Brain kininogen following experimental brain injury: evidence for a secondary event

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Previous studies have shown that following experimental brain injury cerebral arterioles dilate and display endothelial lesions and reduced responsiveness to hypocapnia. These abnormalities are caused by cyclooxygenase-dependent free radical generation. There is evidence that the kallikrein-kinin system may in part stimulate the cyclooxygenase-dependent damage since bradykinin is a powerful stimulator of prostaglandin formation and it has recently been shown that a specific kinin receptor blocker decreases the arteriolar abnormalities caused by injury. In order to further examine the hypothesis that the kallikrein-kinin system is important in inducing damage, rat brain tissue was examined for kininogen, the precursor of kinins, at 10 minutes and 1, 3, 6, 15, 24, 48, and 72 hours after injury. A fluid-percussion brain injury device was attached over the right cerebral cortex of rats and a 1.6-atmosphere pressure injury was administered. The kininogen content was determined by a radioimmunoassay procedure in tissues which were free of intravascular blood.

After injury, bleeding was confined mainly to the right hemisphere. The kininogen content in the right hemisphere was significantly elevated by one hour after injury, continued to rise until 15 hours after injury, then was significantly decreased by 2 days after injury. In the left hemisphere, kininogen was significantly elevated at 1 hour postinjury, returned toward control levels over the 3- to 6-hour period after injury, then was again elevated at 15 hours after injury. These studies also show that brain water and cerebrovascular permeability were greater at 15 hours postinjury than at earlier time points. The data further support a role for the kallikrein-kinin system in brain injury and, when considered with the results of other studies, suggest that a secondary event is occurring in the 12- to 24-hour period after neural injury. The authors hypothesize that this secondary event is related to endothelial and vascular repair and may be important for the return of normal cerebrovascular function.

KEY WORDS head injury • kallikrein-kinin system • cerebrovascular permeability • brain edema • rat

Numerous investigators have shown elevated prostaglandin (PG) levels in the brain and spinal cord following various types of neural injury. We have extensively studied the fluid-percussion model of brain injury and have shown that, following injury, oxygen radicals forming concomitantly with increased PG synthase activity cause cerebral arteriolar abnormalities; these abnormalities include dilation, endothelial lesions, and reduced arteriolar smooth-muscle responsiveness to changes in blood pCO2. Evidence that these arteriolar abnormalities are caused by cyclooxygenase-dependent free radical formation is derived from the fact that indomethacin or superoxide dismutase plus catalase block their formation. A question remains as to the precise biochemical signals or substances which initiate PG formation following injury.

One system that may contribute to the activation of the arachidonic acid cascade following neural injury is the kallikrein-kinin system. Several lines of evidence support its potential involvement. First, bradykinin is known to be a potent stimulator of PG formation. Also, we and others have shown that bradykinin causes cerebral arteriolar dilation via a cyclooxygenase-dependent free radical mechanism since bradykinin-induced dilation is prevented by cyclooxygenase-inhibitors and is decreased by the free radical scavengers superoxide dismutase and catalase. Additional support for a role of the kallikrein-kinin system in injury is the fact that all of the components of this system including bradykinin, the enzyme kallikrein, and its substrate kininogen have been found in the brain. Furthermore, following injury plasma kininogen may gain entry to the extravascular compartment of the brain via frank hemorrhage.

Very recently, we have provided evidence that kinins
contribute to the genesis of the arteriolar abnormalities induced by experimental fluid-percussion brain injury. 2
In that study, pretreatment of cerebral arterioles with a specific bradykinin-receptor blocker prevented the loss of arterial reactivity to hypocapnia, which normally occurs following injury.

