Superoxide Generation Links Protein Kinase C Activation to Impaired ATP-Sensitive K⁺ Channel Function After Brain Injury

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Background and Purpose—Endothelin-1, in concentrations similar to that present in cerebrospinal fluid after fluid percussion brain injury (FPI), increases superoxide anion (O₂⁻) production. Endothelin-1 also contributes to altered cerebral hemodynamics after FPI through impairment of ATP-sensitive K⁺ (K_ATP) channel function through protein kinase C (PKC) activation. Generation of O₂⁻ additionally occurs after FPI. Nitric oxide and cGMP elicit pial artery dilation through K_ATP channel activation. The present study was designed to determine whether PKC activation generates O₂⁻, which, in turn, could link such activation to impaired K_ATP channel function after FPI.

Methods—Injury of moderate severity (1.9 to 2.1 atm) was produced by the lateral FPI technique in anesthetized newborn pigs equipped with a closed cranial window. Superoxide dismutase-inhibitable nitroblue tetrazolium (NBT) reduction was determined as an index of O₂⁻ generation.

Results—Phorbol 12,13-dibutyrate (10⁻⁶ mol/L), a PKC activator, increased superoxide dismutase-inhibitable NBT reduction from 1±1 to 37±5 pmol/mm². Staurosporine (10⁻⁷ mol/L), a PKC antagonist, blocked the NBT reduction after phorbol 12,13-dibutyrate and blunted the NBT reduction observed after FPI (1±1 to 15±2 versus 1±1 to 5±1 pmol/mm² after FPI in the absence versus presence of staurosporine). Exposure of the cerebral cortex to a xanthine oxidase O₂⁻-generating system increased NBT reduction in a manner similar to FPI and blunted pial artery dilation to the K_ATP channel agonists cromakalim and calcitonin gene–related peptide, the nitric oxide releasers sodium nitroprusside and S-nitroso-N-acetylpenicillamine, and the cGMP analogue 8-bromo-cGMP (10⁻¹% and 21±1% versus 4±1% and 9±1% for 10⁻⁸ and 10⁻⁶ mol/L cromakalim before and after activated oxygen-generating system exposure).

Conclusions—These data show that PKC activation increases O₂⁻ production and contributes to such production observed after FPI. These data also show that an activated system that generates an amount of O₂⁻ similar to that observed with FPI blunted pial artery dilation to K_ATP channel agonists and nitric oxide/cGMP. These data suggest, therefore, that O₂⁻ generation links PKC activation to impaired K_ATP channel function after FPI. (Stroke. 1999;30:153-159.)

Key Words: cerebral circulation ■ newborn ■ nitric oxide
observed that coadministration of ET-1 with K<sub>ATP</sub> channel agonists blunted dilation to such stimuli, suggesting that this peptide directly interacts with and impairs the K<sub>ATP</sub> channel. However, pathways involved in linking PKC activation to K<sub>ATP</sub> channel impairment are uncertain.

Superoxide anion (O<sub>2</sub><sup>-</sup>) production is thought to antagonize NO function and to contribute to altered cerebral hemodynamics after FPI because O<sub>2</sub><sup>-</sup> scavengers partially restored decreased NO-dependent dilator responses after FPI. Moreover, ET-1, in concentrations present in CSF after FPI, has been observed to release O<sub>2</sub><sup>-</sup>. Therefore, ET-1 may link O<sub>2</sub><sup>-</sup> generation to altered NO-dependent dilation after FPI. Interestingly, ET-1 is known to activate PKC, while PKC activation may lead to the generation of O<sub>2</sub><sup>-</sup>. However, the effect of O<sub>2</sub><sup>-</sup> on cerebrovascular K<sup>+</sup> channel function is uncertain.

Therefore, the present study was designed to determine whether PKC activation generates O<sub>2</sub><sup>-</sup>, which, in turn, could link such activation to impaired K<sub>ATP</sub> channel function after FPI. Three major types of experiments were performed to address this hypothesis. First, the ability of PKC activation to generate O<sub>2</sub><sup>-</sup> was explored. Second, the role of PKC activation in O<sub>2</sub><sup>-</sup> generation after FPI was investigated. Third, the effects of an exogenous activated oxygen-generating system applied to the cerebral cortical surface on vascular responses to K<sub>ATP</sub> channel agonists were explored.

