Metabolic reprogramming of osteoclasts represents a therapeutic target during the treatment of osteoporosis

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Osteoclasts are specialised bone resorbing cells that control both physiological and pathological bone turnover. Functional changes in the differentiation and activity of osteoclasts are accompanied by active metabolic reprogramming. However, the biological significance and the in vivo relevance of these events has remained unclear. Here we show that bone resorption of differentiated osteoclasts heavily relies on increased aerobic glycolysis and glycolysis-derived lactate production. While pharmacological inhibition of glycolysis did not affect osteoclast differentiation or viability, it efficiently blocked bone resorption in vitro and in vivo and consequently ameliorated ovariectomy-induced bone loss. Our experiments thus highlight the therapeutic potential of interfering with osteoclast-intrinsic metabolic pathways as possible strategy for the treatment of diseases characterized by accelerated bone loss.

Bone is a dynamic tissue undergoing constant remodeling that is orchestrated by osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Osteoclasts (OCs) are formed from precursor cells (OCPs) of the monocyte/macrophage lineage in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL)1,2. OCs are the primary bone-resorbing cells in both physiological and pathological states and thus play a key role in regulating bone mass. Pathologically enhanced osteoclast activity and accelerated bone resorption are observed during postmenopausal osteoporosis and diseases such as rheumatoid arthritis leading to decreased bone mass and increased risk of fractures3.

The molecular events underlying OC differentiation have been extensively studied. RANKL has been identified as key cytokine during osteoclastogenesis and its therapeutic targeting reduces osteoclast numbers and bone resorption during the treatment of osteoporosis. Mechanisms that control and adjust the function of mature osteoclasts are less well understood. However, insights into these pathways would be highly relevant to design novel therapeutic approaches to fine tune osteoclast function.

Recent data indicate that active metabolic reprogramming occurs during RANKL-induced osteoclastogenesis, where these multinucleated cells show an increase in mitochondrial content, biomass and mitochondrial respiration as well as accelerated glycolytic metabolism4–6. More recently, human osteoclasts were described to directly increase glycolysis during bone resorption in vitro. However, the in vivo consequences of these events remain unclear7. In this study, we used cell culture medium containing bone powder to induce a bone-resorbing phenotype in OCs and to study the consecutive functional and metabolic changes. We observed an active metabolic switch in bone resorbing OCs, characterized by increased glycolysis and lactate production. Notably, both

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glycolysis and its metabolite lactate were essential for efficient bone resorption. Functional inhibition of glycolysis by 2-Desoxy-D-glucose (2-DG) blocked bone resorption by OCs, which could be rescued by supplementation of either pyruvate or lactate suggesting lactate as an active metabolic intermediate that drives bone resorption. In accordance with a key role of glycolysis during OC-mediated bone resorption in vivo, we observed effective amelioration of ovariectomy-induced bone loss in mice that received 2-DG or a small molecular inhibitor of lactate dehydrogenase A (LDH-A).

Results
Activation of osteoclasts is paralleled by increased aerobic glycolysis. To analyse changes in the metabolic activity of bone resorbing OCs, we isolated murine bone marrow-derived monocytes as OCp and cultured these cells in M-CSF and RANKL containing medium in the absence and presence of bone powder to simulate a bone resorbing metabolic state in such “activated” OCs (aOCs). The application of bone powder medium did not affect OC fusion capacity, OC number or the expression of OC differentiation marker genes, such as dc-stamp, oscar and cathepsin k (Fig. 1A, B). By performing extracellular flux assays, we compared changes in the extracellular acidification rate (ECAR) as measure of the glycolytic activity between OCp, non-activated “resting” OCs (rOCs) and bone resorbing aOCs. OC differentiation resulted in a reduced glycolytic activity in rOCs in comparison to OCp (Fig. 1C). However, aOCs displayed increased glycolysis, glycolytic reserve (GR) and glycolytic capacity when compared to rOCs, confirming that bone resorption was paralleled by accelerated extracellular acidification and an enhanced aerobic glycolysis (Fig. 1C). Both rOCs and aOCs populations revealed marked elevation of their oxygen consumption rate (OCR), indicated by increased spare respiratory capacity (SRC) and maximal respiration. Moreover, rOCs and aOCs showed an elevated proton leak and basal respiration demonstrating increased mitochondrial respiration during OC differentiation and bone resorption (Fig. 1D).

