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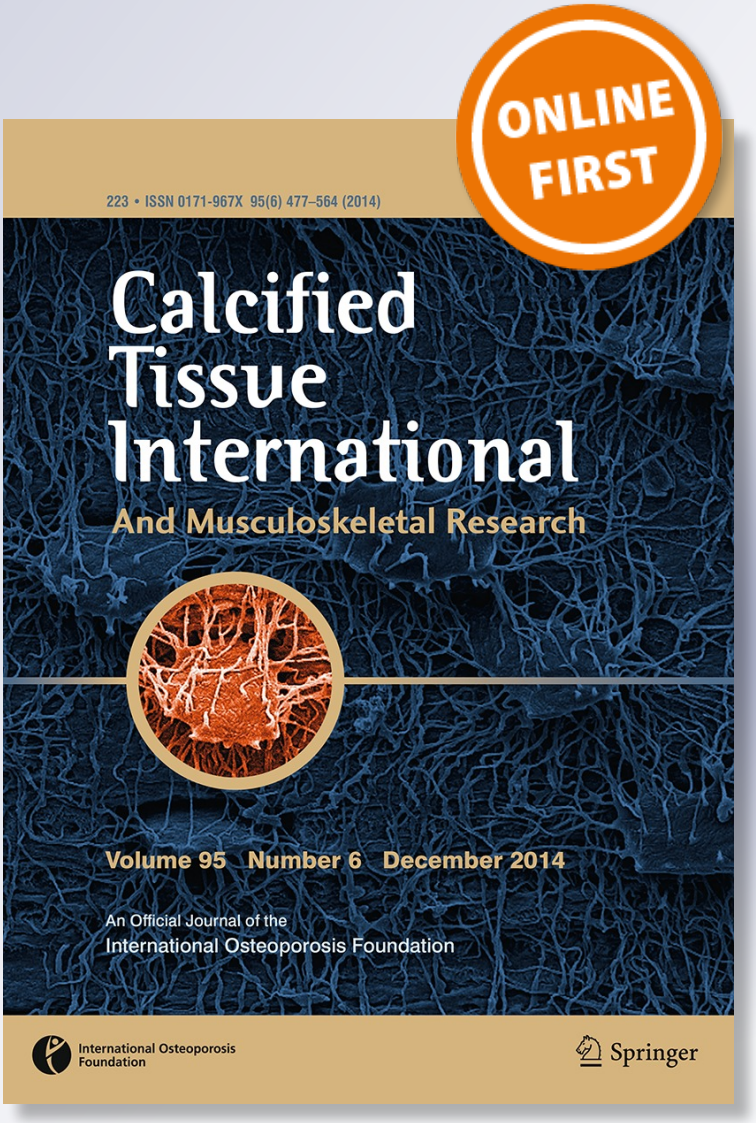

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ORIGINAL RESEARCH

Increased Endoplasmic Reticulum Stress in Mouse Osteocytes with Aging Alters Cox-2 Response to Mechanical Stimuli

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Abstract Aging reduces bone mass as well as the anabolic response of bone to mechanical stimuli, resulting in osteopenia. Endoplasmic reticulum (ER) stress impairs the response of myogenic cells to anabolic stimuli, and is involved in sarcopenia, but whether ER stress also contributes to osteopenia is unknown. Therefore, we tested whether ER stress exists in bones of aged mice, and whether this impairs the osteocyte response to mechanical stimulation. Primary osteocytes were obtained from long bones of adult (8 months) and old (24–26 months) mice, treated with or without the pharmacological ER stress inducer tunicamycin, and either or not subjected to mechanical loading by pulsating fluid flow (PFF). The osteocyte response to PFF was assessed by measuring cyclooxygenase-2 (Cox-2) mRNA levels and nitric oxide (NO) production. mRNA levels of ER stress markers were higher in old versus adult osteocytes (+40 % for activating transcription factor-4, +120 % for C/EBP homologous protein, and +120 % for spliced X-box binding protein-1, $p < 0.05$). The Cox-2 response to PFF was fourfold decreased in cells from old bones ($p < 0.001$), while tunicamycin decreased PFF-induced Cox-2 expression by threefold in cells from adult bones ($p < 0.01$). PFF increased NO production by 50 % at 60 min in osteocytes from old versus adult bones ($p < 0.01$). In conclusion, our data indicate that the expression of several ER stress markers was higher in osteocytes from bones of old compared to adult mice. Since ER stress altered the response of osteocytes to mechanical loading, it could be a novel factor contributing to osteopenia.

