accumulation and dephosphorylation of the pro-apoptotic Bad, and thus increases cytochrome *c* release (175).

At this point, the unavoidable question is why nature has evolved such distinct temporal and opposing roles for these very similar enzymes. One possible explanation is that PKCε is activated when there is only a mild ischemic insult and a reasonable chance of cellular survival. Conversely, PKCδ is called upon to activate apoptotic and necrotic killing in the case of cells severely damaged by ROS, thus preventing the spread of injury. Research into the underlying cardioprotective signaling pathways is ongoing. Harnessing the distinct temporal relationship between PKCε and PKCδ is already a realistic therapeutic goal in the treatment of myocardial ischemia/reperfusion injury.

VIII. PKC in Diabetes

Diabetes mellitus is a medical chronic condition characterized by high blood glucose levels (hyperglycemia) and caused by inherited and/or acquired deficiency in pancreatic secretion of insulin, as well as a decrease of insulin action (insulin resistance). Hyperglycemia has been shown to be the major causal factor in the development and progression of diabetic vascular complications in different parts of the body, in particular the eyes, kidneys, nerves, and heart. Systemic complications are the major cause of morbidity and mortality in patients with diabetes. The molecular mechanisms inducing the development of vascular dysfunction due to hyperglycemia are being intensively studied, and multiple theories have been proposed to explain the pathogenesis of the various complications. Our main goal in this section is to explain and encompass most of the data that have accumulated in this area, highlighting the essential role of ROS and PKC activation in diabetes (Fig. 11). A possible linking element between many of these phenomena might be an increased production of ROS within vascular cells under conditions of hyperglycemia (182). During diabetes or insulin resistance, failure of insulin-stimulated glucose uptake by fat and muscle causes the glucose concentration in blood to remain high. Consequently, glucose uptake by insulin-independent tissues increases. Increased glucose flux both enhances oxidant production and impairs antioxidant defences. Different groups have shown that hyperglycemia increases mitochondrial ROS production and subsequent oxidative stress, and that this occurs before the onset of other complications in diabetes (229). There is also evidence, both from *in vivo* and *in vitro* studies, that lowering oxidative stress with antioxidants like vitamin E can attenuate or even prevent several diabetic complications, such as nephropathy and retinopathy. Chronic or excessive increase in the production of oxidants can adversely affect cellular physiology, interfering with the normal function of the tissues affected. Indeed, hyperglycemia-induced oxidative stress does not only damage cells directly by oxidizing DNA, proteins and lipids, but also indirectly by activating a variety of stress-sensitive intracellular signaling pathways (such as NF-κB, p38 MAPK, JNK, and p53) (10, 125, 145, 180). Chronic activation of these pathways is associated with the late complications of diabetes.

Of all the serine kinase cascades activated by ROS, several studies have suggested that PKC is one of the most important in diabetes (51,137). Oxidants are known to activate PKC either directly (84) or by increasing the intracellular concentration of the lipid DAG (183), an intermediary in glucose and fat metabolism, partly due to a *de novo* synthesis from glycolytic intermediates (272). In particular, recent studies have identified that the activation of PKC and increased DAG levels are associated with endothelial dysfunction in retinal, renal, and cardiovascular tissues. Increasing evidence supports the fact that PKC is involved not only in microvascular complications but also plays a role in several mechanisms that promote atherosclerosis (218). The mechanisms by which glucose/ROS-induced activation of PKC causes diabetic complications are manifold and include: the increase in the production of extracellular matrix and cytokines; the enhancement of contractility, permeability, and vascular cell proliferation; the induction of the activation of cytosolic phospholipase A2; and the inhibition of Na^+-K^+-ATPase (132).

More than one PKC isoform is activated by hyperglycemia, since many isoforms are DAG sensitive, and each cell usually

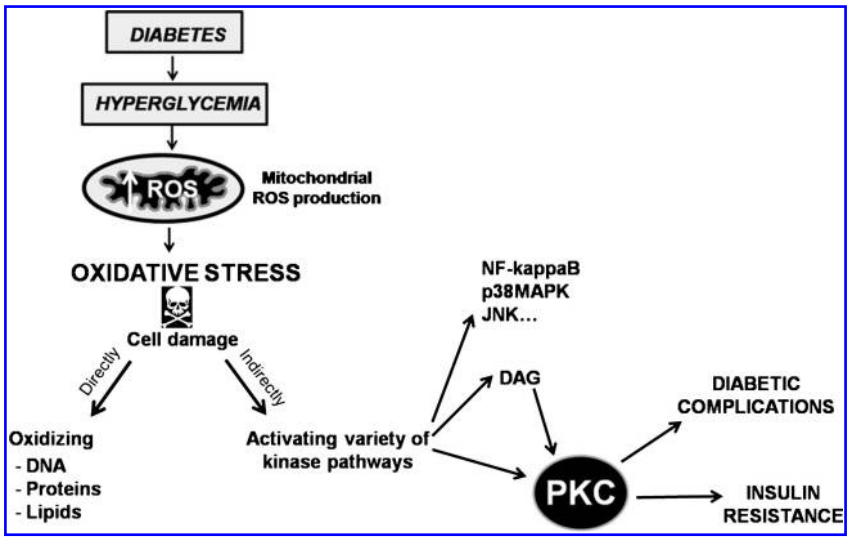

FIG. 11. Oxidative stress-dependent activation of PKC in diabetic conditions. Diabetes causes hyperglycemia that induces oxidative stress through increase of mitochondrial ROS production. Oxidative stress causes cell damage both directly and indirectly through activation of different pathways. One of these involves PKC that in turn promotes insulin resistance and diabetic complications.

contains several PKC isoforms (184). The molecular mechanisms that induce the development of vascular dysfunctions are currently the object of intense studies but are still poorly defined. In recent years, it has been shown that the PKC (isoform is predominantly and persistently activated in all vascular tissues and may be responsible for many of the vascular dysfunctions. In endothelial cells, PKCβ activation leads to basal membrane thickening, extracellular matrix expansion, increased endothelial permeability and cell turnover (132, 272). To determine whether PKCβ activation is important for the development of diabetic complications, *in vitro* and *in vivo* experiments were performed using specific inhibitors of this isoform. Ruboxistaurin, a PKCβ selective inhibitor, has been demonstrated to attenuate diabetes-induced disturbances, showing particular promise in clinical studies of diabetic retinopathy and nephropathy (114, 264). It is known that, in resting conditions, PKCβ is uniformly localized in the cytosol. Upon cell stimulation that induces PKC activation, the kinase translocates from the cytosol to the plasma membrane; in physiological conditions, PKCβ returns completely to the cytosol once the stimulation is over. Avogaro and coworkers demonstrated that in diabetes the (isoform is persistently activated and localized on the plasma membrane (Fig. 12). In other words, hyperglycemia inhibits the return of PKCβ to the cytosol through an unknown mechanism. They also demonstrated that metformin, a widely used antidiabetic agent, inhibits the translocation of PKCβ induced by hyperglycemia, because of a direct antioxidant effect (78). From these data, it is clear that the PKCβ pathway could be a logical candidate as new target for prevention and treatment of diabetes complications.

