

## Introduction

Primary biliary cirrhosis (PBC) is a chronic liver disease that is presumably caused by autoimmunity. The detection of serum antimitochondrial antibodies (AMA) and increased levels of immunoglobulin M (IgM) are biochemical features of this disease. Histopathologically, it is characterized by portal inflammation and the slow progressive destruction of the portal interlobular bile ducts due to chronic non-suppurative cholangitis. The loss of bile ducts leads to cholestasis, which leads to further hepatic damage, fibrosis, cirrhosis, and ultimately, liver failure.<sup>1</sup>

Ursodeoxycholic acid (UDCA) is the only FDA-approved drug and the first-line medicine for the treatment of PBC.<sup>2</sup> UDCA has been shown to improve serum levels of biliary enzymes and IgM, and may slow the histologic progression to liver cirrhosis.<sup>3-6</sup> The mechanisms of the anticholestatic and anti-inflammatory effects of UDCA have been reported to be due to the activation of the canalicular bile salt export pump (BSEP), canalicular multidrug resistance protein 3 (MDR3; ATP-binding cassette transporter B4 [ABCB4]) and basolateral multidrug resistance-associated protein 4 (MRP4 [ABCC4]).<sup>7</sup> In addition, the replacement of hydrophobic bile acids with hydrophilic UDCA appears to attenuate the damage to hepatocytes and biliary cells.<sup>2</sup> It has been reported that about two-thirds of patients treated with UDCA in the early stage of the disease could have a normal life expectancy without additional therapies.<sup>8</sup> However, the remaining patients are not sufficiently controlled with UDCA monotherapy and additional therapeutic approaches have been necessary.

Immunosuppressive medication is not recommended as the first-line, alternative drug for PBC, but budesonide, a non-halogenated glucocorticoid with a high first-pass metabolism, and/or mycophenolate mofeti, an inhibitor of the purine biosynthetic pathway which is critical to

lymphocytic proliferation and activation, are sometimes used in patients who fail to respond to UDCA.<sup>9,10</sup> However, the effects of these immunosuppressive agents remains controversial.<sup>11,12</sup> The farnesoid X receptor (FXR; NR1H4) agonist, 6-ethyl-chenodeoxycholic acid, has been administered to PBC patients that exhibit incomplete responses to UDCA in a phase II clinical trial. This trial exhibited anticholestatic effects and serum ALP levels were reduced, but pruritus occurs at the higher doses.<sup>13</sup>

In 1999, Iwasaki *et al.* introduced the effectiveness of a hypolipidemic agent, bezafibrate, on the reduction of serum ALP and IgM levels in pre-cirrhotic PBC patients,<sup>14</sup> and recently, combination therapy with UDCA and bezafibrate is being recognized as a beneficial treatment for PBC that is refractory to UDCA monotherapy.<sup>15,16</sup> While the mechanisms of anticholestatic action by bezafibrate have not been elucidated completely, it is believed that the induction of MDR3 through activation of the peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ; NR1C1)<sup>17</sup> is the main mechanism, because fibrate class agents are ligands of the PPARs.<sup>18</sup> However, because MDR3 is activated by both the addition of bezafibrate as well as by UDCA monotherapy,<sup>7</sup> the roles of bezafibrate in the combination therapy remain unknown.

The current study was undertaken to explore the mechanisms of the remission of cholestasis by bezafibrate in PBC patients who failed to respond to UDCA monotherapy. Our *in vivo* and *in vitro* studies demonstrated that bezafibrate was a dual PPARs/pregnane X receptor (PXR; NR1I2) agonist with potent anticholestatic efficacy.

## Materials and Methods

**Patients.** Thirty-one Japanese patients with asymptomatic and untreated PBC (4 males and 27 females; aged 37–81 yrs) were enrolled in the study. The diagnosis of PBC was established by laboratory and histological findings, and all patients were classified as early-stage PBC (Scheuer's classification I or II). Informed consent was obtained from all subjects, and the study protocol was approved by the ethics committee of Tokyo Medical University Ibaraki Medical Center.

