

Neutrophil protein kinase C δ as a mediator of stroke-reperfusion injury

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Thrombolysis is widely used to intervene in acute ischemic stroke, but reestablishment of circulation may paradoxically initiate a reperfusion injury. Here we describe studies with mice lacking protein kinase C δ (PKC δ) showing that absence of this enzyme markedly reduces reperfusion injury following transient ischemia. This was associated with reduced infiltration of peripheral blood neutrophils into infarcted tissue and with impaired neutrophil adhesion, migration, respiratory burst, and degranulation in vitro. Total body irradiation followed by transplantation with bone marrow from PKC δ -null mice donors reduced infarct size and improved neurological outcome in WT mice, whereas marrow transplantation from WT donors increased infarction and worsened neurological scores in PKC δ -null mice. These results indicate an important role for neutrophil PKC δ in reperfusion injury and strongly suggest that PKC δ inhibitors could prove useful in the treatment of stroke.

Introduction

Ischemic stroke is the most common fatal neurological disease and the leading cause of long-term disability in the United States (1). Fibrinolytic agents such as tissue plasminogen activator (t-PA) are currently the only drugs approved for pharmacological intervention to reverse acute ischemic stroke. Early treatment can be effective in reducing ischemic damage, but a limiting complication is the occurrence of tissue injury caused by reperfusion following recanalization of the occluded vessel (2, 3). Therefore, there is great interest in developing treatments that can limit reperfusion injury.

During reperfusion, reintroduction of oxygenated blood to ischemic tissues initiates processes leading to the generation of free radicals and, importantly, infiltration of polymorphonuclear leukocytes, also referred to as neutrophils (4). Under quiescent conditions, neutrophils circulate freely and do not interact significantly with the endothelium. In response to tissue ischemia, neutrophils adhere and migrate through the endothelium of the cerebral microvasculature. At extravascular sites neutrophils produce free radicals (O₂⁻; OH⁻), release proteolytic enzymes, and stimulate cytokine release from neighboring cells, thereby promoting further recruitment of neutrophils and other leukocytes. Depletion of neutrophils (5), inhibition of neutrophil adhesion (5), or inhibition of proteolytic enzymes such as elastase that are released from neutrophils (6) reduces injury size and improves neurological deficits in experimental stroke models.

It is clear that multiple steps of neutrophil recruitment provide potential targets for therapeutic intervention. Studies using phorbol esters that activate protein kinase C (PKC) suggest that several neutrophil functions, including superoxide anion generation, cell adhesion, and degranulation, are stimulated by PKC (7).

The PKC family of serine/threonine kinases is composed of at least ten isozymes with distinctive means of regulation and tissue distribution (8). Five isozymes (PKC α , β I, β II, δ , and ζ) are known to be present in human neutrophils (7). The exact functional role of these different PKC isozymes in neutrophils remains to be specified.

PKC δ is a member of the novel PKC subfamily that is activated by diacylglycerol (DAG) but not by calcium. PKC δ promotes apoptosis in various types of cultured cells (9), including neuronal cells (10). In mice that lack PKC δ , reduced cell death is associated with smooth muscle cell accumulation, which leads to accelerated arteriosclerosis of vein bypass grafts (11) and increased B cell proliferation associated with impaired antigen-induced B cell self-tolerance and autoimmune disease in later life (12, 13). Studies using PKC δ peptide inhibitors and activators indicate an important role of PKC δ in cardiac ischemia and reperfusion (14). The mechanism by which PKC δ promotes reperfusion damage in the heart remains to be demonstrated.

Despite evidence linking PKC δ with smooth muscle and B cell death and reperfusion injury in cardiomyocytes, very little is known about its role in CNS disease. To investigate the potential role of PKC δ in nervous system ischemic injury, we generated mice that lack PKC δ and studied their response to cerebral ischemia. Using a model of transient middle cerebral artery (MCA) occlusion (MCAO), we found that PKC δ -null mice show a 70% reduction in stroke size compared with WT mice. This was associated with impaired neutrophil function and reduced neutrophil migration into ischemic tissue in PKC δ -null mice. Treatment with total body irradiation followed by transplantation with bone marrow from the opposite genotype reversed the stroke phenotypes in WT and PKC δ -null mice, confirming an important role for neutrophil PKC δ in reperfusion injury.

Results

Infarct size is reduced in PKC δ -null mice after transient, but not permanent, MCAO. We generated mice that lack PKC δ using homologous recombination in ES cells (Figure 1). Neither full-length nor truncated PKC δ -like immunoreactivity was detected in PKC δ -null mice cells by Western blot analysis (Figure 1D and data not shown). These mice

Nonstandard abbreviations used: anterior cerebral artery (ACA); common carotid artery (CCA); diacylglycerol (DAG); formyl-Met-Leu-Phe peptide (fMLP); internal carotid artery (ICA); leukocyte functional antigen 1 (LFA-1); middle cerebral artery (MCA); middle cerebral artery occlusion (MCAO); protein kinase C (PKC); tissue plasminogen activator (t-PA); 2,3,5-triphenyltetrazolium chloride (TTC).

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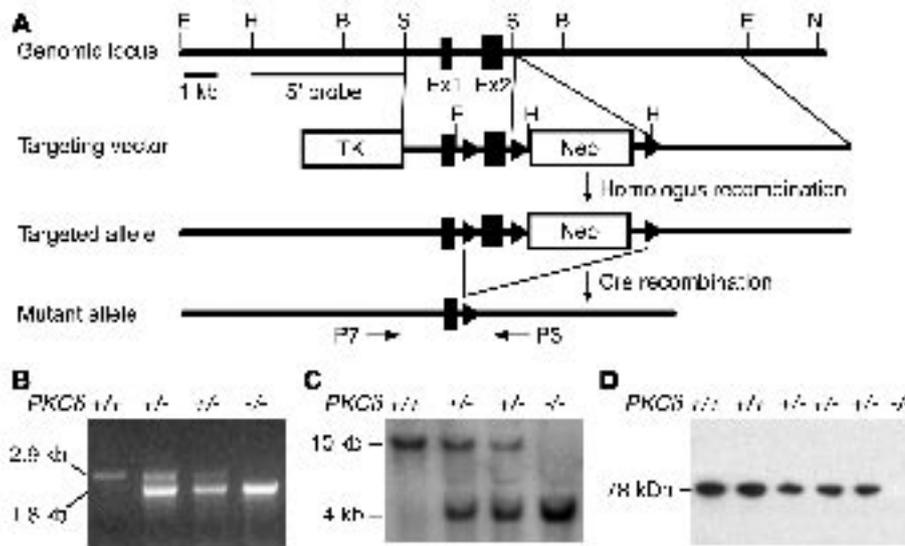


