Modulation of ABCA1 by an LXR Agonist Reduces Beta-Amyloid Levels and Improves Outcome after Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) increases brain beta-amyloid (A β) in humans and animals. Although the role of A β in the injury cascade is unknown, multiple preclinical studies have demonstrated a correlation between reduced A β and improved outcome. Therefore, therapeutic strategies that enhance A β clearance may be beneficial after TBI. Increased levels of ATP-binding cassette A1 (ABCA1) transporters can enhance A β clearance through an apolipoprotein E (apoE)-mediated pathway. By measuring A β and ABCA1 after experimental TBI in C57BL/6J mice, we found that A β peaked early after injury (1–3 days), whereas ABCA1 had a delayed response (beginning at 3 days). As ABCA1 levels increased, A β levels returned to baseline levels—consistent with the known role of ABCA1 in A β clearance. To test if enhancing ABCA1 levels could block TBI-induced A β , we treated TBI mice with the liver X-receptor (LXR) agonist T0901317. Pre- and post-injury treatment increased ABCA1 levels at 24 h post-injury, and reduced the TBI-induced increase in A β . This reduction in A β was not due to decreased amyloid precursor protein processing, or a shift in the solubility of A β , indicating enhanced clearance. T0901317 also limited motor coordination deficits in injured mice and reduced brain lesion volume. These data indicate that activation of LXR can reduce A β accumulation after TBI, and is accompanied by improved functional recovery.

Key words: A β clearance; amyloid precursor protein processing; beta-amyloid; liver X receptor; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is considered a risk factor for the development of Alzheimer's disease (AD; Bazarian et al., 2009; Heyman et al., 1984). In post-mortem studies of TBI victims, approximately 30% have beta-amyloid (A β) deposits (Roberts et al., 1991). Evidence suggests that these deposits are formed rapidly after injury, occurring within 24 h after the initial impact (Ikonovic et al., 2004). The TBI-induced increase in A β has been replicated in animal models of brain trauma (Iwata et al., 2002; Loane et al., 2009; Stone et al., 2002; Uryu et al., 2002). Not only does A β accumulate, but so do the necessary amyloid precursor protein (APP) enzymes responsible for A β production: BACE1 protein (β -secretase) and the γ -secretase complex protein pre-

senilin-1 (PS-1; Blasko et al., 2004; Chen et al., 2004; Nadler et al., 2008). We have recently found that targeting the APP secretases after TBI reduces lesion volume and improves behavioral outcome in mice (Loane et al., 2009).

A β homeostasis is maintained by balancing production (APP processing) and clearance (A β degradation). Clearance of A β is positively enhanced by activity of the cholesterol efflux transporter ATP-binding cassette A1 (ABCA1). Pharmacological elevation of ABCA1 levels with liver X-receptor (LXR) agonists decreases A β levels *in vivo* and *in vitro* (Burns et al., 2006; Koldamova et al., 2005b; Sun et al., 2003). Recently, three independent groups concurrently published studies on the effects of ABCA1 knockout in APP-transgenic mice. Each group found that A β deposition was increased in ABCA1 knockout mice, despite no change in APP processing

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(Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Similarly, knocking out LXR enhances A β deposition in APP-transgenic mice (Zelcer et al., 2007), again with no appreciable effect on APP processing. Conversely, overexpression of ABCA1 in APP-transgenic mice reduces A β deposition (Wahrle et al., 2008). ABCA1 enhances lipidation of apolipoprotein E (apoE). Lipidated apoE binds to A β and delivers it to microglia for degradation by neprilysin (Fitz et al., 2010; Jiang et al., 2008). The richer the apoE lipidation, the more A β will be delivered for degradation. ABCA1 is the primary factor in the brain that regulates apoE lipidation (Fitz et al., 2010; Jiang et al., 2008).

As elevated ABCA1 enhances A β clearance, we hypothesized that increasing ABCA1 could prevent the TBI-induced increase in A β . To test this we administered an LXR agonist prior to, and following, experimental TBI, and examined A β , ABCA1, inflammatory markers, functional outcome, and lesion volume.

Methods

Controlled cortical impact injury

All procedures were carried out in accordance with protocols approved by the Georgetown University Animal Care and Use Committee. The controlled cortical impact (CCI)-injury device was designed and built at Georgetown University, and consists of a microprocessor-controlled pneumatic impactor with a 3.5-mm-diameter tip. Moderate injury was induced by an impactor velocity of 6 m/sec and deformation depth of 2 mm, as previously described (Loane et al., 2009). C57BL/6J mice (23–25 g) were anesthetized with isoflurane (induction at 4% and maintenance at 2%) in a gas mixture containing 30% oxygen/70% nitrous oxide, and administered through a nose mask. Depth of anesthesia was assessed by monitoring respiration rate and pedal withdrawal reflexes. The mouse was placed on a heated pad, and core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and the surgical site was shaved and cleaned with chlorhexidine diacetate and ethanol scrubs. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned on the surface of the exposed dura, and reset to impact the cortical surface. After injury, the incision was closed with interrupted 6-0 silk sutures, anesthesia was terminated, and the animal was placed in a heated cage to maintain normal core temperature for 45 min post-injury. All animals were monitored carefully for at least 4 h after surgery, and then daily thereafter. Sham animals underwent the same procedure as injury mice, except they did not receive an impact.