In addition, we performed metabolic profiling of rOCs and aOCs. In accordance with the data derived from the extracellular flux assays, we observed an increase of metabolites of both glycolysis and the Krebs cycle such as glucose 6-phosphate (G6P) or citrate (Cit), respectively, upon initiation of bone resorption in aOCs (Fig. 1E). Direct measurement of glucose consumption and lactate accumulation in the growth media of rOCs and aOCs revealed that both OC populations substantially consumed glucose. However, lactate production was only significantly elevated in bone-resorbing aOCs, which was in accordance with their enhanced state of aerobic glycolysis (Fig. 1F). When we analysed the metabolic gene expression profile, we found several genes of the glycolytic pathway induced in aOCs (Fig. 1G). In particular, expression of pdk4, which catalyses the conversion of lactate and NAD + to pyruvate, was downregulated in aOCs, indicating a metabolic shift towards active aerobic glycolysis and lactate production during bone resorption.

 Glycolysis-derived lactate essentially supports bone resorption. Next, we aimed to address the functional consequences of the observed metabolic reprogramming of aOCs including the increase in aerobic glycolysis and lactate production on osteoclast differentiation and bone resorption. We therefore isolated monocytes, assessed OC differentiation and quantified their bone resorbing capacity in the presence of specific inhibitors of oxidative phosphorylation and glycolysis, respectively. Notably, 2DG-mediated inhibition of glycolysis efficiently blocked bone resorption, but did not affect OC differentiation and fusion, whereas rotenone as an inhibitor of mitochondrial complex I, decreased osteoclastogenesis but did only moderately affect resorption activity (Fig. 2A, B). These results indicated that aerobic glycolysis in OCs was essential for OC activation and bone resorption, but did not affect earlier steps of osteoclastogenesis such as differentiation and fusion events. To determine downstream mediators and metabolites that were responsible for the glycolysis-mediated increase in bone resorption, we assessed the impact of the glycolysis products lactate and pyruvate on the bone resorption capacity of aOCs in the absence and presence of 2DG. While lactate and pyruvate alone did not affect the bone resorbing activity of aOCs, both metabolites rescued the decreased bone resorption caused by inhibition of glycolysis via 2DG (Fig. 2C), which suggested lactate as the active metabolite mediating bone resorption in response to enhanced glycolysis in aOCs.

Inhibition of glycolysis and lactate production ameliorate ovariectomy-induced bone loss. To assess the potential of OC-mediated glycolysis as therapeutic target for diseases characterized by a pathologi-
cally enhanced bone resorption, we decided to study the effect of blocking glycolysis and lactate production during a mouse model of ovariectomy-induced bone loss. Therefore, we treated mice that displayed an established osteoporosis (treatment start 2 weeks post ovariectomy) with the glycolysis inhibitor 2-Deoxy-d-glucose (2dG; 10 µM) or rotenone (0.02 µM) on day 3 of OC culture. (B,C) OCs were generated on bone resorption plates for 5 days and stimulated with 2dG (10 µM) or rotenone (0.02 µM) in the presence or absence of lactate (5 mM) or pyruvate (2 mM) as indicated. The percentage of the resorbed area was quantified via Photoshop and pictures show representative images of resorption pit formation.

Figure 2. Glycolysis routes bone resorptive activity in vitro. (A) Fusion index (left) and representative TRAP staining of OCs differentiated in the absence or presence of the glycolysis inhibitor 2-Deoxy-d-glucose (2dG; 10 µM) or rotenone (0.02 µM) on day 3 of OC culture. (B,C) OCs were generated on bone resorption plates for 5 days and stimulated with 2dG (10 µM) or rotenone (0.02 µM) in the presence or absence of lactate (5 mM) or pyruvate (2 mM) as indicated. The percentage of the resorbed area was quantified via Photoshop and pictures show representative images of resorption pit formation.
and an increased trabecular thickness in ovariectomized mice, which was in accordance with a block of pathologic bone resorption in these mice.

We additionally validated these results and our in vitro findings in a second round of experiments where we treated mice with the LDHA inhibitor GSK2837808A to selectively interfere with lactate production in vivo. GSK2837808A inhibited the bone resorbing activity of osteoclasts to a similar extend as 2dG without cytotoxic effects in vitro (Suppl. Fig. 1A,B). Treatment was started 2 weeks after ovariectomy in mice that already had developed bone loss (Fig. 3E). In accordance with our previous results, block of lactate synthesis resulted in a slight decrease in bone density in sham-operated mice, whereas GSK2837808A treatment resulted in a significant improvement of ovariectomy-induced bone loss (Fig. 3F,G). Here, we observed an increased BV/TV, an increased trabecular thickness as well as an increased BMD in ovariectomized mice that had received GSK2837808A.