The source of the involved kininogen and the site of its conversion to kinins by kallikrein is uncertain. Kininogen is found in both brain and plasma; kallikrein may be found in plasma and also is known to exist in the vascular wall. Researchers in Munich have previously implicated bradykinin in brain injury20,32,33 and, more recently, other investigators have shown that kininogen is elevated in brain tissue following ischemic injury and is elevated in spinal cord following traumatic injury.1,15 The purpose of the present investigation was to determine whether kininogen levels are altered following experimental concussive brain injury. A secondary goal was to probe whether changes in kininogen levels correlated with brain edema or alterations in vascular permeability.

Materials and Methods
Animal Preparation
A total of 84 adult male Sprague Dawley rats, each weighing 375 to 400 gm, were used in these studies. The method for producing fluid-percussion brain injury has been published in detail previously.4 The protocols were approved by our institutional Animal Care and Use Committee. Briefly, the animals were anesthetized and a craniectomy was made over the right parietal area. The dura was left intact. An injury attachment device was implanted into the craniectomy and held in place with dental acrylic. The rats were then given a dose of antibiotics and allowed to recover for 2 days. On the 2nd day the animals were again anesthetized and injured with the fluid-percussion technique. The fluid-percussion device consists of a fluid-filled cylinder which has a movable piston on the end. The device is attached to the animal such that the cranial vault is continuous with the fluid-filled cylinder. A weighted pendulum is dropped and strikes the piston, thus producing a transient pressure pulse which enters the cranial vault and produces injury. The pressure pulse wave is 20 msec long and is highly reproducible. The pressure injury used in these experiments was 1.6 atmospheres and thus comparatively light in intensity. The duration and intensity of the fluid-percussion pressure wave were recorded on a storage oscilloscope.

Kininogen Studies
At various times after injury the animal’s chest cavity was opened while under anesthesia, a blood sample was collected, and then the left ventricle was perfused with cold saline in order to eliminate intravascular blood from the brain and liver. The vena cava was cut to allow efflux of circulated perfusate and blood. The perfusion pressure was maintained at 100 mm Hg and approximately 100 ml of perfusate was required to eliminate all blood. The brain and a piece of the liver were removed, weighed, frozen, and stored at −70°C, along with the plasma sample. The samples were shipped on dry ice by air freight to Charleston, South Carolina, where the kininogen was assayed by radioimmunoassay using methods which have previously been published in detail.2

Edema and Permeability Studies
In another series of experiments, the effect of injury on brain water content and cerebrovascular permeability to 125I-labeled human serum albumin (HSA) was studied utilizing a technique similar to those previously reported.12 Basically, 30 μCi/kg 125I-HSA was injected via a tail vein 1 hour before sacrifice. As in the kininogen studies, the animals were anesthetized, a blood sample was taken, and the brain was perfused. The brain was removed, dissected, and weighed, and the tissue was dried to a constant weight in an oven. The wet and dry weights were then used to calculate the percentage of water in the brain. The plasma and tissue samples were then counted in a gamma counter, and the permeability was expressed as counts per minute (cpm)/gm of dry brain weight divided by cpm/ml of blood, and multiplied by 100.

Statistical analyses were performed by analysis of variance, followed by a multiple range test. A p value of 0.05 or less was considered to indicate a statistically significant difference between groups.

Results
Previous investigators have shown that surgical stress can elevate kininogen levels.21 Therefore, kininogen levels in control, unoperated animals were compared to those in animals which were prepared for injury but were not brain injured and then analyzed at 15 hours after the sham injury. Brain, liver, and plasma kininogen equivalents (KE) in control animals were (mean ± standard error of the mean): 60 ± 11 pg KE/mg protein, 1653 ± 253 pg KE/mg protein, and 2.3 ± 0.6 μg KE/ml plasma, respectively. The respective values in sham-injured controls were: 555 ± 175 pg KE/mg protein, 3783 ± 877 pg KE/mg protein, and 17.8 ± 3.8 μg KE/ml plasma; thus, all levels were significantly elevated (p < 0.05) compared to unoperated controls. These increases in plasma kininogen due to surgical stress are very similar to those which have been previously reported.21