Drug administration

The LXR agonist T0901317 (Cayman Chemicals, Ann Arbor, MI) was suspended in 0.5% methylcellulose (Sigma-Aldrich, St. Louis, MO) with 2% Tween-20 (Sigma-Aldrich). The animals received 25 mg/kg administered once daily by oral gavage at a final volume of 5 mL/kg.

Study 1. T0901317 was administered using a pretreatment dosing regimen. Mice were administered either vehicle or T0901317 for 3 days prior to injury, then at 15 min post-injury.

Study 2. T0901317 was administered as a pretreatment as described for study 1, and compared to a post-injury treatment dosing regimen. The post-treatment mice received vehicle for 3 days prior to injury, and either T0901317 or vehicle 15 min post-injury.

Study 3. For behavioral and lesion volume analysis at 7 days post-injury, at 3 days post-treatment mice received either T0901317 or vehicle, and were treated as in study 2. They continued to receive daily administrations of T0901317 until day 7.

The timing of the drug dosing regimens was based on our previous work with T0901317 (Burns et al., 2006; Eckert et al., 2007; Hoe et al., 2007). We have shown that a single oral dose of T0901317 is sufficient to cause changes in ABCA1 at 24 h after injection (Hoe et al., 2007); however, we also included a pretreatment regimen as a proof-of-concept study to ensure full penetration and priming of ABCA1 prior to TBI induction and A β production. For study 3, we administered T0901317 on a daily basis to ensure that ABCA1 remained elevated throughout the critical period of A β elevation (1–3 days). We have previously shown that daily treatment with T0901317 for 7 days maintains elevated LXR-responsive proteins, including ABCA1 (Burns et al., 2006; Eckert et al., 2007).

Tissue homogenization

The ipsilateral cortex was homogenized in 10 volumes of ice-cold tissue homogenization buffer containing 250 mM sucrose, 20 mM Tris-base, 1 mM EDTA, and 1 mM EGTA (pH 7.4), with mammalian tissue protease inhibitor cocktail. The homogenate was mixed 1:1 with 0.4% diethylamine (DEA) and 100 mM NaCl solution using a ground glass pestle in a dounce homogenizer. This mixture was centrifuged at 135,000g for 1 h. The supernatant was removed (DEA-soluble fraction) and neutralized with 10% 0.5 M Tris-HCl (pH 6.8). The pellet was resuspended in RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and protease inhibitor cocktail (pH 7.4) (all chemicals were from Sigma-Aldrich), sonicated for 10 sec, and the supernatant was removed (RIPA-soluble fraction) and frozen until needed for Western blotting. The remaining pellet was resuspended in 1 volume of 70% formic acid, sonicated, and centrifuged at 135,000g for 1 h at 4°C. The supernatant was neutralized in 20 volumes of neutralization buffer containing 1 M Tris-base and 0.5 M Na₂PO₄.

A β and IL-1 β enzyme-linked immunosorbent assay

A β levels were quantified in the DEA and FA soluble fractions using a commercially available ELISA kit (Wako Chemicals, Richmond, VA), which uses three antibodies to specifically detect A β ₄₀ and A β ₄₂ (BAN50/BA27 and BNT77/BA27), as first described by the Yonkin laboratory (Suzuki et al., 1994). A β levels were measured according to the manufacturer's instructions.

IL-1 β was measured from the DEA soluble fraction using a commercially available IL-1 β ELISA (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions. All readings were converted to picomoles per milligram protein.

Western immunoblot analysis

Protein levels were measured using the RIPA soluble fraction. The protein concentration was determined by BCA assay (Pierce, Rockford, IL), and the samples were equalized. Protein samples were resolved by either 7%, 10%, or 4–12% SDS-PAGE gel, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and blocked for a minimum of 1 h in blocking buffer (5% skim milk in PBS containing 0.05% Tween 20 [PBS-T]). The membranes were incubated overnight at 4°C with antibodies for LDLR (1:1000, a kind gift from Dr. Guojun Bu, Washington University, St. Louis, MO), ABCA1 (1:1000; Novus, Littleton, CO), full length APP and APP C-terminal fragment (1:1000 APP-CTF, antibody C1/6.1 against the C-terminus of APP, a gift from Dr. Paul Mathews, Nathan S. Kline Institute, Orangeburg, NY), BACE1 (1:1000; Millipore, Billerica, MA), COX-2 (1:1000; Cayman Chemicals), and β -actin (1:10,000; Sigma-Aldrich), in PBS containing 1% skim milk. The membranes were washed (4×10 min in PBS), and incubated in the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG; 1:3,000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The membranes were washed, and protein complexes were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). Protein bands were quantified by densitometric analysis using QuantityOne Basic software (Bio-Rad). The data presented are expressed as percentages of control.

