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Attenuation of oxidative injury after induction of experimental intracerebral hemorrhage in *heme oxygenase–2* knockout mice

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Object. Experimental evidence suggests that hemoglobin degradation products contribute to cellular injury after intracerebal hemorrhage (ICH). Hemoglobin breakdown is catalyzed in part by the heme oxygenase (HO) enzymes. In the present study, the authors tested the hypothesis that *HO-2* gene deletion is cytoprotective in an experimental ICH model.

Methods. After anesthesia was induced with isoflurane, 3- to 6-month-old *HO-2* knockout and wild-type mice were stereotactically injected with 15 μl autologous blood and a group of control mice were injected with an equal volume of sterile saline. Striatal protein and lipid oxidation were quantified 72 hours later using carbonyl and malondialdehyde assays. Cell viability was determined by performing a 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. Following blood injection, the investigators found a 3.4-fold increase in protein carbonylation compared with that in the contralateral striatum in wild-type mice; in knockout mice, the investigators found a twofold increase. The mean malondialdehyde concentration in injected striata was increased twofold in wild-type mice at this time, compared with 1.5-fold in knockout mice. Cell viability, as determined by MTT reduction, was reduced in injected striata to 38 ± 4% of that in the contralateral striata in wild-type mice, compared with 66 ± 5% in *HO-2* knockout mice. Baseline striatal HO-1 protein expression was similar in wild-type and *HO-2* knockout mice, but was induced more rapidly in the former after blood injection.

Conclusions. Deletion of *HO-2* attenuates oxidative cell injury after whole-blood injection into the mouse striatum. Therapies that specifically target *HO-2* may improve outcome after ICH.

KEY WORDS • stroke • intracerebral hemorrhage • free radical • hemoglobin • iron • lipid peroxidation • mouse

CONSIDERABLE experimental evidence supports the hypothesis that tissue injury after ICH may be produced by toxic substances released from the hematoma. One putative neurotoxin is hemoglobin, the most abundant protein in blood, which is released from lysing erythrocytes and in vitro is a potent oxidant.[32] Authors of prior studies have demonstrated that the neurotoxicity of hemoglobin is due at least in part to its breakdown products,[13] particularly iron.[11,20,27]

Release of iron from heme is catalyzed by the HO enzymes in a reaction that also releases equimolar biliverdin and carbon monoxide. Two heme isoforms have been characterized to date in the CNS. Heme oxygenase–1 is induced, mainly in glia, by extravascular blood, hemoglobin, or heme, and by ischemia or trauma.[9,16,19,22,39] Heme oxygenase–2 is constitutively expressed in most central neurons;[17] its level is not altered by HO-1 inducers, although it is upregulated by corticosteroids.[36] The HOs have been identified as therapeutic targets after ICH. Administration of HO inhibitors attenuated edema in perihematomal tissue after injection of autologous blood or hemoglobin[13,14,34] and reduced neuronal death. The interpretation of these key observations is complicated by the myriad nonspecific effects of HO inhibitors, which are non-iron analogs of heme and appear to be quite reactive. In addition to inhibiting HO-1 and HO-2, these compounds also may inhibit the activity of guanyl cyclase, nitric oxide synthase, and caspases.[3,10] Their structural similarity to heme also raises the possibility that

Abbreviations used in this paper: CNS = central nervous system; DNP = 2,4-dintrophenylhydrazone; HO = heme oxygenase; ICH = intracerebral hemorrhage; MTT = 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PCR = polymerase chain reaction.

they may compete with its uptake into CNS cells after release from hemoglobin.

Genetic modification of HO expression provides an alternate, nonpharmacological method for investigating the effect of HO on hemorrhagic CNS injury. To this end, we have established a colony of *HO-2* knockout mice,[24] which are fertile and phenotypically indistinguishable from their wild-type littermates. In prior studies, we have reported that neurons from these knockout mice were more resistant to the toxic effect of hemoglobin in cell cultures and in vivo.[25,28] In the present study, we tested the hypothesis that *HO-2* knockout mice would sustain less tissue injury than their wild-type counterparts after striatal injection of autologous blood.