**Study Design.** All patients (n=31) were treated with UDCA (600 mg/day; 10-13 mg/kg/day) alone for at least 3 months (maximum 6 months) until serum ALP and GGT became stable (Supporting Figure). Then, bezafibrate (400 mg/day) was administered with UDCA (600 mg/day) to patients (n=19; 1 male and 18 females) who exhibited an incomplete biochemical response to UDCA monotherapy (defined as ALP or GGT level of above the upper limit of normal) and treated for 3 months. Before and after UDCA monotherapy and after the addition of bezafibrate, blood samples were collected in the morning before breakfast after an overnight fasting, and serum was stored at  $-20^{\circ}\text{C}$  until analyzed. Control sera from 49 healthy Japanese volunteers (11 males and 38 females; aged 22–79 yrs) were obtained from another study group (courtesy of Prof. T. Teramoto, Teikyo University School of Medicine, Tokyo, Japan) and were stored likewise as mentioned above.

**Determination of Serum Markers for Cholesterol and Bile Acid Metabolism.** Serum sterol concentrations were determined by LC-MS/MS, as described previously.<sup>19</sup> Serum fibroblast growth factor 19 (FGF19) levels were measured using a commercially available ELISA kit (Quantikine Human FGF-19 Immunoassay, R&D systems, Minneapolis, MN). Serum bile acid profiles were determined by LC-MS/MS according to the method by Ando *et al.*<sup>20</sup>

**Cell Culture.** The human hepatoma cell line, HepaRG, was obtained from Biopredic International (Rennes, France). On day 0, a 24-well plate was seeded with  $4.8 \times 10^5$  differentiated HepaRG cells/well using HepaRG Thawing and Seeding Medium 670. On day 3, the medium was replaced with 500  $\mu$ l/well of HepaRG Induction Medium 640 containing bezafibrate, rifampicin, carbamazepine or GW4064 dissolved in 1% acetonitrile. Cells were incubated for 48 hours at 37°C in a humidified incubator containing 5% CO<sub>2</sub> and 95% air.

**Assays of CYP3A4 Activity and PXR Activation.** CYP3A4 activities were measured by cell-based P450-Glo™ CYP3A4 Assay Kit (Luciferin-IPA) purchased from Promega (Madison, WI). The activation of PXR was determined by a Human PXR Activation Assay System (Puracyp, Carlsbad, CA) utilizing DPX2 hepatoma cells harboring the human PXR and luciferase-linked CYP3A4 promoters.

**RNA Measurements.** Total RNA was extracted from the HepaRG cells using an RNeasy Plus Mini Kit (QIAGEN, Tokyo, Japan). Reverse transcription and real-time quantitative PCR were performed as described previously.<sup>21</sup> The sequences of some primer pairs have been described in the same report.<sup>21</sup> The other primer sequences used in this study are listed in the Supporting Table.

**Statistics.** Data are reported as the mean $\pm$ SEM for human data and as the mean $\pm$ SD for cell data. The statistical significance of differences between the results in the different groups was evaluated by non-parametric Mann-Whitney test for human data (Table 1 and 2) and Student's two-tailed *t*-test for cell data (Fig. 4 and 5). On the other hand, the data obtained before and after treatment were compared by Wilcoxon signed-ranks test (Fig. 1-3). In all statistical tests, significance was accepted at the level of  $P < 0.05$ .

## Results

The characteristics of the PBC patients enrolled in the present study are shown in Table 1. In patients before UDCA treatment (n=31) and those responded to UDCA insufficiently and before additional bezafibrate treatment (n=19), serum AST, ALT, GGT, ALP and IgM levels were significantly elevated compared with healthy controls. Serum LDL cholesterol and triglyceride concentrations were increased and HDL cholesterol concentration was decreased significantly in the patients before UDCA treatment compared with controls. In the patients before additional bezafibrate treatment, a similar tendency was observed, but the differences were not statistically significant.

Baseline biomarker levels for lipid metabolism in the three groups are compared in Table 2. In this study, cholesterol metabolism in PBC patients was assayed by measuring serum sterol biomarkers. Because most non-cholesterol sterols are transported in serum with cholesterol, the expression of each sterol level relative to the total cholesterol concentration tends to be more reliable compared with the absolute concentration, especially when dyslipidemia is present.<sup>22</sup> Serum concentrations of sitosterol, 4 $\beta$ -hydroxycholesterol (4 $\beta$ -HC) and 24S-hydroxycholesterol (24S-HC) expressed relative to total cholesterol were significantly elevated in both patients' groups, compared with controls. However, other sterols, 7 $\alpha$ -hydroxy-4-cholesten-3-one (C4), lathosterol, campesterol and 27-hydroxycholesterol (27-HC), and FGF19 concentrations did not differ significantly among the three groups.

