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Localisation of Prostaglandin Endoperoxide H Synthase (PGHS)-1 and PGHS-2 in Bone Following Mechanical Loading In Vivo

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ABSTRACT

Recent data suggests that induction of prostaglandin endoperoxide H synthase-2 (PGHS-2) is critical for the anabolic response of lamellar bone elicited by mechanical strain in vivo. The aim of the present study was to localise PGHS-1 and PGHS-2 in rat tibiae following four-point bending in vivo. Right tibiae of 19 adult female rats were subjected to 300 cycles of bending or sham loading at 2.0 Hz with an applied load of 65 N. At 0, 6, and 24 hr postloading, rats were anaesthetised and perfused with Bouin's fixative. Left and right tibiae were dissected, postfixed for 4 hr at 4°C, decalcified in EDTA, and embedded in paraffin. Serial 5 μM sections were stained for PGHS-1 and PGHS-2 using standard immunoperoxidase procedures. For the first time, immunoreactivity for both PGHS-1 and PGHS-2 was localised in bone cells in situ, in the rat tibia. PGHS-1 was distributed widely in all tibiae, while PGHS-2 showed sparse localisation. At the endocortical surfaces (EcS), osteoblasts, lining cells, and osteocytes close to the surface reacted strongly for PGHS-1, as did intracortical osteocytes. At the periosteal surface (PsS), osteoblasts and cells of the osteogenic region were immunopositive. Immediately after loading, the numerical density ($n.mm^{-2}$) of osteocytes labeled with PGHS-1 was significantly greater in loaded tibiae compared to controls. This increase was not seen after sham loading. At 6 and 24 hr postloading, this difference was no longer evident. Staining for PGHS-2 was sparse compared to PGHS-1. Light to moderate reactivity was observed in osteocytes and canaliculae, but the numerical density of labeled cells was significantly less than that for PGHS-1. Moderate staining was seen in lining cells and osteoblasts at the EcS and PsS of some tibiae. Osteoclasts at the PsS reacted strongly for both PGHS-1 *and* PGHS-2. There was a similar load-related increase in the density of PGHS-2-labeled osteocytes 0 hr postloading. The labeled osteocyte density had decreased at 6 hr, but remained significantly greater in loaded bones. These results show that both forms of PGHS can be localised in bone cells, with PGHS-1 expressed to a greater extent than PGHS-2. The data also suggest that both PGHS-1 and PGHS-2 may play important roles in the early response of bone to mechanical loading in vivo. Anat. Rec. 252:580–586, 1998.

Key words: mechanical loading; rat; adaptation; bone formation; prostaglandin endoperoxide H synthase; immunohistochemistry

Prostaglandin endoperoxide H synthase (PGHS) is a key enzyme in the conversion of arachidonic acid and oxygen to PGH_2, the committed step in prostaglandin (PG) formation. This enzyme has two isoforms, a constitutive form present in tissues such as kidney and stomach (PGHS-1) and an inducible form typically associated with inflammation (PGHS-2; Fu et al., 1990; Masferrer et al., 1990;

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Raz et al., 1988; Sano et al., 1992). Prostaglandin endoperoxide H synthase-1 can be detected in most tissues, whereas PGHS-2 is usually undetectable (Smith et al., 1996). In the latter case, its expression can be induced rapidly in some cells in response to different stimuli. To date, neither of these enzymes has been localised in situ in bone cells.

Induction of PGHS-2 is also associated with PGE_2 produced by bone cells in response to mechanical stress, in vitro (Klein-Nulend et al., 1997), and recent data suggests that induction of PGHS-2 is critical for the anabolic response of lamellar bone elicited by mechanical strain in vivo (Forwood, 1996). For example, load-related bone formation at the endocortical surface (EcS) was significantly inhibited in rats treated with NS-398 (a specific PGHS-2 inhibitor) 3 hr before loading the right tibia in four-point bending (Forwood, 1996). Although mechanically induced bone formation was inhibited by treatment with PGHS inhibitors, neither PGHS-1 nor PGHS-2 were localised in the tissues in that experiment. The aims of the present investigation were to localise PGHS-1 and PGHS-2 in bone cells of rat tibiae in situ, and determine the extent of labeling following four-point bending in vivo.

