Green tea catechin EGCG inhibits ileal apical sodium bile acid transporter ASBT

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Annaba F, Kumar P, Dudeja AK, Saksema S, Gill RK, Alrefai WA. Green tea catechin EGCG inhibits ileal sodium bile acid transporter ASBT. Am J Physiol Gastrointest Liver Physiol 298: G467–G473, 2010. First published January 7, 2010; doi:10.1152/ajpgi.00360.2009.—Green tea catechins exhibit hypocholesterolic effects probably via their inhibitory effects on intestinal bile acid absorption. Ileal apical sodium-dependent bile acid transporter (ASBT) is responsible for reabsorption of bile acids. The present studies were, therefore, designed to investigate the modulation of ASBT function and membrane expression by green tea catechins in human embryonic kidney HEK-293 cells stably transfected with ASBT-V5 fusion protein and intestinal Caco-2 monolayers. Our data showed that ASBT activity was significantly decreased by (−)-epigallocatechin-3-gallate (EGCG) but not other green tea catechins. Inhibition of PKC, phosphatidylinositol 3-kinase, and MAPK-dependent pathways failed to block the reduction in ASBT activity by EGCG. Kinetics studies showed a significant decrease in the Vmax of the transporter, whereas total ASBT content on the plasma membrane was unaltered by EGCG. Concomitant with the decrease in ASBT function, EGCG significantly reduced ASBT pool in the detergent-insoluble fraction, while increasing its presence in the detergent-soluble fraction of plasma membrane. Furthermore, EGCG decreased the association of ASBT with floating lipid raft fractions of cellular membrane on Optiprep density gradient. In conclusion, our data demonstrate a novel role of lipid rafts in the modulation of ASBT function by the dietary component EGCG, which may underlie the hypocholesterolic effects of green tea.

lipid rafts; (−)-epigallocatechin-3-gallate; hypercholesterolemia

GREEN TEA, A POPULAR BEVERAGE derived from the tea plant Camellia sinensis, contains large quantities of the biologically active polyphenols, catechins (20). Four types of catechins, namely (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG), comprise ~30% of the dry weight of green tea (20, 25). EGCG is the most abundantly present catechin and is mainly responsible for the biological properties of green tea such as its potent antioxidant effects (29). Interestingly, green tea consumption has been shown to confer numerous health benefits including protection against cholesterol-related disorders (20, 22). Previous epidemiological studies have shown green tea drinking to be associated with a decreased risk of cardiovascular disease in humans (20, 29). Also, green tea has been demonstrated to reduce the progression of vascular lesions in an apoE-knockout mouse model of atherosclerosis and to lower plasma cholesterol in rats fed with cholesterol-rich diet (11, 17, 20, 24, 30).

Although cholesterol-lowering effects of green tea are well established, the molecular mechanisms by which green tea catechins alter cholesterol metabolism are not fully understood (20). Recent studies indicated that modulation of bile acid absorption and metabolism might underlie a major mechanism by which EGCG reduces cholesterol level (22). Intriguingly, green tea catechins including EGCG are poorly absorbed from the intestine (20). This observation suggests that green tea catechins in the intestinal lumen may lower cholesterol levels by directly interacting with intestinal epithelial bile acid absorption.

Active bile acid absorption occurs predominantly in the distal ileum via the apical sodium-dependent bile acid transporter (ASBT) expressed on the luminal membrane of enterocytes predominantly in the distal ileum (4). Intestinal absorption of bile acids represents a critical process for the maintenance of bile acid and cholesterol homeostasis (4). ASBT function is a key step in the process of bile acid circulation from the intestine to the liver (21). Bile acid flux across intestinal epithelial cells as well as hepatocytes plays a major role in regulating hepatic bile acid synthesis from cholesterol (4, 21). The inhibition of ASBT activity, consequently, reduces the amount of circulating bile acids, leading to stimulation in their synthesis and reduction in cholesterol level (4). This is evident by the fact that ASBT-knockout mice have low levels of plasma cholesterol and blockers of bile acid absorption also reduce plasma cholesterol (13). Whether green tea catechins directly affect ASBT function to lower cholesterol has not been investigated.

