



**Calcitonin gene-related peptide (CGRP) is a survival factor  
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Calcitonin gene-related peptide (CGRP) is a survival factor inhibiting apoptosis in human osteoblasts through cAMP/PKA  $\beta$ -catenin stabilization

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**Running title:** CGRP and Wnt signaling in osteoblasts

**Keywords:** CGRP, human bone cells,  $\beta$ -catenin, apoptosis, intracellular signaling.

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## Abstract

Bone is innervated by CGRP-containing nerve fibers that are increased in number during bone regeneration. Transgenic mice over-expressing CGRP in osteoblasts have increased bone density due to increased bone formation, whereas mice lacking CGRP expression display osteopenia, thus suggesting that CGRP plays a role in bone metabolism. In this study we determined the relationship between CGRP, the canonical Wnt signaling and apoptosis in human osteoblasts (hOBs) in consideration of the well documented involvement of this pathway in bone cells. Primary cultures of hOBs were treated with CGRP  $10^{-8}$ M and  $\beta$ -catenin, which is the cytoplasmic protein mediator of canonical Wnt signaling, was determined. CGRP increases the levels of cytoplasmic  $\beta$ -catenin by binding to its receptor, as this effect is blocked by the antagonist CGRP<sub>8-37</sub>. This facilitatory action on  $\beta$ -catenin appears to be mediated via PKA activity, which inhibits the enzyme GSK-3 $\beta$ . GSK-3 $\beta$  is a glycogen synthase kinase that, by phosphorylating  $\beta$ -catenin, promotes its degradation by the proteosomal machinery. Thus CGRP, besides favouring  $\beta$ -catenin accumulation, also induces its nuclear translocation. Moreover, the peptide is able to inhibit hOBs apoptosis stimulated by dexamethasone or by serum deprivation, possibly through the accumulation of  $\beta$ -catenin, since the inhibitor of PKA activity H89 inhibits the anti-apoptotic effect of the peptide. In conclusion, Wnt appears to be one of the mediator of the anabolic action of CGRP on bone. The peptide, released by nerve fibers, could favour local bone regeneration.

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**Introduction**

**Calcitonin gene-related peptide (CGRP)** is a 37 amino acid product resulting from the tissue-specific processing of the mRNA derived from the calcitonin gene (Amara et al., 1982). CGRP is widely distributed throughout the central and peripheral nervous systems. Bone contains CGRP-immunoreactive nerve fibers whose increased number during bone development and repair suggest that they are directly involved in the local regulation of bone remodeling (Hukkanen et al., 1993; Imai et al., 1997; Irie et al., 1997). Further evidence shows that CGRP plays a role both in bone formation and resorption. Transgenic mice over-expressing CGRP in osteoblasts have increased bone density due to increased bone formation (Ballica et al., 1999), whereas mice lacking specifically  $\alpha$ CGRP expression display osteopenia, caused by decreased bone formation (Schinke et al., 2004). The peptide inhibits bone resorption directly by inhibiting osteoclast activation (Akopian et al., 2000) and indirectly by inhibiting interleukin-1 mediated bone resorption (Zhang et al., 1994). Recently it was shown that CGRP directly stimulates stromal cells differentiation towards mature osteoblast and inhibits RANKL induced osteoclastogenesis (Wang et al., 2009). Expression of mRNAs for CGRP (Drissi et al., 1997) and its receptors was detected in various human osteoblast cell lines and normal human osteoblast-like cells (Villa et al., 2003). CGRP stimulates cell proliferation in these cells (Villa et al., 2000) and inhibits osteoprotegerin secretion, thus suggesting that the peptide modulates the balance between osteoblast and osteoclast activities (Villa et al., 2006). Activated CGRP receptors stimulate adenylyl-cyclase activity (Villa et al., 2000), which induces cAMP production and activation of protein kinase A (PKA), as well as phospholipase C (Drissi et al., 1999), this results in increased intracellular free calcium levels (Burns et al., 2004) with subsequent protein kinase C activation, which is involved in CGRP-induced osteoblast proliferation (Villa et al., 2003).

Overall these studies indicate that CGRP in bone could influence the maintenance of bone mass, however the mechanism by which CGRP exerts its anabolic action on bone is not completely understood.

Wnt signaling has been shown to play an important role in bone development (Rawadi and Roman-Roman, 2005). **Wnt genes** encode for a large family of secreted proteins that bind to a membrane receptor complex of a **Frizzled (FZ)**, G-protein coupled receptor and to **low-density lipoprotein receptor-related proteins (LRP-5/6)**. Depending on the specific nature of the Wnt and of the Fz that are complexed with LRP, three independent pathways can be activated: the canonical Wnt/ $\beta$ -catenin cascade, the non-canonical planar cell polarity (PCP) and the **Wnt/calcium pathway**. The **Wnt canonical signaling pathway relies on the cytosolic stabilization of  $\beta$ -catenin**, which is a 130 amino acid protein that, in the absence of Wnt proteins, **is phosphorylated by a glycogen synthase kinase**

(GSK-3 $\beta$ ) and degraded by the proteosomal machinery. When not degraded, stable  $\beta$ -catenin accumulates in the cytoplasm and upon reaching a certain concentration level translocates into the nucleus to stimulate gene transcription (Novak and Dedhar, 1999). The signaling of  $\beta$ -catenin, required for the canonical Wnt pathway, is essential for osteoblast commitment (Baron and Rawadi, 2007) and survival (Almeida et al., 2005). Several lines of evidence have established that the canonical Wnt pathway is involved in bone mass accrual (Krishnan et al., 2006) and in bone metabolism. This is supported by the results obtained in mice with targeted disruption of LRP-5 that develop a low bone mass phenotype, which is secondary to decreased osteoblast proliferation and function (Kato et al., 2002). Conversely, gain of function of LRP-5 is associated with increased bone mass (Babij et al., 2003). Several other mouse models support the notion that activating Wnt signaling leads to increased bone mass. Inactivating or reducing the expression of Wnt antagonists, such as dickkopf1, leads to increased bone formation and bone mass (Morvan et al., 2006). Loss or gain of function due to mutations affecting LRP-5, either in humans or in mice, alter bone formation without affecting the resorption parameters, indicating that osteoblasts are the main cellular targets of Wnt effects (Baron and Rawadi, 2007). An LRP-5 mutation mouse model, leading to increased Wnt signaling, results in a significant increase in osteoblast number and a reduced number of apoptotic osteoblasts (Babij et al., 2003). Wnt signaling thus controls osteoblasts at different levels: commitment, proliferation, apoptosis and function. During the late phase of osteoblast differentiation, Wnt signaling antagonists are upregulated, suggesting the existence of a Wnt negative feedback loop that controls the last steps of osteoblast maturation (Baron and Rawadi, 2007).

