INTRODUCTION

Vitamin D3 is a prohormone that is required for calcium and phosphorous homeostasis. It is naturally produced in the skin from 7-dehydrocholesterol by exposure to ultraviolet-B (UVB) irradiation (Esvelt et al., 1978). As a secondary source, vitamin D3 can also be obtained orally from fish oil, supplements, or fortified foods. Activation of vitamin D3 requires enzymatic hydroxylation at the 25- and 1-carbon positions to produce the active hormone, 1α,25-dihydroxyvitamin D3 (1,25-(OH)2D3). These hydroxylation steps take place in the liver and kidney, respectively (Fraser & Kodicek, 1970; Ponchon et al., 1969). Once activated, 1,25-(OH)2D3 stimulates intestinal absorption of calcium and phosphorous enabling normal skeletal mineralization. Furthermore, together with parathyroid hormone, 1,25-(OH)2D3 induces calcium mobilization from bone to prevent lethal hypocalcemia.

Although it has traditionally been assumed that oral and cutaneous-derived vitamin D3 are biologically
equivalent, they have never been directly compared. Furthermore, work by our laboratory and others have described distinct differences in their transport and delivery to metabolic tissues. Specifically, oral vitamin D₃ is absorbed from the small intestine and transported to the liver in chylomicrons, whereas we have shown that skin-generated vitamin D₃ is transported from skin by vitamin D binding protein (DBP; Avioli, 1969; Duchow et al., 2019; Dueland et al., 1991; Schachter et al., 1964). Upon entering general circulation, oral vitamin D₃ is rapidly taken up into the liver with a half-life of ~4–6 h (Mawer et al., 1971). In contrast, blood vitamin D₃ increases gradually following UVB exposure, peaking at approximately 24 h following exposure and decreases thereafter with a half-life ranging from 36 to 72 h (Haddad et al., 1993; Stamp et al., 1977).

In the current study, we examined the impact of these differences on the biological activity of vitamin D₃. We report that the biological activity is greater from skin-generated vitamin D₃ than that given orally. By examining the excretion of radiolabeled vitamin D₃ injected unbound or pre-bound by DBP, we determined that DBP significantly reduces the amount of vitamin D₃ excreted in the bile. We also demonstrate that DBP improves the biological activity of oral vitamin D₃ by comparing the activity in DBP knockout (DBP−/−) and wild-type (DBP+/+) mice. Thus, DBP must be considered a major component of the vitamin D system and is essential for naturally produced vitamin D in skin.

2 | METHODS

2.1 | Experimental mice

All experiments were conducted in accordance with the Research Animal Resources Committee of the College of Agricultural & Life Sciences, University of Wisconsin-Madison. Animals were maintained in the Department of Biochemistry vivarium with a 12:12 h light: dark cycle. Fluorescent bulbs in animal housing and procedure rooms were covered by filters, which eliminate the wavelengths that result in vitamin D₃ production in skin. DBP+/− mouse embryos were provided by the Cooke laboratory (Perelman School of Medicine, University of Pennsylvania) and rederived at the University of Wisconsin Genomic Editing and Animal Models Core (University of Wisconsin-Madison; Safadi et al., 1999). DBP+/− breeders were maintained on standard laboratory chow 5051 (Purina Mills). Genotyping of offspring was performed by Transnetyx. To generate vitamin D-deficient animals, DBP+/+ and DBP−/− mice were placed on purified diets devoid of vitamin D at the time of weaning and were fed purified diets containing either 0.47% Ca/0.3% P or 0.02% Ca/0.3% P during depletion. The mice were maintained for 1 week on the 0.47% Ca/0.3% P diet, followed by 3 weeks on the 0.02% Ca/0.3% P diet. This was repeated until mice were determined to be deficient by serum calcium and serum 25(OH)D₃ measurements.

2.2 | UVB radiation

UVB radiation was carried out as previously described (Irving et al., 2017). The dorsal surface of each mouse was shaved using an electric razor ~24 h before each experiment. Irradiation was performed using a bank of 4 UVB lamps that emit from 280 to 330 nm with a peak at 310 nm (Solorc Systems). The radiation output was measured by placing a UV radiometer equipped with a UVX-31 sensor with a calibration point of 310 nm and bandpass 280–340 nm (UVP LLC) at 3 locations within the cage to reproduce the positions of the animals. The average output was calculated, and the time was adjusted to ensure exposure to 2.6–8 kJ/m² per treatment.

