Selective Inhibition of Plasma Kallikrein Protects Brain from Reperfusion Injury

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ABSTRACT

We have studied the effect of DX-88, a selective recombinant inhibitor of human plasma kallikrein, in transient or permanent focal brain ischemia (with or without reperfusion, respectively) induced in C57BL/6 mice. Twenty-four hours after transient ischemia, DX-88 administered at the beginning of ischemia (pre) induced a dose-dependent reduction of ischemic volume that, at the dose of 30 µg/mouse, reached 49% of the volume of saline-treated mice. At the same dose, DX-88 was also able to reduce brain swelling to 32%. Mice treated with DX-88 pre had significantly lower general and focal deficit score. Fluoro-Jade staining, a marker for neuronal degeneration, showed that DX-88-treated mice had a reduction in the number of degenerating cells, compared with saline-treated mice. Seven days after transient ischemia, the DX-88 protective effect was still present. When the inhibitor was injected at the end of ischemia (post), it was still able to reduce ischemic volume, brain swelling, and neurological deficits. DX-88 efficacy was lost when the inhibitor was given 30 min after the beginning of reperfusion (1 h post) or when reperfusion was not present (permanent occlusion model). This study shows that DX-88 has a strong neuroprotective effect in the early phases of brain ischemia preventing reperfusion injury and indicates that inhibition of plasma kallikrein may be a useful tool in the strategy aimed at reducing the detrimental effects linked to reperfusion.

Kallikreins are serine proteases present in biological fluids and tissues. They are divided in plasma and tissue kallikreins that differ in structural characteristics and function. They enzymatically mediate the release of kinins from their precursors (kininogens). Kinins include the vasoactive nonapeptide bradykinin (BK) and the decapeptide kallidin (or Lys-BK) that are produced by two major biochemical cascades, one occurring within the plasma and leading to BK generation and the other within the tissue leading to kallidin production. Kinin act via binding to B1 or B2 receptors whose signaling through nitric oxide-cGMP and prostacyclin-cAMP results in a wide spectrum of actions as part of the inflammatory response that develops after tissue injury (Raidoo and Bhoola, 1998). Recently, a G protein-coupled orphan receptor has been identified as a novel kinin receptor; however, no information is presently available on its functions and roles (Boels and Schaller, 2003; Meini et al., 2004).

The kinin system is involved in ischemia/reperfusion injury in heart, intestine, and kidney (Souza et al., 2004; Veeravalli and Akula, 2004; Chao and Chao, 2005), and a few studies indicate that it is involved also in the pathogenesis of ischemic injury in the brain (Relton et al., 1997; Zausinger et al., 2002; Lehmbach et al., 2003; Xia et al., 2004; Groger et al., 2005). Actually, all the components and the activation products of this system have been demonstrated also in the brain (Raidoo and Bhoola, 1998), and its activation in stroke patients has also been proved (Wagner et al., 2002).

On the whole, the role of kinin system in the pathogenesis of brain ischemia/reperfusion injury is not clear yet. Kallikrein action and/or BK release, during brain ischemia, are known to stimulate the production and release of eicosanoids, cytokines, nitric oxide, free radicals, and excitatory amino acid neurotransmitters. On one hand, release of such mediators may induce cerebral arterial dilatation, loss of cerebrovascular autoregulation, endothelial cell lesions, increased capillary permeability, and breakdown of the blood-brain barrier, causing vasogenic edema formation and possibly neuronal injury and death (Relton et al., 1997; Groger et al., 2005). On the other hand, they may also result in vasoactive, anticoagulant, and proangiogenetic properties, thus inducing protection (Shariat-Madar et al., 2002; Xia et al., 2006).
In experimental brain stroke models, attempts to interfere with this system to obtain protection from brain damage have yielded controversial data. To date, the role of B₁ and B₂ bradykinin receptors is not clearly understood. Reiton et al. (1997), Zausinger et al. (2002), Lumenia et al. (2006), and Ding-Zhou et al. (2003) found a tissue protective effect and an improvement of neurological outcome via selective inhibition of B₂ receptors on brain tissue. Therefore, B₂ knockout mice are significantly protected from the ischemic insult (Groger et al., 2005). On the other hand, Lehmbre et al. (2003) did not find any improvement of neurological deficits or increase of vital neurons when either B₁ or B₂ receptors were inhibited by specific antagonists, and when both receptors were inhibited, an increase in mortality rate was observed. As a further degree of complexity, tissue kallikrein gene transfer has been reported to be protective against cerebral ischemia, an action mediated by B₂ receptors and nitric oxide production (Xia et al., 2004, 2006).

