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Research paper

Metformin increases liver accumulation of vitamin B12 – An experimental study in rats

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ABSTRACT

Aims/hypothesis: Patients treated with metformin exhibit low levels of plasma vitamin B_{12} (B_{12}), and are considered at risk for developing B_{12} deficiency. In this study, we investigated the effect of metformin treatment on B_{12} uptake and distribution in rats.
Methods: Sprague Dawley rats ($n = 18$) were divided into two groups and given daily subcutaneous injections with metformin or saline (control) for three weeks. Following this, the animals received an oral dose of radio-labeled B_{12} (^{57}[Co]-B_{12}), and urine and feces were collected for 24 h. Plasma, bowel content, liver, and kidneys were collected and analyzed for B_{12}, unsaturated B_{12}-binding capacity, and ^{57}[Co]-B_{12}.
Results: Three weeks of metformin treatment reduced plasma B_{12} by 22% or 289 [47-383] pmol/L (median and [range]) ($p = 0.001$), while no effect was observed on unsaturated B_{12}-binding capacity. Compared with controls, the amount of B_{12} in the liver was 36% ($p = 0.007$) higher in metformin-treated rats, while the B_{12} content in the kidney was 34% ($p = 0.013$) lower. No difference in the total amount of absorbed ^{57}[Co]-B_{12} present in the tissues and organs studied was found, suggesting that metformin has no decreasing effect on the B_{12} absorption.
Conclusions/interpretation: These results show that metformin treatment increases liver accumulation of B_{12}, thereby resulting in decreases in circulating B_{12} and kidney accumulation of the vitamin. Our data questions whether the low plasma B_{12} observed in patients treated with metformin reflects impaired B_{12} status, and rather suggests altered tissue distribution and metabolism of the vitamin.

1. Introduction

Metformin, an oral drug commonly used to treat patients with type 2 diabetes, is believed to cause vitamin B_{12} (B_{12}) deficiency [1,2]. B_{12} is involved in DNA synthesis and cell proliferation and is necessary for the formation of red blood cells and to sustain the function of the nervous system. Low B_{12} status can lead to B_{12} deficiency and cause anemia and neurological manifestations [3]. The biochemical signs of B_{12} deficiency are increased plasma levels of methylmalonic acid (MMA) and homocysteine (tHcy) [3].

The association between metformin treatment and decline in plasma B_{12} is amply illustrated in a multitude of randomized control trials and cross-sectional surveys [1,2,4,5]. The mechanism underlying the effects of metformin on plasma B_{12} status remains to be elucidated; however, impaired B_{12} absorption has been proposed [4,6]. Recently, Leung et al. (2010) [7] suggested that metformin reduces only the level of non-functional plasma B_{12}, the part bound to haptocorrin, and not the active part bound to transcobalamin (holotranscobalamin or holoTC).

Consequently, there is a need to understand whether metformin causes B_{12} deficiency, or whether the B_{12} plasma concentration is altered despite adequate tissue supplies.

In this study, we examined the effect of three weeks metformin treatment on the absorption and tissue distribution of B_{12} in a rat model.

2. Methods

2.1. Animals

Female Sprague Dawley rats ($n = 18$) from Charles River Laboratories were used. The experiment was authorized by the University of California, Davis (UC Davis) Institutional Animal Care and

Abbreviations: B_{12}, vitamin B_{12}; HoloTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, homocysteine; $UB_{12}BC$, unsaturated B_{12} binding capacity; ^{57}[Co]-B_{12}, ^{57}[Co]-labeled B_{12}.

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Use Committee, and conducted at the animal facility at UC Davis Medical Center, Sacramento, CA, USA, where the rats were housed in a controlled environment (22.0 ± 0.5 °C) with a 12 h light–dark cycle. The rats were fed a normal stock laboratory diet (Laboratory Rodent Diet 500I, LabDiet, PMI Nutrition International, Richmond, Indiana, USA) containing 50 μg/kg B_{12} throughout the study. Food and water were administered *ad libitum*. The rats weighed 267 [228-282] g (median and [range]) at the beginning of the experiment.

