Prednisolone-induced Ca\textsuperscript{2+} malabsorption is caused by diminished expression of the epithelial Ca\textsuperscript{2+} channel TRPV6

Sylvie Huybers, Ton H. J. Nabers, René J. M. Bindels, and Joost G. J. Hoenderop

1Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre; and 2Departments of Gastroenterology, Radboud University Nijmegen Medical Centre and Internal Medicine, Hilversum Hospital, Nijmegen, The Netherlands

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Prednisolone-induced Ca\textsuperscript{2+} malabsorption is caused by diminished expression of the epithelial Ca\textsuperscript{2+} channel TRPV6. Am J Physiol Gastrointest Liver Physiol 292: G92–G97, 2007. First published August 10, 2006; doi:10.1152/ajpgi.00317.2006.—Glucocorticoids, such as prednisolone, are often used in clinic because of their anti-inflammatory and immunosuppressive properties. However, glucocorticoids reduce bone mineral density (BMD) as a side effect. Malabsorption of Ca\textsuperscript{2+} in the intestine is supposed to play an important role in the etiology of low BMD. To elucidate the mechanism of glucocorticoid-induced Ca\textsuperscript{2+} malabsorption, the present study investigated the effect of prednisolone on the expression and activity of proteins responsible for active intestinal Ca\textsuperscript{2+} absorption including the epithelial Ca\textsuperscript{2+} channel TRPV6, calbindin-D\textsubscript{9K}, and the plasma membrane ATPase PMCA1b. Therefore, C57BL/6 mice received 10 mg/kg body wt prednisolone daily by oral gavage for 7 days and were compared with control mice receiving vehicle only. An in vivo \textsuperscript{45}Ca\textsuperscript{2+} absorption assay indicated that intestinal Ca\textsuperscript{2+} absorption was diminished after prednisolone treatment. We showed decreased duodenal TRPV6 and calbindin-D\textsubscript{9K} mRNA and protein abundance in prednisolone-treated compared with control mice, whereas PMCA1b mRNA levels were not altered. Importantly, detailed expression studies demonstrated that in mice these Ca\textsuperscript{2+} transport proteins are predominantly localized in the first 2 cm of the duodenum. Furthermore, serum Ca\textsuperscript{2+} and 1,25-dihydroxyvitamin D\textsubscript{3} [1,25(OH)\textsubscript{2}D\textsubscript{3}] concentrations remained unchanged by prednisolone treatment. In conclusion, these data suggest that prednisolone reduces the intestinal Ca\textsuperscript{2+} absorption capacity through diminished duodenal expression of the active Ca\textsuperscript{2+} transporter TRPV6 and calbindin-D\textsubscript{9K} independent of systemic 1,25(OH)\textsubscript{2}D\textsubscript{3}.

2,5-dihydroxyvitamin D\textsubscript{3}; duodenum; epithelial calcium channel 2; calcium transporter 1

Reduced bone mineral density (BMD) is particularly present in the elderly and women, which implies that age and gender are important risk factors for developing low BMD. However, in clinical practice low BMD is frequently observed in several patient groups, including inflammatory bowel disease (IBD) patients. Estimates of osteopenia in IBD range from 31 to 59% and osteoporosis from 5 to 41%. Various studies exploring the cause of low BMD in IBD found a significant correlation between glucocorticoid treatment and decreased BMD (3, 5, 17, 28, 29). Glucocorticoids, such as prednisolone, are well-known drugs for their potent anti-inflammatory and immunosuppressive properties. As a consequence, glucocorticoids are widely used in clinic as drugs to treat inflammatory conditions such as IBD. To date, glucocorticoids are generally accepted to reduce BMD, despite the fact that in a number of studies an effect on bone mass could not be observed (5, 11) or could be observed only in male glucocorticoid users (31).

This effect of glucocorticoids on BMD is caused by the combined activity of increased bone resorption and malabsorption of vitamin D and Ca\textsuperscript{2+} from the intestine. Reduced vitamin D levels, as observed in IBD patients, are associated with impaired Ca\textsuperscript{2+} absorption and a compensatory increase in serum parathyroid hormone levels that, in turn, stimulates bone resorption (31). Furthermore, in most studies high glucocorticoid dosage and long-term treatment are positively associated with low BMD. Glucocorticoids are generally prescribed in high doses for a short period in acute exacerbations and are subsequently given in low maintenance doses for a prolonged time. Given these facts, prevention of low BMD in glucocorticoid-treated patients deserves serious attention and treatment.