Materials and Methods

Newborn pigs (1 to 5 days old) of either sex were used in these experiments. All protocols were approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with ketamine hydrochloride (33 mg/kg) and acepromazine (3.3 mg) intramuscularly. Anesthesia was maintained with α-chloralose (30 to 50 mg/kg, supplemented with 5 mg/kg per hour IV). A catheter was inserted into a femoral artery to monitor blood pressure and to sample for blood gas tensions and pH. Drugs to maintain anesthesia were administered through a second catheter placed in a femoral vein. The trachea was cannulated, and the animals were mechanically ventilated with room air. A heating pad was used to maintain the animals at 37°C to 38°C.

One or 2 cranial windows were placed in the parietal skull of these anesthetized animals. The window consists of 3 parts: a stainless steel ring, a connective segmental plastic reservoir, and a piston with a 4.8-kg pendulum. The intensity of the blow (usually 1.9 to 2.3 atm with a constant duration of 19 to 23 milliseconds) was controlled by varying the height from which the pendulum was allowed to fall. The pressure pulse of the blow was recorded on a storage oscilloscope triggered photoelectrically by the fall of the pendulum. The amplitude of the pressure pulse was used to determine the intensity of the injury.

Protocol

Drug effects on the diameter of 2 types of pial arterial vessels—small arteries (baseline diameter, 120 to 160 μm) and arterioles (baseline diameter, 50 to 70 μm)—were examined to determine whether segmental differences in the actions of O<sub>2</sub><sup>-</sup> could be identified. Pial arterial vessel diameter was determined every minute for a 10-minute exposure period after infusion onto the exposed parietal cortex of artificial CSF containing no drug and after infusion of artificial CSF containing a drug.

Ten major types of experiments were performed: (1) generation of O<sub>2</sub><sup>-</sup> with phorbol 12,13-dibutyrate (n=7); (2) generation of O<sub>2</sub><sup>-</sup> with phorbol 12,13-dibutyrate in the presence of staurosporine (n=7); (3) generation of O<sub>2</sub><sup>-</sup> with an activated oxygen-generating system (n=7); (4) generation of O<sub>2</sub><sup>-</sup> with an inactivated oxygen-generating system (n=5); (5) generation of O<sub>2</sub><sup>-</sup> with FPI (n=7); (6) generation of O<sub>2</sub><sup>-</sup> with FPI in the presence of staurosporine (n=7); (7) vascular response to agonists before and after generation of O<sub>2</sub><sup>-</sup> with an activated oxygen-generating system (n=7); (8) vascular response to agonists before and after generation of O<sub>2</sub><sup>-</sup> with an inactivated oxygen-generating system (n=7); (9) time control for agonist responses (n=5); and (10) sham control for O<sub>2</sub><sup>-</sup> generation (n=7).

In the vascular response experiments, responses of arterial vessels to the synthetic K<sub>ATP</sub> channel agonist (–) cromakalim (10<sup>-8</sup> and 10<sup>-6</sup> mol/L, SmithKline Beecham), the endogenous K<sub>ATP</sub> channel activator calcitonin gene–related peptide (CGRP) (10<sup>-7</sup> and 10<sup>-5</sup> mol/L, Sigma Chemical), the cGMP analogue 8-bromo-cGMP (10<sup>-7</sup> and 10<sup>-5</sup> mol/L, Research Biochemical International), and the NO donors SNP (10<sup>-7</sup> and 10<sup>-5</sup> mol/L, Sigma) and S-nitroso-N-acetylpenicillamine (SNAP) (10<sup>-7</sup> and 10<sup>-6</sup> mol/L, Research Biochemical International) were obtained before and 20 minutes after exposure to the active or inactive oxygen-generating system for 20 minutes. The active oxygen-generating system consisted of 0.2 U/mL of xanthine oxidase, 0.6 mmol/L hypoxanthine, and 0.02 mmol/L FeCl<sub>3</sub>, administered repeatedly at 5 minutes intervals over a 20-minute period. Piglets treated with the inactivated oxygen-generating system were initially treated with oxypurinol (50 mg/kg 30 minutes before experimentation) to inhibit endogenous xanthine oxidase. They were treated as above, but the xanthine oxidase in the system was replaced with xanthine oxidase that had been boiled for 30 minutes to inactivate the enzyme. Each of the agonists was applied in a randomized ascending concentration manner. There was a period of 20 minutes after the highest concentration of one agonist was washed off before a different agonist was infused. The percent changes in artery diameter values were calculated on the basis of the diameter measured in the control period for each agonist to obtain oxygen-generating system exposure values. Time control experiments were conducted in a separate series of animals and were designed to obtain responses to agonists initially and then 20 minutes later.