Considering the current understanding of the role of CGRP in bone formation and repair and the critical role of canonical Wnt signaling in the control of bone development (Rawadi and Roman-Roman, 2005), it is likely that CGRP could promote bone formation through  $\beta$ -catenin signaling modulation. The present study was designed to investigate the effect of CGRP on  $\beta$ -catenin production and expression in primary cultures of human osteoblast-like cells (hOB). As the balance between cell proliferation, cell differentiation and apoptosis determines the size of osteoblast population, it was evaluated whether or not the peptide acts as a survival factor for hOB by inhibiting their apoptosis induced by dexamethasone (Dex) or by serum deprivation, and if this effect was mediated by canonical Wnt signaling.

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**Materials and methods**

Drugs

Human  $\alpha$ CGRP was purchased from Phoenix Europe GmbH (Karlsruhe, Germany); bovine 1-34 parathyroid hormone (PTH), Forskolin (FSK), LiCl and H89 were purchased from Sigma (Milan, Italy).

*Human osteoblast-like cells (hOBs) cultures.*

Human bone cell cultures were established by means of a modified version of the Gehron-Robey and Termine procedure (Gehron Robey and Termine, 1985) using trabecular bone samples obtained from waste material during orthopedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy. None of the operated patients (aged 51–73 yr) had any malignant bone diseases and all of them gave their written consent for the use of the waste material. No significant trend related to donor age was observed in any of the effects studied.

Briefly, the trabecular bone was cut into small pieces and thoroughly washed with commercial standardized Joklik's modified MEM (Sigma, Milan, Italy) serum-free medium, to remove nonadherent marrow cells. The pieces were incubated with rotation at 37°C for 30 min with the same medium containing 0.5 mg/ml type IV collagenase (Sigma, Milan, Italy), and collagenase digestion was stopped by the addition of Iscove's modified Dulbecco's medium (IMDM, Lonza, Walkersville, MD, USA) containing 10% fetal bovine serum (FBS, Euroclone, Milan, Italy). Between eight and ten pieces from each patient were then placed in 25cm<sup>2</sup> flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 U/ml mycostatin, and 0.25µg/ml amphotericin B; the culture medium was changed every 2–3 days. The cells began to migrate within 1–2 weeks and reached confluence after 1 month. The cell population was tested for alkaline phosphatase and osteocalcin production after 1,25(OH)<sub>2</sub>D<sub>3</sub> 10<sup>-8</sup> M to ensure that the cells were endowed with osteoblast characteristics. Cells were used at first passage to reduce the possibility of phenotype changes.

Western Blot analysis

*β-catenin:* Confluent cells were treated with CGRP 10<sup>-8</sup>M for 15, 30 and 60 min; LiCl 20mM and PTH 10<sup>-8</sup>M for 60 min; Forskolin 5µM for 5, 15, 30, 60 and 90 min. The antagonist of CGRP, CGRP<sub>8-37</sub> (10<sup>-8</sup>M) and the inhibitor of PKA activity, H89 (2x10<sup>-6</sup>M) were added 30 min before CGRP and/or PTH.

After removing the medium, adherent cells were gently scraped into 75µl lysis buffer (Hepes 50mM, NaCl 250mM, MgCl<sub>2</sub> 5mM, 1% Triton, 10% Glycerol, protease and phosphatase inhibitors cocktail (1:100, Sigma, Milan, Italy). The lysates were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected and the total protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA). 35µg of total protein extract was mixed with the appropriate

volume of Laemmli's sample loading buffer, heated at 100°C for 5 min and loaded onto 10% SDS polyacrylamide gels. Western blots were performed using a specific antibody against human  $\beta$ -catenin (Cell Signaling Technology, Boston, MA, USA) diluted 1:1000 in 5% milk in Tris HCl with 0.1% Tween20. An antibody against human actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was used as a control.

Nuclear fractions were isolated from untreated cells or cells treated with CGRP ( $10^{-8}$ M) or LiCl (20mM) by the Qproteome Cell Compartment Assay (Qiagen SpA, Milan, Italy). This allows one to selectively isolate proteins in different cell compartments by sequential addition of different extraction buffers to a cell pellet. Scion Image software (Scion Corp., Frederick, MD, USA) was used to quantify the Western blots.  $\beta$ -actin was used as an endogenous control to avoid incorrect estimations of the signal from the protein of interest. The intensity of the treated samples were normalized to that of the untreated samples (set as 1) and presented as a ratio of untreated controls.

Active caspase 3: Serum deprivation was used to induce apoptosis. hOB were treated with CGRP ( $10^{-8}$ M) alone for 6h or pre-treated for 30 min with H89  $2 \times 10^{-6}$ M and then challenged with CGRP for 6h. Cell apoptosis was analysed by measuring the levels of active caspase 3 in the cell lysates according to the above described protocol. Western blots were performed using a specific antibody against human active caspase-3 (Sigma, St Louis, MO, USA) raised in rabbit and diluted 1:500 in 5% milk in Tris HCl with 0.1% Tween20, and a secondary HRP conjugated anti-rabbit antibody (Jackson ImmunoResearch, Baltimore Pike, PA, USA) diluted 1:2000 in 5% milk in Tris HCl 0.1% Tween20. An antibody against human actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was used as a control.

#### Immunofluorescence Staining

Human OB were seeded on coverslips in 24 well plates and allowed to attach overnight. After 24h of starvation, cells were treated for 30 min with CGRP ( $10^{-8}$ M) alone or in association with its receptor antagonist, CGRP<sub>8-37</sub> ( $10^{-8}$ M,) 30 min before CGRP, or with LiCl (20mM). At the end of the treatment cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After permeabilization in 0.25% TritonX100 in PBS (PBST) for 10 min, cells were incubated in 1% BSA in PBST for 30 min to avoid nonspecific binding of the antibody, washed three times in PBS and incubated overnight at 4°C with rabbit anti-human  $\beta$ -catenin antibody (Cell Signaling Technology, Boston MA, USA) diluted 1:50 in PBST. After 3 washes, the cells were incubated for 1h with FITC-labelled anti-rabbit antibody diluted 1:100 (Jackson ImmunoResearch, West Grove, PA, USA). Cells were rinsed 3 times in PBS and mounted on slides using DAPI UltraCruz Mounting Medium (Santa Cruz Biotechnology Inc., Heidelberg, Germany) to identify nuclei. Slides were examined by fluorescent microscopy (Leica DMIRE2, Leica SpA, Milan, Italy) and images were acquired by Leica IM50 image manager software.

