Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 343-351

HAPTOGLOBIN SYNERGISTICALLY POTENTIATES BRADYKININ AND THROMBIN INDUCED PROSTAGLANDIN BIOSYNTHESIS IN ISOLATED OSTEOBLASTS

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Received May 24, 1991

Abstract: Haptoglobin of two different phenotypes (Hp 1-1 and Hp 2-1) dose-dependently (1-4 mg/ml) stimulated the formation of prostaglandin E_2 (PGE_2) in osteoblast-like cells isolated from neonatal mouse calvarial bones. The degree of stimulation obtained by haptoglobins (4 mg/ml) on PGE_2 biosynthesis was in the same range as that caused by bradkykinin (1 μmol/l). Pretreatment of osteoblasts with Hp 1-1 or Hp 2-1 (1-4 mg/ml) resulted in a dose-dependent, synergistic potentiation of the stimulatory effect of bradykinin (1 μmol/l) on PGE_2 formation. Thrombin (7 U/ml) stimulated PGE_2 formation in the osteoblast-like cells by a mechanism that was also synergistically potentiated by haptoglobin (2 mg/ml). These data show that haptoglobin per se stimulates PGE_2 biosynthesis in isolated osteoblasts and, in addition, synergistically potentiates the effect of bradykinin and thrombin. Consequently, the enhanced production of haptoglobin seen in different inflammatory processes may contribute to the destruction of bone by inducing the formation of prostanoids capable of stimulating bone resorption.

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Bone destruction is a common finding in association with inflammatory lesions located within or in the vicinity of the skeleton (e.g. rheumatoid arthritis, periodontitis and osteomyelitits). The stimulation of bone resorption in such conditions has been suggested to be due to local formation of inflammatory mediators which, in addition to their local effects on the inflammatory response, have the capacity to activate bone resorbing osteo-

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clasts. Thus, interleukin 1 (IL-1; 1,2), tumor necrosis factors α and β (TNF α and β; 3,4), bradykinin (5), thrombin (6) and prostanoids (7) have been suggested as candidates responsible for osteoclast activation in inflammation induced bone resorption.

In inflammatory conditions, however, not only the production of local factors is induced, but also the formation of acute phase reactants in the liver. Although the induction of these liver proteins in association with inflammatory lesions has been known for several years little is known about their actions. Haptoglobin is one of the classical acute phase proteins. Recently, we found that haptoglobin can stimulate bone resorption and prostanoid formation in cultured neonatal mouse calvarial bones (Lerner & Fröhlander, unpublished observations). Consequently, the possibility may exist that not only paracrinally acting factors may be involved in the activation of osteoclasts in inflammation induced bone resorption, but that also systemic factors may contribute to the process by an endocrine mechanism. Since several stimulators of bone resorption (e.g. parathyroid hormone, $1,25(OH)_2$-vitamin D3, IL-1 and TNFs) act primarily via specific receptors on osteoblasts (8,9), we have in the present investigation studied if these cells are responsive to haptoglobin by analyzing the effect of haptoglobin on the biosynthesis of prostaglandin E_2 (PGE_2) in osteoblast-like cells from neonatal mouse calvaria.

Materials and Methods

Purification of haptoglobins. Human plasma was obtained from the Blood Bank at the University Hospital of Umeå. Haptoglobin phenotypes were determined by horizontal 7% polyacrylamide gel electrophoresis in a discontinous buffer system followed by non-specific protein staining with Coomassie blue. Each haptoglobin phenotype was purified by affinity chromatography. Hydrazide activated Sepharose (Affi-Gel Hz; Biorad, Ca, USA) was prepared accoring to the manufactures specification using anti-Hp (Dakopatts, Copenhagen, Denmark) as ligand. Whatman filtered plasma was applied and the column was washed with phosphate buffered saline (PBS) pH 7.4 until $A_{280}<0.005$, after which haptoglobin was eluted with 5 mol/l urea. Elution fractions with

A_{280}>0.1 were pooled and dialyzed against 4 L 0.02 mol/l $(NH_4)_2CO_3$ with several changes. Each retentate was concentrated using immersible ultrafilters (CX-30; Millipore, Corp, Bedford, MA, USA). Column regeneration was accomplished by two column volumes 0.5 mol/l NaCl in PBS pH 7.4, followed by two column volumes PBS.