MATERIALS AND METHODS

Experimental Animals

A total of 20 virgin female Sprague-Dawley rats (6-month-old) weighing 294 ± 4 g were used for this study. Rats were housed two per cage, had a 12:12-hr light-dark cycle and were fed water and standard rat chow ad libitum. The rats were monitored daily and no signs of leg injury or anxiety were observed. All procedures performed throughout the experiment conformed with the guidelines of The University of Queensland Animal Experimentation Ethics Committee

Experimental Design

The rats were randomly divided into four groups of five rats. Three groups were subjected to applied bending loads and one group to sham loading. Due to attrition, the sham group was reduced to four rats. Bending loads were applied as a sine wave, with a frequency of 2 Hz, to the right tibia through a four-point bending device as previously described (Forwood, 1996; Turner et al., 1994). Each rat was subjected to a single period of loading, consisting of 300 cycles applied at a magnitude of 65 N. Sham loading was applied at 65 N, but the loading pads were arranged so that they squeezed the leg without creating significant bending moments. Before the loading session, rats were anaesthetised by halothane inhalation, and afterwards were allowed normal cage activity.

Tissue Preparation

At 0, 6, and 24 hr postloading, five rats were anaesthetised with pentobarbitone sodium (Nembutal, 50 $mg.kg^{-1}$) and perfused intracardially with phosphate buffered saline (PBS pH 7.4) until the tissues blanched, followed by Bouin's solution (0.9 % v/v picric acid, 9 % v/v formaldehyde, and 5% v/v glacial acetic acid). Rats subjected to sham loading were anaesthetised and perfused immediately after loading. Left and right tibiae were dissected, postfixed in Bouin's solution for 4 hr at 4°C, rinsed four times in 50% ethanol (EtOH) for 1 hr, and washed overnight in 70% EtOH. Bones were then washed twice in 10% EDTA at 4°C, decalcified for 2 weeks in EDTA at 4°C, dehydrated in EtOH, and a 6 mm transverse section from the region of loading was embedded in paraffin under vacuum using standard methods. To reduce variability in section thickness between left and right tibiae, both bones were embedded in the same block. Sections cut at 5 µM were collected onto glass slides and coated with 3-aminopropyl triethoxy silane (APES, Sigma, Australia).

Immunohistochemistry

Monoclonal mouse antiserum against ovine PGHS-1, and polyclonal rabbit antiserum against murine PGHS-2 were obtained commercially (Cayman Chemical Co, Ann Arbor, MI). These antibodies have been well characterised, react with rat tissues, and positive staining is abolished by incubation with pre-immune sera (Iseki, 1995). Positive controls were performed on sections of rat gastric mucosa in which cells of the pyloric gland and mucous neck cells are a source of PGHS-1 and the apical cytoplasm of the surface mucous cells a source of PGHS-2 (Iseki, 1995). Negative controls consisted of the following: a) omission of the primary antiserum; b) omission of the secondary antiserum; c) omission of the streptavidin HRP; d) omission of primary and secondary antisera, and streptavidin HRP.

Sections were deparaffinised and subjected to immunohistochemical staining according to the following schedule: a) elimination of endogenous peroxidase with peroxidase-block (Dako Corporation, Carpinteria, CA) for 15 min at room temperature; b) elimination of nonspecific protein binding by incubation with 10% (v/v) normal horse serum (NHS) for 20 min; c) incubation overnight at 4°C with primary antibody diluted 1:1,000 (PGHS-1) and 1:100 (PGHS-2) in PBS with 1% NHS and 0.01% tween 20; d) incubation with donkey anti-mouse (PGHS-1) and donkey anti-rabbit (PGHS-2) biotinylated IgG (Dako Corporation, diluted 1:300 (donkey anti-mouse) 1:500 (donkey anti-rabbit) in PBS with 1% NHS and 0.01% Tween-20 for 1 hr at room temperature; e) incubation with streptavidin horseradish peroxidase complex (Amersham, Australia), diluted 1:100 in PBS containing 0.01% tween 20 for 1 hr at room temperature; f) treatment with diaminobenzidine (Liquid DAB, Dako Corporation for 5 min. Between each step sections were washed three times in PBS. Sections were left uncounterstained, or counterstained in Mayer's haematoxylin for 15 sec followed by rinsing in running tap water for 10 min, dehydrated, cleared, and mounted.