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*Apoptosis assay*

Human OB, seeded in 96 black well plates and were allowed to attach overnight. Cells were then treated 1h before dexamethasone (Dex,  $10^{-6}$ M) with CGRP ( $10^{-8}$ M), or LiCl (20mM) or PTH and apoptosis was measured after 6h with. In order to antagonize CGRP effect, cells were pretreated with CGRP<sub>8-37</sub> ( $10^{-8}$ M) or H89 ( $2 \times 10^{-6}$ M) 30 min before CGRP. Apoptosis was assessed by means of Homogeneous Caspases Assay (Roche Diagnostics GmbH, Mannheim, Germany), which is a fluorimetric assay for the in vitro determination of caspases activity

*Statistical Analysis*

Statistical analysis was performed with the statistical package Prism vs 4.00, (GraphPad Software, SanDiego, CA, USA). The significance of the differences among the groups was assessed by means of one way ANOVA for nonparametric values (Kruskal-Wallis) and a multiple-comparison test (Dunn's test).

**Results**

Treatment with  $10^{-8}$ M CGRP for 15 and 60 min, increased cytoplasmic  $\beta$ -catenin levels at all times examined (Fig.1 A,B). Human OB were treated with LiCl (20mM) and PTH ( $10^{-8}$ M) for 60 min as positive controls for the rise in  $\beta$ -catenin protein. As expected, both LiCl and PTH induced an increase in  $\beta$ -catenin levels that were comparable to that induced by CGRP at the corresponding times (Fig.1A,B). Increasing concentrations ( $10^{-11}$ M- $10^{-7}$ M) of CGRP induced a dose-dependent rise in cytoplasmic  $\beta$ -catenin levels 15 min after treatment (Fig.2A,B) reaching statistical significance at  $10^{-8}$ M and  $10^{-7}$ M. Pre-treatment of hOBs with the specific receptor antagonist of CGRP, CGRP<sub>8-37</sub>, at  $10^{-8}$ M, reduced the observed effect of CGRP on  $\beta$ -catenin, thus indicating that the peptide stabilizes  $\beta$ -catenin cytoplasmic protein levels by activating its specific receptors (Fig. 3A,B)

~~To investigate whether CGRP also promotes  $\beta$ -catenin nuclear~~ translocation, nuclear fractions from total cell lysates of hOBs exposed to  $10^{-8}$ M CGRP for 15, 30 and 60 min were obtained and  $\beta$ -catenin levels were measured by Western blot. Nuclear  $\beta$ -catenin levels were significantly enhanced 30 and 60 min after CGRP exposure (Fig. 4A,B). This result was further confirmed by immunofluorescence staining of hOBs. The  $\beta$ -catenin fluorescent signal appeared in the nuclei after 30 min exposure to  $10^{-8}$ M CGRP (Fig. 5C). No stained nuclei were detected when hOBs were pre-treated with equimolar concentrations of CGRP<sub>8-37</sub> and then challenged with ( $10^{-8}$ M) CGRP (Fig.5E) 30 min after. We observed similar nuclear staining as with CGRP when hOBs were treated with 20mM LiCl (Fig.5G).

As Forskolin (5 $\mu$ M), an activator of the cAMP/PKA pathway, significantly increased cytoplasmic  $\beta$ -catenin levels (data not shown), in order to determine if PKA was involved in the effects of CGRP on  $\beta$ -catenin, hOBs were pre-treated with H89, a specific PKA inhibitor. H89 (2 $\times 10^{-6}$ M) pre-treatment prevented CGRP induced  $\beta$ -catenin stabilization as well as the PTH-induced increment of  $\beta$ -catenin levels (Fig.6A,B), thus providing evidence that PKA signaling is involved in the stabilization of the  $\beta$ -catenin levels induced by CGRP. Since it has been reported that PKA is able to inactivate GSK-3 $\beta$  in several tissues, the effect of CGRP on phosphorylation of GSK-3 $\beta$  in the presence or absence of H89 (2 $\times 10^{-6}$ M), was evaluated. CGRP induced GSK-3 $\beta$  phosphorylation after 15 and 60 min, and this effect was prevented by H89, indicating that CGRP modulates  $\beta$ -catenin protein levels by inhibiting GSK-3 $\beta$  through PKA activation (Fig.7A,B). When hOBs were treated for 1h with CGRP (10 $^{-8}$ M) and then challenged for 6h with Dex (10 $^{-6}$ M) apoptosis was significantly reduced as evaluated by the activation of caspases. A similar effect was observed with LiCl (20mM) and PTH (10 $^{-8}$ M) (Fig.8A). Pretreatment with H89 or CGRP<sub>8-37</sub> reduced significantly the antiapoptotic activity of CGRP on Dex induced apoptosis (Fig.8B). When apoptosis was induced by serum deprivation CGRP reduced significantly the protein levels of cleaved caspase 3 as measured by western blot and this effect was inhibited by pretreatment of H89 (Fig.9A,B). These data suggest that PKA/ $\beta$ -catenin pathway are involved in the antiapoptotic effect of CGRP.

## Discussion

The present study demonstrated that CGRP is able to regulate  $\beta$ -catenin signaling by the induction of RNA synthesis as well as by favouring the cytoplasmic accumulation of the protein. This effect is mediated by the activation of the receptor for CGRP, as it is blocked by its antagonist. Human OB express several components of the CGRP receptor: calcitonin receptor-like receptor (CLR) and receptor activity modifying proteins of type 1 and 2 (RAMP1, RAMP2; Villa et al., 2006). The co-expression of CLR and RAMP 1 as a unitary complex, give rise to a receptor with greater affinity for CGRP than for other peptides of the calcitonin family, and is important for the internalization of the ligand-receptor complex after CGRP binding and activation of second messengers (Hilairet et al., 2001).

The effect of CGRP on  $\beta$ -catenin accumulation involves the inactivation of GSK-3 $\beta$  activity via a cAMP/PKA signaling pathway with subsequent enhancement of  $\beta$ -catenin nuclear translocation. Consistent with this observation, other authors have described the involvement of cAMP/PKA signaling in the inactivation of GSK-3 $\beta$  in an osteoblast cell line (Suzuki et al, 2008) and in other cell types (Jensen et al., 2006). GSK-3  $\beta$  phosphorylates  $\beta$ -catenin at Ser33/Ser37/Thr41 sites (Fang et al., 2000), so that the protein is ready for proteosomal degradation and is maintained at low levels

1 in the cytoplasm (Rubinfeld et al., 1996). Like the GSK-3 $\beta$  inhibitor LiCl, CGRP increases  $\beta$ -  
2 catenin levels, leading to the nuclear translocation of the protein, which is the essential step for the  
3 activation of Wnt-related gene transcription (Novak and Dedhar, 1999). Recently, it has been  
4 demonstrated that PKA, besides inactivating GSK-3 $\beta$  activity, can phosphorylate  $\beta$ -catenin at  
5 Ser552 and Ser675 sites and this phosphorylation promotes transcriptional activity of  $\beta$ -catenin in  
6 vascular smooth muscle cells (Taurin et al., 2008). This molecular mechanism of PKA-dependent  
7 activation of  $\beta$ -catenin represents a new mode of  $\beta$ -catenin regulation that could be involved in  
8 CGRP induced  $\beta$ -catenin cytoplasmic accumulation.