The degree of injury produced in the experimental animals was highly consistent and averaged 1.57 ± 0.01 atmospheres for all animals. Brain injury at all postinjury times examined had no significant effect on liver or plasma kininogen levels compared to sham-injured controls (data not shown). Subdural bleeding invariably occurred on the right cortex, the side to which the brain injury device was attached. Occasional minor bleeding
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**FIG. 1.** Kininogen levels in the right hemisphere following fluid-percussion brain injury. The injury device was implanted over the right hemisphere. Values are means ± standard error of the means. Significance of difference: a indicates p < 0.05 vs. sham-injured control levels; b indicates p < 0.05 vs. level at 10 minutes postinjury; and c indicates p < 0.05 vs. level at 15 hours postinjury. Results are for four to nine rats in each group. Kin eq = kininogen equivalents; prot = protein.

**FIG. 2.** Kininogen levels in the left hemisphere following fluid-percussion brain injury. The injury device was implanted over the right hemisphere. Values are means ± standard error of the means. Significance of difference: a indicates p < 0.05 vs. sham-injured control levels. Results are for four to nine rats in each group. Kin eq = kininogen equivalents; prot = protein.

occurred in deeper brain structures of the right hemisphere. Comparatively little bleeding was observed in the left hemisphere. At 2 days after injury visible blood on the right hemisphere was reduced, and this was even more evident at 3 days indicating active repair mechanisms.

Figure 1 shows the kininogen levels in the right brain hemisphere, the side on which the brain injury device was attached. The average kininogen level increased soon after injury and was significantly elevated by 1 hour after injury. Kininogen levels continued to rise after 1 hour and peaked at 15 hours, at which time the level was significantly elevated compared to 10 minutes after injury. Kininogen levels declined after 15 hours and were significantly reduced from their peak 15-hour value by 2 days after injury.

The kininogen levels in the left hemisphere are depicted in Fig. 2. Although the injury device was placed over the right hemisphere, the fluid-percussion pulse wave traveled throughout the cranial vault and affected both the left and right brain halves. Similar to the right hemisphere, the kininogen level in the left hemisphere was increased at 1 hour postinjury. However, unlike the right hemisphere the kininogen level was not significantly elevated at 3 or 6 hours postinjury, indicating a transient effect. At 15 hours postinjury, the kininogen content of the left hemisphere was again significantly increased, suggesting some secondary event influencing kininogen levels. Interestingly, this secondary elevation of kininogen in the left hemisphere corresponded to the time of the maximal kininogen level in the right hemisphere. The left hemisphere kininogen level then declined and was not significantly elevated at 24, 48, or 72 hours after injury, compared to the 15-hour sham-injured controls.

Because we were intrigued by the secondary increase in kininogen levels at 15 hours after injury, we decided to determine in another series of animals whether the kininogen increases noted 15 hours postinjury were restricted to cortical areas or were also found in other areas of the brain. Figure 3 compares kininogen levels in the right cortical area under the site of attachment of the injury device, in the adjacent remaining cortex, and in all other brain areas of the right hemisphere combined. Similar areas were also measured in the left hemisphere. While the right cortex under the injury device attachment showed the greatest levels of kininogen and subdural blood, kininogen in other areas

**FIG. 3.** Kininogen levels in the different brain areas at 15 hours after injury. Units are pg kininogen equivalents/mg protein, expressed as means ± standard error of the means in a group of six animals. All left brain values are statistically significantly different from the corresponding right brain levels and the right cortex under the injury device is different from the other right brain areas at p < 0.05.
FIG. 4. Cerebrovascular permeability to $^{125}\text{I}$-human serum albumin following fluid-percussion brain injury. Permeability is calculated by dividing the counts per minute (cpm)/gm of dry brain weight by the cpm/ml of blood and multiplying by 100. Results (means ± standard error of the means) are for seven or eight rats in each group. Significance of difference: a indicates $p < 0.05$ vs. sham-injured control level.