Very recently, it has been demonstrated that the same mechanism of glucose/ROS-induced PKCβ translocation represents also the signaling route in stem cell differentiation to adipocytes (Fig. 12). As reported in more detail below, high glucose *per se* has an adipogenic potential on stem cells residing in both adipose tissue and skeletal muscle via ROS. Indeed, ROS, through downstream effectors and in particular PKCβ translocation, lead to the neoformation of adipose cells. Currently, the search for the signal transduction pathways that operate in obesity and diabetes is of great interest. Moreover, a direct link between hyperglycemia and the increase in adiposity highlights a feed-forward cycle, which may play a key role in the progression of metabolic dysfunction into an irreversible diabetic state (1).

As an antidiabetic drug, metformin is used for its ability to lower the blood glucose level in diabetes, mainly through suppression of hepatic glucose production (111, 275). It has been shown that metformin controls glucose production in the liver inducing phosphorylation of the transcriptional coactivator CREB binding protein (CBP) through the activation of aPKCι/λ (102). Thus, CBP phosphorylation suppresses gluconeogenesis and increases glucose transport in muscle, and it could thus represent a critical step for pharmaceutical intervention. These data are in agreement with those reporting that muscle-specific PKCλ knockout mice mimic most of the features of the metabolic syndrome, including impaired glucose tolerance and diabetes (67). This scenario shows clearly the important role of PKC in the pathogenesis of diabetes, as well as the double molecular effect of metformin as an antidiabetic agent. Indeed, metformin on one hand acts on vascular tissues attenuating PKCβ translocation and, on the other hand, controls hepatic glucose production activating aPKCι/λ.

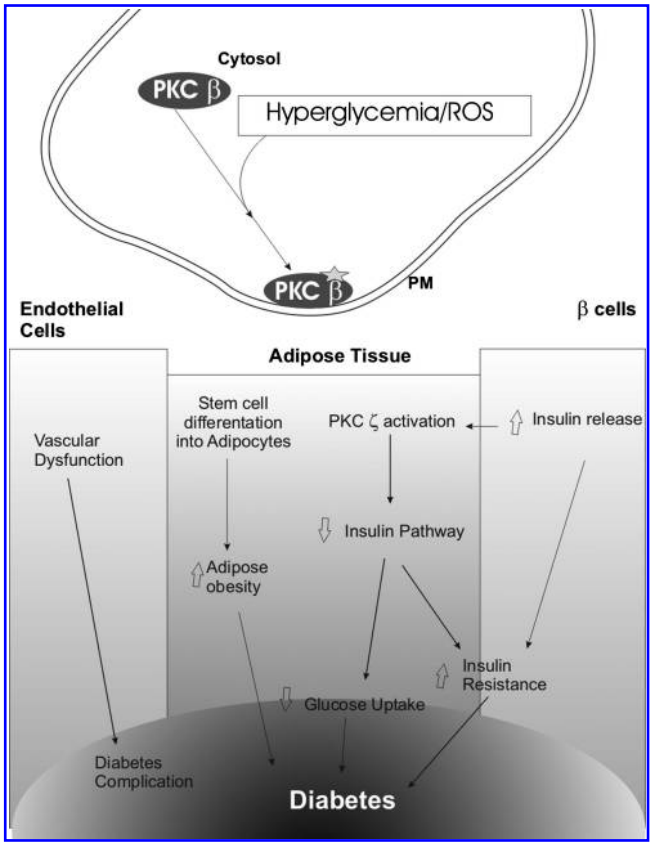

FIG. 12. Role of PKC pathways in diabetes and insulin resistance. A condition of hyperglycemia/ROS causes activation and translocation of PKCβ to plasma membrane (PM). This effect reflects on diabetes development in different ways on distinct cell lines. In endothelial cells, a condition of PKC hyperactivated results in vascular dysfunctions with consequent diabetes complications. In β-cells, that condition defines an increase in insulin release following activation of PKCζ in the adipose tissue. Moreover, in this tissue occurs a higher stem cell adipose differentiation that shapes in adipose obesity. All these effects modified the insulin pathway promoting diabetes aggravation. 112×146 mm (200×200 DPI).

In addition to these pathways, there is a large number of reports about the effect of PKC activation by insulin (66, 74, 116). Moreover, emerging evidence supports also an important role of ROS production and oxidative stress in various forms of insulin resistance (63). Both insulin resistance and decreased insulin secretion are major features of the pathophysiology of type 2 diabetes (221). In a person with normal metabolism, insulin is released from the (cells of the Islets of Langerhans located in the pancreas and tells insulin-sensitive tissues in the body (*e.g.*, muscle, adipose tissue) to absorb glucose. It has been demonstrated that an elevated glucose concentration stimulates insulin secretion from (cells through the activation and translocation of PKCβ to the plasma membrane. Thus, localized changes in PKCβ activity play a role in the spatial control of insulin exocytosis, interacting with proteins of the secretory machinery (209) (Fig. 12).

Insulin resistance can be defined as an attenuated effect of insulin in target tissues, mainly muscle, fat, and liver (122). In

these tissues, insulin resistance is manifested as impaired glucose uptake with the result that glucose levels stay higher than normal. To compensate for this, the pancreas is stimulated to release more insulin. The elevated insulin levels then cause further biological effects throughout the body. Although immense efforts have been made to elucidate the mechanisms underlying insulin resistance, there is still no consensus regarding the nature of the exact defect at the cellular and molecular level. At the cellular level, it has been proposed that excessive circulating insulin contributes to insulin resistance via the downregulation of insulin receptors and GLUT4 receptors (type four glucose receptors); this leads to a greater need for insulin, which in turn decreases the number of receptors further. It was observed that the activity of membrane-bound PKC, in particular the (isoform, was increased in insulin-resistance conditions. Since it was demonstrated that insulin receptor tyrosine kinase activity is impaired in skeletal muscle from insulin-resistant patients, Dohm and colleagues proposed that PKC may phosphorylate that receptor, thus decreasing its activity (116). Moreover, it has been proposed that PKCζ can phosphorylate insulin receptor substrate (IRS) proteins on their serine residues; these are the primary substrates for the insulin receptor (142, 151, 220). This phosphorylation by PKCζ results in a down-regulation of IRS function and its dissociation from the insulin receptor, providing a possible mechanism for the induction of an insulin-resistant state (Fig. 12).

