Osteoporos Int (2013) 24:2123–2126
DOI 10.1007/s00198-012-2229-x

SHORT COMMUNICATION

Soft tissue calcification in the Ossabaw miniature pig: experimental and kinetic modeling studies

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Received: 23 July 2012 / Accepted: 13 November 2012 / Published online: 6 December 2012

Abstract
Summary Calcium (Ca) deposition into vascular tissue was measured in Ossabaw miniature pigs with and without metabolic syndrome (MetS) using Ca tracer kinetics and coronary atherosclerosis measured with intravascular ultrasound. Pigs with MetS had higher Ca uptake into coronary arteries than lean pigs.
Introduction Ca deposition into arteries is a common disease in humans. The Ossabaw pig develops MetS when fed an atherogenic diet. The aim of this study was to measure Ca deposition into arteries of lean vs. MetS pigs.
Methods Male pigs were fed for 5 months with chow diet (healthy, lean; $n=7$) or atherogenic diet ($n=8$) consisting of chow supplemented with 2 % cholesterol, 43 % kcal from fat, and 20 % kcal from fructose. Pigs were verified to have MetS by obesity, insulin resistance, impaired glucose tolerance, dyslipidemia, and hypertension. Two pigs received 50 nCi of ^{41}Ca i.v. and blood was drawn frequently for 24 h, and 2, 3, 6, 8, 10, 15, 20, and at sacrifice at 28 days after injection. Peripheral arteries were biopsied four times per pig over the 28th day and coronary artery sampled at sacrifice. Tissues were analyzed for ^{41}Ca:Ca. A compartmental model was used to estimate rates of Ca deposition into the arteries.
Results The MetS swine had higher ^{41}Ca and atherosclerosis in coronary arteries than lean pigs.
Conclusions This pig model is a suitable model for studying vascular calcification in humans.

Keywords ^{41}Ca · Metabolic syndrome · Pig · Vascular calcification

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Introduction

Recent reports suggest that cardiovascular risk of myocardial infarction is increased in humans consuming calcium (Ca) supplements with total Ca intake of >1,800 mg/day [1], presumably through increased coronary artery calcification although this finding has been challenged [2]. Calcium deposition in mature atheromatous plaques in the coronary arteries is almost universal. It relates to severity of the atheromatous disease and its presence can be used to assess disease prevalence and progression. As calcium supplements are a standard of care for bone health, concern over their overall safety necessitates understanding if calcium intake is causally related to soft tissue calcification. The mouse Apo E knockout [3] is a useful model for studying atherosclerosis, but there is a need for a large animal model that is more representative of human metabolism. The Ossabaw miniature pig develops MetS and coronary atherosclerosis when fed excess calorie atherogenic diet [4].

To determine whether dietary Ca directly affects the rate of Ca deposition in arteries, a method to measure these rates is needed. A Ca tracer, ^{41}Ca with a long half-life ($t_{1/2}>10^5$ year), has been used to measure Ca metabolism in humans [5, 6]. Administration of this tracer enables Ca kinetics to be monitored sensitively (10^{-18}M) at the low levels expected in soft tissues. In addition to the safety of ^{41}Ca over a radioisotope

such as ^{45}Ca, it can be measured for a longer period, and there is also a 2 order of magnitude sensitivity improvement for ^{41}Ca over ^{45}Ca in terms of the number of atoms used in the doses [7]. Thus, the aim of this study was to measure artery Ca deposition in atheromatous plaques using ^{41}Ca tracer and to compare the rates of Ca deposition in lean and MetS pigs using ^{41}Ca and compartmental modeling.

Methods

Animals and diet

Protocols were approved by Purdue University and Indiana University Animal Use Committees. Male pigs aged 9–11 months were fed for 5 months with chow diet (healthy, lean; n=7) or atherogenic diet (n=8) consisting of chow supplemented with 2 % cholesterol, 43 % kcal from fat, and 20 % kcal from fructose. The diets contained Ca at 1.03 %, phosphorus at 0.81 %, available phosphorous was 0.65 %, and vitamin D3, 1.1 IU/g. Pigs were verified to have MetS by presence of obesity, insulin resistance, impaired glucose tolerance, dyslipidemia, and hypertension and, by ultrasound, to have atherosclerosis as described previously [4].