In the first 2 series of experiments designed to investigate generation of O<sub>2</sub><sup>-</sup>, phorbol 12,13-dibutyrate (10<sup>-6</sup> mol/L, Sigma) was applied to the cerebral cortex for 20 minutes in either the absence or presence of staurosporine (10<sup>-7</sup> mol/L, Calbiochem). In the second set of such series of experiments, O<sub>2</sub><sup>-</sup> generation in the presence of either an active or inactive oxygen-generating system was investigated. In the final pair of such experiments, generation of O<sub>2</sub><sup>-</sup> with FPI was investigated in the absence and presence of staurosporine (10<sup>-7</sup> mol/L) pretreatment. In these experiments,
When appropriate. If the value was significant, Fisher’s exact test was performed. A value of $P<0.05$ was considered significant. The $n$ values reflect data for 1 vessel in each animal. Values are represented as mean±SEM of absolute values or as percentages of change from control values. Data presented as percent change were compared by nonparametric means with the Wilcoxon signed rank test.

**Results**

**Role of PKC Activation in O$_2^-$ Generation During Non–Brain Injury and Brain Injury Conditions**

Topical application of phorbol 12,13-dibutyrate ($10^{-6}$ mol/L), a PKC activator, to the cerebral cortical surface of non–brain injured animals increased SOD-inhibitable NBT reduction (Figure 1). Such NBT reduction by this PKC activator was blocked by topical administration of staurosporine ($10^{-7}$ mol/L), a PKC antagonist (Figure 1). Under brain injured conditions, SOD-inhibitable NBT reduction was increased 60 minutes after FPI. Such enhanced NBT reduction after FPI was blunted by staurosporine pretreatment before FPI (Figure 1).

**Influence of a Xanthine Oxidase O$_2^-$-Generating System on O$_2^-$ Production and Pial Artery Dilation to Vasoactive Stimuli**

Exposure of the cerebral cortex to an active xanthine oxidase $O_2^-$-generating system increased NBT reduction in a manner similar to FPI (Figure 1). However, similar exposure of the cerebral cortex to an inactive oxygen-generating system did not change NBT reduction compared with control (Figure 1). Cromakalim and CGRP ($10^{-5}$, $10^{-6}$ mol/L), synthetic and endogenous $K_{ATP}$ channel agonists, respectively, elicited reproducible pial small-artery (120 to 160 $\mu$m) and arteriole (50 to 70 $\mu$m) vasodilation (data not shown). These increases in vessel diameter were attenuated after exposure of the cerebral cortical surface to the active oxygen-generating system (Figure 2). The inactive oxygen-generating system, however, had no effect on pial artery dilation to the $K_{ATP}$ channel agonists (Figure 2). Similarly, the NO releasers, SNP and SNAP, and the cGMP analogue 8-bromo-cGMP elicited reproducible pial artery dilation. As with the $K_{ATP}$ channel agonists, the active oxygen-generating system attenuated pial dilation to SNP, SNAP, and 8-bromo-cGMP, while the inactive oxygen-
generating system had no effect on these responses (Figures 3 and 4). Treatment with the active oxygen-generating system increased pial small-artery diameter from 136 ± 6 to 166 ± 8 μm, while pial arteriole diameter was increased from 61 ± 1 to 84 ± 2 μm (n = 7). Such increases in diameter were rapid in onset, peaked at ∼10 minutes of active oxygen-generating exposure, but were reversed (returned to control diameter) within 20 minutes of the end of the exposure. The inactive oxygen-generating system exposure had no effect on pial artery diameter.

