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Cytoprotective efficacy and mechanisms of the liposoluble iron chelator 2,2'-dipyridyl in the rat photothrombotic ischemic stroke model.

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Running title

Iron chelation protects brain against infarction

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NAA, N-acetyl-aspartate, DP, 2,2'-dipyridyl, ROS, radical oxygen species, HIF-1α, hypoxia inducible factor 1α, HO-1, heme oxygenase-1, BBB, blood brain barrier, rt-PA, recombinant tissue-type plasminogen activator

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Abstract

We examined the efficacy of the liposoluble iron chelator 2,2'-dipyridyl (DP) to reduce histological damage in rats submitted to cerebral ischemia and the mechanisms involved in the potential cytoprotection. For this purpose, DP (20 mg/kg, i.p.) was administered 15 min before and 1h after induction of cortical photothrombotic vascular occlusion in rat. Histological studies were performed for assessment of infarct volume (at days 1 and 3 post-ischemia) and astromicroglial activation (at day 3 post-ischemia). Damage to endothelial and neuronal cells were evaluated at day 1 post-ischemia by quantitative measurements of Evans blue extravasation and N-acetyl-aspartate levels, respectively. Cerebral blood flow (CBF) was recorded in the ischemic core by laser-Doppler flowmetry within the 15min-2h period following photothrombosis. At 4h post-ischemia, radical oxygen species (ROS) production was evaluated by measuring brain glutathione concentrations. The cortical expression of the proteins heme oxygenase-1 (HO-1) and hypoxia inducible factor-1 α (HIF-1α) was analyzed by Western blotting at day 1 post-ischemia. Infarct volume and ischemic damage to endothelial and neuronal cells were significantly reduced by DP treatment. This cytoprotection was associated with a reduction in ROS production, perfusion deficits and astrocytic activation. DP treatment also resulted in significant changes in HO-1 (+ 100%) and HIF-1α (- 50%) protein expression at the level of the ischemic core. These results report the efficacy of the liposoluble iron chelator DP in reducing histological damage consecutive to permanent focal ischemia.

Introduction

In ischemic stroke, the territory normally supplied by the occluded vessels, the ischemic core, will inevitably die but there is also tissue which balances on the edge between death and recovery, the penumbra. Recanalization operated with the fibrinolytic rtPA is the only therapy approved for the treatment of acute ischemic stroke. However, the need of new therapeutic strategies appropriate for patients who cannot benefit from fibrinolysis is evident, in order to save as much ischemic brain tissue as possible.

In brain as in other organs, the level of intracellular free iron is tightly regulated thus indicating the importance for cells to maintain free iron concentration in physiological range. Arguments exist for the existence of disruption of the cerebral iron homeostasis after ischemia. Thus, ischemic brain tissue was reported to accumulate ultrafiltrable iron (Oubidar et al, 1994, Lipscomb et al, 1998) and to express high level of ferritin (Chi et al, 2000), an indirect marker of free iron excess. Indeed, the main mechanism elaborated by cells to keep intracellular free iron level within normal range is the regulation by free iron level itself of the synthesis of ferritin (Eisenstein, 2000), the main intracellular iron storage protein. Given the role of free iron in the production of radical oxygen species (ROS) including superoxide anion and hydroxyl radical (Minotti and Aust, 1992) and the damaging effect of these ROS in brain ischemia (Baker et al, 1998, Kim et al, 2001, Imai et al, 2003) iron chelators are of obvious interest as therapeutic agents in this pathology. However, effective treatments require an iron chelator capable of entering brain cells since the availability of free iron at critical intracellular space appears to be essential to the cellular damage caused by ROS (Lesnefsky and Ye, 1994, Oubidar et al, 1996). This may explain why histological data to support a cytoprotective effect of desferoxamine (the most used iron chelator in clinical and

experimental settings) in brain ischemia are lacking. Indeed, as a result of its high hydrosolubility, desferoxamine cannot rapidly reach the intracellular space of brain after acute systemic treatment (Lloyd et al, 1991). To our knowledge, whether liposoluble iron chelator can decrease histological damage consecutive to brain ischemia has never been reported. However, it was previously shown that the liposoluble iron chelator 2,2'-dipyridyl (DP) decreased edema consecutive to focal brain ischemia (Oubidar et al, 1994).

Heme oxygenase-1 (HO-1) is a heat-shock protein that is induced in brain in response to permanent focal ischemia (Geddes et al, 1996, Panahian et al, 1999, Bidmon et al, 2001). It is the enzyme that converts the heme molecule into bilirubin, carbon monoxide, and iron. HO-1 serves as an adaptive mechanism to protect brain from ischemic injury as evidenced by studies conducted on transgenic mice that overexpress HO-1 in neurons (Panahian et al, 1999). HO-1 was demonstrated to be a HIF-1 regulated gene (Lee et al, 1997). In addition, iron chelation like hypoxia results in the accumulation of HIF-1α (Wanner et al, 2000) enabling it, upon dimerization with the HIF-1β subunit, to bind to a specific DNA sequence within the hypoxia responsive element of an array of target genes. Thus, increased HO-1 expression has been proposed to be a putative mechanism by which iron chelating agents given around the induction of ischemia can protect brain from infarction (Sorond and Ratan, 2000). However, whether iron chelating agents can modify ischemia-induced expression of HIF-1α and HO-1 is not known.

The goal of this study was to examine the efficacy of the liposoluble iron chelator DP to reduce histological damage in rats submitted to permanent focal cerebral ischemia and the mechanisms involved in the potential cytoprotection including decreased ROS production and HO-1 overexpression. Ischemic damage to brain was assessed by histological determination

of infarct volume. Moreover, lesions to neurons and endothelial cells were quantified by measuring cerebral N-acetylaspartate levels and quantitating the opening of the blood brain barrier to circulating proteins, respectively. We then examined residual cerebral blood flow, tissue glutathione loss as a marker of ROS production and expression of the proteins HO-1 and HIF-1α in ischemic rats treated or not with the iron chelator. Importantly, most of the measurements were separately performed in the ischemic core and the penumbra.

Methods

Adult male Wistar rats (Depré, Saint-Doulchard, France) weighing 350-450 g were used in these experiments. Animals were kept under a 12h-12h light:dark cycle and allowed free access to food and water. Animal care was in accordance with the French Department of Agriculture guidelines (license n° 21CAE035). Anesthesia was induced by chloral hydrate (4% in saline, 400 mg/kg, i.p., Sigma).

