Lack of Effect of Ipriflavone on Osteoclast Motility and Bone Resorption in In Vitro and Ex Vivo Studies

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Summary. Using in vitro and ex vivo experimental procedures specifically designed to visualize pharmacological effects on parameters of bone resorption, studies were performed to elucidate whether ipriflavone's reported effect in osteoporosis is due to an effect on the motility and resorptive activity of osteoclasts, as has been shown to be the case with salmon calcitonin. Concentrations of ipriflavone used were higher by a factor of >100 than peak blood levels measured in patients given standard therapeutic doses. Despite this, neither quantitative nor qualitative changes were observed in the motility of isolated rat osteoclasts or in their resorptive activity when incubated with bone slices. The conclusion is that ipriflavone does not possess antosteoclastic and antiresorptive activity of the type documented for salmon calcitonin in the models employed and that further investigation of its mode of action is therefore necessary.

Key words: Ipriflavone – Osteoclast motility – Bone resorption.

Bone tissue is maintained through a homeostatic mechanism in which formation is balanced by resorption. The process takes place within basic multicellular units where osteoclasts, responsible for bone formation, work alongside osteoblasts, responsible for bone resorption. These parallel but opposite activities are closely coupled when bone is in its normal physiological steady state, as overall bone mass changes little relative to bone turnover. The regulatory mechanism involved remains unknown, although a number of factors have been identified as acting on osteoclasts, osteoblasts, or both. Thus, calcitonin inhibits resorption [1] primarily through a direct inhibitory effect on osteoclasts [2], thereby conserving bone mass. Estrogens are also recognized clinically, for example in postmenopausal osteoporosis, as having a preservative effect on bone [3, 4], though the mechanism by which they do so remains disputed and their use is restricted by a number of risks—notably endometrial cancer—particularly if they are given without progesterone.

In contrast to these basically physiological substances, ipriflavone (7-isopropoxy-3-phenyl-4H-1-benzopyran-4-one) is an isoflavone derivative of plant origin that was synthesized in the late 1960s. It belongs to a large class of natural compounds, the flavonoids, which have a wide range of pharmacological properties [5], the precise mechanisms of which remain unknown. Some flavonoids possess estrogenic activity [6, 7], but this has been reported not to be the case with ipriflavone [8]. Ipriflavone has, however, been reported inter alia to possess osteotrophic activity, which has been characterized in laboratory studies as inhibition of resorption [9, 10] and clinically as inhibition of osteoporosis [11–20].

Because effects on bone are difficult to objectify adequately in vivo, in vitro techniques had been devised to demonstrate the effects of putatively active substances on the motility and resorptive activity of isolated osteoclasts. These had provided evidence of a dramatic effect of calcitonins—especially the fish varieties—and it was felt that the evidence adduced in support of the antiresorptive activity of ipriflavone could best be verified by subjecting the compound to the same protocols as being the most objective currently known for the visualization of osteoclast response. Studies were therefore set up using salmon calcitonin (SCT) as a reference to investigate the effect of ipriflavone on these parameters in vitro and, in an ex vivo study, on the same parameters and on adhesion in osteoclasts from rats dosed once or for 8 days. This latter protocol was specifically designed to verify the suggestion that the compound's resorptive activity might be due to one or more metabolites [10] or to a possible increase in endogenous calcitonin secretion [21].

Materials and Methods

Materials

The ipriflavone used was taken from batch 8991, synthesized in house as described in German patent publications 2166458 and 2125245 [22, 23]; the SCT was from batch 90035 synthesized in house in accordance with the method of Guttmann et al. [24]. Ipriflavone was identified by proton nuclear magnetic resonance and mass spectrometry, the analysis showing it to have a purity of the order of 93%. The potency of the salmon calcitonin used, as assessed by bioassay, was 5120 IU/mg. All other chemicals were of reagent grade.

In Vitro Assessment of Effect on Osteoclast Motility

Osteoclasts were obtained from the long bones of 2- or 3-day-old Wistar rats killed by cervical dislocation. The bones concerned were removed, cleaned of adherent soft tissue, and cut across their epiphyses. They were then mechanically disaggregated with a scalpel blade into a medium (= M199) composed of Hepes-buffered medium 199 (2 ml/4 bones) (Flow Laboratories, Irvine, UK) supplemented with 1-glutamine (2 mM), benzylpenicillin (100 IU/ml) and strepto-
mycin (100 μg/ml). The suspension of bone particles was agitated with a pipette (Movette®) and larger fragments were allowed to sediment (~10 seconds). One hundred microliters of the suspension was placed in each of the wells of microtiter plates (Linbro®), into which 13-mm coverslips (Gibco, Paisley, UK) were placed. The plates were incubated for 20 minutes at 37°C in 5% CO₂ in humidified air to allow the osteoclasts to sediment and adhere. The coverslips were then removed, washed in M199, and placed in new wells already containing 2 ml of M199 with ipriflavone (10 μg/ml) or the corresponding vehicle; this was 0.1% ethanol and a 0.1% solution of acetate buffer with 0.75% NaCl, respectively. The plates were reincubated for 2 hours under the conditions already described and the procedure was terminated by fixing the cells in 3% buffered formalin (Gibco; Sigma, St. Louis, Mo, USA). The total number of osteoclasts on each coverslip was counted on a phase-contrast microscope and the percentage of those that were motile was determined using the criteria established by Chambers and Magnus [25], based primarily on the presence or absence of smooth-contoured pseudopods. A total of eight experiments were performed for each compound and control.