***Effects of UDCA and Bezafibrate on Serum Liver Enzymes and Lipids.*** As shown in Fig. 1a, serum AST, ALT, GGT, ALP and IgM levels were all reduced significantly by treatment with UDCA. In patients who responded incompletely to UDCA monotherapy, the combination of bezafibrate and UDCA further reduced serum levels of ALT, GGT, ALP and IgM. The changes in

serum lipid concentrations by UDCA and bezafibrate treatment are presented in Fig. 1b. UDCA monotherapy did not change the serum lipid levels significantly. However, the addition of bezafibrate significantly decreased serum concentrations of total cholesterol, LDL cholesterol and triglyceride in those patients whose cholestasis was not sufficiently improved by UDCA alone.

**Effects of UDCA and Bezafibrate on Bile Acid Metabolism.** C4 and FGF19 are markers of bile acid production<sup>23</sup> and transintestinal flux,<sup>24</sup> respectively. As shown in Fig. 2a, UDCA did not change C4 or FGF19 concentrations, but bezafibrate significantly reduced both C4 and FGF19 levels. In Fig. 2b and 2c, serum bile acid concentrations and UDCA proportion in UDCA treated patients before and after addition of bezafibrate are shown. The addition of bezafibrate significantly reduced the serum chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) concentrations. The serum cholic acid (CA) and lithocholic acid (LCA) concentrations also tended to be reduced by bezafibrate, but the differences were not statistically significant. The serum proportion of UDCA was significantly increased by the addition of bezafibrate compared with UDCA monotherapy, presumably due to its inhibitory effect on *de novo* bile acid biosynthesis. The proportion of UDCA in serum is usually higher than that in bile in patients treated with UDCA, but it appears to reflect the biliary proportion of UDCA to some extent.<sup>25</sup>

**Effects of UDCA and Bezafibrate on Sterol Metabolism.** Cholesterol biosynthesis and intestinal absorption were studied by measuring serum concentrations of lathosterol and plant sterols (sitosterol and campesterol), respectively. As shown in Fig. 3a, UDCA treatment did not affect cholesterol biosynthesis but significantly increased cholesterol absorption. In contrast, bezafibrate significantly inhibited cholesterol biosynthesis but did not change cholesterol absorption.

Serum concentrations of major oxysterols that are potential ligands of Liver X receptor  $\alpha$  (LXR $\alpha$ , NR1H3) were compared between UDCA and bezafibrate treatments (Fig. 3b). UDCA

treatment did not affect serum 4 $\beta$ -HC or 24S-HC concentrations but increased the 27-HC concentration significantly. Treatment with bezafibrate clearly increased serum 4 $\beta$ -HC levels, while it significantly reduced the 24S-HC and 27-HC levels.

***Effects of Bezafibrate on CYP3A4.*** Differentiated HepaRG cells exhibit a gene expression pattern similar to primary human hepatocytes and human liver tissues and maintain significant levels of hepatic cell functions, including CYP and transporter activities.<sup>26</sup> Rifampicin and carbamazepine are classical inducers of CYP3A4 via the activation of PXR<sup>27</sup>, while GW4064 is one of the most potent agonists of FXR.<sup>28</sup> As shown in Fig. 4a, bezafibrate, as well as rifampicin and carbamazepine, induced both CYP3A4 mRNA expression and activity in a dose-dependent manner.

***Effects of Bezafibrate on PXR Activation.*** The DPX2 cell-based luciferase reporter gene assay demonstrated that in comparison with rifampicin, bezafibrate was a weak but significant activator of human PXR as well as carbamazepine (Fig. 4b). It is noteworthy that GW4064 activated human PXR at the concentrations higher than 3  $\mu$ M.