We chose to explore the effects of plasma kallikrein inhibition on brain ischemic injury on the basis of the following considerations: kallikrein, being the precursor of BK, results in a wide activation of the whole system irrespective of the receptors involved; and although the selective involvement in ischemia/reperfusion pathogenesis of the plasma versus tissue cascade has not been elucidated yet, plasma compartment and the activation of the plasmatic cascade occurring on brain endothelial cells should be the earliest step in this pathological condition.

We took advantage of the potent and selective plasma kallikrein inhibitor, DX-88 (Dyax Corp., Cambridge, MA), a 60-amino acid Kunitz domain (molecular mass, 7054 D) with high specificity and affinity for human kallikrein (DX-88 Kᵢ, 44 pM; C1-INH Kᵢ, 1.3 × 10⁴ pM; aprotinin Kᵢ, 3.0 × 10⁴ pM) (Williams and Baird, 2003; Zuraw, 2005). This inhibitor is presently used in humans for hereditary angioedema, a condition due to deficiency of C1-inhibitor and characterized by recurrent episodes of angioedema that are thought to be due to activation of plasma kallikrein (Williams and Baird, 2003; Agostoni et al., 2004; Zuraw, 2005).

We studied DX-88 effects on infarct volume, neurodegeneration, brain swelling, and neurological deficits in a mouse model of cerebral transient ischemia. To specifically address the involvement of reperfusion in DX-88 action, we compared these results with those obtained in a model of permanent ischemia (without reperfusion).

Materials and Methods

Animals. Male C57BL/6 mice (26–28 g; Charles River, Calco, Italy) were housed five per cage and kept at constant temperature (21 ± 1°C) and relative humidity (60%) with regular light/dark schedule (7:00 AM to 7:00 PM). Food (Altromin pellets for mice) and water were available ad libitum.

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (Decreto Legge n.116, Gazzetta Ufficiale supplemento 40, 1992 18 February) and international laws and policies (EEC Council Directive 86/609, OJ L 358/1; Dec.12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, U.S. National Research Council 1996).

Physiological Parameters. Selected physiological parameters (pH, pCO₂, pO₂, and oxygen saturation) were measured in naif mice receiving saline (150 µl) or 30 µg of DX-88 in the same volume i.v. Blood was withdrawn from the retro-orbital plexus 5 min after the drug injection and loaded on G3+ cartridges (i-STAT Corporation, East Windsor, NJ). The hemogas analyses were then performed by the i-STAT 1 analyzer (i-STAT Corporation).

Transient Ischemia. Transient focal cerebral ischemia was achieved by middle cerebral artery occlusion (MCAO) as described previously (De Simoni et al., 2003). Anesthesia was induced by 5% isoflurane in N₂O/O₂ (70/30%) mixture and maintained by 1.5 to 2% isoflurane in the same mixture. To confirm the adequacy of the vascular occlusion in each animal, blood flow was measured continuously before, during, and for 10 min after occlusion by laser Doppler flowmetry (Transonic BLF-21) using a flexible 0.5-mm fiberoptic probe (Transonic, Type M, 0.5-mm diameter) positioned on the brain surface and secured with impression material on the skull at the following coordinates: AP, −1 mm; and L, −3.5 mm (Yang et al., 1999). In brief, the right common carotid artery was exposed, and the external carotid artery and its branches, including the occipital artery and the superior thyroid artery were isolated and cauterized. The pterygopalatine artery was ligated, and the external carotid artery was cauterized. A 6-0 monofilament nylon suture, blunted at the tip by heat and coated in poly-L-lysine, was introduced into the internal carotid artery through the external carotid artery stump and advanced to the anterior cerebral artery so as to block its bifurcation into the anterior cerebral artery and the middle cerebral artery (MCA). The filament was advanced until a >70% reduction of blood flow, compared with preischemic baseline, was observed. At the end of the 30-min ischemic period, blood flow was restored by carefully removing the nylon filament. Intraoperative rectal temperature was kept at 37.0 ± 0.5°C using a heating pad (LSI Letica, Barcelona, Spain). Sham-operated mice received a midline neck incision and the subsequent exposure of the carotid sheath. The external carotid artery and its branches were isolated without being ligated or cauterized. Similar to ischemic mice, sham-operated mice were maintained at 37°C during surgery.