Induction of brain ischemia

Permanent focal ischemia was induced by cortical photothrombotic vascular occlusion as recently described (Demougeot et al, 2003). Briefly, anesthetized rats were infused with Rose bengal (20 mg/kg, i.v., Aldrich) and a laser beam was focussed through the skull on the right hemisphere (1 mm posterior and 3 mm lateral relative to the bregma) by means of an optic fiber (1 mm interior diameter, emerging power 90 mW), using a diode-pumped solid-state laser (LCS-DLT-312, Opton Laser International, Orsay, France) working at 532 nm. The skull was irradiated for 5 min, the irradiation beginning 30 seconds before the dye injection. Body temperature was kept at 37°C using a thermoregulated pad during irradiation. Rats were then placed in individual cage with free access to food and water. All rats survived from brain ischemia.

Measurement of cerebral blood flow

Preischemic and postischemic cerebral blood flow (CBF) were successively monitored in the same anesthetized rat by laser-Doppler flowmetry (Transonic System Inc, Itheca, USA). For postischemic recording, the probe needle was placed after the irradiation period in contact with the dura at the level of the center of the irradiated region. Postischemic values were

recorded within the 15min-2h period following irradiation and expressed as percentage of preischemic values which were obtained from the corresponding region of the left hemisphere 30 to 45 min before photothrombosis initiation. Mean arterial blood pressure (MABP) was recorded from a femoral artery throughout the experiments. The measurements were performed by a manipulator blind to the treatment.

Evaluation of ischemic damage to endothelial and neuronal cells

The ischemic damage to endothelial cells was investigated using Evans blue extravasation. Evans blue (1% in saline, 3 ml/kg, Société Chimique Pointet, Villeneuve la Garenne, France) was injected in the jugular vein and allowed to circulate for 24h. Rats were then anesthetized and perfused with saline through the left ventricle at 100 mm Hg pressure until colorless perfusion fluid was obtained from the right atrium. Lesioned cortices were then dissected, weighed, homogenized in cold 50% trichloroacetic solution (5 ml for 100 mg of fresh tissue), and kept at 4°C for 30 min. Following centrifugation, the concentration of the extracted dye was measured by spectrophotometry (λ=610 nm). The amount of Evans blue was quantified from a linear standard curve obtained from known amounts of the dye and was expressed as µg per g of proteins which were measured by the method of Lowry et al (1951).

The ischemic damage to neuronal cells was investigated by quantifying brain NAA loss, a reliable indicator of neuronal loss/dysfunction within the acute stage of ischemia (Demougeot et al, 2001, 2003). NAA analysis was performed in cortex from rats transcardially perfused with saline using high-performance liquid chromatography coupled to ultraviolet detection (206 nm) after anion exchange separation as previously described in detail (Demougeot et al, 2001). The values were expressed as nmoles per mg of proteins.

HO-1 and HIF-1 α protein expression

Cortical HO-1 and HIF-1α protein expression was studied using Western blotting analysis. Briefly, brain tissues were homogenized in 5 volumes of lysis buffer (PBS containing 1% SDS, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride, 2µg/ml aprotinin and leupeptin, 1µg/ml pepstatin) sonicated and centrifuged at 10,000g for 10 min. Based on protein assay, equivalent amounts of total proteins (e.g. 40µg/well) were dissolved in 62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 10% glycerol and 0.01% bromophenol blue and were heated at 85°C for 10 min. Proteins were separated on 8-12% SDS PAGE according to Laemmli (1970) and electrophoretically transferred to PVDF membrane (0.2 µm pore size, Amersham Pharmacia Biotech) in cold transfer buffer. The composition of the buffers for HO-1 and HIF-1 α was the following: 10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol, pH 9.9, and 25 mM Tris-HCl, 192 mM glycine and 20% methanol, pH 8.3, respectively. After the transfer step, the membrane was soaked in blocking solution (non-fat dry milk in TBS buffer containing 0.1% Tween 20) and washed. Membranes were then incubated for 4h at room temperature with the monoclonal anti-HO-1 and HIF-1α antibodies at 1:1000 (Stressgen and Biomol, respectively, from TEBU, Le Paray en Yvelines, France) and for 1.5h with horseradish peroxidase-conjugated anti-mouse IgGs (1:25000, Jackson Immunoresearch, Interchim, Montluçon, France). Proteins antibody complexes were visualized using the enhanced chemiluminescence Western blotting detection system (ECL^+, Amersham, Orsay, France). The band densities were determined by scanning densitometry (Vilbert-Lournat, Marne la Vallée, France). Values were expressed as arbitrary units.

Histology

For determination of infarct volume, brains of anesthetized rats were transcardially perfused before removal as described above. The removed brains were frozen in isopentane at -40°C

and stored at -80°C. Coronal sections (50-µm thick) were cut in a cryostat (–13°C) at 200-µm interval, collected on slides and stained with Cresyl violet (0.4%). Injured cortical areas (unstained tissue) were measured using a computer image analysis system (Scion image, NIH, USA) and the distances between respective coronal sections were used to calculate a linear integration for the lesion volume.

For astroglia and microglia detection, brains of anesthetized rats were first transcardially perfused with saline and then with the fixative FAM (formol 37%, acetic acid 100%, methanol 1:1:8 volume) solution. The removed brains were postfixed in the same fixative for 1 week, dehydrated in ethanol, and embedded in paraffin. Microglial and astroglial activation was detected on deparaffinized and rehydrated coronal section (10-µm thick) including the lesion using isolectin B4 (ILB4) histochemistry and glial fibrillary acidic protein (GFAP) immunochemistry, respectively, and as described in detail earlier (Demougeot et al, 2003). Histologic examination was performed by two manipulators blind to the treatment.

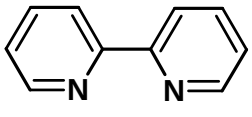
Glutathione assay

Glutathione loss in brain tissue (cytoplasmic and mitochondrial compartments) was used as an indirect marker of ROS production. Reduced and oxidized glutathione level (GSH and GSSG) was measured according to the recycling assay of Tietze (1969). Briefly, cortical samples were homogenized in water containing 5-sulfosalicylic acid (5%). After centrifugation (10,000 g, 5 min, 4°C), the supernatant was collected and stored (-30°C) until analysis. GSH and GSSG levels were determined by measuring the rate of formation of 5-thio-2-nitrobenzoate from 5-5'-dithio-bis-2-nitrobenzoate (Sigma) in the presence of nicotinamide adenine dinucleotide phosphate (Sigma) and glutathione reductase (Sigma). The reaction was performed at room temperature and followed spectrophotometrically at 410 nm. A standard

curve with known amounts of reduced glutathione (Sigma) was established and employed for estimating total glutathione content of each sample.

