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Pentoxifylline prevents spontaneous brain ischemia in stroke-prone rats

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ABSTRACT

Anti-inflammatory properties of pentoxifylline (PTX) have recently been described. Spontaneously hypertensive stroke-prone rats (SHRSP) constitute an animal model which develops an inflammatory condition that precedes the appearance of brain abnormalities. The aim of the present investigation was to assess (a) the efficacy of PTX treatment in protecting the neural system in SHRSP and (b) how its anti-inflammatory properties might be involved in this effect. Male SHRSP fed with a permissive diet received no drug or PTX (100 or 200 mg/kg/day). Brain abnormalities detected by magnetic resonance imaging developed spontaneously in control rats after 42 ± 3 days, whereas in rats treated with 100 mg/kg/day PTX, abnormalities developed in only 80% of the animals, and only after 70–80 days. Treatment with a higher dose of PTX (200 mg/kg/day) completely protected the brain from abnormal development. The drug treatment prevented the accumulation of macrophages or $CD4^+$ positive cells, the activation of glia in brain tissues, and the appearance of inflammatory proteins and thiobarbituric acid-reactive substances in body fluids. PTX treatment did induce a greater increase of serum TNF-alpha, but not of IL-1beta and IL-6 induced by *in vivo* administration of lipopolysaccharide (LPS), which suggests a protective role for TNF-alpha. PTX also exerted protective effects when it was administered after the first occurrence of proteinuria (>40 mg/day). These data indicate that PTX treatment dose-dependently prevents the occurrence of spontaneous brain damage by reducing inflammatory events. We also hypothesize that the increase of TNF-alpha by PTX treatment represents a protective mechanism in SHRSP.

Pentoxifylline (PTX), a methylxanthine derivative and non-specific type 4 phosphodiesterase inhibitor, is a drug widely used in the management of peripheral arterial disease and in particular for intermittent claudication (Creager, 2001; Labs et al., 1997). The mechanism underlying its beneficial effects appears to be related to the improvement of cellular functions and modifications in the plasma that improve microcirculatory perfusion in both peripheral and cerebral vascular beds (Seiffge, 1997; Windmeier and Gressner, 1997).

In recent years, *in vitro* and *in vivo* experiments indicated an additional therapeutic potential for PTX as an anti-inflammatory and immunomodulator agent (Teixeira et al., 1997; Haddad et al., 2002; Laurat et al., 2001). This newly described pharmacological effect can be ascribed to an inhibition of the functional responses of circulating mononuclear phagocytes, neutrophils and T lymphocytes and the decreased synthesis of several pro-inflammatory cytokines (Laurat et al., 2001; Neuner et al., 1997; Bahra et al., 2001; Dong et al., 1997; Marcinkiewicz et al., 2000; Samardzic et al., 2001). Studies of the anti-inflammatory effects of PTX *in vivo* have focused attention on tissue injury after ischemia. Thus, *in vivo* PTX treatment reduced ischemia-reperfusion injury in the lung (Thabut et al., 2001), intestine (Sener et al., 2001), liver (Iwamoto et al., 2002), kidney (Kim et al., 2001), spinal cord (Savas et al., 2002), and brain of different animal species including rats, mice, and dogs (Eun et al., 2000; Sirin et al., 1998; Toung et al., 1994). Inflammatory processes accompany tissue injury regardless of the organ system involved, and chronic inflammation may predispose to ischemia in peripheral organs and to brain damage (Lindsberg and Grau, 2003).

We have previously reported (Sironi et al., 2001; Guerrini et al., 2002) that spontaneously hypertensive stroke-prone rats (SHRSP) subjected to salt loading

develop an inflammatory condition characterized by the accumulation of acute-phase proteins in plasma and urine, and that this event precedes and predicts the appearance of brain abnormalities. This seems to be a suitable experimental model to explore if the effect of PTX on tissue damage depends on anti-inflammatory properties. This study was designed (1) to evaluate the neuroprotective effect of PTX in salt-loaded SHRSP and (2) to understand whether its anti-inflammatory properties are involved. We used the salt-loaded SHRP, a spontaneous model of cerebral damage, and measured the onset and development of brain damage and increase in survival time after PTX treatment in either prophylactic or rescue mode. We found that both regimens delay brain damage and attenuate the associated inflammatory responses.

