Biochemical and Biophysical Research Communications 394 (2010) 6–11

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Further evidence for direct pro-resorptive actions of FSH

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ARTICLE INFO

Article history:
Received 12 February 2010
Available online 19 February 2010

Keywords:
Osteoporosis
Osteoclast
FSH
Gonadotropin
Bone loss

ABSTRACT

We confirm that FSH stimulates osteoclast formation, function and survival to enhance bone resorption. It does so *via* the activation of a pertussis toxin-sensitive G_i-coupled FSH receptor that we and others have identified on murine and human osteoclast precursors and mature osteoclasts. FSH additionally enhances the production of several osteoclastogenic cytokines, importantly TNFα, likely within the bone marrow microenvironment, to augment its pro-resorptive action. FSH levels in humans rise before estrogen falls, and this hormonal change coincides with the most rapid rates of bone loss. On the basis of accumulating evidence, we reaffirm that FSH contributes to the rapid peri-menopausal and early post-menopausal bone loss, which might thus be amenable to FSH blockade.

1. Introduction

Since Fuller Albright first discovered the action of estrogen on bone [1], it has become *dictum* that hypogonadal osteoporosis is caused solely to low circulating estrogen levels. We recently challenged this view, and suggested that high FSH levels, which normally accompany ovarian failure, offer a contribution [2]. We found that mice devoid of either FSH or its receptor, FSHR, failed to display hyper-resorption, despite severe hypogonadism [2]. This was associated with dysfunctional osteoclasts observed both *in vivo* upon histomorphometry, and *ex vivo*, in isolated osteoclast cultures [2]. While the absence of hyper-resorption in chronic FSHR deficiency can be attributed, in part, due to the accompanying hyperandrogenemia [3], this does not exclude the possibility that high FSH levels *per se* contribute to hypogonadal bone loss. This view is buttressed by estrogen-independent correlations between peri-menopausal bone loss and rising FSH levels noted over 4 years [4], and from observations that women with high FSH levels suffer greater bone loss than those with low-to-normal FSH levels, estrogen levels being the same [5].

Consistent with our hypothesis that FSH does directly cause bone loss *in vivo*, we have shown that FSH stimulates the formation and resorptive activity of osteoclasts *in vitro* [2]. This action, we found, is exerted *via* FSHRs coupled to $G_{i2\alpha}$ that appears upstream of the pro-osteoclastogenic MAP kinase and NF-κB pathways, as well as through the enhanced production of tumor necrosis factor-α (TNFα), which expands the osteoclast precursor pool [2,6]. The current study provides further, more compelling, evidence for a direct action of FSH on both murine and human osteoclasts, as has been examined in terms of effects on their genesis, function, and fate. The accompanying manuscript studies in depth the effects of FSH on TNFα expression.

2. Methods

For the osteoclastogenesis experiments, murine bone marrow cells were extracted from femurs and tibias and washed and cultured in α-MEM with FBS (10%), M-CSF (30 ng/ml) and penicillin/streptomycin (1%) for 24 h. Non-adherent hematopoetic stem cell precursors were removed for purification with Ficoll-Paque Plus (Amersham Biotech). The interface cell layer was isolated for culture in α-MEM with FBS (10%), M-CSF (50 ng/ml), and RANK-L (60 ng/ml) at 5×10^4 cells/well in 96-well plates. A tartrate-resistant acid phosphatase (TRAP) kit (Sigma–Aldrich) was used to stain osteoclasts for counting. RAW264.7 and RAW-C3 cells were also cultured with RANK-L (30 ng/ml) for 5–8 days to form TRAP-positive osteoclasts.

For osteoclast survival experiments, RAW264.7 cells were cultured at 5×10^5/6 cm dish for 24 h in α-MEM ad FBS (10%). Cells were treated with camptothesin (0.1 μM) with and without FSH or M-CSF for 24 h, and live cells detected by trypan blue uptake. In separate experiments, apoptotic cells were detected by flow cytometry (FACS Calibur) using Annexin V FITC Apoptosis Detection Kit *per* manufacturer's directions (BD Biosciences, San Diego, CA), and results analyzed with FlowJo software (Treestar, Inc.) as described previously [20].

