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Neutrophil protein kinase Cδ as a mediator of stroke-reperfusion injury

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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 isoforms with distinctive means of regulation and tissue distribution (8). Five isoforms (PKCα, βI, βII, δ, and ζ) are known to be present in human neutrophils (7). The exact functional role of these different PKC isoforms in neutrophils remains to be specified.

PKCδ is a member of the novel PKC subfamily that is activated by diacylglycerol (DAG) but not by calcium. PKCδ promotes apoptosis in various types of cultured cells (9), including neuronal cells (10). In mice that lack PKCδ, 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 PKCδ peptide inhibitors and activators indicate an important role of PKCδ in cardiac ischemia and reperfusion (14). The mechanism by which PKCδ promotes reperfusion damage in the heart remains to be demonstrated.

Despite evidence linking PKCδ 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 PKCδ in nervous system ischemic injury, we generated mice that lack PKCδ and studied their response to cerebral ischemia. Using a model of transient middle cerebral artery (MCA) occlusion (MCAO), we found that PKCδ-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 PKCδ-null mice. Treatment with total body irradiation followed by transplantation with bone marrow from the opposite genotype reversed the stroke phenotypes in WT and PKCδ-null mice, confirming an important role for neutrophil PKCδ in reperfusion injury.

Results

Infarct size is reduced in PKCδ-null mice after transient, but not permanent, MCAO. We generated mice that lack PKCδ using homologous recombination in ES cells (Figure 1). Neither full-length nor truncated PKCδ-like immunoreactivity was detected in PKCδ-null mice cells by Western blot analysis (Figure 1D and data not shown). These mice...
Interestingly, infarct size was decreased by about 65% in PKCδ-null mice (Figure 3A). The distribution of the MCA territory was also similar in WT and PKCδ-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).

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

**Attenuated neutrophil function in PKCδ-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 PKCδ-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 PKCδ-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 PKCδ-null mice compared with WT littermates. In addition, superoxide anion (O₂⁻) 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 and data not shown), were reduced in neutrophils from PKCδ-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 PKCδ-null mice when compared with WT littermates (Table 1). The abundance of other PKC isoforms expressed in neutrophils was also not altered in PKCδ-null mice compared with

**Figure 1**

Generation of PKCδ-null mice. (A) Organization of the mouse PKCδ 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 PKCδ. The migration of PKCδ immunoreactivity (78 kDa) is indicated.
WT mice (Figure 5, E and F). These findings indicate an important role for PKCδ in several aspects of neutrophil function.

**Exchange of bone marrow between PKCδ WT and null mice reverses their stroke phenotypes.** To demonstrate in vivo whether neutrophils contributed to the differences in reperfusion injury observed between PKCδ-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 PKCδ-null bone marrow showed reduced infarct size and improved neurological scores compared with PKCδ-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 PKCδ-null bone marrow compared with PKCδ-null mice transplanted with mouse 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-PKCδ Ab’s (Figure 6D).

**Discussion**

We believe that this study is the first to demonstrate that PKCδ is a major mediator of brain damage following transient ischemia and reperfusion in mice. Our findings also provide clear evidence that PKCδ 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 PKCδ 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-1β and TNF-α (23). Given that PKCδ-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 PKCδ disturbed several aspects of neutrophil function, suggesting that PKCδ regulates neutrophils through several mechanisms. Particularly striking in our results was the nearly complete reduction of infarct size following transient ischemia (28), PKCδ 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 αβ) results in PKCδ-mediated phosphorylation of the LFA-1 β2 integrin cytoplasmic domain.
and of cytohesion, thereby promoting c-Jun–mediated transcription and activation of ERK-1 and ERK-2 MAPKs (29). These events promote differentiation of naïve T cells into IFN-γ–producing Th1 cells. Since cytokine-induced neutrophil adhesion and respiratory burst can be mediated by LFA-1 (30, 31), PKCδ may also act to transduce integrin-dependent signals in neutrophils.

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

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 μm.

Figure 4
Expression pattern of PKCδ in the brain and extravascular neutrophils after transient MCA occlusion. (A and B) Immunocytochemical localizations of PKCδ 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 μm, respectively. *P < 0.05 compared with WT littermates.
residents of 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 PKCε-null mice.