Figure 1 Generation of PKC δ -null mice. **(A)** Organization of the mouse PKC δ gene, the targeting construct, and the allele resulting from homologous recombination. Boxes represent exon 1 (123 bp) and exon 2 (200 bp). Arrowheads represent loxP sequences. Only relevant restriction enzyme sites are indicated. E, EcoRI; H, HindIII; B, BamHI; S, SacI; N, NotI. **(B)** Verification of genotypes by PCR using the primers P7 and P3. The WT allele (+/+) generates a 2.9-kb and the mutant allele (-/-) a 1.8-kb band, as indicated. **(C)** Verification of genotypes by Southern blot analysis of genomic DNA digested with EcoRI from WT (+/+), heterozygous (+/-), and homozygous mutants (-/-) using a 3.2-kb 5'-probe (HindIII-SacI fragment). **(D)** Verification of genotypes by Western blot analysis of whole brain lysates using a mAb against PKC δ . The migration of PKC δ immunoreactivity (78 kDa) is indicated.

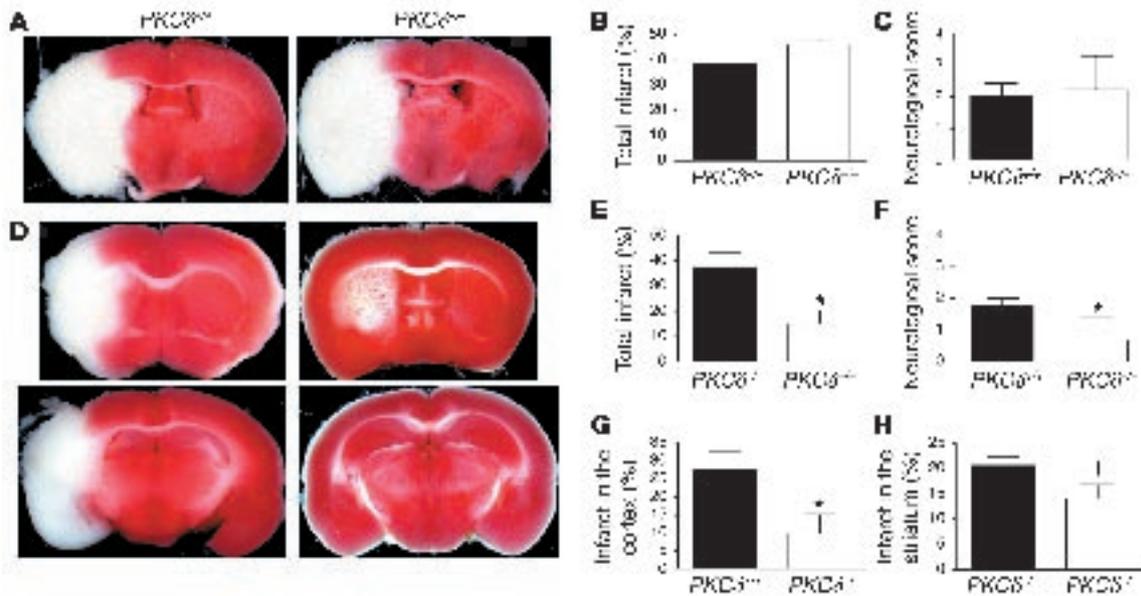
showed no gross or microscopic neuroanatomical abnormalities and no motor deficits on open field testing or on an accelerating rotarod (data not shown). To study the response of these animals to stroke, we first used a model of permanent cerebral ischemia in which an infarction in the MCA distribution is produced by occluding the vessel lumen with a suture. Placement of the suture for 20 hours resulted in cerebral infarcts of similar size in PKC δ -null and WT littermates (Figure 2, A and B). The neurological examination also showed a similar degree of impairment in both genotypes 20 hours after initiation of ischemia (Figure 2C). Since permanent occlusion, when severe, induces cell death that is mainly necrotic (15), these findings suggested that PKC δ does not play an essential role in ischemia-induced necrosis. Given the evidence for PKC δ as a mediator of apoptosis, we next subjected mice to a transient ischemia-reperfusion paradigm, in which apoptosis plays an important role in cell death (16). Interestingly, infarct size was decreased by about 65% in PKC δ -null mice compared with WT littermates (Figure 2, D and E) after 1 hour of ischemia followed by 24 hours of reperfusion. This correlated with a better neurological score in PKC δ -null mice compared with WT mice 24 hours following the ischemic period (Figure 2F). The infarct size was decreased more in the cortex than in the striatum of PKC δ -null mice (Figure 2, G and H). The attenuated infarct size in PKC δ -null mice was not due to a delay in the progression of infarction, since infarct size in PKC δ -null mice after 1 hour of ischemia and 7 days of reperfusion was similar to infarct size measured after 24 hours of reperfusion (data not shown).

To investigate whether the attenuated infarct size in PKC δ -null mice was due to an alteration in cerebrovascular anatomy, we examined the distribution of cerebral vessels by India ink injection. No differences were observed in the origins of the MCA or other major

blood vessels of the circle of Willis (Figure 3A). The distribution of the MCA territory was also similar in WT and PKC δ -null mice (Figure 3, B and C). Additionally, cerebral blood flow was similar in both genotypes before and during ischemia and after reperfusion (Figure 3D). The appearance of the brain microvasculature was also similar in both genotypes (Figure 3E).