The arterial blood gas and pH values for the piglets at the beginning, during O2 generation, and at the end of the experiment were no different between all the experimental groups (eg, 7.43 ± 0.01, 33 ± 1, and 93 ± 4 versus 7.44 ± 0.01, 34 ± 2, and 95 ± 5 versus 7.43 ± 0.01, 34 ± 1, and 92 ± 5 mm Hg for pH, P CO2, and P O2 for the beginning, during active oxygen-generating exposure, and at the end of the experiment, respectively; n = 7).

Discussion

Results of the present study show that topical administration of phorbol 12,13-dibutyrate, a PKC activator, results in increased SOD-inhibitable NBT reduction by newborn pig brains, indicating that O2− was generated. Since staurosporine, a PKC inhibitor, coadministered with the phorbol 12,13-dibutyrate blocked such elevation in SOD-inhibitable NBT reduction as well as the phorbol-induced pial artery vasoconstriction, these data indicate that PKC activation generates O2− in a selective manner. Moreover, this antagonist also attenuated brain injury–induced elevated SOD-inhibitable NBT reduction. Earlier observations that staurosporine blocked piglet pial artery vasoconstriction induced by phorbol 12,13-dibutyrate are supportive of the specificity of staurosporine for inhibition of PKC. Previously, FPI has been observed to be associated with generation of O2− on the cerebral cortical surface, and ET-1 released into CSF by FPI appears to be at least one mechanism whereby brain injury generates such radicals. Interestingly, ET-1 is known to activate PKC. Taken together, then, these data suggest that ET-1 released into CSF after FPI contributes to the generation of O2− after injury through activation of PKC. It should be cautioned, however, that FPI also increases CSF levels of a
number of other vasoactive substances, which, in turn, may also contribute to impaired reactivity of cerebral arteries. Additionally, it should be cautioned that concerns related to the accuracy of the NBT assay have recently been raised.17

The cerebrovascular consequences of free radical production are not fully understood. However, there is a significant amount of evidence that supports a role of oxygen radicals in brain injury. For example, brain injury in cats has been reported to cause the generation of superoxide for at least 1 hour after injury.19 In that study, the sustained dilation and abnormal responsiveness of pial arterioles observed after injury could be reversed by treatment with the free radical scavengers (SOD) and catalase.18 Oxygen radicals also have been shown to increase blood-brain barrier permeability,19 produce ultrastructural changes in pial vessel endothelium,19 and cause abnormal arteriolar reactivity.20 In addition, oxygen radical scavengers have been shown to improve vascular function and blood flow during focal ischemia in rats, which may account for the observed reductions in infarct size.21 Recently, a trial with SOD in humans with severe head injuries showed that death and vegetative state were increased in patients receiving a placebo compared with those receiving polyethylene glycol and SOD.22 Intracellular generation of superoxide or other species could alter structures and/or production of nucleotides, second messengers, receptors, and membranes, and the movement of superoxide out of the cell through anion channels could result in high concentrations of activated oxygen species at cell surfaces, including endothelium. Such oxygen species are thought to antagonize NO function and to contribute to altered cerebral hemodynamics after FPI in the piglet because free radical scavengers partially restored decreased CSF cGMP concentration and decreased responses to NO-dependent dilator stimuli such as opioids.11

The role of the systemic presser response after FPI in altered adult cerebral hemodynamics has been investigated. For example, it was hypothesized that acute elevations of blood pressure after injury in the adult result in the release and metabolism of arachidonic acid, which would generate oxygen free radicals, causing cerebral functional abnormalities.15,18,19,23 However, in contrast to studies performed in adult and juvenile animals, there was no acute elevation in blood pressure after FPI in the newborn pig.4 Since the elevation in systemic blood pressure was thought to be an absolute requirement for cerebral generation of free radicals after injury,15,18,19,23 the observed decrease in blood pressure was initially perplexing. More recent studies, however, have shown that the peptide ET-1 is released after FPI in the piglet.12 Topical administration of ET-1 in the same concentration observed after FPI resulted in the generation of substantial amounts of superoxide on the cerebral cortical surface.12 These results, therefore, link the cerebral release of this peptide to superoxide generation after FPI in the piglet. Interestingly, decreased opioid-induced dilation and associated CSF cGMP release after FPI were partially restored in animals pretreated with the ET-1 antagonist BQ123.12 These data, then, suggest that ET-1 contributes to altered cerebral hemodynamics after FPI, at least in part, through elevated superoxide production.