Methods

Animal model of brain damage

Male SHRSP (8-10 weeks), purchased from Charles River (Calco, Como, Italy), were fed a permissive diet containing 18.7% protein, 0.63% potassium and 0.37% sodium (Laboratorio Dr. Piccioni, Italy), and 1% NaCl in drinking water *ad libitum* (salt-loaded). Their food and liquid intakes were measured weekly, as was proteinuria. T2-weighted MRI (T2W-MRI) assessments were repeated every other day in rats after 24-hour proteinuria exceeded 40 mg/day, and daily after a brain abnormality had been detected (Sironi, 2001). Three days after brain damage was detected, the rats were anesthetized with chloral hydrate (400 mg/kg ip) and the brain was removed for immunohistochemical analysis. At the same time PTX-treated rats with no sign of brain abnormalities were also sacrificed. Procedures involving animals and their care were in accordance with the Guide for the Care and Use of Laboratory Animals.

Drug treatment

In each experiment animals were matched for age and weight and randomly divided into the different treatment groups. Pentoxifylline (PTX; 3,7 dimethyl-1-(5-oxohexyl) xanthine), obtained from Sigma (St. Louis, CO), was dissolved daily in a small amount of drinking water to achieve doses of 100 (n = 12) or 200 mg/kg/day (n = 12). Each rat was kept in a separate cage and an operator ensured that each animal drank the entire drug solution daily, after which the animal had free access to 1% NaCl. Control rats (n = 12) received the vehicle in the same way as PTX.

Drug treatments were started simultaneously with the permissive diet in the prophylactic experiment and soon after proteinuria exceeded 40 mg/day in the rescue therapy. Rats were weighed weekly and their arterial blood pressure was measured, then housed individually in metabolic cages for 24 h and urine was collected for measurement of proteinuria and for proteomic studies.

MRI evaluations of brain damage

The rats were anesthetized with 2% isofluorane in 70% N_2 / 30% O_2, fixed to an animal holder by means of a rod beneath the teeth, and placed inside the magnet (4.7T, vertical 15-cm bore) of a Bruker spectrometer (AMX3 with micro-imaging accessory). A 6.4-cm diameter birdcage coil was used for the imaging. A T2 multi-slice image was obtained after a 3-orthogonal plane gradient echo scout. Sixteen contiguous 1-mm thick slices were analyzed caudally to the olfactory bulb using a field of view (FOV) of 4 x 4 cm^2. A turbo spin echo sequence was used with 16 echoes per excitation, 10 ms inter-echo time, 85 ms equivalent echo time, and 4 s repetition time. The images were 128 x 128 points (zero filled to 256 x 256), and eight images were averaged in 8'30". The occurrence of lesions was identified as the presence of areas of high signal intensity on T2-weighted MRI.

Immunohistochemistry of brain tissue

For histological examination, the brains were fixed in Carnoy reagent (Merck, Darmstadt, D) and embedded in Paraplast (Sigma, St.Louis, CO), and coronal sections (5 µm) were stained with hematoxylin/eosin and examined by light microscopy. For immunohistochemical studies paraffin-embedded brain coronal sections were dewaxed in xylene and dehydrated. Endogenous peroxidase was blocked by adding 1% H_2O_2 in 50% methanol. Nonspecific binding sites were saturated with goat serum. The sections were incubated overnight at 4°C with the primary antibodies, then with biotinylated secondary antibodies and streptavidine peroxidase (LSAB2 kit, DAKO, Glostrup, DK). HRP was detected with H_2O_2 and diaminobenzidine (Sigma, St.Louis, CO). The primary antibodies used were: anti-CD4 which react with T helper cells (1:10; Cymbus Biotechnologies,UK), anti-ED1 which react with myeloid cells (1:20;Serotec, Oxford, UK) and anti-ED2 which react with resident macrophages (1:20; Serotec, Oxford, UK). Microglia were specifically visualized by lectin histochemistry using peroxidase-labeled isolectin B_4 from *Griffonia simplicifolia* seeds (Sigma, St Louis, CO).

Myeloperoxidase (MPO) tissue activity

MPO activity was measured by a modification of the Hillegass technique (Hillegass et al., 1990). Briefly, 0.1 g of rat brain tissue was homogenized in 4 ml of 50 mM potassium phosphate buffer, pH 6.0 and centrifuged at 16,000 rpm for 30 min at 4°C. The pellet was resuspended in the same buffer with 0.5% hexadecyltrimethylammonium bromide. The samples were alternately frozen and thawed three times and sonicated between cycles before another centrifugation (16,000 rpm at 4°C). For the measurement, 0.1 ml of supernatant was mixed with 0.1 ml tetramethylbenzidine 16 mM in dimethylsulfoxide and 0.8 ml H_2O_2 0.3 mM.