Purity of each haptoglobin preparation was checked by two-dimensional immunoelectrophoresis (IEP), with anti-human serum (Dakopatts) in the second dimension gel. Preparations with only one peak, corresponding to the peak of a two-dimensional IEP using anti-haptoglobin in the second dimension gel, were subsequently used. Using double diffusion, immunoglobulins were occasionally detected. However, in the absence of any detectable peak on the two-dimensional IEP, these admixtures were considered negligeable. To eliminate the possibility of contaminating endotoxins, the preparations were passed twice through an endotoxin-binding column (Detoxi-gel, Pierce, Rockford, Ill., USA).

Haptoglobin preparations of each phenotype were pooled and concentrated using Amicon ultrafilters (YM 30). The retentates were then reconstituted in α-MEM culture medium and the ultrafiltration process was repeated twice followed by sterile filtration. Prior to experiments, the concentration of haptoglobin in the culture medium was analyzed by immunonephelometry at the Clinical Chemistry Laboratory, University Hospital of Umeå.

Isolation of osteoblasts. Calvarial bones from 2-3 days-old mice were dissected out and bone cells were isolated according to the time sequential enzymatic digestion method described by Boonekamp et al. (10). In brief, the calvaria were incubated in phosphate-buffered saline (PBS) containing EDTA (0.4 mmol/l) for 3 x 10 min, washed with PBS and subsequently treated with bacterial collagenase (180 mU/ml) in PBS without EDTA for 4 x 10 min. The cells from the last two digestion fluids were collected, centrifuged, resuspended in a-MEM with 10% fetal calf serum (FCS), seeded in 25 cm^2 tissue culture flasks and grown to confluency. The cells were then reseeded in 2 cm^2 multiwell culture dishes and grown in monolayers to approximately 80% confluency. These cells showed an abundant cyclic AMP response after challenge with parathyroid hormone and were insensitive to calcitonin, indicating an osteoblastic phenotype.

Determination of prostaglandin production. Prior to experiments, the cell layers were rinsed twice with Tyrode's solution and then preincubated for 30 min in α-MEM without FCS. The media were then removed and serumfree media with or without different test substances were added. At the end of the experiments, the media were withdrawn, acidified, frozen and stored at -20°C. The amounts of PGE_2 were later determined using a commercially available radioimmunoassay kit with ^{125}I-PGE_2 as tracer. After the experiments the cells were detached and counted in an haemocytometer.

Statistics. Statistical evaluation was performed with Student's two-tailed t test for unpaired samples.

Results

Treatment of osteoblast-like cells from mouse calvarial bones for 30 min with Hp 1-1 or Hp 2-1, at concentrations from 1-4 mg/ml, resulted in a dose-related enhanced formation of PGE_2 (Fig. 1 a,b). The effect of the haptoglobins was statistically significant ($P<0.01$) at all concentrations tested. The magnitude of the PGE_2 response to haptoglobin was indentical to (Hp 2-1) or slightly above (Hp 1-1) that induced by an optimal concentration of bradykinin (1 μmol/l; Fig. 1 a,b). The stimulatory effect of haptoglobins on PGE_2 formation in osteoblast-like cells was observed in three independent experiments.

When osteoblasts were pretreated with Hp 2-1 (2 mg/ml) for 30 min and then incubated with or without bradykinin (1 μmol/l) for 5 min, we found that pre-exposure to haptoglobin resulted in a synergistic potentiation of bradykinin induced PGE_2 formation (Fig. 2). A similar synergistic potentiation