PKC δ has been postulated to be involved in ischemic brain injury since PKC δ mRNA and protein are induced in rat brain tissue surrounding an ischemic lesion several hours to days after transient cerebral ischemia (17). If neuronal PKC δ is responsible for the difference in stroke size between PKC δ WT and null mice, then PKC δ should be abundant in brain regions within the MCA territory of WT mice that were spared in PKC δ -null mice. In rodent brain, PKC δ is expressed predominantly in neurons of the thalamus and their axonal projections throughout the forebrain, especially in the neocortex, although much lower expression levels are also present in other areas (18). Several brain regions that were spared in PKC δ -null mice (Figure 2D), however, contain neurons with little or no PKC δ immunoreactivity in WT mice (Figure 4, A and B). The mismatch between patterns of ischemic infarction and PKC δ expression in WT mice led us away from studies of PKC δ and apoptosis in neural tissues. Instead, we considered whether other tissues outside of the brain explain differences in stroke size between WT and PKC δ -null mice.

Attenuated neutrophil function in PKC δ -null mice. Neutrophils from the peripheral blood infiltrate into ischemic brain tissue during reperfusion and may contribute to reperfusion injury, since inhibition of neutrophil adhesion or depletion of neutrophils with anti-neutrophil adhesion Ab's reduces apoptosis following transient ischemia (4, 19). To examine whether neutrophil infiltration was altered in PKC δ -null mice, we identified neutrophils in brain sections by chloroacetate esterase staining 24 hours after 1 hour of transient ischemia. We found approximately 80% fewer neutrophils in the cerebral cortex and 65% fewer neutrophils in the striatum of PKC δ -null mice compared with WT littermates (Figure 4, C-E). These findings correlated with in vitro studies showing reduced adhesion (Figure 5A) and migration (Figure 5B) of neutrophils from PKC δ -null mice compared with WT littermates. In addition, superoxide anion (O $_2^-$) release in response to TNF- α , formyl-Met-Leu-Phe (fMLP) peptide, or phorbol ester (Figure 5C and data not shown), and degranulation measured as lactoferrin release in response to fMLP (Figure 5D) or phorbol ester (data not shown), were reduced in neutrophils from PKC δ -null mice compared with WT mice. Despite these differences in function, there was no difference in the number of neutrophils or other cells in the peripheral blood of PKC δ -null mice when compared with WT littermates (Table 1). The abundance of other PKC isozymes expressed in neutrophils was also not altered in PKC δ -null mice compared with

**Figure 2**

Infarct size is reduced in PKC δ -null mice after transient, but not permanent, MCAO. (A) Shown are representative images of TTC-stained brain slices after 20 hours of permanent MCAO from PKC $\delta^{+/+}$ and PKC $\delta^{-/-}$ mice. Viable tissue is stained red, whereas the ischemic area remains unstained (white). (B) Infarct size measured as a percentage of the area of the nonischemic hemisphere after 20 hours of permanent MCAO from PKC $\delta^{+/+}$ ($n = 8$) and PKC $\delta^{-/-}$ mice ($n = 8$). (C) Neurological deficit scores after 20 hours of permanent MCAO from PKC $\delta^{+/+}$ ($n = 8$) and PKC $\delta^{-/-}$ mice ($n = 8$). (D) Representative images of TTC-stained brain slices after 1 hour of MCAO and 24 hours of reperfusion from PKC $\delta^{+/+}$ and PKC $\delta^{-/-}$ mice. (E) Total infarct size after 1 hour of MCAO and 24 hours of reperfusion from PKC $\delta^{+/+}$ ($n = 10$) and PKC $\delta^{-/-}$ ($n = 9$) mice. (F) Neurological deficit scores after 1 hour of MCAO and 24 hours of reperfusion from PKC $\delta^{+/+}$ ($n = 8$) and PKC $\delta^{-/-}$ mice ($n = 8$). (G and H) Infarct size in the cerebral cortex (G) and striatum (H) after 1 hour of MCAO and 24 hours of reperfusion from PKC $\delta^{+/+}$ ($n = 10$) and PKC $\delta^{-/-}$ ($n = 9$) mice. * $P < 0.05$ compared with WT littermates (two-tailed, unpaired t test). † $P < 0.05$ compared with WT littermates (one-tailed, unpaired t test).

WT mice (Figure 5, E and F). These findings indicate an important role for PKC δ in several aspects of neutrophil function.

Exchange of bone marrow between PKC δ WT and null mice reverses their stroke phenotypes. To demonstrate in vivo whether neutrophils contributed to the differences in reperfusion injury observed between PKC δ -null and WT mice, we treated mice of both genotypes with total body irradiation followed by transplantation with bone marrow from the opposite genotype. Mice were then subjected to 1 hour of cerebral ischemia followed by 24 hours of reperfusion. WT mice transplanted with PKC δ -null bone marrow showed reduced infarct size and improved neurological scores compared with PKC δ -null mice transplanted with bone marrow from WT donors (Figure 6, A–C). In addition, the number of neutrophils present in the cerebral cortex and striatum was less in WT mice transplanted with PKC δ -null bone marrow compared with PKC δ -null mice transplanted with bone marrow from WT mice (Figure 6, E and F). Survival of the transplanted tissue was verified by blood cell counts 45 days after transplantation (Table 1) and by immunoblotting with anti-PKC δ Ab's (Figure 6D).

Discussion

We believe that this study is the first to demonstrate that PKC δ is a major mediator of brain damage following transient ischemia and reperfusion in mice. Our findings also provide clear evidence that PKC δ mediates neutrophil adhesion, migration, degranulation, and superoxide generation. Impaired neutrophil function can reduce reperfusion brain injury as demonstrated, for example, in mice made deficient in Mac-1 (CD11b/CD18) (20, 21). The protective

effect of PKC δ deletion was more pronounced in the cortex than in the striatum (Figure 2, G and H), which also agrees with previous studies indicating a greater role for neutrophils in damage to the cerebral cortex (22). Neutrophils are proposed to damage ischemic brain tissue by adhesion to endothelial cells and transmigration into brain parenchyma where they release oxygen-derived free radicals, phospholipases, proteases, and proinflammatory cytokines such as IL-1 β and TNF- α (23). Given that PKC δ -null mice showed reduced brain injury in the setting of impaired neutrophil function, our results support further testing of inhibitors of neutrophil function as therapeutic agents to reduce infarct size and improve neurological outcome after ischemia-reperfusion injury (24, 25).

