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Authors

Trexler, Andrew W
Knudsen, Gabriel A
Nicklisch, Sascha CT
et al.

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2,4,6-Tribromophenol Exposure Decreases P-glycoprotein Transport at the Blood-Brain Barrier

Andrew W. Trexler[1], Gabriel A. Knudsen[1], Sascha C. T. Nicklisch[2,3], Linda S. Birnbaum[1], Ronald E. Cannon[1]

[1]NCI Laboratory of Toxicology and Toxicokinetics, Research Triangle Park, NC, USA. [2]Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA. [3]Environmental Toxicology Department, University of California Davis, Davis, CA, USA.

Corresponding author: Ronald E. Cannon, Ph. D

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Telephone: 984-287-3937

Email: cannon1@niehs.nih.gov

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Abstract

2,4,6-Tribromophenol (TBP, CAS No. 118-79-6) is a brominated chemical used in the production of flame-retardant epoxy resins and as a wood preservative. In marine environments, TBP is incorporated into shellfish and consumed by predatory fish. Food processing and water treatment facilities produce TBP as a byproduct. TBP has been detected in human blood and breast milk. Biologically, TBP interferes with estrogen and thyroid hormone signaling, which regulate important transporters of the blood-brain barrier (BBB). The BBB is a selectively permeable barrier characterized by brain microvessels which are composed of endothelial cells mortared by tight-junction proteins. ABC efflux transporters on the luminal membrane facilitate the removal of unwanted endobiotics and xenobiotics from the brain. In this study, we examined the *in vivo* and *ex vivo* effects of TBP on two important transporters of the BBB: P-glycoprotein (P-gp, ABCB1), and Multidrug Resistance-associated Protein 2 (MRP2, ABCC2), using male and female rats and mice. TBP exposure *ex vivo* resulted in a time- (1-3 h) and dose- (1-100 nM) dependent decrease in P-gp transport activity. MRP2 transport activity was unchanged under identical conditions. Immunofluorescence and western blotting measured decreases in P-gp expression after TBP treatment. ATPase assays indicate that TBP is not a substrate and does not directly interact with P-gp. *In vivo* dosing with TBP (0.4 μmol/kg) produced decreases in P-gp transport. Co-treatment with selective Protein Kinase C (PKC) inhibitors prevented the TBP-mediated decreases in P-gp transport activity

Introduction

2,4,6-Tribromophenol (TBP, CAS No. 118-79-6) is an environmentally persistent brominated chemical used in the production of brominated flame retardant (BFR) epoxy resins and as a wood preservative [1, 2]. TBP is one of the most widely produced brominated phenol with production volume estimated at ~10,000 tons/yr in both 2001 and 2012 [1, 3]. Anthropogenic production and marine algae secretion of TBP lead to its release into the environment where it exists as a vapor and particulate in both salt and fresh water [1]. TBP has been found in indoor air in excess of 600 pg/m^3 with the areas of highest average concentrations being office computer rooms (310 pg/m^3), workplace dining areas (230 pg/m^3), and houses (120 pg/m^3) [4]. TBP released into water has a half-life of 20-40 days, while TBP released into air partitions into soil where it persists [1]. Inhalation, dermal, and oral disposition and kinetics studies suggest that TBP is readily absorbed by mammals and has a half-life of 4 h in rats [5]. Dietary exposure to TBP was detected in breast milk and serum in Japan [6]. In southeast India, average serum concentrations of TBP in electronics waste recyclers was measured at 0.3±0.2 pmol/mL (0.9 pmol/mL, maximum concentration), while coastal residents with high pelagic fish intake had an average serum concentration that was more than 3 times higher (mean: 1±0.8 pmol/mL, maximum: 3.6 pmol/mL) [7]. TBP interacts with both thyroid and estrogen signaling [8-10]. TBP disruption of thyroid homeostasis occurs primarily through dysregulation of thyroid mediated gene expression and enzyme inhibition, while TBP is thought to be antagonistic to estrogen receptor signaling pathways. Thyroid and estrogen signaling are also known to modulate P-glycoprotein (P-gp, ABCB1) transport activity [11].

P-gp and Multidrug Resistance-associated Protein 2 (MRP2, ABCC2) are highly conserved ATP Binding Cassette (ABC) transporters that actively restrict the access of toxic endobiotics, drugs and xenobiotics to sensitive tissues. P-gp and MRP2 are present in barriers at the brain, intestine, kidneys, liver, testes, ovaries, and placenta [12, 13]. An important quality of P-gp and MRP2 resides in their ability to transport a large and structurally diverse range of chemicals. Interestingly, the regulation of their expression and activities are linked with the cytochrome P450s. This linkage is logical and offers insight to why oral absorption and drug elimination is altered or impaired when transport function is inhibited or dysregulated [14]. This association is especially important for P-gp in intestine enterocytes and liver hepatocytes. The broad range of substrates for P-gp, of which some are shared with MRP2, include most of the widely prescribed drugs used for immunosuppression, chemotherapy, hypertension, allergies, infection and inflammation [14]. Endogenous substrates of P-gp include lipids, steroids, bilirubin, bile acids, platelet activating factor (PAF) and numerous dietary flavonoids [14]. With respect to the BBB, P-gp and MRP2 with other ABC transporters, share the essential role of preventing drugs, metabolites, xenobiotics and bile conjugates from reaching toxic levels in the brain. Exposure to BFRs are known to alter the expression of ABC transporters in the liver, and TBP alters ABCB5 transport in murine melanoma cells B16F1 [15, 16]. At the blood-brain barrier (BBB), increases in P-gp transport activity reduces the efficacy of its associated pharmaceutical substrates. Alternatively, decreases in transporter activity impact the protective function of the BBB and can lead to neurotoxicity. This is best exemplified by Ivermectin, an antiparasitic P-gp substrate associated with neurotoxicity and death in animals with impaired P-gp function [17].

ABC transporter activity is modulated by several signaling pathways that TBP is known to interact with. Aberrant Thyroid Stimulating Hormone (TSH)signaling associated with hypo- and hyperthyroidism has been linked to aberrant levels of tumor necrosis factor-α (TNF-α) [18]. Furthermore, TNF-α signaling downstream through PKC produces a time-dependent biphasic response in P-gp activity at the BBB [19-23]. Several BFRs modulate TNF-α, including tetrabromobisphenol A (TBBPA) which modulates P-gp activity in a sex-specific manner at the BBB [24-27]. In addition to interfering with thyroid signaling, BFRs are known to interact with estrogen signaling and phase I and phase II chemical metabolism pathways [28]. TBP displaces 43% of 17-ß-estradiol (E2) in estrogen-dependent cells at 1 M TBP [10, 29, 30].