Whole cell lysates and/or nuclear sub-fractions were obtained as described previously with modifications [25]. Western blotting

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doi:10.1016/j.bbrc.2010.02.113

was performed using enhanced chemiluminescence detection (Active Motif, or Amersham, Piscataway, NJ). Antibodies for c-*fos*, c-*jun* were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and antibodies against phospho-Erk1/2, phospho-JNK, and phospho-Akt were from Cell Signaling Technologies (Beverley, MA). In separate experiments cAMP levels were measured in RAW264.7 cell extracts exposed to FSH and/or pertussis toxin using a method described previously [2].

CD14 monocytes were isolated as described [2] plated at 3.75×10^6 cells/cm^2 in human M-CSF (25 ng/ml), human RANK-L (30 ng/ml) (Research Diagnostics, Flanders, NJ), vitamin D_3 (10^{-8} M) (Sigma), dexamethasone (2×10^{-8} M) (Sigma), heat inactivated FBS (10%) (HyClone) in DMEM (Cambrex, East Rutherford, NH). Cultures were maintained for 7 days and fixed in citrate/acetate buffer, pH 4.5, with acetone (40%). TRAP was determined using napthol AS-BI phosphate (12.5 mg/ml) in the presence of tartaric acid (0.67 mM) with fast garnet GBC to produce red precipitate. For pit forming activity, CD14 monocytes were plated on dentine slices for 15 days in the presence of human M-CSF (25 ng/ml) and human RANK-L (30 ng/ml). The slices were then sonicated (ammonium hydroxide, 1 M, 2 min) and stained with toluidine blue (0.5%), pH 7.4, for 2 min. Phalloidin labeling and acid secretion assays were performed as described [26].

3. Results

Confirming our previous results [2] with new cell preparations and reagents, we show that graded concentrations of FSH stimulate, between 50% and 2-fold, the genesis of TRAP-positive osteoclasts from cloned RAW264.7 and RAW-C3 cells, as well as murine bone marrow osteoclast precursors (Fig. 1A–C). Fig. 1D further illustrates that pertussis toxin, which eliminates receptor-G_i coupling, prevents the increase in TRAP-positive osteoclast formation from RAW264.7 cells, consistent with data using $G_{i2\alpha}$-deficient murine osteoclast precursors [2]. Additionally, the expected decrements in cAMP with FSH due to $G_{i2\alpha}$ coupling noted in murine osteoclast precursors [2] were also fully reversed by pertussis toxin (Fig. 1E). Overall, therefore, the findings provide further, more definitive evidence using cloned RAW cells that the FSH-induced osteoclastogenesis arises through a pertussis-toxin-sensitive G_i protein.

We next utilized RAW264.7 cells to explore, in considerable depth, the effect of FSH on two signaling molecules, the MAP kinase Erk and Akt kinase, which we have previously shown are activated in murine osteoclast precursors [2]. Western immunoblotting of RAW264.7 cell extracts displayed increased phosphorylation of Akt at 60 min (Fig. 2Aa), in stark contrast to that seen at 10 min in primary cells [2]. However, this increase was impressively inhibited by pertussis toxin (Fig. 2Ab) indicating that the effect of FSH on Akt was also downstream of the pertussis toxin-sensitive G_i. Phosphorylation of Erk1/2 was also enhanced at 30 min (Fig. 2Ba), again in contrast to a rapid effect noted in primary cells [2]. The effect of FSH was, however, less profound than that due to RANK-L, and no effects of FSH on JNK phosphorylation were observed in RAW264.7 cells (Fig. 2Ba). Importantly, however, the effect of maximal (50 ng/ml) and sub-maximal (12.5 ng/ml) concentrations of RANK-L were additive to those of FSH (30 ng/ml), suggesting common mechanisms for downstream activation. This was confirmed by examining the nuclear localization of c-fos in response to FSH and RANK-L. FSH stimulated c-fos localization in RAW264.7 cells, as before with murine cells [2], but with a more protracted time course, peaking at 120 min (Fig. 2Ca). The effects of sub-maximal (15 and 30 ng/ml) and maximal (60 and 60 ng/ml) of RANK-L and FSH, respectively, were again additive (Fig. 2Cb).