2.2. Experimental design

Prior to the experiment (day 0), rats were weighed, and a blood sample was taken by puncture of the sublingual vein with a 23 gauge needle. For 21 days, the animals were given daily subcutaneous injections (23 gauge needle) in the skin of the neck with either metformin or saline. The experimental group ($n = 9$) received 250 mg/kg/day metformin (Actavis, Stjerneapoteket (Danish pharmacy), Aarhus, Denmark) dissolved in 1 ml of 0.9% saline water, while the control group ($n = 9$) received injections with 1 ml of 0.9% saline water. On day 21, all rats (non-fasted) received an oral dose of 1 pmol ^{57}[Co]-labeled B_{12} (^{57}[Co]-B_{12}) (~5000 Bq) (MP Biomedical, Santa Ana, CA, USA) in a total volume of 0.75 ml 1% sugar water. The oral dose was given by gastric gavage using a curved 20 gauge feeding needle, and the rats were subsequently placed in individual metabolic cages designed to collect urine and feces. After 24 h, the rats were anaesthetized by an intraperitoneal injection of 200 μl Beuthanasia-D special (Schering-Plough Animal Health, Elkhorn, Nebraska, USA), and blood was taken from the dorsal aorta to exsanguination. Immediately after the blood was drawn, liver and kidneys from each animal were excised, cut into smaller pieces, and frozen in liquid nitrogen.

2.3. Sample preparations and protein extractions

Blood samples, taken before (day 0) and after (day 21) treatment, were collected into lithium heparin tubes, and plasma was removed after centrifugation at room temperature at 1200 rpm for 15 min and stored at −80 °C until analysis. Protein extractions of liver and kidney tissues (approx. 1 ml buffer per 1 g tissue) were carried out by homogenization on ice as previously described [8].

2.4. Biochemical measurements

Measurement of radioactivity (^{57}[Co]) in rat tissues and fluids was performed on a Cobra II Auto-Gamma Counter (Packard, Meriden, CT, USA). Total B_{12} was measured using the Cobas 6000 E immunoassay system (Roche Diagnostics, Hvidovre, Denmark) with a detection range of 55–1476 pM. Protein extracts were diluted 1:100 (liver) and 1:1000 (kidney) in the dilution reagent supplied by the manufacturer prior to B_{12} measurements.

Unsaturated B_{12}-binding capacity ($UB_{12}BC$) was measured as described by Gottlieb et al. [9]. In brief, 100 μl sample was incubated with 25 μl ^{57}[Co]-B_{12} (5 nM) (Kem-En-Tec, Taastrup, Denmark) in 200 μl 0.1% phosphate-buffered bovine albumin (PBA) for 15 min, followed by addition of 500 μl hemoglobin-coated charcoal solution to precipitate unbound B_{12}. The charcoal solution was prepared by mixing equal volumes of a 5% aqueous suspension of activated charcoal (Sigma–Aldrich, Broendby, Denmark) with a 0.5% aqueous solution of bovine hemoglobin (Becton Dickinson, Broendby, Denmark). After an additional 10 min of incubation, charcoal-bound B_{12} was precipitated by centrifugation for 10 min at 2600 × g. Finally, the supernatants were measured for radioactivity.

2.5. Statistics

The D'Agostino-Pearson omnibus test was used to determine if data followed the Gaussian distribution. For normally distributed data, differences in ^{57}[Co] and B_{12} content in tissues and fluids between the metformin group and the control group was determined by a two-tailed t-test for unpaired data. To compare the changes between "before treatment" and "after treatment" within the respective groups, we used a two-tailed paired t-test. Values of $p \leq 0.05$ were accepted as statistically significant. Only the data on liver size (g) for the metformin-treated rats was found not to follow the Gaussian distribution, and this could not be achieved by logarithmic transformation. For determining differences in liver size between the metformin group and the control group, the non-parametric Mann–Whitney test for unpaired data was used.