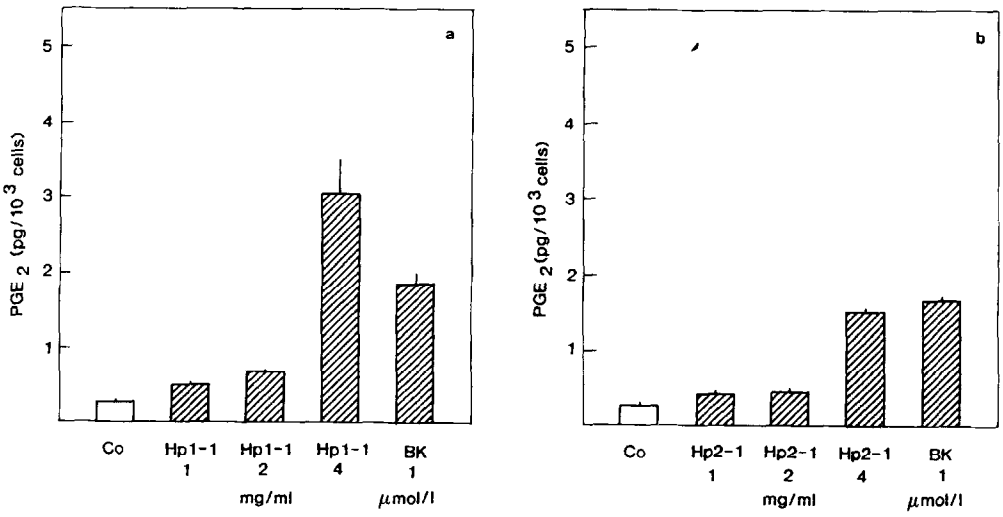

Figure 1. The effect of Hp 1-1 (a) and Hp 2-1 (b), at different concentrations, on prostaglandin E_2 biosynthesis in osteoblast-like cells from neonatal mouse calvarial bones. For comparison is shown the effect of bradykinin.
The effects of haptoglobin, at all concentrations, and bradykinin were statistically significant ($P<0.01$).

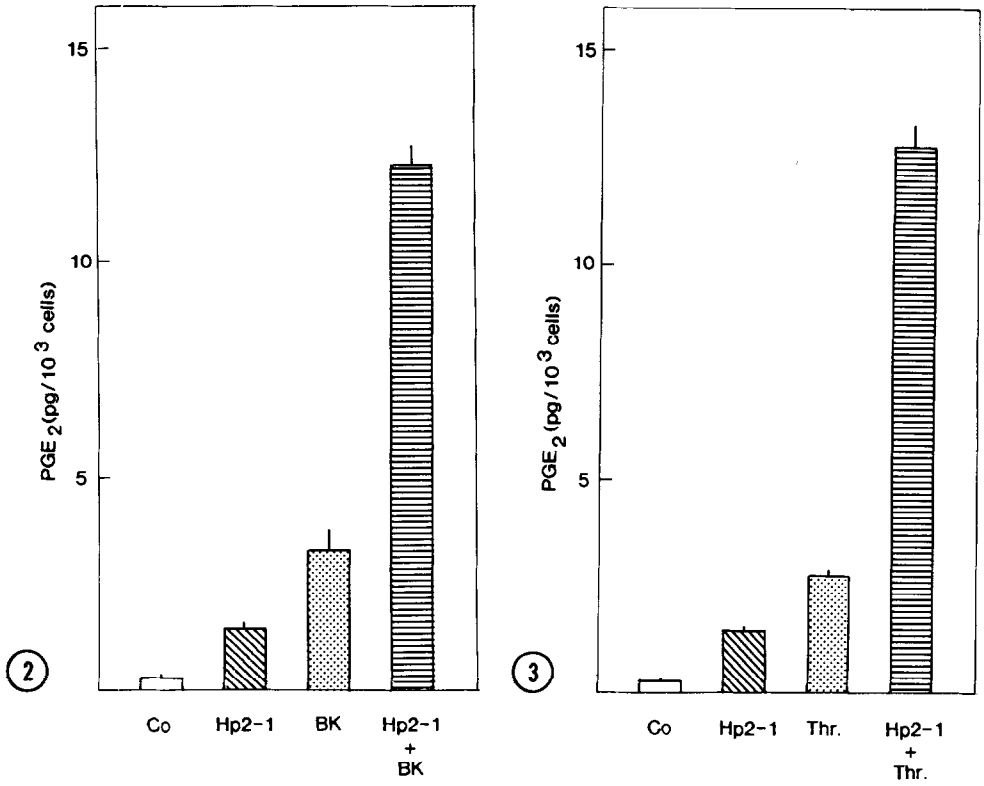

Figure 2. The effect of Hp 2-1 (2 mg/ml) and bradykinin (1 μmol/l) and their combinations on prostaglandin E_2 biosynthesis in mouse osteoblast-like cells. Cells were pretreated with haptoglobin for 30 min and then challenged with bradykinin in the absence and presence of haptoglobin.
The effects of bradykinin per se and haptoglobin without and with bradykinin were statistically significant ($P<0.01$). The effect of haptoglobin + bradykinin was statistically significant from those of bradykinin and haptoglobin per se ($P<0.01$).