Absence of PKC δ disturbed several aspects of neutrophil function, suggesting that PKC δ regulates neutrophils through several mechanisms. Particularly striking in our results was the nearly complete absence of TNF- α -induced O $_2^-$ production in neutrophils from PKC δ -null mice (Figure 5C). This is especially interesting since Brown and colleagues (26) recently found that neutrophils immunodepleted of PKC δ show deficient O $_2^-$ production by NADPH oxidase. PKC δ is likely to stimulate NADPH oxidase by phosphorylating the p47^{phox} subunit, leading to assembly and activation of the enzyme complex (27). Since mice deficient in NADPH oxidase also show reduced reperfusion injury following transient ischemia (28), PKC δ regulation of NADPH oxidase is likely to be particularly important in reperfusion injury. In addition, recent evidence demonstrates that integrin-dependent T cell activation by leukocyte functional antigen 1 (LFA-1, also known as CD11a/CD18 or $\alpha_L\beta_2$) results in PKC δ -mediated phosphorylation of the LFA-1 β_2 integrin cytoplasmic domain

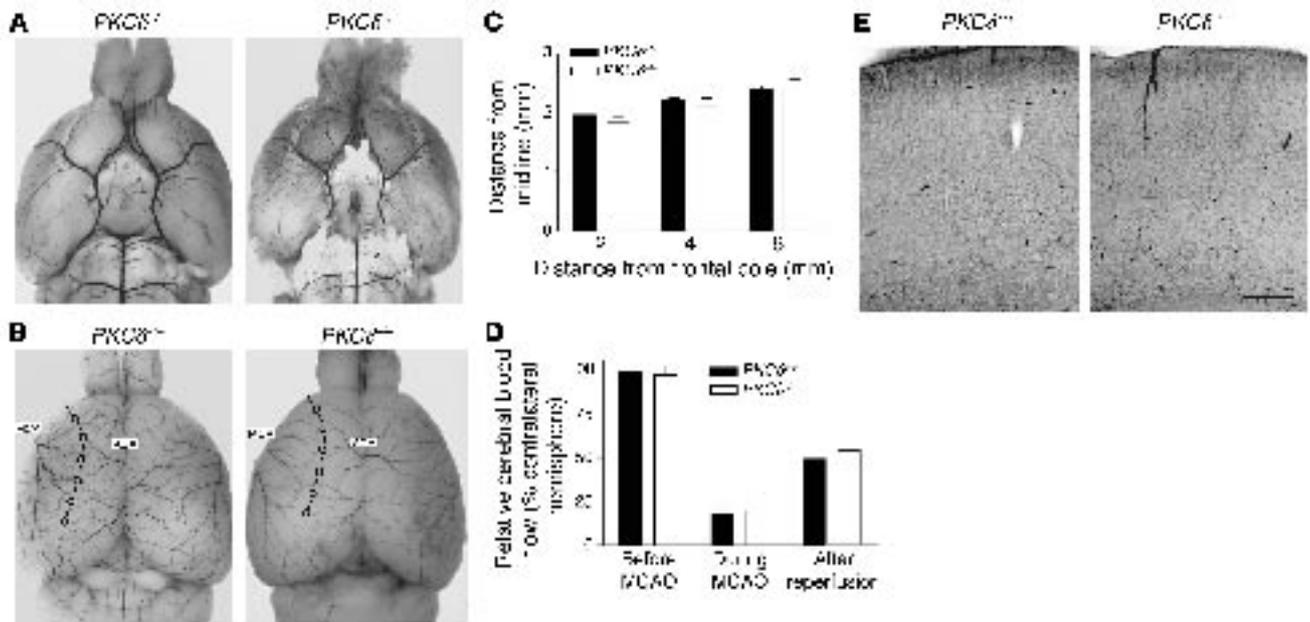


Figure 3 Cerebrovascular anatomy and cerebral blood flow. (A and B) Shown are representative images of the ventral (A) and dorsal (B) surfaces of brains from *PKCδ^{+/+}* and *PKCδ^{-/-}* mice perfused with black ink. The points of anastomoses between the MCA and the ACA are circled and connected by the line of anastomoses to define the respective vascular territories. (C) Distances from the line of anastomoses to the midline in *PKCδ^{+/+}* ($n = 3$) and *PKCδ^{-/-}* ($n = 3$) mice were measured at coronal planes 2, 4, and 6 mm from the frontal pole. (D) Regional cerebral blood flow before and during 1 hour of MCAO and during the first hour of reperfusion in *PKCδ^{+/+}* ($n = 3$) and *PKCδ^{-/-}* ($n = 3$) mice. Relative cerebral blood flow was expressed as the percentage of the Doppler signal intensity of the ischemic compared with the contralateral hemisphere. (E) The microvasculature in the cortex of *PKCδ^{+/+}* and *PKCδ^{-/-}* mice revealed by NADPH diaphorase histochemistry. The scale bar corresponds to 250 μ m.

and of cytohesion, thereby promoting c-Jun-mediated transcription and activation of ERK-1 and ERK-2 MAPKs (29). These events promote differentiation of naive T cells into IFN- γ -producing Th1 cells. Since cytokine-induced neutrophil adhesion and respiratory burst can be mediated by LFA-1 (30, 31), PKC δ may also act to transduce integrin-dependent signals in neutrophils.