FIG. 5. Percent water content in various parts of the brain following fluid-percussion brain injury. Percent water was calculated by the wet weight-dry weight method. Values are means ± standard error of the means for seven or eight rats each group. Significance of difference: a indicates $p < 0.05$ vs. sham-injured control level; b indicates $p < 0.05$ vs. level at 6 hours postinjury.

of the right hemisphere was also elevated compared to the left side. Also, all three regions of the left hemisphere had elevated levels of kininogen compared to 15-hour sham-injured samples (Fig. 2). This indicates that the secondary increase in kininogen at 15 hours postinjury appears to be a whole-brain phenomenon, thus suggesting some generalized response to brain trauma.

Shohami, et al., and Hsu, et al., have shown maximum increases in some PG's at 18 hours after neural injury, just as we have found maximum increases in kininogen at 15 hours postinjury. Shohami, et al., also reported brain specific gravity to be most affected at 18 hours postinjury. In order to determine whether we might observe similar changes, the brain water content and permeability were examined in the right hemisphere at 6 and 15 hours postinjury in an additional two groups of animals. Six hours was chosen because maximum hemorrhage has occurred by that time, but not the maximum or secondary increase in kininogen. Figures 4 and 5 show that just as kininogen was elevated at 15 hours, compared to 6 hours, so were cerebrovascular permeability and brain water content.

**Discussion**

The results of this study further support a role for the kallikrein-kinin system in early and delayed events following experimental brain injury. The early increases in kininogen in the right hemisphere are likely due to frank hemorrhage and thus to plasma kininogen entering the brain. In fact, Hsu, et al., have shown that protein extravasation is greatest in the first 2 hours after spinal cord injury. Evidence suggests that increased pinocytosis may also be transporting plasma kininogen across the vascular wall. Povlishock, et al., have shown that, following experimental fluid-percussion brain injury, there is a dramatic increase in pinocytic vesicular transport of the protein horseradish peroxidase across the blood-brain barrier. They reported that this increase in pinocytosis was greatest in the 1st hour after injury and was greatly reduced after the 1st hour. Interestingly, bradykinin has recently been reported to stimulate pinocytosis of horseradish peroxidase in cerebral vessels, and a recent report by van Iwaarden, et al., shows that cultured endothelial cells cannot synthesize kininogen, which has a high molecular weight, but instead take it up from the culture media. Our results, considered with the findings of others, invite the speculation that kinins formed immediately after injury may further stimulate pinocytotic activity in cerebral endothelial cells.

Very minor bleeding occurred in the left hemisphere after brain injury. However, kininogen was significantly increased in the left hemisphere at 1 hour after injury. This further suggests that some mechanism other than hemorrhage is increasing extravascular kininogen levels. Fluid-percussion brain injury causes a transient episode of hypertension which lasts only 1 to 5 minutes. This hypertension is known to be caused by catecholamine release from the adrenal medulla. Previous investigations have shown that acute hypertension alone will cause blood-brain barrier opening to horseradish peroxidase. Additionally, norepinephrine or epinephrine released following injury may be acting directly to increase permeability, since Westergaard has shown that agents that affect cyclic adenosine monophosphate (cAMP) cause alterations of the blood-brain barrier. Furthermore, we have previously shown that PG's are elevated following injury, and PG's are also known to affect cAMP.