In conclusion, although the mechanisms that cause insulin resistance and diabetes are far from being fully understood, it appears clear that a complex interplay of different mechanisms converge on a common pathway with oxidative balance and PKC activity being highly deregulated.

IX. PKC and Calcium

In all eukaryotic cells, Ca^{2+} regulates a number of different cellular functions. The cytosolic concentration of Ca^{2+} ions ($[Ca^{2+}]_c$) is tightly controlled by complex interactions among transporters, pumps, channels, and binding proteins (44, 228). When the systems responsible for the regulation of cellular Ca^{2+} homeostasis are irreversibly compromised, a cell is condemned to die (204). Ca^{2+} is a crucial activator of some PKC isoforms, and PKC-dependent phosphorylation reactions have been shown to modify the spatio-temporal pattern of cellular Ca^{2+} responses (205). One of the first links between PKC and Ca^{2+} was provided by Montero *et al.* in 2003 (171) during a study about the modulation of histamine-induced Ca^{2+} release from the endoplasmic reticulum (ER) in HeLa cells. The kinetics of ER Ca^{2+} release is biphasic, with a fast initial phase, followed a few seconds later by a much slower one (170). In the same study, it was shown that PKC is responsible for the shift from the fast to the slow phases of Ca^{2+} release during histamine action, and that inhibition of PKC with staurosporine and other selective inhibitors (GF109203X and Ro-31-8220) transformed the biphasic kinetics into a monophasic one. Inhibition of PKC increases Ca^{2+} release during each oscillation, leading to an increase in the amplitude of the oscillations. In this condition, mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) was much more sensitive to changes in ER-Ca^{2+} release induced by PKC modulation than $[Ca^{2+}]_c$. Indeed, PKC inhibitors increased the histamine-induced $[Ca^{2+}]_m$ peak by 4-fold but increased the $[Ca^{2+}]_c$ peak only by 20%. On the contrary, stimulation of PKC with phorbol-12,13-dibutyrate produced a large inhibition of Ca^{2+} release and inhibited the $[Ca^{2+}]_m$ peak by 90% and the cytosolic one by only 50%. As a conclusion, it was suggested that activation of PKC by histamine could modulate Ca^{2+} release through IP3 receptors (IP3Rs) (Fig. 13).

IP3Rs are a family of Ca^{2+} channels of the ER, widely distributed in different tissues. In mammalian cells, three subtypes of IP3R exist (IP3R-1, IP3R-2, and IP3R-3), and are derived from three distinct genes. The actual role of each subtype in physiological Ca^{2+} signaling remains to be established (77). Despite their relatively high sequence homology, the three IP3R subtypes differ in many points, including their affinity for IP3, their tissue distribution, and possibly their regulatory mechanisms and specific physiological roles (202). These channels become activated when agonists acting on specific plasma membrane receptors activate phospholipase C, which cleaves PI 4,5-bisphosphate to generate IP3 and DAG. IP3 rapidly diffuses inside the cell and triggers the opening of the IP3R, thus releasing the Ca^{2+} stored in the ER into the cytosol and generating a transient increase in $[Ca^{2+}]_c$. Simultaneously, a part of the released Ca^{2+} is taken up by mitochondria in close proximity to the ER, thus generating also a transient peak in $[Ca^{2+}]_m$. At the same time, DAG activates PKC and within a few seconds the kinase phosphorylates a modulatory protein to down-regulate Ca^{2+} release. It has been proposed that one possible candidate target of PKC phosphorylation is the IP3R itself.

The first correlation between PKC and IP3R was demonstrated in the early 1990s: originally, it was reported that the IP3R was phosphorylated by cyclic AMP-dependent protein kinase (PKA) (252); afterwards, Ferris *et al.* demonstrated that the IP3R was stoichiometrically phosphorylated by PKC and Ca^{2+} calmodulin-dependent protein kinase II (CaM kinase II) (68). Recently, it was also demonstrated that PKC can phosphorylate all three IP3R isoforms and, as a consequence, modulates Ca^{2+} release (68). Caron *et al.* (29) investigated a regulatory mechanism by which PKC may influence IP3R-3-mediated Ca^{2+} release using RINm5F cells which express almost exclusively ($\sim$90%) IP3R-3. Using spectrofluorometric Ca^{2+} measurement assays, it was shown that PKC decreases IP3-induced Ca^{2+} release and it was concluded that PKC exerts a negative regulatory role on intracellular Ca^{2+} mobilization. This effect of PKC is likely a protection against potential harmful consequences of excessive Ca^{2+} elevation. Arguin *et al.* (8) demonstrated that PKCs influence IP3R-2-mediated Ca^{2+} release. Using AR4-2J cells, which express almost exclusively (86%) IP3R-2, it was shown that that endogenous PKC phosphorylates IP3R-2 and decreases IP3-induced Ca^{2+} release, thus exerting a negative feedback mechanism through which it controls intracellular Ca^{2+} elevation. At the same time, it was hypothesized that PKCs phosphorylate also IP3R-1, but with an opposite effect [*i.e.*, increasing Ca^{2+} release (210)].

These results suggest that the regulatory effect of PKC on Ca^{2+} release is qualitatively different depending on the specific subtype of IP3R that is phosphorylated. It is important to remember that IP3R phosphorylation represents a key event in signal transduction of ER stress. As described previously, ER stress is a condition that triggers ROS production that in turn results in apoptosis. Although direct evidence exists regarding the involvement of PKC in IP3R phosphorylation,