Motor function evaluation

We evaluated motor recovery of the animals using a modified beam-walking task, a method that is particularly good at discriminating fine motor coordination differences between injured and sham-operated animals (Loane et al., 2009). The modified device consists of a narrow wooden beam 8 mm wide and 120 mm long, which is suspended 300 mm above a tabletop. The mouse was placed on one end of the beam and the number of footfaults for the right hindlimb was recorded over 50 steps. No training periods were performed, and the first trial was performed 1 day after CCI surgery, and again on days 3, 5, and 7. All studies were performed double-blind.

Magnetic resonance imaging

T2-weighted magnetic resonance imaging (MRI) at 7 days was used to measure lesion volume following TBI, as previously detailed elsewhere (Faden et al., 2003). Lesion volume analysis by MRI closely correlates with that calculated from histological sections (Faden et al., 2003). At 7 days after TBI all animals were anesthetized using isoflurane (induction at 4% and maintenance at 1.5%) in a gas mixture containing 30% oxygen/70% nitrous oxide applied through a nose-mask, and subjected to MRI using a Bruker 7T/21 cm Biospec-Avance system (Bruker, Ettingen, Germany). Briefly, the animals were placed in an acrylic glass animal bed with a heating pad warmed to 37°C to maintain body temperature. Respiratory gating to reduce motion artifacts was achieved using a respiratory monitor. The animal bed was positioned so the animal's head was in the center of the magnet within a 72-mm ¹H birdcage resonator (Bruker). Field homogeneity across the

brain was optimized and a sagittal scout image acquired (RARE image, FOV = 4×4 cm, 128×128 resolution, TR/TE 1500/10 msec with a rare factor of 8, making the effective TE = 40 msec). Multi-slice T2-weighted images were then acquired to obtain 16 contiguous slices commencing at the end of the olfactory bulb and working caudally (FOV = 2.5×2.5 cm, slice thickness = 0.75 mm, 256×256 resolution, TR/TE 1500/20 msec, four echo images, and four averages). Lesion volume was estimated from the summation of the damaged areas in each slice (including hypersensitivity), multiplied by slice thickness (ImageJ analysis software, National Institutes of Health). Average lesion volume in cubic millimeters was calculated for each treatment group.

Statistical analysis

Data obtained from independent measurements are presented as the mean ± SE, and analyzed using Student's *t*-test or analysis of variance (ANOVA), followed by the *post-hoc* Newman-Keuls multiple comparison test. For the beam-walk test we used a two-way ANOVA with repeated measures, followed by a Bonferroni *post-hoc* test. All statistical tests were performed using GraphPad Prism software, version 3.02 for Windows (GraphPad Software, Inc., San Diego, CA). Differences were considered significant when *p* < 0.05.

Results

TBI increases endogenous $A\beta_{40}$ and $A\beta_{42}$ levels in non-transgenic C57BL/6 mice

Studies in APP-transgenic mice and other animal models have shown that $A\beta$ increases following experimental TBI (Iwata et al., 2002; Stone et al., 2002; Uryu et al., 2002), and we have recently shown, using non-transgenic mice, that endogenous mouse $A\beta_{40}$ is also increased following CCI (Loane et al., 2009). Here, we measured levels of both endogenous mouse $A\beta_{40}$ and $A\beta_{42}$ in the ipsilateral cortex following TBI. We found that both species of $A\beta$ were increased after brain trauma (Fig. 1A). $A\beta_{40}$ was increased by 87% at 1 day post-injury (*p* < 0.05), and remained elevated through 3 days (92% increase; *p* < 0.05), before returning to baseline levels by 7 days. A similar profile was observed with $A\beta_{42}$, which was increased by 100% at 1 day (*p* < 0.01), remained significantly elevated at 3 days (78%; *p* < 0.05), and returned to baseline by 7 days. These changes in $A\beta$ were accompanied by increases in protein levels of full-length APP (flAPP), and in the production of APP C-terminal fragments (APP-CTF), which are produced following α - and β -secretase cleavage of APP (Fig. 1B). There was a similar increase in protein levels of the β -secretase enzyme 1 (BACE1), as previously reported (Loane et al., 2009; Fig. 1B). These proteins displayed profiles similar to the changes seen in $A\beta$, peaking at 1–3 days post-trauma.