The present studies, therefore, were undertaken to examine the direct effects of green tea catechins on human ASBT activity. Human embryonic kidney HEK-293 cells stably transfected with human ASBT-V5 fusion protein as well as human intestinal Caco-2 cells were utilized as in vitro cellular models. Our data showed that short-term exposure to EGCG but not other catechins significantly reduced ASBT activity. EGCG treatment decreased ASBT association with the lipid rafts of plasma membrane. Our findings suggest that changes in lipid rafts of plasma membrane underlie the inhibitory effect of EGCG green tea catechin on ASBT function and bile acid absorption.

MATERIALS AND METHODS

Cell culture models. HEK-293 cells stably transfected with human ASBT-V5 fusion protein (designated as 2BT cells) were previously described by us (6). Human intestinal epithelial Caco-2 cells were obtained from American Type Culture Collection (ATCC). Cells were

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cultured in MEM medium supplemented with FBS (10% for 2BT and 20% for Caco-2 cells). For uptake experiments, 2BT cells were plated at a density of 2 × 10^5/well in 24-well Falcon plates (treated by vacuum gas plasma, Becton-Dickinson, Franklin Lakes, NJ). 2BT cells reached 90–100% confluence after 2–3 days in culture and were utilized for uptake experiments. Caco-2 cells were cultured for 14 days on 24-well culture plates and then utilized for uptake studies as previously described by us (5).

**[3H]taurocholic acid uptake.** Sodium-dependent taurocholic acid (TC) transport in transfected HEK-293 or Caco-2 cells was assessed as previously described by us (5). Briefly, medium was removed and cells were incubated for 5 min at 37°C with buffer containing (in mM) 110 NaCl (with sodium) or choline chloride (without sodium), 4 KCl, 1 MgSO_4_, 1 CaCl_2_, 50mannitol, and 10 HEPES (pH 7.4). Cells were then incubated with the same buffer containing 10 μM of [3H]TC (Perkin Elmer, Boston, MA) for the designated period of time. The transport process was terminated by washing the cells twice with ice-cold PBS. Cells were then solubilized with 0.5 N NaOH for at least 4 h. The protein concentration was measured by the method of Bradford (8), and the radioactivity was counted by Packard liquid scintillation analyzer Tri-CARB 1600-TR (Packard Instrument, Downers Grove, IL). Sodium-dependent TC uptake was expressed as picomoles per milligram protein per 5 min. For the kinetic parameters of TC uptake values were analyzed for simple Michaelis-Menten kinetics by utilizing a nonlinear regression data analysis from a computer model (GraphPad PRISM, San Diego, CA).

**Cell surface biotinylation.** Cell surface biotinylation was performed with sulfo-NHS-SS-biotin (0.5 mg/ml; Pierce, Rockford, IL) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 CaCl_2_, and 10 H_2BO_3_, pH adjusted to 9.0) as previously described, with labeling for 30 min at 4°C to stop endocytosis and internalization of antibodies (6). After immunoprecipitation of biotinylated antigens with streptavidin agarose, biotinylated proteins were released by incubation in 50 mM DTT reconstituted in Laemmli buffer. The immunoprecipitates were subjected to SDS-PAGE, and blots were then probed with anti-V5 antibodies.

**Isolation of DS and DI fractions from 2BT cell membranes.** Detergent-soluble (DS) and insoluble (DI) fractions of 2BT cell membranes were prepared essentially as previously described by us (6). Briefly, 2BT cells were treated with EGCG then washed with PBS, scraped, and collected by centrifugation for 5 min at 500 g. Cells were then lysed in homogenization buffer containing (in mM) 20 Tris·HCl, pH 7.5, 250 sucrose, 4 EDTA, and 2 EGTA with complete proteases inhibitors (Roche). Postnuclear supernatant was collected and then centrifuged for 30 min at 100,000 g at 4°C, the pellet (membrane fraction) was resuspended in MES buffer containing (in mM) 50 MES (pH 6.5), 60 NaCl, 3 EDTA, 5 MgCl_2_, 1% Triton X-100, and supplemented with Complete proteases inhibitors. Membranes were then incubated with MES buffer on a rotary shaker for 30 min at 4°C. At the end of the incubation, membranes were centrifuged at 100,000 g for 30 min, and supernatant was designated as DS fraction. The pellet was resuspended in buffer containing (in mM) 15 HEPES (pH 7.4), 150 NaCl, 10 EDTA, 1 DTT, 1% Triton X-100, 0.1% SDS, supplemented with complete protease inhibitors, and was designated as DI fraction. Both DS and DI fractions were stored at −80°C until further analysis by Western blotting.