3. Results

We investigated the effect of metformin treatment on the absorption and tissue distribution of B_{12} in a rat model. Rats were treated with subcutaneous injections of metformin (metformin group) or saline (control group) for three weeks before given an oral dose of ^{57}[Co]-B_{12}. Urine and fecal matter were collected for 24 h after the per-oral administration. At this time, the rats were killed, and organs and fluids were harvested for measurement of total B_{12}, $UB_{12}BC$, and ^{57}[Co]-B_{12}. Table 1 compares the physiological parameters for the two groups of rats. The metformin rats gained a little less weight than the control rats.

Three weeks of metformin treatment reduced plasma B_{12} by 22% or 289 [47-383] pmol/L (median and [range]) ($p = 0.001$). No change in plasma B_{12} was observed in the control rats (Fig. 1A). No change in $UB_{12}BC$ was observed in either group (Fig. 1B). Total B_{12} present in the liver was an average of 36% or 170 [(−)140-871] pmol/g wet tissue higher ($p = 0.007$) in metformin-treated rats, while the B_{12} content in the kidney was an average of 34% or 1211 [750-5500] pmol/g wet tissue lower ($p = 0.013$) than in the control rats (Fig. 2).

The metformin-treated rats showed a small increase in plasma ^{57}[Co]-B_{12} 24 h after the oral ^{57}[Co]-B_{12} administration. The increase was an average of 7 Bq (0.17% [0.03–0.19]% of the given dose) ($p = 0.036$). No differences in levels of ^{57}[Co]-B_{12} between the metformin-treated rats and the control rats were found in feces, bowel content, urine, kidney and liver 24 h after oral administration of ^{57}[Co]-B_{12} (data not shown).

Table 1
Physiological parameters for metformin-treated rats and control rats. Values are given as median and [range]. For kidneys, the weight is given as the mean of the sum of both kidneys. Differences between the two groups were compared using unpaired t-tests. *p*-values ≤ 0.05 are considered statistically significant.

	Weight (g) (before study)	Weight (g) (after study)	Weight increase (%)	Kidneys (g) (after study)	Liver (g) (after study)
Metformin-treated rats ($n = 9$)	258 [228-282]	280 [235-312]	8.4 [3-10]	3.4 [2.6-3.7]	7.8 [7.3-8]
Control rats ($n = 9$)	269 [241-282]	297 [268-319]	10.3 [7.3-14.7]	3.6 [3.1-3.7]	7.5 [7.3-8]
p-value	0.27	0.10	0.02	0.14	0.27

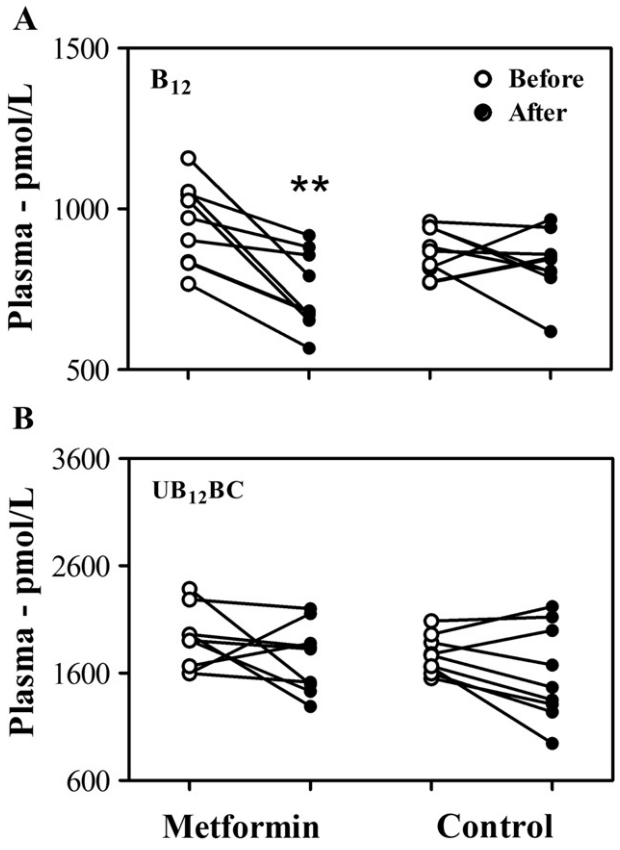

Fig. 1. B_{12} concentration and unsaturated B_{12}-binding capacity ($UB_{12}BC$) in rat plasma before and after treatment with metformin. Plasma B_{12} concentration (A) and $UB_{12}BC$ (B) in metformin-treated and control rats are given as pmol/L plasma. Differences before and after treatment were compared by paired t-test. $^{**}p = 0.001$. No other p values were ≤ 0.05.

