JCI The Journal of Clinical Investigation

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J Clin Invest. 2021;131(5):e147072. https://doi.org/10.1172/JCI147072.

Commentary

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Nitric oxide and bone: The phoenix rises again

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The exploration of nitric oxide in bone biology

Almost 30 years ago, *Science* declared nitric oxide (NO) "molecule of the year," based on a host of breakthrough discoveries and widespread use of drugs such as nitroglycerin, nitroprusside, and sildenafil (1). Subsequent to that proclamation, an even larger, second wave of research followed to delineate the mechanism of NO actions at the molecular, cellular, and tissue level. The skeleton was one focus of those efforts. By the mid 1990s, the Osdoby and Brandi laboratories independently reported that NO synthase (NOS) was present in osteoclasts and osteoblasts, and that NO prevented osteoclast attachment and resorption (2, 3). Other studies described how NO was upregulated in osteoblasts by estrogen in a cell-autonomous manner and could drive both osteoblast proliferation and differentiation (4). An important breakthrough came with the discovery that mechanical loading of the skeleton led to a brisk NO response in osteocytes similar to that induced by estrogen, and this occurred via activation of soluble guanylyl cyclase (sGC) and cGMP-dependent protein kinases (PKGs) (5).

Taken together, the strong in vitro evidence, the ease and previous widespread clinical use of NO-donor drugs, and promising observational data sustained a two-decade effort to establish organic nitrates as a preventive therapy for osteoporosis. But, the case-control, cohort, and clinical trials were either underpowered or were confounded by reverse causation (6, 7). More importantly, the reports from the definitive NO trial, heralded in several editorials, showing strong beneficial effects of nitroglycerin on areal and volumetric bone mineral density, and a reduction in bone resorption, were ultimately retracted because of scientific fraud in 2011 and 2017 (8, 9). Those developments, particularly the former one, basically sealed nitroglycerin's fate as a novel and cost-effective osteoporosis therapy. Unfortunately, it also slowed studies on the basic biology of NO in bone.

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How NO drives bone formation

In this edition of the *JCI*, NO rises again. Jin et al. provide compelling evidence that argininosuccinate lysate (ASL) regulates arginine synthesis intracellularly in osteoblasts, which leads to enhanced NO production with subsequent upregulation of glycolytic capacity in bone-forming cells (ref. 10 and Figure 1). This aspect of the report alone provides a unique mechanism that explains how NO drives bone formation, and places the anabolic target of this molecule, i.e., glycolysis, in the same realm as parathyroid hormone effects on osteoblasts. In addition, the authors demonstrate elegantly, with induced pluripotent stem cells (iPSCs) from a single patient with ASL deficiency, that heterozygous isogenic iPSCs, through homologous recombination with a helper adenovirus, could rescue the autosomal recessive impairment in mineralization and bone formation of ASL-deficient cells (10).

Other important insights emerge from this report and add to our understanding of the complexities surrounding osteoblast differentiation as well as providing a framework for approaches to treat osteoporosis. Jin et al. used distinctive genetic models, such as the hypomorphic ASL-deficient ($Asl^{Neo/Neo}$) mouse, the caveolin 1 heterozygote ($Cav1^{+/-}$), and the osteoblast-specific conditional deletion of ASL to test precisely how NO regulates osteoblast differentiation (10). Those models linked NO induction and activation to both positive (arginine) and negative regulators (e.g., CAV1) that target the glycolytic pathway in osteoblasts. Notably, suppressors of NOS activity failed to rescue the cortical bone phenotype, although trabecular bone mass was restored in the cross between $Cav1^{+/-}$ and $Asl^{Neo/Neo}$ mice (10). As such, a future goal should be to define those determinants of NOS activity in osteocytes, particularly since osteocytes are the most abundant cells in the skeleton, are sensitive to mechanical loading, and could be a target for future NO-mediated antiosteoporotic agents.

Conflict of interest: The authors have declared that no conflict of interest exists.

Reference information: *J Clin Invest*. 2021;131(5):e147072. https://doi.org/10.1172/JCI147072.