Although the aforementioned studies indicate that generation of superoxide contributes to altered cerebral hemodynamics after FPI, the role of more distal signal transduction mechanisms, such as impaired K+ channel function, was not considered. For example, the membrane potential of vascular muscle is a major determinant of vascular tone, and activity of K+ channels is a major regulator of membrane potential.8 Activation or opening of these channels increases K+ efflux, thereby producing hyperpolarization of vascular muscle. Membrane hyperpolarization closes voltage-dependent calcium channels and thereby causes relaxation of vascular muscle.8 Because opioids elicit dilation through KATP channel activation,24 altered dilation to such stimuli after FPI could relate to impaired K+ channel function. Interestingly, global cerebral ischemia has been observed to impair pial artery responses to the KATP channel opener aprikalim in piglets.25 Similarly, another study found that FPI blunted pial artery dilation elicited by the KATP channel agonists CGRP and cromakalim.9 Additionally, although CGRP has been linked to cAMP-dependent dilator mechanisms by others,9 results of that study did not support this idea since pial responses were not associated with changes in cortical periarachnoid CSF cAMP and were also not altered by Rp 8-bromo-cAMP, a cAMP antagonist.9 Since glibenclamide blocked dilation to CGRP and cromakalim while responses were unchanged in the presence of iberiotoxin,9 these data indicate that these agents are selective endogenous and synthetic activators of the KATP channel, respectively, consistent with previous studies.7,26 Similarly, dilator responses to the NO releasers SNAP, SNAP, and the cGMP analogue 8-bromo-cGMP were also blunted after FPI.9 However, responses to brain natriuretic peptide, an activator of particulate guanylate cyclase, and the nonselective dilator papaverine were unchanged. These changes in responsiveness of pial arteries after FPI were observed in the presence of moderate vasoconstriction (∼17% reduction in vessel diameter). Similar reductions in pial artery diameter in the presence of coadministered thromboxane mimics (U46619) did not alter responses to cromakalim, CGRP, SNAP, SNAP, and 8-bromo-cGMP. Thus, there were marked alterations in dilator mechanisms that were not the result of indirect effects of increased vascular tone. Therefore, these data indicate that KATP channel function was selectively impaired after brain injury. Activation of KATP channels has been observed to contribute to the dilation of pial arteries in the newborn pig in response to the NO releaser SNP.7 However, others do not ascribe such a role for KATP channels in NO dilation since pial responses to SNP were unchanged by glibenclamide.27,28 While the reasons for such differences are uncertain, such observations could result from differences in species, age, or experimental conditions. Nonetheless, impaired KATP channel function could serve as a common mechanism for altered cerebral hemodynamics after FPI. Because the ET-1 antagonist BQ123 and the PKC inhibitor staurosporine partially restored decreased dilation to KATP agonists, NO releasers, and a cGMP analogue after FPI, ET-1 appears to contribute to altered cerebral hemodynamics after FPI through impairment of KATP channel function via activation of PKC.10 In separate experiments under non–brain injury conditions, it was observed that coadministration
of ET-1 with \(K_{\text{ATP}}\) channel agonists blunted dilation to such stimuli, suggesting that this peptide directly interacts with and impairs the \(K_{\text{ATP}}\) channel,\(^{10}\) consistent with other studies.\(^{29,30}\) The mechanism by which this impairment occurs is currently uncertain.