15 The modulation of the Wnt/ $\beta$ -catenin signaling pathway in hOBs by CGRP further supports the  
16 view that the peptide plays a role in the physiology of bone cells and particularly of osteoblasts (see  
17 Naot and Cornish, 2008 for review). If it is generally accepted that the Wnt pathway contributes to  
18 the promotion of osteoblastogenesis from mesenchymal cells, to the activation of osteoblasts and to  
19 bone mass accrual, the CGRP anabolic effect on bone could be explained, at least in part, by the  
20 cross-talk between CGRP and Wnt signaling.

26 In the present study, CGRP exhibited an anti-apoptotic effect in hOB comparable to that observed  
27 with LiCl and PTH. It is likely that CGRP promotes hOB survival through  $\beta$ -catenin cytoplasmic  
28 accumulation induced by PKA activation since inhibition of the enzyme activity by H89 reduces  
29 CGRP effect. The finding that CGRP anti apoptotic effect is receptor mediated is shown by the  
30 inhibition obtained with the CGRP antagonist and fits with previous studies that demonstrated that  
31 CGRP receptor activation prevents osteoblast apoptosis in MC3T3-E1, a cloned mouse osteoblast  
32 line (Uzan et al., 2008), and that adrenomedullin, a member of the calcitonin family of peptides,  
33 inhibits rheumatoid fibroblast-like synoviocyte apoptosis through activation of CGRP receptor  
34 (Uzan et al.,2006).

42 Although it is not yet clear to what extent the effect of Wnt/ $\beta$ -catenin signaling on osteoblast  
43 apoptosis contributes to the overall impact of this pathway on bone formation (Bodine, 2008), it is  
44 likely that the CGRP modulation of  $\beta$ -catenin signaling and its anti-apoptotic effect could be  
45 involved in the anabolic activity of the peptide on bone mass. Cell apoptosis plays a critical role  
46 during skeletal development and maturation, bone turnover, fracture repair and bone degeneration.  
47 The balance between osteoblast proliferation, differentiation and apoptosis determines the amount  
48 of competent osteoblasts which subsequently affects the activity of the bone multicellular units at  
49 each remodeling site. Consistent with this notion, the high bone mass phenotype of mice expressing  
50 an activating mutation of LRP5 is associated with decreased osteoblast apoptosis, increased  
51 osteoblast number and activated bone formation rate (Boyden et al., 2002). Also, the increase of  
52 cancellous bone following PTH treatment is associated with inhibition of osteoblast apoptosis (Jilka  
53 et al., 1999).

The modulatory function of CGRP on bone remodeling together with the anti-apoptotic activity could favour the gain of bone mass, depending on the local demand. This may be important at areas of high osteogenic capacity, such as the periosteum, bone marrow and along the osteochondral junction of the epiphyseal growth plate (Sisask et al., 1995), where the neuronal presence of CGRP is detected in a dense net-like meshwork (Martin et al., 2007). This evidence, combined with the present results, suggests an afferent-efferent loop involving the perception of a local demand for bone formation. In fact, CGRP can be released by nerve fibers and could exert paracrine biological effects on bone cells, having functional significance in the local regulation of bone remodelling.

The peptide could be important in reaction to bone fractures, where an increase of CGRP positive nerve fibers was detected (Hukkanen et al., 1993). Fracture repair is a dynamic process that involves the coordination of cell proliferation and differentiation, apoptosis, angiogenesis and remodelling: all events potentially affected by CGRP and, at the same time, under the regulatory action of Wnt/ $\beta$ -catenin signaling. Besides an increment of neuronal CGRP immunoreactivity at the fracture site (Li et al., 2007), Wnt/ $\beta$ -catenin signaling is activated during the fracture healing process (Silkstone et al., 2008). CGRP-positive nerve fibers were found in close proximity to osteoblasts at active sites of bone formation (Imai et al., 1997; Irie et al., 2002). And, during fracture healing, nerve fibers containing CGRP were observed in fracture callus, with more pronounced immunoreactivity in the concave side of angulated fractures where the main osteogenic activity is operative (Li et al., 2007). It is interesting to note that PTH, which activates Wnt/ $\beta$ -catenin signaling through PKA, administered during fracture repair leads to increased fracture callus (Kakar et al., 2007).

In conclusion, from the present results it emerges that CGRP in hOBs activates the Wnt pathway by inhibiting GSK-3 $\beta$  through PKA signaling, thus promoting osteoblasts' survival via  $\beta$ -catenin nuclear translocation. This study has further characterized the cellular events that underlie the anabolic action of CGRP in osteoblasts, suggesting an involvement of CGRP in the modulation of bone tissue metabolism and repair.

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## References

- Akopian A, Demulder A, Ouriaghli F, Corazza F, Fondu P, Bergmann P. 2000. Effects of CGRP on human osteoclast-like cells formation: A possible connection with the bone loss in neurological disorders? *Peptides* 21:559-564.
- Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. 2005. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *J Biol Chem* 280:41342-51.
- Amara SG, Jones V, Rosenfeld MG, Ong ES, Evans RM. 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptides products. *Nature* 29: 240-249.
- Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Bouxsein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA, Bex F. 2003. High bone mass in mice expressing a mutant LRP5 gene. *J Bone Miner Res* 18:960-74.
- Ballica R, Valentijn K, Khachatryan A, Guerder S, Kapadia S, Gundberg C, Gilligan T, Flavell RA, Vignery A. 1999. Targeted expression of calcitonin gene-related peptide to osteoblasts increase bone density in mice. *J Bone Miner Res* 14: 1067-1074.
- Baron R, Rawadi G. 2007. Targeting the Wnt/beta-catenin pathway to regulate bone formation in the adult skeleton. *Endocrinology* 148:2635-43.
- Bodine PV. 2008. Wnt signaling control of bone cell apoptosis. *Cell Res* 18:248-53.
- Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP. 2002. High bone density due to a mutation in LDL-receptor-related protein 5. *N Engl J Med* 346:1513-21.
- Burns DM, Stehno-Bittel L, Kawase T. 2004. Calcitonin gene-related peptide elevates calcium and polarizes membrane potential in MG-63 cells by both cAMP-independent and -dependent mechanisms. *Am J Physiol Cell Physiol* 287:C457-C467.
- Drissi H, Hott M, Marie PJ, Lasmoles F. 1997. Expression of the CT/CGRP gene and its regulation by dibutyryl cyclic adenosine monophosphate in human osteoblastic cells. *J Bone Miner Res* 12:1805-14.
- Drissi H, Lieberherr M, Hott M, Marie PJ, Lasmoles F. 1999. Calcitonin gene-related peptide (CGRP) increases intracellular free  $\text{Ca}^{2+}$  concentrations but not cyclic AMP formation in CGRP receptor-positive osteosarcoma cells (OHS-4). *Cytokine* 11: 200-207.
- Fang X, Yu SX, Lu Y, Bast RC jr, Woodgett JR, Mills GB. 2000. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc Natl Acad Sci USA* 97:11960-11965.
- Gehron-Robey P, Termine JD. Human bone cells in vitro. 1985. *Calcif Tissue Int* 37: 453-460.