Although PKC δ has been implicated in radiation-induced injury (32), such a mechanism could not account for the differences we observed since the stroke phenotype was determined by the genotype of the nonirradiated donor bone marrow and not by the genotype of the irradiated recipient. Moreover, stroke volume is not altered by lethal irradiation in WT mice subjected to transient MCAO after bone marrow transplantation (28). We also consid-

ered if other bone marrow-derived cells besides neutrophils mediate reperfusion injury in transplanted WT and PKC δ -null mice. Although macrophages can be observed in and surrounding ischemic lesions in the rodent brain, they appear to be derived from

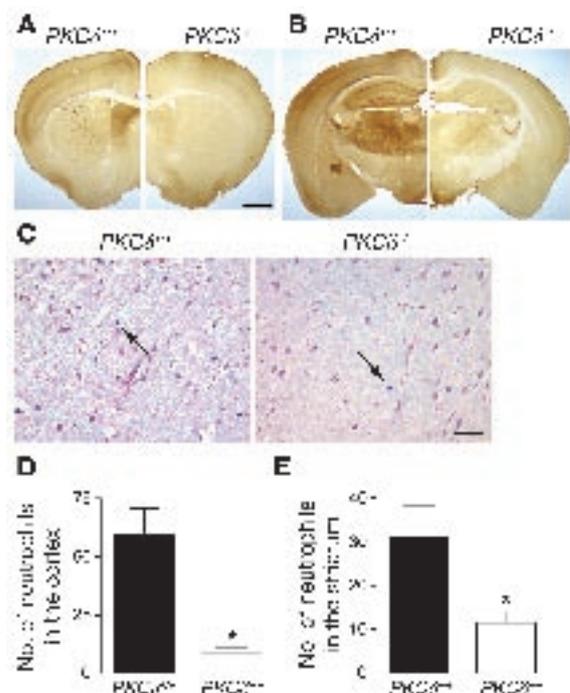


Figure 4 Expression pattern of PKC δ in the brain and extravascular neutrophils after transient MCA occlusion. (A and B) Immunocytochemical localizations of PKC δ in the mouse brain are shown in coronal sections at bregma 0.38 mm (A) and a more posterior section at bregma -1.58 mm (B) for *PKCδ^{+/+}* (left) and *PKCδ^{-/-}* (right) mice. (C) Representative sections from *PKCδ^{+/+}* and *PKCδ^{-/-}* mice showing reduced neutrophil accumulation within infarcted tissue in the cortex of *PKCδ^{-/-}* mice after 1 hour of MCAO and 24 hours of reperfusion. Blue infiltrated neutrophils were identified in the ischemic cortex by staining for esterase activity with dichloroacetate (arrows). (D and E) Number of extravascular neutrophils in the ischemic cortex and striatum of *PKCδ^{+/+}* ($n = 7$) and *PKCδ^{-/-}* mice ($n = 6$) after transient MCAO. No esterase staining was seen in sections from nonischemic animals (data not shown). The scale bars in A and C correspond to 1 mm and 25 μ m, respectively. * $P < 0.05$ compared with WT littermates.

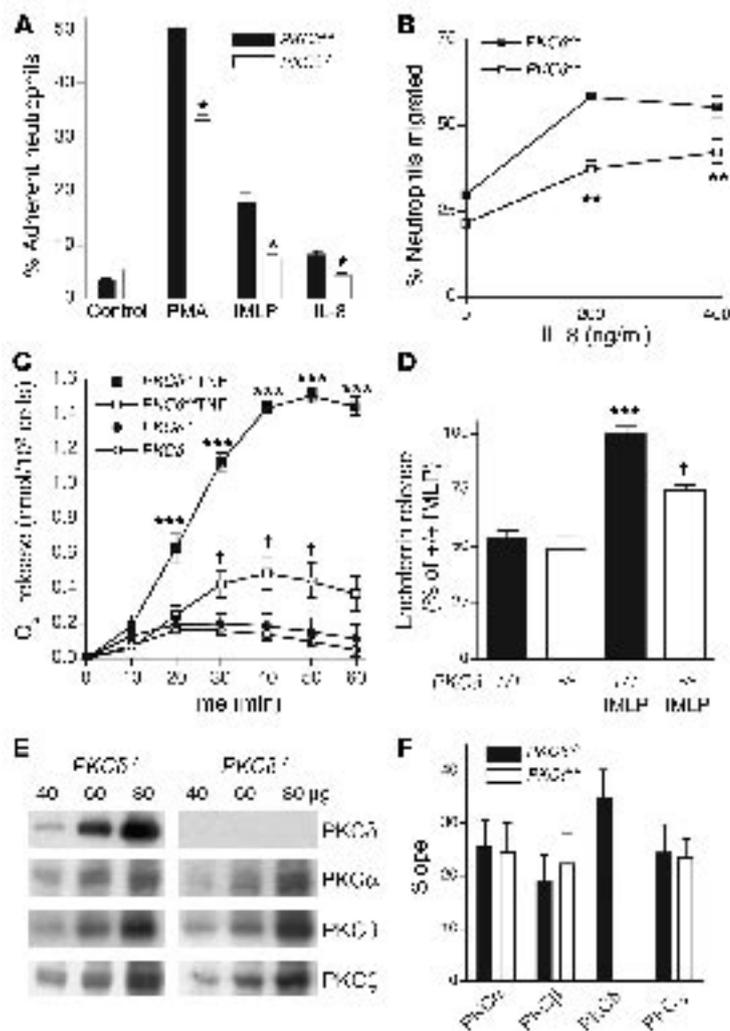


Figure 5

Decreased neutrophil function in PKC δ -null mice. (A) Neutrophil adhesion stimulated by PMA (100 nM), fMLP peptide (1 μ M), or IL-8 (200 ng/ml). * P < 0.05 compared with WT littermates (two-tailed, unpaired t tests). (B) IL-8-stimulated neutrophil migration differed by genotype [F(1,6) = 54.8; P = 0.0003] and treatment [F(2,6) = 63.08; P < 0.0001] without significant interaction between these factors [F(2,6) = 3.9; NS]. ** P < 0.05 compared with PKC $\delta^{+/+}$ at same dose (Bonferroni test). (C) TNF- α -stimulated (20 ng/ml) superoxide anion production showed main effects of treatment [F(1,224) = 236; P < 0.0001], genotype [F(1,224) = 506; P < 0.0001], and time [F(6,224) = 64.6; P < 0.0001] with an interaction between these factors [F(6,224) = 14.7; P < 0.0001]. † P < 0.05 compared with unstimulated PKC $\delta^{-/-}$ neutrophils, and *** P < 0.05 compared with unstimulated PKC $\delta^{+/+}$ and stimulated PKC $\delta^{-/-}$ neutrophils at the same time (Newman Keuls test). (D) fMLP-stimulated (10 μ M) lactoferrin release differed by genotype [F(1,12) = 15.2; P = 0.0021] and treatment [F(1,12) = 89.01; P < 0.0001] with an interaction between these factors [F(1,12) = 7.24; P = 0.0197]. † P < 0.05 compared with unstimulated PKC $\delta^{-/-}$ neutrophils, and *** P < 0.05 compared with unstimulated PKC $\delta^{+/+}$ and stimulated PKC $\delta^{-/-}$ neutrophils (Bonferroni tests). (E) PKC isozyme immunoreactivity in neutrophils. (F) PKC isozyme immunoreactivity determined by regression analysis of protein concentrations and corresponding OD values on Western blots (n = 3). Assays of neutrophil function (A–D) were performed in triplicate or quadruplicate from at least three animals of each genotype.