**Flotation on a discontinuous Optiprep density gradient.** Lipid rafts were isolated by flotation on Optiprep density gradient as previously described by us (6). Briefly, cellular membrane preparations were ultracentrifuged at 100,000 g for 30 min at 4°C and then incubated for 30 min at 4°C in TNE buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 supplemented with 1× Complete protease inhibitor cocktail. The membranes were then adjusted to 40% final concentration of Optiprep (Nycomed, Oslo, Norway) and layered at the bottom of density gradient with steps of final concentrations of 35, 30, 25, and 20% of Optiprep in TNE buffer. The gradient was then centrifuged at 215,000 g for 4 h at 4°C. Fractions were collected from the top of the gradient and then analyzed by Western blotting.

**Western blotting.** Proteins were solubilized in Laemmli sample buffer (2% SDS, 10% glycerol 100 mM DTT, 60 mM Tris, pH 6.8, 0.01% bromphenol blue) and separated on 10% Tris-glycine SDS polyacrylamide gel. Separated proteins were then electrotransferred onto nitrocellulose membranes, and Western blotting was performed by washing the nitrocellulose membranes three times and then blocking them overnight in blocking buffer containing 5% nonfat dry milk in PBS. The blots were then incubated with the anti-V5 horseradish peroxidase-conjugated antibody diluted in the blocking solution for 3 h at room temperature, or left overnight at 4°C. Blots were washed extensively after that with PBS containing 0.1% Tween 20, and then the bands were visualized by enhanced chemiluminescence ECL kit according to the manufacturer’s instructions (Amersham, Arlington Heights, IL).

**Statistical analysis.** Results are expressed as means ± SE. Student’s t-test was utilized in statistical analysis. P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Effect of EGCG on ASBT function.** To examine the effect of green tea catechin EGCG on ASBT function, we utilized HEK-293 cells stably transfected with ASBT-V5 fusion protein (designated as 2BT) as an in vitro cellular model. EGCG incubation significantly decreased sodium-dependent [3H]TC uptake into 2BT cells. As shown in Fig. 1, treatment with EGCG decreased ASBT function in a time- (Fig. 1A) and a dose-dependent manner (Fig. 1B).

We next investigated the effects of other green tea catechins on ASBT function in 2BT cells. As shown in Fig. 2, 90-min incubation with 50 μM of (−)-catechin, (−)-catechin gallate, EC, ECG, and EGC had no effect on sodium-dependent [3H]TC uptake in 2BT cells. These data clearly show that EGCG but not other green tea catechins acutely inhibits ASBT activity.

**PKC, PI3K, ERK1/2, or tyrosine-phosphatase signaling pathways are not involved.** Recent studies have shown the modulation of ASBT activity by various signal transduction pathways (3, 27). EGCG is known to activate protein kinase C (PKC), ERK1/2, phosphatidylinositol 3-kinase (PI3K), and protein-tyrosine phosphatase-dependent pathways (2, 23, 26). We therefore investigated the possible involvement of these signaling intermediates in the EGCG-induced inhibition of ASBT function. As shown in Fig. 3, incubation with 5 μM BIM (PKC inhibitor), 50 μM LY294002 (PI3K inhibitor), 5 μg/ml dephostatin (inhibitor of protein-tyrosine phosphatase), and 10 μM of PD98095 (inhibitor of ERK1/2) failed to abrogate the reduction of ASBT activity by EGCG. These data rule out the involvement of these signaling molecules in mediating the inhibitory effect of EGCG on ASBT function.

