Published in final edited form as:

Acta Neurochir Suppl. 2008 ; 105: 7–12.

Bilirubin oxidation products, oxidative stress, and intracerebral hemorrhage

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Summary

Hematoma and perihematoma regions after intracerebral hemorrhage (ICH) are biochemically active environments known to undergo potent oxidizing reactions. We report facile production of bilirubin oxidation products (BOXes) *via* hemoglobin/Fenton reaction under conditions approximating putative *in vivo* conditions seen following ICH.

Using a mixture of human hemoglobin, physiological buffers, unconjugated solubilized bilirubin, and molecular oxygen and/or hydrogen peroxide, we generated BOXes, confirmed by spectral signature consistent with known BOXes mixtures produced by independent chemical synthesis, as well as HPLC-MS of BOX A and BOX B. Kinetics are straightforward and uncomplicated, having initial rates around 0.002 μM bilirubin per μM hemoglobin per second under normal experimental conditions. In hematomas from porcine ICH model, we observed significant production of BOXes, malondialdehyde, and superoxide dismutase, indicating a potent oxidizing environment. BOX concentrations increased from 0.084 ± 0.01 in fresh blood to 22.24 ± 4.28 in hematoma at 72 h, and were 11.22 ± 1.90 in adjacent white matter (nmol/g). Similar chemical and analytical results are seen in ICH *in vivo*, indicating the hematoma is undergoing similar potent oxidations.

This is the first report of BOXes production using a well-defined biological reaction and *in vivo* model of same. Following ICH, amounts of unconjugated bilirubin in hematoma can be substantial, as can levels of iron and hemoglobin. Oxidation of unconjugated bilirubin to yield bioactive molecules, such as BOXes, is an important discovery, expanding the role of bilirubin in pathological processes seen after ICH.

Keywords

Bilirubin oxidation products; edema; reactive oxygen species; pathology; stroke

Introduction

Radical processes are well-known in biochemistry and are proposed to occur in living organisms, creating many known disease states. Oxidation of common metabolic products by oxygen, hydrogen peroxide, free iron, and other simple compounds have been widely studied and reported [16,21–23]. The nature of these oxidation products is not well understood, even though they may have significant physiological impact and lead to subsequent tissue damage.

There is growing interest in the oxidations that occur in intracerebral hematoma after intracerebral hemorrhage (ICH), because there appears to be a compromised region around the hematoma that is refractory to current therapies or even hematoma evacuation [6,9,20,22,26].

We previously reported that bilirubin is oxidized in a hemorrhagic medium and *in vivo* [4,8,12,17]. Bilirubin oxidation products (BOXes), -(1,5-dihydropyrrole-2-ylidene) acetamide and related compounds, are small vasoactive molecules with demonstrated clinical importance [17]. They are found in the cerebrospinal fluid of patients who have had hemorrhagic strokes such as subarachnoid and intracerebral hemorrhage. These compounds exhibit biological activity *in vivo* and *in vitro* and have been postulated to be a major contributor to cerebral vasospasm, a pathological constriction of arteries. BOXes have been synthesized *in vitro* by the oxidation of unconjugated bilirubin at room temperature with a large excess of hydrogen peroxide. The conversion of bilirubin to BOXes is associated with a biochemical state that may cause or contribute to pathological sequelae after ICH. In this report, we outline the conversion of unconjugated (free) bilirubin and other relevant compounds to compounds well-known to have physiological and pathological effects. This was achieved using an *in vitro* system for modeling chemical oxidations, and *in vivo* using a porcine ICH model. Our results suggest that potent oxidations occur in the hematoma, and that the oxidized products penetrate into the perihematoma region with possible detrimental effects.

Experimental methods

Reagents and chemicals

All reagents were American Chemical Society grade or better. Chemicals and biomaterials were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise stated. BOXes were prepared as previously described [4,8,12]. Gasses used were technical grade or better. Optical spectra were obtained on a micro-Quant microwell plate reader using ultraviolet transparent 96-well plates. All optical densities (OD) are reported as uncorrected for plate. Aliquots of the reaction mixture were taken directly and read for OD within a short period of time from sampling and analyzed for kinetics data. Reaction mixture consisted of 100 μ M solubilized bilirubin, 1 g sodium carbonate, 200 mL deionized water, 1 mg human hemoglobin, μ M oxygen, and/or 100 μ L of 30% hydrogen peroxide. Figure 1 is a schematic of how the *in vitro* system was designed. Figures 2A and B show data collected using this reaction system.