Histomorphometry

Sections were viewed on an Olympus BX60 microscope at ×400 magnification, and measurements of cortical area were made using KSS Stereology (KSS, Salt Lake City, UT). Classification of cells at bone surfaces was made on the basis of morphology, and osteoblast identification was confirmed by the presence of histochemical staining for alkaline phosphatase. Osteoclasts were observed on the periosteal surface of some specimens, and identified on the basis of morphology. Because they were not the subject of quantification in this experiment, no additional staining was performed for verification. Noncounterstained sections were used to determine the numerical density of labeled osteocytes (L.Ot.Dn, $n.mm^{-2}$) within the entire tibial cortex. At the endocortical surface (EcS), counter-

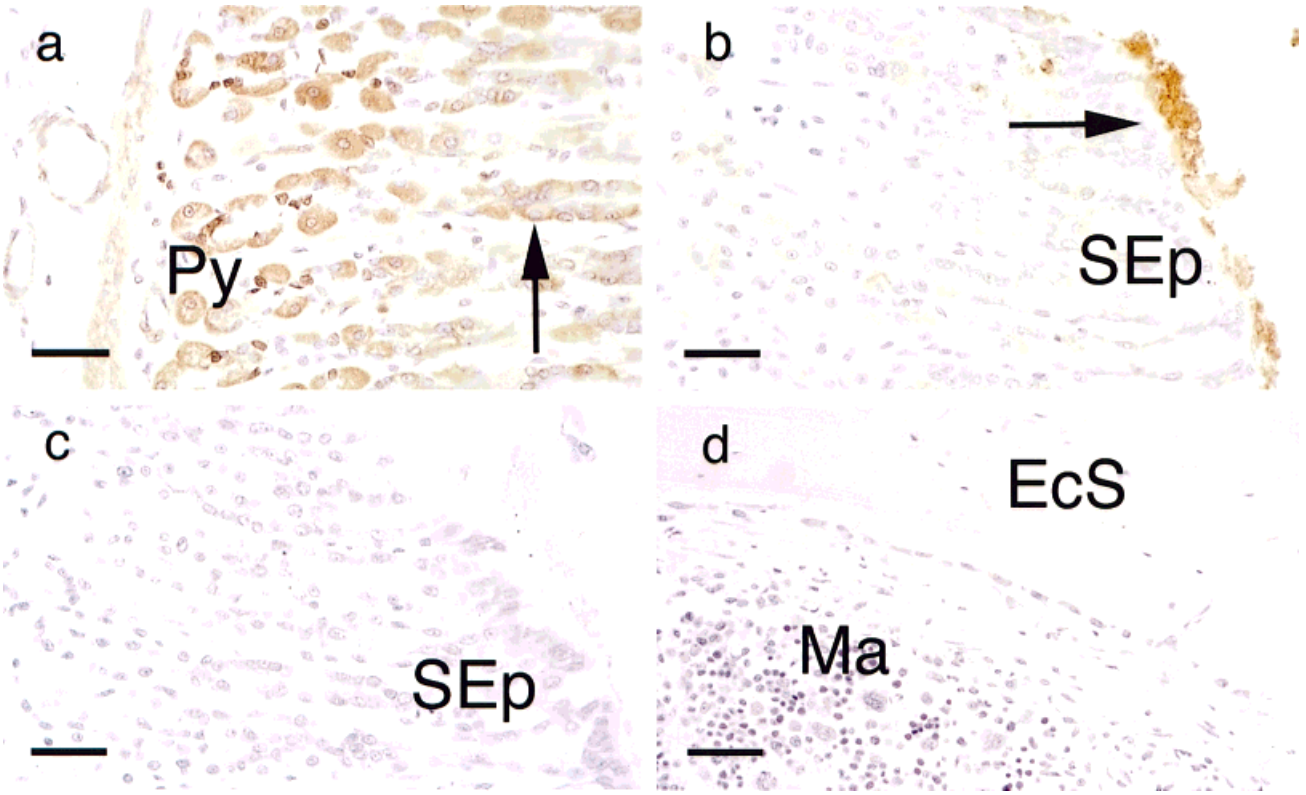

Fig. 1. **a**: Positive staining for PGHS-1 in pyloric glands (Py) and mucous neck cells (arrow) of the rat gastric mucosa. **b**: Positive staining for PGHS-2 (arrow) in the apical cytoplasm of mucous cells of the surface epithelium (SEp) of rat gastric mucosa. **c**: This section of rat gastric mucosa shows absence of immunoreactivity following omission of primary antiserum against PGHS-2. This is the negative control for the section illustrated in b. **d**: Section from the endocortical surface (EcS) of rat tibial diaphysis also shows absence of immunoreactivity in the bone and marrow (Ma) following omission of primary antiserum against PGHS-1. This is the negative control for the section illlustrated in Figure 2a. Sections counterstained with Mayer's haematoxylin. Original magnification was ×400. Scale bar = 50 µm.