***Effects of Bezafibrate on Gene Expression of Nuclear Receptors, Transporters and Enzymes.*** Among the nuclear receptors and related coactivators (Fig. 5a), PXR expression was induced by bezafibrate, to a greater degree than that by rifampicin, which suggests that PXR is a target gene of PPARs, as reported previously.<sup>29</sup> In contrast, the small heterodimer partner (SHP; NR0B2), a target of FXR, and LXRA were downregulated by bezafibrate, as well as rifampicin and carbamazepine. FXR and peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ ) expressions were significantly downregulated by rifampicin and carbamazepine but not by bezafibrate.

The MDR1 (ABCB1) and MRP2 (ABCC2) transporters (Fig. 5b) were upregulated by bezafibrate, similar to rifampicin, whereas MDR3, ABCG5 and ABCG8 were upregulated by

bezafibrate but not by rifampicin. In addition, Na<sup>+</sup>/taurocholate cotransporting polypeptide (NTCP) was downregulated by bezafibrate but did not change significantly by rifampicin. It is notable that significant mRNA expression of BSEP was observed in HepaRG cells treated with GW4064, whereas only trace amount of BSEP expression was detected in control cells and those treated with other compounds.

Enzymes involved in cholesterol, bile acid and fatty acid syntheses and LDL receptor expression are summarized in Fig. 5c. CYP7A1, CYP7B1 and CYP27A1 were downregulated and CYP8B1, fatty acid synthase (FAS) and LDL receptor (LDLR) were upregulated by bezafibrate, which was the same as the effects of rifampicin. HMG-CoA reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthetic pathway, was downregulated by rifampicin but was slightly upregulated by bezafibrate.



## Discussion

Our results clearly showed that the combination therapy of bezafibrate and UDCA significantly improved cholestasis in early-stage PBC patients who were refractory to UDCA monotherapy. The mean levels of ALP and GGT during UDCA monotherapy were further reduced from  $597\pm 51$  to  $324\pm 27$  IU/L and  $178\pm 59$  to  $99\pm 41$  IU/L, respectively, by the additional administration of bezafibrate (Fig. 1). It is known that UDCA not only improves cholestasis but also the serum IgM concentrations.<sup>4,6</sup> The combination therapy of bezafibrate and UDCA further reduced the IgM concentration from  $306\pm 60$  (UDCA alone) to  $232\pm 41$  mg/dL (UDCA + bezafibrate), consistent with the findings reported by Iwasaki *et al.*<sup>16</sup> Furthermore, our results showed that the combination therapy significantly reduced serum total cholesterol, LDL cholesterol and triglyceride concentrations compared with UDCA alone.

The mechanisms of the anticholestatic effect of bezafibrate remain unclear. Because MDR3 is a target gene of PPAR $\alpha$ <sup>17</sup> and bezafibrate is a ligand of PPAR $\alpha$ ,  $\beta/\delta$  and  $\gamma$ ,<sup>18</sup> stimulation of biliary phospholipid secretion due to the up-regulation of MDR3 has generally been believed to be the main mechanism of the action. In fact, our experiment using HepaRG cells showed significantly elevated expression of MDR3 mRNA following the addition of bezafibrate (Fig. 5b). However, MDR3 is activated by both bezafibrate as well as UDCA.<sup>7</sup> Furthermore, recent reports have demonstrated that the expression of MDR3 was already markedly upregulated in PBC patients<sup>30</sup> and it was not significantly affected by bezafibrate treatment.<sup>31</sup> Therefore, the anticholestatic effect of bezafibrate may be caused by mechanisms independent of phospholipid secretion.

Other possible anticholestatic mechanisms of bezafibrate via PPAR $\alpha$  activation include down-regulation of NTCP,<sup>17</sup> CYP7A1<sup>32,33</sup> and CYP27A1.<sup>33</sup> NTCP transports basolateral

(sinusoidal) bile acids into hepatocytes, while CYP7A1 and CYP27A1 are key enzymes in the classic and alternative bile acid biosynthetic pathways, respectively. Coordinate down-regulation of these 3 proteins leads to a decrease in hepatic bile acid concentration and may protect hepatocytes against cytotoxic bile acids. In addition, the reduction of hepatic bile acid levels attenuates the activity of FXR. It is known that deactivation of FXR upregulates MRP4,<sup>34</sup> one of the important basolateral transporters for the efflux of bile acids from hepatocytes to the sinusoid in cholestasis. The transcription of MRP4 is positively controlled by the constitutive androstane receptor (CAR; NR1I3)<sup>35</sup> and a CAR responsive element is embedded within an FXR responsive element in the human MRP4 promoter. Therefore, activated FXR competes with CAR for binding to this overlapping binding site, which downregulates MRP4.<sup>36</sup>

