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N-tosyl-L-phenylalanyl-chloromethylketone reduces hypoxic–ischemic brain injury in rat pups

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Abstract

N-tosyl-L-phenylalanyl-chloromethylketone (TPCK) in vitro blocks apoptotic pathways leading to cell death. We wished to see if TPCK would reduce brain injury in vivo. Seven-day-old rat pups had the right carotid artery ligated and then received either vehicle or TPCK (5 to100 mg/kg i.p.). They were then given 8% oxygen for 2.25 h. Twenty-two days later, the cerebral hemispheres were weighed to determine the reduction in size in the right hemisphere. TPCK decreased the reduction in right hemisphere weight from $15 \pm 3\%$ (vehicle, $n = 20$), to $4 \pm 2\%$ (10 mg/kg, $n = 19$, $P < 0.01$). TPCK reduced the number of cells staining for DNA breaks 3 days after injury from 1729 ± 275 mm^{-2} (vehicle, $n = 8$) to 550 ± 236 mm^{-2} (10 mg/kg TPCK, $n = 9$, $P < 0.01$), decreased the amount of DNA fragmentation 3 days after injury by gel electrophoreses (20 mg/kg, $n = 16$, $P < 0.01$) and eliminated the increase in nitric oxide metabolites 6 h after injury (vehicle 1.5 ± 0.4, $n = 10$; and 20 mg/kg TPCK 0.0 ± 0.1 nM/mg protein, $n = 10$, $P < 0.001$). TPCK pretreatment in the newborn rat model of hypoxic–ischemic brain injury reduces DNA fragmentation, nitric oxide production and brain injury.

Keywords: Chloromethyl ketone; Stroke; Focal ischemia; Apoptosis; Serine protease inhibitor; Nitric oxide (NO)

1. Introduction

Apoptosis describes the programmed death of cells that are no longer needed by the embryo during development (Sulston et al., 1983). Necrotic cell death is the rapid death of cells after injury. However, a subset of injured cells in some protocols follows a pattern of delayed cell death that morphologically and biochemically resemble apoptosis (Ramachandra and Studzinski, 1995). Cytoplasmic proteases play an important role in the pathways leading to apoptotic cell death (Fearnhead et al., 1995; Higuchi et al., 1995). Intranuclear DNA fragmentation into low molecular weight segments (DNA laddering) is important evidence for an apoptotic pattern of cell death (Ramachandra and Studzinski, 1995). Terminal deoxynucleotidyl transferase mediated dUTP biotin nick end-labeling (TUNEL staining), although less specific, provides in situ evidence for DNA fragmentation (Ramachandra and Studzinski, 1995).

N-tosyl-L-phenyalanyl-chloromethylketone (TPCK) is a serine protease inhibitor, originally developed as an irreversible chymotrypsin inhibitor (Schoellmann and Shaw, 1993). TPCK is lipid soluble and enters cells easily. It inhibits apoptotic DNA laddering (Takauji et al., 1996; Dong et al., 1997; Mansat et al., 1997) and cell death in a number of different cell culture systems. As a cell permeate serine protease inhibitor, TPCK has proved useful in determining the chemical pathways involved in DNA laddering (Takauji et al., 1996; Dong et al., 1997; Mansat et al., 1997). TPCK also reduces inducible nitric oxide synthetase production in macrophages (Griscavage et al., 1995) by inhibiting nuclear factor κB (NF-κB) activation (Ruetten and Thiemermann, 1997). TPCK has recently been shown to reduce hippocampal injury in an adult gerbil model of severe forebrain ischemia (Hara et al., 1998). Hypoxic–ischemic brain injury is an important cause of death and disability in human newborns. The developmental stage of the brain of the 7-day-old rat pups resembles that of a human newborn (Palmer et al., 1990). Newborns,

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who are just leaving the normal physiologic period of neuronal apoptosis, might well have more active apoptotic pathways after cell injury than adults. We hypothesized that treatment with TPCK would reduce hypoxic–ischemic brain injury in newborns.