Figure 3. The effect of Hp 2-1 (2 mg/ml) and thrombin (7 U/ml) and their combinations on prostaglandin E_2 biosynthesis in mouse osteoblast-like cells. Cells were pretreated with haptoglobin for 30 min and then challenged with thrombin in the absence and presence of haptoglobin.
The effects of thrombin per se and haptoglobin without and with thrombin were statistically significant ($P<0.01$). The effect of haptoglobin + thrombin was statistically significant from those of haptoglobin and thrombin per se ($P<0.01$).

of bradykinin induced PGE_2 formation was seen also after pretreatment of osteoblast-like cells with Hp 1-1 or 2-2 (2 mg/ml; data not shown).

Cells pre-exposed to Hp 2-1 (2 mg/ml) for 30 min responded to thrombin (7 U/ml) with an abundant enhanced stimulation of PGE_2 release due to a synergistic interaction between haptoglobin and thrombin (Fig. 3). We also found that Hp 1-1 (2 mg/ml) and Hp 2-2 (2 mg/ml) synergistically

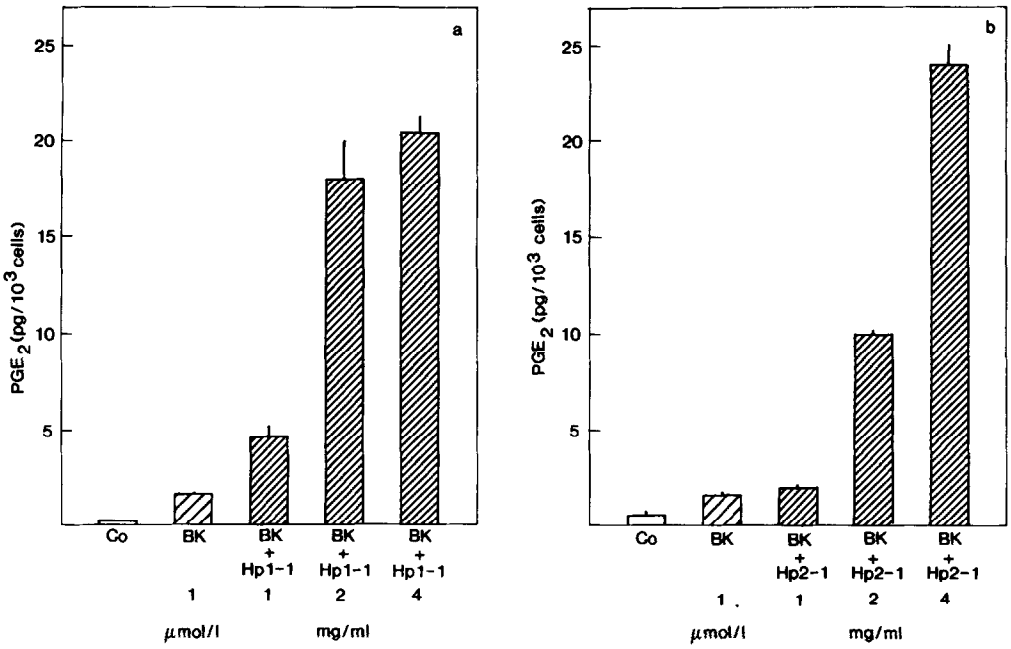

Figure 4. The potentiation by Hp 1-1 (a) and Hp 2-1 (b), at different concentrations, on bradykinin induced prostaglandin E_2 biosynthesis in mouse osteoblast-like cells.

potentiated the stimulatory effect of thrombin (7 U/ml) on PGE_2 formation in mouse calvarial osteoblasts (data not shown).

The degree of synergistic interaction between bradykinin and haptoglobins (Hp 1-1 and Hp 2-1) on PGE_2 formation was dependent upon the concentration of haptoglobins from 1-4 mg/ml (Fig. 4 a,b).