Pig surgery and tissue sampling

Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Methods to induce ICH have been previously described [18–20]. Briefly, 3 mL of autologous blood was infused into frontal hemispheric white matter of pentobarbital-anesthetized pigs. Brains were frozen *in situ* with liquid nitrogen at various time points up to 72 h. Approximately 20 mg of perihematoma, edematous white matter was sampled and homogenized in 200 μ L of homogenization buffer, as previously described [11].

Bilirubin determination

Total bilirubin was assayed using a method based on those developed by Michaelsson *et al.* [14] and adapted for use in a microtiter plate, as previously described [17]. Briefly, bilirubin in the sample is first treated with a caffeine solution to release all bilirubin moieties, followed by a diazo reagent to yield a colored product. This is then alkalized until the color is in a range that is not overlapping the absorption range of hemoglobin, and the absorption is recorded at 600 nm. Sample absorbance is compared to a concomitantly run standard curve constructed from commercially available bilirubin standard solutions (Wako Chemicals USA, Inc., Richmond, VA) to determine total bilirubin concentrations.

BOXes determination

BOXes were quantified by spectroscopic analysis at 320 nm, as previously described [17]. Chemically prepared BOXes were used to construct a standard curve. Samples were subjected to a chloroform extraction and evaporation, and re-suspended in 0.9% saline for analysis.

Hemoglobin determination

The sample was exposed to Drabkin's reagent ($\text{NaHCO}_3\text{:K}_3\text{Fe(CN)}_6\text{:KCN}$, 100:20:5) in order to convert all hemoglobin moieties into cyanomethemoglobin [5]. A surfactant (Brij-35) was added to prevent protein- or lipid-induced turbidity. Concentration of cyanomethemoglobin is determined by comparison to a standard curve constructed with commercially available lyophilized hemoglobin (Sigma-Aldrich), treated with Drabkin's reagent, and the absorbance read at 540 nm.

Malondialdehyde (MDA) determination

MDA was assessed using a commercially available assay kit from Calbiochem (San Diego, CA). Briefly, MDA reacts with N-methyl-2-phenylindole in acetonitrile and ferric ions to form a colored molecule that has maximum absorbance at 568 nm [5].

Superoxide dismutase (SOD) assay

SOD activity was measured using a commercially available kit (Fluka, St. Louis, MO) according to the manufacturer's instructions. Upon reaction with superoxide, Donjino's tetrazolium salt, WST-1, produces a water soluble formazan dye with an absorbance of 450 nm [27,28]. Samples were treated with Donjino's and xanthine oxidase to produce superoxide. The percent inhibition of formazan formation by SOD was measured.

Total protein determination

Total protein was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Side chains of several amino acids reduce cupric copper to the cuprous oxidation state, and this change is measured at 562 nm. The total protein concentration is interpolated from a standard curve using bovine serum albumin as the standard.

Statistics

Statistical differences were determined using ANOVA or Student *t*-test where indicated. A *p*-value of ≤ 0.05 was considered statistically significant.

Results

Figures 2A and B indicate the facile conversion of bilirubin (loss of OD at 425 nm) and conversion to BOXes (OD at 310 nm). This conversion is rapid and takes only 5 min at room temperature under the experimental conditions described (Fig. 2B). This indicates that the small molecules of BOXes have extreme membrane permeability and high biological activity, and appear to be formed in the model system under pseudo-physiological conditions. Both peroxide and hemoglobin are necessary for the reaction to occur. Carbon monoxide significantly inhibits the reaction; thus, the reaction involves the heme moiety in the hemoglobin (Fig. 3). These observations are indicative of a free radical reaction. Also, deferoxamine has little effect on the reaction, indicating that free iron is not involved in, or essential for, the reaction. Thus, this *in vitro* reaction produces significant amounts of BOXes in about 10 min. Figure 3 shows that there are both activators and inhibitors of this simple reaction.

