

Effect of Animal Bedding on Rat Liver Endosome Acidification

Ajay Kumar V. Buddaraju, MD¹ and Rebecca W. Van Dyke, MD^{1,2,*}

Animal beddings, such as pine products, and environmental factors are known to induce liver drug-metabolizing cytochrome P450 enzymes. We observed that a change to pine-based rat bedding altered baseline and cAMP-stimulated rates of acidification in rat liver endosomes, apparently by decreasing ATP-dependent proton transport in the presence and absence of chloride. Although cAMP altered phosphorylation of protein kinase B and extracellular signal-regulated kinases 1 and 2 (ERK 1,2) and p38 mitogen-activated protein kinases, changes in housing conditions did not affect baseline or cAMP-stimulated values of these or other selected signaling molecules. We conclude that compounds in rat bedding may alter not only drug metabolism, but also aspects of endocytosis.

Previous studies have documented effects of environmental changes, especially animal bedding, on rat liver drug metabolism (22, 25). The best characterized effects appear to be induction of cytochrome P450 (CYP) enzymes by exposure to volatile organic compounds in pine- or cedar-based bedding (22, 25), that are potentially mediated by nuclear receptors (9). In our studies of endocytosis, we have documented that the rates of ATP-dependent proton transport (acidification) in rat liver endosomes are increased while endosome maturation and trafficking are altered by cAMP and agents that affect heterotrimeric G-protein signaling, including cholera (CTX) and pertussis toxins (16, 17). Subsequent to institutional changes in animal husbandry practices, we observed that rat liver endocytosis appeared to be altered. We undertook the study reported here to identify and characterize environmental effects on liver endosomes and their ion transport properties. Previous studies by ourselves and others have identified cAMP-dependent changes in various signal transduction pathways in rat liver (19, 20, 23, 24) and have suggested that cAMP, phosphoinositide 3 kinase (PI-3 kinase), protein kinase B (PKB), glycogen synthase kinase-3 α , β (GSK-3 α , β), and the mitogen-activated protein kinase (MAPK) p38 may regulate endosome trafficking and function in liver and other cell types (1-5, 8, 10, 19, 20, 23, 24). Further, estrogen and aromatic hydrocarbons, both of which activate nuclear receptors, have been reported to alter MAPK signaling (13). Therefore, we also explored whether changes in endosome acidification due to housing conditions were associated with changes in representative members of several major signal transduction pathways.

Materials and Methods

Materials. A 70,000-Da fluorescein isothiocyanate (FITC)-dextran conjugate and other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), fluorescein antibody was purchased from Molecular Probes, Inc. (Eugene, Oreg.), CTX

was obtained from List Biological Laboratories, Inc. (Campbell, Calif.), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblot analysis (western blot) supplies were purchased from Bio-Rad Laboratories (Hercules, Calif.), Amersham Life Science (Little Chalfont, England), and Pierce Chemicals (Rockford, Ill.). Polyclonal antibodies to total and phosphorylated PKB, GSK-3 β (total) or GSK-3 α , β (phospho), p38 MAPK, and c-Jun (including antibodies that detect phosphorylation at either Ser63 or Ser73), polyclonal antibodies to total extracellular signal-regulated kinases 1 and 2 (ERK1,2) MAPK, to phosphorylated Raf-1 (at Ser259) and monoclonal antibodies to phosphorylated ERK1,2 MAPK and to phosphorylated p70 S6 kinase/p85 S6 kinase were obtained from Cell Signaling Technology (Beverly, Mass.). Secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (HRP) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa.). Primary (1^o) antibodies were used at a dilution of 1:1,000, and secondary (2^o) antibodies were used at a dilution of 1:1,250, except for antibodies to c-Jun (1^o, 1:100; 2^o, 1:500), phospho p70 S6 kinase/p85 S6 kinase (1^o, 1:800; 2^o, 1:1,000), phospho ERK1,2 (1^o, 1:1,250) and phospho RAF-1(Ser259) and total GSK (1^o, 1:2,000).

Animals. Male Wistar-Furth rats (200 to 250 g) were obtained from a single breeding colony from Harlan, Inc. (Indianapolis, Ind.) and were designated specific pathogen-free by the supplier on the basis of results of serologic studies for 14 virus and bacteria species, polymerase chain reaction (PCR)-based tests for four virus and bacterial species, culturing for respiratory and enteric bacteria, and microscopic examination for endo- and ectoparasites, as detailed at <http://www.harlan.com/us/index.htm>.

Animals received humane care according to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, 1996). All work was approved by the IACUCs at the University of Michigan and the Ann Arbor VA Hospital.