Permanent Ischemia. For the permanent MCAO, a vertical midline incision was made between the left orbit and tragus. The temporal muscle was excised, and the right MCA was permanently occluded by electrocoagulation just proximal to the origin of the maxillary branch. Intraoperative rectal temperature was kept at 37.0 ± 0.5°C using a heating pad (LSI Letica). Sham-operated mice received an incision between the left orbit and tragus and the subsequent exposure of the temporal bone. The bone was drilled, but the dura mater and the middle cerebral artery were left intact. Similar to ischemic mice, sham-operated mice were maintained at 37°C during surgery.

Drug Treatment. Mice received an i.v. injection of saline (150 µl) or the same volume of DX-88 (discovered by Dyax Corp. using its proprietary phage display technology) at different doses (10, 30, or 90 µg/mouse). The dose of 30 µg/mouse (1.2 mg/kg) of DX-88 corresponds to the maximal effective dose used in humans to treat acute attacks in patients with hereditary and acquired angioedema (Williams and Baird, 2003; Agostoni et al., 2004; Zuraw, 2005). The dose of 30 µg/mouse was given at different times from ischemia, i.e., at the beginning of the ischemic period (pre), at reperfusion (post), and 1 h after the beginning of ischemia (1 h post).

Neurological Deficits. Twenty-four hours after ischemia, each mouse was rated on two neurological function scales unique to the mouse (Clark et al., 1997; De Simoni et al., 2003). For both scales, mice were scored from 0 (healthy mouse) to 28 (the worst performance in all categories). The score given represents the sum of the results of all categories for each scale. The general deficit scale evaluates hair, ears, eyes, posture, spontaneous activity, and epileptic behavior. The focal deficit scale evaluates body symmetry, gait, climbing on a surface held at 45°, circling behavior, front limb symmetry, compulsory circling, and whisker response to a light touch. All the experiments were run by a trained investigator blinded to the experimental conditions.
Quantification of Infarct Size and Brain Swelling. Mice were deeply anesthetized with Equitensin (120 µl/mouse i.p.), and brains were rapidly frozen by immersion in isopentane at −45°C for 3 min before being sealed into vials and stored at −70°C until use. For lesion size determination, 40-micron coronal brain cryosections were cut serially at 320-µm intervals and stained with neutral red (Neutral Red Gurr Certistain, BDH, Poole, Dorset, UK) (De Simoni et al., 2003). On each slice, infarcted areas were assessed blindly and delineated by the relative paleness of histological staining tracing the area on a video screen. The infarcted area and the percentage of brain swelling were determined by subtracting the area of the healthy tissue in the ipsilateral hemisphere from the area of the contralateral hemisphere on each section (Swanson et al., 1990; De Simoni et al., 2003). Infarct volumes were calculated by the integration of infarcted areas on each brain slice as quantified with computer-assisted image analyzer and calculated by Analytical Image System (Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada).

Brain Transcardial Perfusion. Mice were deeply anesthetized with Equitensin (120 µl/mouse i.p.) and transcardially perfused with 20 ml of PBS, 0.1 mol/liter, pH 7.4, followed by 50 ml of chilled paraformaldehyde (4%) in PBS. After carefully removing the brains from the skull, they were transferred to 30% sucrose in PBS at 4°C overnight for cryoprotection. The brains were then rapidly frozen by immersion in isopentane at −45°C for 3 min before being sealed into vials and stored at −70°C until use.