Table 1 summarizes the production of BOXes as well as related metabolites produced in and around the hematoma after ICH. Of note is that concentrations of BOXes reach substantial

levels in hematoma and perihematoma white matter on the ipsilateral side. Bilirubin levels are increased in the hematoma as well as perihematoma region and have not reached a plateau at 72 h. Production of MDA appears to plateau at 24 h and is present in both ipsilateral and contralateral brain. SOD activity was consistently highest in the hematoma brain and was relatively constant following hemorrhage. The relative location for obtaining samples presented in Table 1 can be seen in Fig. 4, where we show a representative section of pig brain demonstrating the hematoma, perihematoma region, and contralateral white matter. The edema present in the porcine brain after ICH is visible at 1 h and appears to peak at 48 h (Fig. 5).

Discussion

ICH is a devastating event, with high mortality from the initial hemorrhage, and a tragically high death and disability rate for patients who survive the initial ictus. Complications after ICH include, but are not limited to, increased intracranial pressure, breakdown of blood–brain barrier, clot mass effect, hydrocephalus, necrosis, atrophy, activation of complement, spreading depression, ischemia, and death [2,7,9,10,15,24,25]. Less than 30% of patients who survive an ICH return to normal, and many of the survivors require dependent care [1]. Thus, ICH and complications after ICH are a substantial health burden to the nation and to the families of survivors. Better understanding of the mechanisms associated with the complications will be important in treating these patients and providing hope for improved outcomes.

Our findings include: 1) the chemical environment found after ICH produces a potent oxidizing environment; 2) oxidations in the hematoma produce a cocktail of dangerous metabolites; and 3) these metabolites are quickly found in the perihematoma white matter. Taken together, these data suggest that the chemical environment of the hematoma is a substantial cause of the pathological sequelae seen after ICH, and that damage from the hematoma manifests rapidly after ICH. These 3 major findings and how they contribute to the body of evidence concerning the toxic environment produced by the hematoma are discussed below.

The chemical environment produces BOXes and toxic metabolites

In solution, we found that bilirubin is quickly oxidized in minutes, using several single electron reactions. We were able to confirm significant production of BOXes in the presence of the Fenton reaction as well as a carbon dioxide/carbonate 1-electron donor system (Fig. 3). Importantly, but not surprisingly, we found that there was an apparent pH optimum for this reaction of 6.8. This is a pH that is quite plausible after ICH. Reactive oxygen species can also produce an oxidizing environment to produce BOXes and other metabolites. We found that BOXes are produced with a similar time course when hydrogen peroxide is added to the system as compared to a 1-electron donor system. These data suggest that reactive oxygen species, single electron oxidations, and some radical reactions will produce BOXes. Should BOXes and other toxic metabolites be oxidized in this way, we believe that this oxidizing environment may cause or contribute to the pathological sequelae seen after ICH by allowing these compounds to leech into the perihematoma region and have their toxic effects.

Oxidations in the hematoma produce a cocktail of dangerous metabolites

The potent oxidizing environment seen in the hematoma after ICH can produce substantial concentrations of BOXes and other oxidized metabolites relatively quickly after ICH. These concentrations are in the range that has been reported previously [12,13,17], and are associated with toxicity responses *in vitro* [13]. We also found that substantial amounts of MDA, SOD, and BOXes are produced in the hematoma at 1 h after ICH. These molecules are associated with oxidative damage and are considered to be indicators of hemorrhagic complications. Some of these are also directly biologically active [3].

Metabolites are found in the perihematoma white matter soon after ictus

If one believes that there are toxic compounds produced in the hematoma and that these toxic compounds cause or contribute to the pathological sequelae seen after ICH, it is necessary to assume that these compounds are diffusing out of the hematoma into the surrounding parenchyma. Therefore, we closely examined the concentration of metabolites and compounds in the perihematoma white matter. Ipsilateral brain consistently had elevated levels of BOXes, MDA, and SOD in our studies, with variations in the time course of these metabolites. It is unclear from our data whether the edema and breakdown of the blood–brain barrier is caused by the diffusion of these compounds out of the hematoma, or if these compounds are contributing to this breakdown. There does, nonetheless, seem to be a positive correlation of oxidative damage, oxidative stress, and BOXes with some of the pathological sequelae seen after ICH.

We also believe it is significant that substantial changes in histopathology as well as chemistry can occur within minutes and are manifested within hours. This might be highly relevant when designing strategies to treat these patients. Our data suggests that damage to the perihematoma white matter is occurring in as little as an hour after ICH, and that simple evacuation strategies might be less than successful if they do not address the harmful metabolites that have already diffused from the hematoma.

