Lipoxygenase metabolites are mediators of PTH-dependent human osteoblast growth

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Abstract

PTH-induced osteoblast proliferation may contribute to its anabolic effects in bone. Since PTH-dependent osteoblast-like cell (Ob) growth is mediated via protein kinase C (PKC) and MAP kinase-kinase (MEK) and since lipoxygenase (LO) products activate PKC in a number of cell types, we assessed the expression of LO pathways in primary human cultured Ob. Ob from pre- or post-menopausal women were cultured and were treated with PTH and assayed for the expression of 12-LO and both type I and type II 15-LO mRNA and for the release their enzymatic products, 12- and 15-hydroxyeicosatetraenoic acid (HETE). Cells were also treated with PTH for stimulation DNA synthesis. First, Ob express platelet type- 12-LO and both type I and type II 15-LO mRNA and release their enzymatic products, 12- and 15-hydroxyeicosatetraenoic acid (HETE). Second, in female Ob, PTH induced a rapid increase in 12-HETE (50 fold increase) and 15-HETE (80 fold increase) and increased the expression of 12-LO mRNA but not of the two isoforms of 15-LO. PTH as well as 12 and 15-HETE stimulated DNA synthesis in Ob. The LO inhibitor baicalein inhibited PTH-stimulated DNA synthesis, which was reversed in the presence of either 12- or 15-HETE. A PKC inhibitor (bisindolylmaleimide I) as well as a MEK inhibitor (PD 98059) completely inhibited the stimulation of DNA synthesis by PTH, 12-HETE and the combination of PTH and 12-HETE. In contrast, 15-HETE-induced DNA synthesis was not abolished by these inhibitors. Further, 15-HETE partially restored the stimulatory effect of PTH on DNA synthesis in cells treated with PKC or MEK inhibitors. Finally, PTH- induced ERK1/2 phosphorylation, was blocked by a MEK inhibitor. These results demonstrate a novel mechanism of PTH-induced human bone cell proliferation operating through LO enzymes.

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Introduction

The use of PTH as a means to increase bone density and reduce susceptibility to fracture in human osteoporosis has intensified the interest in the mechanisms underlying its anabolic effects. One aspect of the multifaceted effect of PTH is that it stimulates osteoblast accumulation through diversion of the proliferative machinery in pre-osteoblasts towards accelerated maturation into secretory osteoblasts [1–3], whose products then trigger massive proliferation of osteoprogenitor cells, promote reversal of bone lining cells to osteoblasts, and prevent osteoblast apoptosis [4]. Another less studied effect of PTH is its ability to directly increase osteoblastic proliferation, as shown in cells derived from human trabecular bone [5], osteoid cell lines [6] and primary rat osteoblasts [7].

We have previously reported that cultured human bone cells harvested during orthopedic surgery retain typical osteoblastic
characteristics. They have a high level of alkaline phosphatase (ALP) activity, which is dose-dependently increased by 1,25(OH)₂D₃, and they display 1,25(OH)₂D₃ inducible osteocalcin production, and they express active PTH receptors and have high PTH-sensitivity as reflected by PTH-induced cyclic AMP generation [8,9]. In addition, the growth of these cells was stimulated by both estradiol-17β and PTH [8,9].

PTH may elicit either differentiation or proliferation by interaction with distinct osteoblastic subpopulations, targeting osteoblasts at different phases of the cell cycle and/or through the activation of alternative signaling cascades. PTH stimulates osteoblast differentiation via arrest of cell cycle progression and activation of PKC δ, independent of PLC [1,10]. PTH-induced bone protein expression depends on cAMP-dependent activation of PKA [1,11], which in turn, can down regulate MAPK, thus retarding cell growth. Additionally, PTH also induces the expression of MAPK-phosphatase 1, which reduces phosphorylated MAPK levels, primarily through the cAMP-PKA pathway [12]. In periodontal osteoblast-like cells, inhibition of PKA results in enhanced proliferation, whereas inhibition of MAPK reduces cell growth [13]. PTH was also shown to stimulate cell proliferation via PKC but not via cAMP and/or cAMP-PKA pathway, both in vitro and in vivo [14–16]. Finally, low concentrations of PTH were reported to stimulate cell growth via MAPK and PKC-dependent mechanisms [3].

