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Forum Review

Role of Reactive Oxygen Species and Protein Kinase C in Ischemic Tolerance in the Brain

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ABSTRACT

It is now understood that the mechanisms leading to neuronal cell death after cerebral ischemia are highly complex. A well established fact in this field is that neurons continue to die over days and months after ischemia, and that reperfusion following cerebral ischemia contributes substantially to ischemic injury. It is now well accepted that central to ischemic/reperfusion-induced injury is what occurs to mitochondria hours to days following the ischemic insult. For many years, it has been established that reactive oxygen species (ROS) and reactive nitrogen species (RNS) promote lipid, protein, and DNA oxidation that affects normal cell physiology and eventually leads to neuronal demise. In addition to oxidation of neuronal molecules by ROS and RNS, a novel pathway for molecular modifications has risen from the concept that ROS can activate specific signal transduction pathways that, depending on the insult degree, can lead to either normal plasticity or pathology. Two examples of these pathways could explain why lethal ischemic insults lead to the translocation of protein kinase Cδ (δPKC), which plays a role in apoptosis after cerebral ischemia, or why sublethal ischemic insults, such as in ischemic preconditioning, lead to the translocation of ϵPKC, which plays a pivotal role in neuroprotection. A better understanding of the mechanisms by which ROS and/or RNS modulate key protein kinases that are involved in signaling pathways that lead to cell death and survival after cerebral ischemia will help devise novel therapeutic strategies. *Antioxid. Redox Signal.* 7, 1150–1157.

CEREBRAL ISCHEMIA

Most organs and tissues are dependent on normal blood supply for maintaining normal cell homeostasis. Among all body organs, the brain and heart are the most susceptible to lack of blood supply, as it occurs during ischemia. In particular, brain energy consumption is large because of its inherent physiological activities, which use the predominant fraction of energy supplied in the body. Thus, it is well known that energy failure due to ischemic insults has rapidly damaging consequences in the brain. In fact, insults such as anoxia, ischemia, and hypoglycemia all cause the brain to become isoelectric within a few minutes (4, 40, 83). If these insults continue, the loss of electrical activity is followed by depletion of high-energy intermediates (52), and by the loss of ion gradients (depolarization) (39). As ion gradients are lost, neurons release excitatory neurotransmitters that amplify or cause irreversible pathologies during and after energy failure (18, 36).

Many groups have attempted to scrutinize the key events that lead to neuronal cell death that result as a consequence of energy failure and during recovery. It is now understood that the mechanisms leading to neuronal cell death after cerebral ischemia are highly complex. A well established fact in this field is that cells continue to die over days and months after ischemia, a phenomenon that has been termed delayed cell death (23, 65). Although not clearly defined, neuronal cell death may result from either apoptosis, necrosis, or a cell death mechanism that is a mixture of these processes (50, 51, 54, 59).

In recent years, a specific cellular site, namely, mitochondria, has received special attention as a key player in cell death pathways (24, 50, 60). This is evidenced by numerous studies that support the hypothesis that reperfusion following

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cerebral ischemia contributes substantially to ischemic injury (14, 17, 85, 92) and that mitochondrial dysfunction plays a central role (1, 3, 24, 28, 84, 95, 96).

Evidence of mitochondrial dysfunction following cerebral ischemia was described in previous studies from our laboratory and others, as a prominent change in redox activity of mitochondrial respiratory chain components in postischemic brain (73, 74, 81, 82, 93, 94). This hyperoxidation of electron carriers is indicative of either a response to decreased substrate availability (81) and/or a reaction of mitochondrial complexes to reactive oxygen species (ROS) (74). Postischemic mitochondria may also be a major source of ROS, and free radical-mediated damage has been linked to reperfusion injury following brain ischemia (25, 29, 38, 47, 86, 91). However, recent findings suggest that this hyperoxidation may result from loss of electron carriers from mitochondria following cerebral ischemia, such as cytochrome *c* and NADH (75, 80). The loss of cytochrome *c* from mitochondria might affect respiratory chain activity and/or it may trigger an abnormal signal transduction pathway (10, 80) that may promote the apoptotic cascade (16, 65). This is suggested by findings that apoptosis (programmed cell death) may be linked to mitochondria and their release of cytochrome *c* (3, 46, 84, 97) and the activation of a proapoptotic protein kinase C [δ isoform (δPKC)] (10, 80).