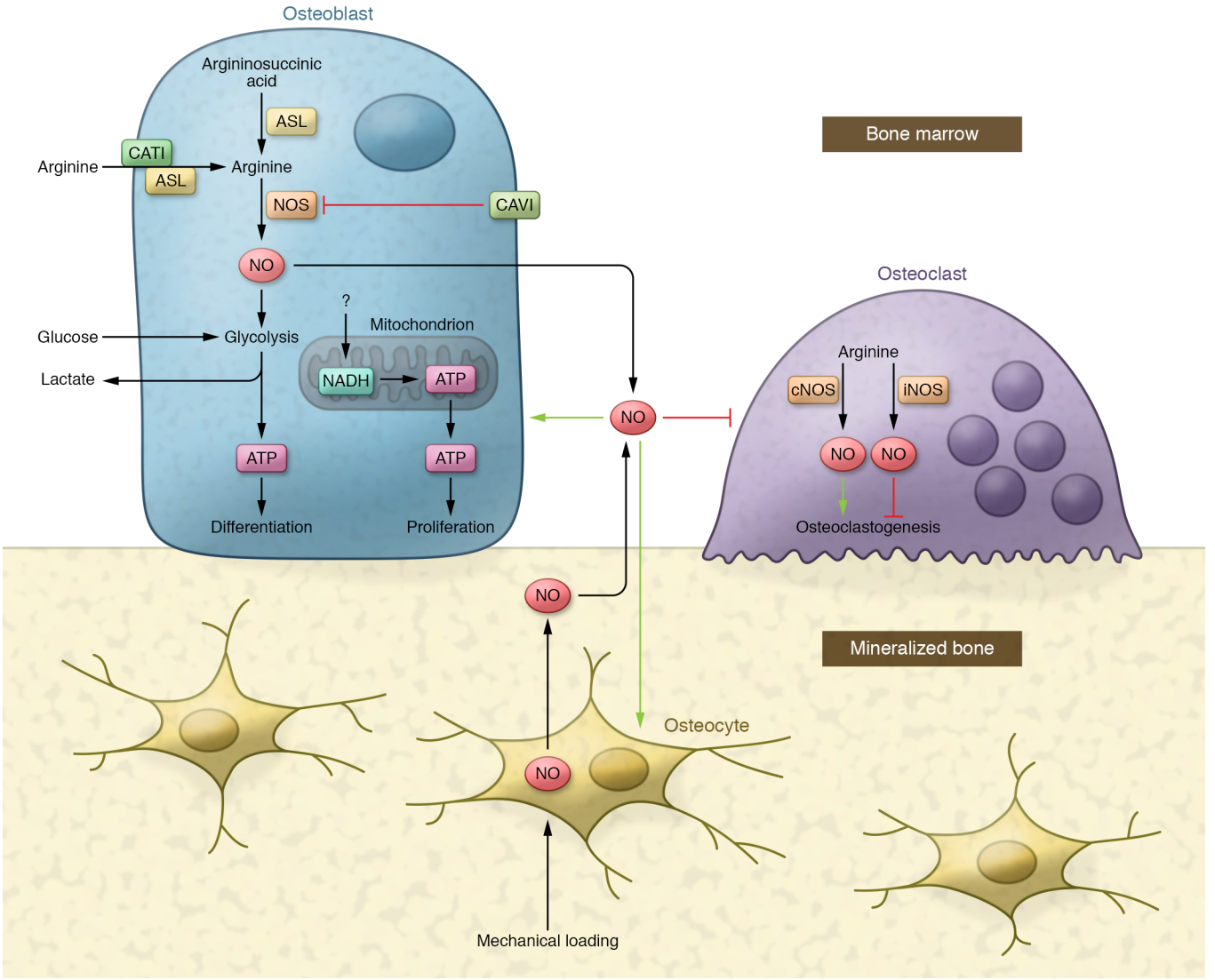

Figure 1. Model for skeletal remodeling with NO positioned at the nexus. In bone marrow, NO is produced by osteoblasts to regulate (green/red arrow) osteoblasts, osteoclasts, and osteocytes. Mechanical loading of the skeleton could also lead to a brisk NO response in osteocytes to regulate surrounded cells. The ASL enzyme generates intracellular arginine and fumarate from argininosuccinate and provides arginine for NO production in osteoblasts, while CAV1 serves as an inhibitor of NOS to decrease the generation of NO. ASL together with cationic amino acid transporter 1 (CAT1) could also help transport arginine across the membrane to increase NO production. Intracellular NO in pre- and differentiated osteoblasts promotes aerobic glycolysis, which has been considered the principal source of energy in fully differentiated osteoblasts. Exogenous NO released by osteoblasts and osteocytes prevents osteoclast attachment and resorption. However, NO generated by osteoclasts has dual effects on osteoclasts, depending on constitutive or inducible NOS activity.

