Inhibition of prostaglandin action and bone resorption by copper

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SUMMARY Mouse calvaria were maintained in organ culture without serum additives. The effects of Cu^{2+} on bone resorption and on the synthesis and action of prostaglandins were studied. Non-toxic concentrations of copper sulphate ($5 \mu\text{M}$) were found to decrease active resorption, measured by ^{45}Ca release, to 54% control values ($p < 0.001$), while prostaglandin F (PGF), prostaglandin E_2 (PGE_2), and 6-keto-prostaglandin $\text{F}_{1\alpha}$, (6-keto-PGF $_{1\alpha}$), determined by radioimmunoassay, were increased above controls ($p < 0.05$). These effects of Cu^{2+} on prostaglandin synthesis were confirmed by the isolation and quantitation of [^3H]-labelled metabolites released from calvaria which had been pre-labelled with [^3H]-arachidonic acid. PGE_2 , PGF $_{2\alpha}$, 6-keto-PGF $_{1\alpha}$, and thromboxane B_2 (TxB_2) were all higher in copper-exposed calvaria, but their relative amounts remained unchanged. There was no evidence that Cu^{2+} influenced the mobilisation of [^3H]-arachidonic acid from prelabelled calvaria. The stimulation of bone resorption by exogenous prostaglandins was decreased in the presence of Cu^{2+} ($p < 0.005$), while parathormone-mediated bone resorption was virtually unaffected. Cu^{2+} also increased the inhibition of bone resorption seen with indomethacin ($p < 0.05$). In addition to the effects of the metal on prostaglandin action Cu^{2+} also decreased β -glucuronidase activity in the media to 86% of the control values ($p < 0.001$). The action of Cu^{2+} in inhibiting bone resorption in vitro appears complex but does not involve inhibition of prostaglandin synthesis. It is likely that Cu^{2+} has more than one inhibitory locus.

Key words: prostaglandins F biosynthesis, prostaglandins E biosynthesis, copper pharmacodynamics, rheumatoid arthritis, bone resorption.

Elevated serum copper levels are present in a number of inflammatory conditions including rheumatoid arthritis,¹ ankylosing spondylitis,² psoriasis,³ adjuvant arthritis,⁴ and periodontal disease.⁵ Despite these observations a number of hypotheses have proposed an 'anti-inflammatory' role for endogenous copper^{6,7} and copper is used in various forms as an anti-inflammatory agent.^{8,9}

Previous studies in this laboratory have shown that copper is a potent inhibitor of bone resorption in vitro.¹⁰ While its mechanism of action is unknown in bone, in cell-free systems there is evidence that copper alters prostaglandin biosynthesis, enhancing

PGF $_{2\alpha}$ production at the expense of PGE_2 .¹¹ Prostaglandins, particularly those of the E series and the F series, are known to stimulate bone resorption in vitro,¹² and the prostaglandin synthetase system has been identified in cultured bone.¹³ The following study examines the possibility that the decreased bone resorption observed in the mouse calvarium in the presence of copper in vitro is associated with altered prostaglandin biosynthesis or action.

Material and methods

ORGAN CULTURE OF BONE

The organ culture procedure has been described in detail.^{10,14} Unless otherwise stated, calvaria were dissected from six-day old mice and then pooled and randomised into treatment groups and one control group. The bones were cultured singly in 5 ml Gibco

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199 medium (without serum additives) and incubated in an atmosphere of 20% O₂, 5% CO₂, and 75% N₂. Calvaria were precultured for 24 h after dissection and then cultured in fresh medium for a further 48 h under experimental conditions. This procedure removes the large quantities of prostaglandins released after dissection and renders the system sensitive to stimulators of resorption.¹⁴ Indomethacin and prostaglandins were added to the culture medium in absolute ethanol solutions. The alcohol added to both control and treated bones did not exceed a final concentration of 0.3% and as such had no effect on active resorption.

