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Short-term resveratrol supplementation stimulates serum levels of bone-specific alkaline phosphatase in obese non-diabetic men

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ABSTRACT

Despite the substantial preclinical evidence for a positive effect of the polyphenolic compound resveratrol, human data are very scarce, and currently no clinical data addressing the potential impact on bone metabolism have been published. In the present study we addressed this issue in order to identify potential bone metabolic effects of resveratrol in human subjects. In a randomised, placebo-controlled, double-blinded and parallel-group design, 24 obese [BMI (kg/m^2): 34.2 ± 0.7] non-diabetic men were randomly assigned to 500 mg resveratrol or placebo treatment three times daily for four weeks. Biomarkers of bone metabolism, inflammatory parameters and circulating hormones were measured before and after the intervention period. Plasma levels of bone-specific alkaline phosphatase increased significantly in the resveratrol group as compared to placebo [delta changes (U/l); resveratrol: 4.9 ± 0.6 vs. placebo: −1.7 ± 0.7; $P < 0.001$]. This was paralleled by a tendency of total alkaline phosphatase to rise within the resveratrol group ($P = 0.061$), whereas no changes were detected in other biomarkers of bone and calcium metabolism, including PINP, osteocalcin, CTx, or PTH. We suggest that resveratrol influences bone metabolism possibly representing a primary anabolic modality in preserving bone integrity. However, the clinical implications remain to be evaluated.

1. Introduction

During the last decade the polyphenolic compound resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) has gained increased scientific attention. A major biological role of resveratrol is activation of the silent mating type information regulator 2 homolog 1 (SIRT1) which is a NAD^+-dependent de-acetylase that modifies various target proteins post-translationally and is physiologically upregulated by calorie restriction and fasting (Pedersen, Olholm, Paulsen, Bennetzen, & Richelsen, 2008). Transcription factors involved in the regulation of energy metabolism such as NF-kB, PGC1α, FOXO and SREBP1 have been identified as resveratrol-sensitive targets of SIRT1 action (Vang et al., 2011). In experimental animals resveratrol-mediated modification of these transcription factors translates into a plethora of physiological effects, including

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anti-inflammation, anti-atherogenetic, cancer prevention, improvement of glucose metabolism, and prevention of non alcoholic fatty liver disease (Baur, Ungvari, Minor, Le Couteur, & de Cabo, 2012; Chang, Lee, & Sheu, 2012; Kopeć, Piątkowska, Leszczyńska, & Koronowicz, 2013; Olholm, Paulsen, Cullberg, Richelsen, & Pedersen, 2010; Poulsen et al., 2012).

Recently, an effect of resveratrol on bone metabolism has been reported adding to the many potential beneficial effects of clinical use of this compound. Resveratrol promotes osteoblastogenesis and antagonises osteoclasts *in vitro* (Boissy et al., 2005), and recently these initial pioneer findings have been substantiated by diverse animal models of osteoporosis, in which resveratrol prevents bone loss due to e.g. ovariectomy (Liu et al., 2005) and immobilisation (Momken et al., 2011). Besides, in a rodent model resembling age-related bone-loss in normal mice, long-term resveratrol supplementation prevented the age related deterioration in bone mineral density (Pearson et al., 2008).

Given the putative beneficial effects on both osteoblast-mediated bone formation and osteoclast-mediated bone resorption seen *in vitro*, resveratrol may have implications for the prevention and treatment of osteoporosis. At present, however, no clinical data on the effects of resveratrol on bone metabolism have been published. In the present study we have therefore investigated the effect of resveratrol on a panel of biomarkers of bone turnover and calcium metabolism in order to identify potential bone metabolic effects of resveratrol in humans.

The data provided in the present publication represent secondary outcome measures from a clinical trial where potential physiological effects of resveratrol treatment were investigated as the primary outcome (Poulsen et al., 2013).

2. Materials and methods

2.1. Subjects and study design

In an investigator-initiated, randomised, double-blinded, placebo-controlled, parallel-group trial design 24 obese (BMI > 30 kg/m^2) but otherwise healthy men, taking no prescription medicine, and without overt endocrine disorders, participated (Poulsen et al., 2013). The subjects were randomly assigned to resveratrol ($N = 12$) or placebo ($N = 12$) treatment for four weeks. Tablets containing 500 mg trans-resveratrol (Fluxome Inc., Stenlose, Denmark) or placebo (Robinson Pharma, Santa Ana, CA, USA) were administered three times daily for four weeks. During the intervention, the participants were instructed to maintain their usual way of living and to abstain from any nutritional supplements (including calcium and vitamin D) and food sources suspected to contain significant amounts of resveratrol.