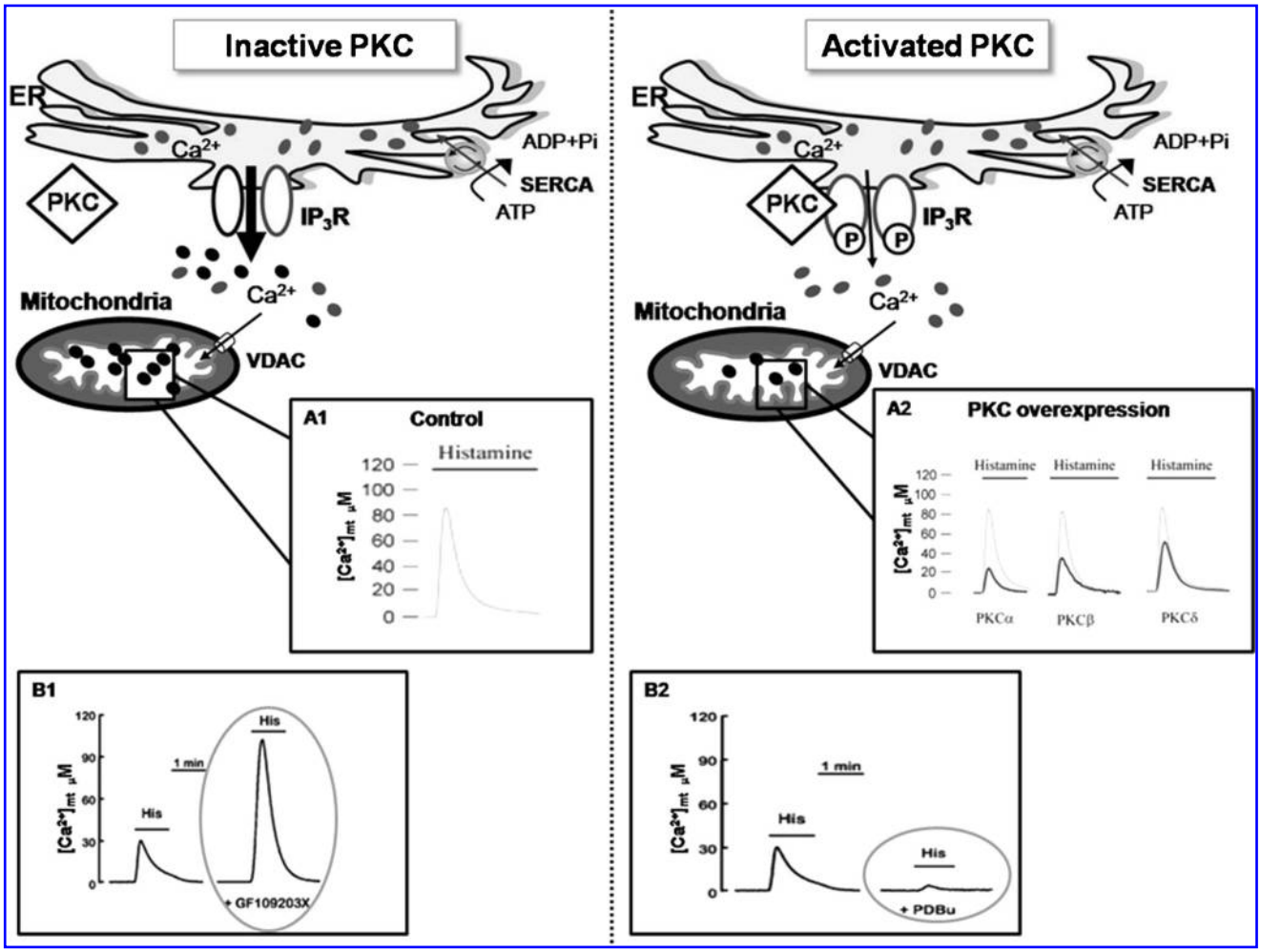

FIG. 13. Schematic representation of PKC effects on Ca^{2+} homeostasis. (**A1**, **A2**) PKC is normally inactivated, after its activation/overexpression the IP3R is phosphorylated; consequently, there is a reduced release of Ca^{2+} from the ER and also a smaller Ca^{2+} uptake into mitochondria, as compared to the control (217). (**B1**, **B2**) Effects of GF109203X (specific inhibitor) and phorbol 12,13-dibutyrate (activator) on histamine-induced $[Ca^{2+}]$m peaks (180).

leading to cell death, a recent work shows that inhibition of PKC activity (*e.g.*, by β-catenin) leads to apoptosis via ER stress (214). This controversial point could potentially be explained by demonstrating different phosphorylation sites in the IP3R.

The modulation of mitochondrial Ca^{2+} signals by PKC isozymes has also been the object of several studies by our group. In particular, using intracellular targeted Ca^{2+} probes of aequorin (207), the effects of various isoforms on organelle Ca^{2+} handling, with special focus on mitochondria, were analyzed (see also Fig. 13). It was concluded that the overexpression of different PKC isoforms induces specific alterations of cellular Ca^{2+} homeostasis (205). In the case of PKCε, no alteration was observed in any of the investigated compartments. However, when the activity of PKCβ, δ, and ζ was enhanced (by overexpression) or inhibited, a large effect on mitochondrial Ca^{2+} uptake was observed; this effect was exclusive to this organelle: although no significant alteration was detected in ER Ca^{2+} release and in cytosolic responses, $[Ca^{2+}]_m$ was reduced by PKCβ, δ and ζ. PKCα overexpressing cells have yielded particularly interesting results, showing a very marked reduction of mitochondrial and cytosolic responses to agonists. Accordingly, a substantially reduced Ca^{2+} release from ER was detected, suggesting a possible direct inhibitory effect of this isoform on IP3R, consistent with data reported before.

X. The PKC–ROS Interplay in the Hormone-Signaling Pathway

Many human hormones transmit their signal through specific GPCRs that lead to the activation of phospholipase C, causing intracellular Ca^{2+} and diacylglycerol release and further activation of PKC members (198). In recent years, an increasing number of studies have pointed at the selective binding and activation of specific PKC isoforms by steroid hormones. Several studies have shown that binding and activation of specific PKC isoforms by steroid hormones is critical for their rapid responses, suggesting that these kinases could act as receptors for these hormones, playing a fundamental role in these specific nongenomic effects (243). One of the first demonstrations of this direct link was the activation

of PKCα, γ, and ε by 1,25(OH)2-vitamin D3, lowering the Ca^{2+} requirements for activation of these isoforms (243).

Likewise, conventional PKCs and PKCζ can be activated by the adrenal androgen dehydroepiandrosterone and dexamethasone. In particular, treatment with this synthetic glucocorticoid led to PKC translocation to the plasma membrane, especially the α and β isoforms (173). Moreover, an article by Harvey and co-workers showed the direct binding of specific PKC isoforms (PKCα and PKCδ) by aldosterone and 17β-estradiol, involving autophosphorylation of these kinases upon direct binding by these hormones (57).

Taken together, these experimental data explain how PKCs may work as receptors for steroid hormones, providing a new rapid mechanism for transducing the rapid effects of these hormones, in addition to the well-known activation of PKC by membrane receptor mediated activation of phospholipase C.

Another class of steroid hormones, highly expressed in the central nervous system, called "neurosteroids", (such as dehydroepiandrosterone and pregnenolone), seems to have an indirect link with PKCs, and this relationship may play an important role in the decline of cognitive and memory functions, typical events that characterize brain aging (199, 215).

Recently, it has been also extensively demonstrated that in the signal transduction system of many human hormones, both PKC and ROS are involved as key players. Below, we will elucidate what is currently known about the interplay between PKC and ROS in the pathways of angiotensin II, thyroid hormones and leptin (Fig. 14).

A. Angiotensin II

The vasopressor octapeptide angiotensin II (Ang II, an endocrine, autocrine/paracrine, and intracrine hormone) exerts homeostatic responses in cardiovascular tissues, including heart, blood vessel walls, adrenal cortex, and liver. Effects of Ang II in responsive tissues are primarily mediated by the seven transmembrane-spanning type 1 angiotensin receptor (AT1). Ang II has been shown to stimulate mitochondrial dysfunction in cardiac, renal, and vascular smooth muscle cells (131), and ROS seems to play key roles in these processes (267).