Materials and Methods

Experimental Animals

The breeding and housing of mice were conducted exclusively at our animal care facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal care and treatments performed were in compliance with the guidelines described in *Principles of Laboratory Animal Care* (NIH publication No. 80-23, revised 1996), and were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. A 12-hour light/dark cycle and unlimited access to food and water were consistently provided.

The *HO-2* knockout mice we used were descended from those produced by Poss et al.;[24] a colony has been maintained at our facility for 7 years. These mice are active, fertile, and grossly indistinguishable from their wild-type littermates. Homozygous *HO-2* knockout and wild-type mice with a B6129 genetic background were used exclusively in this study. Heterozygotes were used for breeding purposes only, and were not included in any experimental group. Genotype was determined by PCR using genomic DNA extracted from tail clippings; the primer sequences have already been published.[28]

Blood Injections

The mice were anesthetized with 2% isoflurane in oxygen, which was delivered with a tightly fitted mask. Blood was collected from a tail vein into a heparinized syringe. After the mice were secured into a stereotactic frame (David Kopf Instruments), a 30-gauge stainless-steel needle was introduced through a bur hole into the right striatum at the following coordinates relative to the bregma: 2 mm lateral, 1 mm anterior, and 3.5 mm below the surface of the skull. Each mouse was then injected with 15 μl blood over 45 minutes via a microinfusion apparatus (2 μl/5 minutes). The injection needle was slowly withdrawn 15 minutes later and the wound was sutured. Mice in the control group were treated in the same manner, but were injected with an equal volume of sterile physiological saline rather than blood.

At 72 hours after injection, the mice were again anesthetized with isoflurane and then killed by cervical dislocation. The brains were rapidly removed and placed in a 60-mm culture dish containing 3 ml Hanks balanced salt solution. Injected and contralateral striata were excised with the aid of a dissecting microscope, the hematoma was removed, and tissue samples were collected for analysis.

Assessment of Cell Injury

Striatal cell viability was assessed using the MTT assay, modified for in vivo use as recently described.[25] The MTT is reduced by viable cells to a colored formazan product, which is then extracted from the cells and quantified by spectrophotometry. The in vivo method makes use of the fact that CNS tissue can be dissociated into individual cells or small groups of cells by gentle trituration without loss of cell viability, as determined by trypan blue exclusion or the ability to reduce tetrazolium salts to formazan. This dissociation technique has been used for many years to prepare primary cell cultures, and ensures that a uniform concentration of tetrazolium is delivered to all cells. In a prior study from our laboratory, this method correlated well with cell counts of neurons in striatal sections.[25]

Striatal tissue was minced with forceps and placed into separate 15-ml centrifuge tubes containing 1 ml Hanks balanced salt solution. Tissue was then dissociated by gentle trituration through a Pasteur pipette, followed by repeated passage through another pipette with a narrowed (flamed) tip. One milliliter of 0.25 mg/ml MTT in Dulbecco modified Eagle medium (Invitrogen) was added to the cell suspension, which was then mixed by brief vortexing. After incubation in a 37°C water bath for 4 minutes, cells were collected using low-speed centrifugation for 2 minutes, and the supernatant was completely removed and discarded. Formazan was then extracted from the cells by adding 2 ml isopropanol containing 0.04 N HCl and vortexing. Cellular debris was removed by centrifugation, and the absorbance of the supernatant was determined at 562 nm with a reference wavelength of 650 nm. The formazan signal in the injected striatum was divided by that in the contralateral striatum and expressed as this ratio.

Protein Oxidation Assay

Injected and contralateral striata were homogenized in ice-cold cell lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM ethylenediaminetetraacetic acid, and 0.1% sodium dodecyl sulfate). After sonication, centrifugation, and protein assay (Pierce BCA kit), proteins were denatured in 12% sodium dodecyl sulfate. Carbonyl groups were then converted to DNP derivatives by treatment with 2,4-dinitrophenyhydrazine using the Oxyblot kit (Chemicon, Inc.) and following the manufacturer's instructions. Proteins were separated on a 12% polyacrylamide gel and were transferred to a polyvinylidene difluoride transfer membrane filter (Imobilon-P, Millipore) using a semidry transfer apparatus (Bio-Rad). Carbonylated proteins were detected with rabbit anti–DNP primary antibody (1:150 dilution; Chemicon) followed by horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin G secondary antibody (1:300 dilution). Immunoreactive proteins were visualized using Kodak ImageStation 400 and SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Malondialdehyde Assay