2.2. Blood sampling and analysis

Blood samples were drawn following an overnight fast at baseline and after four weeks treatment. To avoid potential diurnal changes, samples were drawn between 7.30 AM and 8.00 AM at both occasions. Samples were frozen and stored (−80 °C) immediately after being drawn and centrifuged.

Calcium, albumin and total alkaline phosphatase were determined at the University Hospital Department of Clinical Biochemistry using standard methods (ROCHE cobas 6000C, Roche Applied Science). Total calcium levels were adjusted for albumin based on Payne's formula (Payne, Little, Williams, & Milner, 1973): albumin corrected calcium = total calcium [mmol/l] + 0.020 × (41.3 – albumin[g/l]).

The following proteins were analysed by means of commercially available kits: bone-specific alkaline phosphatase (BAP): MICROVUE BAP EIA kit, Quidel Corporation; San Diego, CA, USA (catalog# 8012); N-terminal propeptide of type 1 procollagen (PINP): UniQ PINP RIA, Orion Diagnistica, Espoo, Finland (catalog# 67034); Osteocalcin: N-MID osteocalcin, Roche Diagnostics, Hvidovre, Denmark (catalog# 12149133 122); C-terminal telopeptide (CTx): β-CrossLaps/serum, Roche Diagnostics, Hvidovre, Denmark (catalog# 11972308 122); parathyroid hormone (PTH): PTH, intact, Roche Diagnostics, Hvidovre, Denmark (catalog# 11972103 122).

2.3. Statistics

Comparison of treatment effects between the two groups were assessed by two way repeated measures analysis of variance (ANOVA). Normality was checked by QQ-plots, and test for equal variance was assessed by the Levene's test for equal variances. If revealing significant differences, *post hoc* pairwise multiple comparison procedures were performed using the Student–Newman–Keuls method.

Within-group comparisons were analysed by paired *t*-test or Wilcoxon signed rank test.

Results are presented as means ± SEM, and *P*-values <0.05 were considered significant. Power calculations were based on changes in insulin sensitivity (*M*-value), which was the primary outcome measure (Poulsen et al., 2013). To detect a treatment difference of 1.0 mg/kg/min at a two-sided 0.05 significance level with a power of 0.90, nine participants had to be included in each group, assuming a SD of 0.6.

3. Results

3.1. Baseline and internal validity

The participants were not stratified according to age at inclusion and at baseline the mean age differed slightly between the two groups. However, the two groups were comparable on all other vital baseline parameters (Poulsen et al., 2013), including the plasma levels of calcium, PTH and markers of bone turnover (Table 1).

Enrollment log, adverse events, compliance, and documentation of absorption and excretion of the ingested resveratrol product has been reported previously (Poulsen et al., 2013).

3.2. Biomarkers of bone turnover, calcium metabolism, circulating hormones, and clinical biochemistry

Plasma levels of bone-specific alkaline phosphatase (BAP) were increased 15% by resveratrol supplementation ($P = 0.001$) (Fig. 1A). In fact, plasma BAP levels increased in

Table 1 – Baseline characteristics.