Disturbance of the intestinal Ca\textsuperscript{2+} absorption likely plays an important role in glucocorticoid-induced bone problems. Ca\textsuperscript{2+} is absorbed by two distinct mechanisms including passive (paracellular) and active (transcellular) transport, and the relative importance of each pathway is set by the dietary Ca\textsuperscript{2+} content (20). Active Ca\textsuperscript{2+} absorption is mainly localized in the duodenum and tightly regulated, enabling the organism to adapt to changes in Ca\textsuperscript{2+} demands. Transcellular Ca\textsuperscript{2+} transport can be described in three sequential cellular steps including transfer of luminal Ca\textsuperscript{2+} into the enterocyte by the epithelial Ca\textsuperscript{2+} channel TRPV6, translocation of cytosolic Ca\textsuperscript{2+} toward the basolateral membrane by calbindin-D\textsubscript{9K}, and finally active extrusion into the circulatory system by the plasma membrane ATPase 1b (PMCA1b) (20, 34). Active Ca\textsuperscript{2+} absorption is predominantly regulated by 1,25-dihydroxyvitamin D\textsubscript{3} [1,25(OH)\textsubscript{2}D\textsubscript{3}], the active form of vitamin D in the body. This is exemplified in 1,25(OH)\textsubscript{2}D\textsubscript{3}-deficient 1x-hydroxylase knockout mice. This strain has a deletion in the enzyme 25-hydroxyvitamin D\textsubscript{3}–1x-hydroxylase responsible for the biosynthesis of active 1,25(OH)\textsubscript{2}D\textsubscript{3} in the kidney. As a consequence, these mice show impaired intestinal Ca\textsuperscript{2+} absorption, decreased serum Ca\textsuperscript{2+} concentration, and a compensatory increase in serum parathyroid hormone levels (10, 21).

Glucocorticoids diminish active Ca\textsuperscript{2+} absorption; however, the responsible molecular mechanism has not been elucidated. Hypothetically, glucocorticoid-induced Ca\textsuperscript{2+} malabsorption is exerted through reduced levels of intestinal proteins involved in active Ca\textsuperscript{2+} transport. The aim of the present study was, therefore, to investigate the effect of prednisolone on the...
expression level of the Ca\(^{2+}\) transport proteins TRPV6, calbindin-D\(_{9k}\), and PMCA1b in mouse duodenum. To this end, mice were treated with prednisolone for 7 days and characterized by in vivo \(^{45}\text{Ca}^{2+}\) absorption assays. Expression levels of the intestinal Ca\(^{2+}\) transporters were measured by real-time PCR analysis and immunoblotting.

**MATERIALS AND METHODS**

**Animal protocol.** Twelve-week-old C57BL/6 mice were kept in a light- and temperature-controlled room with ad libitum access to standard pellet diet and water. For studying the localization of duodenal Ca\(^{2+}\) transporters in mice (\(N=4\)), the first 5 cm of the small intestine were sampled and divided in three parts of 2 cm each. In the prednisolone experiment, mice were randomly assigned to either the control group receiving vehicle only (\(N=8\)) or the treatment group (\(N=8\)), receiving 10 mg/kg body weight prednisolone-hemisuccinate sodium salt (Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in PBS daily in two doses by oral gavage during 7 days (14, 15). Mice were housed individually in metabolic cages for 24 h to collect urine samples. Subsequently, blood samples were taken and the mice were killed. The first 2 cm of the duodenum (whole segment) were sampled and immediately frozen in liquid nitrogen. Samples were stored at \(-80^\circ\text{C}\) until further processing. The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

**Serum and urine biochemistry.** Serum and urine Ca\(^{2+}\) concentrations were determined by a colorimetric assay as described previously (19). Serum 1,25(OH)\(_2\)D\(_3\) concentrations were analyzed by immunoassay method as described by the manufacturer (Am-NO) (1:10,000). Immunoreactive protein was detected by the chemiluminescence (ECL) method as described by the manufacturer (Am-NO) (1:5,000) or (1:10,000) or (1:5,000) or (1:10,000).