Keywords ER stress · Unfolded protein response · Nitric oxide · Cyclooxygenase-2 · Osteopenia · Tunicamycin · Pulsating fluid flow

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Introduction

Osteopenia and osteoporosis are aging-associated diseases characterized by failure to achieve peak bone mass, excessive bone resorption, and/or decreased bone formation during remodeling [18]. While peak bone mass is highly dependent on genetics factors [17], bone formation and bone resorption rely more on environmental factors [18]. Modulation of the latter may thus be used to reduce loss of bone mass. Therefore, the determination of the factors and mechanisms that alter bone formation and resorption is highly important. Mechanical loading is one of the most efficient tools to increase bone mass, since it stimulates bone anabolism and represses bone catabolism

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[5]. As a result of aging, bone cells respond less favorably to mechanical loading [5, 21], although contrasting results have been found as well [14, 15].

Endoplasmic reticulum (ER) stress seems a potential candidate in the search for uncovering the mechanisms that alter bone formation and resorption during aging. ER stress impairs the response to anabolic stimuli in myogenic cells by inhibiting the mammalian target of rapamycin (mTOR) pathway [9, 10]. It also has been proposed to play a role in the loss of muscle mass during aging [8]. The ER is a key organelle where folding and post-translational modifications of proteins occur. Certain stress conditions disrupt ER homeostasis and lead to the accumulation of misfolded proteins in the ER lumen. To cope with this stress, cells activate the so-called unfolded protein response (UPR) [19]. The function of the UPR is the restoration of normal ER function through, amongst others, increased expression of genes such as X-box binding protein-1 (XBP1), C/EBP homologous protein (CHOP), and activating transcription factor-4 (ATF4), which encode proteins that augment the ER protein-folding capacity. In many cell types, when UPR fails, this results in apoptosis [19].

Although exacerbated in old myogenic cells, neurones and hepatocytes [7], the presence of ER stress has never been assessed in aging bone tissue. Therefore, the aim of the present work was to test whether ER stress increases with aging in bone cells, and whether elevated ER stress contributes to an altered bone response of osteocytes to mechanical stimulation. For this purpose osteocytes were subjected to pulsating fluid flow resulting in fluid shear stress on the cells.

Materials and Methods

Animals Care and Cell Cultures

Briefly, long bones from C57BL/6 J male adult (8 months) and old mice (24–26 months, Janvier Labs, Saint-Berthevin, France) were isolated, and primary osteocyte cultures were grown as described previously [20]. These osteocyte cultures also contain cells with a more osteoblastic phenotype but as the latter cells respond similarly to mechanical stimuli as osteocytes, the terms 'osteocyte cultures' were chosen. Once confluent, cells were seeded on slides incubated in a Petri dish, and used for pulsating fluid flow (PFF) experiments. On the day before performing a PFF experiment, osteocytes were incubated for 17 h in serum-free medium supplemented with or without 1 μg/ml tunicamycin (TN, Sigma-Aldrich, Zwijndrecht, Netherlands) to induce ER stress [9]. Tunicamycin prevents the first committed step of *N*-linked glycosylation of proteins in the ER, which causes extensive protein misfolding and activation of the UPR [4]. Cell survival after TN treatment was quantified using the WST-1 assay kit (Roche, Almere, Netherlands).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from the cultured osteocytes using TRIzol® reagent (Invitrogen, Bleiswijk, Netherlands). cDNA was synthesized using 750 ng RNA, 0.08 A260 units of random primers (Roche), 1 mM of each dNTP (Invitrogen), and 1× Transcriptor RT reaction buffer (Roche) in a final volume of 20 μl. Real-time quantitative PCR reactions were performed using the LightCycler® SYBR Green reaction Mix based on the manufacturer's instructions (Roche). Mouse-specific primers for Cyclooxygenase-2 (Cox-2), ATF4, CHOP, and sXBP1 were designed (Table 1). Expression values were normalized to the housekeeping gene porphobilinogen deaminase (PBGD).

