Bone health is an important medical concern in rapidly aging demographics worldwide. Excessive bone resorption, due to enhanced activity of osteoclasts, is a major underlying cause of bone disorders such as osteoporosis. Inflammation and oxidative stress are key factors contributing to increased osteoclastic activity. Like increased activity of osteoclasts, depletion of osteoblasts also contributes to weakened structural integrity of bone. Considering the epidemiology of bone disorders and aging demographics there is a substantial need for novel bone health therapeutics. IRW (Ile-Arg-Trp), an egg-derived tripeptide, exhibits a spectrum of pharmacological activity. In our recent work, we have shown that IRW inhibits osteoclastogenesis and promotes osteogenesis in the mouse macrophage RAW 264.7 and MC3T3-E1 cells. IRW treatment (25 and 50 μM) significantly inhibited osteoclastogenesis-associated factors [TRAF6 (TNF Receptor Associated Factor 6), Fos Proto-Oncogene (c-Fos), Nuclear Factor of Activated T Cells 1 (NFATc1), and cathepsin K] and upregulated osteogenesis-associated factors [RUNX2 (Runt-related transcription factor 2) and RANKL (Receptor activator of nuclear factor kappa-B ligand)] in the two cell lines. Currently, we are conducting studies to analyze the impact of IRW on Angiotensin II (Ang II)-induced stress in vitro and in vivo. In summary, our recent work presents the ability of IRW to prevent LPS-induced inflammatory bone resorption and activation of osteogenesis activity via multiple signaling pathways.

**Key Words**  bone health, IRW, osteoporosis, bioactive peptides, inflammation

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**Summary** Bone is a mineralized connective tissue that serves important mechanical and metabolic functions, such as supporting body structure, providing locomotion, protecting the internal organs, storing minerals as a reservoir to maintain mineral homeostasis, and functioning as an endocrine organ to regulate energy expenditure (1). Thus, bone health is critically important to the overall health and quality of life. Therefore, any imbalance between the two major bone homeostasis activities, i.e., bone formation and bone resorption can cause bone disorders (2). Osteoblasts differentiate from mesenchymal progenitor cells through factors such as RUNX2 activation leading to their proliferation and differentiation (3). During osteoblast differentiation, RUNX2 expression is crucial to driving the transition of osteoprogenitor to mature osteoblasts (4). Osteoclasts come from monocyte/macrophage lineage, which undergoes osteoclastogenesis to form specialized bone-resorption cells (5). Molecular factors such as the tumor necrosis factor α (TNFα) and related cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) play an essential role in the differentiation and activation of osteoclasts. Moreover, the synergistic action of the immune system also plays a vital role in osteoclastogenesis (6). In bone disorders such as rheumatoid arthritis and periodontitis, inflammatory stress occurs adjacent to bone resorption (7). Considering the aging demographics and rise in bone disorders there is an urgent need for novel therapeutics and nutraceuticals to meet the challenge. Interestingly, research from our lab has shown that bioactive tripeptide IRW (Ile-Arg-Trp), derived from ovotransferrin, displays osteoblastic activity, and inhibits LPS-induced osteoclast formation and bone resorption (8, 9). Our results revealed the positive effects of tripeptide IRW on regulating osteogenesis and collagen synthesis, indicating its potential for the prevention or treatment of osteoporosis (9).