- Hilairt S, Belanger C, Bertrand J, Laperrière A, Foord SM, Bouvier M. 2001. Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1) and  $\beta$ -arrestin. *J Biol Chem* 276: 42182-42190.
- Hukkanen M, Konttinen YT, Santavirta S, Paavolainen P, Gu XH, Terenghi G, Polak JM. 1993. Rapid proliferation of calcitonin gene-related peptide-immunoreactive nerves during of rat healing fracture suggests neural involvement in bone growth and remodeling. *Neuroscience* 54:969-979.
- Imai S, Rauvala H, Konttinen YT, Tokunaga T, Maeda T, Hukuda S, Santavirta S. 1997. Efferent targets of osseous CGRP-immunoreactive nerve fiber before and after bone destruction in adjuvant arthritic rat: an ultramorphological study on their terminal-target relations. *J Bone Miner* 12: 1018-1027.
- Irie K, Hara-Irie F, Ozawa H, Yajima T. 2002. Calcitonin gene-related peptide (CGRP)-containing nerve fibers in bone tissue and their involvement in bone remodeling. *Microsc Res Tech* 58: 85-90.
- Jensen J, Brennesvik EO, Lai YC, Sheperd PR. 2006. GSK-3 $\beta$  regulation in skeletal muscles by adrenaline and insulin: Evidence that PKA and PKB regulate different pools of GSK-3. *Cell signal* 19:204:210.
- Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. 1999. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J Clin Invest* 104:439-446.
- Kakar S, Einhorn TA, Vora S, Miara LJ, Hon G, Wigner NA, Toben D, Jacobsen KA, Al-Sebaei MO, Song M, Trackman PC, Morgan EF, Gerstenfeld LC, Barnes GL. 2007. Enhanced chondrogenesis and Wnt signaling in PTH-treated fractures. *J Bone Miner Res* 22:1903-1912.
- Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass 2<sup>nd</sup> DA, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G, Chan L. 2002. *Cbfa1* independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in LRP5, a Wnt coreceptor. *J Cell Biol* 157: 303-314.
- Krishnan V, Bryant HU, Macdougald OA. 2006. Regulation of bone mass by Wnt signaling. *J Clin Invest* 116:1202-9.
- Li J, Kreicbergs A, Bergstrom J, Stark A, Ahmed M. 2007. Site-specific CGRP innervation coincides with bone formation during fracture healing and modeling: a study in rat angulated tibia. *J Orthop Res* 25: 1204-1212.
- Martin CD, Jimenez-Andrade JM, Ghilardi JR, Mantyh PW. 2007. Organization of a unique net-like meshwork of CGRP+ sensory fibers in the mouse periosteum: implications for the generation and maintenance of bone fracture pain. *Neurosci Lett* 427:148-52.
- Morvan F, Boulukos K, Clément-Lacroix P, Roman Roman S, Suc-Royer I, Vayssi re B, Ammann P, Martin P, Pinho S, Pognonec P, Mollat P, Niehrs C, Baron R, Rawadi G. 2006. Deletion of a

- single allele of the *Dkk1* gene leads to an increase in bone formation and bone mass. *J Bone Miner Res* 21:934-45.
- Naot D, Cornish J. 2008. The role of peptides and receptors of the calcitonin family in the regulation of bone metabolism. *Bone* 43:813-8.
- Novak A, Dedhar S. 1999. Signaling through Beta-catenin and Lef/Tcf. *Cellular and Molecular Life Science* 56: 523-537.
- Rawadi G, Roman-Roman S. 2005. Wnt signaling pathway: a new target for the treatment of osteoporosis. *Expert Opin Ther Targets* 9:1063-77.
- Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. 1996. Binding of GSK3 $\beta$  to the APC- $\beta$ -catenin complex and regulation of complex assembly. *Science* 272: 1023-1026.
- Schinke T, Liese S, Priemel M, Haberland M, Schilling AF, Catala-Lehnen P, Blicharski D, Rueger JM, Gagel RF, Emeson RB, Amling M. 2004. Decreased bone formation and osteopenia in mice lacking alpha-calcitonin gene-related peptide. *J Bone Miner Res*. 19:2049-56.
- Silkstone D, Hong H, Alman BA. 2008. Beta-catenin in the race to fracture repair: in it to Wnt. *Nat Clin Pract Rheumatol* 4:413-9.
- Sisask G, Bjurholm A, Ahmed M, Kreicbergs A. 1995. Ontogeny of sensory nerves in the developing skeleton. *Anat Rec* 243:234-40.
- Suzuki A., Ozono K., Kubota T., Kondou H., Tachikawa K., Michigami T. 2008. PTH/cAMP/PKA signaling facilitates canonical Wnt signaling via inactivation of glycogen synthase kinase-3 $\beta$  in osteoblastic Saos-2 cells. *J Cell Biochem* 104:304-317.
- Taurin S, Sandbo N, Yau DM, Sethakorn N, Dulin NO. 2008. Phosphorylation of beta-catenin by PKA promotes ATP-induced proliferation of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 294:C1169-74.
- Uzan B, Ea HK, Launay JM, Garel JM, Champy R, Cressent M, Lioté F. 2006. A critical role for adrenomedullin-calcitonin receptor-like receptor in regulating rheumatoid fibroblast-like synoviocyte apoptosis. *J Immunol* 176:5548-58.
- Uzan B, Vilemin A, Garel JM, Cressent M. 2008. Adrenomedullin is anti-apoptotic in osteoblasts through CGRP1 receptors and MEK-ERK pathway. *J Cell Physiol* 215:122-128.
- Villa I, Melzi R, Pagani F, Ravasi F, Rubinacci A, Guidobono F. 2000. Effects of calcitonin gene-related peptide and amylin on human osteoblast-like cells proliferation. *Eur J Pharmacol* 409: 273-278.
- Villa I, Dal Fiume C, Maestroni A, Rubinacci A, Ravasi F, Guidobono F. 2003. Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity. *Am J Physiol Endocrinol Metab* 284: E627-E633.