The injected and contralateral striata were dissociated in a 5% trichloroacetic acid solution, homogenized, and sonicated on ice. After removal of debris by centrifugation, the supernatant was collected, and a thiobarbituric acid/acetate solution was added to a concentration of 0.3% (pH 3.5). The tubes were heated in a boiling water bath for 15 minutes and then cooled to room temperature. Fluorescence was determined using a Perkin–Elmer fluorescence spectrometer (excitation wavelength 515 nm, emission wavelength 553 nm, slit width 5 mm). Fluorescence intensities were compared with those produced by defined malondialdehyde standards to determine sample concentrations.

Cell Counts

After anesthesia had been induced using pentobarbital (75 mg/kg), the mice were perfused with 4% paraformaldehyde in 100 mM phosphate-buffered saline (pH 7.4). Following fixation for 48 hours with 4% paraformaldehyde, the brains were embedded in paraffin. Ten-micrometer sections were cut coronally through the needle entry site and 2 mm anterior and 2 mm posterior to that plane. Sections were then deparaffinized with xylene, rehydrated, and stained with H & E. After examination of sections by light microscopy, 400× images were captured of the 5th, 25th, and 45th sections from the needle entry site. The dimensions of all images were 340 × 250 μm; the inner border was 340 μm from the site of blood injection or situated in the corresponding area of the contralateral striatum. The cells in each image were counted using a grid. Neurons with condensed and/or irregular nuclei or eosinophilic cytoplasm were considered injured.

Immunoblot Analysis of HO-1 and HO-2

Striata were dissociated by trituration and sonicated in an ice-cold lysis buffer as described earlier. Proteins (15 μg/sample) were sepa-

rated on a 15% polyacrylamide gel and transferred onto a polyvinylidene difluoride transfer membrane filter (Immobilon-P, Millipore) using a semidry transfer apparatus (Bio-Rad). After nonspecific sites were blocked by incubating with 5% nonfat dry milk in a buffer containing 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 hour at 37˚C, membranes were incubated at 4˚C overnight with a 1:2000 dilution of rabbit anti–HO-1 or anti–HO-2 (Stressgen Biotechnologies), followed by horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin G (1:2000 dilution) at 37˚C for 1 hour. Immunoreactive proteins were visualized with Kodak ImageStation 400 and SuperSignal West Femto Maximum Sensitivity Substrate.

Statistical Analysis

The data were analyzed using a one-way analysis of variance, followed by the Bonferroni multiple comparisons test to assess the differences between the defined treatment groups. Probability values less than 0.05 were considered significant.

Results

Mice were genotyped at 3 to 5 weeks of age. Wild-type and homozygous knockout mice were easily distinguished by PCR analysis, because the knockout PCR product is 90 bp heavier than the wild-type product and migrated separately on a 2% agarose gel (Fig. 1A). The accuracy of this method was confirmed on HO-2 immunoblotting, which demonstrated the absence of the protein in the knockout mice (Fig. 1B). Consistent with previous observations,[28] baseline HO-1 expression was not altered by *HO-2* gene deletion.

Mice were 3 to 6 months old when they were injected. The mean weight of the wild-type mice prior to injection was 26.9 ± 0.3 g, whereas that of the knockout mice was 26.0 ± 1.1 g. One of 28 wild-type mice and one of 24 knockout mice injected with blood died within the first 24 hours postinjection. No deaths occurred in the mice injected with saline. Seventy-two hours after injection with blood, the animals' mean weight had significantly decreased in both groups of mice: 24.1 ± 0.3 g in the wild-type group and 24.4 ± 0.4 g in the knockout group. There was no significant difference in the pre- and postinjection weights in the mice in the control group after striatal injection of saline rather than blood.