	Placebo	Resveratrol	P value
Age (years)	31.9 ± 2.9	44.7 ± 3.5	0.01
Weight (kg)	115.9 ± 3.6	107.1 ± 2.7	0.065
Body mass index (kg/m^2)	35.9 ± 1.2	32.5 ± 0.6	0.053
Lean mass (kg)	73.4 ± 1.8	69.1 ± 1.6	0.083
Fat mass (kg)	37.0 ± 2.6	31.7 ± 1.4	0.078
Fat percentage (%)	32.3 ± 1.4	30.2 ± 0.7	0.37
Visceral fat volume (cm^3)	4285 ± 585	4996 ± 876	0.51
Abdominal subcutaneous fat volume (cm^3)	10,737 ± 1086	8103 ± 585	0.051
Systolic blood pressure (mmHg)	126.1 ± 2.3	124.3 ± 2.9	0.64
Diastolic blood pressure (mmHg)	74.4 ± 1.6	75.6 ± 2.1	0.61
Glucose (mmol/l)	5.3 ± 0.1	5.6 ± 0.1	0.18
Haemoglobin A1c (%)	5.6 ± 0.1	5.6 ± 0.1	0.55
Cholesterol (mmol/l)	5.3 ± 0.4	5.5 ± 0.3	0.65
HDL cholesterol (mmol/l)	0.9 ± 0.1	1.0 ± 0.1	0.84
LDL cholesterol (mmol/l)	3.2 ± 0.2	3.6 ± 0.2	0.22
Triacylglycerols (mmol/l)	2.0 ± 0.3	2.0 ± 0.3	0.97
Plasma calcium (mmol/l)	2.32 ± 0.02	2.31 ± 0.01	0.62
PTH (pmol/l)	6.2 ± 0.5	5.4 ± 0.5	0.35
BAP (U/l)	34.6 ± 4.2	32.6 ± 2.1	0.68
Total AP (U/l)	70.8 ± 4.7	72.1 ± 4.2	0.85
PINP (μg/l)	6.5 ± 1.0	5.4 ± 1.2	0.53
Osteocalcin (μg/l)	25.0 ± 2.5	22.8 ± 1.8	0.49
CTx (ng/ml)	0.45 ± 0.08	0.37 ± 0.06	0.42

Twenty-four volunteers were randomly assigned 4 weeks of placebo ($N = 12$) or resveratrol ($N = 12$) treatment. Values are shown as group means ± SEM. Statistical comparison was performed by students unpaired *t*-test.
HDL, high density lipoprotein; LDL, low density lipoprotein; PTH, parathyroid hormone; BAP, bone-specifc alkaline phosphatase; AP, alkaline phosphatase; PINP, N-terminal propeptide of type 1 procollagen; CTx, C-terminal telopeptide.

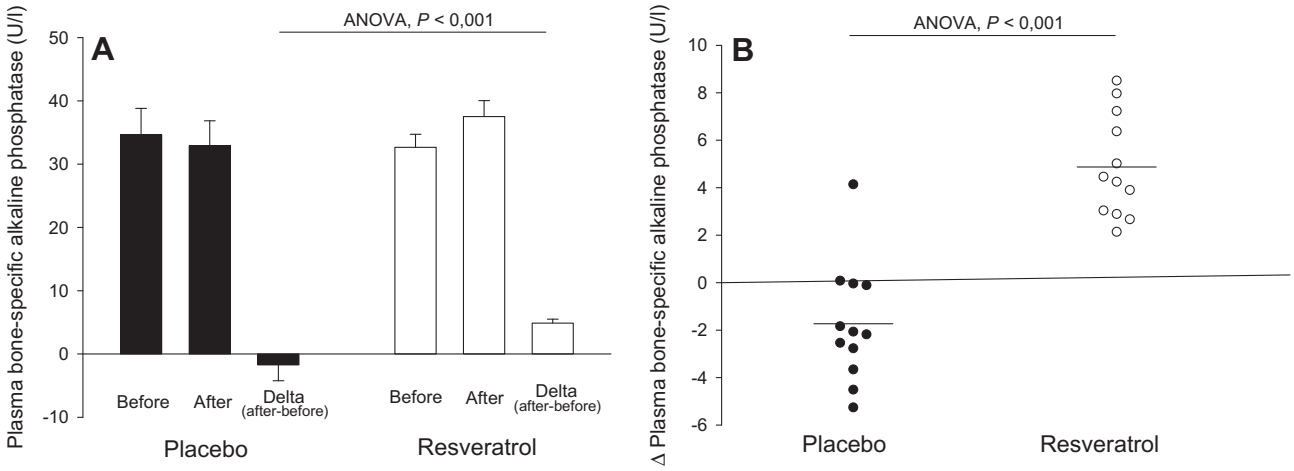

Fig. 1 – Bone-specific alkaline phosphatase. Plasma levels of bone specific alkaline phosphatase were examined before and after 4 weeks of resveratrol or placebo supplementation. Filled bars and dots indicate placebo group ($N = 12$) and open bars and dots indicate resveratrol group ($N = 12$). (A) Absolute changes. Results are presented as group means ± SEM. (B) Individual delta changes (after–before). Horizontal lines indicate group means. *P*-values reflect between-group differences assessed by two way repeated measures ANOVA.

all subjects during resveratrol treatment (Fig. 1B). The increase in BAP was paralleled by a rise in total alkaline phosphatase (AP) in the resveratrol group, which did, however, only tend to reach statistical significance (within-group comparison: $P = 0.061$) (Table 2). The increased BAP was not accompanied by significant changes in plasma levels of other markers of bone formation: N-terminal propeptide of type I procollagen (PINP) and osteocalcin, or resorption: C-terminal telopeptide (CTx), or by changes in plasma levels of calcium or parathyroid hormone (PTH) (Table 2).

