The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations

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We have considered the extracellular serine protease thrombin and its receptor as endogenous mediators of neuronal protection against brain ischemia. Exposure of gerbils to prior mild ischemic insults, here two relatively short-lasting occlusions (2 min) of both common carotid arteries applied at 1-day intervals 2 days before a severe occlusion (6 min), caused a robust ischemic tolerance of hippocampal CA1 neurons. This resistance was impaired if the specific thrombin inhibitor hirudin was injected intracerebroventricularly before each short-lasting insult. Thus, efficient native neuroprotective mechanisms exist and endogenous thrombin seems to be involved therein. In vitro experiments using organotypic slice cultures of rat hippocampus revealed that thrombin can have protective but also deleterious effects on hippocampal CA1 neurons. Low concentrations of thrombin (50 pM, 0.01 unit/ml) or of a synthetic thrombin receptor agonist (10 μM) induced significant neuroprotection against experimental ischemia. In contrast, 50 nM (10 units/ml) thrombin decreased further the reduced neuronal survival that follows the deprivation of oxygen and glucose, and 500 nM even caused neuronal cell death by itself. Degenerative thrombin actions also might be relevant in vivo, because hirudin increased the number of surviving neurons when applied before a 6-min occlusion. Among the thrombin concentrations tested, 50 pM induced intracellular Ca2+ spikes in fura-2-loaded CA1 neurons whereas higher concentrations caused a sustained Ca2+ elevation. Thus, distinct Ca2+ signals may define whether or not thrombin initiates protection. Taken together, in vivo and in vitro data suggest that thrombin can determine neuronal cell death or survival after brain ischemia.

The extracellular serine protease thrombin, a well-known key player in blood coagulation and platelet activation, has been found to be expressed in different brain regions (1, 2). Its physiological importance in the central nervous system is emphasized further by the parallel expression of the highly specific thrombin inhibitor protease nexin-1 (3, 4) and PAR-1, the classical thrombin receptor (2, 5–7). Some recent evidence indicates that thrombin and its receptor might be involved in neurodegenerative processes observed after different insults such as stroke, traumatic brain injuries, and heart arrest or as a frequent consequence of bypass surgeries (8–11). Normal brain function depends critically on a permanent supply of glucose and oxygen. Depending on its source, a disruption of the cerebrospinal blood flow leads to global or focal ischemia (hypoxia/hypoglycemia) and irreversible neuronal damage. Prothrombin as well as the classical thrombin receptor are expressed in brain regions that are particularly vulnerable to ischemia, e.g., neocortex, cortex, striatum, hypothalamus, hippocampus, and cerebellum (2). Furthermore, studies performed on isolated cells (neurons, astrocytes) have demonstrated that nanomolar concentrations of thrombin exert cytotoxic effects (12–14). However, it has been discussed controversially whether thrombin represents a general death signal or whether this protease also can initiate neuroprotection (8, 12, 13, 15).

To examine the role of thrombin in ischemia-induced neurodegeneration, we employed in vivo and in vitro models of experimental ischemia: (i) transient global ischemia by occlusion of both common carotid arteries in Mongolian gerbils and (ii) exposure of organotypic rat hippocampal slice cultures to oxygen and glucose deprivation (OGD).

Methods

Materials. Recombinant hirudin (10,000 antithrombin units/mg protein) and high-activity thrombin (3,000 National Institutes of Health (NIH) units/mg protein) from human plasma were purchased from Calbiochem and Sigma, respectively. NIH units are defined by Sigma by direct comparison with a specific NIH thrombin reference standard lot (manufacturer information). Thrombin was essentially free of other known nonactivated and activated clotting factors as well as of plasminogen and plasmin (manufacturer information). All thrombin treatments were performed by using units per milliliter assuming that 200 units/ml thrombin is approximately equivalent to 1 μM thrombin (9). The synthetic thrombin receptor agonist peptide Ala-pFluoro-Phe-Arg-Cha-HomoArg-Tyr-NH₂ was obtained from Neosystem (Strasbourg). If not stated otherwise, all other compounds used were from Sigma. Experiments involving animals have been approved by the author’s institutional review board. Statistical analysis was performed by using the heteroscedastic t test.

In Vivo Ischemia. Male Mongolian gerbils (Meriones unguiculatus, Tumblebrook farm strain; Charles River Breeding Laboratories) ca. 3 months old, weighing 60–80 g, were used. Global cerebral ischemia was performed by transient occlusion of both common carotid arteries. Briefly, 7 days before the final experiment, an injection cannula (0.6-mm diameter) was implanted into the lateral ventricle (0.6 mm posterior to bregma, 1.2 mm lateral to midline) of anaesthetized animals (80 mg/kg pentobarbital). Before the ischemic procedure, gerbils were anaesthetized with 3% halothane in 30% O₂/70% N₂O. Drugs or vehicle was applied intracerebroventricularly (ICV) 20 min before the occlusion. During all operations, the rectal temperature was maintained at 37 ± 0.5°C by using a heating pad. Surgical clips were used to occlude transiently both common carotid arteries.