Pulsating Fluid Flow

A roller pump was employed to generate fluid shear stress (5 Hz, 0.7 ± 0.7 Pa) for 1 h through a parallel-plate flow chamber containing the glass slide seeded with primary osteocytes [1]. Control slides with osteocytes were kept static under similar conditions. One ml flow or static control medium was collected during PFF or static condition after 0, 5, 15, 30, and 60 min to quantify nitric oxide (NO) production using the Griess assay [12]. Cox-2 mRNA expression and NO production were determined as parameters of bone cell responsiveness since both are essential for the osteogenic response to mechanical loading in vivo [2, 3, 11].

Statistics

A one-way ANOVA (Systat software, San Jose, US) was used to compare basal mRNA expression levels of UPR

Table 1 Sequences of primers used for mRNA quantification by real-time RT-PCR

	Forward	Reverse
CHOP	CCT AGC TTG GCT GAC AGA GG	CTG CTC CTT CTC CTT CAT GC
ATF4	GAG CTT CCT GAA CAG CGA AGT G	TGG CCA CCT CCA GAT AGT CAT C
sXBP1	GAG TCC GCA GCA GGT G	GTG TCA GAG TCC ATG GGA
Cox-2	CCA GCA CTT CAC CCA TCA GTT	ACC CAG GTC CTC GCT TAT GA
PBGD	AGT GAT GAA AGA TGG GCA ACT	TCT GGA CCA TCT TCT TGC TGA

markers between osteocytes obtained from adult and old bones. A two-way ANOVA was used to compare basal mRNA levels of UPR markers as well as PFF-induced Cox-2 mRNA expression between osteocytes from adult and old bones with or without TN incubation. A three-way ANOVA was used to compare PFF-induced NO production in osteocytes from adult and old bones with or without TN incubation at different time points. The significant threshold was set at $p < 0.05$.

Results

Unfolded Protein Response Markers are Up-regulated in Osteocytes from Old Bones

In cultured osteocytes obtained from old bone compared to those from adult bone, mRNA levels of ATF4, CHOP, and sXBP1 were increased by 40, 120, and 100 %, respectively ($p < 0.05$, Fig. 1a). To determine the optimal concentration of TN needed to increase ER stress in bone cells, several concentrations of TN were tested on osteocytes for 17 h (Fig. 1b). mRNA levels of UPR markers increased in response to TN concentrations up to 1.5 μg/ml, with no further increases at higher concentrations. At all TN concentrations tested, cell survival was not altered (Fig. 1c). A TN concentration of 1 μg/ml was therefore chosen for the next experiments. TN increased ATF4 mRNA levels in osteocytes from old bone only (+50 %, $p < 0.01$, Fig. 1d) and CHOP and sXBP1 mRNA levels in osteocytes from adult and old bone (three to eightfold, $p < 0.001$, Figs. 1e–f). TN-induced ATF4 and CHOP mRNA levels were higher in osteocytes from old than adult bone ($p < 0.05$ and $p < 0.01$, respectively).