2.3 | Skin vitamin D measurement

DBP−/− mice underwent single UVB treatment at the desired dose levels. Mice were euthanized 24 h after treatment by CO₂ asphyxiation and skin was collected from the entire dorsal surface exposed to UVB light including shaved dorsal skin, ears, and tail. No vitamin D₃ was detectable in ventral skin. The tissue was minced using razor blades and homogenized in phosphate-buffered saline (PBS) using a Beadmill24 homogenizer (Fisher Scientific). The entire volume was subjected to a Bligh-Dyer extraction using CH₂Cl₂ in place of CHCl₃. The CH₂Cl₂ layer was dried under argon gas. The remaining oil was dissolved in 2 ml of 5% KOH in 95% methanol/5% H₂O and saponified for 2 h at 70°C. The lipid soluble saponification products were isolated following the addition of equal volumes of water and hexane. Samples were centrifuged at 3000 × g for 5 min. The hexane layer was transferred to a fresh tube, and the sample was washed twice more with equal volumes of hexane. The hexane layers were combined and dried under argon gas. The residual oil was dissolved in 99% hexane/1% isopropanol. 1,2-[3H]-vitamin D was added prior to each extraction to monitor extraction efficiency. Lipid extracts were applied to a straight-phase HPLC column (Zorbax SIL, 4.6 × 250 mm, Agilent) run at a flow rate of 0.75 ml/min of 99% hexane/1% isopropanol and monitored at 265 nm. The retention time was based on comigration with the tritiated vitamin D₃. The limit of detection for this method was 1 ng and the limit of quantification was 2.5 ng.
2.4  |  Serum calcium analysis

Blood was collected from the retro-orbital sinus for all reported measurements. Blood was collected for baseline serum calcium measurement 24–48 h prior to each experiment and at all indicated timepoints. Serum calcium was determined by atomic absorption using a PerkinElmer 900H spectrophotometer following 1:40 dilution of the serum with 0.1% LaCl₃. The biological activity was calculated as the area under the curve of the serum calcium plotted over time using GraphPad Prism software.

2.5  |  Dosing solutions and administration

Vitamin D₃ was purchased from Sigma. Oral vitamin D₃ was administered in 1% ethanol in Neobee oil via oral gavage. Intravenous injections of free and bound vitamin D were administered in sterile PBS containing 1% ethanol, 0.01% Tween-20. Recombinant DBP was synthesized by Lytic Solutions, LLC. Briefly, the protein bearing a cleavage his-tag was expressed by transient transfection of CHO cells and affinity purified by immobilized metal affinity chromatography (IMAC). The purified protein was buffer exchanged and treated with tobacco etch virus (TEV) protease (New England Biolabs) to remove the His-tag. The cleaved tag, uncleaved protein, and TEV protease (his-tagged) were removed by passing the digest over an IMAC column.

Radiolabeled vitamin D₃ was synthesized by Moravek Inc., To generated [3H]-vitamin D-DBP complexes, binding was carried out at the desired dosing solution concentration with 6 µg of DBP in 0.01% Tween-20, 1% ethanol in sterile PBS overnight at 4°C. Unbound [3H]-vitamin D was removed on a Sephadex G-25 spin column and flow-through was used as the dosing solution. For the “free vitamin D” dosing solution, [3H]-vitamin D was incubated in 0.01% Tween-20, 1% ethanol in sterile PBS overnight at 4°C. The dosing solution concentrations were confirmed by liquid scintillation counting using a TRI-CARB 4810 TR liquid scintillation analyzer (PerkinElmer). Intravenous injections were administered via retro-orbital injection under ether anesthesia. Oral doses were administered via oral gavage.

2.6  |  Fecal analysis for biliary excretion

Mice were housed in metabolic cages for feces and urine collection for 24–48 h. Dried feces were collected, rehydrated in 0.5 ml of H₂O, and solubilized in 2 ml tissue solubilizer for 2 h at 50°C. Samples were decolorized with 0.2 ml 30% H₂O₂. The tritium content of solubilized samples was determined by liquid scintillation counting using a TRI-CARB 4810 TR liquid scintillation analyzer (PerkinElmer).

2.7  |  Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using the mixed model procedure with Tukey’s adjustment using SAS Version 9.4 (SAS Institute). A value of $p < 0.05$ was considered statistically significant.

3  |  RESULTS

3.1  |  UVB-generated vitamin D₃ is more biologically active than orally administered vitamin D₃

The biological activity of an equivalent amount of vitamin D₃ generated either from a single UVB exposure or delivered as a single oral dose was compared in vitamin D₃-deficient, hypocalcemic mice. The biological activity was assessed based on the area under the curve of the serum calcium response over time for individual animals and averaged within each group.

The biological activity was evaluated for “low” and “high” dosages. To determine the required oral dose, the total amount of vitamin D₃ produced by the UVB conditions was measured in vitamin D₃-deficient DBP−/− mice since the vitamin D₃ does not leave the skin once generated in these mice (Duchow et al., 2019). To obtain this measurement, lipids were isolated from skin collected from the entire dorsal surface exposed to UVB light including dorsal skin, ears, and tail skin. No vitamin D₃ was detectable in ventral skin. Vitamin D₃ content was determined to be 10 ng/mouse and 34 ng/mouse at the low and high UVB dose levels, respectively ($n = 5$ for each dose level).