resident microglia rather than from peripheral blood monocytes during the first 24 hours of reperfusion (33). Additionally, current evidence indicates that adult microglia arise from fetal macrophages rather than from adult peripheral monocytes (34). Given that anti-platelet agents such as aspirin and clopidogrel can reduce the incidence of stroke in humans (35) and reduce injury size in mouse stroke models (36, 37), we also considered whether platelet number or function was decreased in PKC δ -null mice. Although platelet counts, bleeding times, and cerebral blood flow during reperfusion were all similar in WT and PKC δ -null mice, these findings do not exclude a subtle deficit in platelet function. Considering that all clinically effective anti-platelet agents used to treat stroke prolong the bleeding time, however, our findings make it unlikely that a deficit in platelet function or number contributed to reduced reperfusion injury in PKC δ -null mice. On the other hand, in both genotypes there was a moderate decrease in platelet number following total body irradiation and bone marrow transplantation. This was more apparent in WT mice transplanted with marrow from PKC δ -null donors, which may indicate a role for PKC δ in megakaryocyte differentiation and platelet production. This difference in platelet number may have contributed to the more striking differences in stroke volume observed between the genotypes after transplantation (compare Figures 2 and 6).

In the heart PKC ϵ contributes to protection from ischemic damage and absence of PKC ϵ is associated with loss of ischemic preconditioning and with increased expression of PKC δ (38). Cerebral reperfusion injury in PKC δ -null mice, however, is not due to increases in PKC ϵ since PKC ϵ is not expressed in mouse neutrophils, and its expression in the brains of PKC δ -null and WT mice is similar (data not shown). Inhibition of PKC δ in intact rat hearts ex vivo also reduces injury due to ischemia and reperfusion; this does not involve neutrophil PKC δ , since the hearts are perfused with oxygenated buffer lacking blood cells (39). Furthermore, myocardial ischemia/reperfusion injury is similar in WT mice and in mice deficient in NADPH oxidase, whereas significant differences are observed in the cerebral ischemia/reperfusion injury between these genotypes (28). Taken together with our results, these findings indicate a unique role for neutrophil PKC δ in cerebral ischemia-reperfusion injury. This raises the intriguing possibility that PKC δ inhibitors could be useful as adjuncts to fibrinolytic therapy of acute stroke to reduce the risk of reperfusion injury and possibly lengthen the window of time during which drugs such as t-PA can be administered safely.

Methods

Generation of PKC δ -null mice. PKC δ mutant mice were derived by homologous recombination using a targeting vector encoding loxP sequences flanking exon 3 of the mouse PKC δ gene (ENS-MUSG00000021948; NCBI m32), which was introduced into 129SvJ ES cells (Figure 1A). Exon 2 includes the translation start codon. Recombinant cells were then transfected with the plasmid pPac-CRE (40), and ES cell clones lacking exon 2 were identified by Southern blot analysis and PCR. F₁ generation hybrid C57Bl/6J \times 129/SvJ heterozygous progeny of chimeric mice were intercrossed to generate F₂ generation hybrid WT and PKC δ mutant littermates for studies. Unless otherwise stated, we used mice of both genders. The

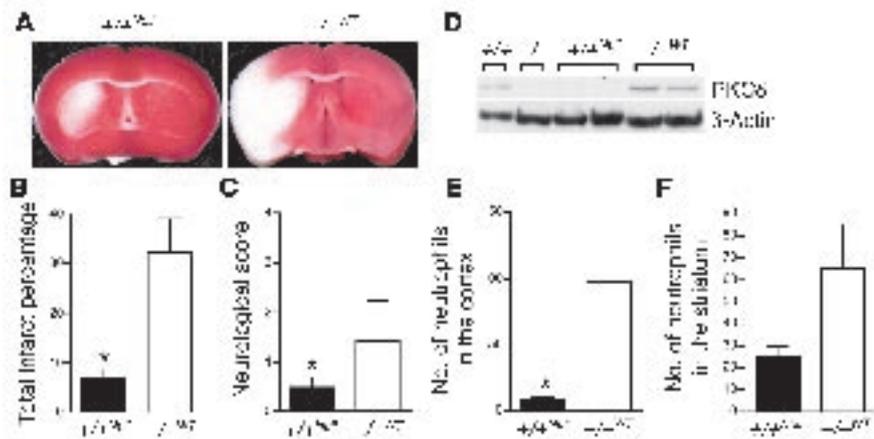


Figure 6

Bone marrow transplantation reverses phenotypes in PKC δ WT and null mice. (A–C) Representative images of TTC-stained brain slices (A), infarct sizes (B), and neurological scores (C) after transient MCAO of irradiated WT mice transplanted with PKC δ -null bone marrow [(+/+^{NULL}), n = 7] and irradiated null mice transplanted with WT marrow [(-/-^{WT}), n = 8]. (D) PKC δ immunoreactivity in bone marrow cells from transplanted mice to confirm the success of bone marrow transplantation. PKC δ immunoreactivity was detected in the marrow of WT (+/+) mice and irradiated null mice transplanted with WT marrow (-/-^{WT}), but not in PKC δ -null mice (-/-) nor in irradiated WT mice transplanted with PKC δ -null bone marrow (+/+^{NULL}). Shown are samples from one WT, one PKC δ -null, two +/+^{NULL}, and two -/-^{WT} mice. Samples were normalized to β -actin immunoreactivity. (E and F) Number of extravascular neutrophils in the ischemic cortex (E) and striatum (F) of irradiated WT mice transplanted with PKC δ -null bone marrow [(+/+^{NULL}), n = 6] and irradiated null mice transplanted with WT marrow [(-/-^{WT}), n = 6] after 1 hour of MCAO and 24 hours of reperfusion. *P < 0.05 compared with WT littermates.

experimental mice were fed standard lab chow ad libitum. Animal care and handling procedures were approved by the Institutional Animal Care and Use Committees of the University of California San Francisco and Gallo Center in accordance with NIH guidelines.