ISCHEMIC PRECONDITIONING

In contrast to the damaging consequences of cerebral ischemia, an intrinsic neuroprotective metabolic state can be reached when the brain and other tissues undergo a sublethal ischemic insult prior to a lethal ischemic insult, referred to as ischemic preconditioning (IPC). Mechanism(s) underlying IPC-induced tolerance remains unclear, although multiple possible induction pathways have been implicated, including neuroactive cytokines (62, 68), activation of glutamate receptors (78 79), adenosine receptors, the ATP-sensitive potassium channel (5, 6, 8, 37, 42, 48, 72), nitric oxide (NO) (13, 30, 89, 90), oxidative stress (67), and hypothermia (63), among others.

Studies of these putative triggering factors and putative links among themselves have centered on the roles of several signal transduction pathways. For example, increases in cytosolic Ca^{2+} played a key role, because Ca^{2+} activated several important enzymes, which initiated different signaling pathways for IPC-induced tolerance. Some of these enzymes include PKC, inducible NO synthase, and mitogen-activated protein kinases (MAPKs) (19). PKC activation might be a common signal transduction factor in both heart and brain, because Ca^{2+} can directly or indirectly activate selective PKC isozymes, and also activation of the adenosine A1 receptor promotes PKC activation via production of diacylglycerol (DAG) and inositol trisphosphate (48, 64, 79).

Support for the possible role of different PKC isozymes during IPC emerged from our recent studies showing that IPC neuroprotection could be emulated with the agonist of ϵPKC (Ψϵ receptor of activated C kinase (ΨϵRACK)], whereas IPC protection could be blocked with its antagonist ϵV1-2 (79). These results demonstrated that the preconditioning-induced neuroprotective effect of the ΨϵRACK is caused by selective activation of ϵPKC.

Further, we have recently demonstrated that the ϵPKC isozyme is a common mediator in IPC-, *N*-methyl-D-aspartate (NMDA)-, and adenosine-induced tolerance (see 48, 71, 79).

FREE RADICAL FORMATION AFTER ISCHEMIA/REPERFUSION

It is well known that ROS are formed during normal aerobic metabolism (15, 47). ROS production, in turn, increases oxidation of lipids, proteins, and DNA (25, 26, 55, 70). However, during pathological events, such as during cerebral ischemia and reperfusion, ROS formation is more pronounced (25, 29, 47). Considerable evidence has accumulated that suggest that oxidative injury occurs almost exclusively during the reperfusion phase (see above), and that ROS accumulation plays a major role in this pathology (26).

Multiple sources of ROS have been suggested. Mitochondria are a major site for ROS formation. In particular, superoxide is produced in mitochondria under normal and pathological conditions (24, 60) and are a likely source of this anion for the generation of peroxynitrite (see below) during ischemia and reperfusion. This normal ROS formation from mitochondria is greatly enhanced during the abnormal state created by the ischemic insult (86). Interestingly, this increase in ROS formation could arise from different patterns of mitochondrial respiration provoked by the ischemic insult and the reperfusion phase. For example, we recently showed that isolated brain mitochondria exhibited a different type of mitochondrial dysfunction early during the reperfusion phase (30 min) and after a day of reperfusion (21, 76). Mitochondrial respiration was significantly diminished at the level of complex IV at the early reperfusion phase (76), but was exacerbated in complexes I and II after 24 h, suggesting that perhaps the loss of either cytochrome *c* and/or NADH during the early reperfusion phase could enhance ROS production that at 24 h elicited increased oxidation of other mitochondrial complexes and further enhanced mitochondrial dysfunction (21).

The main ROS produced by mitochondria are superoxide and hydrogen peroxide (26). Hydrogen peroxide causes little direct damage, but if it is not degraded by the antioxidant defense system, it can generate highly reactive hydroxyl radicals (49). Increases in hydroxyl radicals have been described during early reperfusion following short-term global ischemia (77). Complexes I and III are two possible sources for the increases in hydroxyl radicals. A recent study in organotypic hippocampal slice cultures demonstrated that inhibition of mitochondrial complex I respiration with rotenone reduced free radical generation resulting from ischemia/reoxygenation (27). Similar increases in ROS were described during focal ischemia and particularly during reperfusion (87). Formation of ROS during reperfusion after ischemia and its role in pathology are supported by studies in transgenic mice, which implicated superoxide produced from mitochondria. Mice overexpressing manganese superoxide dismutase, the mitochondrial form of this enzyme, developed smaller infarcts in response to 60 min of middle cerebral artery occlusion (45).