Absorbance was measured at 655 nm for 5 min. The MPO activity values were expressed as percentage change in OD per minute per gram over tissues from that in non-salt-loaded rats (100%).

Determination of urinary thiobarbituric-acid-reacting substances (TBARS)

TBARS were measured by the method of Valenzuela, 1991. Briefly, 500 µL of urine was combined with 500 µL of a 0.67% thiobarbituric acid (TBA) aqueous solution and 500 µL of 20% trichloroacetic acid solution. The samples were vortexed and incubated at 100°C for 1h, and the absorbance at 532 nm was measured spectrophotometrically. The quantity of TBARS is proportional to the amount of malondialdheide (MDA), a lipid peroxidation product generated by the oxidation of membrane lipids by reactive oxygen species. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient $\varepsilon = 1.56 \times 10^5$ cm^{-1} M^{-1}) and expressed as % increase over that in non-salt-loaded rat urine.

Lipopolysaccharide induction of serum TNF-α, IL-1β and IL-6

SHRSPs subjected to the permissive diet were not treated (n=8) or treated with PTX (200 mg/kg/day; n=8). Three days after detection of brain abnormalities in control rats, all the animals were injected with LPS (dissolved in sterile pyrogen-free saline at 5 mg/kg) to trigger the formation of inflammatory cytokines. Three hours later, blood samples were collected, serum was prepared by centrifugation at 1200×*g* for 10 min at 4°C and stored at –80°C until assay. TNF-α, IL-1β and IL-6 levels were determined using an ELISA kit (NIBSC, Hertfordshire, UK) following the manufacturer's instructions.

Proteomic studies

Urine proteins were concentrated by trichloroacetic acid–acetone precipitation. One-dimensional electrophoresis was run on urine proteins in the presence of SDS, without sample reduction, in a discontinuous buffer system on a 4–20% polyacrylamide gradient. The sample load was 3.75 µg per lane. Proteins were stained with 0.3% w/v Coomassie and the protein patterns were digitalized with a scanner.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were computed by ANOVA for repeated measurements, followed by Bonferroni's post-hoc test. $p<0.05$ was taken as statistically significant.

Results

Effects of pentoxifylline on physiological parameters

Body weight increased similarly in the three groups of salt-loaded SHRSPs up to the sixth week of treatment (Table 1). After that, body weight decreased in control rats only. In PTX-treated animals the growth curves constantly increased over the entire experimental period (not shown). The severe hypertension that developed in salt-loaded SHRSPs was not influenced by either dose of PTX used (Table 1).

Effects of pentoxifylline on proteinuria and inflammatory proteins in urine

In control rats proteinuria progressively increased, and rose sharply after 4 weeks of salt loading, reaching a maximum of 166 ± 31.7 mg/day on the 42nd day of treatment. At either dose of PTX, proteinuria was lower than in the untreated animals. The lower dose (n = 12) resulted in a delayed rise in proteinuria (not shown) while the higher dose (n = 12) virtually prevented the loss of protein (Figure 1A). The mixture of proteins excreted by control SHRSP and by those given 200 mg/kg/day of PTX, was

identified by running the urine on one-dimensional gels (example in Figure 1B**)**. The appearance of high molecular weight proteins, markers of an inflammatory response in control animals, were dramatically delayed in the drug-treated rats.

Effects of pentoxifylline on the appearance of brain damage

All control rats (n = 12) developed brain abnormalities after 42 ± 3 days of permissive diet, whereas in the group treated with PTX (100 mg/kg/day, n = 12), the abnormalities developed in 80% of the animals after 70–80 days. Treatment with the higher dose of PTX (n = 12) completely protected the brain (animals free from MRI-detectable lesions after 4 months) (Figure 2).