Delayed increase in cortical ABCA1 following TBI

Given the role of ABCA1 in the clearance of $A\beta$, we wanted to measure the levels of this cholesterol transporter following TBI. We also measured another transporter, the low-density lipoprotein receptor (LDLR), a receptor involved in cellular uptake of cholesterol. We found that LDLR increases in the days immediately following injury (32% increase at day 1, and 70% increase at day 3; *p* < 0.05), before dropping down below baseline levels at day 7 (28% decrease; Fig. 1D and E). This

FIG. 1. Traumatic brain injury (TBI) increases amyloid precursor protein (APP) processing, beta-amyloid ($A\beta$), and ATP-binding cassette A1 (ABCA1) levels in non-transgenic C57BL/6 mice. (A) Soluble $A\beta_{40}$ and $A\beta_{42}$ peptides were measured in ipsilateral cortex 1, 3, and 7 days post-TBI. (B) TBI-induced changes in protein levels of full-length APP (flAPP) and APP-C-terminal fragments (APP-CTF) in mouse cortex. The panel on the left shows mouse brain compared to an APP-over-expressing mouse neuroblastoma cell line treated with a γ -secretase inhibitor to accumulate APP-CTF as a positive control. The asterisk denotes a non-specific band in mouse brain that does not occur in cell lines. The panel on the right shows changes in flAPP, APP-CTF, and β -secretase enzyme 1 (BACE1) after injury. (C) ABCA1 and low-density lipoprotein receptor (LDLR) protein levels in the ipsilateral cortex of controlled cortical impact (CCI)-injured mice after 1, 3, or 7 days compared to sham animals. Representative Western blots are shown (* $p < 0.05$, ** $p < 0.01$ versus sham animals by analysis of variance and Neuman-Keuls *post-hoc* analysis; mean $\pm$ standard error of the mean; $n = 3$).

temporal profile was not shared with ABCA1, however, with protein levels unchanged at 1 day post-injury, but increased by 82% at 3 days ($p < 0.01$), and 96% at 7 days post-injury ($p < 0.01$; Fig. 1C).

LXR activation increases ABCA1 protein levels following injury, and prevents the TBI-induced increase in $A\beta$

Our data show that $A\beta$ levels return to baseline as ABCA1 increases, indicative of the role of ABCA1 in $A\beta$ clearance (Fitz et al., 2010; Jiang et al., 2008). We therefore examined whether increasing ABCA1 levels immediately after trauma could alter the TBI-induced increase in $A\beta$ using T0901317. T0901317 is an LXR agonist that we have previously shown to increase cortical ABCA1 levels within 24 h of a single oral administration (Hoe et al., 2007). We administered a dose of 25 mg/kg, which we have previously shown can increase ABCA1 levels without directly impacting baseline levels of cortical $A\beta$ (Burns et al., 2006). We pretreated mice with T0901317 for 3

days prior to injury, and again 15 min after TBI surgery. Ipsilateral cortical tissue was collected 24 h after injury for biochemical analysis. T0901317 administration caused a significant increase in ABCA1 protein levels in sham-injured mice ($p < 0.01$), and TBI-injured mice ($p < 0.01$; Fig. 2A and B). As we saw in Figure 1, TBI also increased $A\beta_{40}$ and $A\beta_{42}$ levels at 24 h post-injury. $A\beta_{40}$ was increased by 46% ($p < 0.05$), and $A\beta_{42}$ levels were increased by 42% ($p < 0.05$; Fig. 2C). However, T0901317 administration attenuated the TBI-induced increase in $A\beta$ ($p < 0.05$; Fig. 2C), such that $A\beta_{40}$ or $A\beta_{42}$ levels were not significantly different between T0901317-treated injured mice and uninjured sham mice. As previously observed in an earlier study, 25 mg/kg T0901317 did not alter $A\beta$ levels in sham-injured mice ($A\beta_{40}$: 11.98 ± 1.5 fmol/mg protein for sham animals versus 11.42 ± 1.6 fmol/mg protein for T0901317 animals; $A\beta_{42}$: 3.9 ± 0.3 fmol/mg protein for sham animals versus 4.36 ± 0.6 fmol/mg protein for T0901317 animals; Burns et al., 2006). To test if the reduction of TBI-induced $A\beta$ by T0901317 was due to changes in APP processing, we probed for protein levels of flAPP,

FIG. 2. T0901317 pretreatment prevents the accumulation of beta-amyloid (Aβ) following traumatic brain injury (TBI). (A) The effects of TBI and T0901317 (T0) on APP processing, BACE1, and ABCA1 protein levels. The asterisk denotes a non-specific band. (B) Bar graph representing quantification of ABCA1 after TBI, and the effect of T0901317 on these changes. (C) Percent increases in Aβ₄₀ and Aβ₄₂ following TBI, and the effects of T0901317 on the Aβ response, as measured by ELISA. Baseline levels are indicated in the results section. Bar graphs representing the effects of T0901317 on the TBI-induced changes in (D) full-length APP (flAPP), (E) APP-CTF, and (F) BACE1 protein levels (**p* < 0.05, ***p* < 0.01 versus sham animals; +*p* < 0.05 versus TBI + vehicle by analysis of variance and Neuman-Keuls *post-hoc* analysis; mean ± standard error of the mean; *n* = 6; ELISA, enzyme-linked immunosorbent assay; BACE1, β-secretase enzyme 1; ABCA1, ATP-binding cassette A1; APP-CTF, APP-C-terminal fragment; APP, amyloid precursor protein).