2. Materials and methods

2.1. Animal protocol

This protocol was approved by our institutional committee on animal use. Rats were cared for in accordance with National Institutes of Health guidelines. Using the well-characterized Rice et al. (1981) immature rat hypoxic–ischemic brain Injury model, 7-day-old Sprague–Dawley rat pups were anesthetized with isoflurane and had the common right carotid artery isolated from the nerve and vein, ligated and divided. The pups were returned to their dam for at least 3 h recovery after surgery. Pups were randomly assigned to the control group ($n = 22$) or treatment with TCPK (Sigma, St. Louis, MO) in doses of 5 ($n = 21$), 10 ($n = 20$), 20 ($n = 23$), 50 ($n = 9$) or 100 mg/kg ($n = 7$) dissolved in castor oil (with the concentration adjusted so the dose of castor oil was always the same) and administered i.p. The vehicle group was given castor oil alone. Pups were then placed in sealed jars in a 37°C water bath (internal jar temperature approximately 33°C) and subjected to a warmed, humidified mix of 8% oxygen and 92% nitrogen delivered at 4 l/min for 2.25 h. Rectal temperature was taken with a 36-gauge flexible thermocouple (Omega Engineering, Stamford, CT) in a sub-set of pups (six from the control group and six given 20 mg/kg of TPCK) prior to placing the pups in the jar, at the time of removal from the jar and at 1.5, 3, 6, 15, and 24 h later. Rectal temperatures were not significantly different for the control and TPCK groups. Pups were returned to their dams after removal from the jars and allowed to recover and grow for 22 days. Pups were weighed prior to injury and at 4, 7, 11, 14 and 22 days after. Body weights of the treated groups were not significantly different from controls at any of these times. Body weight increased significantly with time in all groups.

2.2. Gross neuropathologic grading

After 22 days, the pups were anesthetized with Pentobarbital and decapitated. Brains were scored normal, mild, moderate or severe by the method of Palmer et al. (1990) by a blinded observer. Normal is no reduction in the size of the right hemisphere, mild is visible reduction in right hemisphere size, moderate is large reduction in hemisphere size with a visible infarct in the right parietal area, and severe is near total destruction of the hemisphere (see Fig. 1). After removing the cerebellum and brainstem, the brain

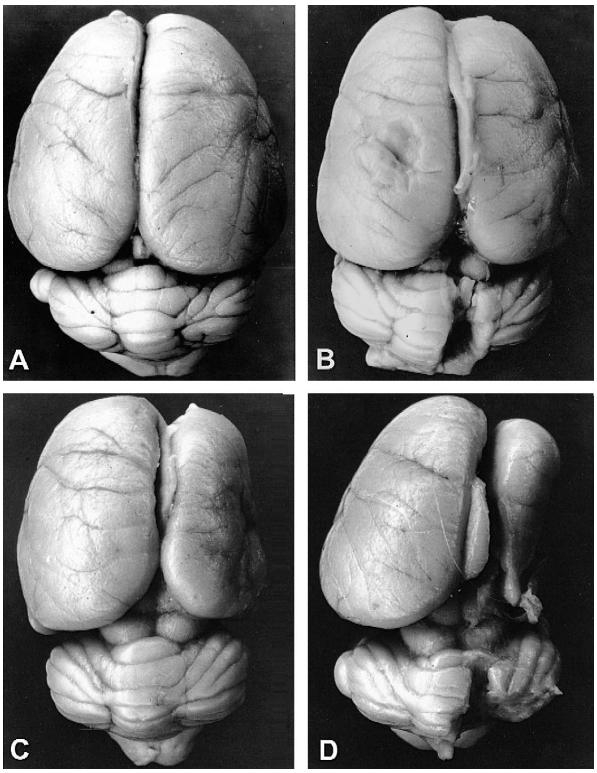

Fig. 1. Dorsal view of representative rat pup brains graded in four distinct categories: normal (A), no difference in size between the two hemispheres; mild (B), some discrepancy in size with the right being smaller than the left; moderate (C), large reduction in hemisphere size with a visible infarct in the right parietal area; and severe damage (D), near total destruction of the hemisphere.

was divided into two hemispheres and weighed. Results are presented as the percent loss of hemispheric weight of the right side relative to the left [(left–right)/left × 100]. The loss of hemispheric weight can be used to measure brain damage in this model, if enough time has elapsed to allow resorption of the dead tissue (Hagberg et al., 1994; Bona et al., 1997). In a preliminary experiment, we measured the hemispheric weight in 30 normal 7-day-old rat pups that had not been subject to carotid ligation and hypoxia. The mean difference between the left (244 ± 19 mg S.D.) and right (242 ± 18 mg) hemispheric weight was 0.6 ± 1.2%.