That a G_i protein was upstream of FSH-induced c-fos nuclear localization was confirmed using pertussis toxin. The effect of FSH in inducing nuclear localization of c-fos was strongly attenuated in the presence of pertussis toxin (Fig. 2Cc). Notably,

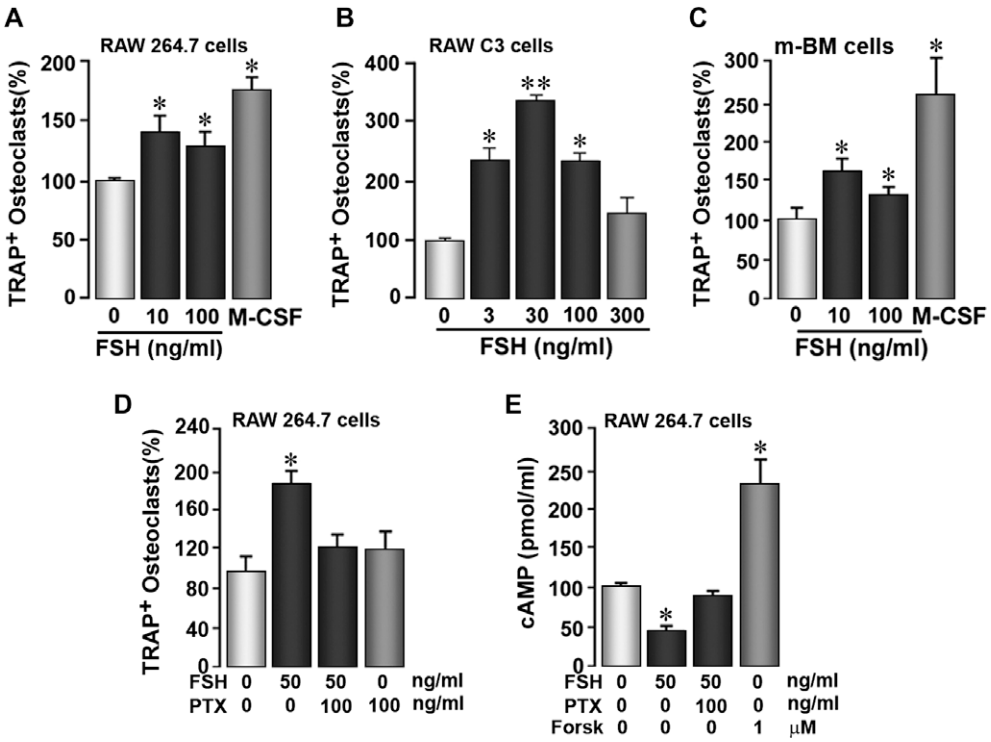

Fig. 1. The effect of FSH on tartrate-resistant acid phosphatase-positive (TRAP$^+$) osteoclast formation from, and cyclic AMP (cAMP) levels in, RAW264.7 or RAW-C3 cells, or primary mouse bone marrow (m-BM) cells, as indicated, in the presence or absence of pertussis toxin (PTX). Abbrev: For – forskolin. Statistics by Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, 3–8 wells/treatment for TRAP$^+$ osteoclast formation assays and $n = 5$ wells/treatment for the cAMP assay.