Effects of pentoxifylline on the appearance of inflammatory cells in the brain

In control rats inflammatory cells were found in the damaged brain hemisphere 3 days after MRI first detected the damage. ED1- and ED2-positive macrophages, $CD4^+$ T-lymphocytes and activated microglia (isolectin B4 positive) accumulated significantly (Figure 3). ED2-positive cells localized preferentially around the vessels, while ED1-positive cells, $CD4^+$ T lymphocytes and activated microglia were spread over the whole damage tissue. Conversely, in PTX treated animals sacrificed at the same time as the controls no inflammatory, immunocompetent or activated cells could be detected (Figure 3). In brain homogenates of control rats we documented increased MPO activity (188 ± 27 % vs. non-salt-loaded SHRSP), an index of accumulation of polymorphonuclear neutrophils; this was not attenuated by PTX treatment (218 ± 16 % vs. non-salt-loaded SHRSP).

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Effects of pentoxifylline on inflammatory parameters

Three hours after *in vivo* administration of LPS, the induction of TNF-α was significantly greater in PTX-treated rats (n = 8) than in controls (n = 8) (Figure 4). However, the induction of IL-1β and IL-6 was not affected by PTX treatment (Figure 4). The urinary increase in TBARS found in control rats after the detection of damage (257 ± 12.2 % vs. non-salt-loaded SHRSP) was almost nullified by PTX treatment (114 ± 24.7 % vs. non-salt-loaded SHRSP).

Effects of pentoxifylline administered after increase of proteinuria

To determine whether the PTX reversed the increase in proteinuria, SHRSPs subjected to salt loading and permissive diet (n=10) were treated with drug (200 mg / kg / day; n = 10) after >40 mg protein/day were lost in the urine. This treatment also protected the animals from loss in weight and any further increase in proteinuria and from the development of MRI-detectable brain lesions (not shown).

Discussion

We have shown here that prophylactic treatment with PTX delays, in a dose-dependent manner, the onset and development of brain damage and increases survival in salt-loaded SHRSP. These effects are independent of blood pressure and are linked to a mitigation of the local and systemic inflammatory responses always associated with brain damage in this animal model. Similar results were obtained when PTX administration was delayed.

We have reported earlier that the gray matter of rat brain that develops damage after salt loading is markedly spongy, with loss of neurons, accumulation of astrocytes, and deposition of fibrinoid-eosinophilic material (Sironi et al., 2001; Guerrini et al., 2002; Sironi et al., 2003). Now we have shown a local response, close to the brain-damaged area of salt-loaded SHRSP, with the accumulation of inflammatory as well as immunocompetent cells. Resident macrophages (ED2) in the damaged area may be activated by proinflammatory cytokines, which in turn may alter the biochemical and physical properties of the blood–brain barrier (BBB). Circulating macrophages (ED1) migrate through the vasculature of the impaired BBB and surround the damaged tissue, thus favoring local progression of the ischemic process. $CD4^+$ T lymphocytes and neutrophils are also recruited into the inflammatory brain area. PTX treatment suppressed recruitment within the brain of inflammatory cells, with the exception of polymorphonuclear neutrophils, and maintained thiobarbituric acid-reactive substances at their normal level.

Several complementary anti-inflammatory effects of PTX could be responsible for its neuroprotective effect. One possible mechanism is the inhibition of cellular functions involved in inflammatory and immune diseases: inhibition of adhesion and activation of peripheral blood T-lymphocytes, suppression of T-cell proliferation and

differentiation, and inhibition of polarization and migration of human leukocytes and neutrophils (Gonzalez-Amaro et al., 1998; Dominguez-Jimenez et al., 2002; Laurat et al., 2001; Dong et al., 1997). A second neuroprotective mechanism of PTX probably includes the inhibition of the generation of oxygen radicals. Oxidative stress may damage different tissues such as the brain and the kidney in SHRSP (Tanito et al., 2004). That PTX protects against lipid peroxidation both in *in vitro* and *in vivo* models of ischemia has been documented (Bhat and Madyastha, 2001).

Lipopolysaccharide injection induced higher levels of TNF-α (but not of IL-1β or IL-6) in PTX-treated rats than in controls. TNF-α has been implicated in brain damage, but also in neuroprotective effects in stroke (Hallenbeck, 2002) and several recent studies postulate an important role for TNF-α in ischemic preconditioning (Ginis et al., 2002). Ischemic tolerance associated with increased plasma levels of TNF-α has recently been reported in human stroke (Castillo et al., 2003). On this basis we hypothesize that the propensity of PTX-treated rats to produce TNF-α may represent a protective mechanism.