2.3. Other assays

A second set of experiments was performed to determine the effect of TPCK on Apoptotic markers. The neonatal hypoxia–ischemia procedure was as described above using a 2.5-h period of hypoxia. Pups were randomly assigned to a control group (vehicle treated) or treatment with TPCK in doses of 10 or 20 mg/kg dissolved in castor oil and administered i.p. prior to hypoxia. After the hypoxic exposure, pups were returned to their dams and allowed to recover and grow for 3 days. Evi-

dence of apoptosis is apparent in this model between 18 and 96 h after injury (Hill et al., 1995; Torres et al., 1997; Pulera et al., 1998).

2.3.1. Histologic evaluation for DNA breaks

Seventeen pups were randomized to treatment with 10 mg/kg of TPCK or vehicle prior to hypoxia. Seventy-two hours after the hypoxic–ischemic brain injury, pups were anesthetized with pentobarbital and received transcardiac perfusion with saline and then with buffered 10% formalin. Brains were removed and embedded in paraffin. Five-micrometer coronal sections were cut at the level of the dorsal hippocampus and then used for TUNEL staining.

The TUNEL staining (Ramachandra and Studzinski, 1995) was performed using ApopTag Kit (Oncor, Gaithersburg, MD) after deparaffinizing the section. Slides were counterstained with 0.5% methyl green. Negative control slides were processed with terminal deoxynucleotidyl transferase enzyme excluded. The slides were examined by light microscopy at 450 × and the number of cells with nuclei stained brown in 40 high power fields (180 μm diameter) were counted and averaged for each pup.

2.3.2. Detection of DNA fragmentation

Detection of DNA fragments in the brain cortex was performed as described by Strauss (1998) with some modifications. Sixteen pups were randomized to be treated with 20 mg/kg of TPCK or vehicle immediately prior to hypoxia. Seventy-two hours after hypoxic–ischemic brain injury, pups were anesthetized with pentobarbital, and the brain was quickly excised into an ice-cold Petri dish. The lateral parietal cerebral cortex of both hemispheres was dissected and stored at −80°C until the time of processing and assay. DNA was isolated from 100 mg of cortex using standard techniques (Strauss, 1998). Resolving agarose gel electrophoresis was performed with 1.25% gel strength containing 1.0 μg/ml ethidium bromide. Thirty micrograms DNA per well was loaded. DNA standards (Promega, Madison, WI) were included to identify the size of the DNA fragment. The 100 base pair fragment of 2 μg of a single batch of standard was used as an internal flourescence standard. Electrophoresis was performed for 2 h at 70 V, and DNA was visualized by ultraviolet fluorescence. Fluorescence was quantitated on photographic negatives by densitometer (Molecular Dynamics, Personal Densitometer) making sure that an equal number of TPCK and vehicle-treated pups were represented on each negative.

2.3.3. Total nitrates and nitrites

Total nitrates and nitrites (NO_x) were measured on the brains of 126 pups randomized to treatment with 20 mg/kg of TPCK or vehicle. This included 13 vehicle-treated shams and six TPCK-treated shams treated with TPCK 3 h prior to sampling and six TPCK-treated shams treated 8 h prior to sampling. Six vehicle and six TPCK pups were sampled immediately after a 1-h period of hypoxia. The rest of the pups, six to 10 in each subgroup by time and treatment, were subjected to a 2.25-h period of hypoxia and sampled at 0.5, 3, 6, 12, and 24 h after the end of the hypoxia. At each time point, the pups were decapitated, and the brains were removed and separated into two hemispheres and frozen at −80°C. Brain samples were homogenized in ice cold buffer (0.1 M potassium phosphate, pH 7.5, 20 mM EDTA) and centrifuged at 14 000 rpm for 30 min at 4°C. Total nitrates and nitrites were measured using the Griess reaction (Titheradge, 1998) after enzymatic conversion of the nitrates in the sample to nitrites using NADPH-dependent nitrate reductase and standardized to sample protein concentration (Bradford, 1976).