To investigate the impact of TBP on two transporters at the BBB, we utilized an *ex vivo* transport assay to measure changes in P-gp and MRP2 transport activity in purified, functional rat brain microvessels [31]. Using this transport assay, we characterized dose- and time-dependent responses in brain microvessels in rats and mice of both sexes and explored possible mechanisms for these changes. Western blots and immunofluorescence were used to quantify protein expression changes after exposure to TBP.

Materials and Methods

Materials

P-gp fluorescent substrate [N-ε-(4-Nitrobenzofurazan-7-yl)-D-Lys^{8}] cyclosporine A (NBD-CSA) was custom-synthesized by R. Wenger (Sandoz, Basel, Switzerland)[32]. TBP (99% pure), MRP2 fluorescent substrate Texas Red (Sulforhodamine 101), corn oil, and ß-actin mouse monoclonal antibody A1978 were purchased from Sigma Aldrich (St. Louis, MO). P-gp inhibitor PSC-833, pan-PKC inhibitor GF109203X, were purchased from Tocris Bioscience (Bristol, United Kingdom). PKC $ß_{I+II}$ inhibitor LY333531, PKC $α+ß_{I}$ inhibitor GO6976, and P-gp rabbit monoclonal antibody ab170904 were purchased from Abcam (Cambridge, United Kingdom). Secondary antibodies Alexafluor 647 goatαmouse IgG and Alexafluor 647 goatαrabbit IgG were purchased from Thermo Fisher Scientific (Waltham, MA). IRDye® 800CW goatαrat IgG was purchased from Licor (Lincoln, NE). Western tissues were processed in CelLytic MT Mammalian Tissue Lysis/Extraction Reagent with complete Mini protease inhibitor. Western blotting materials, including 10-well Invitrogen NuPAGE 4-12% Bis-Tris Gels NP0321, Novex Nitrocellulose membranes LC2001, and the immunofluorescence antibodies, P-gp mouse monoclonal antibody C219 and Alexa Fluor 488 goatαmouse IgG antibody, were obtained from Thermo Fisher (Waltham, MA).

Animals

The animal experiments were approved by the Animal Care and Use Committee at the National Institute of Environmental Health Sciences according to the guidelines from the National Institutes of Health. All data are reported in compliance with the Animal Research Reporting *In Vivo* Experiments (ARRIVE) guidelines. Male and female Hsd: Sprague

Dawley® (SD) rats (age 12-50 weeks) were purchased from Envigo (Raleigh, NC). Rats were used due to their tissue availability and to match previous work. Male and female C57BL/6J mice (age 14-18 weeks) and male estrogen receptor alpha (ERα) knock-out mice (age 15 weeks) derived from the C57BL/6J strain were a kind gift from the laboratory of Dr. Ken Korach of the NIEHS, RTP, NC [33, 34]. Animals were housed in an AAALAC-approved animal care facility (~49% humidity, ~72ºF room temperature, 12 h light/dark cycle, polycarbonate shoebox cages (Techniplast, West Chester, PA, USA) and Sani-Chip bedding (PJ Murphy Forest Products, Montville NJ, USA) for at least 1 week prior to use and allowed food (NIH #31) and water (Durham, NC) *ad libitum*. Animals were euthanized by CO_2 inhalation followed by decapitation.

Ex vivo Transport Assay

Brain tissue (N=3-6 animals) was immediately harvested following euthanasia and placed in 1x PBS (pH 7.4) supplemented with 900 mg/L of glucose and 110 mg/L of sodium pyruvate on ice. Capillaries were isolated as described previously [31]. Briefly, gray matter of the cortex was minced, lipid fraction was removed by gradient centrifugation, capillaries were captured by 30 μM filter, and capillaries were then treated with vehicle control, inhibitor, or TBP in 2 mL of PBS in sterile borosilicate chamber slides purchased from Thermo Scientific, catalog #155380. Serial dilutions of TBP were prepared in DMSO immediately before each experiment using crystalline TBP. Time courses were staggered so that all slides were consecutively at 10 min/slide. Transport activity was measured as steady-state luminal accumulation of a fluorescent substrate specific to each transporter, 2 μM in PBS. In our P-gp and MRP2 transport assays, non-specific background fluorescence

was measured using the chemical inhibitors 10 μM PSC-833 and MK571, respectively. Capillaries were imaged using a Zeiss 710 confocal microscope and fluorescence was quantified using FIJI/ImageJ analysis software (N= 15-20 capillaries/group).

In vivo dosing

Animal dosing was blinded and matched with a vehicle control group. Doses were chosen based on previous work [5]. A single dose of TBP by oral gavage (PO) at dose levels of 0.4, 1, or 5 μmol/kg (~132-1654 μg/kg; 4 mL/kg in corn oil; N=4 animals/dose group) was administered to groups of male and female SD rats. 4 h post dosing, animals were euthanized, microvessels were isolated, and a transport assay was performed on P-gp as described above.

ATPase activity of purified mouse P-gp

For the determination of mouse P-gp ATPase activity, we used a modified malachite green assay as described earlier [35]. For pre-incubation experiments, the assay buffer containing mouse P-gp was supplemented with TBP 30 min prior to the addition of verapamil or ATP. 2 μg of purified and solubilized mouse protein (kindly provided by Dr. Geoffrey Chang from Skaggs School of Pharmacy and Pharmaceutical Sciences in the Department of Pharmacology at UC San Diego, CA) was added to a 96 well PCR plate, each well containing 60 μL of ATP-free reaction buffer (10 mM MgSO4, 0.05 % w/v DDM, 1 mM TCEP, 0.1 mg/mL of *E. coli* Polar Extract lipids in 50 mM Tris-Cl buffer pH 7.5) and serial dilutions of verapamil or TBP with 100 μM verapamil. Then 60 μL of ATP solution (5 mM Na-ATP, 10 mM MgSO4, 0.05 % w/v DDM, 1 mM TCEP, 0.1 mg/mL of *E. coli* Polar Extract