Villa I, Mrak E, Rubinacci A, Ravasi F, Guidobono F. 2006. CGRP inhibits osteoprotegerin production in human osteoblast-like cells via cAMP/PKA-dependent pathway. *Am J Physiol Cell Physiol* 291 : C529-C537.

Wang L, Shi X, Zhao R, Halloran BP, Clark DJ, Jacobs CR, Kingery WS. 2009. calcitonin-gene-related peptide stimulates stromal cell osteogenic differentiation and inhibits RANKL induced NF- $\kappa$ B activation, osteoclastogenesis and bone resorption. *Bone*, doi :10.1016/j.bone.2009.11.029.

Zhang WZ, Yu SF, Zheng LF. 1994. Effects of calcitonin gene-related peptide on bone resorption mediated by interleukin-1. *Chin Med J (Engl)* 107:351-4.

For Peer Review

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**Legends to figures**

**Fig. 1.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of  $\beta$ -catenin protein after treatment, at different times (15, 60 min) with CGRP( $10^{-8}$ M), LiCl (20mM) or PTH ( $10^{-8}$ M), in the total protein extracts of hOBs. Since LiCl and PTH are generally acknowledged as activators of  $\beta$ -catenin signaling, they were used as positive controls. Results are expressed as ratio of  $\beta$ -catenin amount measured in treated cells to that obtained in untreated cells. Data are the means  $\pm$  SEM of four experiments performed with cells obtained from four different donors; \*  $p<0.05$  vs untreated cells, ANOVA for non parametric data with multiple comparison test.

**Fig. 2.** Representative Western blot (A) and western blot quantification (B, as described in Materials and Methods) of the effect of 15 min treatment with increasing concentrations of CGRP ( $10^{-11}$ M- $10^{-7}$ M) on  $\beta$ -catenin protein in total hOB protein extracts. Results are expressed as a ratio of  $\beta$ -catenin amount measured in treated cells to the amount obtained in untreated cells. Data are the mean  $\pm$  SEM of three experiments performed with cells obtained from three different donors; \*  $p<0.05$  vs untreated cells

**Fig. 3.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of  $\beta$ -catenin measured in total hOB protein extracts. Results of cells treated with CGRP ( $10^{-8}$ M) after pre-treatment with the antagonist CGRP<sub>8-37</sub> ( $10^{-8}$ M, 30min before). Results are expressed as a ratio of  $\beta$ -catenin amount measured in treated cells to the amount obtained in untreated cells. Data are the mean  $\pm$  SEM of five experiments performed with cells obtained from five different donors; \*\*  $p<0.01$  vs untreated cells, §  $p<0.05$  vs cells treated with CGRP<sub>8-37</sub> plus CGRP; ANOVA for non parametric data with multiple comparison test.

**Fig. 4.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of  $\beta$ -catenin protein at different times after CGRP treatment in the nuclear extract of hOBs. LiCl was used as control of  $\beta$ -catenin nuclear localization. Results are expressed as a ratio of  $\beta$ -catenin amount measured in treated cells to the amount obtained in untreated cells. Data are the mean  $\pm$  SEM of six experiments performed with cells obtained from six different donors; \*\*  $p<0.01$ , \*  $p<0.05$  vs untreated cells, ANOVA for non parametric data with multiple comparison test.

**Fig. 5.** Immunofluorescence staining of  $\beta$ -catenin in hOBs after treatment with CGRP. A, untreated cells; B, hOBs treated with CGRP  $10^{-8}$ M for 30 min; C, hOBs treated with CGRP<sub>8-37</sub> 30 min before CGRP exposure; D, hOBs treated with LiCl 20mM as positive control of  $\beta$ -catenin nuclear localization. E, F, G, H, DAPI staining of the nuclei of the corresponding cells.

**Fig. 6.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of the effect of pre-treatment with a PKA inhibitor, H89 ( $2 \times 10^{-6}$ M) 30 min before CGRP ( $10^{-8}$ M) or PTH<sub>1-34</sub> ( $10^{-8}$ M), used as a positive control, on  $\beta$ -catenin levels in total hOB protein

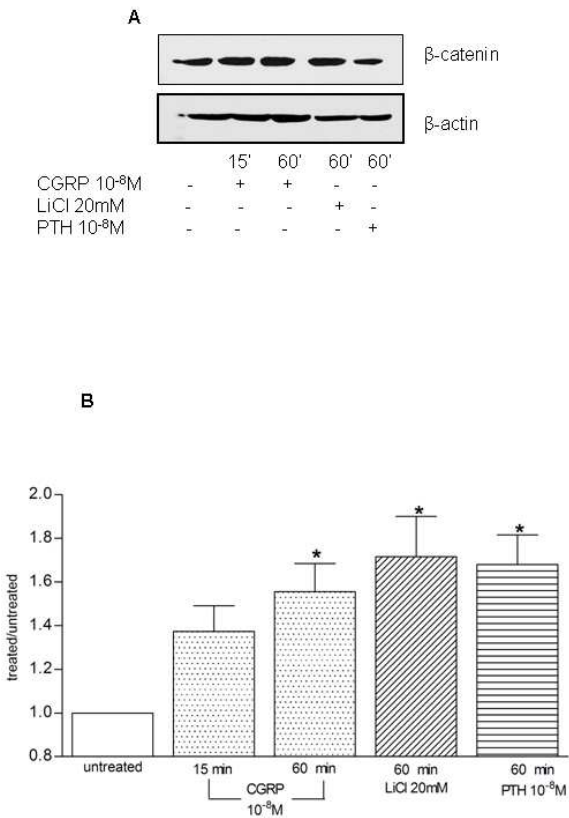
extracts. Data are the mean  $\pm$  SEM of four experiments performed with cells obtained from four different donors; \*\*  $p < 0.01$ , \*  $p < 0.05$  vs untreated, §  $p < 0.05$  vs CGRP treated cells, ●  $p < 0.05$  vs PTH treated cells; ANOVA for non parametric data with multiple comparison test.

**Fig. 7.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of the effect of pre-treatment with H89, 30 min before CGRP ( $10^{-8}$ M) treatment for 15 and 60 min on the phosphorylated form of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). Data are the mean  $\pm$  SEM of three experiments performed with cells obtained from three different donors; \*  $p < 0.05$  vs untreated, §  $p < 0.05$  vs CGRP treated cells; ANOVA for non parametric data with multiple comparison test.