Treatment

2,2'-dipyridyl (2,2'-bipyridine, (1)) was used as a liposoluble iron chelator. 2,2'-dipyridyl (DP, Sigma) was dissolved in absolute ethanol (0.4 g/ml). The alcoholic solution was then diluted in NaCl 0.9 % (1/200). DP (20 mg/kg) was applied systemically (i.p., 10 ml/kg) before (15 min) and after (1h) brain ischemia induction. The effects of DP (treated rats) were compared to that of corresponding vehicle (untreated rats). The doses of DP used in this study were chosen on the basis of two previous experiments with this agent (Ikeda et al, 1989, Oubidar et al, 1994). The authors reported the efficacy of 20 mg/kg DP to reduce brain edema when given 15 min before and 1h after induction of cortical freezing or photothrombotic ischemic lesion.



(1)

Statistical analysis

The values were expressed as means ± SEM of n rats. Comparisons between two groups were performed using the unparametric Kruskal-Wallis test. For multiple comparaisons, the Kruskal-Wallis test was followed by the test of Mann-Whitney. Statistical significance was set at $p< 0.05$.

Results

Effect of DP on infarct volume and astromicroglial activation

Infarct volume and maximal infarct area are presented in Fig.1. DP treatment resulted in a significant decrease in histological damage. The reduction of infarct volume reached 42% at day 1 ($p<0.01$) and 25% at day 3 (NS) post-irradiation. This was accompanied with a 25% decrease in the maximal infarct area at days 1 ($p<0.01$) and 3 ($p<0.05$). Note that in DP-treated rats, infarct did not enlarge with time.

Astromicroglial activation at day 3 post-irradiation is illustrated in Fig.2. In both groups of rats, the cortical infarct was well-demarcated from the unlesioned tissue by a rim of ameboid microglial cells and no difference in ILB4 immunostaining was observed between groups (Fig. 2A, B). The fact that the thickness of the rim formed by ameboid microglial cells was not affected by DP suggests that DP did not interfere with the mechanisms involved in the transformation of resting microglia into phagocytic microglia. At this stage of ischemia, activated astrocytes were detected not only at the vicinity of the infarct (Fig. 2C, D) but also in remote ipsilateral regions and more especially in the parietal cortex in regard with the corpus callosum (Fig 2E, F). GFAP immunostaining was found less intense in treated than in untreated rats by two investigators blind of the treatment.

Dimensions of the ischemic lesion and cortical tissue sampling

In order to separately examine the biochemical events occurring in the ischemic core (unsalvageable tissue) and the penumbra (salvageable tissue), we determined the dimensions of the lesion 24h after ischemia in treated and untreated rats. In both groups of rats, the cortical lesion was truncated, cone-shaped, with ellipsoidal upper and bottom sections, the upper section being larger than the bottom section. The large and the small axis (mm) of the upper section were 6.5 ± 0.3 and 5.5 ± 0.2 in untreated rats and 5.0 ± 0.3 and 4.5 ± 0.2 in rats

treated with DP, respectively. For the bottom section, the values were 4.7 ± 0.2 and 4.0 ± 0.1 in treated rats and 3.1 ± 0.2 and 2.8 ± 0.2 in DP-treated rats. Three concentric pieces of cortical brain tissue (P1, P2 and P3) were then collected using punch with increasing internal diameter (2.8, 4.6 and 9.5 mm, respectively) as indicated in Fig.3. The first punch (P1) was centered on the infarct that was macroscopically readily observable (petechial hemorrhages). P1 corresponded to infarcted tissue both in untreated and DP-treated rats. P2 corresponded to penumbra i.e. infarcted tissue in untreated rats and tissue rescued from infarction in DP-treated rat. P3 corresponded to unlesioned tissue in two groups of rats. In certain experiments, the remaining cortex (P4 sample) was kept.

Effect of DP on ischemia-induced damage to endothelial and neuronal cells

DP treatment protected endothelial cells from ischemia. Indeed, the amount of extravasated Evans blue (µg/g proteins) at 24h post-irradiation was 142.6 ± 16.4 in untreated rats (n=9) and 84.1 ± 14.1 in rats treated with DP (n=7, -41%, $p<0.01$, data not shown). Neurons were also protected from ischemia by DP. As shown in Fig. 4, ischemia-induced NAA loss was significantly less severe in DP-treated rats than in untreated rats in P1(- 67% vs –47% after DP), P2 (-28% vs –13% after DP), and P3 (-13% vs no significant changes after DP). NAA content of P4 was not affected by ischemia.

Effect of DP on CBF changes induced by photothrombosis

The preischemic CBF expressed as tissue perfusion unit was 21.2 ± 0.4 and 21.0 ± 0.5 in untreated and treated rats, respectively (data not shown). The time course of CBF following photothrombosis is illustrated in Fig.5. In untreated rats, CBF gradually decreased from 50% to 33% of the preischemic values within the 15 min-2h interval following photothrombosis as previously reported by Kim et al (2000). In rats treated with DP, CBF was 49% of the

preischemic value at 15 min after photothrombosis initiation and remained close to this value thereafter. During CBF recording, mean arterial blood pressure was not different between the two groups of rats (data not shown).

Effect of DP on ischemia-induced ROS production

Glutathione levels in the cortex contralateral to irradiation was about 11-12 nmoles/mg of proteins in all examined regions (P1, P2, P3 and P4) both in treated and untreated ischemic rats (not shown). Such a value is within normal range (Lerouet et al, 2002). In untreated rat, ischemia resulted in oxidative stress as evidenced by the significant glutathione loss in P1 and P2 of the ischemic cortex as compared to contralateral corresponding values. Conversely, no difference was observed between the two sides for P3 and P4. Ischemia-induced glutathione loss was less in DP-treated rats than in untreated rats. Thus, as summarized in Fig.6, glutathione loss in ipsilateral P1, expressed as percentage of contralateral values, was 63.3 ± 5.2 in untreated rats and 44.3 ± 5.9 in rats treated with DP ($p<0.05$). The corresponding values for P2 were 29.4 ± 3.5 and 6.3 ± 1.1 ($p<0.01$).