DETERMINATION OF ACTIVE RESORPTION

Resorption of bone was determined by the release of ⁴⁵Ca into the medium from calvaria obtained from mice which were prelabelled with 5 μ Ci ⁴⁵CaCl₂ at 2 days of age. Non-cell-mediated release of ⁴⁵Ca was determined from four calvaria of each litter, which were killed by triple freezing and thawing prior to incubation. The percentage active resorption (⁴⁵Ca) was quantified and calculated as described.¹⁰

MEASUREMENT OF PROSTAGLANDINS

The culture medium was sampled to determine ⁴⁵Ca release and then acidified (pH 3.0) prior to extraction of the prostanoids. Prostaglandin F (PGF), prostaglandin E₂ (PGE₂), and 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) were measured by specific radioimmunoassays. The method of extraction, assay procedures and cross-reactivity data for PGF and PGE₂ radioimmunoassays have been described in detail.^{14 15} Extraction with ethyl acetate improved the recovery of 6-keto-PGF_{1 α} , and was therefore used in preference to diethyl ether when this prostanoid was assayed. An antiserum to 6-keto-PGF_{1 α} conjugated with bovine serum albumin (BSA) was raised in rabbits. This antiserum cross-reacted 14.7% with PGD₂, 9.3% with PGF_{2 α} , 8.4% with PGE₂, and 22.9% with 13, 14-dihydro-PGF_{2 α} .¹⁵ Samples were redissolved in phosphate buffer (0.1 M) pH 7.4, containing NaCl (0.15 M), NaN₃ (0.015 M), and gelatin (0.1%) and assayed in duplicate. The assay method and cross-reactivity data are fully described.¹⁵ The standard curve was constructed over a range of 0–500 pg. Blank values of 20.8 $\pm$ 1.8 (SD) pg were obtained by extracting medium and processing it as for the samples.

Further purification of the samples was not required unless exogenous arachidonic acid (10⁻⁵ M) was present, resulting in high blank values. Column chromatography of these samples with silicic acid eliminated this non-specific binding effect. All results presented have been corrected for recovery from extraction.

UPTAKE OF (³H)ARACHIDONIC ACID INTO CALVARIA

[³H] Arachidonic acid ([³H]-AA) was purified by column chromatography prior to use.¹⁶ The following procedure optimised the uptake of [³H]-AA into bone. After dissection each whole calvarium was inverted and cultured for 24 h in 5 ml medium containing no serum additives. The cranial space was filled with 80 μ l medium containing 2–5 μ Ci [³H]-AA to give a concentrated source of isotope close to the tissue surface. After 24 h incubation with the isotope the tissue was rinsed to remove excess [³H]-AA and then dissected into paired hemicalvaria. One half of each calvarium was used as a control bone and the other cultured in medium containing copper sulphate (5 μ M). Two calvaria were cultured per 5 ml medium. After 48 h the medium from each treatment group was pooled, the radioactivity estimated, and the remaining media frozen rapidly. Calvaria from each treatment group were pooled, demineralised¹⁷ and solubilised to estimate total incorporation of labelled arachidonic acid and to ensure uniform uptake of isotope between treatment groups.