Doughan and co-workers demonstrated how Ang II stimulates NADPH oxidase via PKC-dependent pathways, which in turn increases ROS production (60). Based on their findings, they suggest that $O_2^{\cdot -}$ reacts with endothelial NO, leading to formation of $ONOO^-$, which can damage respiratory complexes, leading to mitochondrial dysfunction, as evidenced by increased mitochondrial ROS production and decreased mitochondrial membrane potential. Through a positive-feedback loop, the increased mitochondrial H_2O_2 production can lead to further activation of cellular NADPH oxidase, resulting in increased intracellular $O_2^{\cdot -}$ production and diminished endothelial NO bioavailability, thereby promoting vascular oxidative stress. Both NADPH oxidase and PKC inhibitors (Apocynin and chelerythrine, respectively) dramatically attenuate mitochondrial ROS generation in response to Ang II. These actions of Ang II on mitochondrial function raise the possibility that mitochondria-targeted antioxidants might prevent endothelial dysfunction and be beneficial in diseases such as hypertension and atherosclerosis.

In hepatocytes, Ang II activates a diacyglycerol-sensitive PKC signal transduction cascade that targets nuclear NF-kappaB1 isoforms (96, 56, and 50 kDa) through a ROS-generating system of an unknown type. This signal activates NF-kappaB1 DNA-binding activity present in the nucleus and is associated with transcriptional activation. It was found that PKC downregulation or addition of the free-radical scavenger dimethylsulfoxide blocked the transcriptional effects of Ang II. These data indicate a requirement for PKC and ROS also in hepatocytes in Ang II-inducible NF-κB transcriptional activity (26).

B. Thyroid hormones

Over the past few years, increasing evidence has been raised which suggests several nongenomic effects of thyroid hormones (52), such as the activation of the signal transduction pathways and the activation of NF-κB by the induction of oxidative stress (258).

The important role of classical PKC isoenzymes in the mechanism of thyroid hormone-induced $O_2^{\cdot -}$ generation was established in the study of Mezosi *et al.* (165). They investigated the rapid nongenomic effects of thyroid hormones (T2, T3, and T4) in human polymorphonuclear leukocytes (PMNLs), which are known as important sources of ROS in the circulation. In these cells, it was found that thyroid hormone is involved in the stimulation of $O_2^{\cdot -}$ production via the activation of the PKC pathway, the induction of $^{125}I^-$ incorporation, and the enhancement of myeloperoxidase activity. Indeed, all tested PKC inhibitors blocked the thyroid hormone-induced $O_2^{\cdot -}$ release in PMNLs, and the most pronounced effect was observed by Ro-32–0432, a specific PKCα and PKCβI inhibitor. These results suggest the existence of a membrane-bound binding site for thyroid hormone in PMNLs and a physiological role for thyroid hormone in cellular defence mechanisms by stimulating free radical production.

C. Leptin

Leptin is a 16-kDa peptide hormone produced mainly by adipocytes that plays a central role in the regulation of body weight (194). Recently, a role for leptin in the control of inflammation was also proposed. Indeed, it has been demonstrated that leptin stimulates the proliferation, differentiation, and functional activation of hemopoietic cells, including monocytes/macrophages, and increases the production of pro-inflammatory cytokines (153, 237). These results and the findings that leptin modulates key processes involved in atherogenesis, including angiogenesis, oxidative stress, vascular calcification, and thrombosis (24, 279), suggest a role for leptin as a potential cardiovascular risk factor in obese and diabetic patients.

Maingrette and Renier demonstrated that leptin increases the expression of human macrophage lipoprotein lipase (LPL, a pro-atherogenic cytokine overexpressed in patients with type 2 diabetes), at both the mRNA and protein levels. Pretreatment of these cells with anti-leptin receptor antibody, PKC inhibitors (calphostin C and GF109203X), or an antioxidant (*N*-acetylcysteine), blocked the effects of leptin (157). In accordance with the results of Takekoshi *et al.* (255) in chromaffin cells, they also found that leptin enhances the expression of PKCα, βI, βII, and γ in macrophages. Overall, these data demonstrate that binding of leptin at the macrophage cell surface increases, through oxidative stress- and PKC-dependent pathways, LPL expression (157). This

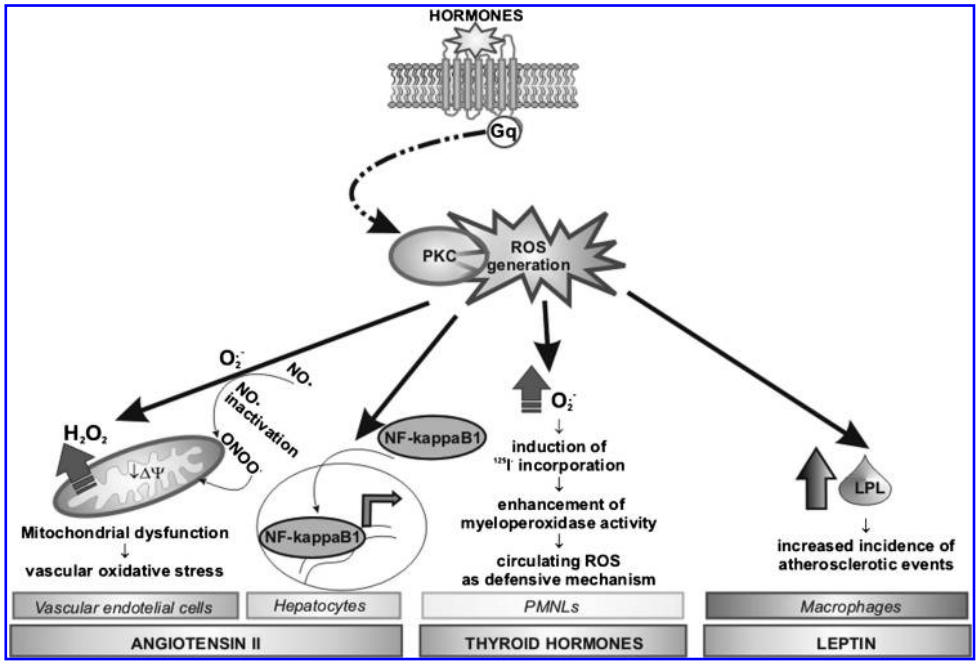

FIG. 14. The PKC–ROS interplay in hormone signaling pathway. In vascular endothelial cells, Ang II mediates endothelial dysfunction and promotes vascular inflammation and atherogenesis via PKC-dependent pathways, increasing ROS production, $ONOO^-$ formation, and consequent decreasing in mitochondrial membrane potential. This mitochondrial abnormality is the triggering event, leading to vascular oxidative stress. In hepatocytes, Ang II activates a diacyglycerol-sensitive PKC signal transduction cascade that targets nuclear NF-kB1 through a ROS-generating system of an unknown type. In human PMNLs, thyroid hormone is involved in the stimulation of $O_2^{\cdot -}$ production via the activation of the PKC pathway, induction of $^{125}I^-$ incorporation and enhancement of myeloperoxidase activity. Leptin increases human macrophage LPL expression through oxidative stress- and PKC-dependent pathways. 112×78 mm (200×200 DPI).

observation is consistent with previous results showing that reactive oxygen species are effective enhancers of macrophage LPL expression (224) and that leptin induces oxidative stress in human endothelial cells (24, 279). Leptin-induced macrophage LPL may contribute to the increased incidence of atherosclerotic events seen in patients with type 2 diabetes.