FIG. 3. T0901317 pretreatment attenuates inflammation markers following traumatic brain injury (TBI). (A) Time course of IL-1 β levels after TBI in mice as quantified by ELISA (* $p < 0.05$ versus sham animals). (B) Effect of T0901317 on the TBI-induced increase in IL-1 β at 1 day post-trauma (** $p < 0.01$ versus sham vehicle animals; ++ $p < 0.01$ versus TBI + vehicle animals). (C) Time course of COX-2 protein levels after TBI in mice (* $p < 0.05$ versus sham animals). (D) Effect of T0901317 (T0) on the TBI-induced increase in COX-2 protein levels at 1 day post-trauma (* $p < 0.05$ versus sham vehicle animals; + $p < 0.05$ versus TBI + vehicle animals; $n = 6$; ELISA, enzyme-linked immunosorbent assay; COX-2, cyclooxygenase-2; IL-1 β , interleukin-1 β).

APP-CTF, and BACE1. Levels of all proteins were significantly increased at 24 h post-injury (flAPP = 63%, $p < 0.05$; APP-CTF = 117%, $p < 0.01$; BACE1 = 74%, $p < 0.01$), and this was not affected by pretreatment with T0901317 (Fig. 2D–F), suggesting a mechanism other than altered APP processing. Levels of soluble A β can also be reduced if there is a shift in A β from soluble to insoluble pools (i.e., fibrilization of A β). To determine if T0901317 was causing such a shift, we measured levels of insoluble A β_{40} after TBI and T0901317 treatment. Insoluble A β_{40} was barely detectable in sham-injured mice (0.0983 ± 0.019 fmol/g tissue), or in TBI mice at 24 h (0.1123 ± 0.025 fmol/g tissue). T0901317 had no effect on insoluble A β_{40} levels after TBI (0.0886 ± 0.041 fmol/g tissue). These data indicate that T0901317 prevents the accumulation of A β following TBI, independent of any effects on APP processing or shifts in solubility.

T0901317 reduces inflammation following TBI

Central inflammatory and immune responses contribute to secondary tissue damage after TBI. The proinflammatory cytokine interleukin-1 β (IL-1 β) is produced in mice within hours of injury, and is a key promoter of neuroinflammation. IL-1 β -mediated neuronal damage does not result from the cytokine itself, but rather from its ability to activate other proinflammatory mediators, such as cyclooxygenase-2 (COX-

2). In our model system we measured IL-1 β at 1, 3, and 7 days post-trauma, and found that IL-1 β was increased by 53% at 1 day after injury ($p < 0.05$; Fig. 3A), and had returned to baseline levels by day 3. As LXR agonists have been shown to have anti-inflammatory properties (Joseph et al., 2003), we next examined if T0901317 could decrease markers of inflammation in our trauma model. We measured IL-1 β in the cytosolic fraction and found that at 24 h post-injury, TBI caused a 40% increase of IL-1 β in vehicle-treated mice ($p < 0.01$). Pretreatment with T0901317 reduced this TBI-induced IL-1 β increase, such that levels were indistinguishable from IL-1 β levels in sham-treated mice ($p < 0.01$; Fig. 3B). Similarly, TBI increased COX-2 levels at 1 and 3 days after TBI ($p < 0.05$; Fig. 3C), and T0901317 pretreatment reduced COX-2 levels at 24 h after injury compared to vehicle-treated mice ($p < 0.05$; Fig. 3D).

Post-injury administration of T0901317 reduces the A β response, but not the IL-1 β response, after TBI

We have shown that a 3-day pretreatment with T0901317 can modulate cholesterol transport protein expression, reduce A β levels, and attenuate the production of proinflammatory mediators following CCI in mice. In order to ascertain if T0901317 would make a plausible treatment for TBI, we performed a second drug treatment study that repeated our

FIG. 4. Post-injury administration of T0901317 reduces A β , but not IL-1 β , following traumatic brain injury (TBI). T0901317 was administered either for 3 days prior to TBI (pre), or as a single injection 15 min after TBI (post). The ipsilateral cortex was collected 24 h after injury. (A) The effects of these regimens on protein levels of ABCA1 and LDLR (* p < 0.05; ** p < 0.01 versus TBI + vehicle animals). (B) The effects of T0901317 treatment regimens on TBI-induced A β ₄₀ levels. Data are shown as percent increase over sham animals. Sham A β ₄₀ levels were 5.7 ± 0.54 fmol/mg protein (* p < 0.05; ** p < 0.01 versus TBI + vehicle animals). (C) The differential effects of pre- and post-injury treatment regimens on TBI-induced IL-1 β levels (* p < 0.05 versus sham + vehicle animals; + p < 0.05 versus TBI + vehicle animals). (D) Effects of TBI and T0901317 treatments on APP processing. The asterisk denotes a non-specific band (mean $\pm$ standard error of the mean; n = 5; IL-1 β , interleukin-1 β ; ABCA1, ATP-binding cassette A1; LDLR, low-density lipoprotein receptor; A β , beta-amyloid; APP, amyloid precursor protein; APP-CTF, APP-C-terminal fragment; flAPP, full-length APP).