stained sections were used to determine the total number of cells (Ce.N) and the number of labeled cells (L.Ce.N) around the endocortical perimeter, in a zone that extended 20 µM into the marrow cavity. A labeling index was then calculated (L.Ce.N/Ce.N, %). Mechanically-induced labeling indices were calculated by subtracting measurements of the left tibia (control) from those of the right (experimental).

Statistical Analysis

Due to inhomogeneity of variance, labeling indices were tested using Kruskall-Wallis ANOVA (SigmaStat, Jandel Scientific, Corte Madera, CA). Multiple comparisons were performed post hoc using Dunn's test. Data from left and right tibiae were compared using a Wilcoxon signed rank test. Significance was assumed at $P < 0.05$. Probability levels between 0.05 and 0.1 were classified as marginal and nonsignificant (NS) at $P > 0.1$.

RESULTS

Incubation of sections after omission of the primary or secondary antisera, streptavidin HRP complex, or all of these, did not produce immunohistochemical staining (Fig. 1). Consistent with earlier reports (Iseki, 1995), positive staining for PGHS-1 was confirmed in the mucous neck cells and pyloric gland of rat gastric mucosa, and apical cytoplasm of surface mucous cells for PGHS-2 (Fig. 1a,b).

Prostaglandin endoperoxide H synthase-1 was distributed widely in all tibiae. At the EcS, osteoblasts, lining cells, and osteocytes close to the EcS showed strong reactivity for PGHS-1, as did intracortical osteocytes (Fig. 2). At the PsS, osteoclasts, osteoblasts, and cells of the osteogenic region were immunopositive. Within 10 min of loading, the numerical density ($n.mm^{-2}$) of osteocytes labeled for PGHS-1 was significantly greater in right tibiae (loaded) when compared to left (control; Table 1). At 6 and 24 hr postloading, this difference was no longer evident; and there was no difference between limbs for sham loading. When normalised, the labeled osteocyte density for PGHS-1 induced by mechanical loading was significantly different among groups ($P < 0.05$), being greatest immediately after loading ($P < 0.05$; Fig. 3). In contrast, the labeling index for PGHS-1 at the EcS was significantly lower in right tibiae, compared to left, immediately after loading, but was no different at 6 and 24 hr (Table 1). The difference among groups for the mechanically-induced labeling index was not significantly different.

Staining for PGHS-2 was sparse when compared to PGHS-1. Light to moderate reactivity was observed in