The appearance of brain damage in salt loaded SHRSP is always preceded by proteinuria and the accumulation of several inflammatory markers in plasma and urine (Sironi et al., 2001). Most of the acute phase proteins (transferrin, hemopexin, albumin and thiostatin) found in serum are also detected in urine, and their levels increase over time until stroke occurs; the total amount and the composition of proteins in the urine changes during the 2 weeks preceding an ischemic event (Sironi et al., 2001). We therefore tried treating the salt-loaded SHRSPs with PTX immediately *after* the appearance of inflammatory proteins in the urine. Even this delayed administration protected the animals from the development of brain damage as well as from the linear increase of urinary protein, which suggests that PTX may

"cure or reverse" the kidney abnormalities reported in this animal model (Abumiya et al., 1996). Indeed, a nephroprotective effect of PTX in experimentally induced renal failure (Kim et al., 2001), and an antiproteinuric effect in patients with nephropathy (Ducloux et al., 2001) has been reported.

Methylxanthine derivatives are of potential interest in the treatment of vascular diseases in general and of stroke in particular because of their vasodilating properties (Kruuse et al., 2000). PTX has been reported to increase peripheral blood flow and walking distance in those with arterial disease (Creager, 2001): results on the effect of PTX on cerebral blood flow are not consistent; the discrepancies could be ascribed either to the different dosages or routes of administration (Kruuse et al., 2000). Preclinical studies suggest that pentoxifylline and propentofylline reduce neural damage following ischemia (Labs et al., 1997; Teixeira et al., 1997). Similarly, uncontrolled open trials have suggested that PTX may improve stroke outcome in humans (Bath et al., 2000). A recent meta-analysis that included five trials with methylxanthines administered either intravenously or orally found insufficient evidence to assess the effectiveness and safety of methylxanthines after acute ischemic stroke (Bath et al., 2000). However, a collaborative group is being formed to assess further the effects of methylxanthine derivatives in stroke patients in view of their multiple pharmacological properties as anti-inflammatories, inhibitors of free radical production, neuroprotectors, vasodilators and antiplatelet agents (Bath et al., 2000).

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Legends for figures

Figure 1. Effects of pentoxifylline on urinary proteins in SHRSP

Panel A: Daily proteinuria as a function of the duration of salt loading, for control SHRSPs ? or 200 mg/kg/day pentoxifylline ¦ (**p<0.01). Panel B: representative one-dimensional electrophoresis of urinary proteins, collected weekly, from a control SHRSP (left) or one receiving 200 mg/kg/day pentoxifylline (right). Samples of 3.75 µg from a 24-h collection from a metabolic cage were loaded per lane. Gels shown are representative of results obtained in four rats. MUP = major urinary protein; SA = albumin; Tf = transferrin.

Figure 2. Effects of pentoxifylline on the appearance of brain damage in SHRSP

Event-free survival as a function of the duration of salt loading for control SHRSPs (n = 12) ? , SHRSP treated with 100 mg/kg/day pentoxifylline (n = 12) **?** and with 200 mg/kg/day (n = 12) ¦ .

Figure 3. Immunohistochemical analysis of inflammatory cells within the brain of SHRSP

Representative immunohistochemical staining of ED1, ED2, isolectin-B4 and CD4 of brain slices obtained from control SHRSP (panel A-D) or treated with pentoxifylline (200 mg/kg/day; panel E-H) and sacrificed 3 days after the MRI detection of brain damage in the control group. Result is representative of data obtained in five independent experiments with brain from different rats (magnification 20x; 40x for C and G).

Figure 4. Effects of pentoxifylline of serum levels of cytokines in SHRSP

Effects on control (n=8) and pentoxifylline-treated rats (200 mg/kg/day; n = 8) on LPS-induced serum levels of TNF-α, IL-1β and IL-6 in salt-loaded-SHRSP. Three days after detection of brain abnormalities in vehicle-treated rats, the animals of both

experimental groups were injected with LPS. Blood samples were collected 3 h after LPS injection. *$p<0.05$

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Table 1. Body weight (grams)and blood pressure (mmHg) for salt–loaded SHRSPs treated with vehicle or pentoxifylline after 6 weeks of treatment

Groups	Body weight	Blood pressure
Not treated	265 ± 9	257 ± 29
PTX 100 mg/kg/day	273 ± 6	235 ± 10
PTX 200 mg/kg/day	275 ± 3	244 ± 18