Assessment of Neurodegeneration. Fluoro-Jade labeling was carried out on perfused brains (Schmued et al., 1997). In brief, 20-µm mounted sections were dried and rehydrated in ethanol (100–75%) and distilled water. Then, they were incubated in 0.06% potassium permanganate, washed in distilled water, and transferred to 0.001% Fluoro-Jade staining solution. After staining, the sections were rinsed in distilled water, dried, immersed in xylene, and coverslipped.

Neuronal Count. At 7 days after ischemia, the lesioned area was not evaluable by the paleness of the tissue following neutral red staining because of the large microglial activation and cell recruitment to the lesion site. Therefore, at this point of time, the ischemic lesion was assessed by neuronal count. After cresyl violet staining, three 20-µm sections at 640-µm intervals from ipsi- and contralateral hemispheres were selected for neuronal count. The first section was at stereotoxic coordinates anteroposterior +0.86 from bregma. Thus, the selected slices include the striatum, the brain area corresponding to the ischemic core. The amount of neuronal loss was calculated by pooling the number of stained neurons in the three sections of both hemispheres and expressed as percentage of contralateral hemisphere. An Olympus BX61 microscope (Olympus, Tokyo, Japan), interfaced with Soft Imaging System Colorview video camera and AnalySIS software, was used. Segmentation was used to discriminate neurons from glia on the basis of cell size. The quantitative analysis was performed at 40× magnification by an investigator blinded to the treatment.

Experimental Groups. Eighteen experimental groups were prepared: two groups receiving 30 µg of DX-88 or saline (n = 4) for physiological parameter analysis; eight groups receiving 10, 30, or 90 µg of DX-88 or saline pre, 30 µg of DX-88, or saline post and 1 h post (n = 8–10) for evaluation of neurological deficits, ischemic volume, and brain swelling at 24 h after ischemia/reperfusion; two groups receiving 30 µg of DX-88 or saline pre (n = 6–8) for determination of neurodegeneration by Fluoro-Jade staining 24 h after ischemia/reperfusion; two groups receiving 30 µg of DX-88 or saline pre (n = 6–8) for evaluation of neuronal loss 7 days after ischemia/reperfusion; two groups receiving 30 µg of DX-88 or saline pre (n = 6–8) for evaluation of ischemic lesion 7 days after permanent ischemia; one group of transient MCAO sham-operated mice for neurological deficits and neurodegeneration evaluation 24 h after surgery (n = 3); and one group of permanent MCAO sham-operated mice for ischemic volume 7 days after surgery (n = 3).

Physiological Parameters. DX-88 did not modify the physiological parameters tested (pH, 7.12 ± 0.005 and 7.13 ± 0.015; pCO₂, 43.1 ± 0.4 and 42.35 ± 2.35; pO₂, 58.5 ± 2.5 and 54 ± 1; oxygen saturation, 80.5 ± 1.5 and 77 ± 2% for saline- and DX-88-treated mice, respectively; data are expressed as mean ± S.E.M.).

Transient MCAO: 24-h Outcome. C57BL/6 mice treated with DX-88, given at the beginning of the ischemic period, had a dose-dependent reduction of ischemic volume to 38.4% of the volume of saline-treated mice (12.11 ± 1.44 and 4.65 ± 0.88 mm³, saline- and DX-88-treated mice, respectively) with the dose of 90 µg/mouse (Fig. 1). In addition, 30 µg/mouse was able to significantly reduce the ischemic lesion (5.94 ± 1.10 mm³, reduction to 49% of saline), whereas 10 µg/mouse was ineffective (Fig. 1). Likewise, DX-88 was also effective in markedly reducing brain swelling at the dose of 30 µg/mouse when given at the beginning of the ischemic period (8.96 ± 1.57% and 2.85 ± 0.54%, for saline- and DX-88-treated mice, respectively, or a reduction to 32%, Fig. 2, lower panel).