Hence, MAPK may serve as a PTH-sensitive integrator of osteoblastic functional trafficking, upon which both proliferative and differentiating signals apparently converge.

In the present study we analyzed the expression and regulation of lipoxygenase (LO) enzymes in PTH-sensitive cultured human bone cells. We focused on these enzymes in osteoblasts since LO products were shown to modulate MAPK activity [17–19] and proliferation or survival [19–22] in a number of cell types and could thus play a role in promoting cell growth, either independently or through PTH action. Additionally, there is recent strong circumstantial evidence linking LO expression to bone mineral content [23–25]. We hypothesized that the growth promoting effects of PTH in human osteoblast-like cells may be associated with accelerated production of LO expression products, which consist of a ×20 mixture of unlabeled PCR primers for the human platelet type 12-LO, the forward primer was 5′-GGTGATGGGGCTGAAATAA; and for human 15-LO type 1 the 5′ primer was ACTGAAATCGGGCTGCAAAGG and the 3′ primer was GGGTGATGGGGCTGAAATAA; and for human 15-LO type 2, the 5′ primer was AACTCAAGGTTGGAAGACTCGGAG and the 3′ primer was ATATAGTCTGCCCCAGACCATATC. For subsequent quantification of the effect of PTH on 12-LO expression, amplification of the resulting cDNA was performed in 25 μl on 96-well plates in a reaction buffer containing Taqman universal PCR master mixture. RNAase P expression served as an internal control for each sample and was measured by assay-on-demand gene expression products, which consist of a ×20 mixture of unlabeled PCR primers and Taqman MGB probe labeled with 5-carboxy fluorescein dye.

**Materials and methods**

**Reagents**

All reagents were of analytical grade. Chemicals, PTH 1–84, bisindolylmaleimide I and PD 98059 were purchased from Sigma Chemicals Co. (St. Louis, MO). 12 Hydroxyeicosatetraenoic acid (HETE) and 15-HETE were purchased from Biomol (Biomol International, Plymouth Meeting, PA). Bisindolylmaleimide I and PD 98059 were purchased from Sigma Chemicals Co. (St. Louis, MO). 12 Hydroxyeicosatetraenoic acid (HETE) and 15-HETE were purchased from Biomol (Biomol International, Plymouth Meeting, PA).

**Cell cultures**

Human bone cells were prepared from biopsies of patients undergoing corrective surgery following accidental injury, hip or knee replacement. All patients signed informed consent and were healthy, non-osteoporotic and not receiving hormonal replacement treatment. We studied both pre-menopausal women, whose ages ranged between 37–50 years (n=4) and post-menopausal women, with an age range of 59–84 years (n=4). The non-enzymatic method for isolation and culture of human bone cells and their characterization as osteoblasts were previously described [8,9]. Briefly, unused fragments of the trabecular surface of the iliac crest used for corrective spinal surgery were cut into 1 mm² pieces and extensively and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants, with no enzymatic digestion, were seeded in 100 mm diameter tissue culture dishes and incubated in DMEM medium containing 10% fetal calf serum (FCS) and antibiotics, in the absence of Ca²⁺, to avoid fibroblastic, chondroblastic and osteoclastic growth [8,26]. Cell outgrowth from the bone explants was apparent after 6–10 days. The culture cell population consisted of osteoblast-like cells (with a negligible number of fibroblasts) showing high basal alkaline phosphatase activity and osteocalcin secretion, both of which increasing in response to 1,25(OH)₂D₃. Additionally, these cells had increased cAMP formation in response to PTH [8,9]. First passage cells were seeded at a density of 3x10⁵ cells per 35 mm tissue culture dish in phenol red free DMEM with 10% charcoal stripped FCS and incubated at 37 °C in 5% CO₂.

**Hormonal treatment**

a. Cells were treated daily with vehicle (saline) or PTH 1–84 at a final concentration of 20 nM [14,15] for 3 days, starting after sub-confluence was reached. On day 4, cultures were collected and prepared for RNA extraction for determination of 12-LO and 15-LO type 1 and type 2 mRNA levels as described [27–29].

b. Cells were incubated for 1 h with serum-free medium, followed by the addition of vehicle (saline) or PTH 1–84, at concentrations ranging from 1–20 nM for 10 min. The medium and cellular layers were then collected for HETE extraction and assessed as previously described [28,29].

