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Zinc protoporphyrin IX attenuates closed head injury-induced edema formation, blood-brain barrier disruption, and serotonin levels in the rat

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Summary

The role of heme oxygenase (HO) in closed head injury (CHI) was examined using a potent HO and guanylyl cyclase inhibitor, zinc protoporphyrin (Zn-PP) in the rat. Blood-brain barrier (BBB) permeability to Evans blue and radioiodine, edema formation, and plasma and brain levels of serotonin were measured in control, CHI, and Zn-PP-treated CHI rats. CHI was produced by an impact of 0.224 N on the right parietal bone by dropping 114.6 g weight from a height of 20 cm in anesthetized rats. This concussive injury resulted in edema formation and brain swelling 5 hours after insult that was most pronounced in the contralateral hemisphere. The whole brain was edematous and remained in a semi-fluid state. Microvascular permeability disturbances to protein tracers were prominent in both cerebral hemispheres and the underlying cerebral structures. Plasma and brain serotonin showed pronounced increases and correlated with edema formation. Pretreatment with Zn-PP (10 mg/kg, i.p) 30 minutes before or after CHI attenuated edema formation, brain swelling, plasma and brain serotonin levels, and microvascular permeability at 5 hours. Brain edema, BBB permeability, and serotonin levels were not attenuated when the compound was administered 60 minutes post-CHI suggesting that HO is involved in cellular and molecular mechanisms of edema formation and BBB breakdown early after CHI.

Keywords: Closed head injury; edema; heme oxygenase; zinc protoporphyrin; blood-brain barrier; serotonin.

Introduction

Closed head injury (CHI) results in instant death in many victims [6, 15, 16, 19]. In the United States, CHI accounts for at least 2000 admissions to hospital per million population [2, 15, 16]. About 400,000 new cases are added each year, and many patients have long-term disabilities [6, 15]. Swelling of the brain in a closed cranial compartment is largely responsible for instant deaths [10]. Clinical cases may show diffuse injury with brain shift, mass lesions, or brain stem injury that are responsible for high mortality rates [18, 19]. Diffuse injuries with brain swelling may leave patients in a persistent vegetative state [2, 7, 9]. Unfortunately, there are few proven therapies available now. Efforts are needed to understand the molecular mechanisms of early pathophysiological events and to explore the therapeutic potentials of neuroprotective agents in order to minimize edema formation and cell death.

It is likely that CHI-induced micro-hemorrhage, oxidative stress, and generation of free radicals contribute to blood-brain barrier (BBB) breakdown and vasogenic brain edema formation [4, 5, 40]. Extravasation of blood and blood degradation products in the brain parenchyma are potential sources of free radical generation and may have key roles in the induction of brain swelling [36]. Hemoglobin is metabolized by the enzyme heme oxygenase (HO) after lysis of red blood cells, releasing iron, carbon monoxide (CO), and biliverdin [14]. CO is a free radical gas, similar to nitric oxide (NO), which can induce profound cell and tissue injury [37]. Since CO, like NO, is a molecule with very short half-life (<5 seconds) [12, 37, 38], its involvement in cell and tissue injury is largely based on studies using its synthesizing enzyme, HO.

The role of HO in CHI is not well understood. We examined HO using zinc protoporphyrin IX (Zn-PP), a potent HO and guanylyl cyclase inhibitor compound [3, 17, 20, 39] in a rat model [5]. Since blockade of serotonin synthesis appears to be neuroprotective in this CHI model [5], plasma and brain levels of the amine were also measured in animals treated with Zn-PP.

Materials and methods

Animals

Experiments were carried out on 48 young male rats (250–300 g) housed at 21 ± 1 °C room temperature, on a 12-hour light, 12-hour dark schedule. Food and tap water were supplied ad libitum before the experiments.

Anesthesia

All experiments were carried out under urethane anesthesia (1.5 g/kg, i.p.). This dose was sufficient to induce a grade IV anesthesia for more than 12 hours [5]. Urethane is a long-lasting irreversible anesthetic that acts mainly at the cerebral cortical level [24]. Thus, arterial blood pressure, heart rate, and respiration were stable throughout the experimental period [22].