**Fig. 8, A.** Effect CGRP ( $10^{-8}$ M), LiCl (20mM) and PTH ( $10^{-8}$ M), used as positive controls, on dexamethasone (Dex,  $10^{-6}$ M) induced apoptosis in hOBs measured as Relative Fluorescence Units. Data are the mean  $\pm$  SEM of the ratio of treated to Dex treated cells. The assay was performed three times using cells derived from three different donors and each experimental point was assayed in quintuplicate. **B.** Effect of pre-treatment with H89 or with the CGRP antagonist, CGRP<sub>8-37</sub> on CGRP antiapoptotic effect on Dex ( $10^{-6}$ M) induced apoptosis in hOBs. The assay was performed six times using cells derived from six different donors and each experimental point was assayed in triplicate; \*\*  $p < 0.01$  vs Dex-treated, §  $p < 0.05$  vs CGRP treated cells; ANOVA for non parametric data with multiple comparison test.

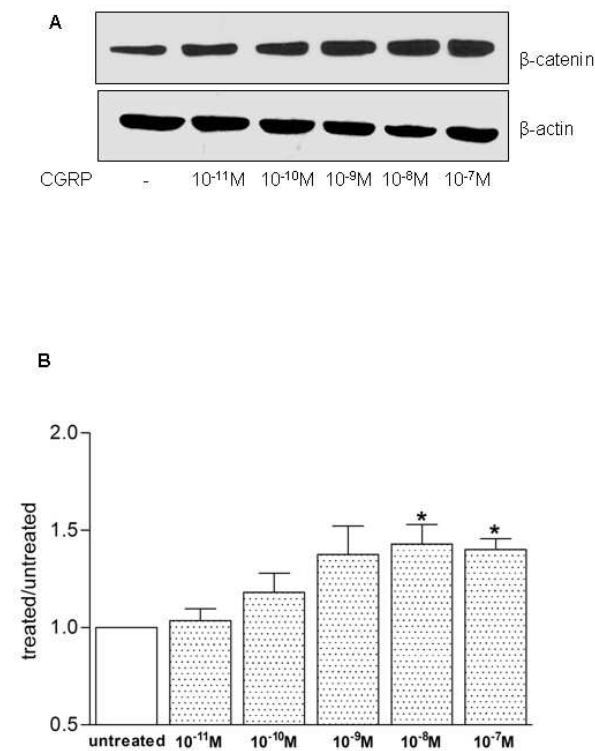
**Fig. 9.** Representative Western blot (A) and quantification (B, as described in Materials and Methods) of the protein levels of the cleaved form of caspase 3, as an index of hOB apoptosis, induced by serum deprivation (w/oFBS). Effect of 6h treatment with CGRP ( $10^{-8}$ M) and of the pre-treatment 30 min before CGRP with the PKA inhibitor H89. Data are the mean  $\pm$  SEM of three experiments performed with cells obtained from three different donors; \*\*  $p < 0.01$  vs w/oFBS; §  $p < 0.05$  vs H89+CGRP treated cells; ANOVA for non parametric data with multiple comparison test.

Fig. 1



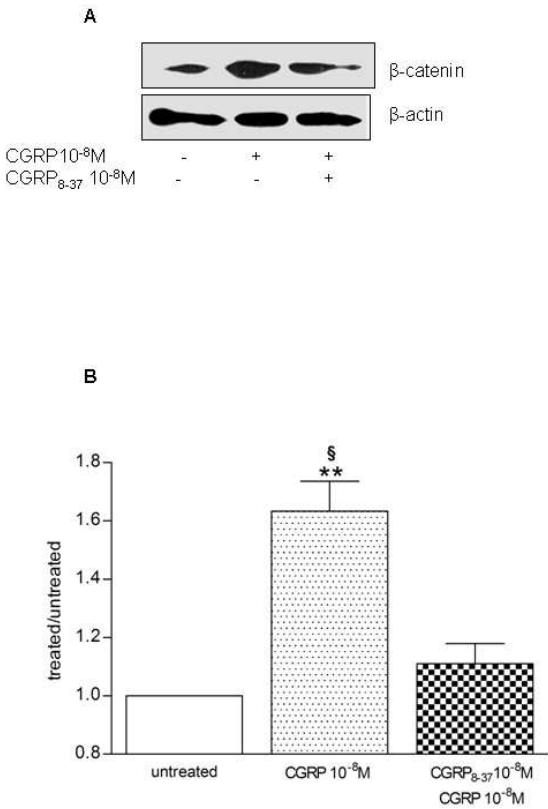
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Fig. 2



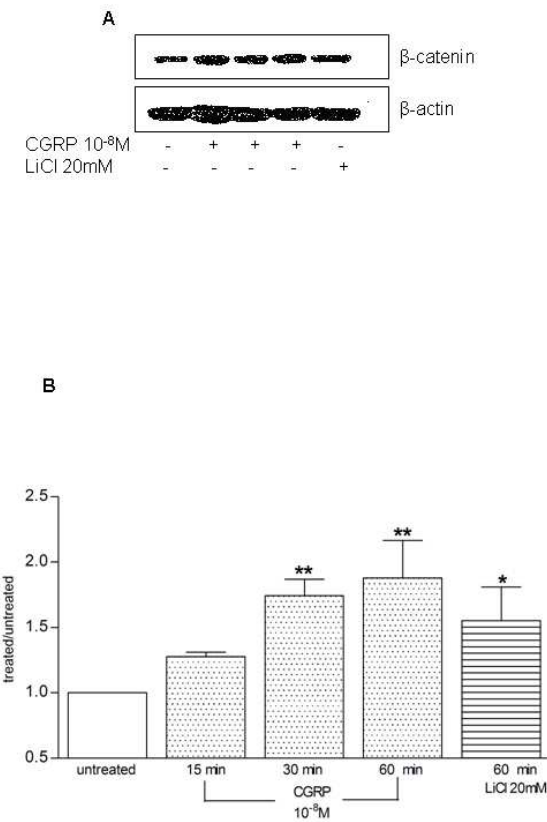
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Fig. 3



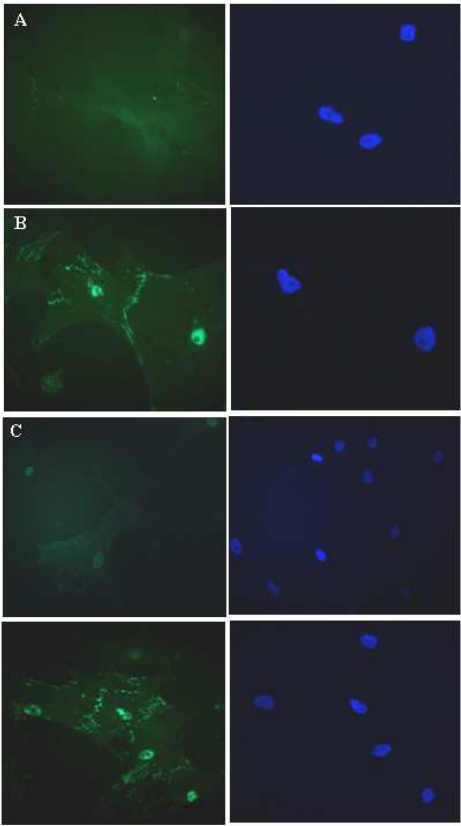
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Fig. 4