XI. Mesenchymal Stem Cell Differentiation: PKCs and Oxidative Stress

Adult human mesenchymal stem cells (MSCs) are somatic stem cells residing in a variety of tissues. MSCs can differentiate into progenies of multiple lineages, including osteoblasts, chondrocytes, adipocytes, muscle cells, and hepatocytes. The differentiation fate of MSCs is determined in large measure by a complex interplay of extracellular mediators such as growth factors, hormones, and nutrients that affect the expression and activation of lineage-specific transcription factors. Within the bone marrow, the differentiation of MSCs into adipocytes or osteoblasts is competitively balanced; mechanisms that promote one cell fate actively suppress mechanisms that induce the alternative lineage. Thus, optimal conditions entailing appropriate levels and spectra of growth factors, nutrients, and hormones are likely required for the normal osteoblastogenesis and the suppression of adipogenesis (177).

This occurs through the crosstalk between complex signaling pathways, including those derived from bone morphogenic proteins, wingless-type MMTV integration site proteins, hedgehogs, delta/jagged proteins, fibroblastic growth factors, insulin, IGF, and transcriptional regulators of adipocyte and osteoblast differentiation, including peroxisome proliferation activated receptor-γ (PPARγ) and runt-related transcription factor 2. Recently, researchers also added ROS and PKC to this long list of candidates.

A. Adipocyte differentiation

Obesity is an energy balance disorder in which nutrient intake chronically exceeds energy expenditure, resulting in excessive white adipose tissue accumulation. Aside from being a social stigma, obesity is frequently associated with several metabolic syndromes such as insulin resistance (in turn linked to the development of type 2 diabetes) and hypertension. Adipocytes arise from mesenchymal precursors whose commitment and differentiation along the adipocytic lineage is tightly regulated. The regulatory factors mediate crosstalk between adipose cells, ensuring that the growth and differentiation of adipocytes are coupled to energy storage demands. (41). In this context, preadipocytes (multipotent adipogenic precursor cells deriving from mature adipose tissue) receive increasing attention regarding nutrition-associated diseases. Indeed, often the role of PKC in adipocyte development has been studied through the analysis of expression levels of different PKC isoforms during the differentiation of preadipocytes. Some PKC isoforms can be activated by membrane-associated DAG, which causes PKC translocation from the cytosol to the plasma membrane. As previously reported, this translocation has been widely used as an indicator for enzyme activation. The translocation

of PKCβI from the cytosol to the membrane fraction during the process of adipogenesis further suggests that PKCβI is an important player. Fleming *et al.* showed in 1998 that overexpression of a dominant negative mutant of PKCβI blocked adipogenesis, suggesting that PKCβI is required in the induction of adipogenesis in 3T3-l1 preadipocytes and adipocytes. A brief and transient expression of this isoform occurs during differentiation, confirming that PKCβ expression is measurable only during the early stages of the differentiation process and not detectable in fully differentiated cells (162). More recently, in 2006, Zhou *et al.* (284) showed that the expression of PKCβI in the early stages of differentiation might be necessary to exert its regulatory effect on adipogenesis. One hypothesis is related to the transcription factor PPARγ. Since PPARγ (a key factor driving adipogenic differentiation) can restore the ability of cells overexpressing the dominant negative mutant of PKCβI to differentiate into adipocytes, it suggests that PKCβI may act as an early signaling molecule that is upstream of PPARγ (284).

Another PKC isoform, PKCζ, has been found to play an essential role in mediating the effect of insulin on glucose transport in preadipocytes (35). Zhou *et al*. (284) showed that PKCδ has an inhibitory effect on adipogenesis. It has also been shown that PKCζ translocation into the nucleus is related to the adipogenic phenotype. More recently, it has been reported that insulin-induced activation of PKCβ is dependent on the phosphorylation of IRS-1 (73). Indeed, Liberman *et al.* (146) provided evidence that, in diabetic fat tissue, the phosphorylation of IRS-1 is coordinated by glycogen synthase kinase 3 and PKCβII. In this context, it must thus be concluded that insulin is a major adipogenic agent. It stimulates the uptake of glucose, which is then converted into triglycerides and stored in fat droplets, leading to adipocyte maturation. Insulin is also known to stimulate ROS production, an overproduction of which is involved in insulin resistance. Aguiari *et al.* investigated the effect of an increase in glucose concentration on the differentiation route of stem cells residing in both adipose tissue and skeletal muscle (1). In both cases, a clear induction of adipogenic transformation was observed with the use of high-glucose culture conditions. Regarding the signals driving the differentiation of muscle stem cells (MDSCs) into adipocytes, the authors suggested a crucial role for ROS. ROS production during high-glucose incubation was measured by loading MDSCs with a ROS-sensitive fluorescent probe and monitoring the fluorescence emission by confocal microscopy. Continuous ROS production was demonstrated, markedly higher in high-glucose conditions than in the control case. Using immunofluorescence, the authors also showed that endogenous PKCβ was mostly cytosolic in MDSCs maintained in low-glucose medium. Incubation for 1 h in 25 m*M* glucose induced a partial translocation of the kinase to the plasma membrane in about 30% of the cells. The simplest interpretation of the data is that ROS produced in response to high-glucose, by stimulating PKCβ (and possibly other effectors), act as a differentiation signal for adipogenic conversion of MDSCs. This hypothesis has been tested using three additional approaches: (i) low-glucose medium was supplemented with H_2O_2 to test whether it could mimic the effect of high glucose and thus trigger adipocytic differentiation; (ii) cells maintained in high-glucose medium were transduced with a vector carrying the PKCβ siRNA, to test whether this could inhibit adipocytic differentiation; and (iii) cells in high-glucose were transduced with a PKCβ expression vector, to verify whether overexpression of this isozyme could synergize with high-glucose. The results strongly support the hypothesis: (i) in low-glucose, addition of H_2O_2 enhanced the differentiation rate to a level comparable to that observed in high-glucose; (ii) PKCβ siRNA drastically reduced adipocytic differentiation to levels below those found in low-glucose; and (iii) a marked increase (300%) of lipid-loaded cells was observed in cells overexpressing PKCβ and incubated in high-glucose. These results demonstrate an important conceptual mechanism by revealing a morphogenetic signaling activity of ROS. In fact, ROS, through downstream effectors and in particular through PKCβ (as revealed by the strong inhibitory effect of siRNA and by the strong synergism of high-glucose with PKCβ overexpression), lead to the neoformation of adipose cells (1).