2.4. Statistics

Statistical comparisons were made using χ^2 or Fischer's exact test for categorical variables and analysis of variance with or without repeated measures for continuous variables. Newman–Keul's test was used where all groups are compared and Dunnett's test was used to compare to shams.

3. Results

Gross neuropathologic damage was scored 22 days after injury. Nine percent (2/22) of the vehicle-treated pups died prior to 22 days. Five percent (1/21) of the pups given 5 mg/kg of TPCK, 5% (1/20) of the pups given 10 mg/kg, and 13% (3/23) of the pups given 20 mg/kg died prior to 22 days (P = ns, vs. vehicle). Sixty-seven percent (6/9) of the pups given 50 mg/kg of TPCK ($P < 0.01$ vs. vehicle) and 100% of the seven pups given 100 mg/kg died prior to 22 days ($P < 0.01$ vs. vehicle). Most of the deaths occurred within 4 days of the hypoxic period in both groups.

All but one of the brains were scored as either normal or mild injury (one 20 mg/kg-pup was scored as moderate). The proportion of pup brains scored as normal by blinded observer was 45% (9/20), 85% (17/20), 89.5% (17/19), and 80% (16/20) for vehicle, 5, 10, and 20 mg/kg TPCK, respectively (Fig. 2). At doses of 5, 10 and 20 mg/kg TPCK increased the number of brains scored as normal ($P < 0.01$ vs. vehicle) with a peak effect at 10 mg/kg. Two of the three surviving pups in the 50 mg/kg group had brains scored as normal, but because of the small numbers, this was not statistically different from the other groups.

Left hemisphere weight was 536 ± 5 mg (mean ± S.E.M., $n = 20$), 520 ± 6 mg ($n = 20$), 529 ± 5 mg ($n = 19$), 528 ± 5 mg ($n = 20$) and 518 ± 40 mg ($n = 3$) in the

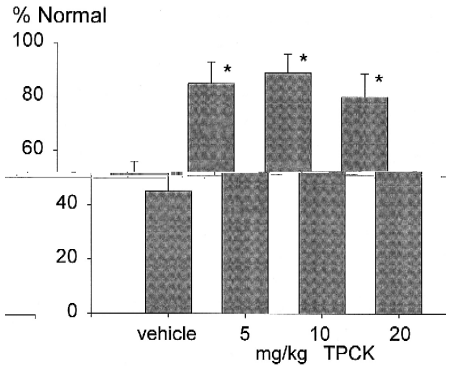

Fig. 2. The dose–response for the proportion of brains in each group scored as normal by a blinded observer 22 days after injury. There were approximately 20 pups in each group. Brains were scored as normal if they had no visible infarcts or decrease in size of the right hemisphere. Treatment was given immediately prior to hypoxia. The error bars are standard deviation of the proportion. The asterisk signifies $P < 0.01$ vs. vehicle. Treatment with 5 to 20 mg/kg of TPCK increased the number of brains scored as normal with a peak effect at 10 mg/kg.

vehicle, 5, 10, 20 and 50 mg/kg groups, respectively. Left hemisphere weight is unaffected by the procedure (Rice et al., 1981) and was the same in all groups. The percent reduction in right hemispheric weight was $14.7 \pm 2.7\%$, $8.5 \pm 2.5\%$, $4.3 \pm 1.8\%$, and $7.8 \pm 2.6\%$ for the vehicle, 5, 10 and 20 mg/kg TPCK treatment groups, respectively (Fig. 3). TPCK at doses of 5 mg/kg, 10 mg/kg and 20 mg/kg produced significant improvement in right hemisphere weight reduction. There were no statistically significant differences between the three treated groups, but all three treated groups showed statistically significant improvement over the vehicle group ($P < 0.01$). TPCK, at a dose of 50 mg/kg, produced the same improvement seen at the 20 mg/kg dose, but with only three pups surviving, this was not statistically significant.

Fig. 3. The dose–response for the percentage reduction in right cerebral hemisphere weight using the left hemisphere weight as the standard. There were approximately 20 pups in each group. Treatment was given immediately prior to hypoxia and injury was evaluated 22 days later. The mean and S.E.M. are graphed. Result for vehicle-treated controls and pups treated with TPCK are compared ($^*P < 0.01$ vs. vehicle). Treatment with 5 to 20 mg/kg of TPCK decreased the percentage reduction in right hemisphere weight with a peak effect at 10 mg/kg.

