Inhibition of cyclo-oxygenase-2 activity does not abolish the anabolic effect of prostaglandin E₂ *in vivo* or *in vitro*

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Abstract

It was previously reported that the expression of cyclo-oxygenase-2 (COX-2) is induced by prostaglandin E₂ (PGE₂) *in vitro* in an osteogenic cell line and organ culture, suggesting an autoamplification mechanism. In this study, we first tested whether this phenomenon also occurs in bone tissue *in vivo* and found that a single anabolic dose of PGE₂ (5 mg/kg) induced (between 30 and 120 min) in rat tibiae, an increase in the mRNA level of COX-2 (2.5- to 9-fold) but not that of COX-1. Secondly, to test whether COX-2 activity in generating endogenous prostaglandins (PGs) is required for the *in vivo* anabolic properties of PGE₂, young male rats were injected daily with either vehicle (8% ethanol) or 5 mg/kg PGE₂ for 21 days. PGE₂-injected rats received, 45 min prior to PGE₂, either dimethyl sulphoxide (as vehicle) or one of two doses of NS-398, a selective COX-2 inhibitor: a low dose (3 mg/kg) or a high dose (10 mg/kg). PGE₂ increased bone formation (measured as cancellous mineralizing surface, mineral apposition rate and bone formation rate) and bone mass (measured as cancellous bone area and surface and cortical width). None of these increases was

suppressed by pre-administration of NS-398. In contrast, the high dose of NS-398 effectively suppressed an increase in rat hind-paw volume induced by a local carrageenan injection. Furthermore, since COX-2 inactivation may affect PG receptor expression, we found that pre-administration of NS-398 did not abolish the induction in EP₄ receptor mRNA levels, caused by PGE₂ in rat bone tissue. For *in vitro* testing, rat femoral bone marrow stromal cell cultures were initiated and were incubated in the absence or presence of PGE₂ at 100 nM (as an inducer) and with increasing concentrations of NS-398 (10⁻⁸ M to 10⁻⁵ M) for 21 days, after which time mineralized (Von-Kossa positive) nodules were counted. PGE₂ increased nodule formation as previously reported; however, NS-398 reduced nodule formation in both control and PGE₂-treated cultures to the same extent.

We conclude that while the level of COX-2 mRNA is increased *in vivo* by administration of PGE₂, inhibition of its activity (i.e. generation of endogenous PGs) does not abolish the anabolic effect of PGE₂.

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Introduction

Prostaglandins of the E series (PGEs) are potent stimulators of bone formation *in vivo* in many species (reviewed by Norrdin *et al.* 1990, Jee & Ma 1997). It is now established that PGE₂-induced bone formation results primarily from the appearance of new osteoblasts and production of new bone. Previously, others and ourselves have reported that PGE₂ is anabolic in young and aging rats (Ueno *et al.* 1985, Jee *et al.* 1987, 1992, Suponitzki & Weinreb 1998). In addition, we have published a detailed analysis of the effects of PGE₂ and have clarified some aspects of the mechanisms involved in the actions of PGE₂: induction of early response genes (Weinreb *et al.* 1997a), stimulation of osteoblast differentiation from bone marrow osteoprogenitors (Weinreb *et al.* 1997b, Keila *et al.* 2001) and involvement of the cAMP signaling pathway and of the EP₄ receptor (Weinreb *et al.* 1999). Recently, we have

also shown that PGE₂ induced the expression of its receptor (EP₄) *in vivo* and *in vitro* and suggested that this effect might amplify the response of bone tissue to PGE₂ (Weinreb *et al.* 2001).

Cyclo-oxygenase (COX, also called prostaglandin endoperoxide synthase) is the key enzyme responsible for PGE production from arachidonic acid (Smith & Marnett 1991). Recently, two different COX isoforms (COX-1 and COX-2) were identified and characterized. COX-1 is expressed constitutively in many cells and COX-2 is induced by serum, pro-inflammatory cytokines, growth factors and other stimuli (Herschman 1996, Smith & Dewitt 1996). Due to their involvement in PGE production in many inflammatory processes, the two isoforms are targets for all non-steroidal anti-inflammatory drugs.