Abbreviations: [Ca2+]i, intracellular Ca2+ concentration; ICV, intracerebroventricularly; OGD, oxygen and glucose deprivation; PI, propidium iodide.

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Global ischemia causes a massive loss of pyramidal cells in the hippocampal CA1 region of Mongolian gerbils. Cellular survival was visualized by staining of paraffin-embedded brain slices with toluidine blue/fuchsin acid 7 days after a sham operation (A) or a transient occlusion (6 min) of both common carotid arteries (B). A layer of intact pyramidal cells can be identified easily within the CA1 region of the control animals (A) whereas most neurons disappeared after ischemia (B). The pyramidal cell layers are indicated by arrows in A and B.

**Fig. 1.** Global ischemia causes a massive loss of pyramidal cells in the hippocampal CA1 region of Mongolian gerbils. Cellular survival was visualized by staining of paraffin-embedded brain slices with toluidine blue/fuchsin acid 7 days after a sham operation (A) or a transient occlusion (6 min) of both common carotid arteries (B). A layer of intact pyramidal cells can be identified easily within the CA1 region of the control animals (A) whereas most neurons disappeared after ischemia (B). The pyramidal cell layers are indicated by arrows in A and B.

Organotypic Hippocampal Slice Cultures. Organotypic hippocampal slice cultures were prepared and maintained according to Stoppini et al. (16). Briefly, hippocampi were prepared from male Wistar rats (approximately 10 days old, 18–20 g) after decapitation. Hippocampal slices (350 μm) were cut by using a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, U.K.) and transferred to ice-cold MEM-Hanks’ solution (pH 7.35 at 6°C, saturated with 100% O₂) containing 25 mM Hepes and 2 mM l-glutamine (GIBCO). Subsequently, undamaged slices were selected by using a binocular microscope and transferred onto Anopore membranes (25-mm diameter, 0.02-μm pore size) of cell and tissue culture inserts (Nunc). Usually, four to six slices per membrane were cultured. The inserts were set into Nunc uncloned and tissue culture six-well plates (Nunc) containing 1.2 ml of culture medium per well. The culture medium (pH 7.35 at 37°C) was 50% MEM-Hanks’ solution (Biochrom, Berlin)/25% HBSS (GIBCO)/25% heat-inactivated horse serum (GIBCO)/350 mg/ml NaHCO₃/12.5 mM Hepes. During culture, the slices were exposed to 95% air/5% CO₂ (36°C). The medium was changed every 2–3 days.

Intracellular Ca²⁺ Imaging. Organotypic hippocampal slice cultures were loaded with 50 μM fura-2/AM at 27°C for 45 min. Slice cultures then were transferred into a perfusion chamber (vol = 3 ml) mounted on the stage of an Axiovert inverted fluorescence microscope with a Fluar objective (×20, 0.75) (Zeiss). An artificial cerebrospinal fluid medium containing 124 mM NaCl, 4.9 mM KCl, 1.3 mM MgSO₄, 2.0 mM CaCl₂, 1.2 mM KH₂PO₄, 25.6 mM NaHCO₃ and 10 mM glucose (pH 7.4) saturated with 95% O₂/5% CO₂ was used for perfusion (1 ml/min). The chamber temperature was adjusted to 27 ± 1°C. The fura-2 fluorescence of single CA1 neurons was captured by using a cooled IMAGO charge-coupled device camera and the Polychrome II System (TILL Photonics, Planegg, Germany). Excit-
tation and emission wavelengths were 340/380 nm and ≥500 nm, respectively. Thrombin additions were performed by switching to another medium reservoir containing the appropriate concentration of the protease. The TILLVISION software package (TILL Photonics) was used for data analysis.

**Results**

**Hirudin Increases the Survival of Hippocampal CA1 Neurons After Ischemia in Vivo.** The number of surviving pyramidal cells in the hippocampal CA1 region was determined 7 days after transient occlusion of both carotid arteries. An occlusion maintained for 6 min caused a massive loss of CA1 neurons. Compared with sham-operated animals, only 6.3 ± 1.1% of the neurons survived (Figs. 1 and 24). To investigate whether endogenous thrombin is involved in ischemia-induced degeneration, we injected the specific thrombin antagonist hirudin ICV (final concentration about 0.1 unit/ml) 20 min before the onset of carotid occlusion. As in the animal group that did not receive hirudin, the survival of hippocampal CA1 cells was determined 7 days after the ischemia. In the hirudin-treated animals, the number of intact CA1 neurons increased about 5-fold to 30.3 ± 2.6% in the animals that did not receive the prior mild occlusions. To elucidate whether endogenous thrombin, besides its role in ischemia-induced degeneration, is also involved in this kind of ischemic tolerance, hirudin again was employed. For these experiments, hirudin (0.1 unit/ml) was injected ICV 20 min before each short-lasting but not before the long-lasting occlusion. This treatment decreased significantly the number of neurons surviving in the hippocampal CA1 region—from 64% to 43.8 ± 6.6% (P = 0.016, Fig. 2B).