4. Discussion

To investigate the effect of metformin on B_{12} metabolism independent of diabetic state, we investigated the absorption and tissue distribution of B_{12} in healthy Sprague Dawley rats. Metformin treatment by subcutaneous injections was chosen to avoid the hazards of giving daily oral administration by force-feeding, and because of this, the study design provides no information on interactions between metformin and the intestinal B_{12} absorption mechanism. Despite this weakness, several interesting results can be extracted from our study. We report an association between metformin treatment and increased accumulation of B_{12} in rat liver. Since a decrease in liver B_{12} is one of the first signs of B_{12} deficiency [10], this observation contradicts the hypothesis that metformin induces B_{12} deficiency.

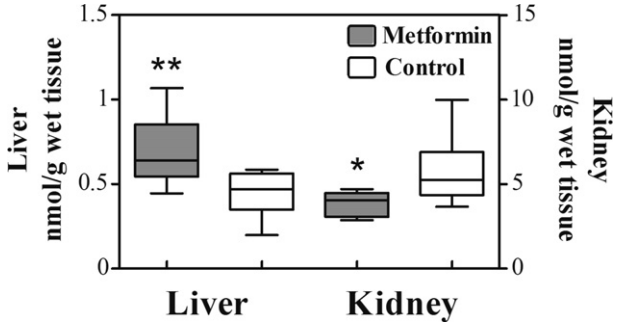

Fig. 2. B_{12} concentration in rat liver and kidney in response to metformin treatment. Protein extracts of liver (left y-axis) and kidney (right y-axis) were measured for B_{12} concentration. Results are shown as box plots with whiskers (range), and values for metformin-treated rats were compared to values obtained for control rats by unpaired t-test. $^{*}p = 0.013$, $^{**}p = 0.007$.

At first, these data appear contradictory. Metformin induced a decrease in plasma B_{12}, but the total amount of absorbed ^{57}[Co]-B_{12} present in tissues and organs studied were comparable between the two groups, suggesting that no decrease in B_{12} absorption took place in the metformin-treated rats. Also contradictory, is the finding of reduced kidney B_{12} combined with an increased level of liver B_{12}. The design of the study did not allow for determination of a mechanism for these changes, but the data indicate that in this rat model, metformin does not decrease the absorptive capacity of B_{12}, nor does it lead to liver depletion of B_{12}. The data suggest that the decline in plasma B_{12} is because of increased accumulation of B_{12} in the liver (and possibly other organs), and not caused by a metformin-induced B_{12} malabsorption. These findings are in accordance with new findings by Obeid et al. (this issue) that diabetes patients on metformin treatment have increased cellular uptake of B_{12} and normal intracellular metabolic markers despite a low B_{12} plasma status [11].

The finding of decreased B_{12} levels in the kidney is coincident with the reduction in circulating B_{12}. The kidneys obtain B_{12} partly due to clearance from blood and partly from traditional cellular uptake seen in all tissues [3]. The finding that kidney B_{12} is an order of magnitude higher than liver B_{12} is consistent with earlier studies on rodents [12,13].

In conclusion, our data suggest that metformin treatment increases the accumulation of B_{12} in the liver, thereby resulting in a decrease in circulating B_{12} and in kidney accumulation of the vitamin. We see no evidence that the B_{12} absorption mechanism in influenced negatively by metformin. This questions whether low plasma B_{12} observed in patients treated with metformin reflects impaired B_{12} status, and rather suggests altered tissue distribution and metabolism of the vitamin.

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