**Immunoblotting.** For protein analysis, frozen duodenal tissues were homogenized in ice-cold solubilization buffer as previously described (35). Protein concentration of the homogenates was determined (Bio-Rad Protein Assay, Bio-Rad Munich, Munich, Germany) and immediately frozen in liquid nitrogen. Samples were stored at \(-80^\circ\text{C}\) until further processing. The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

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**RESULTS**

**Body weight, urine, and serum analysis.** Mice were placed in metabolic cages to collect 24-h urine samples. Prednisolone-treated mice showed equal amounts of urinary Ca\(^{2+}\) loss compared with controls (Table 1). Likewise, no effect of prednisolone was observed on serum Ca\(^{2+}\) and 1,25(OH)\(_2\)D\(_3\) levels. To investigate the effect of prednisolone on body weight, mice were weighed before and after 7 days of oral treatment. Body weight significantly reduced after prednisolone treatment compared with controls (Table 1). body weight, urine, and serum analysis. Mice were placed in metabolic cages to collect 24-h urine samples. Prednisolone-treated mice showed equal amounts of urinary Ca\(^{2+}\) loss compared with controls (Table 1). Likewise, no effect of prednisolone was observed on serum Ca\(^{2+}\) and 1,25(OH)\(_2\)D\(_3\) levels. To investigate the effect of prednisolone on body weight, mice were weighed before and after 7 days of oral treatment. Body weight significantly reduced after prednisolone treatment compared with controls (Table 1).

**Effect of prednisolone on in vivo intestinal Ca\(^{2+}\) absorption.** In addition, we used an in vivo \(^{45}\text{Ca}^{2+}\) absorption assay to evaluate the effect of prednisolone on intestinal Ca\(^{2+}\) absorption. At several time points after oral \(^{45}\text{Ca}^{2+}\) intake, the amount of \(^{45}\text{Ca}^{2+}\) in the blood was measured in controls and prednisolone-treated mice. Figure 1 shows that prednisolone resulted in a diminished uptake of Ca\(^{2+}\) from the intestinal lumen. Two minutes after oral gavage, the Ca\(^{2+}\) absorption in prednisolone-treated mice was 47 ± 8% compared with controls. After 4 min an equilibrium for Ca\(^{2+}\) uptake was reached.

**Localization of active Ca\(^{2+}\) transporters in the duodenum.** Subsequently, the molecular mechanism of prednisolone-induced Ca\(^{2+}\) malabsorption in the intestine was investigated. First, the main localization of active intestinal Ca\(^{2+}\) uptake was determined. Thereafter, the first 6 cm of the duodenum was divided in three equal parts in which the expression level of active Ca\(^{2+}\) transporters was quantified by real-time PCR and immunoblotting (Fig. 2). All Ca\(^{2+}\) transporters displayed a robust mRNA expression gradient, which was highest near the stomach and decreased toward the jejunum. Intriguingly, more than 90% of TRPV6 and calbindin-D\(_{9k}\) mRNA was localized in the first 2 cm directly after the stomach. This was less evident for PMCA1b, where 61% of PMCA1b mRNA was localized in the first 2 cm directly after the stomach.
was expressed in the first 2 cm. Similar results were obtained when analyzing duodenal calbindin-D$_{9K}$ protein abundance (Fig. 2).

**Effect of prednisolone on duodenal expression of active Ca$^{2+}$/H$^{+}$ transporters.** To study the effect of prednisolone on duodenal expression of the Ca$^{2+}$ transport proteins TRPV6, calbindin-D$_{9K}$, and PMCA1b, mRNA levels were measured in the first 2 cm by quantitative real-time PCR (Fig. 3A). Prednisolone treatment induced a twofold decrease in TRPV6 mRNA expression compared with controls, and also calbindin-D$_{9K}$ expression was slightly but significantly reduced. However, no significant effect of prednisolone was observed on PMCA1b mRNA levels and, therefore, we did not perform immunoblot analysis. Unfortunately, there is no appropriate antibody available to measure duodenal TRPV6 expression by immunoblot analysis. Subsequently, immunoblot analysis of duodenal samples consistently demonstrated a reduction in calbindin-D$_{9K}$ protein abundance in the prednisolone-treated group (Fig. 4A). Densitometric analysis of the intensity of the immunocomplexes confirmed this reduction and showed a significant decrease in calbindin-D$_{9K}$ protein expression after prednisolone treatment (Fig. 4B).

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**Fig. 1.** Effect of prednisolone on in vivo intestinal $^{45}$Ca$^{2+}$ absorption. At $t = 0$, $^{45}$Ca$^{2+}$ is loaded in the stomach of the mouse by oral gavage. After the indicated time points blood was collected by orbita puncture and subsequently analyzed for the amount of $^{45}$Ca$^{2+}$ in serum. The control group (●) received vehicle only; the treatment group (▲) received 10 mg/kg body wt prednisolone daily by oral gavage. Data are means ± SE ($N = 8$). *$P < 0.05$ vs. control.