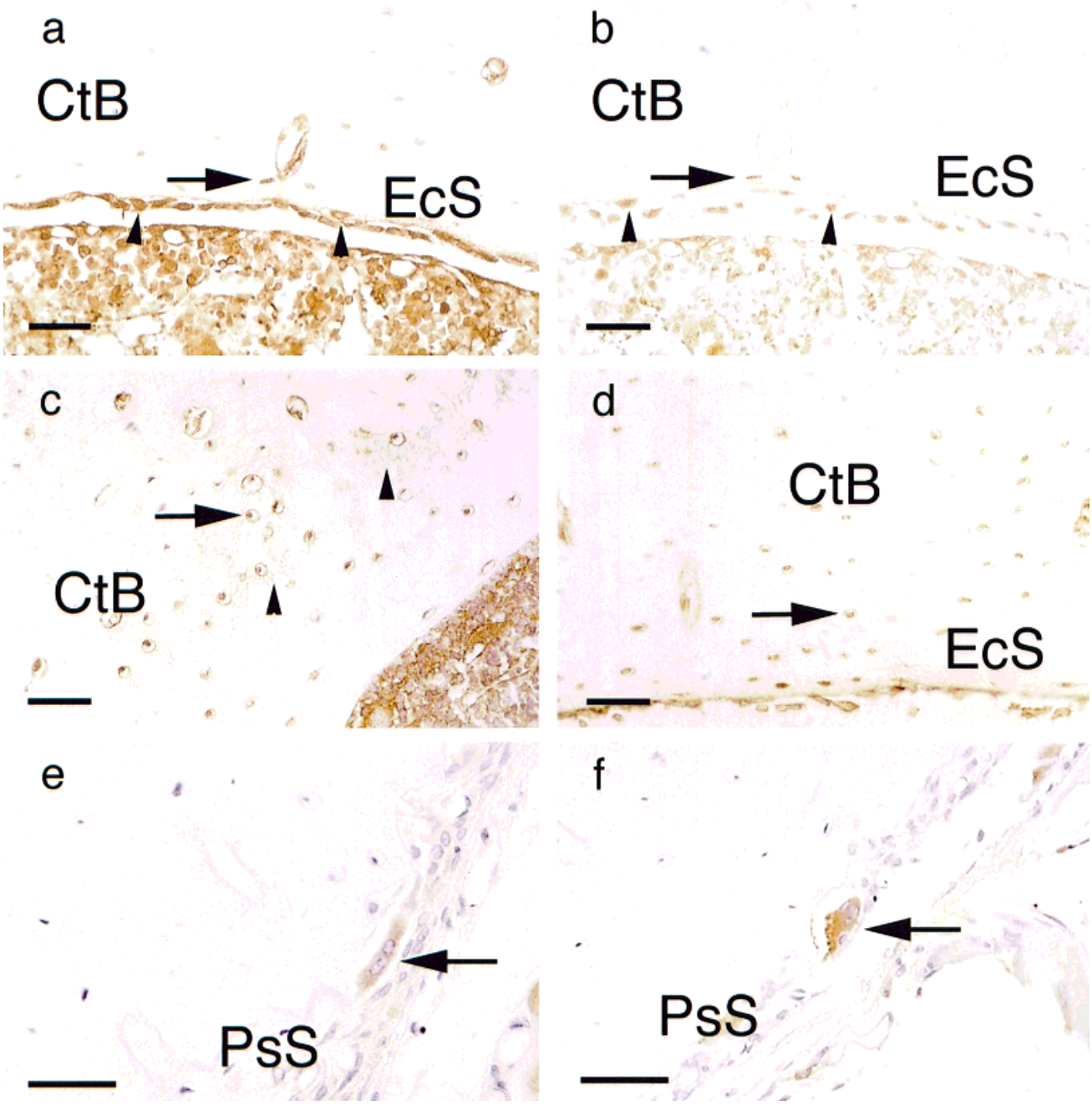

Fig. 2. **a**: Strong reactivity for PGHS-1 demonstrated in osteoblasts (arrowheads) at the endocortical surface (EcS), and osteocytes (arrow) within the cortical bone (CtB). Section not counterstained. **b**: Moderate immunoreactivity is also demonstrated for PGHS-2 in osteoblasts (arrowheads) at the EcS, and osteocytes (arrow). Localisation for PGHS-1 and PGHS-2 is shown in the same osteocytes cut in serial sections (large arrow in a and b). Section not counterstained. **c**: Within the intracortical bone (CtB), osteocytes (arrow), and canaliculi (arrowheads) showed strong reactivity for PGHS-1. **d**: Light to moderate reactivity for PGHS-2 was observed in osteocytes (arrow). **e**: At the PsS, osteoclasts (arrow) showed positive reactivity throughout the cytoplasm. **f**: Osteoclasts also showed strong immunoreactivity for PGHS-2 (arrow), which tended to be polarised towards the ruffled border. Original magnification was ×400. Scale bar = 50 µm.

osteocytes (Fig. 2), but the numerical density of labeled cells was considerably less than that for PGHS-1 (Table 1). Moderate staining was seen in lining cells and osteoblasts at the EcS and PsS of some tibiae. Strong reactivity was observed in canaliculi of loaded tibiae, and osteoclasts at the PsS reacted strongly with both PGHS-1 and PGHS-2. A similar load-related increase in the numerical density of osteocytes labeled for PGHS-2 occurred 0 hr postloading (Table 1). The numerical density of labeled osteocytes was less at 6 hr, but remained marginally greater in right tibiae compared to left ($P < 0.1$). The numerical density of osteocytes labeled for PGHS-2 induced by mechanical

TABLE 1. Immunohistochemical Labelling Indices for PGHS-1 and PGHS-2 (Mean ± SE)