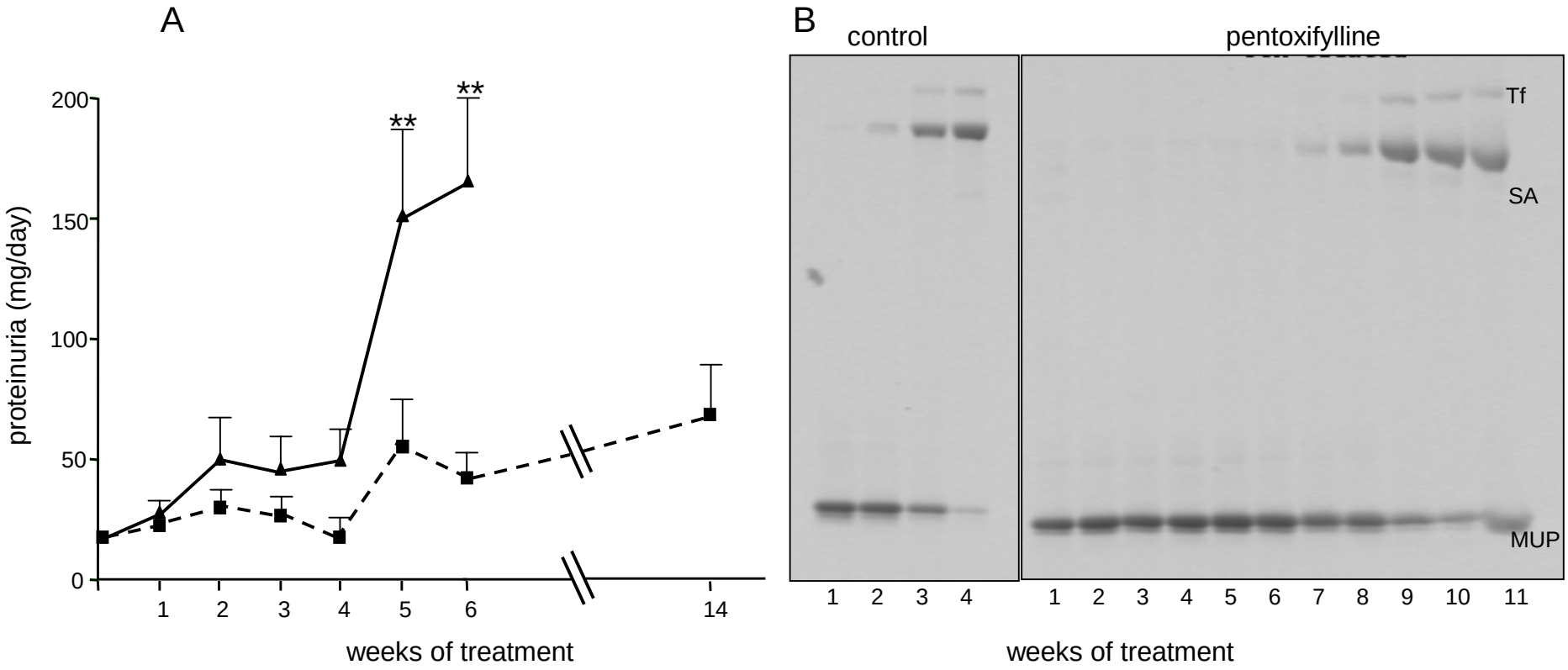

Figure 1