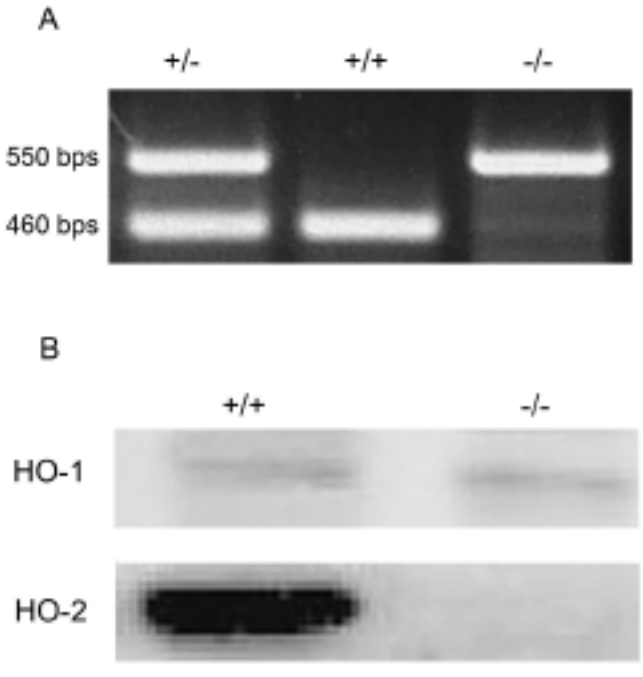

FIG. 1. A: Representative agarose gel demonstrating separate migration of *HO-2* knockout (-) and wild-type (+) PCR products. B: Immunoblots showing no evidence of HO-2 expression in the striatal lysate of a knockout mouse (identified with PCR) and unaltered HO-1 expression.

Increased Striatal Cell Viability After Blood Injection in Knockout Mice

After cell dissociation and suspension, the MTT assay provides an efficient measure of striatal cell viability that correlates well with cell counts in the murine striatum.[25] In wild-type mice, the mean reduction of MTT to formazan in cells from blood-injected striata was 38 ± 4% of that in the contralateral striatum at 72 hours (Fig. 2 *upper*). One wild-

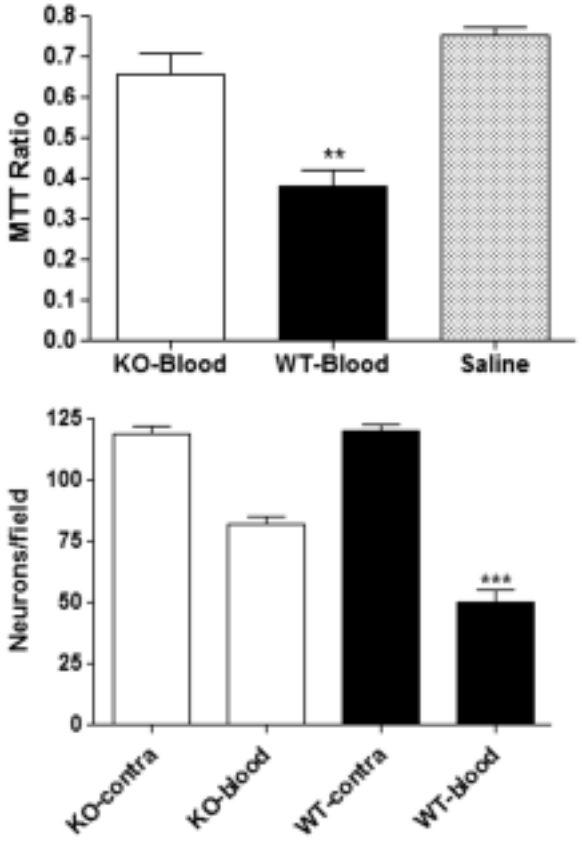

FIG. 2. *Upper:* Bar graph demonstrating that the cytotoxic effect of blood injection is attenuated in *HO-2* knockout mice. Striatal cell viability 72 hours after autologous blood injection in *HO-2* knockout mice (KO), wild-type (WT) mice, and saline-injected control mice (mean ± standard error of the mean [SEM], six or seven mice/condition), as assessed using MTT reduction to formazan. The MTT reduction is expressed as a fraction of that in the hemisphere contralateral to the injection site. **$p < 0.01$ compared with the ratio in knockout mice, Bonferroni multiple comparisons test. *Lower:* Bar graph demonstrating the number of morphologically normal neurons per field (mean ± SEM, five mice/condition) after 72 hours in blood-injected and contralateral (contra) striata. All fields had an inner boundary 340 μm from the injection site. ***$p < 0.001$ compared with the mean number in wild-type mice.

type mouse injected for this cell viability assay died within the first 24 hours and was excluded from this analysis. In the *HO-2* knockout mice, the mean MTT reduction at this time point was 66 ± 5% of that in the contralateral striatum, compared with 77 ± 3% in control mice injected only with saline.

