ELSEVIER

Bone 40 (2007) 895–903

BONE

www.elsevier.com/locate/bone

Galanin treatment offsets the inhibition of bone formation and downregulates the increase in mouse calvarial expression of TNFα and GalR2 mRNA induced by chronic daily injections of an injurious vehicle

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Received 6 June 2006; revised 17 October 2006; accepted 26 October 2006
Available online 8 December 2006

Abstract

We have previously shown that after bone fracture, galanin (GAL) and GAL receptor expression is increased in osteoblast-like cells of callus; however, the role of elevated GAL/GAL receptors in this instance of bone injury is not known. We hypothesize that in injury, GAL may facilitate bone formation by suppressing the production of cytokines such as TNFα and IL-1α, thereby affecting bone collagen formation and collagenolysis by key matrix metalloproteinases (MMPs).

In studies to explore this hypothesis, we used a mouse calvarial injection model to (1) investigate whether mild injury caused by a daily subcutaneous injection of a glycerol-containing vehicle onto calvaria affected osteoblast/bone formation-associated histomorphometric parameters and gene expression (mRNA encoding GAL, GAL receptors, TNFα, IL-1β, collagen type I, MMP-2 and -13) compared to non-injected, control mice and (2) determine the effect of GAL+vehicle treatment on these entities.

Five groups of 4-week-old mice were used: a non-injected control group; a vehicle (50/50 solution of 10 mM PBS+0.025% BSA/5.4 M glycerol)-treated group; and 3 GAL-treated groups (0.2, 2 and 20 ng doses). Solutions were injected subcutaneously onto calvaria in a 10 μl volume, every day for 2 weeks.

Vehicle injection reduced calvarial periosteal osteoblast cell height ($P<0.001$), osteoblast number ($P<0.001$) and osteoid thickness ($P<0.01$), relative to values in non-injected animals at 2 weeks. Vehicle injection also inhibited BFR in this periosteal bone relative to values in non-injected animals at both 1 and 2 weeks ($P<0.05$ and $P<0.001$, respectively). Increasing concentrations of GAL reversed the above-listed inhibitory effects caused by vehicle. This reversal was demonstrated by a dose-dependent effect of GAL on osteoblast cell height (Pearson's $r=0.330$; $P<0.05$), osteoblast number (Pearson's $r=0.715$; $P=0.000$), osteoid thickness (Pearson's $r=0.516$; $P=0.000$) and BFR (Pearson's $r=0.525$; $P<0.05$) after 2 weeks of GAL+vehicle treatment; with the 20 ng/day GAL+vehicle injection schedule returning these measured parameters toward non-injected control values.

All GAL+vehicle treatments had no effect on calvarial expression of GAL, GALR1, GALR3, collagen type 1 and MMP-2 mRNAs compared to levels in vehicle-injected controls. GAL treatment did, however, produce dose-dependent effects on calvarial expression of GALR2 (Pearson's $r=0.763$; $P=0.000$), MMP-13 (Pearson's $r=0.806$; $P=0.000$), IL-1β (Pearson's $r=0.807$; $P=0.000$) and TNFα (Pearson's $r=0.542$; $P=0.000$) mRNAs with 20 ng/day of GAL+vehicle producing the strongest reversal of vehicle-associated changes.

Thus, the 20 ng/day GAL+vehicle regimen offset the inhibition of osteoblastic activity, and therefore bone formation caused by daily glycerol-containing vehicle injection. This effect on bone formation may be due in part to the peptide suppressing the formation and associated activity of TNFα, IL-1β and MMP-13, as TNFα and IL-1β are known inhibitors of bone formation and MMP-13 is involved in collagenolysis. Furthermore, these effects may be due to the action of GAL via GALR2, as it was the only GAL receptor affected by this GAL treatment regimen.

These results indicate that GAL can facilitate bone formation associated with injury and reveal potential efficacy for GAL in treating osseous conditions where bone formation may be inhibited due to excess TNFα and IL-1β production.

Keywords: Galanin; Galanin receptors; TNFα; IL-1β; Bone injury; Bone formation; Calvaria

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8756-3282/$ - see front matter.
doi:10.1016/j.bone.2006.10.018

Introduction

Galanin (GAL) is a 29–30 amino acid peptide [46] that is widely distributed in various tissues including the central and peripheral nervous systems [28,29]; the gastrointestinal tract [46]; tendon and ligament [1]; joints [1,52]; cartilage [27]; and bone [27,52]. GAL signals via three G-protein-coupled receptors – GALR1, GALR2 and GALR3 [6] – and is thought to have important and diverse physiological/pathophysiological roles in such modalities as cognition, nociception [17,23], sensory nerve outgrowth [25], neuroendocrine function [53] and inflammation [15].

The role of GAL in normal skeletal function is unknown. Concentrations of GAL are low in rat whole bone samples [27,52] and both GAL-like immunoreactivity (GAL-LI) and GALR1-LI are equivocal in rat cortical bone [27]. Yet, the role of GAL may be significant in areas of active remodeling and in bone injury. Our group has shown that in the rat both GAL-LI and GALR1-LI are intense in osteoblasts lining metaphyseal trabeculae of unfractured bone as well as in osteoprogenitor cells, osteoblasts and chondrocytes of fracture callus [27]. Furthermore, GAL concentrations increase in both fracture callus and plasma after rib fracture in the rat [27]. On this basis, we speculate that significant levels of GAL and its receptor(s) in the skeleton produce similar actions in bone to those described in other tissues.