Animals were housed in one of two animal care facilities (University of Michigan [UM] and Veterans Administration [VA]) for at least five days before the study. Both facilities housed animals in plastic cages with contact bedding and supplied with filtered air and city water; however, the standard

Received: 5/5/03. Revision requested: 6/04/03. Accepted: 7/07/03.

¹Medical Service, 11R Research Service, Ann Arbor VA Hospital, 2215 Fuller Road, Ann Arbor, Michigan 48105 and ²Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109.

*Corresponding author.

bedding and diet differed between the two facilities. The UM facility used corncob bedding (Bed O' Cobs, The Anderson AgriServices, Inc., Maumee, Ohio) and LabDiet chow 5001 (PMI Nutrition International, LLC, Brentwood, Mo.; a combination designated "UM conditions"), whereas the VA facility used pine bedding (SaniChips, PJ Murphy Forest Products, Montville, N.J.) and LabDiet chow 5008 (a combination designated "VA conditions"). The two diets differed only in the percentage of fat (10% versus 14%, respectively). In our initial preliminary studies, animals were housed in the two animal care facilities with the respective food and bedding. For all other experiments, animals were housed only in the VA facility, but were exposed to various combinations of these diets and bedding types. To reduce seasonal variation, comparisons were made between experiments performed on animals obtained from the same breeding colony at the same time.

Study design. Seventeen hours before use, some rats were injected intraperitoneally (i.p.) with 120 μ g of CTX/100 mg of body weight (16, 17). Liver weight and total liver protein content were decreased by 24 to 34% ($P < 0.005$) by overnight exposure to CTX, likely due to the catabolic effects of cAMP, as described (16, 17); however, neither liver weight nor protein content was affected by housing conditions (data not shown).

To load endosomes, rats were anesthetized briefly and 75 mg of 70,000-Da FITC-dextran in saline was administered intravenously (i.v.) 20 min before sacrifice (16, 17). Animals were anesthetized again, the liver was removed for preparation of endosomes and lysates, then the animals were euthanized while under anesthesia by exsanguination and bilateral thoracotomy.

In all studies, animal handling, surgical procedures, preparation of subcellular fractions, measurement of endosome acidification and western blotting were performed by the same individuals in the same laboratory using the same reagents, solutions, and equipment.

Endosomes and lysates. Total liver populations of dextran-loaded endocytic vesicles were isolated from liver homogenates as a microsomal pellet as described (16, 17), re-suspended in isotonic buffer containing 140 mM potassium gluconate and 30 mM Bis-Tris (pH 7.1), and kept at 4°C for up to six hours. Samples (0.5 g) of rat liver were lysed by homogenization in 35 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4 , 10 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10 H_2O$, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoridehydrochloride, 1 μ g of leupeptin/ml, and 1 μ g of aprotinin/ml), followed by centrifugation at 16,000 \times g for 20 min. Supernatants were stored at -70°C, protein content was measured by use of the Lowry assay, and content of phosphorylated and total proteins were analyzed by use of western blotting (14, 19, 20).

Vesicle acidification. The ATP-dependent acidification rates and steady-state ATP-dependent intravesicular pH (pH_i) were measured in fresh endosomes (suspended in buffers with various concentrations of KCl and sufficient potassium gluconate to maintain isotonicity) from changes in the ratio of fluorescein fluorescence as described. (16-18, 21). This procedure yields rates that are independent of the total amount of endosomes present in the assay.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed through 4.4% stacking and 7.5% running gels, with equal amounts of protein in each lane. Proteins were electrophoretically transferred to

nitrocellulose membranes, blots were washed in Tris-buffered saline with 0.3% Tween (TBST), blocked in TBST with 5% dry milk, incubated with primary antibody in TBST with milk overnight at 4°C, washed in TBST, incubated with secondary antibody conjugated to HRP in TBST with milk for 30 min at room temperature, washed in TBST, and developed, using Pierce SuperSignal chemiluminescence. Bands were captured on x-ray film. Films were scanned and band density was determined, using calibrated NIH Image on a Macintosh G4 computer. As CTX decreased liver weight and total liver protein proportionately (by approx. 25 to 35%) but not hepatocyte numbers, lanes containing lysates from CTX-treated livers likely represented the protein content of approximately 25 to 35% more hepatocytes than did lanes containing lysates from control livers.

Calculations and statistics. For plots of acidification rates versus buffer Cl^- concentration, curves were fitted to the data, using nonlinear curve-fitting techniques and a form of the Michaelis-Menton equation ($y = \frac{[ax]/[b + x]}{1 + [ax]/[b + x]} + cx + d$), where x is the concentration of chloride in millimoles; "a" represents $V_{max Cl^-}$, the maximal saturable rate due to Cl^- ; "b", the 50% effective dose (ED_{50}) for Cl^- ; "c" V_{linear} , the linear increase in acidification with Cl^- ; and "d" $V_{0 Cl^-}$, the rate without Cl^- (16, 17). For protein kinase determinations, the optical density of bands from the same blots were compared directly or the optical density of bands from CTX-treated livers were expressed as a percentage of the optical density of bands from control livers from the same blot. These percentages were averaged and compared within and between the various blots. Data were presented as mean \pm SEM. Values were compared by use of Student's t test, with $P < 0.05$ taken to indicate statistical significance, using StatView 5.0.1 software (SAS Institute, Inc., Cary, N.C.).