		L. Ot. Dn (n.mm^{-2})		Ec labelling index (%)	
Group	*n*	PGHS-1	PGHS-2	PGHS-1	PGHS-2
65 N Bending					
0 hr postloading					
Right	5	102.4 ± 44.9[a]	13.8 ± 5.1*	82.7 ± 9.7[b]	9.7 ± 4.3
Left	5	43.6 ± 20.6	4.1 ± 1.6	92.5 ± 6.9	13.0 ± 3.7
6 hr postloading					
Right	5	23.0 ± 5.8	0.9 ± 0.1**	95.6 ± 3.3	11.1 ± 3.4
Left	5	21.7 ± 5.0	0.4 ± 0.1	97.0 ± 1.1	11.4 ± 4.7
24 hr postloading					
Right	5	25.2 ± 9.4	2.0 ± 0.6	92.0 ± 3.7	4.9 ± 2.4
Left	5	28.7 ± 10.3	1.2 ± 0.8	93.0 ± 2.6	3.1 ± 0.9
65 N Sham loading					
0 hr postloading					
Right	4	20.9 ± 4.6	1.6 ± 0.3	97.1 ± 2.9	14.5 ± 3.8
Left	4	37.0 ± 12.2	2.9 ± 0.7	98.4 ± 1.2	16.3 ± 2.9

*$P < 0.05$.
**$P < 0.1$, right compared to left—Wilcoxon signed rank test.

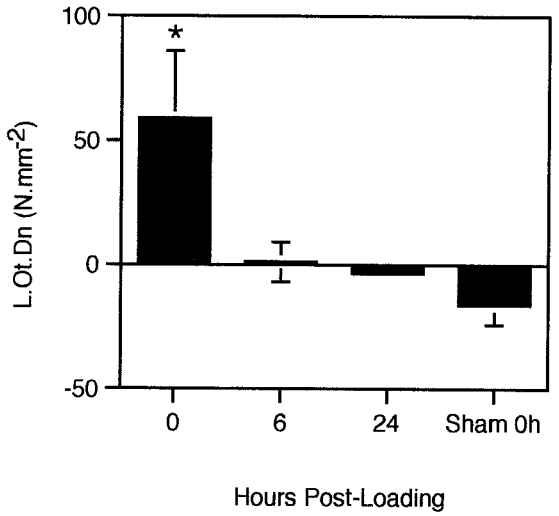

Fig. 3. Numerical density of osteocytes (L.Ot.Dn) labelled for PGHS-1 induced by mechanical loading (data for left tibiae subtracted from right, mean ± SE). *Significantly different from other groups, $P < 0.05$, Dunn's test.

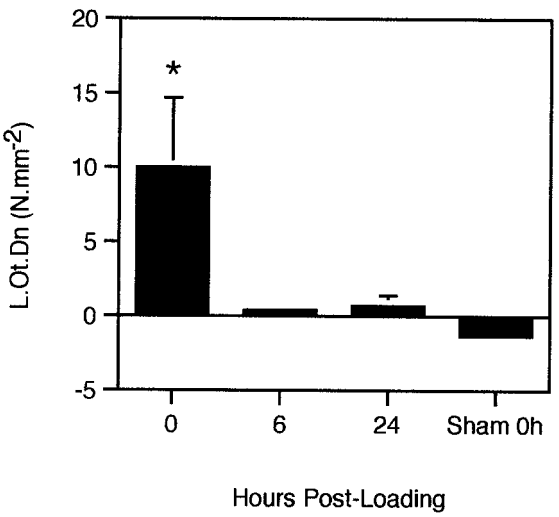

Fig. 4. Numerical density of osteocytes (L.Ot.Dn) labelled for PGHS-2 induced by mechanical loading (data for left tibiae subtracted from right, mean ± SE). *Significantly different from other groups, $P < 0.05$, Dunn's test.

loading was also significantly different among groups ($P < 0.05$), again being greatest immediately after loading ($P < 0.05$) (Fig. 4). At this time-point, there was a trend for a lower Ec labeling index in loaded tibiae, but this was not significant for PGHS-2.