Bone cells are known to produce PGEs in response to various stimuli (Raisz *et al.* 1993) and therefore express one or both of the COX isoforms. Exogenous PGE₂ *in vitro*

was reported to induce COX-2 expression in organ cultures of newborn mouse calvaria and in a cell line derived from this tissue (MC3T3-E1) (Pilbeam *et al.* 1993, 1995, Kawaguchi *et al.* 1994, Takahashi *et al.* 1994). This finding raised the possibility that COX induction is another autoamplification mechanism, whereby exogenous PGE₂ causes an increased production of endogenous PGs. However, whether this effect occurs *in vivo* in bone tissue of young adult rats (where PGE₂ is clearly anabolic) is not known. Furthermore, even if it does, there are no data indicating whether the activity of COX-2 is required for the anabolic effect of exogenous PGE₂.

Finally, a recent report showed that a COX-2 inhibitor (NS-398) increases the expression and abundance of the EP₄ receptor in the murine M-1 kidney cell line (Nasrallah *et al.* 2001). If this effect occurred *in vivo* in bone tissue, COX-2 inactivation could theoretically affect the actions of PGE₂ and even mask its concomitant effect on COX-2. For this reason we tested whether inhibition of COX-2 activity modulates the PGE₂-induced EP₄ expression in bone.

Therefore, the purpose of this study was threefold: to test whether systemically administered PGE₂ increases the mRNA levels of one or both isoforms of COX in rat bone tissue; to examine whether inhibition of COX-2 activity will abrogate the anabolic effect of PGE₂ *in vivo* and *in vitro*; and to determine if COX-2 inactivation interferes with the PGE₂-induced increase in EP₄ mRNA.

Materials and Methods

Northern blot analysis

Five-week-old male Sprague-Dawley (SD) rats were administered subcutaneously a single injection of either vehicle (8% ethanol) or PGE₂ (5 mg/kg in the same vehicle, a generous gift from Dr C Hall, The Upjohn Company, Kalamazoo, MI, USA) and then killed between 15 and 120 min later. This dose is within the range known to be anabolic in rats (Ueno *et al.* 1985, Jee *et al.* 1987, 1992, Suponitzki & Weinreb 1998). Total RNA was extracted from the tibial metaphysis using a guanidinium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987), after pulverizing frozen bone samples by a tissue homogenizer. RNA samples (20 µg) were separated on 0.9% agarose/formaldehyde gels and transferred onto nylon membranes (Boehringer Mannheim, Indianapolis, IN, USA). Membranes were prehybridized in Hybrisol I (Oncor, Gaithersburg, MD, USA) and 0.5 mg/ml sonicated salmon sperm DNA (Boehringer Mannheim) at 42 °C for 3 h. Subsequently, membranes were hybridized at 42 °C for 20 h with a COX-1 or a COX-2 cDNA (generously donated by Dr R DuBois, Vanderbilt University, Nashville, TN, USA; DuBois *et al.* 1994), which were labeled with [³²P]-dCTP (Amersham

International plc, Amersham, Bucks, UK) by random priming using the rediprime kit (Amersham). After hybridization, membranes were washed four times in 2 × SSC + 0.1% SDS at room temperature for a total of 1 h and once with 0.2 × SSC + 0.1% SDS at 55 °C for 1 h and then exposed to Kodak XAR 2 film at -70 °C using intensifying screens. After developing the films, bound probes were removed twice with 0.1% SDS at 80 °C and membranes were hybridized with a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA, USA) for loading control. Following hybridization with the GAPDH probe, densitometric analysis was used to determine the ratio between the optical density of the COX bands to that of the GAPDH bands. In a separate experiment, vehicle, PGE₂ (5 mg/kg) or NS-398 (10 mg/kg in dimethyl sulphoxide (DMSO)) followed, after 45 min, by PGE₂ were injected once subcutaneously to 5-week-old SD rats, which were killed 2 h later. RNA samples were prepared as described above and then hybridized with a cDNA probe for the rat EP₄ prostanoid receptor (Boie *et al.* 1997, a generous gift from Dr Ford-Hutchinson, Merck Frosst, Pointe-Claire-Dorval, Quebec, Canada).