lipids in 50 mM Tris-Cl buffer pH 7.5) was added, mixed, and incubated for 5 min on ice. After incubation, the reaction mixtures were transferred to a thermocycler and the reaction was started for 3 sec at 4°C, 5 min at 37°C, 15 sec at 80°C (heat inactivation), with a final hold at 4°C. Immediately, 30 µL of the reactions were transferred to a 96 well ELISA plate and liberated P_i was measured by adding 150 µL of a color development solution (17 mg malachite green in 3.75 mL MilliQ H_2O, 0.525 g ammonium molybdate tetrahydrate in 12.5 mL of 4 N HCl, activated with 0.02% v/v Triton X-100). The absorbance of each sample was measured at 600 nm in a microplate reader (Spectramax M2, Sunnyvale, CA, US). Control samples containing reaction buffer and DMSO (TBP) or H_2O (verapamil) without P-gp protein were subtracted as background values. Inorganic phosphate standards (KH_2PO_4) from 0.125 to 2 nmol served as internal controls. Dose-response curves of mouse P-gp and verapamil or TBP were created with final concentrations ranging from 20 nM to 167 µM. All graphs were analyzed using the OriginPro 2016 software and graphed using GraphPad Prism (7.05) software.

Immunofluorescence

Isolated capillaries were plated and treated as in the transport assay. At the end of the treatment time, capillaries were fixed for 15 minutes with 1 mL 3% paraformaldehyde/0.2% glutaraldehyde in PBS, rinsed, permeabilized for 30 minutes with 1 mL 0.1% Triton X, rinsed, blocked 30 minutes with 1 mL 1% BSA, and then rinsed. Capillaries were incubated with 1:200 P-gp primary antibody overnight with 0.1% Tween in PBS. Primary antibody was removed, and secondary antibody was incubated for 3 h at 37°C. After removing the secondary antibody, capillaries were washed 3 times with PBS and fluorescence was

quantified as described in the transport assay. Non-specific fluorescence from “no-primary” controls was subtracted before graphing.

Western Blotting

Isolated capillaries were treated with vehicle control, inhibitor, or TBP in 5 mL PBS for 3 h. After treatment, capillaries were centrifuged for 15 minutes at 1860 × *g* and pellets were then stored at -80°C until use. 200 µL lysis buffer with protease inhibitor were added to each pellet. These samples were kept on ice and vortexed for 10 seconds every 10 minutes for 90 minutes. Additionally, samples were sonicated for 20 seconds at the 20, 40, and 60-minute mark. Samples were centrifuged at 10,000 g for 30 minutes to isolate the nuclear pellet. 20 µL of lysis buffet was added to the nuclear pellet. The denucleated supernatant was centrifuged at 100,000 g for 90 minutes. 30 µL of lysis buffer was added to the membrane pellet. The membrane, nuclear, and cytosolic fractions were stored at -80°C until quantification of protein concentration using a modified Bradford assay with BSA as the protein standard based on instructions in Thermo Scientific kit 23236 [36]. Western blotting was performed according to the manufacturer’s instructions [37]. Protein was transferred to membranes and hybridized overnight at 4°C in 1x PBS with 0.1% Tween with 1:200 P-gp primary antibody and 1:5000 ß-actin primary antibody. The membrane was incubated at 25°C for 3 h in 1x PBS with 1:10000 secondary antibodies, and then imaged using a LI-COR Odyssey CLx.

Statistics

Data were analyzed and graphed using GraphPad Prism (v 7.05) software. Data are expressed as mean +/- SEM and significance was determined by one-way ANOVA and Tukey multiple comparison. Significance is as compared to control unless otherwise specified: ***a*** = P < 0.05, ***b*** = P < 0.01, ***c*** = P < 0.001, ***d*** = P < 0.0001.

Results

The effect of increasing doses of TBP on two important ABC efflux transporters at the BBB was measured using fluorescent confocal microscopy coupled with a steady-state based transport assay. Male and female brain capillaries isolated from SD rats were exposed to increasing concentrations of TBP for 3 h. Following exposure, P-gp and MRP2 transport activities were determined by measuring steady state luminal accumulation of the transport specific fluorescent substrates, 2 μM NBD-CSA and Texas Red, respectively. Specific transport was obtained by subtracting the non-specific luminal fluorescence following treatment with inhibitors selective for each transporter, (P-gp: PSC833, and MRP2-MK571). Figure 1 (A-D) graphically illustrates the observed levels of luminal fluorescence in rat brain capillaries exposed *ex vivo* for 3 h to 0.1-100 pM TBP for males and 0.1- -100 nM for females. (For direct comparison TBP doses at 1-100nM are provided in Supplementary Figure 1.) Representative confocal images of capillaries digitally captured from each assay are pictured below their respective graph. TBP exposure decreased P-gp transport activity in both males and females in a dose-dependent manner (Figure 1 A & B). However, male P-gp transport activity was significantly reduced by exposures to 1-100 pM TBP while significant decreases in female P-gp transport activity required 1-100 nM of TBP. Furthermore, we observed no significant changes in MRP2 transport activity in either sex across TBP concentrations of 0.1-1000 nM (Figure 1 C & D).

To investigate the kinetics of the observed changes in P-gp and MRP2 transport, we exposed male and female rat brain capillaries to 100 nM TBP and measured their transport activities at hourly intervals for 4 h. Figure 2 A shows that P-gp transport activity in male capillaries exposed to 100 nM TBP significantly decreased to its lowest level in 1 h and

persisted for the remaining 3 h. In females, P-gp transport activity was significantly decreased in 1 h and continued to decline for 3 h (Figure 2 B). Similarly observed in our *ex vivo* experiments, MRP2 transport in TBP exposed capillaries remained unchanged throughout the 4 h treatment period in both male and female brain capillaries (Figure 2A & B).

Previous studies of chemicals that alter P-gp regulation show that rapid decreases in P-gp transport activity occur by either direct interaction or by activation of a biological based signaling pathway that regulates transport activity or expression [38]. We hypothesized that rapid changes in transport activity, independent of protein degradation or expression, would reverse back to control levels after the chemical is removed. To test this, we performed a reversibility assay by removing TBP (100 nM) after 1 h of pretreatment to the male and female rat brain capillaries. Following its removal, P-gp transport activity was measured at specific intervals to access reversibility of transport activity. As shown in Figure 3 A, P-gp transport activity in males rapidly reverses to basal control levels within 1 h after TBP removal. The TBP-mediated decreases in female P-gp transport also reverses to control basal levels upon removal of TBP, albeit with an increased latency of 2 h (Figure 3 B). Together these data suggest that TBP inhibits P-gp transport activity by one of two mechanisms: direct interaction or activating a signaling pathway.