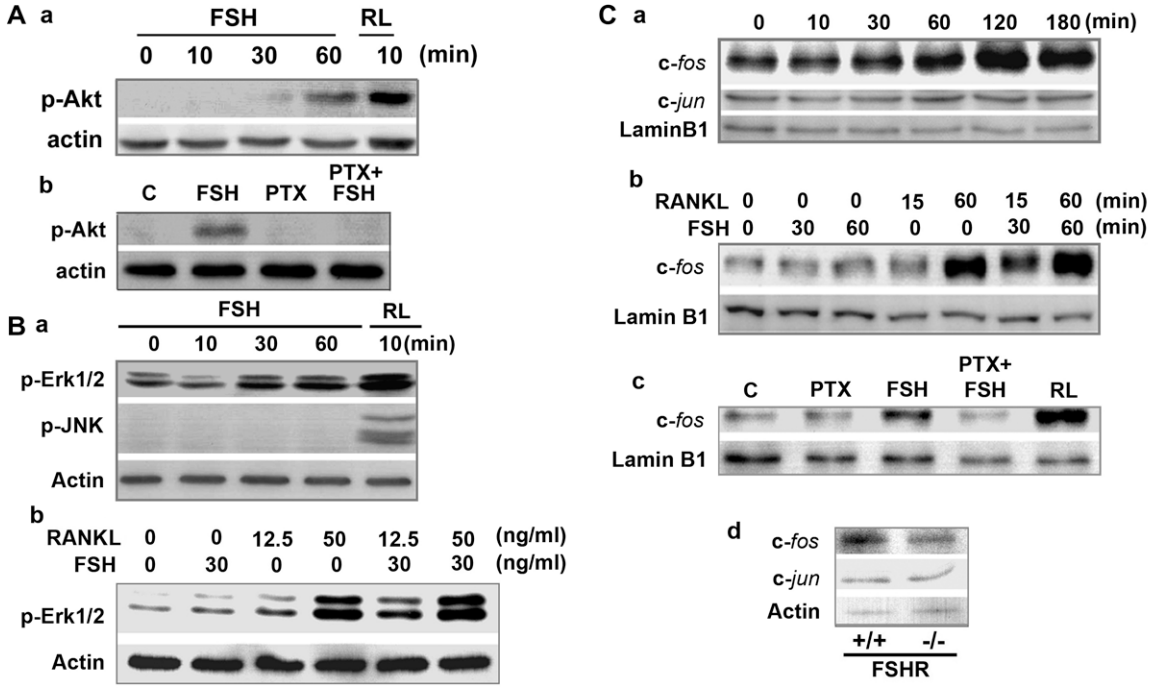

Fig. 2. Western immunoblots showing the effect of FSH and/or RANK-L on the phosphorylation of Akt (p-Akt), Erk (p-Erk) or JNK (p-JNK) measured in total cell lysates (panels: A,B) (loading control: actin), or on c-fos and c-jun measured in nuclear extracts (loading control: lamin B1) in RAW264.7 cells (panels: Ca,Cc) in the presence or absence of pertussis toxin (PTX). Western immunoblots for c-fos and c-jun in cells isolated from bone marrow precursors derived from FSH receptor null mice ($FSHR^{-/-}$) and wild type littermates ($FSHR^{+/+}$) (panel: Cd).

consistent with a lack of effects of FSH on JNK activation (Fig. 2Ba), the nuclear localization of c-jun remained unaltered up to 180 min following exposure to FSH (Fig. 2Ca). Likewise, c-jun levels remained unaffected in $FSHR^{-/-}$ osteoclast precursors, whereas wild type cells displayed lower c-fos levels (Fig. 2Cd). The latter observation attests to the direct regulation of c-fos nuclear localization by FSH.

In addition to stimulating osteoclast formation by activating the aforementioned pathways, we also confirmed and extended previous results on the direct action of FSH on the resorptive function of mature human osteoclasts [2]. In the pit assay, where resorptive lacunae positive for toluidine blue are counted, FSH (30 ng/ml) stimulated resorption (Fig. 3A). This was consistent with a substantial change in the phalloidin-labeled actin rings in osteoclasts on plastic coverslips (Fig. 3B), suggesting that FSH enhances osteoclast activation. Typically, osteoclasts on artificial matrix show punctate attachments (Fig. 3B, top frame), while on bone a complete attachment ring occurs; with FSH enhancement, the cells on plastic show this resorptive ring (Fig. 3B, bottom frame). Accompanied with this, there was a concentration-dependent increase in lysotracker-positive acid lakes under osteoclast surfaces, indicative of increased acid secretion (Fig. 3Ca). When analysed by image intensity comparison in a second experiment (Fig. 3Cb), where GnRH was used as an additional control, considerable variation in lysotracker activity is apparent, although 20–100 cells per group were analysed and the differences without and with FSH were significant. Overall, therefore, FSH stimulated bone resorption by mature osteoclasts by increasing substrate adhesion and extracellular acid secretion.

Finally, we studied the effect of FSH on the fate of RAW264.7 cells and osteoclasts by examining cell number and annexin V staining after treatment with a pro-apoptotic agent, camptothesin. There was a doubling of cell numbers with FSH within 24 h in the presence of camptothesin, although not as dramatic as with M-CSF (Fig. 4A). This was in agreement with parallel flow cytometry studies, where we found a concentration-dependent decrease in annexin-positive cells (Fig. 4B). To differentiate dead cells from cells undergoing early apoptosis, cells were double-labeled with annexin V and propidium iodide, a nuclear stain that labels dead cells. There was a striking decline in annexin V-high, propidium iodide-low cells, concordant with a pro-survival action of FSH (Fig. 4C). Overall, therefore, our studies provide more persuasive evidence for the stimulation of osteoclastogenesis and resorptive activity, and the inhibition of apoptosis by FSH.