Cell injury was also evaluated by examination of tissue sections that had been stained with H & E. Consistent with the MTT results, the number of morphologically normal neurons in the fields adjacent to the hematoma was significantly increased in knockout mice (Fig. 2 *lower*).

Deletion of the HO-2 *Gene Reduces Lipid Peroxidation After Blood Injection*

Malondialdehyde is a sensitive marker of oxidative injury produced by hemoglobin in vitro and in vivo.[31] In wild-type mice injected with blood, mean malondialdehyde increased approximately twofold compared with its level in the contralateral striata (Fig. 3). In knockout mice, its increase in the injected striata was significantly attenuated. No significant increase in malondialdehyde content was observed in the mice injected with saline only.

Deletion of the HO-2 *Gene Reduces Protein Oxidation*

In previous studies, protein oxidation produced by hemin, as detected with an assay for carbonyl groups, was reduced in cultured neurons from *HO-2* knockout mice compared with neurons from the wild-type control group.[26] We therefore tested the hypothesis that protein oxidation would also be attenuated by *HO-2* gene deletion after striatal blood injection. A 3.4-fold increase in protein carbonyl levels was detected in the striata of wild-type mice injected with blood compared with the level in the contralateral striata (Fig. 4). The level of protein carbonyls in the injected striata of the knockout mice increased only twofold.

Expression of HO-1 After Blood Injection is Delayed in HO-2 *Knockout Mice*

Injection of blood or hemoglobin into the brain is followed by induction of HO-1 in the surrounding tissue.[17] In wild-type mice, HO-1 expression was significantly increased compared with its expression in the contralateral striata at both 24 and 72 hours after blood injection (Fig. 5). In knockout mice, HO-1 expression in the ipsilateral striata was reduced at 24 hours compared with that in the wild-type mice; at 72 hours the two groups had similar levels of expression.

Discussion

The results of the present study provide further experimental support for the hypothesis that HO-2 accelerates tissue injury after CNS hemorrhage. Seventy-two hours after striatal injection of autologous blood, a marked increase in lipid and protein oxidation was observed in the surrounding tissue of wild-type mice, along with decreased cell viability; these injury parameters were significantly attenuated in *HO-2* knockout mice. These results are consistent with our previous observations that *HO-2* gene deletion increases resistance to the oxidative toxicity of hemoglobin in the mouse striatum[25] and in cortical cell culture.[28] They also suggest that the protective effect of HO inhibitors in a variety of hemorrhagic injury models[13,14,34] is due to enzyme inhibition itself, rather than to the nonspecific effects of currently available inhibitors.

These results, however, are in sharp contrast to the protective effect exerted by *HO-2* gene deletion in models that are relevant to ischemia and trauma. Doré and colleagues[7] have reported increased neuronal death in the penumbral area of *HO-2* knockout mice after transient focal ischemia, while Chang et al.[4] have reported a similar effect after cortical impact injury. These dichotomous results are consistent with the hypothesis that HO has both antioxidant and