above experiments, but in addition to a 3-day pretreatment group, we also included a post-injury treatment group. For the post-injury treatment group we treated the mice with vehicle for 3 days prior to injury, and T0901317 or vehicle was administered 15 min after surgery. Mice were euthanized 24 h after surgery for measurement of A β ₄₀, ABCA1, and IL-1 β .

Both pretreatment (p < 0.01) and post-injury treatment (p < 0.05) with T0901317 increased levels of ABCA1 in the ipsilateral cortex of injured mice at 24 h (Fig. 4A). Neither treatment regimen had an effect on LDLR levels (Fig. 4A). We again saw increased A β ₄₀ levels in TBI mice (175% increase

compared to sham animals; p < 0.01; Fig. 4B), with both pre- and post-injury treatment attenuating this increase in A β ₄₀ by approximately 40% (p < 0.01 for pre-injury treatment; p < 0.05 for post-injury treatment; Fig. 4B). While both treatment schedules had similar effects on ABCA1 and A β ₄₀ levels, this was not the case when we examined the proinflammatory cytokine IL-1 β . Levels of IL-1 β were increased after TBI (48% increase compared to sham animals; p < 0.05). While T0901317 pretreatment completely blocked this response (p < 0.05), post-injury treatment had no effect on IL-1 β levels after injury (Fig. 4C). We also found that T0901317 had no effect on flAPP or APP-CTF levels after TBI (Fig. 4D).

FIG. 5. T0901317 attenuates behavioral deficits and reduces lesion volume following traumatic brain injury (TBI). **(A)** Fine motor coordination deficits were quantified using a modified beam-walking test. Hindlimb foot placement was recorded and the number of mistakes (footfaults) were recorded from 50 steps ($***p < 0.001$ versus TBI + vehicle animals by repeated-measures two-way analysis of variance [ANOVA] and Bonferonni *post-hoc* analysis; $n = 13$ – 14 for the TBI + vehicle and TBI + pretreatment [pre] groups; $n = 6$ for all other groups; post, post-treatment). **(B)** The lesions were imaged using a 7-Tesla magnetic resonance imaging (MRI) device. 2T-weighted images were generated, and the lesion area can be seen as a hyperintense white region in the upper left cortical area. Representative images from the TBI groups are shown. **(C)** Lesion quantification by MRI ($**p < 0.01$ versus vehicle animals by ANOVA and Neuman-Keuls *post-hoc* analysis; mean $\pm$ standard error of the mean; $n = 8$ for the TBI + T0901317 pretreatment group and the TBI + vehicle group; $n = 5$ for the TBI + T0901317 treatment group).

T0901317 improves functional recovery and reduces lesion volume in TBI mice

Given T0901317's ability to reduce inflammation and A β levels after injury, we wondered if T0901317 could improve TBI-induced behavioral deficits. Beginning with the same pre- and post-injury treatment paradigm as that described above, we continued daily dosing of T0901317 for 7 days after injury, and assessed motor coordination using a beam-walking test. The beam-walking task is a behavioral test that is particularly good at discriminating fine motor coordination differences between injured and sham-operated animals (Loane et al., 2009). Mice were placed on a 8-mm-wide beam and the number of footfaults for the right hindlimb was recorded

during 50 steps counted in either direction on the beam. The mice were not trained prior to injury, with the first trial occurring 24 hours after surgery, and testing was repeated on days 3, 5, and 7. Sham-injured mice were able to perform this task at all time points, having < 5 footfaults on each day of testing (Fig. 5A). T0901317 did not alter the ability of sham-injured mice to perform this test. Brain trauma impairs the ability of mice to perform this task, and vehicle-treated TBI mice made 49 ± 1 footfaults the day after injury ($p < 0.001$ versus sham vehicle animals). Over the 7-day trial period these mice improved slightly, but their performance was still deficient compared to sham-injured mice, with 38 ± 3 footfaults ($p < 0.001$ versus sham vehicle animals). Pretreatment with T0901317 did not improve performance of injured ani-

imals on the day after injury (49.0 ± 0.2 footfaults); however, these mice had significantly fewer faults on day 7 of the trial (22 ± 2 footfaults; $p < 0.001$ versus vehicle + TBI animals; Fig. 5A), indicating that pretreatment with T0901317 significantly improved recovery of fine motor coordination. Mice treated with T0901317 after injury were also impaired on the day after injury (50 ± 0 footfaults), and these mice showed a trend toward improvement in fine motor coordination over 7 days (30 ± 5 footfaults), but it did not reach statistical significance (Fig. 5A).