Tracer kinetics and sampling

After 4 months, each pig received 50 nCi ^{41}Ca i.v. in 3 mL via a catheter inserted into the right or left jugular vein, and blood (10 mL) was drawn at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 7, 10,

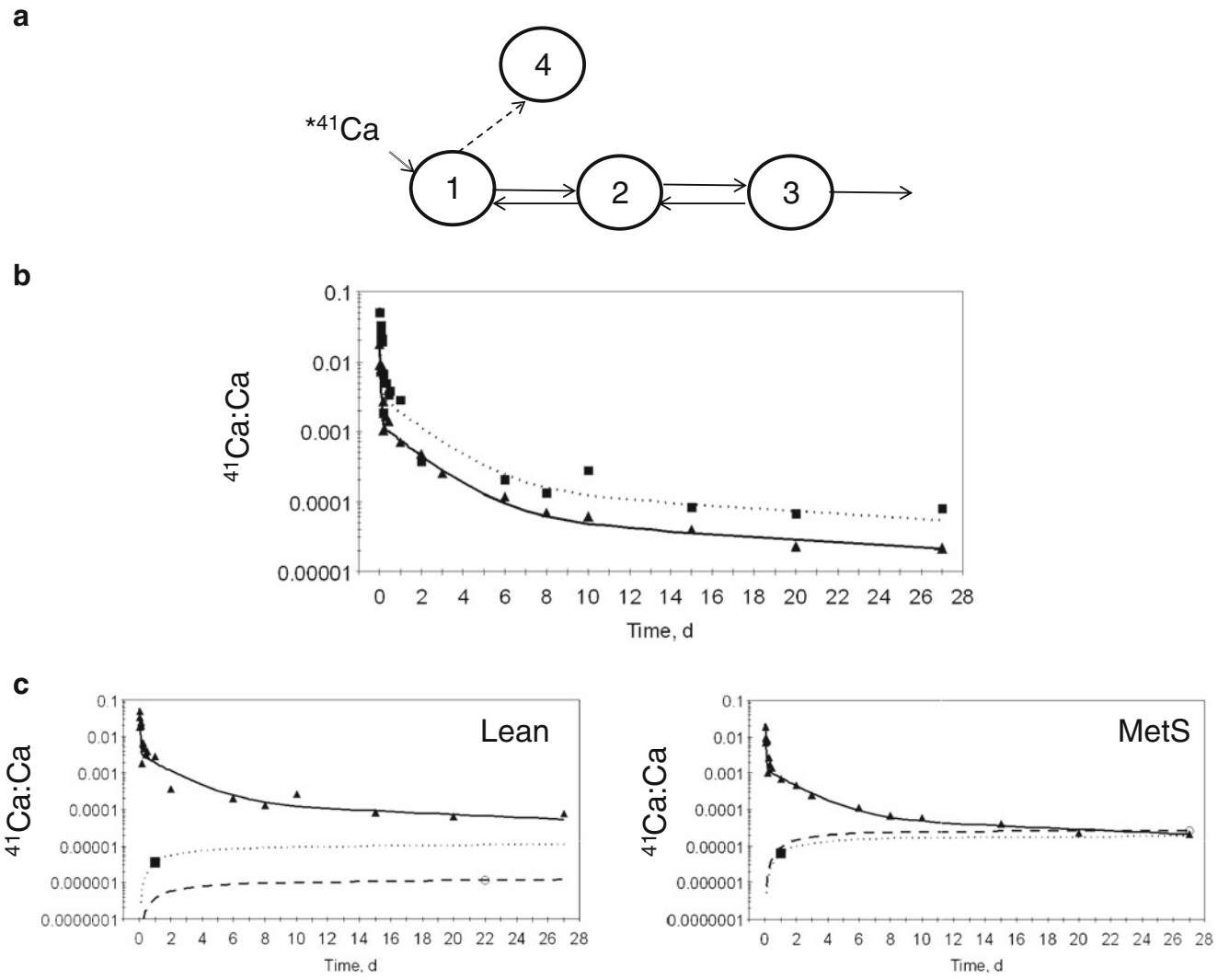

Fig. 1 **a** Model structure used to fit the serum data and to test the source of artery Ca. Compartment *4* and *dashed arrow* represented artery shown here using compartment *1* as the source; the model was also evaluated with compartments *2* and *3* as alternative sources for the arterial data. **b** Serum tracer ($\times 10^{-5}$) in a MetS (*triangle*, *solid line*) and a lean (*box*, *dotted line*) pig. The lines were calculated by the model (**a**). **c** Serum data (*triangles*) and peripheral artery data ($\times 10^{-5}$) obtained at 1 day (*box*) and at sacrifice (*circle*) for a MetS and lean pig. Calculated curves (*solid line* for serum and *dotted and dashed lines* for arteries) were from the model in **a**. Note that the peripheral artery samples were from different sites and so are fitted by separate pathways

and 12 h, and then 1, 2, 3, 6, 8, 10, 15, 20, and at sacrifice at 28 days after injection. Femoral arteries were biopsied four times per pig over the 28th day while pigs were completely anesthetized with 2–5 % isoflurane and coronary artery sampled at sacrifice. Samples weighed 0.003–0.015 g dry weight.