Because PKC activation contributes to \(K_{\text{ATP}}\) channel impairment after FPI, as described above, and also results in \(O_2^-\) generation, as observed in the present study, it was hypothesized that such superoxide generation could link PKC to impaired \(K_{\text{ATP}}\) channel function after brain injury. New data in the present study are the first to show that generation of oxygen free radicals through an activated oxygen-generating system results in attenuated pial artery dilation to \(K_{\text{ATP}}\) channel agonists, NO releasers, and a cGMP analogue. These data suggest, therefore, that ET-1 impairs \(K_{\text{ATP}}\) channel function after FPI through superoxide generation due to PKC activation. These studies extend previous observations that PKC activation inhibits the \(K^+\) current in isolated feline cerebral vascular smooth muscle cells\(^{31}\) to the intact animal and also demonstrate the physiological relevance of such observations in the context of impaired cerebral artery reactivity after FPI. The activated oxygen-generating system had been observed in a previous study to result in pial vessels that were ultrastructurally abnormal.\(^{20}\) Lesions consisted of increased numbers of vascular cytoplasmic inclusions, more numerous surface pits, and mitochondrial injury.\(^{20}\) Although pial artery diameter returned to the pretreatment diameter after removal of the activated oxygen-generating system, pial artery responsiveness was altered. In the earlier study, pial artery dilation in response to hypercapnia and hypotension was reduced, while that to isoproterenol or constriction to norepinephrine was unchanged after activated oxygen-generating system treatment.\(^{20}\) Observations similar to those obtained with a piglet model of global cerebral ischemia.\(^{32,33}\) After FPI, cerebral blood flow and cerebral oxygenation are reduced,\(^{4}\) suggesting that ischemia may occur after such injury in the piglet as well. Results of the present study show that the activated oxygen-generating system produced a reduction of NBT similar to that observed after FPI, indicating that approximately the same amount of superoxide is generated with either intervention. The inactive oxygen-generating system, however, did not cause the reduction of NBT, nor did it alter vascular responses to \(K_{\text{ATP}}\) channel agonists, NO releasers, or a cGMP analogue, thereby giving specificity to the conclusions related to the actions of oxygen free radicals. By repeated application of the activated oxygen-generating system for 20 minutes in the present study, superoxide was generated continuously over the application period. However, the effect of topical application of the activated oxygen-generating system on endothelial cells may be attenuated by intervening tissue, although ultrastructural endothelial alterations appear considerable.\(^{20}\) Intracellular generation of superoxide or other species after FPI could result in higher concentrations of more active species at cell surfaces, including endothelium.

In conclusion, results of the present study show that PKC activation increases \(O_2^-\) production and contributes to such production observed after FPI. These data also show that an activated oxygen-generating system that generates an amount of \(O_2^-\) similar to that observed with FPI attenuated pial artery dilation to \(K_{\text{ATP}}\) channel agonists, NO releasers, and a cGMP analogue. These data, therefore, suggest that \(O_2^-\) generation links PKC activation to impaired \(K_{\text{ATP}}\) channel function after FPI.

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**References**

Editorial Comment

Many investigators have suggested that the formation of oxygen radicals, during a variety of conditions, produces adverse effects on the cerebral circulation. For example, acute increases in arterial blood pressure damage cerebrovascular endothelium through the production of oxygen radicals,1 impaired responses of cerebral blood vessels during brain injury can be restored by scavengers of oxygen radicals,2 and oxygen radicals increase the permeability of the blood-brain barrier.3 In addition, recent studies by Kasemsri and Armstead4,5 have shown that topical application of ET-1, at a level seen during FPI, produces an increase in oxygen radical formation and impairs K<sub>ATP</sub> channel function of cerebral blood vessels through activation of PKC. The purpose of the present study was to determine whether activation of PKC generates oxygen radicals and thus accounts for impaired cerebrovascular reactivity during brain injury.

In the present study, the investigators measured oxygen radical formation during activation of PKC and FPI. In addition, the investigators measured in vivo responses of piglet cerebral arterioles to activation of K<sub>ATP</sub> channels, NO donors, and activators of cGMP before and after generation of oxygen radicals. The authors report that activation of PKC and FPI increased oxygen radical formation, which could be attenuated by pretreatment with staurosporine. Furthermore, the authors report that dilation of cerebral blood vessels to the agonists was inhibited by oxygen radical formation.

Thus, on the basis of the findings of the present study, the authors suggest that activation of PKC during brain injury produces an increase in oxygen radical formation and accounts for impaired responses of cerebral blood vessels to activators of K<sub>ATP</sub> channels, NO donors, and activators of cGMP. These findings may have important implications regarding therapeutic approaches used in the treatment of brain injury in newborns.

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