**Fig. 2.** Localization of Ca$^{2+}$ transporters in the duodenum. The first 6 cm of the duodenum were sampled and divided into 3 parts of 2 cm each. By using quantitative real-time PCR, duodenal mRNA expression of the Ca$^{2+}$ transporters TRPV6 (solid bars), calbindin-D$_{9K}$ (shaded bars), and PMCA1b (open bars) were measured in each of the 3 parts, corrected for the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT), and presented as % of total expression. Calbindin-D$_{9K}$ protein abundance was analyzed by immunoblot analysis. Data are means ± SE ($N = 4$).

**Fig. 3.** Effect of prednisolone on duodenal and renal mRNA levels of genes encoding Ca$^{2+}$ transport proteins. Using quantitative real-time PCR, duodenal mRNA expression of TRPV6, calbindin-D$_{9K}$, and PMCA1b (A) and renal mRNA expression of TRPV5, calbindin-D$_{28K}$, and Na$^{+}$/Ca$^{2+}$ exchanger (NCX1) (B) were measured, corrected for HPRT, and presented as % of the control group. The control group (solid bars) received vehicle only; the treatment group (open bars) received 10 mg/kg body wt prednisolone daily by oral gavage. Data are means ± SE ($N = 8$). #$P < 0.01$ vs. control; *$P < 0.05$ vs. control.
The present study demonstrated that prednisolone impairs Ca2+ absorption, which is accompanied by diminished TRPV6 and calbindin-D9K expression in the early part of the duodenum. These findings strongly suggest that inhibition of active Ca2+ transport is at least in part responsible for the Ca2+ malabsorption during glucocorticoid treatment. This defect likely contributes to the markedly decreased BMD after prolonged glucocorticoid usage.

Prednisolone could exert a direct inhibitory action on Ca2+ transport proteins, which can, however, also be due to a secondary response to decreased 1,25(OH)2D3 serum levels. Importantly, we have previously shown that the expression of intestinal Ca2+ transport proteins is under the tight control of 1,25(OH)2D3 (34, 36, 37). Available data about the effect of glucocorticoids on vitamin D metabolism are, however, inconclusive. Serum levels of 25-hydroxyvitamin D were low in a number of studies (7) and normal in others (39). Serum 1,25(OH)2D3 levels also varied between studies (1, 7, 32). In addition, increased mRNA levels of the vitamin D receptor were measured in both intestine and kidney after dexamethasone treatment (1, 22). Our finding demonstrating a decline in expression of the duodenal Ca2+ transporters TRPV6 and calbindin-D9K in combination with constant serum 1,25(OH)2D3 levels indeed implies a 1,25(OH)2D3-independent effect of glucocorticoids on Ca2+ absorption. These data are in line with a previous study of Hahn et al. (18), who demonstrated that 20 mg of prednisone per day for 14 days had a minor effect on serum 1,25(OH)2D3 levels in 12 patients, whereas intestinal Ca2+ absorption fell by 30%, suggesting that the glucocorticoid-related impairment in Ca2+ absorption may be independent of vitamin D. Moreover, renal 1α-hydroxylase mRNA abundance and enzyme activity were not altered (1), and glucocorticoids did not alter the vitamin D binding protein expression (6). The 1,25(OH)2D3-independent effect of glucocorticoids on Ca2+ absorption, in turn, could implicate a direct effect of prednisolone. Lee et al. (23) examined the effect of mifepristone, a glucocorticoid receptor antagonist. They observed that the decline of calbindin-D9K caused by dexamethasone was completely abolished by mifepristone. Altogether this implicates that the decline of intestinal Ca2+ uptake is 1,25(OH)2D3 independent and might be caused by a direct effect on the glucocorticoid receptor.