Table 2 – Biomarkers of bone metabolism.

	Placebo		Resveratrol		ANOVA
	Before	After	Before	After	P-value
BAP (U/l)	34.6 ± 4.2	32.9 ± 4.0	32.6 ± 2.1	37.5 ± 2.5	<0.001
Total AP (U/l)	70.8 ± 4.7	70.3 ± 4.2	72.1 ± 4.2	76.7 ± 4.5*	0.50
PINP (μg/l)	6.5 ± 1.0	8.6 ± 1.6	5.4 ± 1.2	5.1 ± 1.2	1.00
Osteocalcin (μg/l)	25.0 ± 2.5	24.5 ± 2.3	22.8 ± 1.8	21.0 ± 1.9	0.35
CTx (ng/ml)	0.45 ± 0.08	0.52 ± 0.08	0.37 ± 0.06	0.40 ± 0.04	0.27
Plasma calcium (mmol/l)	2.32 ± 0.02	2.35 ± 0.01	2.31 ± 0.01	2.29 ± 0.03	0.25
PTH (pmol/l)	6.2 ± 0.5	5.7 ± 0.6	5.4 ± 0.5	5.5 ± 0.5	0.51

Pertinent biomarkers of bone metabolism were determined before and after 4 weeks treatment with placebo ($N = 12$) or resveratrol ($N = 12$). All data are presented as group means ± SEM. P-values reflect overall comparison of the groups by two way repeated measures ANOVA.
BAP, bone-specifc alkaline phosphatase; AP, alkaline phosphatase; PINP, N-terminal propeptide of type 1 procollagen; CTx, C-terminal telopeptide; PTH, parathyroid hormone.
* The increase in total alkaline phosphatase within the resveratrol group tended to reach statistical significance when analysed by paired t-test ($P = 0.061$).

We detected no change in various inflammatory markers, leptin, adiponectin, liver function tests or glucose metabolism (Poulsen et al., 2013).

4. Discussion

In the present study, we demonstrated a statistically significant increase in bone-specific alkaline phosphatase after high-dose resveratrol supplementation for four weeks. The rise in BAP was not associated with changes in other biomarkers of bone and calcium metabolism or in markers of inflammation, glucose metabolism or liver function (Poulsen et al., 2013).

In addition to the increase in BAP, we demonstrated a borderline significant increase in total AP ($P = 0.061$). This supports our finding of increased BAP levels, but it should be kept in mind that the total amount of AP found in the circulation is composed of all AP subtypes, i.e. bone, liver and intestine. In adults the bone and liver isoenzymes contribute approximately equally to total AP with the intestinal fraction accounting for less than 10%. Even though cross-reactivity between the isoenzymes is a potential confounder, this possibility does not explain our finding, as the applied antibodies in the BAP assay are selective and have high affinity for the bone isoform; potential cross-reactivity to the liver form is modest 3–8%, and binding of placental and intestinal isoenzymes is negligible. This provides sensitivity and accuracy to a degree which substantially eliminates the possibility of an assay related false positive finding.

Hypothetically, the increased levels of BAP could potentially be driven by alterations in calcium homeostasis. However, the plasma levels of calcium and PTH were unchanged after four weeks of resveratrol treatment ruling out that a permanent resveratrol-induced alteration in calcium homeostasis is causing the increased BAP. Unfortunately, we do not have measurements of plasma levels of calcium and PTH after 1 or 2 weeks of treatment, so we cannot rule out early and temporary changes in calcium homeostasis as a cause for the increase in BAP. We recorded normal levels of the liver enzyme ALT, which to some extend eliminate abnormal liver function as a cause of the increase in BAP, which is important, since circulating BAP is mainly cleared by the liver.