Discussion

These data show that two phenotypes of human haptoglobin (Hp 2-1 and Hp 1-1) cause a rapid and dose-dependent stimulation of prostanoid formation in osteoblast-like cells from neonatal mouse calvaria. The concentrations of haptoglobins causing stimulation of prostaglandin biosynthesis in osteoblasts (1-4 mg/ml) are similar to the amounts found in serum. The magnitude of haptoglobin induced PGE_2 formation was in the same range as that induced by bradykinin, a potent stimulator of prostaglandin formation in mouse and human osteoblasts (11,12). In preliminary experiments, we have

observed that not only Hp 2-1 and Hp 1-1 are capable of activating prostanoid biosynthesis but that also the third common phenotype of haptoglobin (Hp 2-2) can induce prostanoid formation. Our data hitherto obtained, however, do not allow us to compare the relative potency of the three different haptoglobin phenotypes.

In contrast to our findings, it has previosly been reported that haptoglobin inhibits biosynthesis of several prostanoids, using bull seminal-vesicle prostaglandin synthetase as an assay system (13). In accordance with our observation in the present paper, however, Baseler & Burrell (14) recently showed that haptoglobin stimulates PGE_2 formation in intact rabbit alveolar macrophages. The latter observation indicates that haptoblobin induced PGE_2 release is not a specific feature of mouse osteoblasts.

The results presented in the present paper show not only that haptoglobin can stimulate prostaglandin production per se, but also that osteoblasts pretreated with haptoglobin responded to a subsequent challenge to bradykinin with synergistically enhanced formation of PGE_2. The potentiation of bradykinin induced PGE_2 release by haptoglobin was dose-dependent and seen at concentrations of haptoglobin similar to those found in serum. The synergistic interaction between bradykinin and haptoglobin was observed with two different phenotypes of haptoglobin (Hp 2-1 and Hp 1-1). In addition, the capacity of haptoglobin to potentiate prostanoid formation was not restricted only to bradykinin, but was also observed when pretreated cells were challenged with thrombin, another stimulator of prostaglandin biosynthesis in bone (11,15). We do not know at present the signal-transducing pathway involved in the stimulatory effect of haptoglobin on prostanoid formation, nor do we know the molecular mechanism involved in the synergistic interaction between bradykinin, thrombin and haptoglobin. Since both effects of haptoglobin, however, were observed already within 30 min, it is more likely that haptoglobin stimulates prostanoid formation by

enhancing the availability of arachidonic acid via stimulation of phospholipase A2 activity, rather than to increase cyclooxygenase. Interestingly, we have recently observed that also human recombinant IL-1 β synergistically potentiates the stimulatory effect of bradykinin on prostanoid formation in bone (16).

It has been demonstrated by many laboratories that several products of arachidonic acid metabolism via the cyclooxygenase pathway, especially PGE_2 and PGI_2, are potent stimulators of bone resorption (reviewed in ref. 7). In addition, a significant correlation between tissue levels of prostaglandins and the progress of bone resorption in periodontal disease has been found (17). Moreover, the loss of the alveolar bone surrounding the teeth in experimentally induced periodontitis can be reduced by treatment with cyclooxygenase inhibitors (18). Thus, the stimulatory and potentiating effect of haptoglobin on prostaglandin biosynthesis in osteoblasts may be implicated in the pathogenesis of inflammation induced bone resorption. In accordance with this view, we have recently observed that Hp 2-1 can stimulate bone resorption in vitro as assessed by the release of radioactive calcium from prelabeled mouse calvaria (Lerner & Fröhlander, unpublished observations). Our observations indicate that not only paracrine stimulators of osteoclasts (e.g. IL-1, TNF, bradykinin and thrombin), but also humoral factors induced by the inflammatory process (e.g. haptoglobin) may contribute to resorption of bone. Although bone loss in the vicinity of inflammatory processes is generally considered to be a local process, it has been reported that 'non-osseous' inflammation induced by talc powder injections in growing rats is associated with generalized ostepenia (19,20).

Acknowledgments

The present investigation was supported by grants from the Swedish Medical Research Council (project no 7525), the Swedish Association Against Rheumatic Diseases, the Royal 80 Year Fund of King Gustav V, Magnus Bergvall Foundation, Croschinsky Foundation and Craaford Foundation.

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