The duodenum is generally implicated as the main site of active Ca2+ absorption; however, the precise localization of active Ca2+ transporters in the intestine has not been evaluated to date. Interestingly, the Ca2+ transporter expression did not gradually decrease toward jejunum, but Ca2+ transporters were almost exclusively localized in the first part (2 cm) of mouse duodenum. This holds true for TRPV6 and calbindin-D9K and to a lesser extent for PMCA1b. Because the expression level of the Ca2+ transporters was measured in duodenal segments instead of isolated mucosa preparations the presence of PMCA1b in duodenal muscle layers could theoretically contribute to the determined mRNA levels. However, Walters and coworkers (16) demonstrated significant higher levels of the PMCA1b transcript in duodenal mucosa compared with duodenal muscle layers. Thus it is unlikely that PMCA1b contamination from another layer (e.g., muscle cells) does significantly contribute to the PMCA1b expression in duodenal mucosa. Our study suggests that active Ca2+ transport is restricted to the duodenal part directly after the stomach. In addition, previous data indicated that TRPV6 is abundantly expressed in stomach (26, 27), where Ca2+ is ionized by gastric acid enabling absorption in the duodenum. Possibly the stomach plays a role in active dietary Ca2+ absorption next to the duodenum. Overall, the strong duodenal gradient of the Ca2+ transport proteins is a unique finding and suggests that active intestinal Ca2+ absorption occurs only in the first part of the duodenum.

In the etiology of low BMD, it is emphasized that there is interplay between Ca2+ waste from bone, increased renal Ca2+ wasting, and diminished Ca2+ absorption from the duodenum. The involvement of this last pathway is supported by previous studies. With the in situ intestinal loop technique it was evaluated that the net active Ca2+ flow over the duodenal membrane is inhibited by prednisolone (2, 38). A study in children on dexamethasone treatment observed a 61 to 42% fall in Ca2+ absorption (30). Moreover, in rats injected with other glucocorticoid drugs including dexamethasone or methylprednisolone, a decline of duodenal calbindin-D9K mRNA was observed (13, 22). Furthermore, Li and Christakos (25) demonstrated a dose-dependent decrease of duodenal calbindin-D9K after dexamethasone treatment in mice. Recently, Lee et al. (23) showed that dexamethasone reduces duodenal cal-

Effect of prednisolone on renal Ca2+ transporters. To study the effect of prednisolone on renal expression of the Ca2+ transport proteins TRPV5, calbindin-D28K, and the Na+/Ca2+ exchanger (NCX1), mRNA levels were measured by quantitative real-time PCR (Fig. 3B). Prednisolone treatment had no significant effect on mRNA expression level of the renal Ca2+ transporters compared with controls.

DISCUSSION

Fig. 4. Effect of prednisolone on calbindin-D9K protein abundance in mouse duodenum. Immunoblot from mouse duodenal homogenates labeled with antibodies against calbindin-D9K (A). Expression of calbindin-D9K protein was quantified by computer-assisted densitometry analysis and expressed as % of controls (B). The control group (solid bars) received vehicle only; the treatment group (open bars) received 10 mg/kg body wt prednisolone daily by oral gavage. Data are means ± SE (N = 8). *P < 0.05 vs. control.
These results could explain the normal Ca\textsuperscript{2+} cannot be explained by just one mechanism. These studies together with our observation that prednisolone reduces duodenal TRPV6, conclusively demonstrates that disabled active Ca\textsuperscript{2+} uptake is involved in glucocorticoid-induced Ca\textsuperscript{2+} malabsorption. Our findings indicate that the abundance of the renal Ca\textsuperscript{2+} transporters including TRPV5, calbindin-D\textsubscript{28K}, and NCX1 is not affected by prednisolone treatment. These results could explain the normal Ca\textsuperscript{2+} excretion in mice administered with prednisolone.

Although glucocorticoids are not likely to affect 1,25(OH)\textsubscript{2}D\textsubscript{3} levels, 1,25(OH)\textsubscript{2}D\textsubscript{3} supplementation may still be an important treatment to prevent glucocorticoid-induced malabsorption, because of its stimulatory effect on active Ca\textsuperscript{2+} transport. This can protect patients against the negative side effects of glucocorticoids on BMD (24). Furthermore, glucocorticoid treatment can only partly explain decreased BMD. For example, IBD patients who did not receive a glucocorticoid treatment also showed a reduction in BMD (4). Besides Ca\textsuperscript{2+} malabsorption, inflammatory cytokines, which are released during IBD, can directly stimulate osteoclast activity. Dresner-Pollak et al. (12), leaked during IBD, can directly stimulate osteoclast activity.

In conclusion, this study shows that prednisolone decreases intestinal Ca\textsuperscript{2+} absorption via impaired active duodenal Ca\textsuperscript{2+} absorption, mainly through diminished duodenal TRPV6 expression. This process takes place independently of blood 1,25(OH)\textsubscript{2}D\textsubscript{3} levels. The described decline in active Ca\textsuperscript{2+} absorption may explain the Ca\textsuperscript{2+} malabsorption observed in patients using glucocorticoids and may in the long-term contribute to the development of osteoporosis.

GRANTS
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