Addressing skeletal biology challenges

Beyond the translational implications of NO-targeted therapeutics, the work from Jin et al. (10) underlies two ongoing challenges in skeletal biology: (a) the difficulty in defining the relative magnitude of cell-autonomous versus non–cell-autonomous determinants of osteoblast differentiation, particularly in light of the strong dose dependency of secretory factors, such as NO (11); and (b) the trouble assessing the relative balance and type of substrate utilization during osteoblast maturation (12). With respect to the former, Jin et al. created an osteocalcin-Cre targeted deletion of *Asl* that showed very low trabecular bone mass and markedly impaired bone formation but preserved cortical thickness (10). In contrast, the human cross-sectional study, despite including individuals with a homozygous *ASL* deficiency, revealed that spine bone mineral density was low in only a few individuals (10). Interestingly, those modest skeletal phenotypes actually are consistent with the generally negative outcomes from clinical nitrate trials for the prevention of bone loss (6, 7). But these data might also suggest that NO has non–cell-autonomous effects on other tissues that can regulate bone remodeling, thereby balancing potentially adverse cell-autonomous effects from the ASL-deficient state. For example, NO can impact the CNS to drive sympathetic tone or target other cell types within the marrow niche, complicating interpretation of the skeletal phenotype (ref. 13 and Figure 1). In that

same vein, NO can have dual effects on osteoclasts, depending on constitutive or inducible NOS activity (14). IFN-γ induction of NOS by cells in the marrow leads to suppression of osteoclastogenesis, whereas constitutive NOS activity stimulates bone resorption (15). Furthermore, granulocyte colony–stimulating factor, in the hematopoietic niche, induces neutrophils to produce NO, which in turn can inhibit osteoblast differentiation (16). Hence, to better appreciate the full actions of NO in the bone marrow niche, approaches such as three-dimensional organoids, better ex vivo imaging techniques, and specialized lineage tracing methods will be required.

The second challenge to fully understanding bone formation lies in the choice of substrate utilization by the differentiating osteoblast. In general, fuel sources available to stromal cells as they progress down the osteogenic pathway include glucose, glutamate, and fatty acids. Seminal work from the Long group demonstrated that aerobic glycolysis is the principal source of energy (i.e., 80% of the ATP is generated by glycolysis) for fully differentiated osteoblasts (17). There is debate about the preeminent role of glutamate, a precursor component for the Krebs cycle, as a fuel source in normal osteoblasts, but less uncertainty exists about the preference for mitochondrial respiration over glycolysis in preosteoblasts and stromal cells. The work from Jin et al. showed that glycolysis was suppressed in the $Asl^{Neo/Neo}$ differentiating osteoblasts, and this suppression was rescued either by adding back SNAP (an NO donor) or by derepressing NOS inhibition by CAV1, using the heterozygous $Cav1^{+/-}$ mouse crossed to the $Asl^{Neo/Neo}$ hypomorph (10). Indeed, these data fit the current paradigm that normal differentiating osteoblasts, whether stimulated by parathyroid hormone or Wnts, upregulate glycolysis and suppress mitochondrial respiration (18). In fact, even though only two ATP molecules are produced for every glucose entering the system, compared with 36 ATP molecules/glucose from fatty acid oxidation, the speed of glucose utilization and the employment of the malate-aspartate shuttle from pyruvate to prolong glycolysis while reducing mitochondrial oxidation serves to enhance the overall efficiency of the working osteoblast (19). However, osteoblast bioenergetics is temporally specific and depends on its workload (e.g., collagen formation), and as such preosteoblasts primarily rely on oxidative phosphorylation for their energy during proliferation (20). In vitro, a switch to glycolysis occurs sometime between three and four days during osteogenic differentiation, although it is unclear whether that timing is the same in vivo and what signal causes that switch (20). Therefore, it will be important to understand both what happens early in the *Asl*-deficient preosteoblasts and the timing of the switch to glycolysis, to determine whether the reduction in osteoblast number seen in vivo is due to an energy deficit in early oxidative phosphorylation or impaired glycolysis due to greater-than-expected glucose dependency. Those studies might provide further insight into the disposition of carbon skeletons in the absence of NO and could illuminate drug targets beyond NO donors.

In sum, Jin et al. have reopened the exploration of NO in bone biology using a unique translational approach involving a rare mutation in a gene generally associated with urea disposition and hepatic insufficiency (10). The importance of NO in regulating glycolysis during terminal osteoblast differentiation provides opportunities to understand a former "molecule of the year" in the context of bone remodeling. Yes, the phoenix may indeed rise from the ashes of earlier studies, and its presence may lead to exciting translational innovations.

Acknowledgments

This work was supported by the Northern New England Clinical and Translational Research Network (NIH grant U54GM115516).

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