When given at the end of 30 min of ischemia, but before reperfusion, 30 µg/mouse DX-88 was still able to significantly reduce the ischemic volume (5.07 ± 1.83 mm³, reduction to 42%) and brain swelling (4.16 ± 0.96%, reduction to 46.4%, Fig. 2, post). DX-88 efficacy was lost when the inhibitor was given 30 min after the beginning of reperfusion (Fig. 2, 1 h post).

To evaluate whether the marked reduction of the ischemic lesion observed 24 h after ischemia resulted in reduced func-

![Saline vs. DX-88 30µg](http://example.com/saline_vs_dx88.png)

Fig. 1. Infarct volume assessed 24 h after ischemia in mice receiving saline or different doses of DX-88 at the beginning of the ischemic period. Data are expressed as mean ± S.E.M. (n = 8–10 mice per group). A representative ischemic lesion is outlined in the brain slices (upper panel). **, P < 0.01 versus saline, one-way ANOVA, and Dunnett as post hoc test.
Functional impairment, neurological deficits were evaluated at 24 h in mice receiving saline or 30 μg/mouse DX-88 at the beginning of ischemic period (pre) (Fig. 3). Although saline-treated mice showed stable scores, those who received DX-88 had significantly reduced general (11.5 ± 1.0 and 8.87 ± 1.13, means of saline- and DX-88-treated mice, respectively, Fig. 3, upper panel) and focal (18.9 ± 2.14 and 11.62 ± 1.87, means of saline- and DX-88-treated mice, respectively, Fig. 3, lower panel) deficit scores. The same results were obtained when the inhibitor was given at the end of ischemia but before reperfusion (8.17 ± 1.01 means of general and 11.67 ± 2.33 means of focal deficits, Fig. 3). No change in functional impairment was observed when the inhibitor was given after 30 min of reperfusion.

We then analyzed the effect of ischemia and DX-88 treatment on neurodegeneration to assess if neurons were actually spared by the inhibitor treatment. The staining with Fluoro-Jade, a marker for neuronal degeneration, evaluated 24 h after ischemia, showed the presence of degenerating neurons in selected brain areas. As expected, in saline-treated mice, positive cells were consistently observed in hippocampus and striatum where they appeared as extended clusters of fluorescent cells (three of three mice, Fig. 4, A–C). In mice treated with 30 μg of DX-88 pre, the number of degenerating cells was greatly reduced, and the general structure of the brain tissue was well preserved compared with saline-treated mice. These mice showed small groups of Fluoro-Jade-positive cells both in striatum and hippocampus (Fig. 4, B–D). Positive cells could not be found in the other brain regions in these mice, indicating the focal nature of the lesion. Sham-operated mice did not show any neurological deficits nor neurodegeneration (data not shown).

**Transient MCAO: 7-Day Outcome.** To evaluate if the protective effect of DX-88 was long-lasting, we assessed the neuronal loss 7 days after induction of ischemia/reperfusion and drug treatment. DX-88 protective effect was still present at this time point (14 ± 2.18% and 3.7 ± 1.5%, saline- and DX-88-treated mice, respectively, Fig. 5).

**Permanent MCAO: 7-Day Outcome.** Lastly, the effect of plasma kallikrein inhibitor was then assessed in a model of permanent brain ischemia, i.e., without reperfusion. Seven days after permanent ischemia, mice treated with 30 μg/mouse DX-88, at the beginning of ischemia, did not show a reduction of the ischemic volume compared with saline-treated mice (14.71 ± 0.98 and 18.77 ± 3.11 mm³, saline- and DX-88-treated mice, respectively, Fig. 6). Sham-operated mice did not show ischemic cerebral lesion (data not shown).
The results of this study show that DX-88, a selective plasma kallikrein inhibitor, reduced the ischemic volume, both infarct size and swelling, and the functional impairment in mouse transient ischemia, showing a remarkably high degree of neuroprotection. This protection was persistent and related to reperfusion. DX-88 dose dependently reduced the ischemic volume. Thirty and 90 μg of the inhibitor were able to reduce the ischemic volume. The use of Fluoro-Jade staining to evidence degenerating neuronal cells allowed us to investigate whether DX-88 action resulted in sparing of these cells and showed that 24 h after ischemia, Fluoro-Jade-positive cells were markedly decreased in treated mice compared with mice receiving saline.