In vivo anabolic testing

Four-week-old SD rats were divided into four groups (seven rats each). One group received daily s.c. injections of a vehicle (8% ethanol) and three groups received daily injections of PGE₂ at 5 mg/kg per day in the same vehicle for 21 days. All PGE₂-injected rats received another injection, 45 min prior to PGE₂: group 2 received 0.1 ml DMSO/rat, group 3 received a low dose (3 mg/kg) of NS-398 (Cayman Chemical, Ann Arbor, MI, USA) dissolved in 0.1 ml DMSO, and group 4 received a high dose (10 mg/kg) of NS-398. These doses were selected based on published papers (Hirata *et al.* 1997, Mikuni *et al.* 1998, Shigeta *et al.* 1998). All rats were injected with calcein (Sigma, St Louis, MO, USA) at 15 mg/kg, 8 and 2 days prior to death to measure dynamic parameters of bone formation. At the end of the injections, animals were killed with CO₂ and hind limbs were removed. Tibiae were fixed in cold 50% ethanol and embedded in methyl methacrylate for histomorphometry (Weinreb *et al.* 1991, Suponitzki & Weinreb 1998). Six-micrometer thick frontal sections were cut with a Polycut microtome (Leica, Nussloch, Germany) and mounted on gelatin-coated slides. Some sections were deplastified using 2-methoxyethyl acetate (Merck, Darmstadt, Germany) and stained with the Von-Kossa stain for mineralized tissue. The Bioquant Nova Image Analysis System (R&M Biometrics, Nashville, TN, USA) was used for the following measurements: (a) cancellous bone area and surface in the tibial proximal metaphysis in an area extending 0.75–2.75 mm distal to the growth plate and (b) cortical thickness (a mean of ten measurements located 6–9 mm

distal to the growth plate). Other, unstained sections were mounted with Merckoglas (Merck) and analyzed under a fluorescence microscope. The extent of bone surface with single (sL) or double (dL) calcein labels was determined as percentage of the total bone surface (BS) in an area located 0.75–2.25 mm distal to the growth plate using the same system. The mineralizing surface (MS) was calculated according to the formula $MS = (dL + 1/2 \text{ sL}) \times 100/BS$ (Parfitt *et al.* 1987). In addition, the mean distance between the two calcein labels was measured in the same area of all sections and the cancellous mineral apposition rate (MAR) was derived by dividing the inter-label distance by 6 (the number of days between labels).

Foot-pad edema model

In order to ascertain that the dose and route of administration of NS-398 we used were effective *in vivo*, we used the foot-pad edema model. This model involves induction of COX-2 mRNA (Kennedy *et al.* 1993) and is often used to test COX inhibitors (Horizoe *et al.* 1998, Gupta *et al.* 1999).

Five-week-old SD rats were divided into three groups (six rats each). The first group received an i.p. injection of DMSO (the NS-398 solvent) followed, 45 min later, by an injection of 0.1 ml saline into the foot pad of the hind limb (negative control group). The second group received DMSO i.p. followed 45 min later by local administration of 0.1 ml of 1% λ carrageenan (Sigma) into the foot pad (positive control group). The third group received a single i.p. dose of NS-398 (10 mg/kg in DMSO) followed 45 min later by a carrageenan injection (test group). Rats were anesthetized with 80 mg/kg ketalar + 10 mg/kg rompun and the foot volume of each animal was measured using displacement of mercury in a graduated cylinder immediately before and 5 h following the foot-pad injections (Horizoe *et al.* 1998, Gupta *et al.* 1999).

All animal procedures were performed according to the guidelines of the Tel-Aviv University Faculty of Medicine.