Focal cerebral ischemia. The surgical procedure to induce focal cerebral ischemia is a modification based on a published protocol (41). Mice weighing 25–35 g were anesthetized with 3% isoflurane in 100% O₂. Following the induction of anesthesia, isoflurane was reduced to and maintained at 1.5%. Body temperature was maintained at 35.5–37°C with a heating pad throughout the surgery. A 5-0-monofilament nylon suture rounded at the tip was inserted into external carotid artery and advanced 9.0–10 mm in the internal carotid artery (ICA) past the bifurcation of the common carotid artery (CCA), to the proximal MCA. Following 60 minutes of occlusion, the suture was removed from the CCA to induce reperfusion. After 24 hours of reperfusion, the mice were sacrificed for study. Some mice were subjected to permanent MCAO by leaving the suture in the ICA for 20 hours.

Regional cerebral blood flow. Regional cerebral blood flow was measured by laser-Doppler flowmetry with a probe (Vasamedics Inc., St. Paul, Minnesota, USA) placed on the skull 1.5 mm lateral to the midline and 2 mm posterior to the bregma. Relative cerebral blood flow measurements were made during three different periods: after anesthesia, during a 1-hour period of MCAO, and during the first hour of reperfusion. Data are expressed as the ratio of the Doppler signal intensity of the ischemic hemisphere compared with the contralateral hemisphere.

Neurological deficits. Neurological examinations were performed 20 hours after the induction of focal cerebral ischemia by use of a four-tiered grading system: 0, no observed neurological deficit (normal);

1, inability to walk straight (mild); 2, circling toward the paretic side (moderate); 3, falling on the paretic side (moderate-severe); 4, loss of the righting reflex (severe) (20).

Determination of infarct size. After focal cerebral ischemia, the mice were sacrificed and the brains were rapidly removed and sliced coronally at 1-mm intervals. Brain slices were incubated for 20 minutes in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, Missouri, USA) in PBS. Stained slices were fixed in 10% neutral buffered formalin (Sigma-Aldrich) until imaging. Brain slices were photographed using a Leica M420 microscope equipped with a Sony 3CCD color video camera. A blinded observer measured the areas of ischemic infarction and of the normal contralateral half of the brain by tracing outlines of these regions on a computer screen using NIH Image 1.6.1 software. The infarcted area was expressed as a percentage of the contralateral half of the brain to eliminate the contribution of edema to the calculation (42, 43). Total infarct area was calculated by dividing the sum of infarct areas by the sum of contralateral hemi-brain areas.

Visualization of cerebrovascular anatomy. The mapping of cerebrovascular anatomy was performed as described (44). Higgins Black

Magic waterproof ink (200–250 μ l; Sanford Corp., Bellwood, Illinois, USA) was injected into the left ventricle using a 26-gauge needle, and the right atrium was cut open to release the effluent. The mice were decapitated, and the heads were soaked in 10% neutral buffered formalin (Sigma-Aldrich) for 3–4 days. The brains were carefully removed from the skulls and imaged. Points of anastomoses between anterior cerebral artery (ACA) and MCA were located by tracing the peripheral branches of these vessels. Adjacent anastomotic points were connected to identify a “line of anastomoses,” which represents the border of the territories of the ACA and the MCA. The distances from the midline to the line of anastomoses were measured at coronal planes 2, 4, and 6 mm from the frontal pole. Brain capillaries were stained using NADPH diaphorase histochemistry, which reveals both endothelial and neuronal NOS in the rodent brain. This was done by post-fixing formaldehyde-fixed tissue sections overnight in 2% glutaraldehyde in PBS at 4°C, rinsing them in PBS, and incubating them in NADPH (0.5 mg/ml; Sigma-Aldrich), nitroblue tetrazolium (0.2 mg/ml; Sigma-Aldrich), and 0.3% Triton X-100 in PBS for 4 hours at 37°C.

Immunohistochemistry. The brains were processed as described (45). Coronal sections were incubated overnight with a primary Ab (a goat polyclonal Ab recognizing the C terminus of rat PKC δ (1:1,000–1,500; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), or a mAb (1:1,500; BD Biosciences, San Diego, California, USA) generated using a peptide comprised of amino acids 114–289 of human PKC δ , which is encoded by exons 5–10 of the human PKC δ gene (ENST00000330452); this corresponds to exons 4–9 of the mouse gene (ENSMUSG00000021948; NCBI m32). Secondary Ab's were biotinylated donkey anti-goat or anti-mouse Ab's (1:300; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania,



Table 1
Blood cell counts before and 45 days after bone marrow transplantation

	Blood cell counts ^A			
	PKC $\delta^{+/+}$ (n = 5)	PKC $\delta^{-/-}$ (n = 4)	+/ \pm M ^{Null} (n = 4)	-/ \pm WT (n = 5)
wbc's (10 ³ / μ l)	6.09 \pm 0.74	7.78 \pm 0.90	3.35 \pm 0.88	4.06 \pm 0.51
Neutrophils (10 ³ / μ l)	1.03 \pm 0.09	1.29 \pm 0.31	1.12 \pm 0.51	0.96 \pm 0.19
Lymphocytes (10 ³ / μ l)	4.53 \pm 0.67	5.70 \pm 0.77	1.98 \pm 0.30	2.66 \pm 0.28
Monocytes (10 ³ / μ l)	0.40 \pm 0.06	0.43 \pm 0.09	0.13 \pm 0.05	0.22 \pm 0.03
rbc's (10 ⁹ / μ l)	11.32 \pm 0.90	10.47 \pm 0.53	10.25 \pm 0.36	10.47 \pm 0.14
Platelet (10 ³ / μ l)	709.40 \pm 171.80	719.80 \pm 175.00	127.0 \pm 32.63	355.2 \pm 88.00