A new model of CHI

We developed a new animal model of CHI that is easily reproduced and induces severe brain edema in the rat [5]. The model involves an impact of 0.224 N on the right parietal skull bone during anesthesia (Fig. 1), achieved by dropping a 114.6 g weight from a height of 20 cm through a guide-tube [5]. The animals were allowed to survive 1 hour, 2 hours, and 5 hours after injury. The biomechanical forces generated by this impact diffusely penetrate to the underlying brain tissues to induce a powerful concussive brain injury. A few animals (<5%) had minor skull fracture and were not included in this study. Untraumatized urethane-anesthetized rats were used as controls. These experiments were approved by the Ethics Committee of Uppsala University, and Banaras Hindu University.

Treatment with HO inhibitor, Zn-PP

Zn-PP (Tocris Bioscience, Avonmouth, UK) was administered (10 mg/kg, i.p) in a group of rats 30 minutes before CHI [11, 17, 21, 26, 28, 39]. In other groups of animals, Zn-PP was given either 30 or 60 min after brain trauma. The animals were allowed to survive 5 hours after CHI [5].

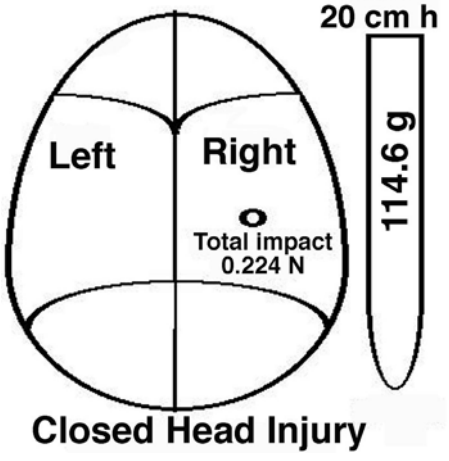

Fig. 1. Closed head injury model in rat. Under anesthesia, a 114.6 g weight (non-piercing) was dropped from a height of 20 cm through a guide-tube on a predetermined location on the right parietal bone. The skull was firmly held to avoid displacement during impact. This procedure generated an impact of 0.224 N on the surface of skull. A few animals (<5%) showed minor skull fracture and were not included in this study [5]

BBB permeability

BBB permeability in the cerebral cortex of both hemispheres was measured using Evans blue albumin (2%, 3 mL/kg, i.v.) and $^{[131]}$Iodine [1, 25, 33, 34]. These tracers were allowed to circulate for 5 minutes, and the intravascular tracer was washed out by transcardiac perfusion with 0.9% saline [22]. About 1 mL of arterial blood was withdrawn via heart puncture for whole blood radioactivity before perfusion [26, 35]. BBB permeability was expressed by percentage increase in the radioactivity in brain over the whole blood concentration [29, 31].

Brain water content

Brain water content in the right and left cerebral cortices was determined using the difference in sample wet and dry weights [30, 32]. The right cerebral cortex and the left cerebral cortex were dissected out, weighed immediately, and placed in an oven maintained at 90 °C for 72 hours or until the dry weight of the samples became constant [33]. The percentage of volume swelling was calculated from changes in the brain water content [24, 33].

Measurement of serotonin in plasma and brain

Plasma and brain serotonin were measured using a fluorometric assay [5, 22, 25]. About 1 mL of whole blood was collected after cardiac puncture. Plasma was obtained by centrifugation. Plasma (0.5 mL) and brain samples were diluted to 4 mL in 0.4 N ice-cooled perchloric acid and were centrifuged at 4 °C to separate out proteins [22]. The extraction of serotonin from 1 mL of aliquots from plasma or brain was performed using butanol in a salt-saturated and alkaline medium (pH 10) and purified using n-heptane (Extra Pure, Merck). The fluoropores were developed by incubating the samples with ninhydrin at 75 °C for 30 minutes and measured in duplicate samples at room temperature using a spectrophotofluorometer at excitation 385 nm and emission 490 nm wave lengths (Aminco-Bowman, USA) [5, 28].

Statistical analysis

ANOVA following Dunnet's test for multiple group comparisons with one control group was applied to evaluate the statistical significance of the data obtained. A p-value less than 0.05 was considered significant.