**Experimental Ischemia in Vitro.** The in vivo experiments presented above indicate that endogenous thrombin might be involved in both ischemia-induced degeneration and ischemic preconditioning. Conceivably, opposite thrombin effects might be caused by different concentrations of the protease present in the brain after mild or severe ischemic conditions. Unfortunately, the thrombin concentration hardly can be controlled in vitro. Therefore, to manipulate the thrombin concentration, we used organotypic hippocampal slice cultures. Because the intercellular communication network remains at least partly intact, slices resemble the in vivo situation better than the dissociated cells in culture used frequently. The organotypic cultures were subjected to experimental “ischemia” by transient OGD. After another 24 h of normal culture conditions, i.e., in the presence of glucose and oxygen, the neuronal survival within each organotypic slice was quantified by the uptake of the fluorescent PI into damaged cells. As shown in Fig. 3, 30 min of OGD induced a widespread neuronal cell death throughout the hippocampal CA1 region. Other regions such as CA4/CA3 and the dentate gyrus usually were less affected (Fig. 3D). In contrast, an OGD lasting for only 5 min was tolerated by the cells in all areas, including the particularly sensitive CA1 region (not shown).

To try to imitate the preconditioning effect observed in vivo, organotypic hippocampal slices were subjected to a mild OGD (5 min), which was followed by a severe OGD (30 min) applied 24 h later. We were not able to detect any protection by preconditioning in vitro (not shown). We have also tested whether thrombin is able to assist a mild OGD in inducing ischemic tolerance in vitro. For that purpose, organotypic hippocampal slice cultures were coexposed to a prior mild OGD (5 min) and different concentrations of thrombin 24 h before applying the severe OGD (30 min). Notwithstanding, even this combined treatment failed to improve neuronal survival.

**Effect of Thrombin Treatment on the Viability of Organotypic Hippocampal Slice Cultures.** Considering the possibility that ischemia-induced neurodegeneration and protection might be regulated...
by different concentrations of endogenous thrombin in vivo, organotypic hippocampal slice cultures were exposed to different concentrations of thrombin. At first, we were interested to see whether thrombin per se affects the viability of organotypic hippocampal slices. For this reason, slices were transiently exposed to 50 pM, 500 pM, 5 nM, 50 nM, or 500 nM thrombin in the presence of oxygen and glucose. In each experiment, the incubation with thrombin was maintained for 1 h. In analogy to the former experiments, the extent of neuronal degeneration was quantified by the uptake of PI into damaged cells 24 h later (C and D). Exposure to a relatively high thrombin concentration (500 nM, 1-h incubation) in the presence of oxygen and glucose caused even greater damage (E and F). In contrast, thrombin at a concentration as low as 50 pM, given immediately before and during the OGD, induced significant neuroprotection (G and H). (Bars = 500 μm.)

Fig. 3. Influence of OGD and/or thrombin on neuronal survival in organotypic hippocampal slice cultures. Transmission images (Left) and the uptake of fluorescent PI (Right) are shown. Neuronal cell damage can be recognized by dark slice areas and red PI fluorescence in the transmission and fluorescence images, respectively. A control slice is shown in A and B. Transient OGD (30 min) was followed by a pronounced neuronal damage in the CA1 region 24 h later (C and D). Exposure to a relatively high thrombin concentration (500 nM, 1-h incubation) in the presence of oxygen and glucose caused even greater damage (E and F). In contrast, thrombin at a concentration as low as 50 pM, given immediately before and during the OGD, induced significant neuroprotection (G and H). (Bars = 500 μm.)

Fig. 4. Thrombin can perform either protective or deleterious effects in a concentration-dependent manner. Organotypic hippocampal slice cultures were exposed to OGD and/or thrombin as indicated. The presence or absence of thrombin is indicated by (+) and (−), respectively. At 50 pM (0.01 unit/ml), thrombin caused protection against experimental ischemia, whereas at 500 nM (100 units/ml) thrombin was cytotoxic per se. Note that even 50 nM (10 units/ml) thrombin was deleterious when applied in combination with OGD. Intermediate thrombin concentrations of 500 pM–5 nM (0.1–1 unit/ml) did not affect the survival of the slice cultures. For each experiment, the mean ± SEM with n between 3 and 22 is shown. *, P < 0.025; **, P < 0.001.