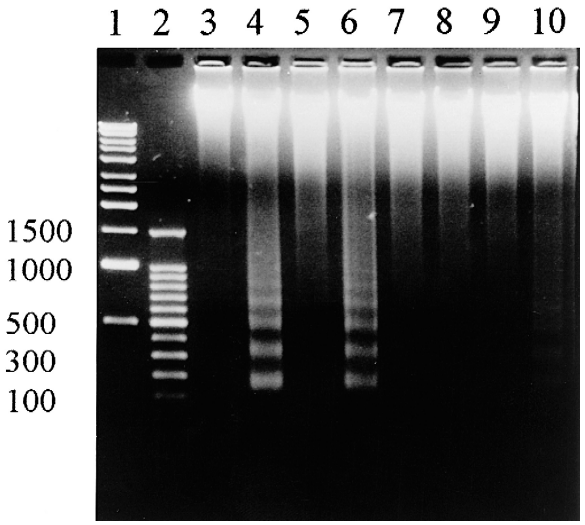

Fig. 4. DNA electrophoresis of cerebral cortex. The first column is an oligonucleotide standard containing fragments in 500 base pair (bp) increments starting at 500 bp. The second column is an oligonucleotide standard containing fragments in 100 bp increments between 100 and 1000 bp. The third and fifth columns are from the left (uninjured) cortex of vehicle-treated pups and show no oligonucleotide fragments. The fourth and sixth columns are from the corresponding right (injured) cortexes and show a prominent pattern of oligonucleotide fragments. The seventh and ninth columns are from the left (uninjured) cortex of pups treated with 20 mg/kg of TPCK and show no oligonuleotide fragments. The eighth and tenth columns are from the corresponding right (injured) cortexes. The eighth column shows no fragment. The tenth column shows a faint pattern of fragments.

A gel showing DNA electrophoresis of two pups pretreated with 20 mg/kg of TPCK and two vehicle-treated pups is shown in Fig. 4. A summary of the quantitation with eight pups in each group is shown in Fig. 5. For all three bands, the right hemisphere in the vehicle group had more prominent bands than either the left hemisphere in

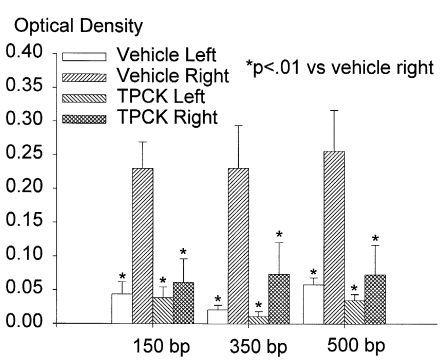

Fig. 5. The optical density of the negative from fluorescence imaging of DNA double stranded fragments of the 150, 350, and 500 bp bands for the left and right cortical specimen of the TPCK- and vehicle-treated pups. Mean and S.E.M. are graphed for eight pups in each group. Statistics compare to the right cortex from the vehicle group ($^*P < 0.01$). For all three fragments, the right cortex of the vehicle-treated animals shows significantly greater fragment density than either the left cortex of the vehicle group or the left or right cortex of the TPCK-treated group.

the vehicle group ($P < 0.01$) or the right hemisphere in the treated group ($P < 0.01$). Thus, the increase in DNA bands caused by the hypoxic–ischemic injury is decreased or eliminated by the pretreatment with TPCK.

The results of the TUNEL staining are shown in Fig. 6. Pretreatment with TPCK significantly reduced the number of TUNEL positive cells seen 3 days after the hypoxic period from 1729 ± 275 cells/mm^2 (vehicle, $n = 8$) to 550 ± 236 cells/mm^2 (TPCK, $n = 9$, $P < 0.01$).