Although platelet counts, bleeding times, and cerebral blood flow during reperfusion were all similar in WT and PKCε-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 PKCε-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 PKCε-null donors, which may indicate a role for PKCε 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 PKCε contributes to protection from ischemic damage and absence of PKCε is associated with loss of ischemic preconditioning and with increased expression of PKCδ (38). Cerebral reperfusion injury in PKCδ-null mice, however, is not due to increases in PKCε since PKCε is not expressed in mouse neutrophils, and its expression in the brains of PKCδ-null and WT mice is similar (data not shown). Inhibition of PKCδ in intact rat hearts ex vivo also reduces injury due to ischemia and reperfusion; this does not involve neutrophil PKCε, 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 PKCδ in cerebral ischemia-reperfusion injury. This raises the intriguing possibility that PKCδ 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 PKCδ-null mice. PKCδ mutant mice were derived by homologous recombination using a targeting vector encoding loxP sequences flanking exon 3 of the mouse PKCδ gene (ENS-MUSG00000021948; NCBI32), 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 x 129SvJ heterozygous progeny of chimeric mice were intercrossed to generate F2 generation hybrid WT and PKCδ mutant littersates for studies. Unless otherwise stated, we used mice of both genders. The
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 the internal carotid artery (ICA) past the bifurcation of the common 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 μl; 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) until imaging. Stained slices were fixed in 10% neutral buffered formalin (Sigma-Aldrich), or a mAb (1:1,500; BD Biosciences, San Diego, California, 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 anastomatic 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 PKCδ, 1:1000–1,500; 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 PKCδ, which is encoded by exons 5–10 of the human PKCδ gene (ENST00000330452); this corresponds to exons 4–9 of the mouse gene (ENSMSUG00000021948; NCBI31. Secondary Ab’s were biotinylated donkey anti-goat or anti-mouse Ab’s (1:300; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania...
The abundance of each isozyme was expressed as the slope of the correlation and corresponding density values was performed, and the number of cells was measured by fluorescence detection 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 PKCδ Ab’s for mouse PKCδ was verified using tissue obtained from PKCδ–/– 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 PKCδ, PKCζ, and PKCβ (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 PKCζ (Santa Cruz Biotechnology Inc.) was used at 0.5 μg/ml. Rabbit polyclonal Ab against PKCe (SN134) was used at 1:2,000 (46). Immuno reactive 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, O2release, 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 calcine 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 μM; 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-μm 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 μM fMLP or 100 nM PMA as a stimulus (51). Degranulation stimulated by fMLP (10 μM) 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-μm-thick sections, mounted on standard microscope slides, deparaffinized, and incubated in solution containing Naphosol 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 x40 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 × 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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**Table 1**

<table>
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<th>Blood cell counts</th>
<th>Blood cell counts*</th>
<th>PKCδ+/+ (n = 5)</th>
<th>PKCδ−/− (n = 4)</th>
<th>1/x Mean (n = 4)</th>
<th>1/x Mean (n = 5)</th>
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<td>wbc’s (10³/μl)</td>
<td>6.09 ± 0.74</td>
<td>7.78 ± 0.90</td>
<td>3.35 ± 0.88</td>
<td>4.06 ± 0.51</td>
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<tr>
<td>Neutrophils (10³/μl)</td>
<td>1.03 ± 0.09</td>
<td>1.29 ± 0.31</td>
<td>1.12 ± 0.51</td>
<td>0.96 ± 0.19</td>
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<td>Lymphocytes (10³/μl)</td>
<td>4.53 ± 0.67</td>
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<td>1.98 ± 0.30</td>
<td>2.66 ± 0.28</td>
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<tr>
<td>Monocytes (10³/μl)</td>
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<tr>
<td>rbc’s (10³/μl)</td>
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<td>Platelet (10³/μl)</td>
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<td>719.80 ± 175.00</td>
<td>127.0 ± 32.63</td>
<td>355.2 ± 88.00</td>
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*Values shown are means ± SEM. wbc’s, white blood cells.


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