Strigov et al. PNAS February 29, 2000 vol. 97 no. 5 2267
The experiment was performed as that which revealed neuroprotection by 50 pM; but, instead of the protease, the agonist peptide was added 30 min before and during the severe OGD (30 min). The agonist peptide exerted significant neuroprotection at a concentration of 10 μM (Table 1). Moreover, the extent of protection was similar to that obtained by 50 pM thrombin (compare Table 1 and Fig. 4). Because of the relatively low efficiency of artificial receptor-activating peptides (21), it was not possible to apply an agonist concentration that could mimic the degenerative effect seen at 500 nM thrombin.

**Thrombin-Induced Ca^{2+} Signals in Organotypic Hippocampal Slice Cultures.** The finding that 50 pM thrombin induced neuroprotection against OGD whereas concentrations of $\geq$500 pM were not effective might be due to distinct signaling cascades that follow receptor activation. Thrombin initiates the generation of inositol 1,4,5-trisphosphate, which regulates the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (11). To explore the possibility that specific Ca^{2+} signals are involved in thrombin-induced neuroprotection, hippocampal CA1 neurons were loaded with fura-2. As shown in Fig. 5, the two thrombin concentrations tested, 50 and 500 pM, affected $[Ca^{2+}]_i$ of CA1 neurons in a characteristic manner. The protective concentration of thrombin (50 pM) induced single or repetitive Ca^{2+} spikes (Fig. 5C). On the other hand, thrombin concentrations that had failed to mediate neuroprotection against ischemia ($\geq$500 pM) caused a delayed single spike of $[Ca^{2+}]_i$ followed by a sustained plateau phase (Fig. 5D). Moreover, in the latter case, the spike occurred synchronously among the different cells of a slice.

**Discussion**

One of the central findings of this work is that the thrombin antagonist hirudin protected hippocampal CA1 pyramidal cells *in vivo* when applied before the onset of a severe global ischemia. Because hirudin is said to be highly thrombin-specific and pharmacodynamically inert (22, 23), it appears that its protective effect is caused by inhibition of endogenous thrombin. This, in turn, strongly suggests that endogenous thrombin participates in ischemia-induced neurodegeneration in the hippocampus. An involvement of thrombin in neurodegeneration is supported further by the *in vitro* experiments that showed that 500 nM exerted degenerative effects whereas concentrations $\leq$50 nM were tolerated. Such a concentration dependence is in accordance with previous studies performed on primary cultures of astrocytes and neurons (12–14). It may be questioned whether thrombin concentrations of 500 nM are reached in the brain, even under pathophysiological conditions. However, it is pertinent to mention here that 50 nM thrombin, although nontoxic *per se*, already impaired further the neuronal survival of the cultures when applied in combination with a 30-min OGD. This combination of factors may reflect the situation encountered during a stroke, where the cells might be exposed simultaneously to OGD and to increased levels of thrombin. That is, under ischemic conditions, thrombin does not need to reach very high concentrations to unleash its deleterious effects.

The development of ischemic tolerance after a prior mild ischemia *in vivo* indicates the existence of endogenous mecha-

### Table 1. Effect of the thrombin receptor agonist peptide on OGD-induced degeneration

<table>
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<th>Agonist, μM</th>
<th>OGD, min</th>
<th>Cellular damage</th>
<th>%</th>
<th>SEM</th>
<th>n</th>
<th>p</th>
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Hippocampal slice cultures were exposed to different agonist concentrations 30 min before and during OGD. The neuronal survival was quantified 24 h after OGD. *, P < 0.05.
neuroprotection against ischemia (18–20). This fact is of paramount importance for the development of new strategies to alleviate the disabling consequences of stroke. Unfortunately, apart from the suggestion that different heat-shock proteins play a role in ischemic preconditioning (24), its mechanism remains widely unknown. In this respect, that intrathalamic thalamic neurons, when injected immediately before or during OGD, suggest that thalamic neurons can mediate rapid neuronal protection against ischemia.

Nevertheless, we were able to induce significant neuroprotection by thrombin in vitro when the protease was applied immediately before and during OGD, suggesting that thrombin, besides its involvement in long-lasting ischemic preconditioning, also can mediate rapid neuronal protection against ischemic insults. It turns out that thrombin receptor activation as well as its involvement in long-lasting ischemic preconditioning, is necessary but not sufficient for an efficacious preconditioning.

Striggow et al. (1997) have shown that endogenous thrombin may be a necessary factor. However, because we failed to reproduce the preconditioning effect in the slices in vitro, it is likely that an increase in thrombin concentration induces cell death within the infarct center, whereas low levels of thrombin may rescue neuronal cells in the periphery.

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