Another suggested player in reperfusion injury following cerebral ischemia is NO (42, 53). However, it is believed that the source of NO is not mitochondrial. Most studies support that NO production occurs via the neuronal NO synthase (nNOS) after cerebral ischemia (43, 69). These conclusions are based on the many studies where neuroprotection was achieved by pharmacological inhibition of nNOS, as well as studies with the transgenic mice expressing knockout mutations of nNOS (43, 69).

Although mitochondria do not produce NO, it is believed that mitochondria are one of the main targets for NO, and perhaps a key cause of mitochondrial pathology. The first evidence comes from the fact that NO by itself at low concentrations (in the nanomolar range) can compete with oxygen to reversibly inhibit cytochrome *c* oxidase (88) and impairs mitochondrial energetics (12). Increases in NO could also explain the inhibition observed at the level of complex IV mitochondrial respiration observed in our study early during the reperfusion phase (76).

In addition to NO-induced mitochondrial respiration inhibition, higher NO concentrations can promote irreversible protein and lipid modifications that can further impair mitochondrial respiration, an effect that may occur hours to days following cerebral ischemia and that might explain the additional mitochondrial dysfunctions described in our group 24 h following cerebral ischemia (21). It has been shown that generation of superoxide anions and NO will produce peroxynitrite (7). Once produced, peroxynitrite can directly produce damage or be converted to other strong oxidants, including the highly reactive hydroxyl radical (49). Some of the targets for peroxynitrite damage can include the major enzyme complexes of the electron transport chain and ATP synthetase (49), which could further impair respiration.

Among the different molecular modifications provoked by ROS and reactive nitrogen species (RNS), one that is known to have profound consequences at different cellular levels is termed membrane lipid peroxidation (MLP) (55). It has been well described that MLP occurs during different neurodegenerative processes both in acute conditions, such as cerebral ischemia in traumatic brain injury, and in chronic disorders, such as Alzheimer's and Parkinson's diseases (55). Different effects of MLP include modification of neurotransmitter release and uptake, ion-channel activity, the function of ionmotive ATPases and glucose transporters, and the coupling of cell-surface receptors to GTP-binding proteins (for review, see 55). It is interesting, however, that MLP can also act on normal physiological states, such as that observed during synaptic plasticity (for review, see 55).

Other well known effects of ROS and RNS are oxidative modification of proteins. Peroxynitrite nitrates protein tyrosine residues to form 3-nitrotyrosine in reoxygenated tissues (49). As in MLP, protein oxidation can have harmful consequences or, if of a mild nature, can have signaling roles for proteins involved in neuronal plasticity (42, 49). It has been shown that protein modification may promote activation of transcription factors that will consolidate the plasticity event (20). NO interacts with two signaling pathways important to neuronal survival: the Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) pathway, and the phosphatidylinositol 3-kinase Akt pathway (42).

SUGGESTED ROLE OF ROS IN ISCHEMIC TOLERANCE

It is now recognized that a small production of ROS or RNS could modify cell-signaling proteins and that these modifications have functional consequences. In the case of IPC, short exposure to hypoxia/ischemia can either directly or indirectly produce a mild degree of oxidative stress, which may be involved in ischemic tolerance in heart and brain. In heart, ischemic tolerance is abolished when heart is perfused with the antioxidant *N*-acetylcysteine, suggesting that redox signaling is key in the survival signal after IPC (20). In brain, administration of diethyldithiocarbamate, which inhibits superoxide dismutase and elicits ROS production, is neuroprotective if global ischemia is induced 2–4 days later in gerbils (67).

In fact, ROS can modulate cell-signaling proteins by acting on redox-sensitive transcription factors, such as nuclear factor-κB and activator protein-1 (20) or redox-sensitive protein kinases (20, 33, 49). These transcription factors, in turn, promote the induction of a number of genes involved in cell survival or plasticity and the increase of the antioxidant system. For example, nuclear factor-κB has been found to control the antiapoptotic gene, Bcl-2, and proapoptotic factors, bax and p53, in the ischemic/reperfused myocardium (11).

Oxidant exposure can also increase the activity of other protein tyrosine kinases, such as the MAPKs and c-Jun N-terminal kinase (2, 56). For example, superoxide and hydrogen peroxide increased ERK 1/2 activity in a dose- and time-dependent manner and in a similar fashion as with tumor necrosis factor stimulation (56).