4. Discussion

The question we ask is whether FSH has a role in hypogonadal, and in humans, peri- and post-menopausal bone loss, which has *hitherto* been attributed solely to declining estrogen levels. And, if so, what is the mechanism through which these effects are exerted? In our earlier study, we showed that FSH is potently osteoclastogenic and pro-resorptive *in vitro* and that the absence of FSH or its receptor protects against hypogonadal bone loss [2]. While the *in vitro* effects of FSH have since been replicated in other laboratories [7,8], it was shown subsequently that part of the osteoprotection in chronic genetic FSHR deficiency arises from the accompanying hyperandrogenemia due to preserved luteal function [3]. However, the clinical paradox still remains: the fundamental observation that women lose bone dramatically and most rapidly during late peri-menopausal and early post-menopausal years [4,9,10]. This precipitous bone loss [11,12] cannot possibly be explained by hypoestrogenemia, particularly because, during this time, estrogen levels are relatively normal, while early follicular FSH levels have risen to over 5-fold of perimenopausal values [13]. Indeed, a change in serum FSH correlates strongly with a change in bone mineral density [4] and women that fall within the highest tertile of serum FSH levels have highest bone resorption markers [14,15]. Although correlative, these clinical

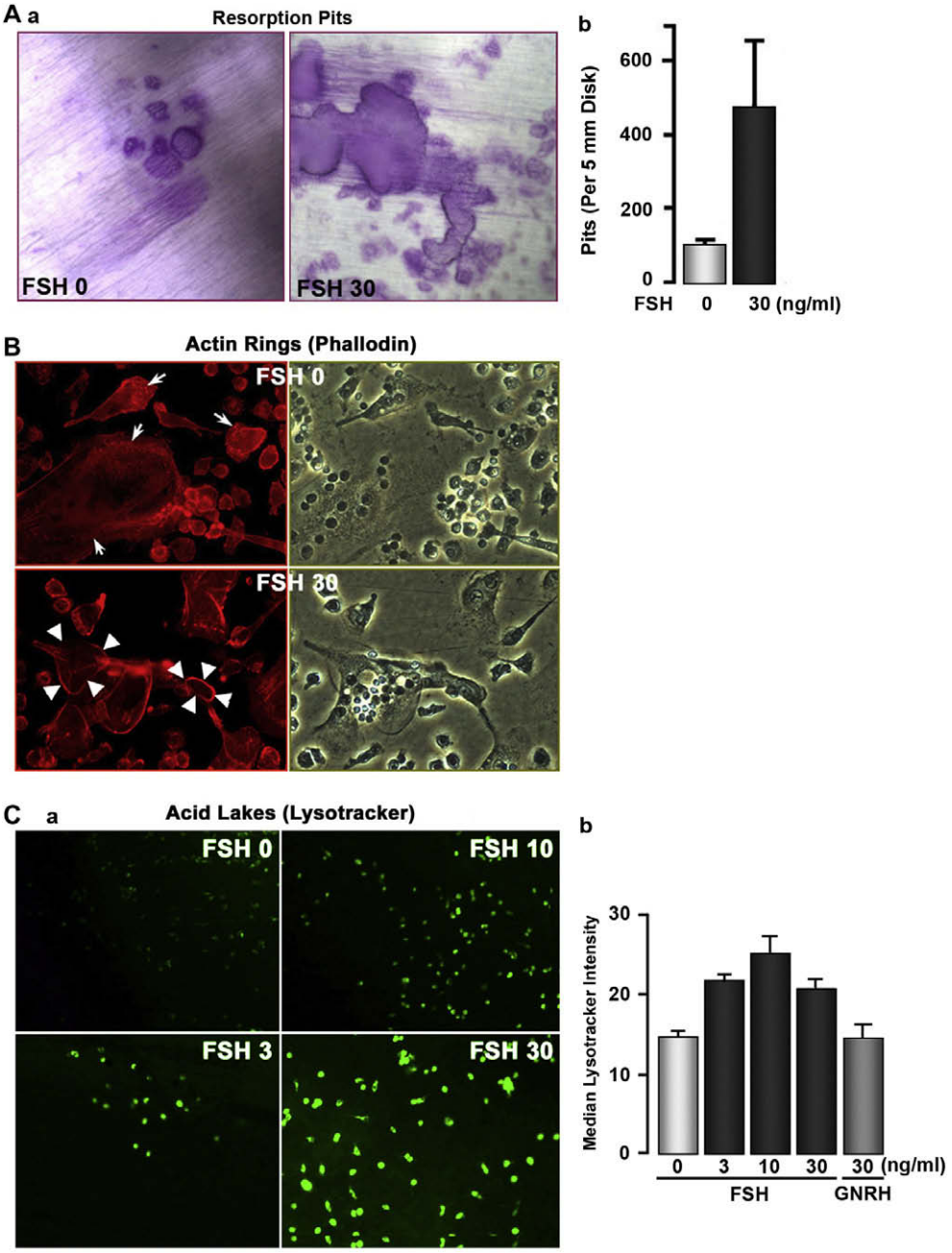

Fig. 3. Response of human peripheral blood mononuclear cell-derived osteoclasts to FSH. (A) Resorption of devitalized dentine substrate (Aa) as photomicrographs and expressed as pits per slice (Ab); this response was variable over several experiments and on average an increase of ~30–50% was seen, although striking increases in activity, as in this case, were not unusual. Photomicrographs are 300 μm^2. (B) Osteoclasts on tissue culture plastic with phalloidin-Cy5 to label actin (red fluorescence, left frames) and in phase (right frames), without (top frames) or with (bottom frames) human FSH (30 ng/ml) added. Note that without added FSH, actin attachments are punctate (associated with podosomes), while after FSH, a continuous actin ring is present, similar to the tight attachment on bone. Fields are 200 μm across. One of triplicate cell cultures showing similar changes is illustrated. (C) Lysotracker labeling of human monocyte cultures on bone 7 days after addition of M-CSF (10 ng/ml) and RANK-L (40 ng/ml) and indicated concentrations of human FSH in ng/ml. Note in the photomicrographs in (a) the trend toward increased labeling of acid compartments with exposure to FSH. Fields are 580 nm across. In a second experiment, labeling was quantified as fluorescence intensity for 20–100 cells of each culture type (b). There was considerable variability, with overlap between individual cells with each type of treatment, but the average increase in acid secretion with FSH addition is clear. An additional control, GnRH addition, is shown (right bar).

observations come not only from SWAN (Study of Women's Health across the Nations) [4,14,15], but also from a Chinese cohort [9], and a younger cohort of American women aged between 20 and 50 years [10]. Most impressive, however, is that the AA rs6166 *FSHR* genotype is associated with low femoral neck and total body bone mineral density, independently of circulating estrogen [16]. We, and more recently others, have shown that human osteoclasts bear FSHRs [2,10]. We also show that FSH stimulates the formation of human osteoclasts, as well as their resorptive function. These *in vitro* and human studies together provide a compelling rationale for further investigations on a role for FSH in the pathophysiology of post-menopausal bone loss.

Demonstrating the attenuation of ovariectomy-induced bone loss by inhibiting the rising FSH will constitute ultimate proof for causality. Our current studies nonetheless provide further affirmative evidence for its mechanism of action in both human