**KEY FINDINGS**

In our first study (9), we presented findings on the ability of IRW to increase osteogenesis via activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/RUNX2 pathway along with an increase in the collagen synthesis pathway. As inflammation and oxidative stress play an underlying role in bone resorption (10) their inhibition by IRW presents first and vital evidence in the preservation of bone health (9). Our first study showed that IRW treatment (50 and 25 μM) for 24 h decreased the expression of interferon-gamma (IFNγ) and increased the expression of the antioxidant enzyme catalase in MC3T3-E1 cells (7, 9). Further, IRW treatment (50 and 25 μM) in MC3T3-E1 cells for 24 h increases osteoblast cells, indicating proliferation of osteoblasts. This was further supported by flow cytometry experiments which confirmed the increase of osteoblast cells in the S phase of the cell cycle after IRW treat-
ment (50 and 25 μM) for 24 h (9, 11). An increase in osteoblast cells and S-phase osteoblasts was confirmed by mineralization assay. Our experiments showed that IRW treatment (50 and 25 μM) over the period of 20 d improved the absorbance of Alizarin S red and significantly increased the mineralized nodes (>2-fold), indicating the formation of new bone cells in vitro (9). Following confirmation of the osteogenesis, we explored the underlying pathways contributing to this increase in anabolism. Research evidence shows that RUNX2, a critical activator of osteoblast differentiation, is activated via the Akt pathway (12). Thus, we investigated the phosphorylation of Akt and the status of RUNX2 expression following IRW treatment (50 and 25 μM). Our results showed that IRW treatment significantly increased phosphorylation of Akt (Ser473) and the PI3K pathway in MC3T3-E1 cells. In line with previous findings (12), the phosphorylation of Akt/PI3K was accompanied by an increase in RUNX2 protein expression as well (9). Next, we further confirmed the increase in downstream efforts of the RUNX2 pathway. Our experiments showed cell differentiation biomarkers such as alkaline phosphatase (ALP) and type I collagen alpha 2 (Col1A2) in MC3T3-E1 cells (9, 13). The upregulation of these critical factors, RUNX2 and Col1A2, by IRW was confirmed using inhibitors of their pathways as well. Collectively, our first study supported our assertion that the PI3K-Akt-RUNX2 activation by IRW stimulates the differentiation of osteoblasts in cell model studies. Another vital finding of our first study was a decrease in the expression of RANKL and an increase in the expression of osteoprotegerin (OPG), both vital factors regulating osteoclastogenesis or bone resorption. These findings paved the way for our next study exploring the ability of IRW to inhibit osteoclast formation and bone resorption using the in vitro murine monocyte cell line RAW 264.7. Using LPS-induced stress, the RAW 264.7 cells were converted to mature osteoclasts and their morphology was confirmed by TRAP (tartrate-resistant acid phosphatase) staining. The tripeptide IRW (50 and 25 μM) inhibited the LPS-induced bone resorption as indicated by a decrease in resorption pits (9). Pretreatment of osteoclasts with IRW (50 and 25 μM) led to a decline in IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and TNFα levels, the vital factors in bone inflammation and resorption (7, 8). Further, we observed depletion in nitric oxide and PGE2 (prostaglandin E2) levels as well (8). Like our first study, we then elucidated the pathways underlying the mechanism of IRW’s anti-resorptive activity. Western blot analysis showed a decline in the ratio of inflammatory osteoclast markers -NF-κB p65 (Ser 536)/NF-κB p65 and p-ERK1/2 (Thr 202/Tyr 204)/ERK1/2 along with TRAF6, Nfatc1, c-Fos, and cathepsin K. These results confirmed the ability of IRW to inhibit bone resorption and enhance bone formation (8, 9).

DISCUSSION

In these two studies, we successfully demonstrated that IRW treatment (1) enhances osteoblast proliferation via activation of PI3K-Akt stimulation and RUNX2 increase along with downstream effectors and (2) decreases LPS-induced bone resorption and the expression of inflammatory markers such as TNF-α, IL-6, iNOS, and COX2 along with their downstream targets, NO and PGE2. Moreover, use of LPS-induced osteoclast formation in RAW 264.7 cells is independent of the treatment of RANKL, indicating the use of an efficient cell model mimicking osteoporosis (14). These studies confirm the dual pharmacological activity of IRW as a stimulator of osteogenesis and inhibitor of osteoclastogenesis. These findings illuminate an encouraging role for bioactive tripeptide IRW in the prevention and/or potential treatment of osteoporosis, as bone remodeling is the most crucial process for bone health management, which is primarily mediated by homeostasis between osteoblasts and osteoclasts (2). IRW also showed potential to prevent the osteoblast-produced bone resorptive RANKL, suggesting its role in regulating the crosstalk between osteoblasts and osteoclasts. Another strong aspect of these studies is the choice of multiple factors, especially in the anti-resorptive study including NFκB and mitogen-activated protein kinase (MAPK; ERK, JNK, and p38) pathways (8). Therefore, the meticulous management of this homeostasis by IRW is a novel and encouraging research finding.

To our best knowledge, these studies are the first to indicate the bioactivity of food-derived peptide(s) towards RUNX2 with minimal off-target effects. Previous studies showing the osteogenic activity in osteoblasts by RANKL-binding peptides WP9QY and OP3–4 support our research findings (15). Thus, dietary intake of IRW may increase the endogenous production of collagen, improving bone mineralization in vivo as well. Further, like IRW’s anti-resorptive activity, many clini-
cally used drugs such as bisphosphonates, estrogen, and calcitonin, target the osteoclastic bone resorption in osteoporosis. However, the dual osteogenic and anti-resorptive ability of IR W is exclusive (Fig. 1). Even more importantly, the use of IR W as a multi-target anti-inflammatory agent may be particularly useful in rheumatoid arthritis and periodontitis. Further, as a novel angiotensin-converting enzyme 2 (ACE2) activator, the use of IR W is supported in metabolically critical bone health. A strong body of literature supports the role of the overactive renin angiotensin system (RAS) pathway in the development of osteoporosis (16). Activation ACE2 can be employed to reduce the levels of angiotensin II (AngII), a vital stress factor contributing to bone disorders (17). We are currently conducting studies to assess the impact of IR W against AngII stress in MC3T3-E1 cells and ovariectomized (OVX) rats. Some of the key findings from IRW-AngII work show the ability of IRW to (1) promote osteoblastic activity and (2) exhibit anti-apoptosis against Ang II stimulation, (16) exhibit anti-inflammatory activity of IR W to (1) promote osteoblastic activity and pre-osteoblastogenesis. These findings also open the future possibilities to explore IRW both for the prevention or treatment of osteoporosis in humans.

**Disclosure of state of COI**

No conflicts of interest to be declared.

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