Total nitrates and nitrites (NO_x) are shown in Fig. 7 for pretreatment with 20 mg/kg of TPCK or vehicle. The two groups of treated shams were not significantly different from each other, so their results were combined. There was an initial increase in NO_x after a 1-h hypoxic period in the right and left cortex of the vehicle group, and in the right cortex of the treated group. NO_x returned to baseline after 2.25 h of hypoxia and 1/2 h recovery in both hemispheres of the vehicle-treated group and was significantly below baseline in both hemispheres of the TPCK-treated pups. There was a secondary rise in NO_x peaking at 6 h after injury ($P < 0.05$) in the right cortex of the vehicle group that was eliminated by pretreatment with TPCK. There was a period of decreased NO_x production in the right cortex of the vehicle group at 24 h after injury ($P < 0.05$) relative to the left hemisphere. At 24 h, the NO_x in the left or right cortex of the TPCK-treated group was less than

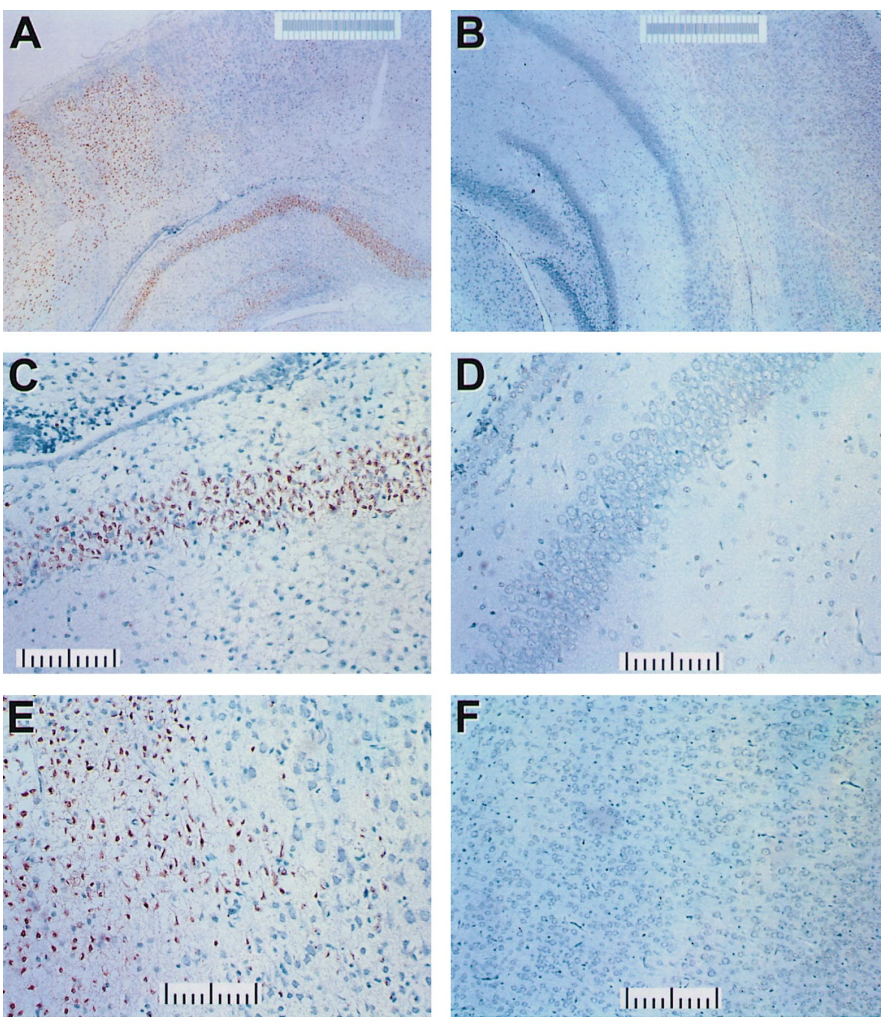

Fig. 6. Five-microns TUNEL-stained slices. (A) and (B) are low power views of the hippocampuses and surrounding cortex from the right hemisphere of vehicle- and TPCK- (10 mg/kg) treated pups, respectively. The scale at the upper right is 1-mm long. (C) and (D) are high power views of the hippocampuses from the right hemisphere of vehicle- and TPCK- (10 mg/kg) treated pups, respectively. The scale is 100 μm in 10-μm increments for (C), (D), (E), and (F). (E) and (F) are high power views of the cortexes from the right hemisphere of vehicle- and TPCK- (10 mg/kg) treated pups, respectively. Extensive brown staining labels double stranded DNA breaks in the condensed nuclei of the vehicle-treated pups. The right hemisphere of TPCK-treated animal shows normal background staining. The left hemispheres of both groups were similar to the right hemisphere in the TPCK-treated group (not shown). Pretreatment with TPCK significantly reduced the number of TUNEL positive cells seen 3 days after the hypoxic period from 1729 ± 275 cells/mm^2 (vehicle, $n = 8$) to 550 ± 236 cells/mm^2 (TPCK, $n = 9$, $P < 0.01$).