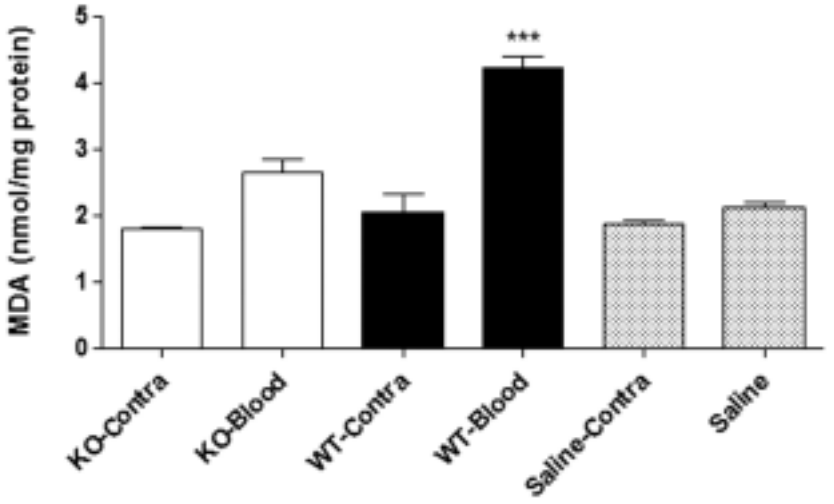

FIG. 3. Bar graph showing attenuation of lipid peroxidation produced by striatal blood injection in *HO-2* knockout mice. Malondialdehyde (MDA) content (mean value ± SEM, four mice/condition) in injected and contralateral striata 72 hours after injection of autologous blood or an equal volume of saline. ***$p < 0.001$ compared with the value in *HO-2* knockout mice, Bonferroni multiple comparisons test.