Discussion

Increasing data highlight the mutual influence of cellular metabolic processes and the functional properties of innate and adaptive immune cells. Among others, macrophages were shown to undergo a defined metabolic reprogramming in response to different activation stimuli. Pro-inflammatory classically-activated macrophages e.g. increase their glycolytic activity and shut down their mitochondrial respiration, whereas anti-inflammatory alternatively-activated macrophages show an increase in mitochondrial respiration. These contrasting metabolic adaptations of individual macrophage subsets seem to support distinct activation and differentiation programs, thereby promoting either onset or resolution of inflammation. Our current data confirm that osteoclast, as mononuclear phagocyte-derived cells that settle the bone microenvironment, undergo specific metabolic adaptations during differentiation and bone resorption as well.

Notably, and in contrast to many other immune cells, bone resorbing and activated osteoclasts are not characterized by an isolated, but by a simultaneous increase of both glycolytic activity and mitochondrial respiration when compared to resting osteoclasts. This observation suggests that the process of bone resorption substantially increases the metabolic and energetic requirements of osteoclasts. This adaptation might be merely linked to an increased demand in energy. Alternatively, it points to the necessity of a provision of specific and essential metabolites that derive from glycolysis and/or mitochondrial respiration and support the functional properties of osteoclasts during bone resorption. Our data primarily support the latter scenario and suggest that glycolysis-derived lactate is essential for the efficient resorption of bone by osteoclasts. Block of oxidative phosphorylation, in turn, only moderately affected bone resorption, although osteoclast differentiation was clearly impaired. Block of glycolysis by 2dG or block of the conversion of pyruvate to lactate by the LDHA-inhibitor GSK2837808A accordingly interfered with regular bone resorption, which could be rescued by the restoration of regular lactate levels. The exact role of lactate during this process remains unclear at the moment. Our in vitro experiments show a substantial increase in extracellular lactate upon initiation of bone resorption. A likely possibility is therefore a role of lactate during the acidification of the osteoclast resorption pit, which is an essential step during bone resorption by osteoclasts, facilitates the activation of acid proteases that brake down bone matrix, and additionally dissolves bone minerals.

Our data additionally show that blocking glycolysis and lactate production represents efficient strategies to interfere with pathological osteoclast-mediated bone loss during ovariectomy as a model of postmenopausal osteoporosis that is usually characterized by increased differentiation and activation of osteoclasts. Although such a strategy significantly increased bone mass in osteoporotic mice, healthy mice displayed a slightly decreased bone density upon block of glycolysis. Although the underlying reasons remain to be elucidated, block of glycolysis likely affects other cell types such as osteoblasts and osteocytes that are equally relevant for a regular bone homeostasis. Especially osteoblast are dependent on glycolysis to form new bone, although they equally require mitochondrial respiration and fatty acids to mineralize newly formed extracellular matrix.

Taken together, our data identify aerobic glycolysis and osteoclast-mediated lactate production as essential steps during bone resorption and suggest that blocking glycolysis and lactate production in osteoclasts represents a potential therapeutic strategy during diseases that are characterised by accelerated osteoclast-mediated bone loss such as postmenopausal osteoporosis and rheumatoid arthritis.

Methods

The authors confirm that all methods were carried out in accordance with relevant guidelines and regulations. The ethical approval for all animal experiments was carried out in accordance with relevant guidelines and regulations by the ethical committee of the Friedrich Alexander University of Erlangen-Nuremberg (FAU), Germany.

Mice. Mice were maintained at the specific pathogen-free animal care facility (FPZ) of the University of Erlangen-Nuremberg and housed in a room at 23 ± 2 °C, with 50 ± 10% humidity and a 12-h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.). All mice were allowed free access to water and regular rodent chow.