To address the possibility that TBP inhibits P-gp by direct interaction with the P-gp protein, we measured ATPase activity in a purified reconstituted *in vitro* system containing active purified mouse P-gp solubilized in *E. coli* lipids incubated with ATP and increasing concentrations of verapamil. Verapamil-stimulated P-gp activity, with or without TBP (10 μM), was determined by measuring inorganic phosphate levels liberated by the hydrolysis

of ATP. Verapamil was able to stimulate mouse P-gp ATPase activity with an EC_{50} of 14 μM (Figure 4 A). When tested with a 30 min pre-incubation containing 10 μM TBP, verapamil stimulates mouse P-gp ATPase activity with an EC_{50} of 11.6 μM (Figure 4 A). Similarly, when TBP was added 30 min after ATP addition, the respective dose response curve had an EC_{50} of 15.5 μM (Figure 4 A). All three EC_{50} values were comparable to previously published values [35], indicating that TBP does not affect the binding and/or stimulation of mouse P-gp by verapamil. Moreover these data indicate that TBP does not hinder ATP binding to P-gp at either of its nucleotide-binding domains [35, 39]. When pre-stimulated with 100 μM verapamil, TBP did not inhibit mouse P-gp ATPase activity up to final concentrations of 167 μM, indicating that TBP does not bind or significantly hinder the binding of the P-gp substrate verapamil (Figure 4 B). Additionally, TBP alone was unable to stimulate mouse P-gp ATPase activity up to a concentration of 167 μM indicating that it is not a P-gp substrate (Figure 4 B). These data taken together support the hypothesis that TBP-dependent decreases in P-gp transport activity do not involve direct interaction or substrate inhibition but are likely due to the activation or inhibition of a signaling pathway that regulates P-gp pump activity.

Next, we examined the effect of TBP on P-gp transport activity in wild type mice. To accomplish this, we isolated brain capillaries from male and female C57BL/6J mice, exposed them to 100 nM TBP for 4 h, and measured P-gp transport activity. Like rats, P-gp-transport activity in male and female mice was significantly reduced after 100 nM TBP exposure (Figure 5 A & B). Significant decreases in P-gp transport activity in male mice were observed at 1-100 nM TBP exposures. Similar to rats, female mice required 10 -1000 nM TBP to significantly reduce P-gp activity compared to control, suggesting that males

were more sensitive to TBP exposure. Interestingly, P-gp transport activity in male mice exposed to 1 μM TBP was not significantly different from vehicle control. .

Knowing that 1 -100 nM TBP exposure decreases P-gp transport activity at the BBB in both rats and mice, we next determined if estrogen signaling through ERα was required in the TBP-mediated response. We exposed freshly isolated brain capillaries from ERα KO male mice to 1 -1000 nM TBP for 4 h and measured P-gp transport activity. As in wildtype mice, we found that 1 -100 nM TBP exposure reduced P-gp transport activity in ERα knockout mice (Figure 5 C), indicating that signaling through ERα was not a requirement for the TBP-dependent decreases in P-gp transport activity.

We expanded our search for potential signaling mechanisms by measuring P-gp transport activity after cotreating capillaries with TBP and inhibitors selective for PI3K, ERK1/2, Src and PKC. We selected these compounds because the pathways they inhibit are known to regulate basal P-gp activity and expression [40-44]. We found only inhibitors selective for PKC were able to fully block the decreased effects of TBP on P-gp transport (Figure 6 A & B). PKC is a large family of protein kinase enzymes consisting of numerous isoforms that regulate the activity of other enzymes by serine or threonine phosphorylation [45]. To better understand which PKC isoforms might be involved, we cotreated capillaries with TBP and PKC inhibitors selective for PKCß$_{1+2}$, PKCα+ß$_1$, PKCα+ß$_1$+δ+ε and ζ. We found each of the three selective PKC inhibitors tested prevented the TBP-dependent decreases in P-gp (Figure 6 B); however, PKCß$_1$ was the only subtype common to each.

We next used immunofluorescence to visualize changes in P-gp expression under vehicle control and 100 nM TBP treatment conditions in rat brain capillaries. In both male and female SD rats, immunofluorescence experiments showed a slight reduction in

measurable fluorescence indicative of decreased P-gp expression with TBP treatment (Figure 7 A & B). Western blots for P-gp in male rats also showed small decreases in P-gp expression after a 3 h 100 nM TBP treatment, mirroring the immunofluorescence results (Figure 7 C).

To confirm our *ex vivo* findings *in vivo*, we dosed SD rats (15-20 weeks old) by oral gavage with 0.4, 1.0 and 5.0 μmol/kg TBP or vehicle control in masked groups of 4 animals each. Noteworthy, the lowest TBP dose, 0.4 μmol/kg, corresponded to an average blood concentration of 100 nM TBP. Animals were euthanized after 4 h, brain capillaries were isolated and P-gp transport was measured. In male rats, P-gp activity was unchanged at the 1 and 5 μmol/kg doses. Consistent with our *ex vivo* findings, P-gp transport activity was significantly lower at 0.4 μmol/kg. Taken together these data indicate that low TBP (1-100 nM) exposures *in vivo* and *ex vivo* produces rapid decreases in P-gp transport activity in rats and mice.

Discussion

We report here that exposing the BBB to the brominated flame retardant, TBP, leads to relatively rapid (1 h) decreases in P-gp transport, which may compromise the protective ability of the BBB. Although TBP is rapidly removed from systemic circulation ($t_{1/2}$= 4 h), TBP exposure in the home and workplace is ubiquitous and continuous, having the potential to increase brain and CNS exposures by allowing harmful P-gp substrates to cross the BBB. The lack of measurable changes in MRP2 transport activity after identical exposures indicates that the TBP-mediated effects on the BBB are transporter specific. They also indicate that capillary membranes and tight junction proteins of the BBB remain intact.