Results

Effect of Zn-PP on brain edema in CHI

Five hours after CHI, the whole brain was considerably edematous, softened, and remained in a semi-fluid state. A marked increase in brain water content and brain swelling was observed that was more pronounced in the contralateral than the ipsilateral cerebral hemisphere (Table 1). Pretreatment with Zn-PP markedly attenuated brain swelling 5 hours after CHI compared to the untreated traumatized group (Table

Table 1. *Effects of HO inhibitor Zn-PP on BBB permeability, brain edema formation, and plasma and brain serotonin levels following CHI in rats.*

Groups	n	Brain water content %		BBB permeability [131]Iodine %		Brain serotonin (μg/g)		Plasma serotonin (μg/mL)
		Right injured	Left intact	Right injured	Left intact	Right injured	Left intact	
Control	6	78.34 ± 0.23	78.03 ± 0.27	0.38 ± 0.06	0.40 ± 0.08	0.68 ± 0.24	0.70 ± 0.32	0.34 ± 0.08
5 h after CHI	8	81.34 ± 0.89**	82.47 ± 0.67**[a]	1.78 ± 0.56**	2.14 ± 0.32**[a]	1.89 ± 0.45**	2.23 ± 0.18**[a]	0.68 ± 0.11**
Zn-PP# + CHI	6	79.32 ± 0.22*[b]	80.16 ± 0.21*[b]	0.67 ± 0.23*[b]	0.76 ± 0.44*[b]	0.93 ± 0.28*[b]	1.16 ± 0.21*[b]	0.48 ± 0.11*[b]

BBB Blood-brain barrier; *CHI* closed head injury; *HO* heme oxygenase; *Zn-PP* zinc protoporphyrin.
= 10 mg/kg, i.p. 30 min after CHI; a = $p < 0.05$ from injured half; b = $p < 0.05$ from CHI; ** = $p < 0.01$ from control.

1). This effect on edema was also evident when the HO inhibitor was administered 30 minutes after CHI (Table 1). However, no significant reduction in brain water content or volume swelling was noted when Zn-PP was given 1 hour after trauma (results not shown).

Effect of Zn-PP on BBB permeability in CHI

Microvascular permeability disturbances to Evans blue and radioiodine tracers were prominent at 5 hours in both cerebral hemispheres as well as in underlying cerebral structures (Table 1). However, the extravasation of protein tracers was higher in the contralateral hemisphere compared to the side ipsilateral to injury. Pretreatment with Zn-PP (10 mg/kg, i.p) 30 minutes before or 30 minutes after CHI significantly attenuated the enhanced BBB permeability to protein tracers seen at 5 hours (Table 1). However, Zn-PP was ineffective when administered 60 minutes after CHI (results not shown).

Effect of Zn-PP on plasma and brain serotonin levels in CHI

There were pronounced increases in serotonin in both traumatized and contralateral hemispheres 5 hours after CHI. This increase in the contralateral hemisphere was higher than the injured cortex (Table 1). The plasma serotonin also increased significantly from the control group.

Pretreatment with Zn-PP markedly attenuated increased plasma and brain serotonin levels 5 hours after CHI. The increase in serotonin levels was diminished by the HO-inhibitor when given 30 minutes after CHI (Table 1). No changes in plasma or brain serotonin were seen when Zn-PP was administered 60 minutes after CHI (results not shown).

Discussion

Treatment with an HO and guanylyl cyclase inhibitor compound, Zn-PP, within 30 minutes of CHI markedly attenuates BBB disruption and brain edema formation. This new observation suggests that CO participates in the early phase of the pathophysiology after CHI, a concept consistent with the fact that administration of Zn-PP 1 hour after CHI did not reduce brain edema formation and/or BBB breakdown.

The increase in brain edema in the contralateral hemisphere suggests that the model can be used to study contre coup mechanisms in the brain. Physical forces following impact on the intact skull will be transmitted to the opposite hemisphere causing massive damage compared to the injured side [5]. Our observations further show a close parallelism between serotonin levels, BBB dysfunction, and brain edema formation in CHI.

Increased levels of serotonin in plasma and brain closely correspond to BBB breakdown. Furthermore, Zn-PP administered either 30 minutes before or after CHI was able to reduce plasma and brain serotonin levels effectively, together with brain edema formation and BBB breakdown. On the other hand, serotonin levels, BBB disruption, and brain edema formation did not fall when Zn-PP was administered 1 hour after CHI, indicating an interaction between HO and serotonin in CHI.