Conclusions

In this study, we found that a potent oxidizing environment exists in the hematoma after ICH, and that oxidative stress produces a cadre of compounds, some of which are known to be toxic and biologically active. These compounds diffuse into perihematoma white matter and may cause or contribute to the pathological sequelae seen after ICH. The time course for these chemical changes is also quite rapid (many seen in less than 24 h), suggesting that ultra-early intervention may be needed to prevent damage caused by the hematoma after ICH.

Acknowledgments

This work was supported by grants NS050569, NS042308, NS049428 and NS30652 from the National Institutes of Health. ML is supported by the Physician Scientist Training Program PSTP.

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Fig. 1.
Simple oxidization reaction vessel showing *in vitro* system for modeling the oxidizing environment in porcine hematoma

Fig. 2.

Fig. 2A. A solution of 1 g sodium carbonate, 105 μ M solubilized bilirubin, saturating levels of carbon dioxide gas and compressed air, 1 mg human hemoglobin, and 42 mM hydrogen peroxide were allowed to react with constant stirring in an open system. Aliquots of reaction were taken at 0 min (*square*), 5 min (*triangle*), 10 min (*X*), and 30 min (*diamond*). Optical density was followed in the visible region. The pH of this reaction is 6.8

Fig. 2B. The apparent kinetics of bilirubin degradation and BOX production using visible spectroscopy and extinction coefficients for bilirubin and BOXes. The rise in BOXes is apparent within 10 min and plateaus at about 10 min, reaching a steady state. Bilirubin falls at 426 nm, mirroring the production of BOXes

Fig. 3.

Relative amounts of BOX production normalized to control condition A. A = hemoglobin (0.64 μ M), bilirubin (105 μ M), peroxide (383 mM); B = incubation A without hemoglobin; C = incubation without hemoglobin and peroxide; D = incubation A without peroxide; E = incubation A with added deferoxamine (65 mM); F = incubation A with carbon monoxide; G = incubation A with benzoic acid (1.2 mM); H = incubation A with added potassium cyanide (6.5 mM); I = incubation A with added arachidonic acid (4.3 mM); J = incubation A with added oxalic acid (550 mM); K = incubation A with added thiourea (110 mM). Error bars are standard deviation. $N=3$ or more for all conditions. Reactions were performed in phosphate-buffered saline at pH 7.5

Fig. 4. Representative cross-section of porcine brain 16 h after ICH. Breakdown of blood-brain barrier is indicated by Evans blue perfusion. Arrows indicate relative areas of sampling for use in generating data presented in Table 1

Fig. 5. Edema in the pig brain after ICH is evident in 8 h after hemorrhage and appears to peak at 2 days

Table 1

Metabolites present post ICH

	Malondialdehyde (mM/mg protein)	Superoxide dismutase (% activity/mg/mL protein)	Bilirubin (mg/dL)	Bilirubin oxidation products (nmol/g)
Fresh blood	0.37 ± 0.06		4.18 ± 0.08	0.084 ± 0.01
Sham ipsilateral	0.51 ± 0.041	0.16 ± 0.010		
Sham contralateral	0.54 ± 0.021	0.17 ± 0.011		
1 h hematoma	0.088 ± 0.034	0.029 ± 0.0021		
1 h contralateral	1.15 ± 0.45	0.19 ± 0.041		
1 h ipsilateral	0.86 ± 0.030	0.16 ± 0.040		
8–12 h hematoma	0.56 ± 0.038		13.6 ± 0.98	3.44 ± 0.37
24 h hematoma	0.082 ± 0.036	0.039 ± 0.011	20.31 ± 3.32	
24 h contralateral	0.99 ± 0.33	0.174 ± 0.017	0.71	0.78
24 h ipsilateral	0.47 ± 0.14	0.181 ± 0.012	10.35	3.10
48 h hematoma	0.11 ± 0.097	0.034 ± 0.011		11.22 ± 1.90
48 h ipsilateral	0.39 ± 0.20	0.18 ± 0.025		
48 h contralateral	0.96 ± 0.23	0.16 ± 0.030		
72 h hematoma	0.098 ± 0.011	0.027 ± 0.002	40.72 ± 3.03	22.24 ± 4.28
72 h ipsilateral	0.63 ± 0.07	0.25 ± 0.060		
72 h contralateral	0.88 ± 0.16	0.17 ± 0.002		