We observed slight differences in P-gp transport activity in response to TBP across sex and species. When measuring the effects of TBP on P-gp transport activity, we observed a 1000-fold increase in the sensitivity in male rats compared to female rats. We also observed an increased sensitivity to TBP exposure in male mice as evidenced by significant decreases in transport activity at lower TBP concentrations and no changes in activity at the highest (1 μM) exposure. This reversal in the response in males may represent an adaptive response at the higher TBP (1 μM) exposure. We speculate that the absence of decreases in P-gp transport in males at the high TBP dose may result from an early pre-toxic response. Alternatively, high doses of TBP in males may activate secondary regulatory pathways responsible for maintaining P-gp basal transport activity. Regardless, additional work will be needed to fully understand the sex specific differences in TBP exposure. Our experiments also show that TBP-dependent decreases of P-gp transport activity are rapid and reversible with only slight changes in protein levels. We initially

hypothesized that the reversible nature of the decreased response in P-gp transport activity following TBP exposure likely resulted from a direct interaction of TBP with the P-gp protein. However, the ATPase assay did not confirm this. TBP did not affect the EC_{50} or IC_{50} of the verapamil-induced ATPase activity in the purified mouse P-gp system, indicating that TBP does not interact with the substrate binding pockets or ATP binding domains of mouse P-gp. Furthermore, rising concentrations (20 nM - 167 μM) of TBP did not modulate the P-gp stimulation by verapamil. Importantly, TBP was unable to stimulate ATPase activity from 20 nM - 167 μM in the absence of a known substrate. We understand that these assays do not categorially eliminate the possibility of direct interaction; however, they are consistent with the notion that TBP is not a substrate and it does not inhibit P-gp transport activity by direct interaction. Immunofluorescence and western blots do show small decreases in the steady state levels of P-gp protein following TBP exposure. However, reversibility experiments clearly indicate that P-gp transport activity can return to control levels after exposure to TBP. This discrepancy likely indicates that significant amounts of P-gp are stored in intracellular reserve vesicles as previously reported [46]. Taken together, these findings strongly support the notion that TBP decreases P-gp transport activity at the BBB through dysregulation of cellular signaling pathways.

In the search for a signaling mechanism TBP treated male ERα receptor KO mice were compared to their isogenic wildtype littermates. We found that P-gp activity was decreased similarly after TBP exposure in both wildtype and ERα knock-out mice, eliminating a role for ERα signaling in the TBP-mediated decreases in P-gp transport activity. A pan-PKC inhibitor prevented the TBP-dependent decrease in P-gp transport and PKC inhibitors selective for each isoform helped determined the subtype involved. Although

all three inhibitors successfully prevented the TBP-dependent decrease in P-gp transport activity, PKC $ß_I$ was the only common subtype. However due to the selectivity of the inhibitors, the contribution of PKC α or $ß_{II}$ cannot be fully eliminated. Additional work will be required to sort this issue and fully understand the role each PKC isoform may play in TBP's modulation of P-gp at the BBB.

In summary, these data demonstrated that exposures to TBP (1 -100 nM), a ubiquitous brominated flame retardant, fungicide, and wood preservative, can compromise blood-brain barrier function by decreasing the activity of the important transporter, P-gp, in both sexes of rats and mice. This work is significant because P-gp substrates include a large proportion of chemotherapeutic and widely prescribed drugs, many of which are neurotoxic. Decreases in P-gp function at the BBB not only elevate the risk of increased deposition of toxic xenobiotic agents to the brain but systemically may contribute to significant changes in absorption and elimination and altered pharmacokinetics of important metabolites throughout the body.

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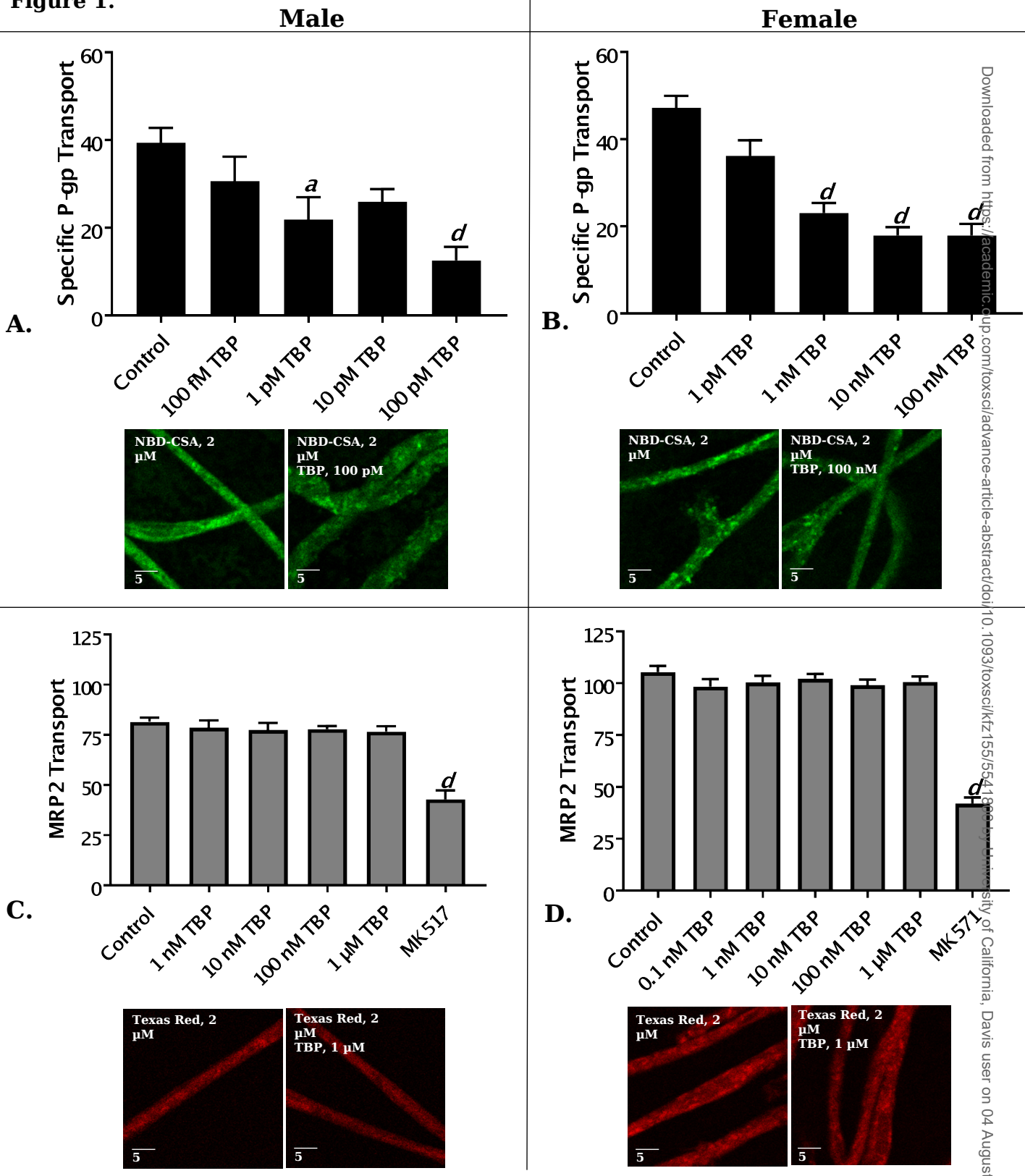
Figure 1.