To our knowledge there is no documented evidence of GAL influencing bone remodeling, but GAL downregulates the production of TNFα in microglial cells *in vitro* [43] and raised concentrations of both TNFα and IL-1β contribute to increased bone remodeling and subsequent bone loss [8] by inhibiting bone formation [7,40,42] and stimulating osseous collagenolytic activity by osteoblasts [19,21]. Therefore, we propose that GAL may suppress the formation of cytokines such as TNFα and IL-1β in bone cells and thus affect bone remodeling.

In relation to bone formation, the net amount of bone formed by osteoblasts is a result of a balance between osteoblastic bone matrix production and breakdown. Type I collagen, the major structural matrix protein produced by osteoblasts, is also lysed by certain osteoblastic-derived matrix metalloproteinases such as MMP-2 (gelatinase A) and MMP-13 [21,24,32,38], which cleave fibrillar type I collagen [2,31].

As GAL and its receptors are upregulated in osteoblast-like cells after injury, we used a mouse model to (1) investigate whether injury caused by a daily subcutaneous injection of a glycerol-containing vehicle onto calvaria (McDonald and Grills, unpublished observations) affected osteoblast-associated histomorphometric parameters (osteoblast cell height (as an indicator of cell size and therefore activity), osteoblast number, osteoid thickness, mineralization lag time, mineral apposition rate and bone formation rate) and gene expression (mRNA encoding GAL, GAL receptors, TNFα, IL-1β, collagen type I and MMP-2 and -13) in calvaria compared to non-injected, control values; and (2) determine if GAL (plus vehicle) treatment affected any of these parameters.

Materials and methods

Animal procedures

This project was approved by the La Trobe University Animal Ethics Committee. Animals were fed a standard pellet diet and were allowed water *ad libitum*. Thirty-five, 4-week-old 129OlaHsd male mice were used and were divided into five groups of seven: group 1, no injection; group 2, vehicle injection; groups 3–5, 0.2, 2 and 20 ng GAL+vehicle injection, respectively. The vehicle was a 50/50 solution of 10 mM PBS+0.025% BSA/5.4 M glycerol (pH 7) and was used on the basis of recommendations from many pharmaceutical and biotechnology companies of the suitability of this solution for peptide dilution to avoid loss of efficacy upon repeated thawing of peptide solutions stored at −20°C. Injected groups received a 10 μl subcutaneous injection of either vehicle or GAL (rat) (Bachem, Bubendorf, Switzerland) with vehicle over calvaria using a fine gauge needle and a Hamilton syringe each day from days 1 to 6 and from days 8 to 13.

MAR and BFR of bone directly adjacent to the periosteal layer of calvaria were determined by subcutaneous injection of the fluorescent dyes 3,3′-Bis[*N*,*N*′-di(carboxymethyl)aminomethyl]-fluorescein (calcein; Sigma, St. Louis, MO, USA) and alizarin complexone (Sigma) at the cervical region of each mouse (both groups) at doses of 6 mg/kg and 20 mg/kg body weight, respectively, on day 0 (calcein), day 7 (alizarin complexone) and day 14 (calcein) (day 1 being the first day of calvarial injection), i.e. on days where vehicle or GAL+vehicle were not injected as these injections may have interfered with deposition of the fluorescent dyes onto mineralizing surfaces. All mice were euthanized by 100% CO_2 asphyxiation on day 15.

Histological preparation and histomorphometry of calvaria

The right parietal bone of each mouse was removed and processed for histology by embedding in LR Gold resin [11]. Briefly, bone tissue was immersion-fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer for 24 h. Following fixation, samples were washed in 0.1 M sodium cacodylate buffer containing 10% sucrose (pH 7.4) and then dehydrated in a graded series of ethanol and placed in a 50/50 mixture of LR Gold resin (London Resin Co. Ltd., Theale, Berkshire, UK) and 100% ethanol. Samples were then placed in 100% LR Gold resin (containing 0.1% Benzil) and left agitating overnight. Each hemi-calvarium was then placed into a size 00 gelatin capsule filled with the 100% resin containing 0.1% Benzil and placed under a fluorescent desk lamp until the resin polymerized. All procedures were performed at 4°C. Sections (5 μm) were cut using a tungsten-carbide knife on an automated microtome (Leica Microsystems, Heerbrugg, Switzerland). For fluorescence microscopy, sections were then heat-mounted onto slides and coverslipped using a glycerol-based mounting media (Gelmount, Biomedia Corp, Foster City, CA, USA). All sections were analyzed both qualitatively and quantitatively using a Leica DRB microscope and high-resolution digital images were captured using a Leica DC 300, 7.3-megapixel digital camera (Leica). For some static histomorphometric measurements, osteoid thickness (O.Th.) and osteoblast cell height (Ob.Ce.Ht) sections were stained with 1% toluidine blue+1% borax (Sigma). For osteoblast number measurements, sections were stained with DAPI (Sigma) and analyzed under fluorescence microscopy. For dynamic histomorphometry, MAR was calculated by measuring the average distance between two fluorescent labels and dividing by the number of days between injections of the labels. Mineralization lag time (Mlt) was calculated by the formula O.Th/Aj.AR, where Aj.AR is the adjusted apposition rate. Aj.AR was calculated as MAR×MS/OS, where OS is the length of osteoid surface. BFR/BS was calculated using the formula BFR=MAR×(dLS+sLS/2)/BS, where dLS is length of double label surface, sLS is length of single-labeled surface and BS is the length of bone surface.

All measurements were performed directly adjacent to the periosteal layer of calvaria and determined over four adjacent fields (×400 magnification) by obtaining ten measurements in each field for each hemi-calvaria. Fluorescent markers were identified under an I3 filter cube (calcein), an N2.1 filter cube (alizarin complexone) and an A filter cube (DAPI) and measurements were made using QWin software (Leica). Histomorphometric analyses were performed by an observer blinded to the identity of the treatment groups.