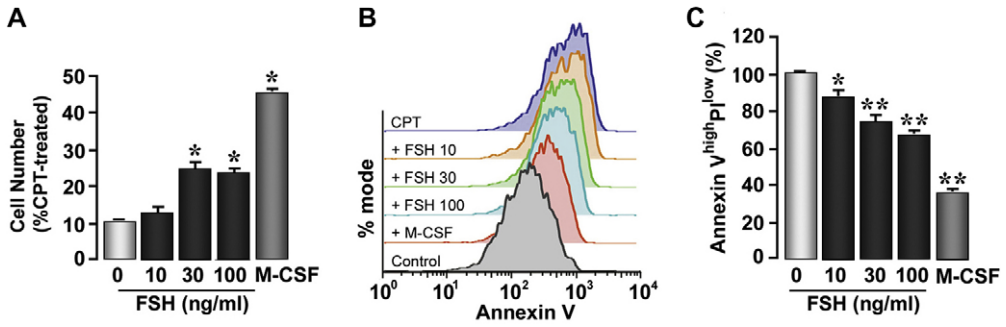

Fig. 4. The pro-survival actions of FSH on RAW264.7 cells and primary murine bone marrow cells. Cell number (A) and annexin V staining (B, C) was assessed after treatment with a pro-apoptotic agent, camptothesin (CPT). Positive control: M-CSF. Propidium iodine labeling was used to differentiate dead cells from cells undergoing early apoptosis; flow cytometry allowed the quantitation of annexin V-high, propidium iodide-low cells (C). Statistics by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, (A) 3 experiments pooled; (C) $n = 4$ wells/treatment.

osteoclasts and RAW264.7 cells. Namely, FSH has both direct and indirect actions to increase resorption. Using RAW264.7 cells, we show that FSH stimulates osteoclastogenesis, proven previously by us [2] and others [7,8] using murine and human osteoclasts. It does this primarily by activating Erk phosphorylation *via* a $G_{i2\alpha}$ protein [2], which we demonstrate here as classically pertussis toxin-sensitive. It also enhances osteoclast activation, acid secretion and overall resorptive activity. Additionally, we show, for the first time, that FSH stimulates osteoclast survival, even in the presence of a potent pro-apoptotic stimulus, camptothesin. Together, we find that the exposure of osteoclast precursors to FSH results in more osteoclasts being formed, each with enhanced function and reduced propensity to undergo apoptosis.

In addition to the direct effects of FSH on osteoclast formation, the hormone also stimulates the production of several osteoclastogenic cytokines, namely TNFα, IL-1β, and IL-6 [6,10]. The accompanying manuscript demonstrating clear effects of FSH on TNFα production from human osteoclasts [17] confirms previous mouse studies [2]. We know that a key function of TNFα is to expand the osteoclast precursor pool [6], thus amplifying overall osteoclast formation. Nonetheless, whether the elevated peripheral blood mononuclear cell TNFα reported during human menopause [18] contributes to the increased osteoclastogenesis remains to be determined. A recent report however shows striking correlations between FSH and TNFα, IL-1β and IL-6 in serum as well as, between monocytic FSHR expression and serum cytokine levels [10]. FSH also stimulated cytokine production from monocyte precursors, confirming our results with both murine and human cells [6,17]. However, serum cytokine levels did not relate to bone mineral density, whereas serum FSH correlated strongly [10].

We have established previously that, in contrast to FSH, thyroid stimulating hormone (TSH) negatively regulates TNFα production through an effect on gene transcription [19]. Elevated TNFα levels in bone marrow supernatants and macrophages from the $TSHR^{-/-}$ mice [20], and the rescue of their enhanced osteoclastogenesis in compound mutants also lacking TNFα [21] together attest to a role for TNFα in synergizing the bone loss of experimental TSH signaling deficiency. However, whether TNFα and other inflammatory cytokines play a role in human osteoporosis has yet to be determined. That said, TNFα blockade is unlikely to become a putative target for either hypogonadal or thyrotoxic osteoporosis, as infliximab administration to patients with Crohn disease, where the osteoporosis is overtly TNFα-driven, fails to prevent bone loss [22]. In contrast, a recent study shows that exogenous FSH enhances periodontitis-induced alveolar osteolysis, as direct evidence for synergism between FSH and locally-released cytokines [23]. Finally, and perhaps most intriguingly, Pacifici and colleagues show that FSH up-regulates the CD40-ligand on antigen presenting cells, in essence attributing the T-cell-mediated, hypogonadal bone loss, in part, to high FSH levels [24]. In all, evidence for a role of FSH in bone loss continues to mount.

Acknowledgments

This work was supported by the National Institute on Aging (AG23176 to M.Z.). M.Z., L.S., and H.C.B. also acknowledge the National Institutes of Health for grant support (DK70526, DK80490, AR053976, AR055208, and AR053566. H.C.B. and J.I. also acknowledges the Department of Veterans Affairs and the American Federation for Aging Research, respectively. M.Z. is a named inventor of a pending patent application related to osteoclastic bone resorption filed by Mount Sinai School of Medicine. In the event the pending or issued patent is licensed, M.Z. will be entitled to a share of the proceeds.

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