At the low dose, treatment with both UVB (2.6 kJ) and oral vitamin D₃ (10 ng) resulted in an increase in serum calcium, with UVB-generated vitamin D₃ being significantly more potent at raising serum calcium in wild-type mice (Figure 1). The area under the curve for serum calcium response over time was approximately twofold higher for UVB-generated vitamin D₃ compared to oral vitamin D₃ ($40 ± 4$ mg × d/dl vs. $17 ± 2$ mg × d/dl). The initial response to the treatment was more rapid for UVB-generated vitamin D₃, occurring within 24 h of dosing, whereas an increase was not detectable until 72 h after oral administration. Vitamin D₃ generated by the low UVB dose was sufficient to normalize serum calcium levels ($8.8 ± 0.1$ mg/dl). However, when administered orally,
the maximum level achieved was 7.8 ± 0.1 mg/dl. Serum calcium gradually returned to baseline level by day 21 for the oral treatment group and day 28 for the UVB group.

Similar results were obtained at the high UVB (8 kJ) and oral (34 ng) dose (Figure 2). The area under the curve was 254 ± 30 mg × d/dl for the UVB group and 127 ± 19 mg × d/dL for the oral dose group. As found with the low dose experiment, a significant increase occurred within 24 h after UVB treatment, whereas an increase was not detectable until the 72-h timepoint for the wild-type oral dose group. After the 24-h timepoint, serum calcium remained comparable between the two groups (difference <0.6 mg%) through week 5. Serum calcium for the oral treatment group began to gradually decline after week 5 and returned to baseline at 12 weeks in DBP+/+ mice. In contrast, serum calcium levels remained elevated in the UVB group through week 7 and returned to baseline at week 15.
The impact of DBP on the biological activity of oral vitamin D$_3$ was also assessed in vitamin D-deficient and hypocalcemic DBP$^{-/-}$ mice at the high oral dose (Figure 2). As expected, activity was significantly reduced in DBP$^{-/-}$ mice compared to DBP$^{+/+}$ mice. The area under the biological activity curve was approximately 2.8 ± 0.6 mg × d/ dl, an amount approximately 40 times lower than DBP$^{+/+}$ mice. Besides the much lower maximal response (7.1 mg/dl vs. 8.8 mg/dl), oral administration in DBP$^{-/-}$ mice also resulted in a very quick return to baseline (1 week vs. 11 weeks).

### 3.2 DBP improves the biological activity of oral vitamin D$_3$

To further investigate the decreased activity of oral vitamin D$_3$ in DBP$^{-/-}$ mice relative to DBP$^{+/+}$ mice, the serum calcium response to daily oral administration of a physiologic dose of vitamin D$_3$ (250 ng) was compared in vitamin D-deficient mice. After 1 week of dosing, serum calcium levels had normalized in DBP$^{+/+}$ mice (6.4 ± 0.1 mg/dl vs. 8.7 ± 0.4 mg/dl) and had increased from 6.3 ± 0.1 mg/dl to 7.5 ± 0.3 mg/dl in DBP$^{-/-}$ mice (Figure 3). DBP$^{-/-}$ mice required 2 weeks of daily dosing to normalize serum calcium (9.0 ± 0.4 mg/dl). At the end of the treatment, serum calcium levels were 9.4 ± 0.1 and 9.0 ± 0.4 mg/dl for the DBP$^{+/+}$ and DBP$^{-/-}$ mice, respectively.

### 3.3 DBP mitigates excessive biliary excretion of [3H]-vitamin D$_3$

To determine if the differences in the potency and duration of activity were due to changes in clearance by the liver, we evaluated the biliary excretion of tritium-labeled vitamin D$_3$ in vitamin D-sufficient wild-type mice. Intravenous injection of 8 ng of [3H]-vitamin D$_3$ pre-bound to DBP resulted in a 2.5-fold reduction in the total amount of tritium detectable in the feces at 24 h relative to injection of 8 ng of unbound [3H]-vitamin D$_3$ (Figure 4). The total fraction of the dose excreted in the feces was 27% in mice that received free vitamin D$_3$, whereas this was only 11% in mice injected with vitamin D$_3$ pre-bound by DBP.
We also examined excretion of a single oral dose of radiolabeled vitamin D$_3$ by DBP$^{-/-}$ mice compared to DBP$^{+/+}$ mice. After 24 h, a larger portion of the dose was detectable in the feces of DBP$^{-/-}$ mice compared to the DBP$^{+/+}$ mice (Figure 5, 38% vs. 59%). By 48 h, this had increased to 41% of the dose in DBP$^{+/+}$ mice and 65% in DBP$^{-/-}$ mice.