Ovariectomy-induced osteoporosis (OVX). Female C57BL/6J(Rj) wild-type mice were obtained from Janvier Labs and experiments were performed according to guidelines of laboratory animal care and use. All efforts were made to reduce the number of animals tested and their suffering. For ovariectomy, mice (12 weeks of age) were anaesthetized with a ketamine-xylazine (KX) solution and bilaterally ovariectomized, while ovaries of the sham group were left intact. The ovariectomized mice were allowed to recover for two weeks to ensure the development of osteoporosis. After 6 full weeks of treatment with vehicle, 2d or GSK 2837808A, all animals were killed via cervical dislocation under CO₂ anesthesia and one tibia was excised for histological analyses and one for microcomputer tomography (µCT) imaging. Successful ovariectomy was examined at the day of preparation by anatomical analysis of the ovaries.
lin/streptomycin) at 37 °C with 5% CO2. OCPs were cultured in OC-growth medium (MEM Alpha Medium 1 × GlutaMAX, GIBCO; #32571-028, containing 10% L929 conditioned medium, 10% FCS and 1% penicillin). Cultured overnight and stimulated with appropriate growth medium (MEM Alpha Medium 1 × Glutamax, GIBCO; #32571-028, containing 10% L929 conditioned medium, 10 ng/ml RANKL, 10% FCS and 1% penicillin).

**Cell culture.** Bone marrow derived monocytes (BMDMs) were isolated as previously described and differentiated into osteoclast (OCs). Briefly, hematopoietic bone marrow cells were purified from tibial bone with a 70-μm cell strainer, cultured overnight and stimulated with appropriate growth medium (MEM Alpha Medium 1 × GlutaMAX, GIBCO; #32571-028, containing 10% L929 conditioned medium, 10% FCS and 1% penicillin/streptomycin) at 37 °C with 5% CO2. OCPs were cultured in OC-growth medium (MEM Alpha Medium 1 × GlutaMAX, GIBCO; #32571-028, containing 10% L929 conditioned medium, 10% FCS and 1% penicillin/streptomycin) for 24 h. Osteoclast differentiation was quantified by counting multinucleated (> 3 nuclei) TRAP + cells on day 4 of OC culture using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, # 387 A). Osteoclast fusion events were quantified by counting the nuclei per osteoclast.

**Calculation of the fusion index.** For analysis of the fusion efficiency, a fusion index calculated. The total number of cell fusion events in one syncytium (number of nuclei/osteoclast) were counted (= fusion index). Multinucleated osteoclasts were defined as > 3 nuclei/cell. The absolute number of cells with the same amount of nuclei were set in ratio with the total number of cells per setting and given in cumulative frequency in percent in order to define the fusion efficiency.

**Bone resorption analysis.** For analysis of osteoclast resorption, osteoclast differentiation was conducted in Osteoplates (Corning Osteo Assay Surface 24-well Multiple Well Plates; #3987). Experiments were stopped by aspirating the medium and adding 10% bleach solution for 5 min at room temperature. Afterwards, bleach solution was aspirated and cells were washed twice with 150 μl of dH2O and air-dried overnight. Pictures were taken with Zeiss Axioskop 2 microskop (5 × enlargement) and converted into black and white with Adobe Photoshop. Osteoclast resorption activity was quantified by measurement of black (= resorption area) versus white (= bone) pixels with a Histogram.

**Bone powder medium.** Bovine bone meal box bone powder (from “Hund und Sport Hungenberg”; #KNM3) was crushed into fine fragments with sterile backed mortar. Bone powder was stored in the freezer. Needed concentrations were measured and filled up with not heat inactivated FCS. Tubes were heat inactivated in a water bath for 1 h at 56 °C. One sample was separated on two bacterial dishes and plated under UV light overnight. Finally 5 ml of heat inactivated and sterilized FCS containing Bone Powder was filled up with 45 ml of growth medium (MEM Alpha Medium 1 × GlutaMAX, GIBCO; #32571-028, containing 10% L929 conditioned medium, 10% FCS and 1% penicillin/streptomycin) to obtain bone powder medium.
RT-PCR analysis. Total RNA was isolated from cells using peqGOLD TRIFast (peqlab, Germany). 1 µg was used for the first-strand complementary DNA synthesis (Amersham Biosciences), which was then used for SYBR Green–based quantitative RT-PCR as described previously12. Triplicates were performed according to the manufacturer's instructions. The following mouse RT-PCR primer sequences were used:

| Primer   | Forward sequence                | Reverse sequence                |
|----------|---------------------------------|---------------------------------|
| beta-actin | TGT CCA CCT TCC AGC AGA TGT    | AGCT CGA TTC TGA ATG CCA GA    |
| HK2      | TTT TAC TAC TTC TGT CCA TCC     | TTT TAG TAC CAG CAG TCA ACG    |
| PDK4     | CCT TCA TCC AGA ACC CAA GGG    | TCC ACT CAG GCA ACC TCA ACG    |
| GLUT1    | TCA ACA GGC CCT TCA ACT         | CGA CTG CAG ATG ACG CAT ACG    |
| LDHA     | GGA CAG TGA GCT GAG GTG TGA    | GTA GAG GCA CTA CCT TCA ACG    |
| LDHB     | GGG GAA GTC TCT GTC GAT GAG TAA  | CTG TAC AAG ATA CTT TAT CCG    |
| DC-STAMP | AAA ACC CTT GGC CCT TCT ATT     | AAT CAG GAC ACT CCT TGG        |
| OSCAR    | TCC GTT GAT TCC AGC GTG TGC    | ACC CGG GAC CGC CAT TGC        |
| CATH.K   | ATAT GGG GGC AGA TGA AAT TTT   | TCC AGA AGC AAT CTA TCT        |

Extracellular flux assay. Real time bioenergetic profile of OCPs were obtained by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using the XF96 extracellular flux analyzer (Seahorse Bioscience, Agilent Technologies, North Billerica, MA). Briefly, OCPs were seeded at a density of 150,000 cells per well (96-well-plate) and left untreated or treated with RANKL (10 ng/ml) in growth medium with and without bone powder for 48 h. On the day of measurement, cells were washed two times with Seahorse XF Base Medium and

growth medium was replaced by Seahorse XF Base Medium and supplemented with glucose (10 mM), sodium-pyruvate (200 mM) and glutamax (200 mM). Further, medium was warmed up to 37 °C and pH was subsequently adjusted to 7.4 ± 0.1. For Glyco Stress assay, no glucose and pyruvate was added to Seahorse XF Basismedium. Following incubation in an incubator without CO2 at 37 °C for 60 min, basal OCR and ECAR were recorded for 105 min. Mito Stress assay was performed by sequential addition of 20 µM oligomycin (inhibitor of ATP synthesis), 10 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, uncoupling agent) and 10 µM rotenone/antimycin A (inhibitors of complex I and complex III of the respiratory chain, respectively). Glyco Stress assay was performed by sequential addition of 100 mM glucose, 20 µM oligomycin and 500 mM 2dG. Parameters such as ATP-linked OCR, maximal OCR, spare respiratory capacity (SRC) and glycolysis were evaluated using the Wave XFe Analyzer software Wave Desktop 2.6 (PC only) and Wave Controller 2.6 (https://www.agilent.com/).

Glucose and lactate measurements. Glucose and lactate content were determined as mg/dl in cell culture supernatants using a SuperGLcompact (Hitado, Möhnesee, Germany) according to the manufacturers standard operating procedure.

Measurement of glycolysis and Krebs cycle metabolites. Phosphorylated intermediates and carboxylates were extracted with perchloric acid from xx to xx mg samples of shock-frozen xxx tissue for four biological replicates as described previously13 applying ionchromatography with an ICS3000 HPLC- system (Dionex) and ESI/MS/MS detection using a QTrap3200 Triple-Quadrupole massspectrometer with turbo V ion source (Applied Biosystems) operated in multiple reaction monitoring mode.

Cytotoxicity assay. Cytotoxicity assay was performed with CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega as a measurement of lactate dehydrogenase (LDH) release into the supernatant according to the manufacturers protocol. During experimental treatment via bone resorption assay, supernatant samples were cleared from non adherent cells and transferred to a 96 plate and an equal volume of CytoTox 96 reagent is added to each well and incubated for 30 min. Stop solution is added and the absorbance signal is measured at 490 nm in a plate reader. Results were calculated by subtracting the average values of the culture medium background from all values of experimental wells to compute percent cytotoxicity.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8 software (https://www.graphpad.com/scientific-software/prism/). Results are depicted as median ± interquartile range (IQR) if not stated otherwise. For a two-group comparison, a Student’s t-test was applied if the pretest for normality (D’Agostino-Pearson normality test) was not rejected at the 0.05 significance level; otherwise, a Mann–Whitney U-test for nonparametric data was used. P values less than 0.05 were considered significant. Results are expressed as mean ± SE (SEM). No statistical method was used to predetermine sample size.

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Author contributions

J.T. designed the study, performed and interpreted experiments. C.St., C.B., J.T., B.K., S.A, D.I.H.M and M.F. performed experiments, collected, and interpreted data. D.M. and M.B. established and performed the metabolic characterization of osteoclasts. U.S and J.H performed metabolic analysis. J.T., G.S., C.S. and G.K. designed the study and experiments and wrote the manuscript. All authors read and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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