In vitro testing

Femora of 5-week-old SD rats were removed, defleshed and the epiphyses cut off. Bone marrow was flushed out and a single-cell suspension was achieved by repeated pipetting (Maniopoulos *et al.* 1988). Cells were counted with a hemocytometer and were cultured in 6-well plates (Nunc, Roskilde, Denmark) at a density of 3×10^7 cells/well in a medium containing α -minimum essential medium (all reagents except where noted were from Biological Industries, Beit Haemek, Israel) + 13% fetal calf serum + 2 mM glutamine + 100 U/ml penicillin + 100 μ g/ml streptomycin + 12.5 U/ml nystatin + 10 mM β -glycerophosphate (Sigma) + 50 μ g/ml ascorbic acid (Merck) + 10 nM dexamethasone (Sigma). After an

attachment period of 24 h, non-adherent cells were removed by rinsing with phosphate-buffered saline (PBS) and cultures were maintained in 7% CO₂ and 37 °C for an additional 20 days with twice-a-week medium changes. Both control cultures (no PGE₂) and cultures to which PGE₂ (100 nM) was added were generated. Additional control and PGE₂-treated wells were incubated with increasing concentrations (10^{-8} M to 10^{-5} M) of NS-398. At the end of the culture period, cells were rinsed in PBS, fixed in a 1:1:1.5 solution of 10% formalin/methanol/water for 2 h and stained with the Von-Kossa method for mineralized nodules (Keila *et al.* 1994, Weinreb *et al.* 1997b). Mineralized nodules (stained black) were counted macroscopically with a magnifying glass over transmitted light and their number per well was determined.

All data are presented as means $\pm$ S.E.M. Comparison between experimental and control group means was performed using non-paired *t*-tests.

Results

COX expression

The basal level of COX-2 expression in rat tibiae was very low. However, a single anabolic dose of PGE₂ induced a significant increase in COX-2 mRNA levels at 30–120 min post-injection (Fig. 1). Densitometry revealed that the fold increase in the COX-2/GAPDH ratio was 2.5–4 at 30 min, 6–9.5 at 60 min and $\sim$ 6 at 120 min. In contrast, basal levels of COX-1 mRNA were significantly higher, but no induction occurred after PGE₂ injection.

In vivo testing

As previously reported, systemic administration of PGE₂ for 3 weeks increased bone formation in the tibial metaphysis. PGE₂ significantly increased MS (which is generally interpreted to reflect the total number of active osteoblasts; Fig. 2) as well as MAR (thought to reflect the activity of existing osteoblasts; Fig. 3) by 16% and 23% respectively. Thus PGE₂ increased both osteoblast recruitment/differentiation and activity. Figure 4 shows that when these two effects are combined (by calculating bone formation rate (BFR)), PGE₂ increased cancellous bone formation by $\sim$ 40%. None of these effects was suppressed by a daily pre-administration of NS-398 (45 min prior to that of PGE₂).

As anticipated, PGE₂-induced increased bone formation resulted in increased bone mass. PGE₂ administration increased cancellous bone area (by 75%) and cancellous bone surface (by 45%) (Fig. 5). It also increased cortical bone thickness of the tibia by 16% (Fig. 6). Again, none of these effects was blocked by NS-398 pre-administration.

Figure 1 Induction of COX-2 but not COX-1 mRNA by PGE₂ *in vivo* in rat bones. Northern blot of total RNA from the tibial metaphysis of rats injected with 5 mg/kg PGE₂ (P) or vehicle (V) and killed 15–120 min later. Note that COX-2 mRNA induction peaks at 30–60 min post-injection while no induction of COX-1 can be seen. The densitometric ratio of the COX/GAPDH bands is given above each band. Data are representative of two similar experiments.

These data suggest that COX-2 inhibition did not abrogate the anabolic effect of systemically administered PGE₂. However, these experiments did not offer positive proof for the *in vivo* effectiveness of NS-398 at these doses, route of administration and timing. However, the foot-pad experiment showed that injection of carrageenan increased significantly the foot-pad volume while that of saline did not (Fig. 7). Compared with DMSO pre-treatment, a single injection

of NS-398 at 10 mg/kg 45 min before that of carrageenan blocked most of this increase, attesting to its efficacy in blocking a COX-2-driven effect.