Altered Response to Pulsating Fluid Flow on Osteocytes from Old Bones

Bone anabolism after PFF was evaluated by measuring Cox-2 mRNA expression, since this is known to increase after PFF treatment [2], and plays an important role in bone formation [11]. PFF increased Cox-2 expression in osteocytes from adult and old bone independent of TN treatment (Fig. 2a). However, the increase was four times lower in osteocytes from old bone compared to those from adult bone ($p < 0.001$). TN reduced PFF-induced Cox-2 expression by threefold in osteocytes from adult bone ($p < 0.01$), but did not further decrease Cox-2 response to PFF in osteocytes from old bone. NO production by osteocytes was measured after PFF, since NO is essential for the anabolic response of bone to mechanical loading, and increased NO production reduces osteoclastic bone resorption in mice [16]. In all conditions, NO production increased over time ($p < 0.001$ at 60 min compared to 0, 10, 20, and 30 min; Fig. 2b). At 60 min, NO production was 50 % higher in osteocytes from old compared to adult bone ($p < 0.01$). TN had no effect on NO production.

Discussion

The primary result of the present study is that UPR markers are increased in cultured osteocytes from old compared to adult bone, indicating that ER stress is increased in osteocytes with aging similarly to myogenic cells, neurones, and hepatocytes [7]. We show for the first time that ER stress alters the osteocyte response to mechanical loading. PFF-induced Cox-2 expression was clearly decreased in osteocytes from old bone, and inhibition of Cox-2 in vivo has been shown to reduce the anabolic response of bone to mechanical loading [11]. The latter result is in line with previous reports in mice showing a lowered anabolic response of bones to exercise with age [13], but they are in contrast to other studies indicating a similar or even an enhanced response to exercise [5]. The reduced increase in Cox-2 expression we observed in osteocytes from old bone compared to adult bone could be due to either a lower sensitivity to mechanical loading of osteocytes from old bone, or could indicate an altered response, downstream of the sensing of the mechanical stimulus. As NO production was increased to a similar extent in osteocytes from adult and old mouse bone, this suggests that the intrinsic capacity for mechanosensing in osteocytes is not impaired with age. The decreased osteocyte response at the level of Cox-2 could result in a decreased osteoblast proliferation and/or activity, as Cox-2 is the rate-limiting enzyme in prostaglandin E2 production, which enhances osteoblast formation and activity. As previously observed in myogenic cells [9], our results suggest that ER stress in osteocytes from old bones could contribute to a decreased anabolic response following mechanical loading, considering that TN decreased PFF-induced Cox-2 expression in osteocytes from adult bone to a level similar as in those from old bone. We did not observe any effect of TN treatment on Cox-2 expression in osteocytes from old bone, possibly because of the already high level of endogenous ER stress.

Noteworthy, TN had no effect on NO production, either or not after mechanical loading, indicating that NO production was not regulated by ER stress, contrary to Cox-2 expression. However, NO production after PFF treatment increased in osteocytes from both adult and old mice, but to a larger extent in the latter group. Aging may thus affect NO response in osteocytes independent of ER stress. NO