The most striking results among our serum biomarker analyses are the elevation of 4 $\beta$ -HC, as well as the reduction of C4 during treatment with bezafibrate. Serum 4 $\beta$ -HC concentration is considered a biomarker of CYP3A4/5 activity<sup>37</sup> while C4 is a marker of CYP7A1 activity or *de novo* bile acid synthesis.<sup>23</sup> Therefore, the changes in 4 $\beta$ -HC and C4 concentrations during bezafibrate treatment suggest that bezafibrate upregulates CYP3A4/5 and downregulates CYP7A1. In fact, our experiments using HepaRG cells clearly demonstrated that bezafibrate induced CYP3A4 mRNA expression and activity (Fig. 4a) and inhibited the expression of CYP7A1 mRNA (Fig. 5c) in a dose-dependent manner. Significant up-regulation of CYP3A4 was caused by at least 10  $\mu$ M of bezafibrate, while the serum peak concentration ( $C_{max}$ ) values after oral administration of 400 mg bezafibrate are 9.1-22.7  $\mu$ M.<sup>38</sup>

Because the expression of CYP3A4 is mainly controlled by PXR,<sup>39</sup> it was strongly suggested that bezafibrate was a ligand of this nuclear receptor, and this hypothesis was proved by the reporter gene assay (Fig. 4b). In addition to PPAR $\alpha$ , PXR also regulates hepatic enzyme and transporter activities to exert protective effects against cholestasis. First, the induced CYP3A4

detoxifies xenobiotics and endogenous substances, including the toxic bile acid LCA.<sup>40,41</sup> The C-6 $\alpha$  or C-6 $\beta$  position of LCA is hydroxylated by CYP3A4 and non-toxic hyodeoxycholic acid (6 $\alpha$ -OH) or murideoxycholic acid (6 $\beta$ -OH) is formed. Second, the activation of PXR upregulates MDR1<sup>42</sup> and MRP2,<sup>43</sup> which was also observed in our HepaRG cells treated with rifampicin and bezafibrate (Fig. 5b). MDR1 transports various toxic metabolites and xenobiotics while MRP2 transports organic anions from hepatocytes to bile canaliculi.

These results further suggest that the down-regulation of CYP7A1 by bezafibrate is caused not only by the activation of PPAR $\alpha$  but also by the activation of PXR. Li and Chiang<sup>44</sup> demonstrated that hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ; NR2A1) interacts with several coactivators including PGC1 $\alpha$ , and that the complex activates the transcription of *CYP7A1* in the absence of ligands.<sup>45</sup> Ligands for PXR activate PXR to promote its interaction with HNF4 $\alpha$ , which disrupts the interaction between HNF4 $\alpha$  and PGC1 $\alpha$  and results in suppression of CYP7A1 expression.

Rifampicin is a more potent ligand of human PXR than bezafibrate (Fig. 4), and has also been shown to have anticholestatic effects in PBC patients.<sup>46</sup> However, continuous administration of rifampicin can sometime result in severe hepatitis.<sup>47</sup> In addition to rifampicin and bezafibrate, budesonide, but not prednisolone, is also an agonist of the human PXR.<sup>48</sup> Therefore, the therapeutic effects of budesonide on PBC patients may be caused at least in part by the anticholestatic effects via the activation of PXR.

Hypercholesterolemia and hypertriglyceridemia are often observed in PBC patients. While it remains controversial whether or not the lipid abnormalities in this disease increase atherosclerotic risk,<sup>49</sup> the administration of bezafibrate significantly reduced the serum concentrations of LDL cholesterol and triglycerides. The mechanism of the cholesterol-lowering effect of bezafibrate has not yet been completely elucidated, and at the very least, it is not likely

due to a direct inhibition of HMGCR<sup>50</sup> (Fig. 5c). Because the concentration of serum lathosterol, a marker for *de novo* cholesterol biosynthesis, was decreased significantly during bezafibrate therapy, inhibition of other enzymes involved in the pathway is strongly suggested. Another mechanism of the cholesterol-lowering effect of bezafibrate may be due to the stimulation of cholesterol efflux from hepatocytes to the bile canaliculi *via* the activation of PPARs. Our experiment using HepaRG cells showed significantly upregulated expression of ABCG5 and ABCG8 mRNA after bezafibrate but not rifampicin treatment (Fig. 5b). A similar effect of bezafibrate on ABCG5 in human liver has been reported previously.<sup>51</sup>