Results

In preliminary studies, we observed that the rates of ATP-dependent acidification in rat liver endosomes decreased after our laboratory moved from the UM Medical School to the Ann Arbor VA research facility, even when animals obtained from the same colony at the same time were housed simultaneously in the two facilities (data not shown). Animal food and bedding were the only identified differences in the two facilities. Further preliminary studies eliminated other environmental factors as endosome acidification rates were identical for animals from the same colony simultaneously housed on corncob bedding with LabDiet 5001 chow: at the UM animal care facility and the VA animal care facility; and in two animal care rooms at the VA animal care facility (date not shown). In all subsequent studies, all animals were housed in the VA animal care facility, using different combinations of food and bedding.

As indicated (Fig. 1A), endosomes prepared from animals housed on cob bedding with 5001 food (cob/5001, "UM conditions") had substantially faster rates of acidification at all buffer Cl^- concentrations, under both control conditions and after treatment with CTX, compared with those from animals housed on pine bedding with 5008 food (pine/5008, "VA conditions"), when all animals were housed in the VA animal care facility. Differences in the acidification rates at each of the nine Cl^- concentrations shown in the figure were calculated and averaged. Compared with cob/5001 conditions, pine/5008 decreased endosome acidification by $16.7 \pm 2.2\%$ in control livers and by $22.8 \pm 1.5\%$ in CTX-treated livers, respectively ($P < 0.0001$ for

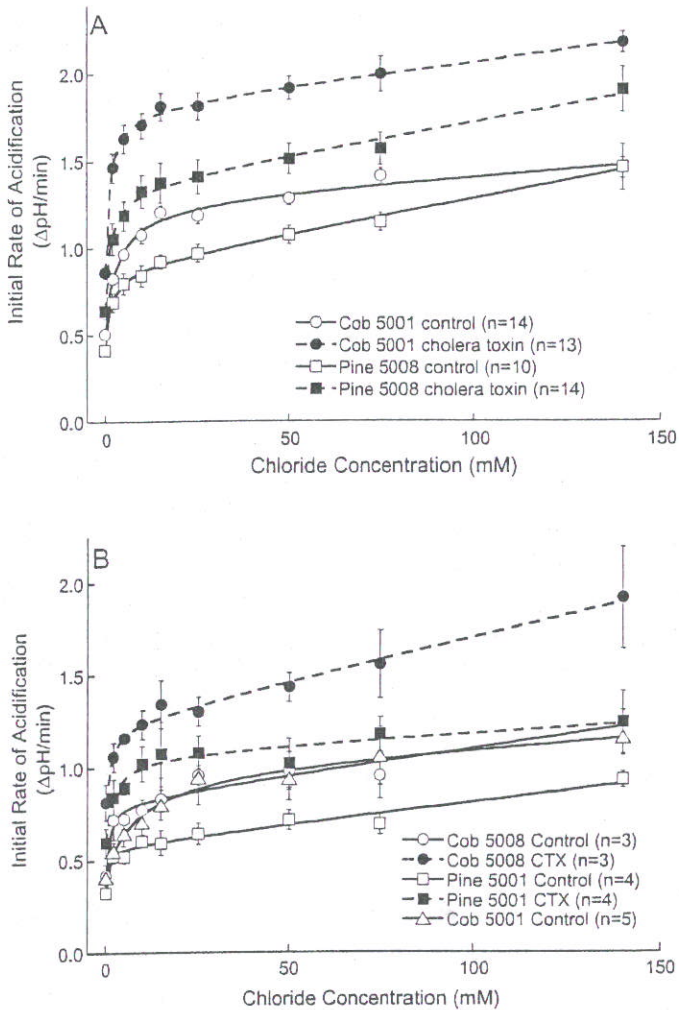


Figure 1. Rat housing conditions altered control and cholera toxin (CTX)-stimulated liver endosome acidification rates. The initial rates of ATP-dependent endosome acidification were plotted against buffer chloride concentration. Endosomes were prepared from rats exposed to the indicated bedding and diet combinations (A: circles, cob/5001; squares, pine/5008) (B: circles, cob/5008; squares, pine/5001; triangles, cob/5001) and untreated (open symbols, solid lines) or administered cholera toxin (closed symbols, dashed lines). Curves are the best fit to the data, using the equation $y = ([ax]/[b + x]) + cx + d$.

both comparisons). However, CTX significantly ($P < 0.0001$) increased acidification rates over control rates by a similar amount, $57.9 \pm 4.0\%$ and $46.4 \pm 3.0\%$ in endosomes from animals housed on cob/5001 and pine/5008, respectively.