In vitro testing

As previously shown by others and ourselves, PGE₂ at 100 nM increased nodule formation by 130% over control cultures (Fig. 8). Increasing concentrations of NS-398

Figure 2 Systemic PGE₂ treatment for 3 weeks increases cancellous % mineralizing surface in the tibial secondary spongiosa. Pre-administration of NS-398 at 3 or 10 mg/kg did not prevent this effect. **P*<0.05, ***P*<0.01 (vs vehicle).

Figure 3 Systemic PGE₂ treatment for 3 weeks increases cancellous MAR in the tibial secondary spongiosa. Pre-administration of NS-398 at 3 or 10 mg/kg did not prevent this effect. **P*<0.05, ***P*<0.01, ****P*<0.005 (vs vehicle).

Figure 4 Systemic PGE₂ treatment for 3 weeks increases cancellous BFR in the tibial secondary spongiosa. Pre-administration of NS-398 at 3 or 10 mg/kg did not prevent this effect. * $P < 0.05$, *** $P < 0.005$ (vs vehicle).

gradually reduced nodule formation in both control and PGE₂-treated cultures. At 10^{-5} M, nodule formation in control cultures (no PGE₂ added) was reduced by 41%, and in PGE₂-treated cultures it was reduced by 47%. Even at this concentration of NS-398, PGE₂ increased nodule formation by 110% over the respective control cultures. Thus, the inhibition of COX-2 activity in these cultures did not block the effect of PGE₂ but did block the effect of some other ingredient of the culture medium, possibly fetal calf serum.

Overall, we have concluded that while COX-2 expression is induced in bone tissue of young rats by a single anabolic dose of PGE₂, the results of the present study do

not support a role for endogenous PGs in mediating the anabolic effect of exogenous PGE₂.

EP₄ expression

We previously reported that PGE₂ induces the expression of its receptor, EP₄, in rat bones (Weinreb *et al.* 2001). Here we tested whether NS-398 alters the expression of this receptor in addition to its inhibition of COX-2 activity. While PGE₂ administration induced EP₄ expression, NS-398 pre-administration did not change this effect (Fig. 9).

Discussion

During the past few years we have conducted a series of experiments aimed at elucidating the mechanisms of the anabolic effect of PGE₂. Histomorphometrically, we repeated the observations of others that systemic administration of PGE₂ increases bone formation and bone mass in long bones and calvariae of young adult rats with a consequent increase in bone strength (Suponitzki & Weinreb 1998). Mechanistically, we showed that PGE₂ induces the expression of various early-response genes such as c-fos, c-jun and egr-1 in bone tissue of these animals as early as 15 min post-injection. At least for c-fos, this induced expression was localized by *in situ* hybridization to bone marrow cells, suggesting that some marrow-resident cells are PGE₂ targets (Weinreb *et al.* 1997a). In support of this hypothesis, we reported that PGE₂ both *in vivo* and *in vitro* increases the osteogenic potential of

Figure 5 Cancellous bone area and surface in the tibial secondary spongiosa are increased by systemic PGE₂ treatment for 3 weeks. Pre-administration of NS-398 at 3 or 10 mg/kg did not prevent this effect. *** $P < 0.005$ (vs vehicle).

Figure 6 Tibial cortical bone thickness is increased by systemic PGE₂ treatment for 3 weeks. Pre-administration of NS-398 at 3 or 10 mg/kg did not prevent this effect. **P*<0.05, ***P*<0.01 (vs vehicle).

bone marrow by stimulating osteoblastic differentiation (Weinreb *et al.* 1997b, Keila *et al.* 2001). We also reported that the stimulatory effect of PGE₂ on bone marrow cells *in vitro* involves cAMP generation and is mediated by the EP₄ prostanoïd receptor (Weinreb *et al.* 1999). A role for EP₄ in mediating the anabolic effects of PGE₂ *in vivo* was also suggested (Machwate *et al.* 2001). Recently, we showed that EP₄ is the most abundant cAMP-related receptor in rat bone tissue and bone cells and that its expression is induced by its ligand, PGE₂ (Weinreb *et al.* 2001). This last observation suggests that the actions of exogenously administered PGE₂ are augmented via induction of its receptor.