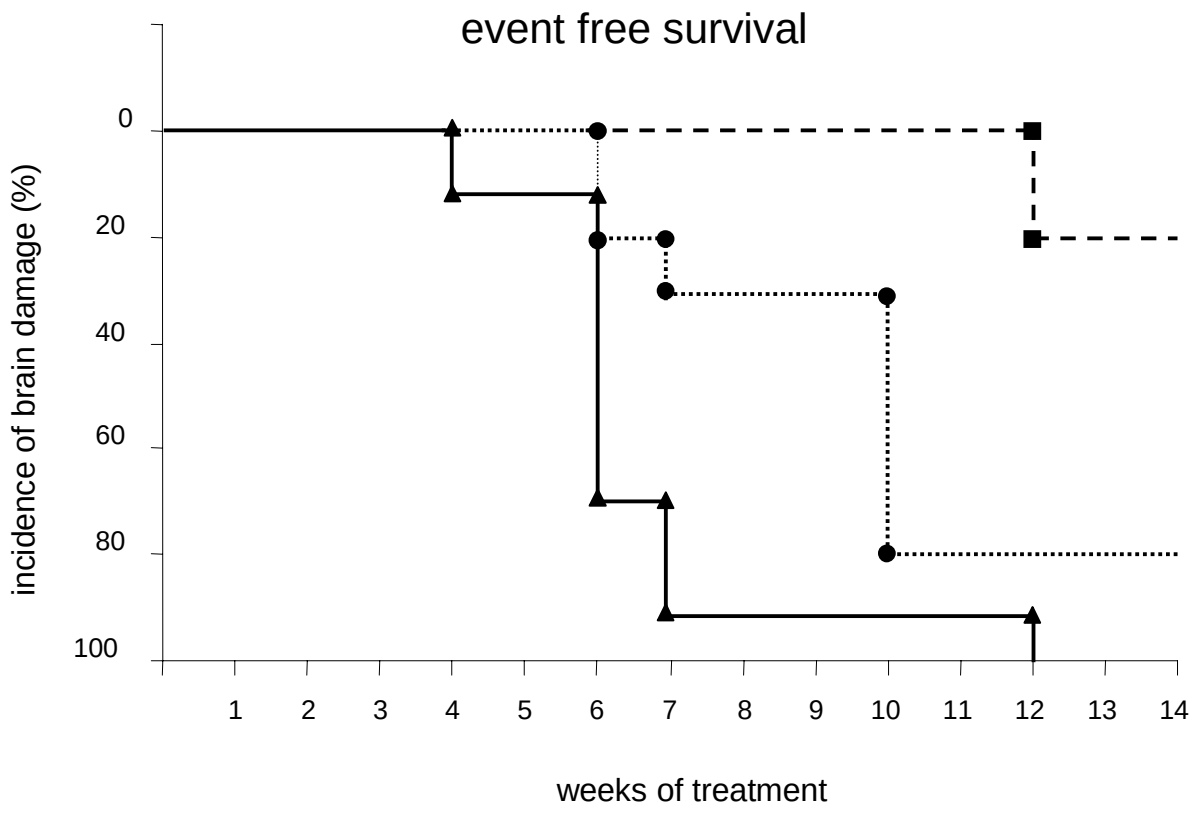

Figure 2

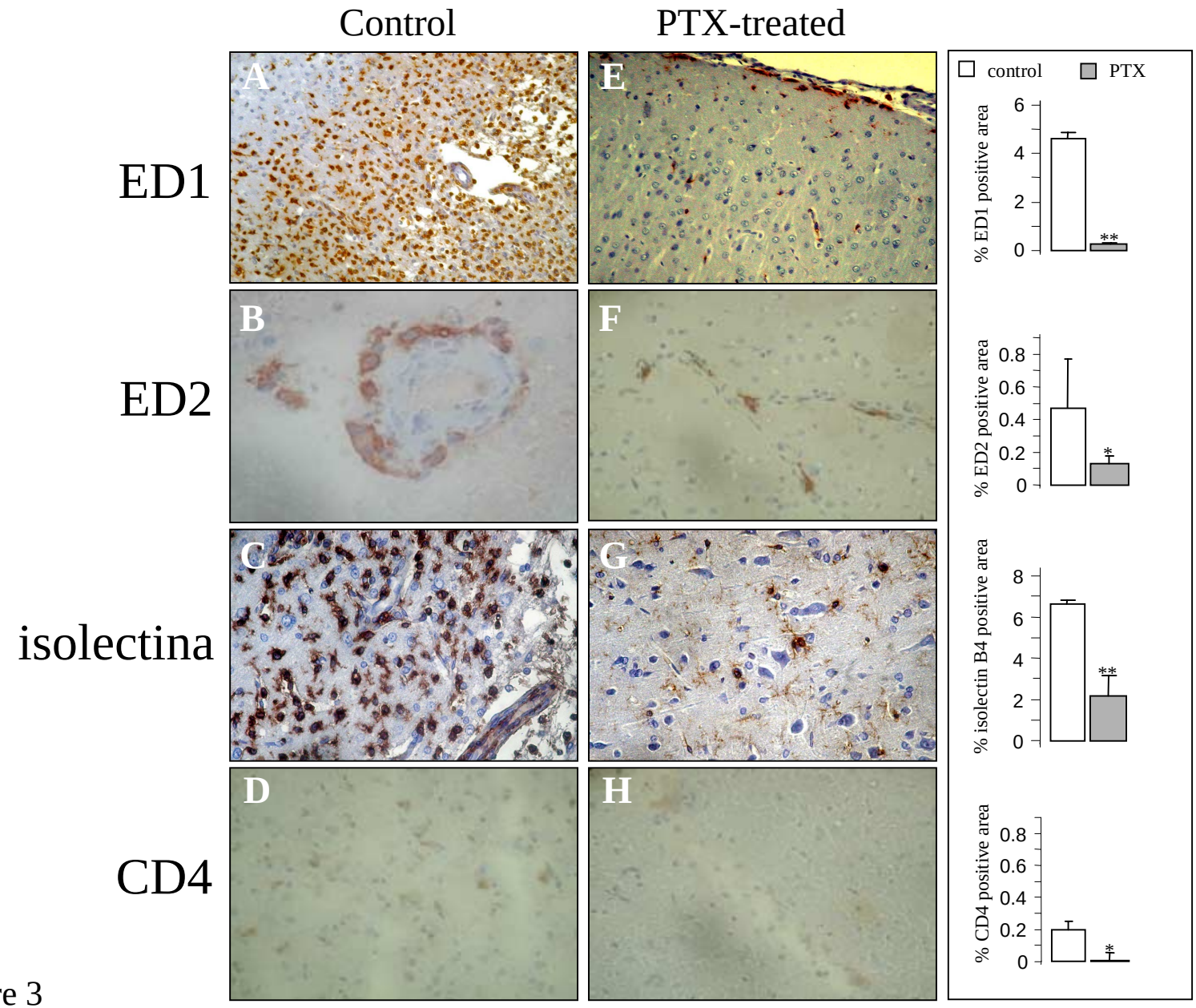