**Determination of mRNA for 12- and 15-LO by RT-PCR**

RNA was extracted from cultured human bone cells as previously reported [26]. Initial detection of the expression of 12- and 15-LO enzymes was carried out by RT-PCR as previously described [27,28], using the following primers for the human platelet type 12-LO, the forward primer was 5’-GATGATCT- TACCTCATTAG-3’ and reverse 5’-CTGGGCCAGGAACTCGT-3; for the human 15-LO type 1 the 5′ Primer was ACTGAAATCGGGCTGCAAAGG and the 3′ primer was GGGTGATGGGGCTGAAATAA; and for human 15-LO type 2, the 5′ primer was AACTCAAGGTTGGAAGACTCGGAG and the 3′ primer was ATATAGTCTGCCCCAGACCATATC. For subsequent quantification of the effect of PTH on 12-LO expression, amplification of the resulting cDNA was performed in 25 μl on 96-well plates in a reaction buffer containing Taqman universal PCR master mixture. RNAse P expression served as an internal control for each sample and was measured by assay-on-demand gene expression products, which consist of a ×20 mixture of unlabeled PCR primers and Taqman MGB probe labeled with 5-carboxy fluorescein dye.

**Determination of levels of 12- and 15-HETE by HPLC**

Cells and medium were extracted for HETE and analyzed by HPLC as described before [27,28]. Protein was assayed by Coomassie brilliant blue dye binding, using BSA as the standard.

**Activation of mitogen-activated protein kinase (MAPK) cascades**

Supertransants from cultured cells exposed to the various treatments for 20 min were collected. After treatment the cells were washed twice with ice-cold PBS and once with ice-cold buffer A (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate). Cells were harvested in 0.3 ml of buffer B (buffer A containing 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) followed by sonication (two times for 7 s, 40 W) and centrifugation (15,000 × g for 15 min at 4 °C). The supernatants were collected, and aliquots (30 μg sample) were separated on 10% SDS-PAGE, followed by Western blotting with mouse monoclonal anti-active MAPK (ERK). Total ERK was detected with polyclonal antibodies for the various MAPK as a control. The blots were developed with horseradish peroxidase-conjugated secondary antibody (Jackson Immunotech).
human female osteoblast-like cells (Fig. 1). Maximal increase was seen at PTH concentration of 20 nM, but significant stimulation was already detectable at low concentrations (1–2 nM). At the highest concentration of PTH used (20 nM), the production of 12- and 15-HETE increased 50 and 80 fold, respectively. At all doses tested, no significant difference was seen in PTH- induced increases in 12- and 15-HETE between pre- and post-menopausal women and therefore the data were averaged together (Figs. 1 and 2).

Additionally, PTH affected the mRNA levels of 12- and 15-LO in human female osteoblasts. Daily treatment of cultured cells with PTH (20 nM) for 3 day s resulted in a 2–3 fold increase in 12-LO mRNA, as expressed by real time PCR, but 30–40% decrease in 15-LO type 2 mRNA expression measured by semi quantitative PCR (data not shown). Also, the effects of PTH on 12- or 15-LO type 2 mRNA expression were not significantly different in pre- and post-menopausal women (data not shown).

**The effect of PTH and the LO products 12- and 15-HETE on DNA synthesis in human female osteoblasts**

Treatment with 20 nM PTH for 24 h elicited an almost 200% change in DNA synthesis in osteoblasts derived from both pre- and post-menopausal women as measured by $^3$H thymidine incorporation. This effect was entirely blocked in the presence of the LO inhibitor baicalein. Both 12-HETE and 15-HETE (100 nM for 24 h) themselves induced a moderate increase in DNA synthesis in either pre- or post-menopausal cells (Figs. 3a and b). Notably, the increase in DNA synthesis observed in response to PTH was not further augmented in the presence of either 12- or 15-HETE. However, both 12- and 15-HETE fully restored the stimulatory effect of PTH on DNA synthesis which was blocked by the LO inhibitor baicalein, suggesting the effect of PTH is mediated via these LO products (Figs. 3a and b). Since there is no significant difference by menopausal status,