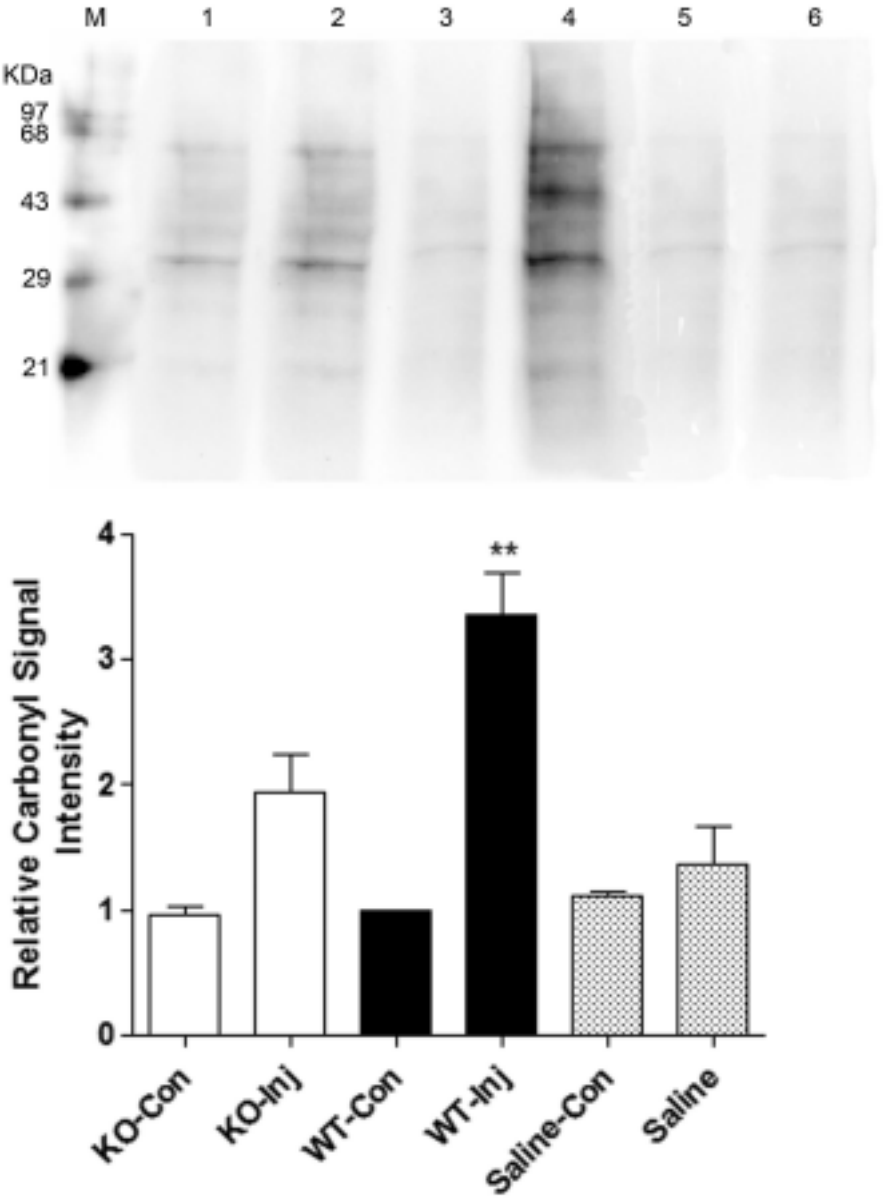

FIG. 4. Striatal protein oxidation after blood injection is reduced in *HO-2* knockout mice. *Upper:* Representative immunoblot of proteins from *HO-2* knockout and wild-type mice 72 hours after injection. Lane M, molecular weight standard with attached DNP residues; Lanes 1 and 2, carbonylated proteins from contra- and ipsilateral striata of a knockout mouse; Lanes 3 and 4, striatal proteins from a wild-type mouse after the same treatment; Lanes 5 and 6, striatal proteins from a mouse injected with saline. *Lower:* Bar graph showing carbonyl signal intensities (mean ± SEM, three to six mice/condition) in injected (Inj) and contralateral striata (Con) 72 hours after blood or saline injection. $^{**}p < 0.01$ compared with mean signal in *HO-2* knockout striata injected with blood.

prooxidant properties.[30] The former may be due primarily to the conversion of a potent oxidant such as oxidized heme (hemin) to the antioxidants biliverdin and bilirubin;[8] the antiinflammatory and cytoprotective effects of low concentrations of carbon monoxide may also contribute.[29] The prooxidant effect of HO-2 in the CNS is probably due, at least in part, to the vulnerability of neurons to iron,[15] which is released in high concentrations after experimental CNS hemorrhage and persists in the tissues surrounding a striatal hematoma for at least several weeks afterwards.[37] The role of iron release in secondary tissue injury after CNS hemorrhage is supported by observations that iron chelators are protective in models of both intracerebal and subarachnoid hemorrhage.[1,20] The potentiation of oxidative injury by HO in models of CNS hemorrhage, as observed in this and other studies,[13,14,34] suggests that the deleterious effect of heme breakdown in the presence of extreme substrate excess after hemorrhage overwhelms other protective mechanisms.

One limitation to using knockout mice in a mechanistic investigation is that cells can compensate for a lack of the protein of interest by increasing expression of another protein with a similar function. This is of particular concern in