Beside the brain ischemic lesion, edema is a serious complication in postischemic injury. It results from local vasodilatation, increased microvascular permeability, and retention of fluid in injured tissue. The increase in blood flow and vascular permeability at inflammatory sites causes plasma to leak into the brain parenchyma, provoking swelling, and the plasma inflammatory mediators, such as free radicals, cytokines, and activated complement and kinin system factors, can directly affect neuronal survival. The plasma kallikrein inhibitor, DX-88, was able to counteract swelling formation, further supporting the hypothesis that the release of bradykinin in brain tissue plays an important role in brain edema after focal ischemia (Groger et al., 2005).

An additional important aspect when assessing the protective effect of a compound on ischemia-reperfusion injury is the evaluation of functional deficits. DX-88 induced a progressive recovery in both general and focal neurological deficits, indicating an amelioration of the appearance of the mice, their motor performance, their reactivity, and response to stimuli. To assess whether the favorable effect of DX-88 treatment was merely due to a delay in degenerating processes or to a real neuroprotection, we analyzed the neuronal loss 7 days after ischemia/reperfusion and fully confirmed the observation that neurodegeneration was actually inhibited by DX-88 treatment.

At variance with this finding, DX-88 did not affect the ischemic volume in the permanent model of ischemia, indicating that the compound was effective only if reperfusion was present, i.e., its protective effect is exerted by inhibiting events related to reperfusion. This is further supported by the observation that the protective effect of DX-88 is not
present when the inhibitor is given 30 min after the beginning of reperfusion in the transient ischemia model. Although reperfusion of ischemic brain induced by thrombolytic agents is an effective therapy for stroke, this phenomenon can also contribute to tissue damage by several mechanisms that include activation of endothelium, increased production of oxygen radicals, inflammatory cytokine induction, leukocyte recruitment, and edema formation (Hallenbeck and Dutka, 1990; Kaur et al., 2004; Latour et al., 2004). Thus, the lack of neuroprotection when reperfusion was not allowed (permanent ischemia model) or when DX-88 was administered after reperfusion (transient ischemia model) indicates that plasma kallikrein plays a pivotal role in the mechanism of ischemic brain injury at reperfusion and supports the idea that blockade of kallikrein may be an effective strategy for prevention of the inflammatory injuries occurring early during reperfusion of ischemic organs, including brain.

Previous data of ours (De Simoni et al., 2003, 2004; Storini et al., 2005) showed a powerful neuroprotective action of C1-INH when given to C1q−/− mice before reperfusion. C1NKH, the endogenous inhibitor of the activated first component of complement classic pathway (C1), is a major inhibitor of plasma kallikrein; thus, our previous data, together with the present data, allow us to hypothesize that part of the powerful neuroprotective effect of C1-INH in ischemia/reperfusion injury is due to an action on the kinin system.

Recently, Xia et al. (2004, 2006) showed a protective effect of delayed tissue kallikrein gene transfer, the production of the peptide occurring 2 to 5 days after brain ischemia. This action was linked to promotion of angiogenesis and neurogenesis. These data, together with ours, indicate that the kinin system, similar to other inflammatory systems and molecules (McIntosh et al., 1996; Walport, 2001a,b), may have a dual role, detrimental and beneficial, mainly depending on the degree and/or time of activation. We can hypothesize that early after the ischemic injury, when the contact-innatin system is fully activated, its inhibition is protective, whereas at later times, kinin vasoactive properties result in protective effects.

In our model of brain ischemia/reperfusion, DX-88 induced a dose-dependent reduction of the ischemic volume and swelling, and it lowered the number of dying neurons and significantly lowered functional neurological impairment. Its protective effect was still present 7 days after ischemia. The experimental data indicate that the inhibition of plasma kallikrein protects from reperfusion injury and support this strategy for the treatment of ischemic stroke aimed at reducing detrimental effects linked to reperfusion.

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References


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