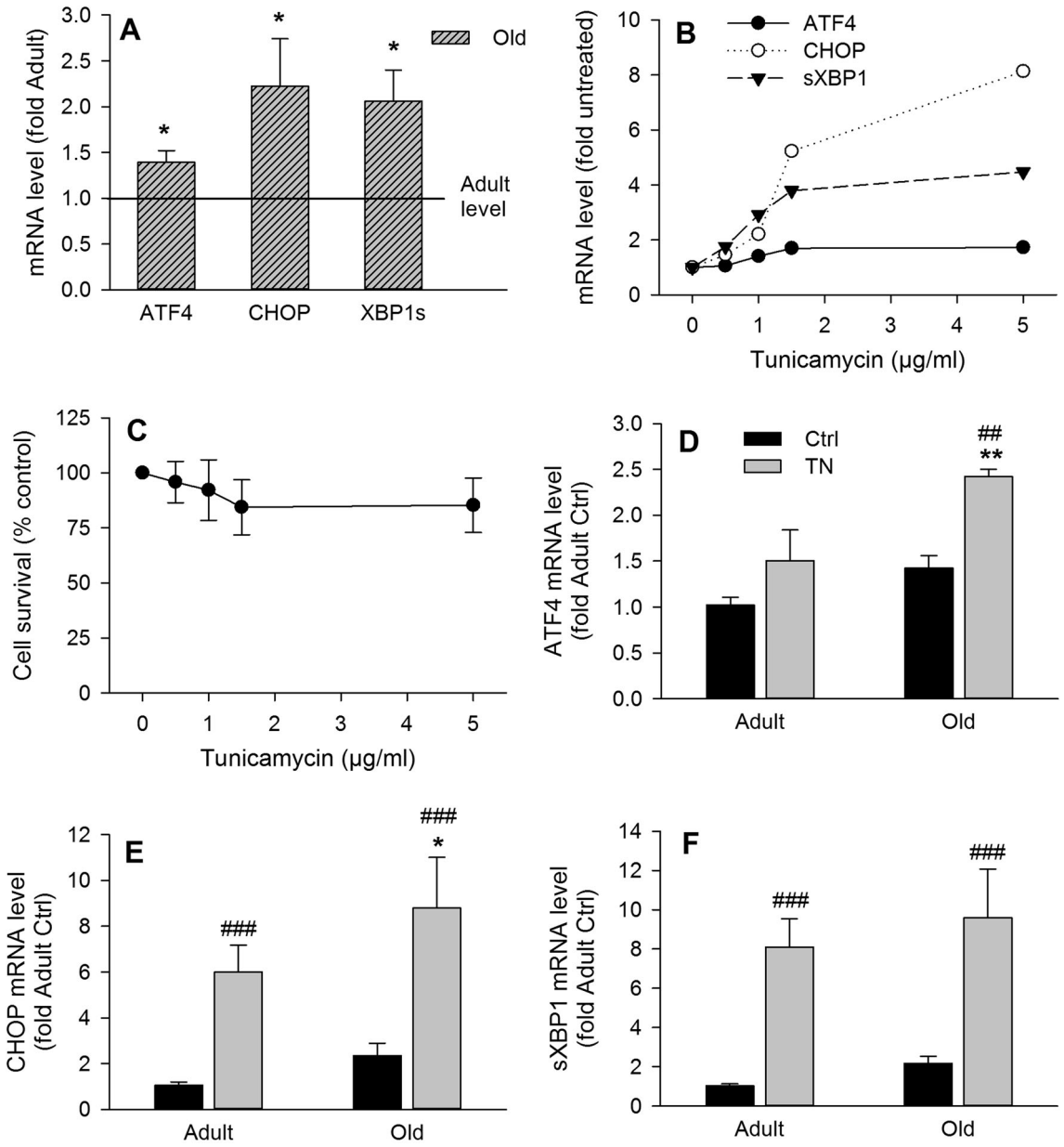

Fig. 1 Unfolded protein response markers are up-regulated in osteocytes from old bones. **a** ATF4, CHOP, and sXBP1 mRNA level in osteocytes from old (*hatched bars*) compared to adult bone (*black line*). **b** ATF4, CHOP, and sXBP1 mRNA levels, and **c** percentage of osteocyte (from old bone) survival after 17 h incubation with increasing concentrations of TN. **d** ATF4, **e** CHOP, and **f** sXBP1 mRNA levels in osteocytes from adult and old bone with and without incubation with 1 µg/ml TN for 17 h. Values are mean ± SEM. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus ctrl; * $p < 0.05$, ** $p < 0.01$ versus adult

production has been shown to reduce osteoclastic bone resorption [16]. Therefore, as reported earlier [5], our results suggest that mechanical loading may induce a strongly decreased osteoclastic bone resorption in osteocytes from old versus adult mice. Since bone mass is decreased in old mice [22], we expected to see an attenuated NO production after mechanical loading rather than an increased production. However, there are many other factors able to affect osteoclastogenesis [18], most notably receptor activator of nuclear factor kappa-B ligand (RANKL), which we did not currently measure. A co-culture of aged osteocytes with osteoclasts could determine whether the inhibitory effect of mechanical stimuli on osteocyte-induced osteoclastogenesis is reduced with aging. Of note, we could not detect any effect of TN or age on PFF-induced c-Fos and CD44 mRNA expression, which are downstream targets of the Wnt/beta-catenin pathway [6] (data not shown).