To further quantitate the effects of housing conditions on acidification rates, we used non-linear curve-fitting techniques

to estimate the kinetic parameters for the relationship of acidification rates to buffer Cl^- concentration from the data shown in Fig. 1A. Our previous studies had indicated that, as endosomes mature, $V_{max Cl^-}$, V_{linear} , and V_{0Cl^-} decrease while ED_{50} increases, and CTX changes all of these parameters in the opposite direction (16-18, 21). As indicated (Table 1), compared with endosomes from animals on cob bedding with 5001 diet, pine bedding with 5008 diet decreased $V_{max Cl^-}$ by 38% (control) and 24% (CTX), decreased V_{0Cl^-} by 19% (control) and 26% (CTX), and increased V_{linear} by 156% (control) and 39% (CTX). The ED_{50} was not consistently altered by changes in housing conditions. Further, pine bedding and 5008 diet did not alter the fractional increase in either $V_{max Cl^-}$ or V_{0Cl^-} that was due to CTX.

To distinguish the effects of bedding changes from potential effects of diet changes, we obtained a further lot of animals and measured rates of ATP-dependent acidification in endosomes from control and CTX-treated rats housed on cob bedding and fed 5008 or 5001 diet or housed on pine bedding and fed 5001 diet. As shown in Fig. 1B, acidification rates were decreased by the pine bedding, but were not altered by the two diets when animals were housed on cob bedding. Similar to the effects of pine/5008 conditions shown in Fig. 1A, pine/5001 housing decreased acidification rates by $26.5 \pm 1.1\%$ (control endosomes) and by $23.7 \pm 2.0\%$ (CTX-treated endosomes), compared with cob/5008 housing ($P < 0.0001$ for both). As observed for cob/5001, compared with pine/5008 conditions (Fig. 1A), CTX significantly ($P < 0.0001$) increased acidification values over control rates by a similar amount in endosomes from animals housed on cob/5008 and pine/5001 (58.4 ± 5.6 and $64.3 \pm 5.5\%$, respectively). Kinetic parameters of acidification were estimated, and the effects of CTX and of pine/5001, compared with cob/5008 data not shown, were similar to the effects of CTX and pine/5008 and cob/5001 shown in Table 1. These findings indicate that animal bedding, rather than rat chow, likely was principally responsible for the changes in endosome acidification.

We also examined the effects of animal housing conditions on a variety of proteins involved in major signal transduction cascades, including some known to be affected by CTX/cAMP (protein kinase A, PKB, GSK-3 α , β and the MAPK extracellular signal-regulated kinases 1 and 2 (ERK1,2) and p38) (19, 20) and postulated to be involved in regulation of endosome function and trafficking (cAMP, PKB, GSK-3 α , β and p38) (1-5, 8, 10, 19, 20, 23, 24). A representation of the critical elements of these signaling pathways is shown in Fig. 2.

Similar to our previous studies (19, 20), CTX treatment increased phosphorylation of PKB and its downstream effector, GSK-3 α , β . The CTX also increased phosphorylation of ERK1,2, but decreased phosphorylation of p38 (Fig. 3A). Liver content of these protein kinases, expressed per milligram of protein (or per

Table 1. Kinetic parameters of endosome acidification

Housing condition	N	Kinetic parameter $V_{max Cl^-}$ ($\Delta pH/min$)	Chloride ED_{50} (mM)	V_{linear} ($\Delta pH/min$)	V_{0Cl^-} ($\Delta pH/min$)
Control					
Cob 5001	14	0.756 ± 0.06	3.26 ± 0.94	0.0016 ± 0.0005	0.511 ± 0.041
Pine 5008	10	0.465 ± 0.029	1.47 ± 0.36	0.0041 ± 0.0002	0.414 ± 0.022
Cholera toxin					
Cob 5001	13	0.937 ± 0.029	1.17 ± 0.15	0.0028 ± 0.0002	0.862 ± 0.022
Pine 5008	14	0.711 ± 0.043	1.45 ± 0.39	0.0039 ± 0.0004	0.639 ± 0.036

Values are the mean \pm SEM estimated parameters from non-linear least squares fitting of the data.

$V_{max Cl^-}$ = maximum saturable rate due to Cl^- ; V_{linear} = linear increase in rate due to Cl^- ; V_{0Cl^-} = rate in the absence of Cl^- ; ED_{50} = median effective dose.