Effect of DP on HIF-1α and HO-1 protein expression

Results are summarized in Fig. 7 and 8. In non-ischemic rats, HIF-1α and HO-1 protein expression was found to be below detectable threshold (not shown). In untreated rats, ischemia resulted in the induction of HIF-1α and HO-1 both in the (left) non-irradiated and the (right) irradiated cortex. In the left cortex, no difference in protein levels was observed among P1, P2 and P3 samples and the values for HO-1 and HIF-1α were pooled. As compared to these contralateral pooled values, ischemia caused an important increase in HIF-1α levels in P1 (x 5) and P2 (x 3) and did not change protein levels in P3. Conversely, HO-1 expression in the irradiated cortex was not different from that observed in the non-irradiated

cortex except in P1 sample where it was twice lower in the irradiated than in the non-irradiated cortex. Iron chelation therapy diminished the effects of ischemia on HIF-1α in all examined regions but selectively increased HO-1 levels in the infarct core (i.e. P1 sample of the irradiated cortex).

Discussion

By using 2,2'-dipyridyl (DP) as a liposoluble iron chelator and the photothrombotic occlusion of cortical vessels as a model of brain infarction, the present study is the first to give histological data to support a cytoprotective effect of iron chelation therapy in brain ischemia. The decreased infarct size consecutive to DP administration was associated with reduced ischemic damage to neuronal and endothelial cells. Interestingly, the cytoprotective effect of DP appears to go beyond the sole reduction of oxidative damage. Indeed, beside the reduction of ischemia-induced glutathione loss as a marker of decreased ROS production, DP treatment resulted in an amelioration of residual blood flow, and in significant changes in the expression of both HIF-1α and HO-1 protein expression.

After photothrombosis, the brain lesion grows and matures as a function of time spreading from the unrecoverable ischemic core to adjacent tissues which represent the salvageable penumbra (Lanens et al, 1995). The lesion reaches maximal size 24h after ischemia (Grome et al, 1988). At this time, the volume of infarcted tissue was reduced by 50% by DP treatment. Moreover, in DP-treated rats, the prolongation of ischemia from 24h to 3 days was not associated with an enlargement of infarct volume, indicating that acute treatment with DP did not delay but rather inhibited the deleterious mechanisms involved in the transformation of penumbra into infarct. In addition, the decreased infarct volume was associated with the preservation of the neuronal marker NAA both in the ischemic core and the penumbra, and also with decreased cortical Evans blue extravasation. These data strongly suggest that increased resistance to ischemia of neurons and blood brain barrier (BBB) are involved in the prevention of infarct growth by iron chelation therapy. One cautionary note is that the attenuated disruption of the BBB in DP-treated rats may be only reflection of

decreased infarct volume. Nevertheless, in the photothrombotic ischemic stroke model, BBB disruption is the preceding event in the ischemic lesion. In this model, vascular occlusion results from the production of singlet oxygen that is reactive with endothelial cells causing very rapid (within 2 min) platelet aggregation (Dietrich et al, 1987). Importantly, DP did not interfere with the platelet response to endothelium damage since DP failed to alleviate the initial perfusion deficit. On the contrary, DP treatment was found to reduce the secondary perfusion deficit. Given that DP was previously demonstrated to prevent edema consecutive to brain ischemia (Oubidar et al, 1994) and that edema by mechanical occlusion of patents vessels is suspected to reduce blood perfusion, decreased vasogenic edema may account for the delayed amelioration of residual blood flow observed after DP treatment. However, it is unlikely that reduced infarct volume consecutive to DP treatment involved a decrease in vasogenic edema. Indeed, whereas the osmotic agent glycerol suppressed edema consecutive to focal ischemia, it failed to reduce infarct volume (Aoki et al, 2001).

The prevention of the participation of iron in ROS formation is suspected to be an important mechanism by which liposoluble iron chelators mitigate cell damage resulting from pathological conditions associated with oxidative stress such as ischemia/reperfusion to heart (Horwitz et al, 1998) and neurodegenerative diseases (Shachar et al, 2004). In our study, ROS formation by ischemic brain was evaluated by measuring glutathione concentrations. In normal conditions, glutathione is present mainly in a reduced form (GSH), while the principal oxidized form GSSG is detected at low levels. GSSG which results from the reaction of GSH with peroxide in the presence of glutathione peroxidase is reduced back to GSH with the consumption of NADPH as electron donor by means of glutathione reductase. During ischemia, the reaction of GSH with superoxide anion, hydroxyl radical and also nitric oxide results in the formation of oxidation products which, conversely to GSSG cannot regenerate

GSH (Camera and Picardo, 2002). Thus, a decrease in tissue total glutathione (GSH and GSSG) concentration is considered as a robust marker of oxidative stress and ROS production. Our results showing glutathione loss both in the ischemic core and penumbra confirm previous studies in which these two regions were reported to produce ROS (Lancelot et al, 1995, Peters et al, 1998) and to exhibit oxidative damage (Imai et al, 2003). The effect of ischemia on glutathione concentrations was reduced by DP treatment, thus giving experimental evidence for the role of free iron in ROS production by ischemic brain. Moreover, beside the preservation of glutathione concentrations, DP treatment was associated with reduced astroglial response to ischemia. This is an additive argument for the *in vivo* antioxidant properties of DP. Indeed, antioxidants such as resveratrol and lipid peroxidation inhibitor were previously reported to decrease brain ischemia-induced astrocytic activation (Oostveen et al, 1998, Wang et al, 2002). Overall data suggest that a mechanism of the cytoprotective action of iron chelator in brain ischemia is attenuation of ROS formation. Decreased ROS formation may account for the neuroprotective effect of DP through different ways including decreased oxidative damage, blockade of apoptotic processes (Kim et al, 2000), and inhibition of inflammatory cytokines signaling (Zhang and Frei, 2003).