**FIGURE 5** Fraction of total dose of radiolabeled vitamin D present feces of DBP$^{+/+}$ and DBP$^{-/-}$ mice after dosing. Excretion of oral [3H]-vitamin D is significantly higher in DBP$^{-/-}$ mice at 24 and 48-h following administration. Vitamin D sufficient mice received a single oral dose of 0.25 µg [3H]-vitamin D ($n = 3$/group, ±SEM, *$p < 0.05$ vs. DBP$^{+/+}$). DBP, D binding protein

4 | DISCUSSION

Our results demonstrate that DBP significantly increases the biological activity of vitamin D$_3$. Although orally administered vitamin D$_3$ is biologically effective, its potency and the duration of activity are reduced compared to that delivered to the body bound to DBP. This difference is at least in part due to the decreased biliary excretion of DBP-bound vitamin D$_3$. As a result, the activity of oral vitamin D$_3$ is significantly reduced compared to UVB-generated vitamin D$_3$ which we previously showed is delivered exclusively by DBP (Duchow et al., 2019). Additional evidence of the importance of DBP for optimal vitamin D$_3$ activity is exemplified in DBP$^{-/-}$ mice where the potency of oral vitamin D$_3$ is dramatically reduced, likely because of rapid clearance of unbound vitamin D$_3$ from the body.

The increased biliary excretion of vitamin D$_3$ observed with free and oral vitamin D$_3$ is likely a result of the rapid accumulation in the liver leading to its degradation. This is consistent with a previous report that excessive accumulation of vitamin D$_3$ in the liver results in the appearance of water-soluble metabolites (Fraser, 1983). Investigation of hepatic uptake through liver perfusion of radiolabeled vitamin D$_3$ bound by its various carriers in rats indicated that uptake is significantly reduced when bound by DBP. Therefore DBP transport may keep vitamin D$_3$ out of the liver, preventing accumulation and protecting it from rapid degradation (Haddad et al., 1988).

We also found that the duration of biological activity is significantly longer with UVB-generated vitamin D$_3$ relative to oral vitamin D$_3$, suggesting a difference in the metabolism. Previous investigations of vitamin D$_3$ metabolism after oral administration report a spike in 25-OHD$_3$ levels shortly after administration (Mawer et al., 1971; Stamp et al., 1977). In contrast, a gradual and sustained increase in 25-OHD$_3$ levels has been reported following UVB treatment (Adams et al., 1982; Mawer et al., 1971; Stamp et al., 1977). The prolonged production of 25-OHD$_3$ likely leads to continued generation of biologically active 1,25-(OH)$_2$D$_3$, thus increasing the bioactivity as seen in these experiments.

In addition to sustained biological activity, the observed potency was higher for UVB-generated vitamin D$_3$ than oral vitamin D$_3$. At the low dose, oral vitamin D did not restore normal blood calcium levels, whereas UV administration did. An oral dose of vitamin D three times higher was necessary to achieve normal circulating levels of calcium. We propose the increased potency is due to a larger pool of bioavailable vitamin D$_3$ resulting in the production of larger amounts of 1,25-(OH)$_2$D$_3$ when transported by DBP. However, 25-OHD$_3$ and 1,25-(OH)$_2$D$_3$ were not measured in these experiments, this cannot be definitively determined.

The observation that physiologic doses of vitamin D$_3$ are approximately half as effective at correcting vitamin D$_3$ deficiency in DBP$^{-/-}$ mice support the concept that DBP is important for the biological activity of oral vitamin D$_3$ albeit not essential. The increased biliary excretion observed in DBP$^{-/-}$ mice relative to DBP$^{+/+}$ is interesting because DBP is not involved in absorption of vitamin D$_3$. Therefore, the rate of delivery to the liver should be comparable between the two genotypes. Considering this, it is possible that DBP may be required for optimal removal of 25-OHD$_3$ following its production in the liver, preventing its degradation. It is also possible that DBP binding in the liver sequesters 25-OHD$_3$, preventing further metabolism.

These data demonstrate that oral vitamin D$_3$ and UVB-generated vitamin D$_3$ are not biologically equivalent, and that vitamin D$_3$ derived through the natural (skin) system
is more biologically active. Our results are suggestive that the non-specific absorption of oral vitamin D₃ and transport to the liver results in excessive waste as biliary excretion, whereas DBP-bound vitamin D₃ is protected.

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CONFLICT OF INTEREST
The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS
E.G.D., L.A.P., and H.F.D designed the experiments. E.G.D. performed research. M.W.D. maintained experimental animals. E.G.D, L.A.P., and H.F.D analyzed the data. E.G.D., L.A.P., and H.F.D wrote the paper.

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