**EGCG effect on kinetic parameters of TC uptake.** To further investigate the mechanisms by which EGCG inhibits ASBT function, we evaluated the kinetics of [3H]TC uptake in the presence of EGCG. [3H]TC uptake was measured in the presence of increasing concentrations of the substrate TC. As depicted in Fig. 4, 90-min incubation with 50 μM EGCG resulted in a significant decrease in the V_max of the transporter (EGCG 137.1 ± 9.11 vs. Control 487.1 μmol/mg protein·5 min⁻¹) with no change in the apparent K_m. These data demonstrate that EGCG-induced inhibition of ASBT func-
tion may result from a decrease in the number of active molecules on the membrane or a reduction in the rate of turnover of the transporter.

**EGCG does not change the surface level of ASBT.** To determine whether the reduction in the $V_{\text{max}}$ of TC uptake is a result of a decreased level of ASBT on the plasma membrane, we utilized cell surface biotinylation to assess changes in the level of ASBT on plasma membrane. As shown in Fig. 5A, total cellular level of ASBT-V5 as well as ASBT-V5 membrane expression were unaltered by EGCG. Additionally, EGCG-mediated inhibition of ASBT function was not blocked in the presence of the inhibitor of endocytosis, phenylarsine oxide, PAO (Fig. 5B). These data clearly indicate that the reduction in ASBT function by EGCG is not mediated by a decrease in ASBT content on the plasma membrane.

**EGCG changes the association of ASBT with the DS and DI fractions of the plasma membrane.** Recent evidence has shown EGCG to be a potent modulator of membrane lipid rafts structure (1, 15, 26). We have recently shown that association of ASBT with plasma membrane lipid rafts is essential for its optimal function. Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains resistant to solubilization by Triton X at 4°C (16). To determine whether EGCG treatment alters ASBT localization to lipid rafts, we first examined the effect of EGCG on the distribution of ASBT-V5 fusion protein in DS and DI membrane fractions of 2BT cells. After treatment with EGCG, crude membranes from 2BT cells were extracted and subjected to solubilization with Triton X. Figure 6 shows that ASBT-V5 fusion protein was detected in both DI and DS fractions of membranes but actin was mainly associated with the DI fraction. Also, as depicted in Fig. 6, 90-min incubation with 50 μM EGCG significantly reduced the association of ASBT-V5 but not actin with the DI fractions, whereas treat-
ment with EC catechin did not significantly alter the level of ASBT-V5 in the DI membrane fractions. The level of ASBT-V5 in the DI fraction was reverted back to normal 24 h after the removal of EGCG (Fig. 6). In parallel, the inhibitory effect of EGCG on ASBT function was also reversed back to baseline 24 h after EGCG removal (Fig. 7). These results indicate that the effects of EGCG green tea catechin on ASBT function and distribution between different membrane domains are reversible.

We further confirmed EGCG-induced disassociation of ASBT protein from lipid raft microdomains by investigating changes in the flotation pattern of membrane fractions containing ASBT on Optiprep density gradient. Total crude membranes were prepared from control and EGCG-treated cells. Membranes were then incubated with Triton X-100 and laid at the bottom of Optiprep density gradient. After ultracentrifugation, fractions were collected along the gradient and the distribution of ASBT-V5 was investigated by Western blotting. As shown in Fig. 8 (top), ASBT-V5 fusion protein was detected in all fractions including floating fractions of lipid rafts (fractions 1–3). A similar pattern was also observed for the lipid raft marker flotillin (Fig. 8, bottom). Upon treatment with EGCG, both ASBT-V5 fusion protein and flotillin shifted to the high-density fractions (Fig. 8), indicating that EGCG increased solubility of lipid raft microdomains containing ASBT-V5 fusion protein. Altogether, these data suggest that EGCG inhibits bile acid uptake by altering the distribution of bile acid transporter ASBT between various domains of plasma membrane.

EGCG decreases ASBT function in intestinal epithelial cells. We next investigated the effect of EGCG catechin on ASBT in human intestinal Caco-2 cells. Caco-2 monolayers 5–7 days postconfluent were treated with different concentrations of EGCG for 90 min, and Na-dependent [3H]TC uptake was then performed as described in MATERIAL AND METHODS. In a separate group of cells, EGCG was washed out and cells were left for additional 24 h after EGCG treatment before lysing (washout). Cellular membranes were then isolated by ultracentrifugation and then incubated for 30 min at 4°C in a buffer containing 1% Triton X-100. Detergent soluble (DS) and DI fractions were then isolated as described in MATERIAL AND METHODS. Proteins were separated on 10% SDS-PAGE gel and blots were probed with anti-V5 antibodies to visualize the ASBT-V5 fusion proteins.