Up-regulation of HO-1 and HO-2 occurs following various types of centra nervous system insult [1, 8, 22, 23, 25–27, 30–34]. Previous reports from our laboratory showed up-regulation of HO-2 5 hours after focal spinal cord injury (SCI) in the rat, which closely corresponds to cell and tissue injury [25, 26, 30–32]. Inhibition of HO-2 expression in the cord caused by either topical application of neurotrophins [31, 32] or by pretreatment with the serotonin synthesis inhibitor, p-

chlorophenylalanine [25], markedly attenuated edema formation, microvascular permeability disturbances, and cell injury [26]. Up-regulation of HO is associated with increased production of CO that may contribute to cell and tissue injury similar to NO [24, 26, 28, 29, 33, 38]. These observations suggest that an up-regulation of HO and subsequent generation of CO appears to be an instrumental factor causing cell and tissue injury in CHI. To confirm this hypothesis further, studies on HO expression in CHI are needed, and are currently being investigated in our laboratory.

Brain injury is a complex event that includes physical destruction of microvessels, alterations in local and global microcirculation, as well as permeability changes in vessel walls leading to leakage of plasma constituents into the brain microenvironment [19, 24]. Early events following focal brain trauma are influenced by a number of compounds which are released or become activated in and around the primary lesion [24, 26]. These chemical mediators of the inflammatory response include biogenic amines, arachidonic acid derivatives, free radicals, histamine, and bradykinin [21–24, 26, 28, 35].

Various neurochemicals interact in vivo influencing cell and molecular functions in synergy, and play important roles in BBB disturbances, edema formation, and cell injury [1, 22, 26, 28]. And, inhibitors of serotonin, prostaglandin, histamine, and NO synthesis before injury attenuate microvascular permeability disturbances and edema formation [24, 28]. Furthermore, blockade of serotonin synthesis prior to SCI attenuates trauma-induced HO-2 up-regulation in the spinal cord [33], indicating an interaction between serotonin and HO in SCI, and suggesting that serotonin somehow influences trauma-induced HO expression. In the present study, HO inhibition attenuated CHI-induced increases in brain and plasma serotonin levels, supporting the concept that there are interactions among various endogenous substances that are released or affected during secondary injury cascades.

Serotonin is a powerful neurochemical involved in BBB disruption and edema formation [22, 25]. A focal incision into the brain or spinal cord induces profound increases in plasma and tissue serotonin levels [5]. Elevated levels of tissue and blood serotonin therefore influence microvascular permeability disturbances and edema formation following CHI. However, the probable mechanism(s) by which serotonin influences HO production or vice versa is still unclear from this study. Micro-hemorrhages are a known inducer of HO-1 expression [13, 14]. Since platelets are also very rich in serotonin content, the possibility exists that extravasation of blood components into the cerebral compartment somehow contributes to HO expression and vice versa [38]. An increased level of brain serotonin following CHI may also result from breakdown of the BBB and from direct release of amines from central monoaminergic neurons following trauma [22, 24].

That Zn-PP attenuates edema formation and BBB breakdown supports the involvement of HO in CHI pathophysiology. Other studies on the effects of HO inhibitors on cell injury and edema formation following injury in vivo and in vitro are in line with this hypothesis [3, 11–14, 17, 20, 39]. The mechanisms by which HO inhibitors confer neuroprotection are not yet clear. It appears that HO inhibitors exert anti-inflammatory effects, since Zn-PP reduces infarct size and edema following cerebral ischemia [11, 13]. The use of Zn-PP in the present study does not indicate that HO-1 or HO-2 expression is related to cell injury in CHI. Previous reports from our laboratory and others suggest that HO-2 expression is injurious to the cell and that HO-1 up-regulation has some beneficial effects [26]. It would be important to examine HO-1 and HO-2 expression in CHI in Zn-PP treated rats to clarify this point.

Conclusion

Our observations indicate that CHI induces profound brain swelling within a short period (5 hours). This swelling appears to be caused by leakage of plasma proteins through a disrupted BBB. Early intervention with HO-inhibitor Zn-PP significantly attenuated brain edema formation, BBB leakage, and elevated circulation and brain serotonin levels, suggesting that HO and serotonin are working in synergy during the early phase of CHI pathophysiology.

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