**Fig. 1. Effect of PTH on 12- and 15-HETE production in bone cell cultures.** Female bone cells were serum deprived for 1 h and exposed to PTH at different concentrations for 10 min. The results are expressed as percent increase in 12-HETE (solid circles) and 15-HETE production (open circles) over control (vehicle), in cells derived from both 4 pre- and 4 post-menopausal subjects. $^*P<0.05$, $^**P<0.01$, $^***P<0.001$, compared with untreated cells.

**Fig. 2. Effect of PTH on 12- and 15-HETE production in cultured human female osteoblasts.**

Female bone cells were serum deprived for 1 h and exposed to PTH at 20 nM for 10 min. The results are expressed as percent change in 12-HETE and 15-HETE production over control (vehicle), in cells derived from pre- and post-menopausal subjects ($n=4$ in each group). $^*P<0.05$, $^**P<0.01$, $^***P<0.001$, compared with untreated cells. Results are expressed as percent change by PTH over untreated cells ($n=4$ in each group). $^*P<0.05$, $^**P<0.01$, $^***P<0.001$. 

**Results**

Human osteoblast-like cells in culture express lipooxygenase enzymes. Identification of lipooxygenase enzymes in human osteoblast-like cells in culture was performed by RT-PCR as detailed in methods. Three different LO species capable of producing either 12- or/and 15-HETE were expressed in these cells: the platelet-type 12-LO, and 15-LO type 1 and type 2 (data not shown).

**The effect of PTH on the production of 12- and 15-HETE and on 12- and 15-LO mRNA in human female osteoblasts**

Under basal conditions, pre-menopausal cells generated both 12-HETE ($0.42\pm0.08$ ng/mg protein) and 15-HETE ($0.60\pm0.04$ ng/mg protein), as did post-menopausal cells (12-HETE $-0.17\pm0.04$ ng/mg protein; 15-HETE $-0.49\pm0.06$ ng/mg protein, respectively). Treatment with PTH, at concentrations ranging between 1–20 nM for 10 min, dose-dependently stimulated synthesis of 12- and 15-HETE in primary cultured

**Human female bone cells**

**ImmunoResearch** in enhanced chemiluminescence reaction, and the phosphorylation was quantitated by densitometry (Bio-Rad 690 densitometer).

**Assessment of DNA synthesis**

Cells were grown until sub-confluence and then treated with various hormones as indicated. Twenty-two hours later, [3H] thymidine was added for two hours. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 ml of 0.3N NaOH, samples were aspirated for 5 min and washed twice with 5% TCA and then with cold ethanol. The [3H] thymidine incorporation into DNA was determined [8,14].

**Statistical significance**

Differences between the mean values obtained from the experimental and control groups were evaluated by analysis of variance (ANOVA). A $P$ value less than 0.05 was considered significant. Values are means±S.E.M. for $n$=number of donors.

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Fig. 3b depicts only pooled data from pre- and post-menopausal women.

The effect of PKC and MEK inhibitors on PTH- induced DNA synthesis in human female osteoblasts: interaction with 12- or 15-HETE

The stimulatory effect of PTH (20 nM for 24 h) was entirely blocked by co-incubation with either the MEK inhibitor PD98059 (1 μM) or the PKC inhibitor bisindolylmaleimide I (1 μM). Additionally, both the MEK inhibitor and the PKC inhibitor entirely blocked the increase in [3H] thymidine incorporation induced by 12-HETE alone (Fig. 4, lower panel) without affecting basal DNA synthesis (PD 20±14% and Bis 9±9%). In the presence of either bisindolylmaleimide I or PD98059, the addition of 12-HETE could not restore PTH-induced DNA synthesis (Fig. 4, lower panel). In contrast, the stimulatory effect of 15-HETE on [3H] thymidine incorporation, while smaller in this set of experiments than in those shown in Fig. 3, was not attenuated by MEK or PKC inhibition (Fig. 4, upper panel). Further, in cells treated with PTH and either the MEK inhibitor or the PKC inhibitor, in which PTH-induced DNA synthesis was completely blocked, the addition of 15-HETE resulted in partial restoration of PTH stimulatory effect on [3H] thymidine incorporation into DNA.