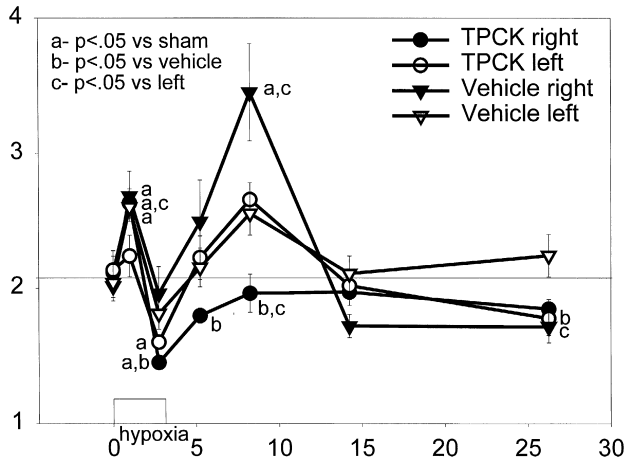

Fig. 7. Total nitrates and nitrites in nanomolar per milligram protein in sham pups cerebral cortex (0 h, both vehicle- and TPCK-treated shams are shown) and in pups treated with 20 mg/kg of TPCK or vehicle prior to hypoxia and sampled at various times after the start of a 2.25-h period of hypoxia (mean ± S.E.M.). The line is the mean value of the shams. Hypoxia results in a primary increase in NO_x after 1 h of hypoxia that returns to baseline by 2.25 h of hypoxia and 1/2 h recovery. There is a larger secondary increase in NO_x in the right cortex at 6 h after hypoxia that is eliminated by treatment with TPCK. There is a reduction in NO_x in the right cortex of the vehicle-treated group at 24 h relative to the left cortex. There is a reduction in NO_x in the right and left cortex of the TPCK-treated animals immediately after hypoxia relative to the shams and at 24 h relative to the left cortex of the vehicle treated pups. $^{a}P < 0.05$ vs. the same side of the sham group, $^{b}P < 0.05$ vs. the same side of the vehicle group, $^{c}P < 0.05$ vs. the left side of the brain in the same group.

the NO_x in the left (uninjured) cortex of the vehicle treated group ($P < 0.05$).

4. Discussion

TPCK given prior to injury reduces the degree of injury in the focal ischemia model of the neonatal rat pup. Results are similar to that reported in the adult gerbil global ischemia model (Hara et al., 1998). Since delayed neuronal injury sometimes requires a prolonged period to develop (Trescher et al., 1997), we extended the time from injury to brain assessment from the 96 h used in Hara's study in gerbils to 22 days without a reduction in efficacy. Since late hypothermia can effect study results (Colbourne et al., 1998), we measured temperature for 24 h rather than the 3 h used in Hara's study and found no effect of the drug on body temperature. TPCK is effective at doses of 5 to 20 mg/kg in the rat pup with a peak effect at 10 mg/kg, while in the gerbil TPCK is only effective at 50 to 100 mg/kg with a peak effect at 100 mg/kg or higher. TPCK becomes lethal in the rat pup hypoxic–ischemia model at 50 mg/kg or higher while 100 mg/kg in the gerbil sometimes causes a non-fatal paralytic ileus at 96 h. Paralytic ileus would be lethal much more rapidly in a rat pup than in a gerbil because rat pups lose water more quickly through their skin. This is both because of the pups smaller size and larger relative surface area, and because of their immature skin (Cartlidge, 1998). The differences in the dose–response range are presumably because of the differences in age, species and protocols.