In the present study, we also investigated to what extent HO-1 can be involved in the cytoprotective effects of iron chelation therapy against brain infarction. First, we evaluated the effects of brain ischemia on HO-1 expression as well as on HIF-1α, a transcription factor involved in the control of the gene encoding HO-1. Our results showing upregulation of these two proteins in the cortex ipsilateral to ischemia are in accordance with recent studies in which brain was reported to express high levels of HIF-1α mRNA (Bergeron et al, 1999, Marti et al, 2000) and HO-1 protein (Panahian et al, 1999, Bidmon et al, 2001) after permanent ischemia. An interesting result of the present study is the difference in the spatial

profile of protein expression between HIF-1α and HO-1. Whereas HIF-1α showed a gradual decreasing expression from the ischemic core to more distant regions, HO-1 protein expression was found to be lower in the ischemic core than in the other regions which express equivalent amount of HO-1 protein. Such a "mismatch" between HIF-1α and HO-1 protein expression suggests that other transcription factors than HIF-1 are involved in the induction of HO-1 after brain ischemia. Then, we examined for the first time the impact of DP on ischemia-induced changes in HIF-1α and HO-1 protein expression. Our results reported that DP dramatically decreased HIF-1α content in all examined cortical regions (ipsilateral and contralateral) but selectively increased HO-1 content of the ischemic core. The inhibition by DP of ischemia-induced HIF-1α expression may be secondary to decreased ROS production (Haddad and Land, 2001) and/or to the amelioration of residual blood flow and subsequent less severe hypoxia. Moreover, the opposite effects of DP on HO-1 and HIF-1α protein expression confirm that HIF-independent mechanisms prevail over HIF-dependent mechanisms in HO-1 induction after brain ischemia. Whereas oxidative stress is presently fully accepted as an HO-1 inducer, HO-1 expression is largely dependent on the severity of oxidative stress. We recently showed that contrary to mild oxidative stress which induced a time-dependent increase in both mRNA HO-1 and protein, moderate oxidative stress caused a transient early reduction of HO-1 mRNA accompanied by a deep and sustained loss of HO-1 protein (Méthy et al, 2004). In accordance with an inhibitory effect of severe oxidative stress on HO-1 induction are our results showing that HO-1 expression was lower in the region where glutathione loss was maximal (i.e. the ischemic core) than in the region where it was less severe (i.e. the penumbra). In fact, when too sustained, oxidative stress certainly limits the expression of HO-1 protein as a result of transcriptional downregulation and/or protein degradation. Thus, in regions where oxidative stress is not compatible with the full expression of HO-1, as in the ischemic core but not in the penumbra, antioxidants may increase HO-1

protein content. This could explain the selective effect of DP on the HO-1 content of the ischemic core.

There are increasing evidence for a beneficial role of HO-1 in ischemic diseases. Both carbon monoxide (a vasodilatory and antiapoptotic agent as well as a derepressor of the fibrinolytic axis) and bilirubin (a potent antioxidant), the two by-products of HO-1 are involved in the resistance of cells to ischemic insults (Clark et al, 2000, Fujita et al, 2001, Vulapalli et al, 2002, Akamatsu et al, 2004). In our study, the reduction of infarct volume by DP was associated with increased HO-1 expression in the ischemic core but not in the penumbra, thus questioning the implication of HO-1 in the cytoprotective effect of DP. However, since carbon monoxide and bilirubin are liposoluble, they can diffuse from the ischemic core to the penumbra thereby preventing infarct growth. Alternatively, the enhancement in HO-1 expression in cells of the ischemic core after DP treatment may increase their tolerance to ischemia thereby delaying the release of cytotoxic compounds by damaged cells. Such a paradigm would allow the penumbra to fully develop its defense mechanisms against infarction.

In summary, by using the clinically relevant photothrombotic ischemic stroke model, the liposoluble iron chelator DP was found to reduce infarct volume and ROS production. Decreases in oxidative damage to neurons and endothelial cells as well as exacerbation of HO-1 expression may be involved in the cytoprotective effects of DP in brain ischemia.

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Footnotes

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Legends for figures

Fig.1. Effect of 2,2'-dipyridyl on infarct volume (mm^3) and maximal infarct area (mm^2).

2,2'-dipyridyl (20 mg/kg, i.p.) was given 15 min before and 1h after photothrombosis. Bars are means ± SEM from 5-6 rats.

*p<0.05, ** p<0.01 vs. untreated rats

Fig.2. Microglial and astroglial activation induced by photothrombosis.

The left panel of micrographs corresponds to untreated ischemic rats and the right panel to rats treated with 2,2'-dipyridyl (20 mg/kg, i.p.,15 min before and 1h after photothrombosis). Five rats were examined in each group. Sections were stained with isolectin B4 (A and B) and glial fibrillary acidic protein (C to F) to visualize microglia and astrocytes, respectively. Then, they were counterstained with hematoxylin. The rim of microglial cells surrounding the infarcted area was found similar between the two groups of rats (A and B, see arrow). Astroglial activation was visible outside and close to the infarcted area (C and D) as well as in the parietal cortex (E and F) and was found in both cases of a lesser extent in treated rats.

Scale bars = 400µm (A and B), 100 µm (C to F).

Fig. 3. Three concentric cortical samples (P1, P2 and P3) were collected using punches with increasing internal diameter (2.8 mm, 4.6 mm, 9.5 mm, respectively). P1 corresponded to the ischemic core (unsalvageable tissue), P2 to the penumbra (salvageable tissue) and P3 to unlesioned tissue. P4 corresponded to the remaining cortex. P1 was centered on the infarct that was macroscopically readily observable (petechial hemorrhages).

Fig. 4. Effect of 2,2'-dipyridyl on photothrombosis-induced NAA loss.

NAA levels were measured before ischemia and at day 1 after ischemia in the irradiated cortex. Ischemic rats received 20 mg/kg of 2,2'-dipyridyl (treated rats) or vehicle (untreated rats) 15 min before and 1h after photothrombosis. P1, P2, P3 and P4 samples were collected as indicated in Fig. 3. Bars are means ± SEM. n = number of rats.

* $p<0.05$, ** $p<0.01$, [a] vs. before ischemia, [b] vs. untreated rats

Fig. 5. Effect of 2,2'-dipyridyl on the time course of CBF following photothrombosis.

Values are expressed as percentage of preischemic contralateral CBF which was recorded before treatment initiation. 2,2'-dipyridyl (20 mg/kg, i.p.) or vehicle was given 15 min before and 1h after photothrombosis. Values are expressed as means ± SEM. n = number of rats.

* $p<0.05$ vs. untreated rats

Fig. 6. Effect of 2,2'-dipyridyl on photothrombosis-induced glutathione loss.

Glutathione levels were measured in cortex contralateral and ipsilateral to irradiation. Bars show glutathione loss in P1 and P2 samples expressed as percentage of contralateral corresponding values. 2,2'-dipyridyl (20 mg/kg, i.p.) or vehicle was given 15 min before and 1h after photothrombosis. Bars are means ± SEM. n = number of rats. See Fig. 3 for details of tissue sampling.

*$p<0.05$, **$p<0.01$ vs. untreated rats.