The effect of PTH on MAPK phosphorylation

PTH induced a marked increase in phospho-ERK1/2, which was clearly blocked as expected by the MEK inhibitor PD98059 (data not shown). Additionally, 12-HETE and 15-HETE each induced a 2–3 fold increase in ERK 1/2 phosphorylation (Fig. 5). Combining PTH and 12-HETE or PTH and 15-HETE had an additive effect on ERK1, but not on ERK2 phosphorylation (Fig. 5).

Discussion

Cumulative evidence in a number of cell types suggests an important role for 12- and/or 15-HETE/ lipoxygenase in cell growth, which is exerted through the RAS/Raf/MEK/ ERK...
pathway and ERK1/2 phosphorylation and via PKC-dependent mechanisms. Several mechanisms of activation of MAPK by 12- or 15-HETE have been elucidated and have linked this interaction to cell growth. For example, in A431 cells, 12-HETE was shown to activate ERK1/2 through MEK and Raf, and to increase the activity of Ras via Src family kinases, thus further stimulating the Raf/MEK/ERK cascade [30]. Additionally, 12-HETE-triggers Src/Shc interaction, which appears necessary for its MAPK activating effect, since Src inhibitors can attenuate 12-HETE-induced ERK phosphorylation [17]. In HCT-116 cells, over expression of 15-LO type 1 induced ERK 1/2 phosphorylation, decreased p21 (Cip/WAF1) expression, and increased cell growth, whereas treatment with the LO inhibitor NDGA decreased ERK 1/2 phosphorylation, and increased p21 [18]. In pancreatic cancer cells, 12-HETE accelerated cell growth and enhanced ERK phosphorylation, whereas the specific MEK inhibitors PD098059 and U0126 suppressed both 12-induced ERK phosphorylation and 12(S)-HETE-stimulated cell proliferation. Because 12-HETE-stimulated ERK phosphorylation could be inhibited by genistein, it appears that tyrosine phosphorylation is essential for 12-HETE-mediated ERK activation [19]. Although these and other reports collectively suggest that 12- and 15-HETE interact with multiple signals promoting cell replication, their effects are probably not redundant, since inhibition of their production hinders normal cell growth in a variety of unrelated cell types [17, 18, 20–22, 31].

Three recent publications have linked 12/15-LO or 15-LO and 12-LO (platelet type) to bone density. In the mouse, absence of 12/15-LO expression appeared to confer increased bone density [23], thus indirectly portraying eicosanoid products of this enzyme, such as 12-HETE, 15-HETE, 13-hydroxyoctadecadienoic acid (13-HODE) or, possibly, their hydroxyeicosatetraenoic acid precursors as potential negative modulators of bone mineral content. In Japanese postmenopausal women, a single nucleotide polymorphism in the 5′-flanking region of the ALOX15 gene (−5299G/A), the human homolog of the mouse 12/15-LO gene (15-LO type 1), was associated with bone density such that subjects bearing at least one variant A allele had lower lumbar spine and total body bone mineral density than did subjects carrying the G allele only [24]. Finally, in white men and women, six different single nucleotide polymorphisms in the human platelet type 12-LO gene (ALOX12) but not 15-LO type 1 (ALOX15), were independently associated with increased spine bone mineral density in a gender-related manner [25]. Although the functional significance of these polymorphisms with respect to lipoxynagen expression and/or activity has not been determined, the possibility that such genetic variations in two different lipoxynagen enzymes translate into altered HETE production in bone, thus affecting bone properties, is of obvious interest.