Figure 3

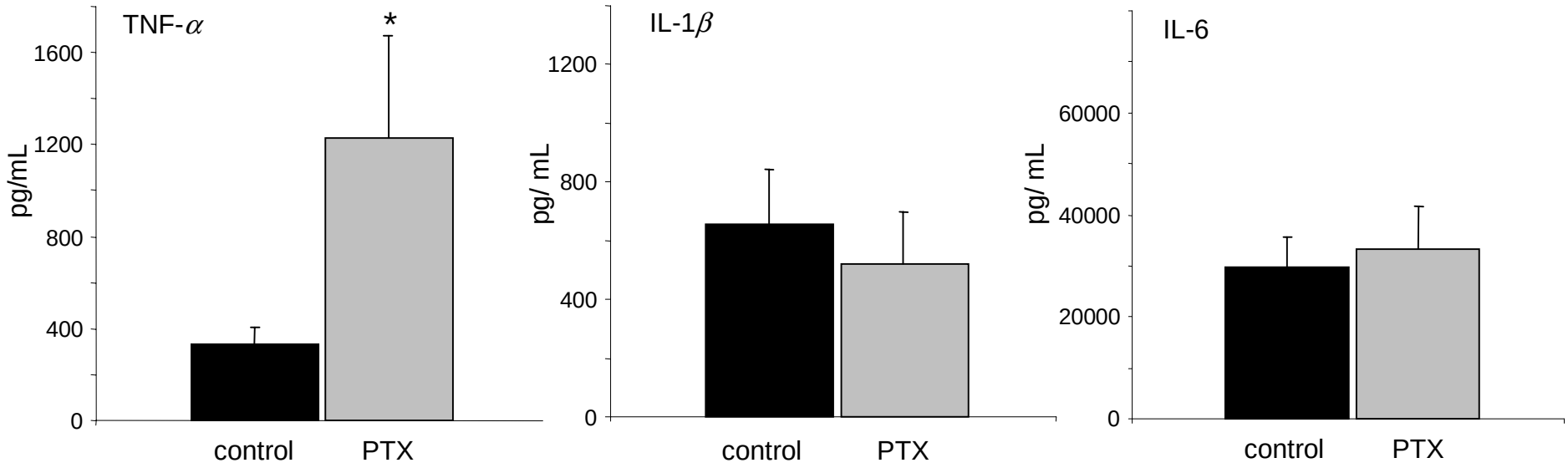

Figure 4