Direct *in vivo* experiments involving PKCβ have been recently described by Bansode *et al.* (12). In this study, the authors demonstrated a prominent upregulation of PKCβ expression in the fat tissues of two different phenotypes (obese animal models, *ob/ob,* and HFD-fed mice) reporting that diet-induced insulin resistance may contribute to the induction of adipose PKCβ gene expression. They evaluated whether PKCβ expression and its distribution is influenced by high-fat feeding and also assessed the impact of PKCβ deficiency on obesity and its accompanying metabolic disorders under conditions of severe dietary stress. It is conceivable that diet-induced insulin resistance may contribute to the induction of adipose PKCβ gene expression. As a consequence of increased insulin resistance, insulin levels are elevated in these mice, which in turn can induce PKCβ expression. These studies suggest that a consequence of PKCβ deficiency is an activation of lipid oxidation, in addition to reduced fatty acid synthesis and storage; furthermore, adipose PKCβ is controlled specifically by factors responding to the consumption of dietary fat and the expression of PKCβ is linked to the development of obesity.

B. Osteogenic differentiation

Recently, Chen *et al.* (34) took advantage of the well-established osteogenic differentiation system of human MSCs to investigate the changes of mitochondria and redox status during stem cell differentiation. They found that in undifferentiated MSCs, mitochondria were maintained at a relatively low activity status but glycolytic activities were upregulated as indicated by the high-level expression of glycolytic enzymes (glucophosphate isomerase and phosphofructokinase) and a high lactate production rate. Upon induction of osteogenic differentiation, mitochondrial respiratory function was enhanced in several aspects, including increase in the mtDNA copy number, protein amount of respiratory enzymes, and oxygen consumption rate. Mitochondrial biogenesis-associated genes such as mtTFA, PGC-1α, and Polγ were upregulated as well. In addition to the respiratory function, they also examined the intracellular ROS levels and were surprised to find that both $O_2^{\cdot -}$ and H_2O_2 were significantly decreased at the beginning of differentiation, which seemed to be contradictory to the activation of aerobic metabolism. However, the immediate and remarkable upregulation of antioxidant enzymes including Mn-dependent SOD (MnSOD) and catalase provided a rational explanation for this phenomenon. This coordinated

regulation of metabolic shift and antioxidant enzyme activation was critical for the differentiation of MSCs since treatment of stem cells with oligomycin (inhibition of ATP synthase) and H_2O_2 suppressed osteogenic differentiation. It is noteworthy that the same treatment was able to induce the adipogenic program by means of activation of PKCβ, as Aguiari *et al.* reported (1). Taken together, a metabolic shift from anaerobic glycolysis to mitochondrial respiration for a more efficient production of ATP is required for the higher energy demand of cells undergoing differentiation. At the same time, the coordinated upregulation of antioxidant enzymes ensures a proper redox environment for differentiating cells to prevent excess ROS and oxidative stress resulting from the exuberant oxidative phosphorylation (Fig. 15). In summary, it has been demonstrated that alterations in mitochondrial biogenesis and antioxidant enzymes are well coordinated during osteogenic differentiation of MSCs.

The relatively underappreciated roles of ROS in stem cells deserve greater attention and the mechanism underlying the coordinated regulation of gene expression pertaining to mitochondrial respiration and antioxidant enzymes merits further investigation. Understanding the roles of mitochondria and ROS during cell differentiation will facilitate the optimization of *in vitro* differentiation protocols by adjusting biochemical properties such as energy production or redox status of stem cells. Control of gene expression of ROS-related PKC proteins will also allow a better design of cell therapy (33).

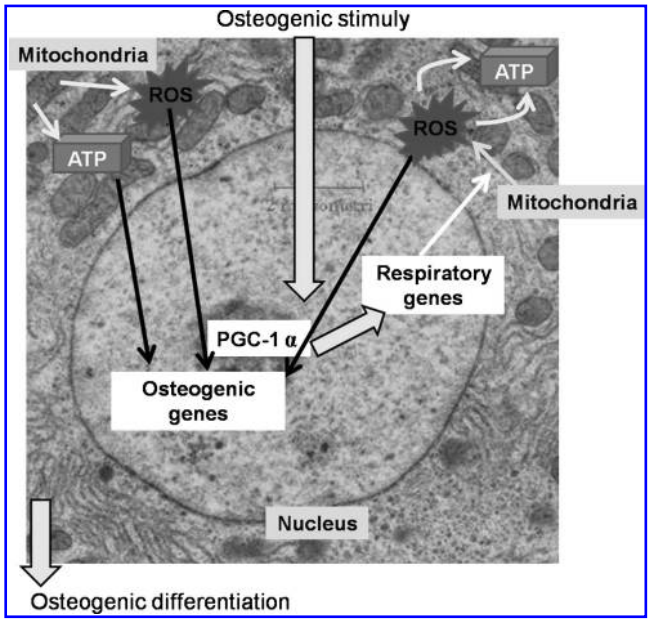

FIG. 15. Osteogenic induction on MSCs. Upon osteogenic induction, the osteogenic marker genes were induced along with a significant "metabolic shift" that reprogrammed the bioenergetic properties of human MSCs from anaerobic glycolysis to mitochondrial respiration to meet the higher energy demand of differentiated osteoblasts. At the same time, an array of antioxidant enzymes such as catalase and SOD were tuned up to protect the differentiating cells against the ROS generated from active respiration.

XII. Conclusions

In this contribution, we attempted to present an updated analysis of what is currently known about PKCs and redox signaling in health and disease. The participation of PKCs as intracellular transducers is vital to numerous physiological processes. Since protein kinases are known to be associated with many diseases, considerable effort has gone into the discovery of protein kinase modulators. Moreover, the knowledge that oxidative stress plays important roles in the modulation of PKC activity naturally makes antioxidant agents the focus of potential therapeutic strategies.