In the present study we provide the first direct evidence for the expression and at least one biological role of lipoxynagen enzymes in bone cell biology in vitro. First, we find evidence for the mRNA expression of three types of LO enzymes in culture human bone cells, i.e., the platelet type 12-LO (“ALOX12”), 15-LO type 1 (“ALOX15”) and 15-LO type 2. We have previously established that cultured osteoblast-like cells derived from surgical specimen by the method used in the present communication retain several key metabolic features of well differentiated human osteoblasts [8, 9]. Here we show that the expression of 12-LO and 15-LO type 2 in these cells is modulated by PTH, such that 12-LO expression is markedly stimulated, whereas the expression of 15-LO type 2 is suppressed by PTH. Additionally, the expression of these enzymes results in the ability of “osteoblast-like-bone cells” to secrete both 12-HETE, a potential product of the two enzymes and 15-HETE, a product of both types of 15-LO. Moreover, the generation of these metabolites is strongly driven by PTH and is functionally linked to PTH-dependent osteoblast growth.

Our results are consistent with the concept that PTH rapidly increases 12-HETE production which in turn activates PKC-dependent mechanisms involving also increased ERK1/2 phosphorylation. These signaling elements, i.e., 12-HETE, PKC and MEK are all required to preserve PTH-induced osteoblast-like proliferation, such that 12-HETE likely activates both PKC and MEK. This is supported by the findings that PTH-dependent [3H] thymidine incorporation in these cells, previously shown by us to precede the increase in cell count [14], is fully blocked by inhibitors of LO, PKC or MEK and that 12-HETE can restore the proliferative effect of PTH in the presence of a LO inhibitor, but not in the presence of PKC or MEK blockers. Our results indicate that 12-HETE per se can increase ERK1/2 phosphorylation, but its effect is markedly smaller than the effect of PTH. Thus, 12-HETE-dependent increase in MEK activity may provide only a partial explanation for the ability of 12-HETE to increase [3H] thymidine incorporation.
incorporation or to restore in full the proliferative effect of PTH in LO-blocked cells. Alternatively, it is possible that the massive increase in endogenous 12-HETE elicited by PTH is compartmentalized within the cell such that it can increase ERK1/2 phosphorylation much more effectively than exogenous 12-HETE. It is of important to notice the difference in the time course of the induction of the different parameters analyzed here, due to the previously established different optimal time for activation.

In this system, 15-HETE apparently assumes a somewhat different role than 12-HETE. In parallel to the effects of 12-HETE, 15-HETE can increase ERK1/2 phosphorylation as well as osteoblast-like cell growth like 12-HETE, however, its stimulatory effect on DNA synthesis is not sensitive to PKC or MEK inhibition. Consistent with the latter is the ability of 15-HETE to restore, in part, PTH-induced DNA synthesis which has been blocked not only by LO inhibition, but also by PKC or MEK inhibition. This would suggest that even though 15-HETE increases ERK1/2 phosphorylation, it can affect DNA synthesis by mechanisms which “bypass”, at least in part, both ERK1/2 and PKC, thus possibly interacting with signaling elements operating downstream to this cascade.

Finally, could the observations in this study be related to the emerging link between ALOXI2/ALOX15 and osteoporosis? We find that PTH-stimulated 12- and 15-HETE formation is linked to an increase in osteoblast-like cell growth, an effect presumed to promote bone formation. Yet, lipooxygenase enzymes are also known to generate obligatory intermediary hydroperoxy metabolites such as 12H-[P]ETE and 15H-[P]ETE and hydroperoxyoctadecadienoic acid (HPODE), which can release reactive oxygen intra-cellularly, thereby increasing oxidative stress [30]. The 15-LO product of linoleic acid metabolism, 13-HODE, is also a potent pro-oxidant capable of accelerating the generation of biologically active oxidized phospholipids [31]. Thus, PTH-induced fluxing of arachidonic and linoleic acid through the 12-LO and/or the 15-LO pathways in bone cells could also lead to transient, albeit excessive formation of 12-HPETE, 15-HPETE and/or 13-HODE, potentially capable of raising local oxidative stress. Because oxidative stress, in turn, may lead to inhibition of differentiation of bone osteoblasts-like cells [32] and acceleration of osteoclast differentiation [33], PTH-induced LO expression and activity may not only comprise a direct signal for osteoblast growth, but also result in the release of oxidizing fatty acids which unfavorably affect overall bone osteoblast/osteoclast homeostasis through enhanced oxidative stress. Presently, however, these potential secondary sequels of PTH-stimulated LO activities in bone remain entirely conjectural and are the subject of ongoing investigation.

References