Because of the inhibition of bile acid synthesis and presumably the stimulation of cholesterol excretion into bile, bezafibrate significantly increases biliary cholesterol saturation.<sup>52</sup> Indeed, increased risk of gallstone formation has been reported in hyperlipidemic patients treated with another fibrate, fenofibrate.<sup>53</sup> However, combination therapy of UDCA and bezafibrate appears to attenuate the adverse effect of bezafibrate, because UDCA markedly lowers biliary cholesterol saturation and dissolves cholesterol gallstones.<sup>2</sup> On the other hand, bezafibrate may augment the anticholestatic and anti-lithogenic actions of UDCA by inhibiting bile acid synthesis and increasing the proportion of UDCA (Fig. 2c).

In addition to anticholestatic effects, activation of PXR<sup>54</sup> and the PPARs<sup>55</sup> has been reported to suppress inflammation through the inhibition of proinflammatory genes, including NF- $\kappa$ B, tumor necrosis factor- $\alpha$  and interleukin-1 $\alpha$ . In this study, although we did not evaluate the contribution of the anti-inflammatory effects of bezafibrate to the improvement of biochemical markers, bezafibrate is suggested to be an ideal drug with anticholestatic, hypolipidemic and even anti-inflammatory actions on PBC *via* the activation of both PXR and PPARs.

In summary, bezafibrate exhibited anticholestatic efficacy on PBC patients who showed an incomplete response to UDCA monotherapy. While UDCA replaces hydrophobic bile acids and

activates canalicular BSEP and MDR3 and basolateral MRP4,<sup>7</sup> bezafibrate inhibits hepatic synthesis and uptake of bile acids, enhances bile acid detoxification, and stimulates canalicular MDR3, MDR1 and MRP2 activities as a dual PPARs/PXR agonist (Fig. 6). These data lend support to the idea that the combination therapy with UDCA and bezafibrate is an excellent method for the treatment of early-stage PBC patients who exhibit an incomplete biochemical response to UDCA monotherapy.

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## Figure legends

**Fig. 1.** Effects of UDCA and additional bezafibrate treatment on serum liver enzymes (a) and lipids (b). B, before treatment; A, after treatment; BF, bezafibrate; T-CHOL, total cholesterol; LDL, LDL cholesterol; HDL, HDL cholesterol; TG, triglyceride. The mean concentrations before treatment were set to 1.0, and the absolute concentrations before treatment are shown in Table 1. Data are expressed as the mean $\pm$ SEM.

**Fig. 2.** Effects of UDCA and additional bezafibrate treatment on bile acid metabolism. (a) C4, 7 $\alpha$ -hydroxy-4-cholesten-3-one; FGF19, fibroblast growth factor 19; B, before treatment; A, after treatment; BF, bezafibrate. Mean concentrations before treatment (ng/mg cholesterol for C4 and pg/ml for FGF19) were set to 1.0, and the absolute concentrations before treatment are shown in Table 2. Data are expressed as the mean $\pm$ SEM. (b) Serum concentrations of bile acids in UDCA treated patients before and after addition of bezafibrate (n=17). (c) Serum proportions of UDCA in UDCA treated patients before and after addition of bezafibrate (n=17). The mean value for each group is indicated by the columns. Free BA, unconjugated bile acids; Glyco-BA, glycine-conjugated bile acids; Tauro-BA, taurine-conjugated bile acids.

**Fig. 3.** Effect of UDCA and additional bezafibrate treatment on cholesterol (a) and oxysterol (b) metabolism. B, before treatment; A, after treatment; BF, bezafibrate; 4 $\beta$ -HC, 4 $\beta$ -hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol. Mean concentrations before treatment ( $\mu$ g/mg cholesterol or ng/mg cholesterol) were set to 1.0, and the absolute concentrations before treatment are shown in Table 2. Data are expressed as the mean $\pm$ SEM.