Analyses

Serum and tissues were analyzed for ^{41}Ca:Ca using accelerator mass spectrometry [6]. Coronary atherosclerosis was quantified with intravascular ultrasound [4].

Data analysis

Data consisted of the ^{41}Ca:Ca ratio and Ca content of serum and peripheral artery samples for two pigs and coronary artery samples from all pigs. Kinetic data were analyzed using WinSAAM [8] and a three-compartment model (Fig. 1a) considered to represent serum, extravascular fluid, and exchangeable calcium in the bone [6]. A single loss pathway was included to represent Ca deposition into the bone, urinary excretion and endogenous excretion into the intestine. Initial condition was the dose of tracer. The model was first fitted to the serum data by allowing transfer coefficients and initial volume of distribution to be adjustable, using the least squares procedure in WinSAAM. The ^{41}Ca:Ca ratio was calculated as the tracer in the first compartment divided by the steady-state mass (the product of the pool mass and volume of distribution). Tissue data were fitted by including a pathway from the first compartment to the fourth compartment representing tissue (Fig. 1a). The pathway into tissue was also tested as originating from compartment 2 or 3. The rate of Ca entry into the tissue pool was calculated in WinSAAM as the product of the fractional transfer from compartment 1 (or compartment 2 or 3) into the tissue compartment and the mass of compartment 1 (or 2 or 3). The initial amount of Ca in the tissue pool was made adjustable so that the measured Ca content at time of sampling and calculated Ca agreed. Separate tissue pools were included for the coronary artery and the four peripheral sites. Model results were the volume of distribution, rates of tracer distribution, and rates of Ca deposition into arterial pools.

Statistics

Values are expressed as mean±SD. Lean and MetS pigs were compared using Student's *t* test. Differences were significant if $P<0.05$.

Results

At the start of the study, there was no difference in weight between the pigs, 37.9±6.7 kg (lean) and 41.1±5.7 kg (MetS), but at sacrifice, the MetS pigs were heavier than the lean (93.0±5.7 vs. 65.1±10.4 kg, $P<0.001$). The Ca content of the coronary artery samples did not differ between MetS and lean pigs (2.313±1.665 for MetS vs. 2.276 ±1.517 for lean pigs (in milligrams per gram dry weight)). However, the ratio of ^{41}Ca:Ca was higher in coronary artery samples from MetS pigs than lean pigs (Fig. 2a). For most samples from the lean animals, values were below detection as carrier had to be added for AMS analysis (Fig. 2a).