In tissue culture, the decrease in cell death from apoptotic agents by TPCK is associated with a reduction in DNA fragmentation (Takauji et al., 1996; Dong et al., 1997; Mansat et al., 1997). We have shown that the effect of TPCK on cell death and DNA fragmentation also occurs in vivo in the neonatal rat focal ischemia model. Histologic examination shows a loss of the large cells with prominent nuclei, the pyramidal neurons, and their replacement with TUNEL positive cells with pyknotic nuclei, indicating that the neurons are probably contributing to the DNA fragmentation that is occurring. TPCK acts downstream from caspase 3 activation in tissue culture (Dong et al., 1997; Wright et al., 1997) and blocks activation of neutral sphingomylinase (Mansat et al., 1997) and the 24-kDa apoptotic protease described by Wright et al. (1997) and thus blocks DNA fragmentation. TPCK also acts proximal to caspase 3 to reduce inducible nitric oxide synthase production (Griscavage et al., 1995) in macrophages in tissue culture by inhibiting NF-κB activation (Ruetten and Thiemermann, 1997). However, NF-κB activation is not thought to be the essential mechanism for inhibiting apoptosis and DNA laddering by TPCK, since the inhibition of NF-κB activation in tissue culture required 10 times the concentration of TPCK that is required for inhibition of DNA laddering (Higuchi et al., 1995). Similarly, Bcl-xL induction by TPCK (Hara et al., 1998) is upstream of caspase 3 and of the protease inhibited by TPCK during inhibition of apoptosis (Schmitt et al., 1997) in tissue culture. We cannot tell from our work which actions of TPCK are most important in vivo.

The effect of TPCK on total nitrates and nitrites is more complex. Higuchi et al. (1998) described a biphasic increase in NO_x in the neonatal rat model. The first increase occurred 1 h into the hypoxic period and resolved by the end of the hypoxic period. This was followed by a second increase in NO_x that was seen 3 h after hypoxia, when their last measurement was taken. In their experiment, the first increase was inhibitable by neuronal nitric oxide synthase inhibitors while the latter increase required both neuronal and inducible nitric oxide synthase inhibitors to block. Blocking the first peak eliminated the second peak, demonstrating it was a secondary peak. Earlier data from this group showed an increase in neurons displaying neuronal nitric oxide synthase in conjunction with the second peak, that is induction of neuronal nitric oxide synthase. Lastly, Higuchi et al. (1996) saw a loss of cells displaying neuronal nitric oxide synthase at 24 h after injury that they attributed to a loss of neuronal nitric oxide positive cells. Our data shows the primary and secondary peaks in NO_x in the vehicle group with the secondary increase continuing to rise until 6 h after hypoxia. We also saw a decrease

in NO_x at 24 h after injury in the ischemic hemisphere relative to the non-ischemic side in the vehicle group, that is similarly timed to the decrease in neuronal nitric oxide synthase positive cells seen by Higuchi et al. (1996). The primary increase in NO_x after 1 h of hypoxia is not effected by TPCK. The secondary increase in NO_x at 6 h after hypoxia is eliminated by TPCK. Based on the above described studies, we would speculate that TPCK eliminates the increase in NO_x at 6 h after hypoxia by blocking activation of NF-κB and consequently the induction of both neuronal nitric oxide synthase and inducible nitric oxide synthase. Since TPCK reduces NO_x at 0.5 and 24 h after hypoxia in both the injured and uninjured cortex, we wondered whether TPCK reduced baseline NO_x. We did not find this to be true when we measured NO_x in uninjured TPCK-treated animals. Based on tissue culture result, we would suggest that the effects of TPCK on NO_x and on DNA fragmentation are independent actions of TPCK (Higuchi et al., 1995). However, inhibiting NO production is neuroprotective in the newborn rat model (Hamada et al., 1994). Nitric oxide production may induce DNA fragmentation by damage to mitochondria resulting in loss of transmembrane potential, induction of the mitochondrial transition pore, release of cytochrome *C* from mitochondria, and initiating caspase activation, thereby initiating the apoptotic cascade (Budd, 1998). Nitric oxide, through the production of peroxynitrate, can cause oxidative DNA damage and single stranded DNA breaks and initiate the apoptotic cascade through stimulation of poly(ADP-ribose)polymerase (Endres et al., 1997).

Further studies are needed to isolate the specific mechanism and site of action of the neuroprotective action of TPCK. Similarly, determining the mechanism and site of action of its toxic effects would also be important if the therapeutic potential of this compound is to be exploited. It would probably be necessary to find a compound with a broader therapeutic window to produce a clinically useful agent.

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