^AValues shown are means \pm SEM. wbc's, white blood cells.

nia, USA). Incubation for 2 hours with a secondary Ab was followed by incubation in ExtrAvidin-peroxidase complex (Sigma-Aldrich), 1:3,000 in PBS, for 2 hours. Peroxidase activity was histochemically visualized with diaminobenzidine. Slides were examined using a Nikon Eclipse E600 microscope equipped with a Spot II digital color camera. Both primary Ab's gave similar patterns of staining in the brains of WT mice. The specificity of the PKC δ Ab's for mouse PKC δ was verified using tissue obtained from PKC δ -null mice in which immunostaining was absent. In control experiments, omitting primary Ab's resulted in the lack of immunostaining.

Western blot analysis. Protein lysates were subjected to SDS-PAGE. After blotting, nitrocellulose membranes were probed with mAb's against PKC δ , PKC α , and PKC β (BD Transduction Laboratories, San Diego, California, USA), which were used at dilutions of 1:500, 1:1,000, and 1:250, respectively. Rabbit polyclonal Ab against PKC ζ (Santa Cruz Biotechnology Inc.) was used at 0.5 μ g/ml. Rabbit polyclonal Ab against PKC ϵ (SN134) was used at 1:2,000 (46). Immunoreactive bands were quantified by densitometric scanning. Linear regression analysis of protein concentrations and corresponding density values was performed, and the abundance of each isozyme was expressed as the slope of the corresponding regression line (47).

Neutrophil adhesion, migration, O₂⁻ release, and degranulation. Neutrophils were isolated by Percoll density gradient centrifugation from mouse bone marrow as described (48). Adhesion assays were performed in 96-well Falcon plates (BD Discovery Labware, Boston, Massachusetts, USA) coated with 20% FCS (49). Purified neutrophils were fluorescently labeled with calcein AM (Molecular Probes Inc., Eugene, Oregon, USA) and incubated in the wells for 30 minutes at 37°C with the chemoattractants phorbol 12-myristate, 13-acetate (100 nM; Sigma-Aldrich), fMLP (1 μ M; Sigma-Aldrich), and IL-8 (200 ng/ml; Sigma-Aldrich). The total number of cells was measured by fluorescence detection using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, Massachusetts, USA) at 485-nm excitation and 530-nm emission wavelengths. The plates were then washed several times to remove nonadherent cells, and the fluorescence remaining on the plates was measured again. The degree of cell adhesion was expressed as a percentage calculated by dividing the amount of fluorescence after washing by the amount of fluorescence before washing.

The neutrophil chemotaxis assay (in vitro migration assay) was performed as described (50). Transwell inserts (5- μ m pore size polycarbonate membranes; Corning Inc., Corning, New York, USA) were filled with neutrophils in suspension and were placed into media containing indicated concentrations of IL-8 in 24-well tissue culture plates. After 1 hour of incubation in a humidified

CO₂ incubator at 37°C, the inserts were removed, and the number of neutrophils that migrated into the bottom of the wells was determined by FACS analysis. Parallel samples of wells loaded with suspension without inserts were included to determine the maximal signal intensity from the number of cells loaded into the Transwell inserts.

Superoxide anion (O₂⁻) release was measured by a cytochrome *c* reduction assay using 20 ng/ml TNF- α (BD Biosciences) and 10 μ M fMLP or 100 nM PMA as a stimulus (51). Degranulation stimulated by fMLP (10 μ M) or PMA (100 nM) was determined by the release of the specific granule marker lactoferrin using anti-human lactoferrin

Ab (Sigma-Aldrich) by ELISA (52).

Blood cell counts. Blood was collected by decapitation, and the profiles of blood samples were analyzed using a HEMAVET 850 cell counter (CDC Technologies Inc., Oxford, Connecticut, USA).

Histological detection of neutrophils in brain. Brain neutrophils were detected as described (21). Coronal brain slices containing the striatum were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and embedded in paraffin. Tissue was cut into serial 7- μ m-thick sections, mounted on standard microscope slides, deparaffinized, and incubated in solution containing Naphesol AS-D and fast blue salt (Sigma-Aldrich) to reveal the presence of chloroacetate esterase activity. Sections were counterstained with nuclear fast red (Vector Laboratories, Burlingame, California, USA). Neutrophils in the cortex and striatum were counted by a blinded observer using unbiased microscopic sampling (optical dissector) in four serial, but nonadjacent, sections representing one, approximately 0.5-mm-thick coronal slice from a forebrain region (bregma coordinates were approximately 0-1) for each animal. Cells were sampled at Nikon Eclipse E600 microscope using \times 40 objective. Neutrophils within blood vessels were not counted.

Bone marrow transplantation. Bone marrow transplantation was accomplished by intravenous injection of the donor's bone marrow into the left retro-orbital sinus of the recipients (52). Bone marrow suspensions were prepared from the cells flushed from the femurs of donors (2-5 months old). The recipients (5-8 weeks old) were given lethal doses of total body radiation with two exposures given 3 hours apart of 6 Gy from a ¹³⁷Cs source. The irradiated recipients were rescued by injecting bone marrow suspensions (5 \times 10⁶ cell in 0.3 ml) from the donors within 1 hour after the irradiation. Polymyxin B (120 U/ml; Sigma-Aldrich) and neomycin (0.6 mg/ml; Sigma-Aldrich) were added into the drinking water for the transplanted mice for 20 days after transplantation to suppress pathogens.

Statistical analysis. Quantitative data were expressed as mean plus or minus SEM. One-, two-, or three-way ANOVA with Bonferroni or Newman Keuls post-hoc tests, or two-tailed, unpaired Student's *t* tests were used to assess statistical significance. In all tests, *P* values less than 0.05 were considered to be statistically significant.

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