Following the final behavioral trial, mice from each treatment group were randomly chosen and imaged using 7-Tesla MRI. Multi-slice, multi-echo T2-weighted images were obtained of contiguous slices through the brain, from the end of the olfactory bulb to the cerebellum. Figure 5B shows representative MRI images from a vehicle-treated mouse, a mouse pretreated with T0901317, and a mouse post-treated with T0901317. The bright areas of hypersensitivity outside of the ventricular areas represent edema and damaged tissue. In T0901317 pretreated mice, the damaged cortical area was smaller than in vehicle-treated mice, and there was a marked decrease in hypersensitivity in the surrounding areas, resulting in a decrease in TBI-induced lesion volume of 40% ($p < 0.01$; Fig. 5C). Similarly, post-treatment with T0901317 resulted in less damage and edema in the injured cortex, and reduced the TBI-induced lesion volume by 55% ($p < 0.01$; Fig. 5C).

Discussion

In this study we found that the LXR agonist T0901317 attenuated the TBI-induced increase in A β without significantly altering APP processing or A β solubility. Rather, it appears that T0901317 enhances clearance of A β after TBI. Furthermore, treatment with T0901317 improved functional recovery after TBI and significantly reduced brain lesion volume.

The precise role of A β following TBI is unknown. In studies of the injured human brain, performed post-mortem or on survivors, accumulation of A β was found in approximately 30% of all patients, and as early as 2 h after injury (Ikonomovic et al., 2004; Roberts et al., 1991). Whether the A β increase seen in these cases contributed to neuronal or patient death is unknown. However, the toxicity of A β has been well documented. It is implicated as the primary neurotoxic factor in Alzheimer's disease pathogenesis, and is toxic to neurons (Yankner et al., 1989), endothelial cells (Thomas et al., 1996), astrocytes (Brera et al., 2000), and vascular smooth muscle cells (Davis-Salinas et al., 1995). A β induces the production of cytokines and reactive oxygen species in microglial cells (Combs et al., 2001), increases sensitivity of primary neurons to excitotoxic damage (Mark et al., 1995; Mattson et al., 1992), and A β -mediated cell death demonstrates morphological and molecular features of apoptosis (Imaizumi et al., 1999). Therefore, the increased production of A β after brain trauma may be involved in numerous secondary injury cascades that contribute to neurological dysfunction after injury, and preventing A β production may be a novel therapeutic strategy for the treatment of TBI.

We have recently reported that targeting the APP secretases after TBI can improve functional recovery, prevent hippocampal neurodegeneration, and reduce lesion volume (Loane et al., 2009). Other reports have also explored the relationship between A β and lesion size following TBI in ro-

dents. Abrahamson and colleagues used the pan-caspase inhibitor BAF to prevent caspases-induced cleavage of APP into A β following injury (Abrahamson et al., 2006). They successfully reduced A β levels 24 h after TBI, and reduced lesion volume 7 days following injury. Another study examined the effects of an apoE-mimetic compound on A β and behavior following closed head injury in rodents (Wang et al., 2007). Their drug reduced trauma-induced A β_{42} , and significantly ameliorated motor deficits following trauma. Finally, a recent report shows that simvastatin administered 3 h after injury can reduce A β levels in an APP-transgenic mouse model (Abrahamson et al., 2009). It should be noted that none of these earlier studies, or the present study, can definitively lead one to conclude that A β is mediating secondary injury after TBI, and it may be that any A β -lowering effects are secondary to cell survival. However, what can be concluded from these studies is that A β is an excellent indicator of functional and histological outcome following TBI.

We examined the profile of APP processing and A β accumulation in non-transgenic mice following TBI. We found that the accumulation of A β is a transient process in these mice, and that it returns to baseline levels by 7 days post-injury. This is the first profile of both A β_{40} and A β_{42} in non-transgenic mice, and these results may help clarify what has previously been reported in mice overexpressing human APP. CCI in PDAPP mice causes a brief spike in A β_{40} and A β_{42} , peaking at 2 h post-injury, and returning to baseline by 6 h (Smith et al., 1998). Longer studies in platelet-derived growth factor promoter expressing APP (PDAPP) mice have shown that CCI can actually decrease deposition of A β in the ipsilateral cortex and hippocampus at 4–8 months after injury compared to the uninjured side of the brain (Nakagawa et al., 1999, 2000). Single and repetitive mild CCI injuries in Tg2576 mice caused elevated levels of soluble and insoluble cortical A β_{40} and A β_{42} (Uryu et al., 2002). Finally, studies of APP^{NLh/NLh} mice, a gene-targeted mouse that expresses normal levels of human APP, have elevated A β_{40} only through the first 24 h after CCI, while A β_{42} levels remained elevated through 14 days (Abrahamson et al., 2006). As studies in transgenic mice have yielded variable results, we focused on non-transgenic mice in our studies. However, there are significant differences between rodent A β and human A β that need to be taken into consideration. Rodent A β does not deposit as amyloid plaques in non-transgenic mice, although it is able to form beta-sheet fibrils *in vitro* (Fraser et al., 1992), albeit not as aggressively as human A β can (Boyd-Kimball et al., 2004). Rodent A β also differs from human A β at 3 amino acid sites: arginine 5, tyrosine 10, and histidine 13 (Yamada et al., 1987). These changes are thought to reduce the ability of rodent A β to reduce Cu (II) to Cu (I), and thus rodent A β is perceived to lack oxidative stress properties. However, rodent A β can still induce protein oxidation and lipid peroxidation in primary neurons, and can trigger apoptosis and cell death (Boyd-Kimball et al., 2004), although at a slower rate than human A β . Thus the differences seen in A β accumulation after TBI in non-transgenic mice compared to APP transgenic mice and human TBI cases may be due to the reduced ability of mouse A β to aggregate as aggressively as human A β . As A β aggregation has been reported in 30% of human cases, translating treatments that target A β from non-transgenic mice to the clinic should be preceded by testing in APP transgenic mouse models.