DISCUSSION

For the first time, immunoreactivity for both PGHS-1 and PGHS-2 was localised in bone cells in situ, in the rat tibia, with PGHS-1 expressed to a far greater extent than PGHS-2. Using serial sections, localisation of these enzymes was also evident in osteoclasts, osteocytes and osteoblastic cells at the periosteal and endocortical surfaces (Fig. 2). In osteoclasts, PGHS-1 was distributed throughout the cytoplasm, but PGHS-2 showed intense localisation towards the ruffled border and resorption lacuna (Fig. 2f). In the rat stomach, PGHS-2 localised in the surface mucous cells (Fig. 1b) which are considered the principal target of cytoprotective actions of prostaglandins (Iseki, 1995; Johansson and Bergstrom, 1982). Similar to gastric mucosa, "digestion" of bone by osteoclasts is facilitated by local acidification. It is possible that PGHS-2 in osteoclasts also contributes to the production of cytoprotective prostaglandins in the region of the ruffled border, where an acid compartment is maintained between the cell membrane and the bone surface (Vaananen, 1996). It is not possible in this experiment, however, to test the functional significance of PGHS-2 in osteoclasts.

Although PGHS-2 is usually expressed at low levels, both enzymes have now been identified in a wide variety of other tissues. For example, PGHS-1 is detectable in most tissues, but not necessarily in all cells (Smith et al., 1996). Expression of PGHS-2 is usually undetectable in most mammalian tissues, but can be induced rapidly in fibroblasts (Dewitt and Meade, 1993), endothelial cells (Jones et al., 1993), and monocytes (O'Sullivan et al., 1992) by

various factors or inflammatory mediators. This constitutive or inducible nature of PGHS-1 and PGHS-2 is not mutually exclusive, however, because there are also some tissues in which PGHS-2 is expressed constitutively (Walenga et al., 1996; Yamagata et al., 1993), and in the present experiment, both PGHS-1 and PGHS-2 immunoreactivity in osteocytes increased after mechanical loading.

Within 10 min after loading, the numerical density of osteocytes with positive immunoreactivity for PGHS-1 and PGHS-2 was significantly greater in right tibiae (loaded) when compared to left (control). At 6 and 24 hr postloading, this difference was no longer evident for PGHS-1, and a small difference remained for PGHS-2 at 6 hr postloading. This transient increase is unlikely to result from increased gene expression, but may be related to stabilisation of the mRNA by signaling molecules such as nitric oxide, or direct alterations in enzyme availability necessary for increased PG synthesis. Within 10 min of mechanical loading or fluid shear stress, osteoblastic cells in organ culture (Rawlinson et al., 1993), or cell culture (Reich and Frangos, 1991; Ajubi et al., 1996) increase their release of PGE_2 or PGI_2. The constitutive isoform of PGHS is typically bound to the cell membrane and may be available for mechanotransduction involving G proteins (Reich et al., 1997). This process may liberate the enzyme for PG metabolism, increasing its immunoreactivity. Nitric oxide also plays a critical role in the release of PGE_2 by direct activation of PGHS-1 and -2 (Salvemini et al., 1993). This occurs by inducing a conformational change in the PGHS protein secondary structure (Hajjar et al., 1994), which could also explain the increased immunoreactivity.

Transient increases in PGHS-2 mRNA expression have previously been observed in rat osteocytes 2 hr after a mechanical loading event in vivo (Nakayama et al., 1996) and in cultures of mouse calvarial bone cells subjected to one hour of pulsating fluid flow (PFF) (Klein-Nulend et al., 1997). These increases may result from autoregulation of PG synthesis following mechanotransduction which amplifies the adaptive response over time. In the fluid flow experiment, PGHS-1 mRNA was expressed constitutively by these cells, but did not respond to the fluid shear induced by PFF. The rapid increase in numerical density of osteocytes labeled for PGHS-1 following loading in this experiment was unexpected, but may be indicative of differences between early mechanochemical transduction and subsequent cell signaling in vivo. The requirement for increased production of different prostanoids for early signaling events in vivo could require utilisation of both enzyme pools. This demand could be quite different to the response of isolated osteoblastic cells subjected to loading in vitro, as demonstrated by Klein-Nulend et al. (1997). Further experiments are now required to determine the time-course of PGHS gene expression in situ following four-point loading.

In conclusion, both forms of PGHS can be localised in mature cells of osteoblast and osteoclast lineage in situ. PGHS-1 is expressed to a greater extent than PGHS-2, and both enzymes demonstrated a response to mechanical loading in vivo. These data also suggest, therefore, that both PGHS-1 and PGHS-2 may play important roles in the early response of bone to mechanical loading in vivo, involving augmentation of prostanoid production for signaling in the osteocyte network, as well as autocrine feedback at the bone surfaces.

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