For qualitative assessment, osteoclasts were disaggregated as before from neonatal rats and incubated in 50 mm tissue culture dishes containing M199. A suitable osteoclast was chosen and recorded by time-lapse cinematography, as previously described [25]. After a control period, vehicle (0.1% ethanol) or ipriflavone (10 μg/ml) was added and recording was continued for a further 2 hours. Osteoclast behavior was assessed at intervals: a playback of the recording viewed at 72 times real time. As a control for these experiments, the behavior of osteoclasts exposed to ipriflavone was compared with their response to salmon calcitonin at doses of up to 50 pg/ml.

In Vitro Assessment of Effect on Bone Resorption

Devitalized slices were prepared from bovine femoral cortical bone, as previously described [26]. The slices (0.4 x 0.4 x 0.01 cm) were prepared from wafers of bone cut with a low-speed saw (Isomet, Buehler, IL, USA), cleaned by ultrasoundication for 20 minutes in distilled water, immersed in acetic acid for 10 minutes followed by alcohol (also 10 minutes), and stored dry at room temperature [27]. Osteoclasts were disaggregated as before from neonatal rat long bones and the suspension was removed and placed in the wells of a 5 x 5 culture plate (Flow Laboratories) containing the bone slices. These were reincubated for 10 minutes at 37°C, washed vigorously in Earle’s minimal essential medium (MEM) (Gibco) containing 10% fetal calf serum (FCS) to remove nonadherent cells, and reincubated in 0.2 ml of the previous medium in humidified air in the presence of ipriflavone (0.1, 0.3, 1, 3, 10 μg/ml) or vehicle (0.1% ethanol). Previous experiments (unpublished) showed that at concentrations up to 0.1% ethanol has no effect on bone resorption. Six bone slices were used for each concentration and each experiment. After 18 hours’ incubation, the bone slices were removed from the wells and placed in 10% NaOCl for 10 minutes in order to remove the cells so that the osteoclastic excavations could be visualized. The slices were then washed in water, dried, and sputter-coated with gold. Bone resorption was measured in the scanning electron microscope, as previously described [28, 29], by inspecting the whole surface of each bone slice for osteoclastic excavations and recording the number of such resorption pits and the plan area of each. The results were expressed in absolute terms as excavations/bone slice and as the total plan area of resorbed bone/slice, and also as percentages of the control values (vehicle).

Ex Vivo Assessment of Effect on Osteoclast Adhesion and Motility

Groups of five young female rats weighing ~20 g were given a single oral dose of 300 mg ipriflavone (two groups), 0.5 ml suspension in 1% hydroxypropylcellulose (HPCC) (Aldrich, Milwaukee, Wis, USA) or vehicle only (two groups), or a single subcutaneous dose of 25 IU SCT in 0.025 ml acetic buffer solution with 0.75% NaCl (one group) or the corresponding vehicle (one group), respectively. The animals comprising the ipriflavone and corresponding placebo groups were sacrificed 60 and 180 minutes after treatment; those in the salmon calcitonin and corresponding placebo groups were sacrificed 60 minutes after treatment. The choice of these intervals was based on data from previous experiments as being most appropriate to the respective absorption properties of and biological response to the two study compounds and their different routes of administration [30-32]. Osteoclasts were disaggregated and counts were made of the absolute number adhering and the percentage of motile osteoclasts, as described above. The same experiment was carried out after daily administration for 8 days in order to assess whether ipriflavone develops an effect in the longer term, possibly through the mediation of a metabolite or metabolites. Treatments were administered in the morning and 0.5 ml milk per animal was given in the late afternoon.

The overall interassay variability was of the order of 6.7% (= coefficient of variation) for the adhesion studies and 8.5% for the motility series.

Results

Osteoclast Motility

The percentage of motile osteoclasts was not reduced at all by ipriflavone, even at the comparatively high dose of 10 μg/ml, but there was marked inhibition with the same dose of SCT (Table 1). Previous experiments [33] have clearly established that much lower doses of salmon calcitonin cause potent inhibition of isolated rat osteoclast motility. Qualitatively, no change was detected in osteoclast behavior in response to the presence of ipriflavone in any of the recordings made, and inspection of the osteoclasts in the same dish that were not recorded revealed no sign of the change in shape that accompanies osteoclast motility inhibition. Osteoclasts incubated in SCT at concentrations up to 50 pg/ml, on the other hand, showed a dramatic change in behavior within minutes, with complete cessation of pseudopodial motility and gradual cytoplasmic retraction over the ensuing hours (Fig. 1).

Bone Resorption

No effect of ipriflavone was found on the parameters of bone resorption investigated, even at the highest concentration used (Table 2).

Osteoclast Motility and Adhesion Ex Vivo

A single oral dose of 300 mg ipriflavone caused no inhibition of osteoclast adhesion or motility at the times tested (1 hour

| Table 1. Percentage of osteoclasts showing motility after treatment with ipriflavone or salmon calcitonin compared with vehicle only (= control) |
|---------------------------------|------------------|-----------------|------------------|
| Treatment                       | Motile osteoclasts (%) | % Control value | Stat. signif.    |
| Control (vehicle)               | 80 ± 2            | 100             | ns               |
| Ipriflavone (10 μg/ml)          | 88 ± 1            | 110             | ns               |
| Control (vehicle)               | 82 ± 1            | 100             | ns               |
| Salmon calcitonin (10 μg/ml)    | 24 ± 2            | 29              | <0.01            |

Each value is the mean (±SEM) for eight assays.