Another ROS-mediated signaling pathway involves the tyrosine kinases. Receptor tyrosine kinases play an important role in redox signaling by autophosphorylation of tyrosines along their own intracellular tails, and phosphorylation of tyrosine kinases was linked with the activation of phospholipases such as C and D, which then leads to the activation of downstream kinases (20). We recently showed that phospholipase C, PKC, and the MAPK, ERK 1/2, were critical in ischemic tolerance in the brain (48, 71, 79), which suggests the possibility that ROS may be mediating this signal transduction pathway. This conjecture is supported by studies in heart, where a ROS-dependent activation of εPKC is considered a key event responsible for the protective effect of IPC in heart (66, 98).

Among the different PKC isozymes we demonstrated that εPKC was a key player in the induction of ischemic tolerance in our models of IPC (Fig. 1). In contrast, we recently demonstrated that after lethal ischemia another PKC isozyme, δPKC, is translocated and involved in the cell death pathway (10, 80) (Fig. 2). What provokes the translocation of one of these isozymes rather than the other, depending on the time of ischemia, remains undefined. It is possible that the difference in ROS production during a sublethal ischemic insult versus a

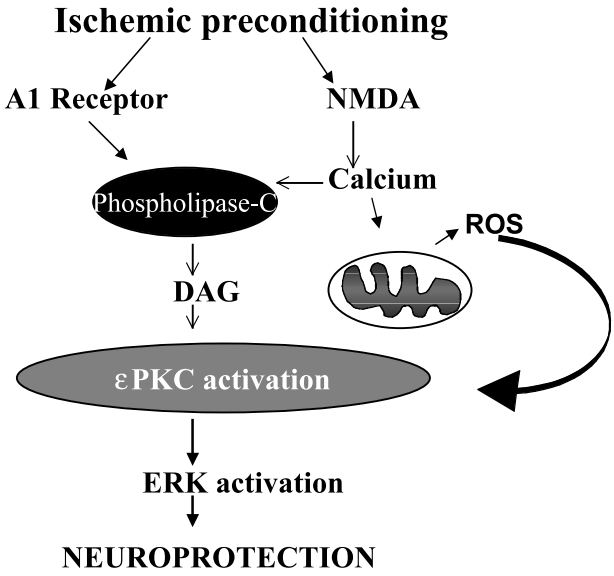

FIG. 1. Current model suggesting a ϵPKC signaling pathway promoting ischemic tolerance in the brain.

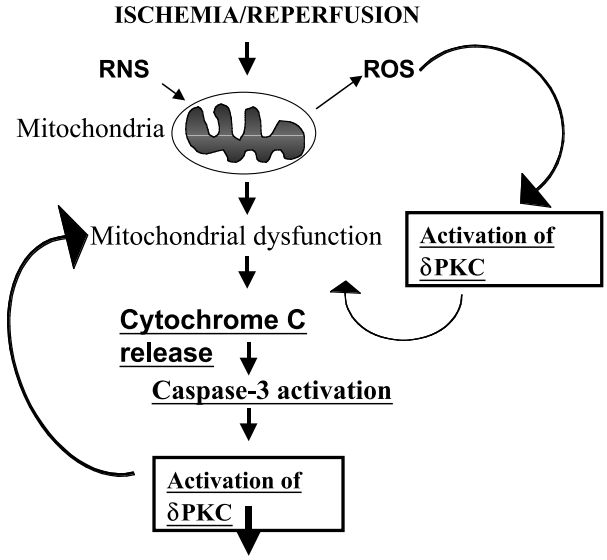

FIG. 2. Current model suggesting a δPKC signaling pathway promoting cell death after cerebral ischemia.

lethal ischemic insult may trigger activation of survival versus cell death pathways, respectively. This is supported by the fact that PKCs are highly sensitive to the redox environment of the cell. For example, it is known that various antioxidants inhibit PKC-dependent cellular responses (33). Therefore, PKC is a logical candidate for redox modification by oxidants and antioxidants (9, 35).