DISCUSSION

In the present studies, we have provided novel data demonstrating a direct effect of EGCG green tea catechin on the function of ileal ASBT. Our data showed that modification of...
lipid rafts of plasma membranes is involved in the reduction of ASBT function without altering the level of ASBT protein on the plasma membranes. Our study strongly suggests an essential role of ASBT inhibition in cholesterol-lowering effects of EGCG green tea catechin.

We utilized two different cell models to investigate the effect of green tea catechins on ASBT: human intestinal cell line Caco-2 and HEK-293 cells. Human intestinal Caco-2 monolayers have been previously demonstrated to be an excellent model to investigate the regulation of ASBT activity (5, 6, 27). Also, we have previously shown that HEK-293 cells stably transfected with ASBT-V5 fusion protein (designated as 2BT cells) also represent an excellent model to examine the acute nontranscriptional regulation of ASBT function (6). Indeed, 2BT cells represent a better cellular model to investigate the regulation of ASBT by lipid rafts for several reasons. First, the regulatory mechanisms appear to be similar to that observed in intestinal epithelial Caco-2 cells (6). Second, 2BT cells provide an excellent model to perform biochemical studies, such as Western blotting and immunoprecipitation, to delineate the mechanisms underlying acute ASBT regulation taking advantage of the availability of commercial anti V5 antibodies. Third, HEK-293 cells lack the endogenous expression of ASBT allowing a direct assessment of the function of the transfected ASBT-V5 fusion protein to determine the impact of changes in the structure or the association with lipid rafts on its transport activity. Therefore, we focused the majority of our investigations on 2BT cells as a model to determine the modulation of ASBT by green tea catechins. EGCG green tea catechin significantly inhibited ASBT activity in both cell lines, indicating that ASBT is altered in 2BT cells in the same manner as in intestinal epithelial cells. The reduction in ASBT by EGCG was dose dependent within a range of concentrations (50–200 μM) similar to those used in a number of previous studies and is also comparable with EGCG concentrations in the intestinal lumen of habitual green tea drinkers (9, 19). The fact that EGCG but not other green tea catechins inhibited ASBT activity indicates a specific effect for EGCG on the ileal bile acid transporter. Interestingly, previous studies demonstrated that EGCG is the major biologically active component mediating the beneficial effects of green tea including hypolipidemic and hypocholesterolemic effects (20, 29). Consistent with these previous observations, our present findings indicate that EGCG but not other catechins of green tea mediates the inhibition of ileal bile acid transporter ASBT.

A number of intracellular signaling pathways have been previously implicated in mediating EGCG influence on cellular functions in various tissues and cell types. For example, PI3K was shown to play a role in EGCG-induced activation of ERK1/2 in prostate cancer PC3 cell line (2). Additionally, EGCG was shown to activate PKC in murine hippocampus (23). ASBT activity was recently shown to be modulated by...
several signaling molecules including PKCζ (3, 27). The finding of the present studies, however, ruled out the involvement of PKC, PI3K, as well as ERK1/2 in mediating the inhibition of ASBT function by EGCG. Further examination of the mechanisms of ASBT inhibition by EGCG depicted a significant decrease in the \( V_{\text{max}} \) of the transporter with no significant alteration in the affinity for the substrate (TC). Although a decrease in the level of ASBT on the plasma membrane may explain the changes in the \( V_{\text{max}} \), a decline in the turnover rate of the transporter across the surface membrane could also lead to a reduction in the \( V_{\text{max}} \) of the transporter. Indeed, biotinylation studies clearly showed that the level of ASBT on the plasma membrane remained unaltered by EGCG treatment. Furthermore, the inhibitor of endocytosis phenylarsine oxide (PAO) failed to block the reduction of ASBT, supporting the observation that reduction of ASBT function by EGCG is not associated with a decrease in the transporter level on plasma membrane. It is worth mentioning that PAO is also an inhibitor of phosphotyrosine phosphatase (PTP) (14) and hence the failure of PAO to block EGCG effect also rules out the involvement of PTP-dependent signaling pathway in the observed inhibition of ASBT function. Collectively, these observations clearly show that ASBT level on plasma membrane remains unaltered by EGCG and the decline in the \( V_{\text{max}} \) is likely due to a decrease in the turnover rate of the transporter.