In the present study, we found that T0901317 reduced A β levels in the absence of any changes in APP processing, or any shift of A β into the insoluble fraction. These data indicate that T0901317 increases A β clearance after TBI. Our findings are supported by multiple studies in APP transgenic mice in which ABCA1 was ablated. These mice had increased A β deposition in the absence of altered APP processing (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Conversely, overexpression of ABCA1 in APP transgenic mice reduces A β deposition (Wahrle et al., 2008). The mechanism behind the role of ABCA1 in A β clearance has recently been shown to be an indirect one, involving apoE as a mediator (Fitz et al., 2010; Jiang et al., 2008). The degradation of A β extracellularly (by insulin-degrading enzyme), and intracellularly (in microglia by neprilysin), is greatly enhanced by apoE; however, the ability of apoE to facilitate A β degradation is dependent on its lipidation status (Jiang et al., 2008). ABCA1 is essential for the proper lipidation of apoE, and has been shown to mediate the effects of T0901317 on A β degradation by microglia (Fitz et al., 2010).

LXR agonists have previously been shown to be neuroprotective in multiple models of neurodegeneration, including spinal cord injury (Paterniti et al., 2010), stroke (Morales et al., 2008; Sironi et al., 2008), and Niemann-Pick type C (NPC) disease (Repa et al., 2007). In spinal cord injury, administration of T0901317 was found to reduce spinal cord inflammation and tissue injury (Paterniti et al., 2010). Following experimental stroke, post-injury administration also reduced the infarct volume, spared cortical tissue, reduced proinflammatory gene expression, and improved neurological scores in rats (Morales et al., 2008). Another group has demonstrated that a single administration of another LXR agonist (GW3965) can reduce lesion volume and improve behavioral outcome (including performance on the balance beam) at 24 and 72 h post-ischemia (Sironi et al., 2008). In NPC mice, administration of T0901317 significantly extended life expectancy, improved motor function, and returned activated microglia to their resting state (Repa et al., 2007). In this article we have examined similar outcome measures, and found improvements in behavioral recovery and reductions in lesion volume. An interesting addendum is that enhanced brain A β levels occur in NPC disease, stroke, and spinal cord injury (Burns et al., 2003; Kobayashi et al., 2010; Zhang et al., 2007), but the ameliorating effect of T0901317 on A β has not been assessed in any of these models.

In vitro reports have shown that IL-1 β can increase mRNA expression and regulate transcription of APP (Forloni et al., 1992; Goldgaber et al., 1989). It is unclear if a similar mechanism is responsible for the increase seen in APP after TBI, but a previous report has demonstrated that the increase in IL-1 β occurs prior to the increase in APP protein, suggesting that IL-1 β is temporally and spatially available to regulate APP metabolism after trauma (Ciallella et al., 2002). In our study we found that pre-injury treatment with T0901317 significantly attenuated the trauma-induced IL-1 β response, without an effect on the trauma-induced APP response. These data suggest that IL-1 β does not regulate the production or transcription of APP after injury. We did find that pretreatment with T0901317 successfully inhibited the TBI-induced IL-1 β increase in two separate studies; however, post-injury treatment did not. As both treatments successfully reduced A β ₄₀

levels, it appears that the APP and IL-1 β pathways are not necessarily dependent on each other after TBI.

In conclusion, we have shown that TBI increases A β ₄₀ and A β ₄₂ in the cortex of injured, non-transgenic mice. Treatment with an LXR agonist enhances ABCA1 levels, and reduces the TBI-induced A β peak. These biochemical changes are accompanied by improvements in functional outcome and a reduction in brain lesion volume. These data, along with the effects of T0901317 seen in other models of neurodegeneration, suggest that LXR agonists are neuroprotective in multiple models of CNS dysfunction.

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Author Disclosure Statement

No competing financial interests exist.

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