Fig. 7. Effect of 2,2'-dipyridyl on photothrombosis-induced changes in HIF-1α.

HIF-1α protein expression was examined in P1, P2, P3 samples collected from the lesioned cortex (right cortex) and the unlesioned contralateral cortex (left cortex) on day 1 after photothrombosis. The values in the left cortex were pooled. 2,2'-dipyridyl (20 mg/kg) or

vehicle was injected (i.p.) 15 min before and 1h after photothrombosis initiation. Bars are means ± SEM. n = number of rats.

*p<0.05, **p<0.01, [a] vs. left cortex, [b] vs. untreated rats.

Fig. 8. Effect of 2,2'-dipyridyl on photothrombosis-induced changes in HO-1.

See Fig. 7 for legend. *p<0.05, ***p<0.001, [a] vs. left cortex, [b] vs. untreated rats.

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Figure 1

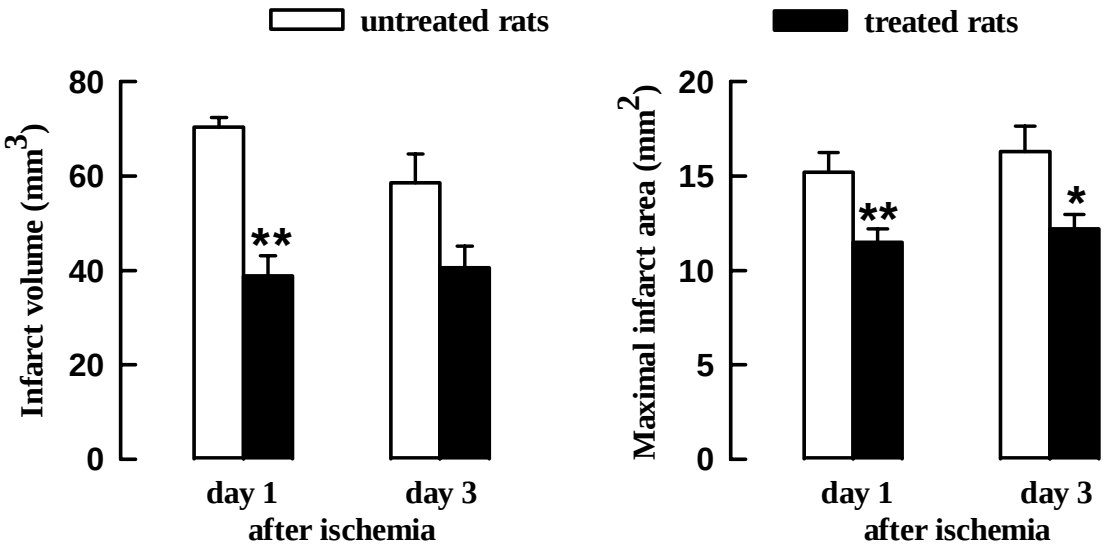

Figure 2

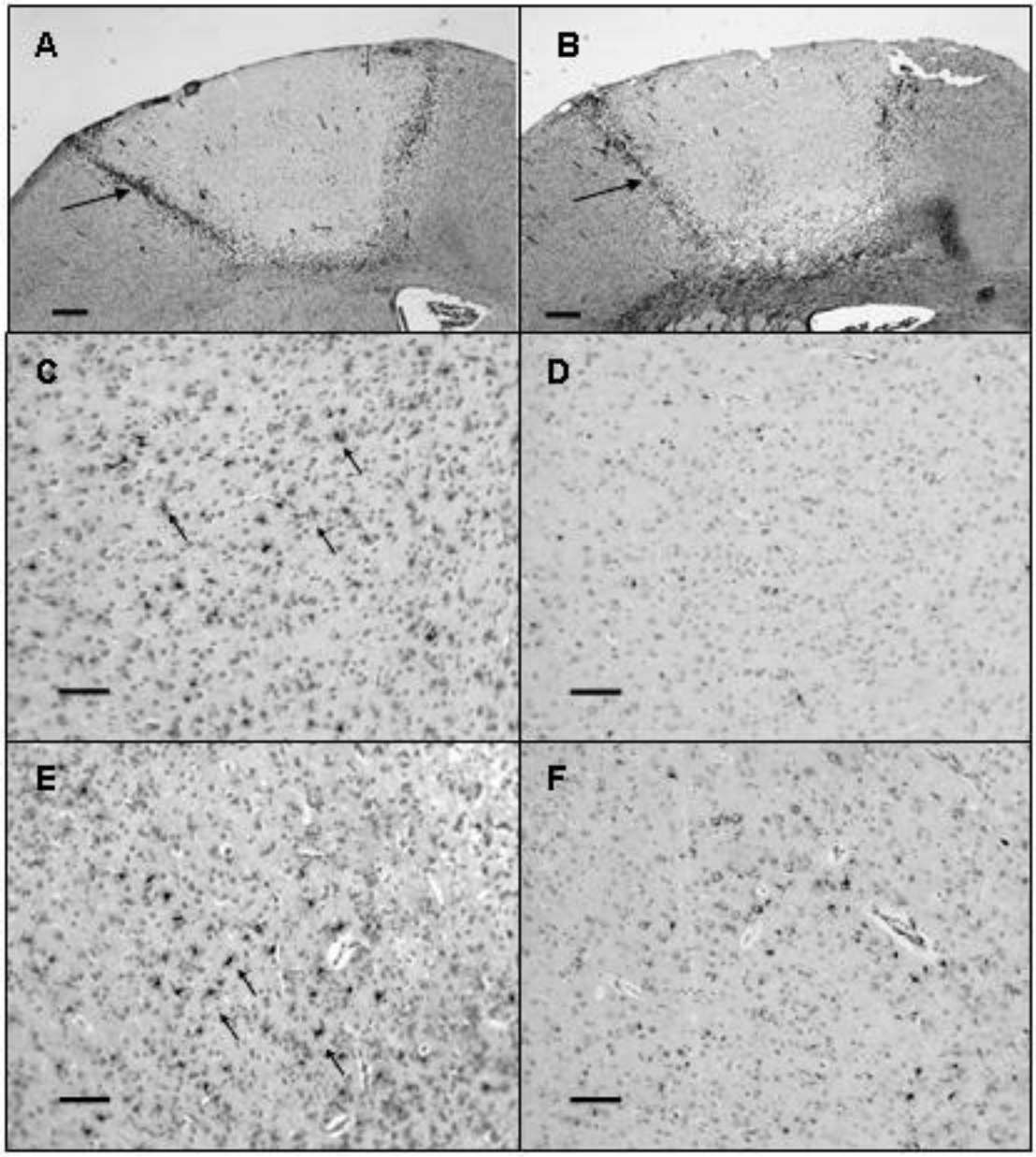