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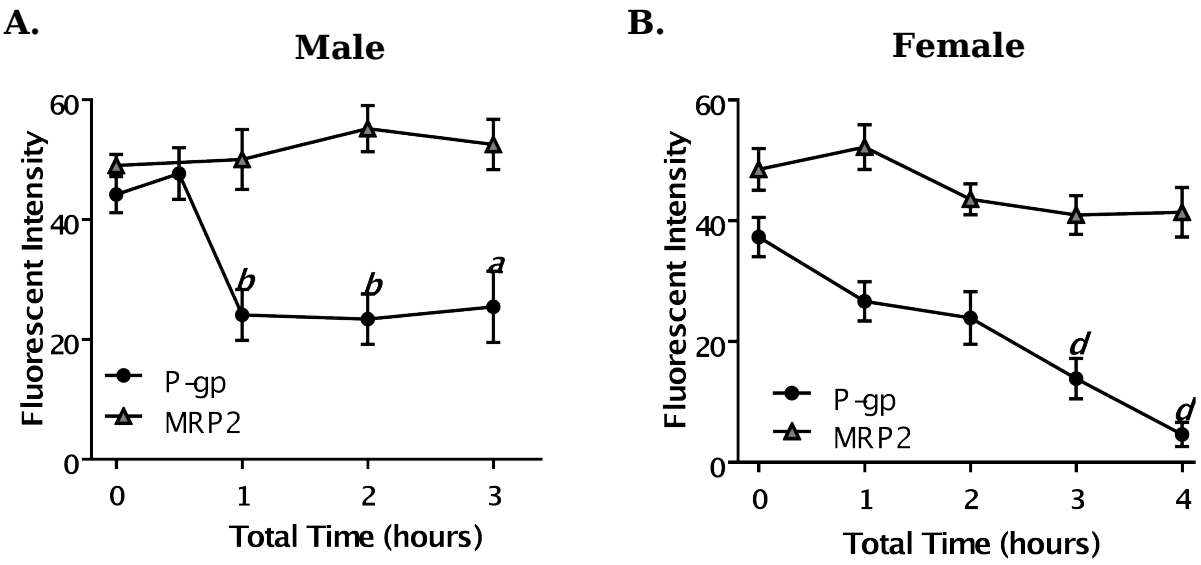

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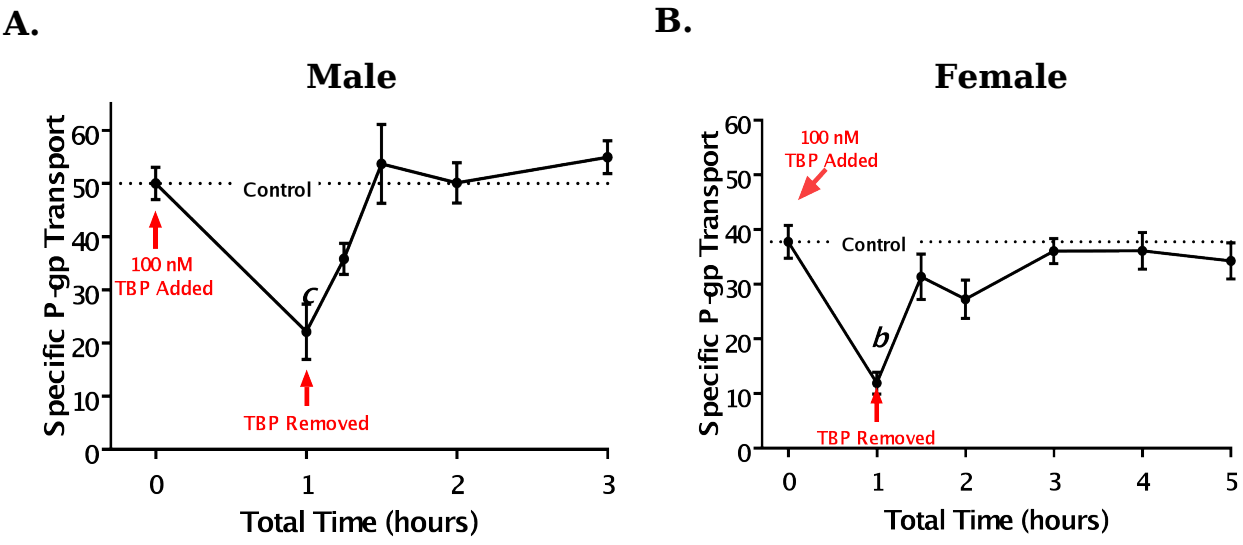