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Kininogen content peaks at 15 hours postinjury in the right half of the brain. Although the average kininogen level at 15 hours is not significantly increased over the previous 6-hour value in the right hemisphere, the 15-hour level is significantly elevated in the left hemisphere. These results indicate a secondary event acting globally within the brain to again increase the kininogen content. The cause for the 15-hour increase in kininogen is uncertain, but it may be due to increased formation within the brain or perhaps increased uptake from the plasma. The fact that 125-I-albumin uptake and water content were elevated at 15 hours compared to 6 hours also suggests an important late-occurring event. Since both kininogen and albumin are high molecular weight proteins, some general phenomenon may be increasing permeability to all proteins. Alternatively, one might hypothesize that an increased formation of kinins at 15 hours postinjury may be stimulating an increased protein permeability or pinocytosis. The question therefore is whether increased kininogen is the cause or the result of increased plasma protein accumulation in the brain. If the kallikrein-kinin system activity is increased, then other indicators of kinin effects may be present. One well-known effect of kinins is their stimulation of PG formation. In this regard, there is interesting evidence that the PG-generating capacity is increased in this time period after injury. Shohami, et al.,26,27 examined the PG-generating capacity in brain tissue removed at various times between 15 minutes and 10 days after impact injury. They found that the PGE2 synthetic capacity was greatest at 18 hours after injury, the same time at which they found gravimetrically measured edema to be the greatest. Their results indicated that this PGE2 synthetic capacity was elevated in both the impacted injured hemisphere and the contralateral hemisphere. In another series of similar experiments, they measured PG levels in tissue frozen at various time periods after injury and reported that PGE2 and 6-keto-PGF1α levels in tissue remote from the site of injury were highest at 18 hours after injury.29 Hsu, et al.,16 have studied the temporal profile of PG levels in experimental spinal cord injury. These investigators also reported that the 6-keto-PGF1α levels in spinal cord was maximal at 18 hours after injury.

Thus, there seems to be abundant evidence that a secondary event occurring between 12 and 24 hours after neural injury is causing increased kininogen levels, increased PG levels, and increased edema formation. Based on the morphological studies of Griffiths, et al.,12,13 and the fact that the vasculature is thought to be the main producer of PGI2 in the brain, Hsu, et al.,16 speculated that endothelial cell activity may be increased at 18 hours postinjury. Further examination of the literature supports this possibility. Endothelial cell injury is a well-documented phenomenon following experimental brain or spinal cord injury. Using tissue culture techniques, Sholley, et al.,31 have examined endothelial cell migration and replication in confluent cultures where a 300- to 400-μm wide line on the culture plate was mechanically denuded of endothelial cells. They examined endothelial cell migration and deoxyribonucleic acid (DNA) synthesis at various intervals up to 72 hours after denudation. They found that migration into the denuded area between 12 and 24 hours after denudation was at least twofold greater than at any other time period examined. Cell replication was greatest between 24 and 36 hours, indicating that the cells first migrate into the denuded area and then divide and cover the injured area. We have previously studied the recovery of PGI2 synthetic activity in the aorta at various times after administration of a single dose of aspirin which eliminated PGI2 synthetic capacity.8 We found that the rate of return of PGI2 synthetic capacity was greatest in the period from 12 to 24 hours after cyclooxygenase inactivation. These results support the possibility that the increased events which we, Hsu, et al.,16 and Shohami, et al.,29,30 have observed at 15 to 18 hours after injury may be related to increased endothelial cell activity. These secondary events following neural injury may therefore be associated with desirable repair and healing of the injured endothelium. This potentially helpful response has been previously suggested by Shohami, et al.26 If this secondary event is associated with healing, then vascular function may be improved in the 2nd day after injury. In fact, recent evidence provided by Marmarou and colleagues (personal communication) shows that cerebrovascular reactivity to changes in pCO2 in traumatically injured humans is altered during the first 24 hours after injury, but returns toward normal values in the 2nd day after injury.

In summary, we have shown that kininogen is increased in brain immediately after and at 15 hours after brain trauma. Our results, when considered with the results of others, strongly suggest that an important secondary event is occurring in the 12- to 24-hour period after injury. Whether this secondary event is harmful or part of the brain’s recovery process is uncertain. We hypothesize that critical endothelial repair mechanisms occur in this 12- to 24-hour period and that the endothelial repair is vital to the return of normal cerebrovascular function. In order to further test this hypothesis, future investigations should examine structural and functional integrity of the cerebral endothelium and vasculature at increasingly long periods after trauma.

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