PGE₂-induced EP₄ expression is only one possible autoamplification mechanism. It is well known that prostaglandins, including PGE₂, are produced in bone tissue, predominantly by cells of the osteoblastic lineage (Raisz *et al.* 1993). *In vitro*, PGE₂ production by osteoblastic cells occurs in response to a variety of stimuli, including serum, pro-inflammatory cytokines, growth factors etc. (Kawaguchi *et al.* 1994, Wadleigh & Herschman 1999). Since osteoblastic cells produce PGE₂, they must express one or both of the COX isoforms. Several reports have indicated that the osteoblastic cell line MC3T3-E1, derived from newborn mouse calvaria, expresses both COX-1 and COX-2 and produces PGE₂ in response to various stimuli via the activity of COX-2, and that exogenous PGE₂ induces the expression of COX-2 (Pilbeam *et al.* 1993, 1995, Takahashi *et al.* 1994). A similar observation was made in newborn mouse calvaria organ cultures (Kawaguchi *et al.* 1994). These observations point to PGE₂-induced COX-2 expression as another possible autoamplification mechanism. However, these data generated two important questions. (1) Does this phenomenon also occur *in vivo* and (2) is the generation of endogenous PGs via COX-2 required for the anabolic effect of exogenous PGE₂?

Figure 7 NS-398 prevents carrageenan-induced footpad edema. NS-398 (10 mg/kg) but not DMSO (=vehicle) pre-treatment prevents an increase in foot-pad volume induced by a local carrageenan injection. ** and ##*P*<0.01 (vs the saline or NS-398 groups respectively).

Production of endogenous PGs *in vivo* in response to exogenous PGE₂ could potentiate either a bone-forming effect or a bone-resorbing effect. Unfortunately, *in vitro* data from cell lines did not allow drawing of (tentative) conclusions on this issue. As to the first question, we clearly show here, for the first time, that the regulation of COX-1 and COX-2 expression in rat bone tissue by PGE₂ is similar to that reported previously for MC3T3-E1 cells (Pilbeam *et al.* 1993, Takahashi *et al.* 1994), i.e. the basal level of COX-1 mRNA is far greater than that of COX-2, but it is COX-2 mRNA that is induced by a single administration of exogenous PGE₂. COX-2 induction occurred as early as 30 min post-injection and was still elevated 2 h post-injection. This dose of PGE₂ is anabolic and therefore we sought to answer the second question, whether COX-2 activity is required for the anabolic effect of PGE₂. As expected, our PGE₂ regimen significantly increased bone formation and bone mass, but none of these effects was abrogated by COX-2 inhibition. NS-398 is known to preferentially inhibit COX-2 and the doses that were used are known to inhibit COX-2 activity in a variety of systems (Hirata *et al.* 1997, Mikuni *et al.* 1998, Shigeta *et al.* 1998). In addition, the foot-pad edema model that we used proved that the dose and route of administration were effective *in vivo*. Thus our *in vivo* studies have suggested that COX-2 activity is not essential for the anabolic action of systemic PGE₂. The same strategy was previously used to determine which of the COX isoenzymes is involved in the effect of mechanical loading on bone formation. It has long been known that mechanical forces induce the production of PGE₂ in bone and bone cells (e.g. Klein-Nulend *et al.* 1997) and increase bone formation. Since NS-398 abrogates the mechanical loading-induced bone formation *in vivo* much more

Figure 8 PGE₂ increases and NS-398 decreases the number of mineralized nodules in bone marrow stromal cultures. Note that nodule formation is reduced in control and PGE₂-treated cultures to about the same degree. ****P*<0.005 (vs the respective control groups, matching for the concentration of NS-398).

efficiently than does indomethacin (a non-selective COX inhibitor), COX-2 activity was implicated in this effect (Forwood 1996).