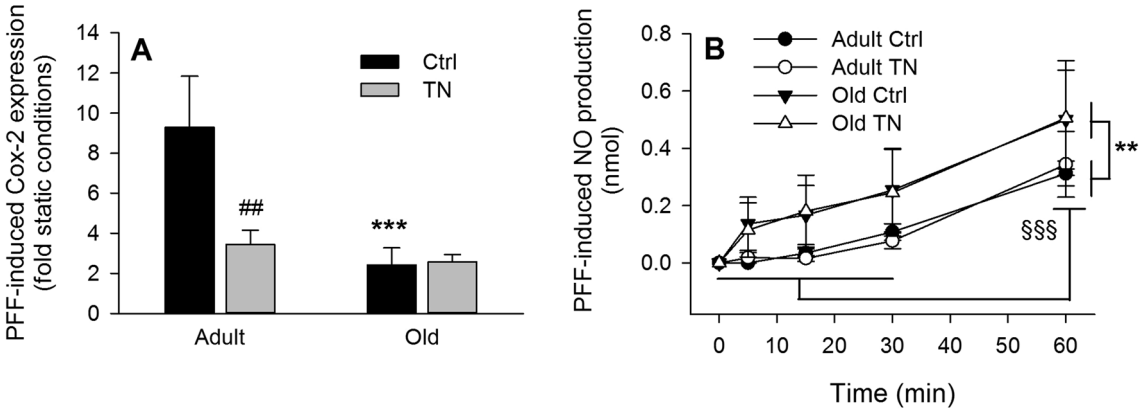

Fig. 2 Effects of age on pulsating fluid flow-induced expression of Cox-2 and NO production by osteocytes from adult and old bone. **a** PFF-induced Cox-2 mRNA expression in osteocytes from adult and old bone with and without incubation with TN (1 μg/ml) for 17 h before the start of PFF. The delta delta Ct method was used and data are reported as fold expression compared to the static conditions of the same age and treatment. **b** PFF-induced NO production in osteocytes from old bone with and without incubation with TN (1 μg/ml) for 17 h before PFF treatment. NO production in static conditions was subtracted from NO production after PFF. Values are mean ± SEM. $^{\#\#}p < 0.01$ versus ctrl; ** $p < 0.01$, *** $p < 0.001$ versus adult; $^{\S\S\S}p < 0.001$ versus 0, 10, 20, 30 min

Taken together, it is clear that osteocytes from old mice do not show a decreased sensitivity to mechanical loading, but Cox-2 response to mechanical loading is different in osteocytes from old versus that of adult mice possibly due to increased ER stress. It seems that osteocytes from old mice compared to those from adult mice exhibit a response to a mechanical stimulus which could result in decreased stimulation of osteoblastic bone formation (via Cox-2), and increased inhibition of osteoclastic bone resorption (via NO).

In conclusion, this study shows that the expression of several UPR markers was increased in osteocytes from old mice compared to those from adult mice. Since ER stress altered the response of osteocytes to mechanical loading, it could be a novel factor contributing to osteopenia.

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Conflict of interest Sreeda Chalil, Richard T Jaspers, Ralph J Manders, Jenneke Klein-Nulend, Astrid D Bakker, and Louise Deldicque declare no conflict of interest.

Human and Animal Rights and Informed Consent The use of animals in this study was approved by the animal care and use committee of the VU University Amsterdam.

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