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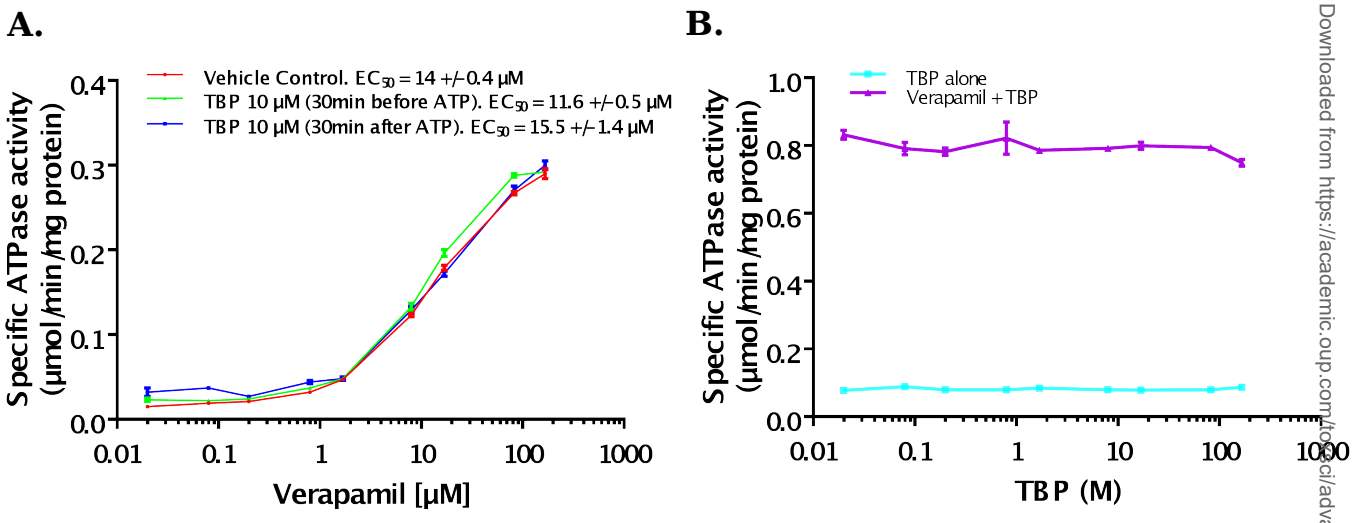

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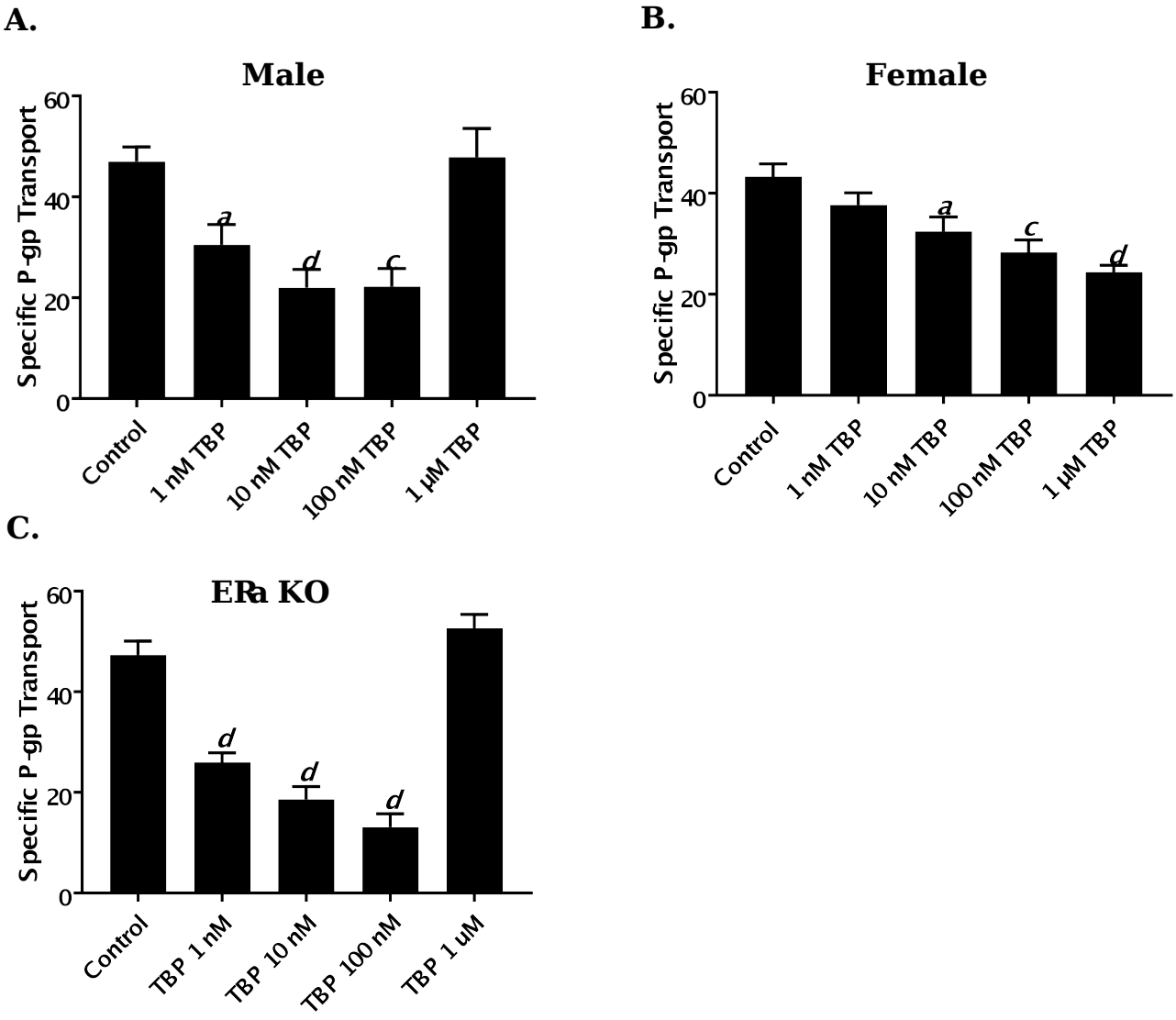