In addition to the *in vivo* studies, we have investigated the effect of NS-398 on nodule formation in bone marrow stromal cell cultures. We and others have shown that PGE₂ at 10–100 nM increases osteoblastic differentiation of these cells as measured by nodule formation and alkaline phosphatase activity (Scutt & Bertram 1995, Scutt *et al.* 1995, Weinreb *et al.* 1997b, 1999, Keila *et al.* 2001). In this study, PGE₂, in the absence of NS-398, increased nodule formation by ~130%. With increasing concentration of NS-398, nodule formation of both control and PGE₂-treated cultures gradually decreased to a similar extent. Even in the presence of 10⁻⁵ M NS-398, PGE₂

increased nodule formation by ~110%, suggesting that endogenous production of PGs is not necessary for exogenous PGE₂ to increase osteoblast differentiation of marrow osteoprogenitors. However, these data also imply that endogenous PG production is required for the effect of other component(s) of the culture medium, possibly fetal bovine serum.

Since COX-2 catalyzes the production of PGH₂, which is the precursor for generations of all PGs (e.g. PGE₂, PGD₂, PGF_{2α} etc. (Pilbeam *et al.* 2002)), the production of all these PGs will be inhibited by an effective dose of NS-398. Therefore it can be concluded that exogenous PGE₂ does not require any of the endogenous COX-2-dependent PGs for its anabolic action.

COX-1 and COX-2 knockout mice have been generated and characterized to a great extent (Dinchuk *et al.* 1995, Langerbach *et al.* 1995). Recently it was reported that bone mass is reduced in COX-2 ^{-/-} mice compared with COX-2 ^{+/+} mice and that marrow stromal cultures from ^{-/-} animals displayed diminished osteoblastic differentiation (Okada *et al.* 2000). These data again point to the importance of COX-2-mediated PG production for normal bone development. However, the reduced osteoblastic differentiation in cultures of COX-2 ^{-/-} cells could be corrected by the addition of exogenous PGE₂. Thus the absence of COX-2 activity does not interfere with the PGE₂-stimulated differentiation of marrow osteoprogenitors, supporting our results. Unfortunately, the study of COX involvement in the *in vivo* anabolic effect of PGE₂ is greatly hampered by the fact that PGE₂ is not anabolic in mice at the same dose range as in rats (Crawford *et al.* 2000, Li *et al.* 2000, authors'

Figure 9 Effect of PGE₂ and NS-398 on EP₄ expression in rat bones. Five-week-old rats were given a single injection of 5 mg/kg PGE₂ (P) or vehicle (V) and were killed 120 min later. Total RNA from the tibial metaphysis was subjected to Northern blot analysis. Note that PGE₂ induces EP₄ expression and that pre-administration of NS-398 (N) does not alter this induction.

unpublished data). Otherwise, analyzing the anabolic effect of PGE₂ in COX-2^{-/-} mice would help to clear this issue.

Furthermore, it was reported that PGE₂ induces COX-2 expression in MC3T3-E1 cells via activation of the EP₁ prostanoid receptor (Suda *et al.* 1998). Since, as mentioned above, there is mounting evidence that the bone anabolic effects of PGE₂ are mediated via the EP₄ receptor and cAMP, this finding also augments the argument that COX-2 induction in bone cells is unrelated to the anabolic action of PGE₂. There are other cells, e.g. lipopolysaccharide (LPS)-stimulated macrophages (Hinz *et al.* 2000) and keratinocytes (Maldve *et al.* 2000), in which exogenous PGE₂ induces COX-2 expression via a cAMP pathway, in contrast with fetal bone cells. Altogether, our study seems to indicate that PGE₂ induces the expression of COX-2 *in vivo* for yet undetermined purposes.

Finally, our data do not indicate that COX-2 inactivation, at least in the time-frame tested, alters the induction of EP₄ caused by PGE₂. Thus the action of NS-398 can be assumed to involve COX-2 inactivation and not interference with the other possible amplification pathway, i.e. induction of EP₄ mRNA.

In summary, our data do not support the hypothesis that endogenous production of PGs via COX-2 is essential for the anabolic effect of PGE₂ *in vivo* or *in vitro*. Nevertheless, it is possible that stimuli, which are weaker than the anabolic dose of PGE₂ used in this study (e.g. local mechanical forces or other mediators), do require endogenous PG production to amplify their effect.

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