As extensively discussed above, during normal cellular activities, various processes inside of cells produce ROS that are involved to some extent as signaling molecules and defense mechanisms. On the contrary, excess generation in oxidative stress has pathological consequences determining various diseases. The purpose of antioxidants in a physiological setting is to prevent these ROS concentrations from reaching a high-enough level within a cell that damage may occur. Cellular antioxidants may be enzymatic (catalase, glutathione peroxidase, superoxide dismutase) or nonenzymatic (glutathione, thiols, some vitamins and metals, or phytochemicals such as isoflavones, polyphenols, and flavanoids). Despite a unifying theory of the molecular basis of oxidative stress and the supportive epidemiologic and animal data, most clinical trials failed to support the putative role of antioxidant treatment in preventing diseases. Vitamins E, C, A, flavonoids, and vitamins cocktail have been widely tested in humans and animal models, resulting in promising data from *in vitro* experimental systems and animals models, but in controversial data from clinical study with no significant effects for the set up of appropriate treatments based on antioxidants (see (81) for a detailed review). Many explanations were provided to justify this insufficiency. An obvious reason could be that the wrong drug or combination of drugs has been used. Most antioxidant therapies that have been tested were selected because of their easily availability and not because they were the ideal treatments. Another explanation may be that, in the majority of studies, antioxidant agents were tested at inappropriate doses or for inadequate durations on wrong patients.

Vitamin E is an excellent example since clinical trials focused mainly on the use of that vitamin, in particular for cardiovascular and diabetes treatments. Recently, it has been postulated that antioxidant potency of vitamin E is limited because it has exhibited also a pro-oxidant effect (201). Moreover, even if vitamin E is the correct agent it could be possible that its correct form has not been properly studied. Most of the clinical studies used a cheap and easily available synthetic vitamin E. Natural Vitamin E is different from the synthetic form and it is composed of different eight isoforms. Another important point to be mentioned, with specific regards to diabetes trials, is that most of the studies are not designed to assess the effect of antioxidant use in a restricted and selected patient population, for example, specifically in diabetic patients. This is an important point because diabetic patients represent a population in whom oxidative stress is much higher than in the general population, and perhaps only patients who have a proven increase in levels of oxidative stress should be treated. This may explain why clinical trials in selected populations, such as renal failure (SPACE trial) and cardiovascular disease, have been more likely to demonstrate a benefit from antioxidant therapies (22). Clinical trials to date

have been conducted without any real understanding of the mechanism of action or the concentration of the various agents used, so hopefully further research will lead to appropriately-designed clinical trials trying to translate understanding of molecular and cellular function into clinical practice.

Based on our knowledge of the pathophysiology of oxidative stress, it is clear that strategies to block the formation of reactive radicals will provide a targeted approach. However, what must be considered is that, even if oxidative stress appears to be a key event in the appearance and progression of different pathologies, ROS are not only an enemy for the correct physiology, but real components of the complex cellular signaling system. A wide spectrum of effectors protein could be modulated by ROS levels with result of even completely opposite effect on cell physiology. The PKC family should be inserted in this context. PKCs have unique structural properties that render them differently susceptible to activation by redox-modifying agents eliciting different responses. Whether PKC is activated or inactivated depends on the type of oxidant, the site of oxidation, and the extent of modification. PKC are only one component of the molecular pathway involved in cellular signaling and actions of antioxidants are mediated by a lot of other critical cellular targets, so the redox-mediated modulation of PKC is only partly responsible for the antioxidant action (88). The use of antioxidants could inactivate all the PKC pool despite the cellular system or the pathologic conditions, and for this reason must be reconsidered or supplemented by selective drugs for different isoforms.

In this contest, a generalized use of antioxidants could be inappropriate, whereas selective drugs for oxidative stress sensitive protein should provide a "calibrated" option in modulation of cellular signaling within the wide complexity of the different disorders. Examination of single PKC isoforms contribution to each disease and their response to antioxidant or selective drugs will provide new basis for therapeutic approach.

For these reasons, although the experimental evidence for an antioxidant therapy is quite promising, further validation work is required. A possible strategy of success could be the use of antioxidant compounds in association with other specific molecules designed to prevent alterations in pathways specific to the different pathologies (*i.e.*, a double-hit model of therapy).

In conclusion, PKCs are predicted to be an increasingly relevant biological target in therapy and management of different human pathologies.

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Abbreviations Used

ACF = aberrant crypt foci
ALDH2 = aldehyde dehydrogenase 2
ALL = acute lymphoblastic leukemia
ANT = adenine nucleotide translocase
APC = adenomatous polyposis coli
aPKC = atypical PKCs
APP = amyloid precursor protein
ara-C = 1-b-D-arabinofuranosylcytosine
Ca^{2+} = calcium ion
$[Ca^{2+}]_c$ = cytosolic calcium concentration
$[Ca^{2+}]_m$ = mitochondrial calcium concentration
CBP = CREB binding protein
CD95L = CD95 ligand
Cdks = cyclin-dependent kinases
COIV, cytochrome c oxidase subunit IV
cPKC = classical (conventional) PKCs
Cx43 = connexin 43
DAG = diacylglycerol
DNR = daunorubicin
ER = endoplasmic reticulum
ERK = extracellular signal-regulated kinase
GPCRs = G-protein coupled receptors
GSH = glutathione
IGF = insulin-like growth factor
IL-6 = interleukin-6
IMM = inner mitochondrial membrane
IP3 = inositol-1,4,5-trisphosphate
IP3R = IP3 receptor
IPC = ischemic preconditioning
IRS = insulin receptor substrate
LPL = lipoprotein lipase
MAPK = mitogen-activated protein kinase
MAPKK (MEK) = mitogen-activated protein kinase kinase
MDSCs = skeletal muscle-derived stem cells
MEFs = mouse embryonic fibroblasts
$mitoK_{ATP}$ = mitochondrial ATP-sensitive K^+ channel
mPTP = mitochondrial permeability transition pore
MPTP = 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine
MSC = mesenchymal stem cells
mTO = mammalian target of rapamycin
nPKCs = novel PKCs
OMM = outer mitochondrial membrane
PAK = p21-activated kinase
PDH = pyruvate dehydrogenase
PDK = pyruvate dehydrogenase kinase 2
PDK1 = PI-dependent protein kinase 1
PI = phosphatidylinositol
PIP = phosphatidylinositol-3,4, 5-trisphosphate
PK = protein kinase B
PKA = cyclic AMP-dependent protein kinase
PKC = protein kinase C
PMA = phorbol-12-myristate-13-acetate
PMNLs = polymorphonuclear leukocytes
PPARγ = peroxisome proliferation activated receptor-γ
PS = pseudosubstrate
RACK = receptors for activated C kinases

Abbreviations Used (cont.)

ROS = reactive oxygen species
SM-CER = sphingomyelin-ceramide
SOD = superoxide dismutase
TCR = T cell antigen receptor
TG2 = transglutaminase
TNF = tumor necrosis factor
TPA = 12-O-tetradecanoylphorbol-13-acetate
TRAIL = TNF-related apoptosis-inducing ligand
VDAC = voltage-dependent anion channel
XIAP = X-linked inhibitor of apoptosis