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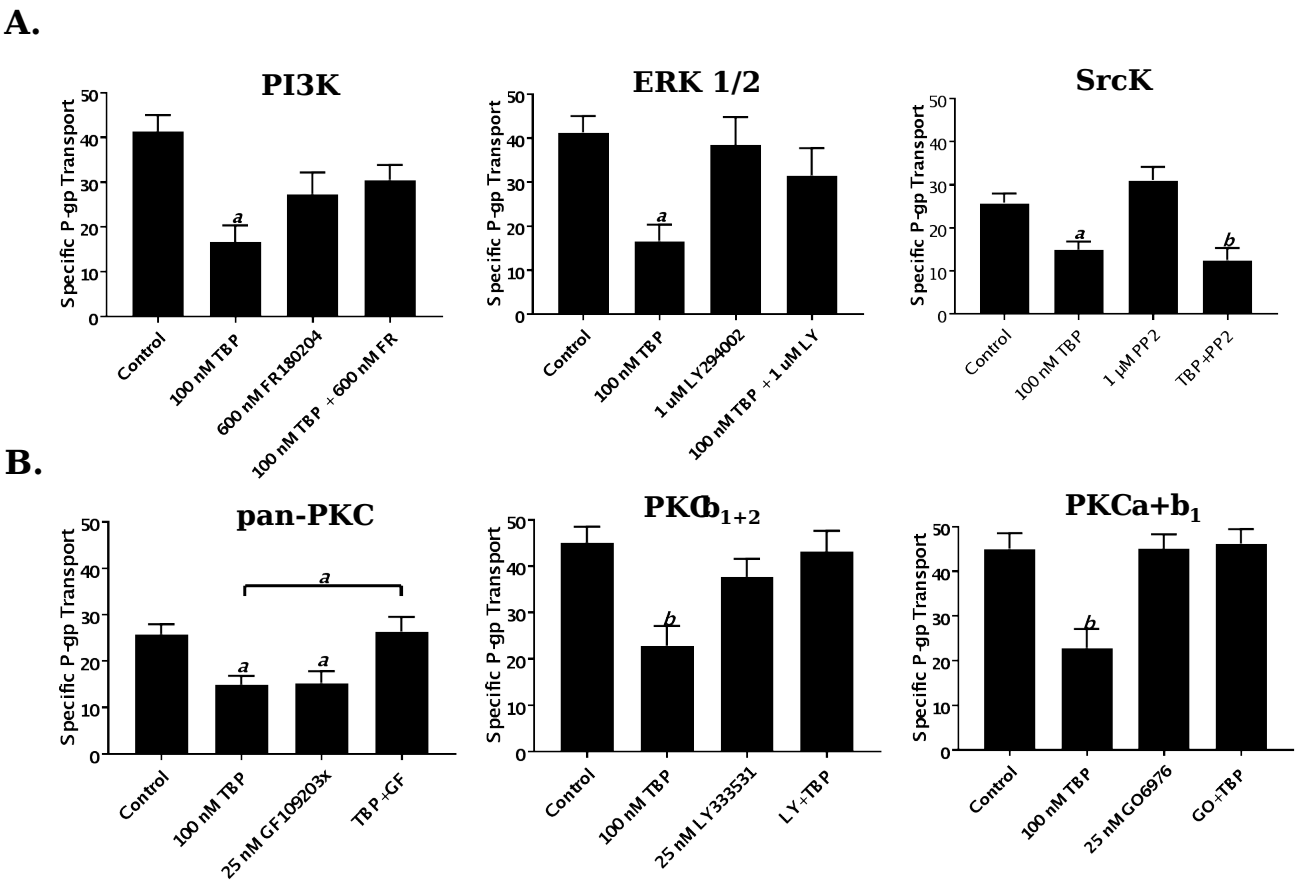

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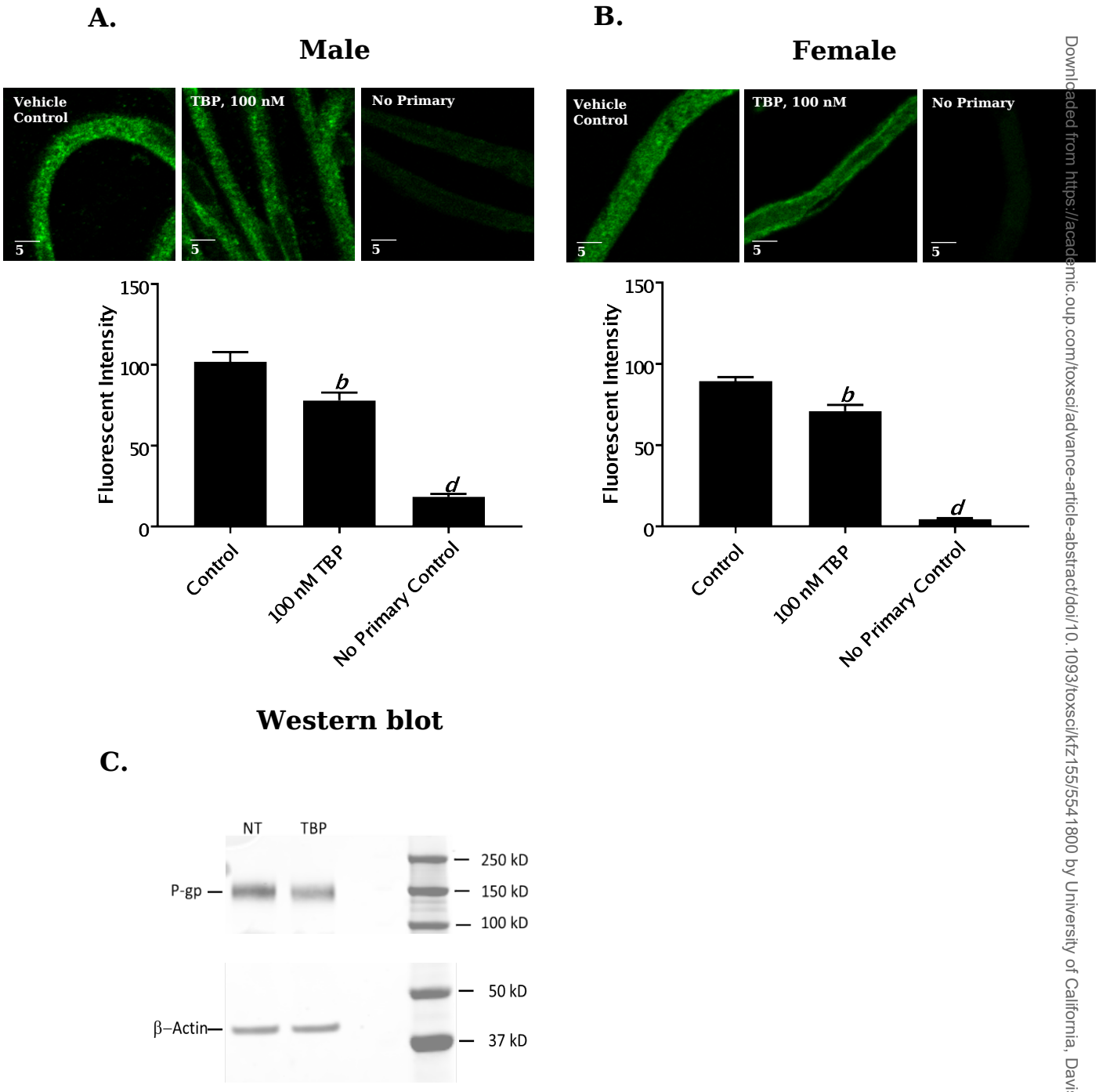

Figure 8.