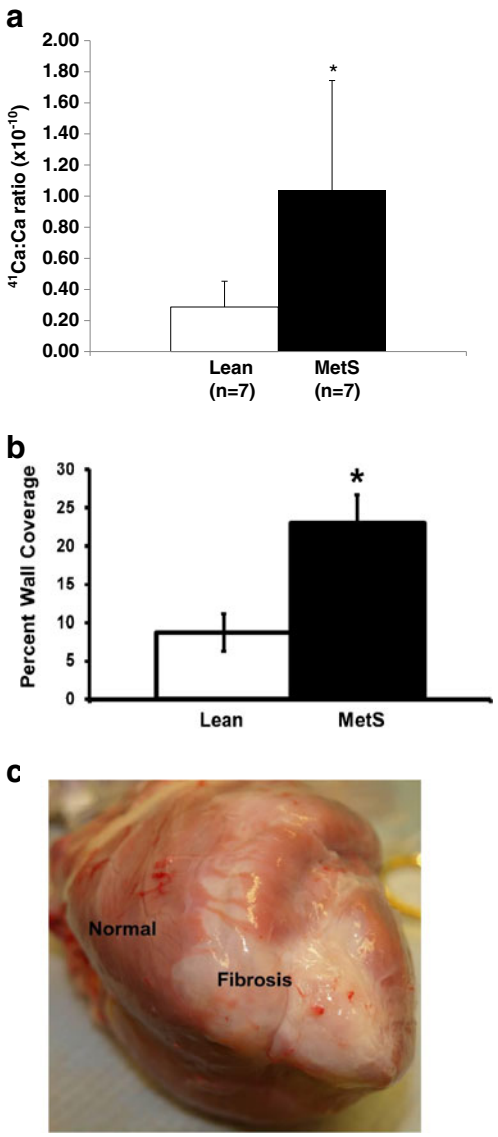

Fig. 2 **a** ^{41}Ca/Ca ratio of right coronary artery samples of lean ($n=7$) and MetS ($n=7$) pigs (mean±SD, $P<0.05$). Values for six lean pigs and two MetS pigs were below detection limit; the upper limit of detection was used for these animals for calculating the means. **b** Coronary atheroma assessed by intravascular ultrasound. Percent wall coverage was obtained by pullback of the ultrasound catheter along the length of the coronary artery. **c** Spontaneous myocardial infarction evidenced by fibrosis (*pale color*) in the myocardium. The fibrosis resulted from a myocardial infarction that occurred in this pig >1 week before the pig was euthanized and the heart harvested

Similar to ^{41}Ca uptake, coronary atherosclerosis quantified as percent wall coverage [4] was negligible for the lean group, while the MetS group had 23±5 % wall coverage (Fig. 2b). Clear evidence of myocardial spontaneous infarction in MetS pigs is shown in Fig. 2c, likely the result of plaque rupture in an epicardial conduit artery [9].

Serum data from the two pigs could be fitted by allowing only the initial volume of distribution to differ between MetS and lean pigs (13.9±0.7 L in MetS pigs vs. 5.4± 0.3 L in lean pigs; Fig. 1b). The coronary sample from the lean pig was below detection. Peripheral artery samples were obtained on days 1, 7, and 22 from the lean animal (the 7th day sample was below detection) and days 1, 2, 8, and 27 from the MetS pig (day 8 was below detection). As there was only one datum per sample site, peripheral artery data (and the aorta sample from the MetS pig) could be fitted equally well using compartment 1, 2, or 3 as the source (Fig. 1c). If we assume that the fractional transfer from a compartment into peripheral tissue was similar across time points, then compartment 1 or 2 was the more likely source (data not shown). Regardless of which compartment was the source of Ca in the arteries, the rate of Ca entry into the peripheral arteries was fivefold higher in the MetS pigs on day 1 (8.7E−4 vs. 1.58E−4 mg/day in the MetS vs. lean pig) and ninefold higher at sacrifice (2.3E−4 vs. 2.55E−5 mg/day in the MetS vs. the lean pig).

Discussion

The kinetic studies showed that the metabolism of Ca was similar between MetS and lean pigs, but that the rate of serum Ca entering the arteries was faster in MetS than lean animals. This suggests that the Ossabaw pig, with tissue sampling and tracer kinetics, is a suitable humanoid model for the study of Ca deposition in soft tissues.

Some limitations of the study were the accurate identification of area of blood vessels containing mature, calcified plaque for sampling, and the number of animals that had both serum and peripheral artery sampling. However, due to the diffuse atherosclerosis shown in MetS Ossabaw swine with our intravascular ultrasound measures of wall coverage along the entire length of the coronary arteries [4], we can be certain that atherosclerosis was at least in early stages. These limitations, however, do not alter the results of tracer measured in the coronary artery. The large variation for the MetS pigs likely relates to uneven distribution of the tracer between tissue with varying amounts of plaque. The lack of difference in total Ca vs. detectable change in ^{41}Ca is related to the vastly higher sensitivity in ^{41}Ca:Ca ratio measurements and possibly due to the tracer reflecting only recent deposition during the period of plaque development. Differences in tracer deposition are not likely the result of differences in blood flow in MetS and lean pigs because blood flow is decreased in MetS [4, 10].

We conclude that the Ossabaw miniature pig, because of its suitable size for laboratory analysis, combined with its natural ability to develop MetS and now the ability to measure Ca deposition in arteries, makes it a suitable model to explore the kinetics of Ca deposition in soft tissues throughout the progression of atherosclerosis. The pig could be used in future studies that mimic the human environment such as the effect of dietary Ca on rate of Ca deposition in soft tissue and plaque formation.

Acknowledgments The authors acknowledge the National Institutes of Health grant HL062552 to M.S.

Conflicts of interest None.

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