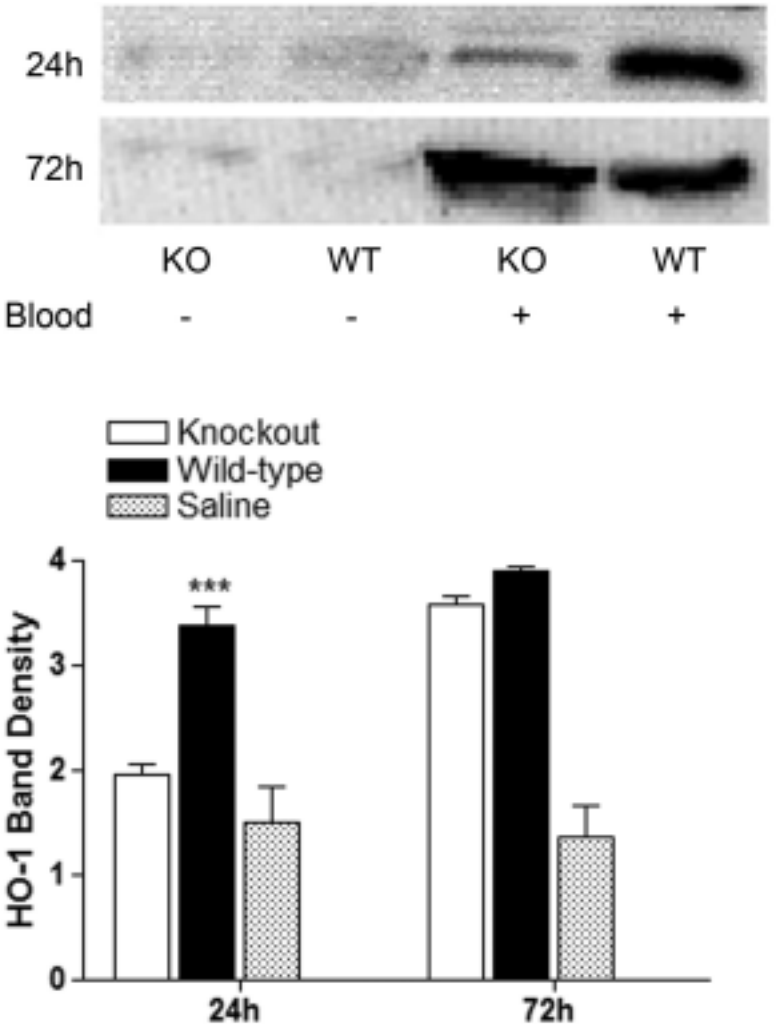

FIG. 5. More rapid induction of HO-1 in injected striata of wild-type mice. *Upper:* Representative immunoblots of injected and contralateral striata of wild-type and *HO-2* knockout mice 24 and 72 hours after blood injection. *Lower:* Mean band densities of injected striata at these time points. Control mice were injected with an equal volume of saline. ***$p < 0.001$ compared with expression in knockout mice at 24 hours postinjection.

HO-2 knockout mice because an alternate isoform, HO-1, is easily induced in most cells, and has been observed to be increased in the lung tissue of *HO-2* knockout mice.[6] However, we have previously reported that HO-1 expression is not increased in *HO-2* knockout neurons and astrocytes at baseline in cell culture.[28] In the present study, HO-1 induction was accelerated in wild-type mice after blood injection, compared with *HO-2* knockout mice. In addition to its induction by heme, HO-1 is induced by oxidative stress due to the presence of the antioxidant response element in the 5′ untranslated region of its gene. Munoz et al.[18] noted rapid induction of HO-1 in the striatum after injection of excitotoxins or surgical corticostriatal deafferentation, and proposed that its early induction is a histochemical marker of tissue injury. The faster induction of HO-1 in wild-type mice is consistent with the more rapid development of striatal oxidative stress in this group.

Although these results support the hypothesis that HO-2 is a therapeutic target after CNS hemorrhage, all currently available inhibitors of this enzyme also block HO-1, the isoform that is induced primarily in glial cells.[16] Although this nonselectivity may serve to further protect neurons from the toxicity of heme degradation products,[28] considerable evidence suggests that HO-1, combined with the induction of ferritin, is a critical component of the defense mechanism of astrocytes and vascular cells against heme.[2,5,23] If this is the case, then the efficacy of nonselective inhibitors may be fairly limited because any benefit to neurons may be negated by exacerbation of glial or vascular injury. To date, authors of published studies of HO inhibitors in small animal models have indicated that their net effect is protective. It is noteworthy, however, that the ratio of neurons to astrocytes in these species is quite high, making injury to the latter difficult to detect due to their relative paucity.[21,33] In humans, the cellular composition of the brain is quite different from that of the rodent or rabbit, because greater than 70% of cells in the middle-aged striatum are astrocytes and other glial cells.[12] It therefore seems essential to test any HO inhibitor under development in large-animal models of hemorrhage prior to any clinical trials in humans. Only one

large-animal study has been conducted to date. Wagner et al.[34] observed that tin mesoporphyrin reduced hematoma volume and edema formation 24 hours after infusion of autologous blood in pigs; however, no effect on lipid peroxidation was observed. These mixed results suggest that further preclinical investigation of HO inhibitors in pigs or other large animal models is indicated.

Other limitations of the murine model are acknowledged. In addition to the different cellular composition of the mouse and human brain, the size of the former necessitated a rather slow blood injection rate of 15 μl over 45 minutes, because at faster rates the relatively small amount of tissue was unable to impede retrograde flow of injected blood around the needle. However, this rate is unlikely to reproduce that of a ruptured arteriole in spontaneous ICH, and so injuries produced by a sudden increase in intracranial pressure were probably minimized in this study. The slow infusion rate also necessitated anticoagulation of collected blood, which may have reduced the component of injury produced by thrombin.[38] As a result, the toxicity of hemoglobin and its degradation products may have contributed a disproportionate amount to the total injury. Local vascular disruption may be best modeled in the mouse brain by injection of bacterial collagenase, which causes hemorrhaging via enzymatic digestion of small vessels. Studies using this method[35] will serve to complement the current approach and should provide further insight into the complex role of HO in the pathogenesis of hemorrhagic CNS injury.

Conclusions

Heme oxygenase–2 increased oxidative cell injury in this murine experimental model of ICH. Pharmacological or genetic therapies that reduce its activity may be protective after clinical ICH. Further investigation in large-animal models that more closely represent the cellular composition of the human CNS seems warranted.

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