Recently, we have shown that the optimal function of ASBT is dependent on its association with lipid rafts and cholesterol content of the plasma membrane (6). Interestingly, the involvement of lipid raft domains in mediating the biological effects of EGCG has previously been reported (1, 15, 26). Lipid rafts, defined as specialized plasma membrane domains rich in cholesterol and sphingolipids and resistant to detergent solubilization (16), have been demonstrated as a platform for the binding and the initiation of EGCG effect on cellular functions (26). For example, EGCG has been demonstrated to prevent the activation of epidermal growth factor receptor (EGFR) via reorganization of the lipid order in the plasma membranes and increasing the detergent solubility of lipid rafts in membranes of human colonic cell line HT29 (1). Our present studies also provided novel evidence indicating the importance of lipid rafts in the modulation of ASBT function by EGCG. Similar to the effect of EGCG on EGFR, the solubility of ASBT-associated lipid rafts was also decreased by EGCG as assessed by the flotation techniques as well as the isolation of detergent soluble and insoluble fractions of cellular membranes. The notion that EGCG but not EC altered the function and the solubility of lipid rafts clearly indicates that these structural microdomains of plasma membrane provide the molecular basis for determining the specificity of ASBT inhibition by green tea catechins. Accordingly, the decrease in ASBT function specifically by EGCG is due to the ability of this potent catechin to induce reorganization of lipids of the plasma membrane. In this regard, depleting cholesterol from plasma membrane with agents such as methyl β-cyclodextrin (MβCD) leads to an increase in solubility of lipid rafts and also decreases the ASBT function (6). In contrast to MβCD, EGCG does not alter the level of cholesterol in plasma membranes (1). These observations strongly suggest that EGCG alters the solubility and functional characteristics of the lipid rafts by a mechanism distinct from that of MβCD. In this regard, EGCG has been previously shown to modulate membrane fluidity (10, 28).

Also, EGCG was demonstrated to directly bind lipids (18). It is possible, therefore, that alterations in the fluidity of lipid rafts by EGCG affects both detergent solubility as well as ASBT function. It is also possible that EGCG binds cholesterol, phospholipids, and sphingolipids, causing changes in the interactions of these lipids with ASBT in plasma membrane and leading to a decrease in its function. These observations warrant future studies to examine the molecular mechanisms by which EGCG alters the composition of lipid rafts and inhibits ASBT function.

The reversal of the inhibitory effect of EGCG on ASBT function indicates that EGCG-induced inhibition is not due to any possible cytotoxic effects. Since the effects of EGCG on the solubility of membrane fractions containing ASBT as well as ASBT function are reversible, we speculate that EGCG is eliminated from the membrane, and, therefore, its actions are reversed by a process that still needs to be determined in future studies. The reversal of EGCG influence on membrane structure and ASBT function is consistent with epidemiological studies demonstrating the beneficial effects are associated with the habitual and continuous drinking of green tea (11, 17, 20, 24, 30).

Blocking intestinal bile acid absorption has been long known as an attractive method to reduce plasma cholesterol (4, 7). Previous studies in animal models demonstrated an increase in the level of fecal bile acids by green tea feeding, suggesting a reduction in intestinal bile acid absorption (12, 30). Our data indicate that EGCG green tea catechin reduces the activity of ileal ASBT, implicating its involvement in the hypocholesterolemic effect of green tea. Our findings also clearly showed that lipid rafts of plasma membrane are essential for the modulation of ASBT by EGCG. These novel observations suggest that lipid rafts of the apical membrane of intestinal epithelial cells represent a platform for the rapid regulation of ileal ASBT and define a mechanism for the beneficial effect of green tea in lowering cholesterol.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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