Until now, at least 12 different PKCs have been identified. They are serine/threonine kinases and can be split into three broad categories: conventional, novel, and atypical (44, 58), and they have multiple cellular roles. The general structure of PKC is depicted in Fig. 3. The conventional PKCs (α, β_I, β_{II}, and γ) require Ca^{2+}, DAG, and phospholipid for activation. The novel PKC isozymes (δ, ϵ, η, θ) lack the calcium-binding region, so these subtypes are not dependent on Ca^{2+} for activation, but may be activated by Ca^{2+} indirectly with DAG. Activation of isozymes of the atypical PKC group (ζ, λ, μ, and ι) are also independent of Ca^{2+}. However, atypical PKC isozymes lack the Zn^{2+} finger region required for binding of DAG or phorbol ester [phorbol 12-myristate 13-acetate (PMA)]. Instead, 3′-phosphoinositides may be the activators of atypical PKCs (44, 58). In general, PKC is regulated by two sequential mechanisms: (a) phosphorylation triggered by the 3-phosphoinositide-dependent kinase (PDK)-1, and (b) binding to DAG (22), and traditionally their activation is detected by measuring intracellular translocation to membrane or cytoskeleton. In the last decade, a better understanding of PKC function has emerged. The importance of location is a key determinant of isozyme specificity. It is now recognized that isozyme specificity is mediated by association of each PKC isozyme with specific anchoring proteins, receptors of activated C kinases (RACKs) (58). In this manner, RACKs promote specific intracellular organization and compartmentalization for individual PKCs (22). The localization facilitates cross-talk between different signaling intermediates, targeted substrate phosphorylation, and regulation of catalytic activity. Overexpression of one isozyme can alter the levels of others (22).

Although Ca^{2+} and DAG are key activators of PKC, this protein contains unique structural features that are susceptible to oxidative modification (33, 34). The N-terminal regulatory domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by peroxide. When oxidized, the autoinhibitory function of the regulatory domain is compromised

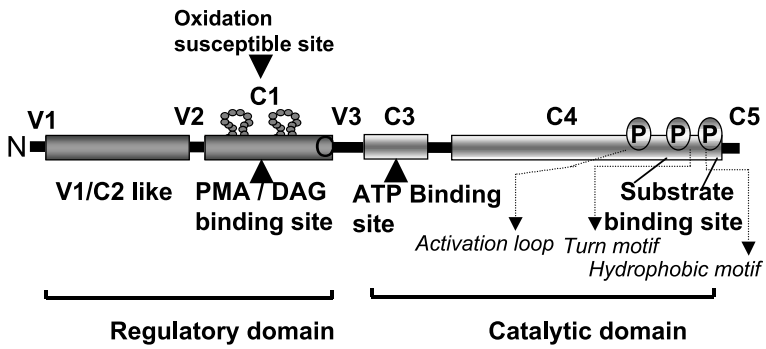

FIG. 3. Schematic structure of the PKC depicting general composition, activators, and sites of oxidation susceptibility.

and, consequently, cellular PKC activity is stimulated (31). The C-terminal catalytic domain contains several reactive cysteines that are targets for various antioxidants (32). Modification of these cysteines decreases cellular PKC activity. Thus, the two domains of PKC respond differently to two different types of agents: oxidants selectively react with the regulatory domain and stimulate cellular PKC; in contrast, antioxidants react with the catalytic domain and inhibit cellular PKC activity (31, 32).

An alternative route for ROS-mediated modulation of PKC is by acting upon upstream activators of these kinases. For example, oxidative stress increases activity of receptor-regulated enzymes such as phospholipases A2, D, and C (57, 61), and the lipid second messengers derived from these reactions (arachidonic acid, phosphatidic acid, and DAG) can activate PKC (33). Thus, both the oxidant-induced increase in kinase activity and redox regulation are important mechanisms for oxidant-induced changes in gene expression that impact the overall cellular response to changes in redox status. It is possible that oxidation and phosphorylation represent alternative mechanisms for stimulating cellular responses. The combined effects of PKC phosphorylation and oxidation on signaling events have not yet been described. However, the possibility of cooperation and amplification of responses should be considered.

In conclusion, the sources, species, and concentrations of ROS and/or RNS may be pivotal to defining the fate of the signal transduction pathway that ensues following cerebral ischemia or IPC. Because we have defined two different profiles in PKC translocation that are linked to either cell death or cell survival, and because PKCs are proteins that are susceptible to ROS modulation, we surmise that definition of the ROS sources, species, and concentrations following these insults may dictate the signal transduction direction that ensues following these insults. Studies are under way in our laboratory to define these pathways.

ABBREVIATIONS

DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; IPC, ischemic preconditioning; MAPK, mitogen-activated protein kinase; MLP, membrane lipid peroxidation; NMDA, *N*-methyl-D-aspartate; nNOS, nitric oxide synthase; NO, nitric oxide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RACK, receptor of activated C kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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