Support for our suggestion of resveratrol having a positive effect on bone metabolism can be found in the literature. Several *in vitro* studies have demonstrated resveratrol-mediated induction of osteoblast-derived BAP, providing evidence of osteoblastic differentiating potential (Mizutani, Ikeda, Kawai, & Yamori, 1998; Su, Yang, Zhao, Kuo, & Yen, 2007). Resveratrol has also been demonstrated to inhibit osteoclast formation (Boissy et al., 2005), and finally, the impact of resveratrol has been studied in rodent models of osteoporosis (Liu et al., 2005; Momken et al., 2011). However, only two animal studies have assessed biomarkers of bone turnover and inflammatory parameters: In the first publication, resveratrol prevented loss of bone mass and strength through inhibition of both the increased bone resorption, measured by urinary excretion of deoxypyridinoline, and the decreased bone formation, measured by osteocalcin, seen in association with mechanical unloading (Momken et al., 2011). Interestingly, in the same study, bone mass and strength were increased in control animals treated with resveratrol without concomitant changes in the biomarkers of bone turnover. Unfortunately, BAP was not measured in this study, but the fact that osteocalcin levels were maintained at the control level supports our finding of an osteogenic potential of resveratrol in addition to an inhibitory effect on mechanical unloading-stimulated bone resorption. In addition, resveratrol supplementation did not affect plasma levels of TNFα, IL1 and IL6 in neither the unloaded, nor the loaded animals, suggesting that the osteogenic effect is not a result of the suggested anti-inflammatory potential of resveratrol. This is also in agreement with our findings, as we did not demonstrate any effects of resveratrol on inflammatory markers, leptin or adiponectin. Furthermore, our finding of resveratrol mediated increased BAP is supported by another recently published paper in which resveratrol preserves bone mass in hindlimb-suspended rodents; the effect is associated with significant increases in AP in rats supplemented with resveratrol (Durbin et al., 2013).

Despite these similarities, it remains to be explained why the increase in BAP was not accompanied by increases in other formative markers. Given the putative crucial involvement in the bone mineralisation process (Urena and de Vernejoul, 1999), one would expect that changes in osteocalcin and/or PINP preceded the changes in BAP, since osteocalcin

and PINP are considered to reflect bone matrix synthesis. Indeed, this is what previous clinical trials on anabolic treatment of osteoporosis with recombinant human parathyroid hormone (rhPTH (1–34)) have demonstrated (Glover et al., 2009; Kurland et al., 2000; Lane, Sanchez, Genant, Jenkins, & Arnaud, 2000; Orwoll et al., 2003; Rubin and Bilezikian, 2005). In the present short-term study, we only measured markers of bone turnover at a single time point and can therefore not evaluate the dynamics of the changes in the measured markers, but we consider it unlikely that an effect of resveratrol on PINP or osteocalcin would already have disappeared after four weeks of treatment. Consequently, the isolated increase in BAP may represent evidence of a possible resveratrol-mediated influence on the mineralisation process. Nonetheless, it remains to be evaluated whether this should be regarded as a beneficial effect or quite the contrary; mpaired mineralisation as seen in for example osteomalacia (Urena & de Vernejoul, 1999). In addition, we cannot rule out that resveratrol influences mineralisation in an osteoblast-independent way, but we are currently studying this aspect using cultures of human primary osteoblasts, and we have indications of a direct effect of resveratrol on osteoblast metabolism, which supports our clinical findings.

Our data represent the first evidence of resveratrol-mediated effect on bone metabolism in human subjects. Strengths of the study are that several markers of bone turnover and calcium homeostasis have been measured and that the study population is homogenous. On the other hand, the study is limited by the study group which comprised younger, obese men with relatively low bone turnover and high bone mass, and that the study is short term (4 weeks) and markers of bone turnover were only measured twice. The difference in mean age between our two treatment groups comprises a potential confounder. However, the difference was due to two outliers in the resveratrol group, aged 60 and 68 years. Repeating all our analysis without these two outliers did not change the overall outcome.

5. Conclusion

On the basis of the present findings, we suggest that resveratrol influences bone metabolism, possibly representing a primary anabolic modality in preserving bone integrity. However, additional clinical studies are required and should comprise trials of longer duration in both genders in addition to inclusion of patients with osteoporosis.

Disclosures

None.

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