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Figure 3

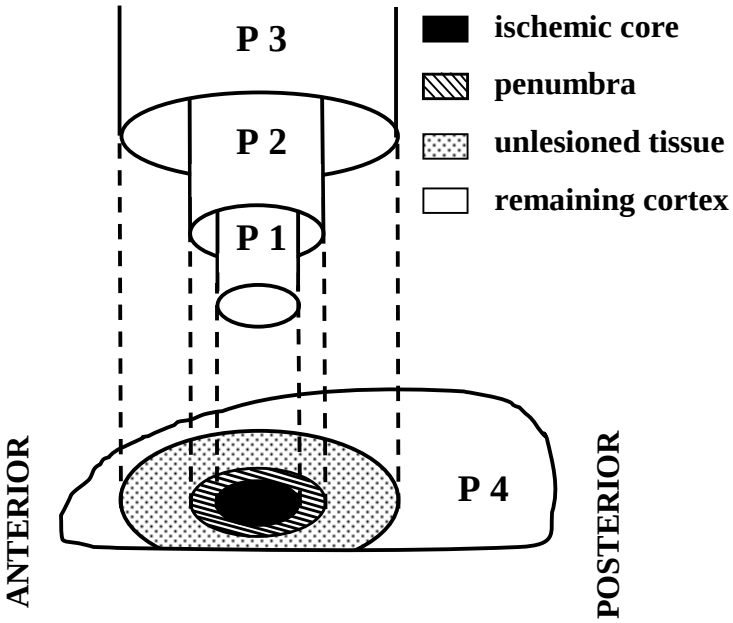

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Figure 4

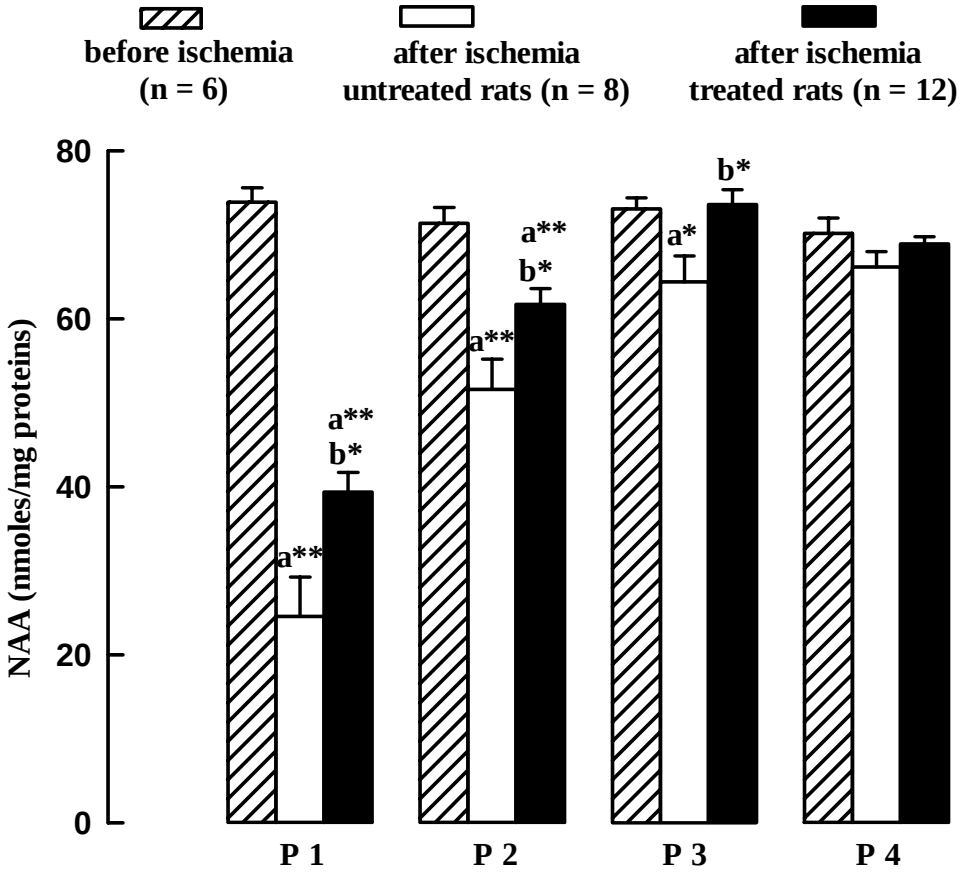

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Figure 5

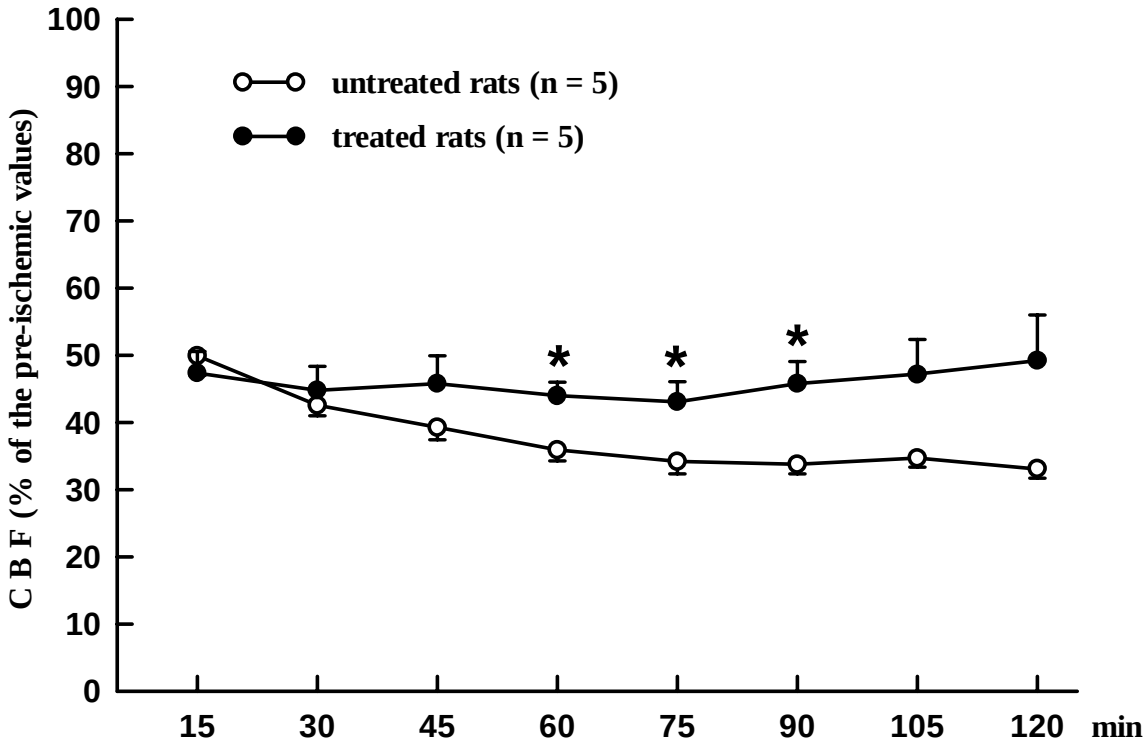

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Figure 6

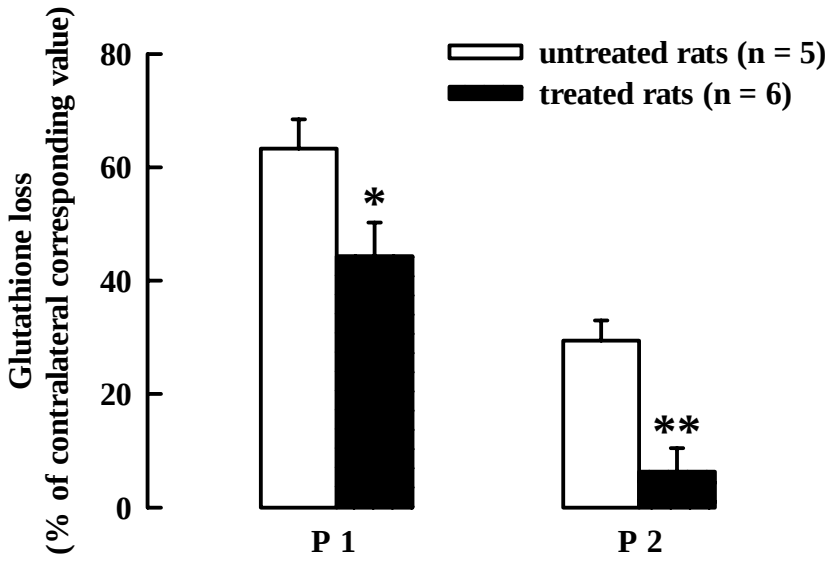

Figure 7

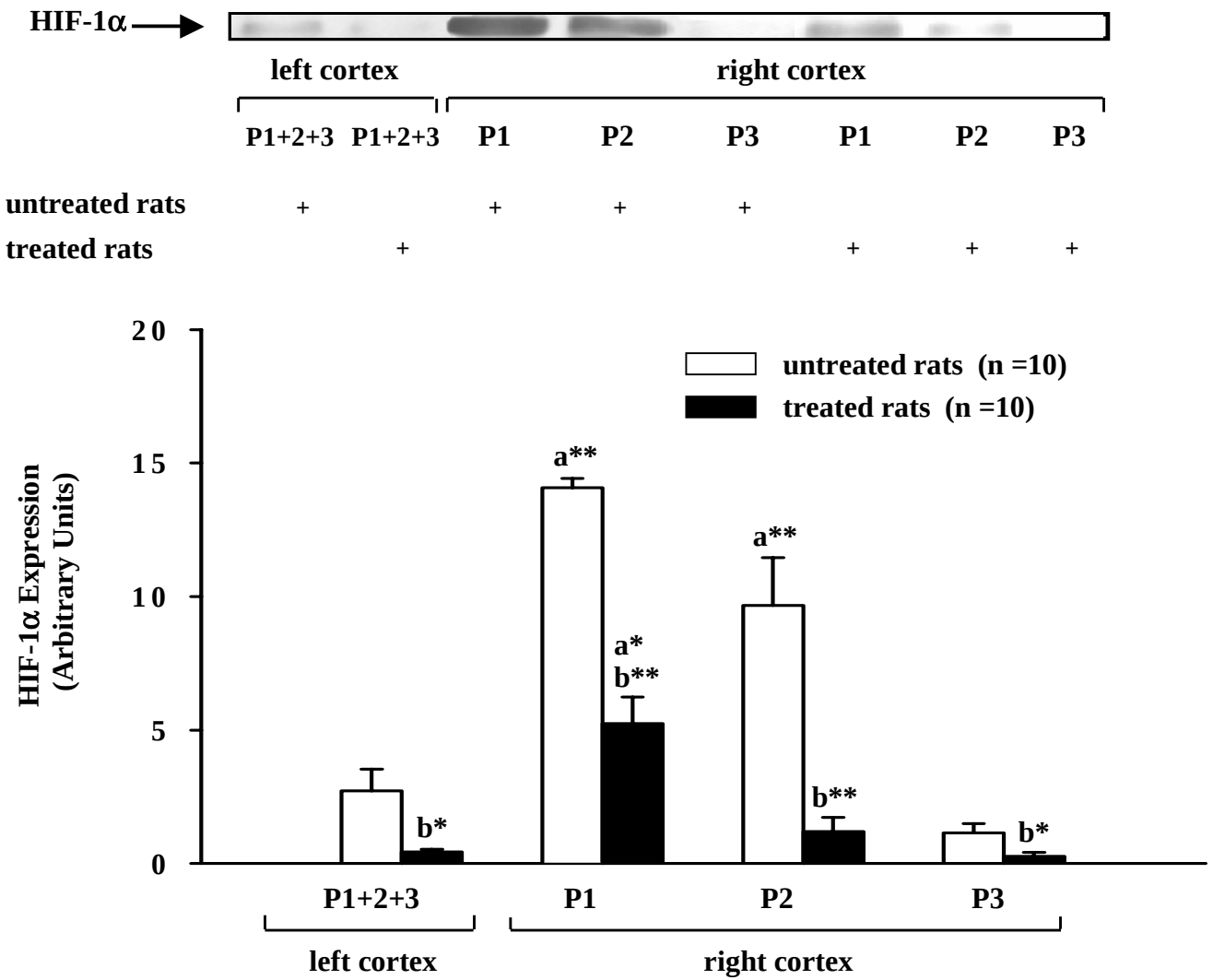

Figure 8