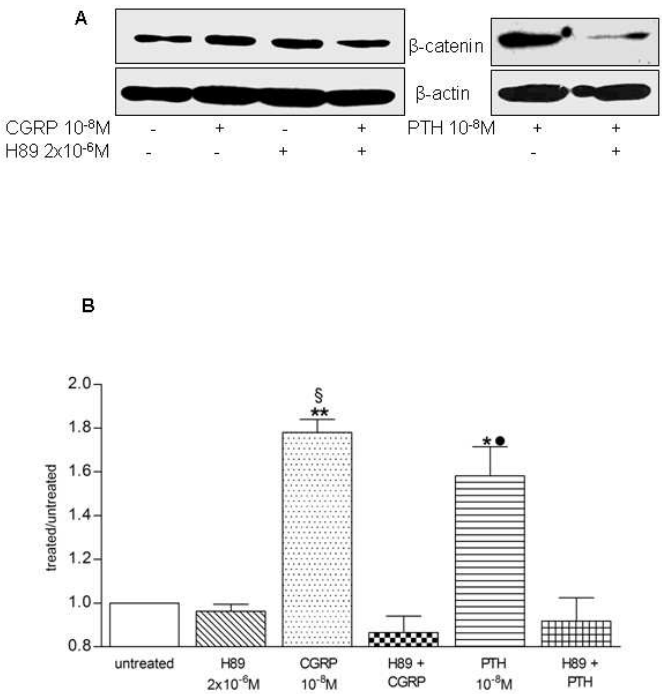
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Fig. 5



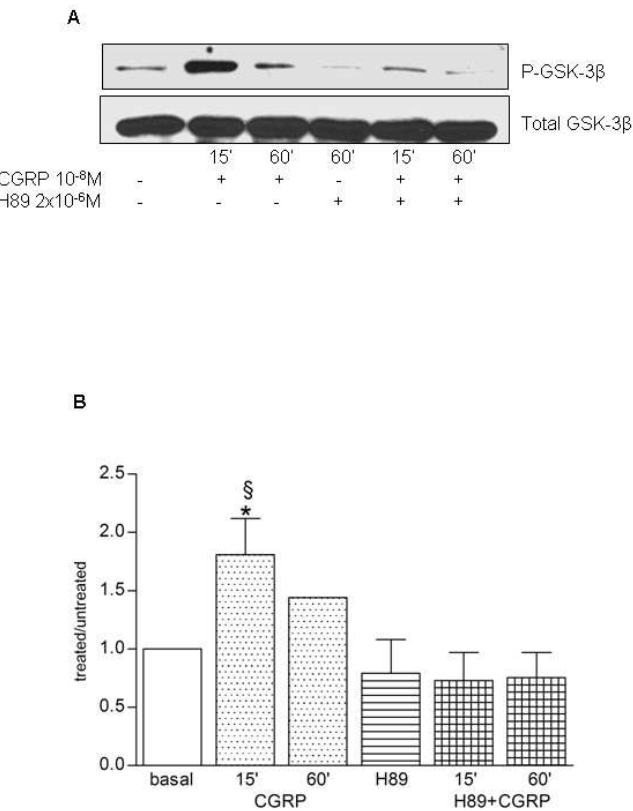
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Fig. 6



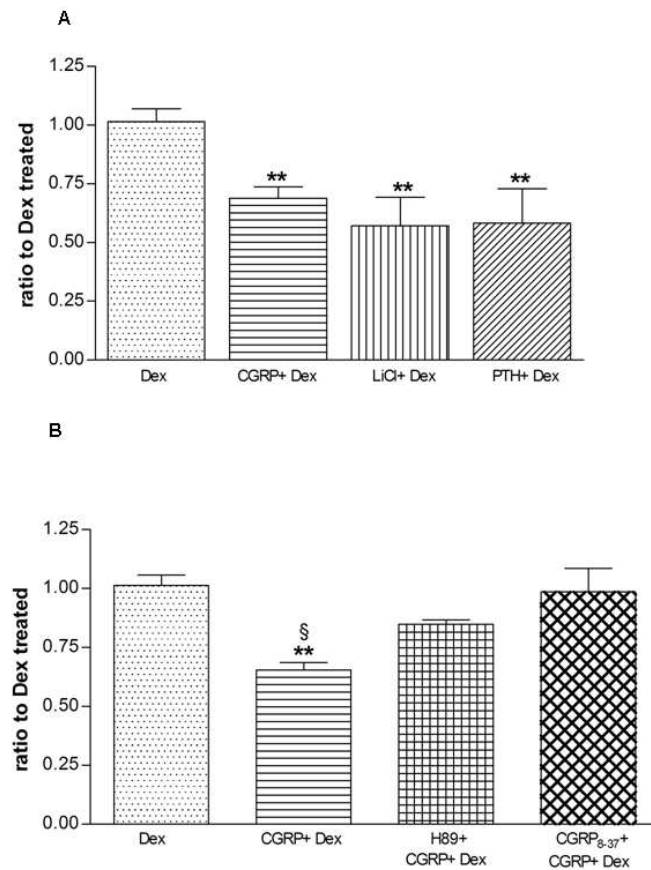
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Fig. 7



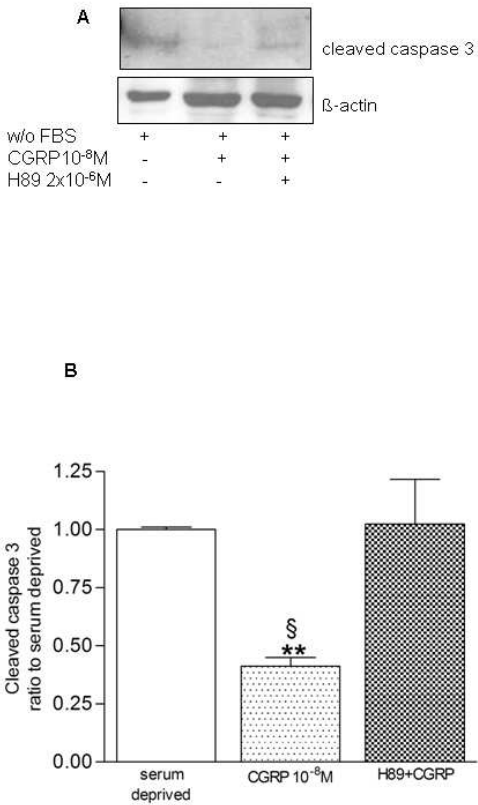
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Fig. 8



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Fig. 9



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