PURIFICATION AND SEPARATION OF LABELLED PROSTANOIDS

Twenty μ g of each of unlabelled PGE₂, PGF_{2 α} , 6-keto-PGF_{1 α} , and thromboxane B₂ (TxB₂) was added to each medium sample to optimise recoveries. Medium (15 ml) was then acidified (pH 3.4) with formic acid and loaded on to a C₁₈ Sep Pak (Waters Assocs, Mass, USA), washed with (1) H₂O, (2) 12% ethanol (3) petroleum ether, and the prostanoids eluted with methyl acetate, similar to the method of Powell.¹⁶ Excess solvent was removed with oxygen-free dry nitrogen and the residue redissolved in 200 μ l of high-performance liquid chromatography (HPLC) column solvent (see below). Prostanoids were separated by HPLC using a C₁₈ μ -Bondapak column and instrumentation from Waters Associates. The separation method was based on that of Alam *et al.*¹⁸ The arachidonic acid metabolites were eluted isocratically with a solvent system consisting of acetonitrile:water:benzene:acetic acid (230:767:2:1) at a flow rate of 1 ml/min. All fractions were collected and counted. Results presented are not corrected for recovery from the column. Authentic [³H]-PGE₂, [³H]-PGF_{2 α} , [³H]-6-keto-PGF_{1 α} , and [³H]-TxB₂ were mixed with 20 μ g of each appropriately unlabelled prostanoid and used as standards to identify the major metabolites. The recoveries were from 69.5 to 80.0%. [³H]-AA was incubated in the absence of calvaria with and without Cu²⁺ under the normal culture conditions and processed as described above. No significant

radioactive material was eluted by HPLC with retention times corresponding to 6-keto-PGF_{1α}, TxB₂, PGF_{2α}, or PGE₂.

MATERIALS

Medium 199 was from Grand Island Biological Company, Grand Island, New York. Indomethacin, activated charcoal, dextran, sodium azide, 4-methyl umbelliferone, and 4-methylumbelliferyl glucuronide were from the Sigma Chemical Co., St Louis, USA. The radioactive chemicals ⁴⁵CaCl₂ (59 µg/mCi), [5,6,8,12,14,15(n)-³H] PGE₂ (160 Ci/mmol), [9-³H] PGF_{2α} (19.4 Ci/mmol), [5,6,8,9,11,12,14,15, -³H] arachidonic acid (100 Ci/mmol) were purchased from the Radiochemical Co., Amersham, England. [5,8,9,11,12,14,15(n)-³H] 6-keto-PGF_{1α} (120 Ci/mmol) was supplied by New England Nuclear, Boston, USA. Unlabelled prostaglandins were kindly provided by Dr J. E. Pike, Upjohn Co., Kalamazoo, USA. Antiserum to PGF and to 6-keto-PGF_{1α} were kindly supplied by Professor G. C. Liggins and antiserum to PGE₂ was purchased from the Pasteur Institute, Paris. All other solvents and reagents used were of analytical grade. Mice were Charles River CD-1 (an outbred strain).

STATISTICS

Results are presented as mean values ± SEM. The data were evaluated by analysis of variance followed by Spjøtvoll and Stoline's T method.¹⁹ Student's *t* test was used for unpaired data as appropriate.

Results

Previous in-vitro studies have shown that Cu²⁺ inhibits bone resorption in a dose-dependent manner.¹⁰ In the experiment described below CuSO₄·5H₂O, used at a concentration of 5 µM, did not influence DNA and protein synthesis within calvaria.¹⁰ The quantities of prostaglandins released into the medium from calvaria cultured with and without Cu²⁺ (5 µM) are shown in Table 1. The

amounts of PGF, PGE₂, and 6-keto-PGF_{1α} released from calvaria were increased significantly in the presence of Cu²⁺ (*p*<0.05), while active resorption was decreased to 54% of controls (*p*<0.001). The relative amounts of the prostaglandins measured were similar in control and Cu²⁺-treated calvaria and were in the order 6-keto-PGF_{1α}>PGE₂>PGF. Cu²⁺ (5 µM) did not appear selectively to enhance the production of any one of the prostaglandins measured (Table 1).

The addition of arachidonic acid (10⁻⁵ M) to calvaria caused increased production of both PGF and PGE₂ compared with the amounts released from calvaria cultured without arachidonic acid. This increase in PGF and PGE₂ was concomitant with a rise in active resorption.¹⁴ When calvaria were cultured with arachidonic acid in the presence of Cu²⁺, active resorption was significantly lowered, while PGF production increased above that seen with arachidonic acid alone (*p*<0.001), and PGE₂ levels were not significantly different (Table 2).

The results obtained by radioimmunoassay measurements (Tables 1 and 2) were confirmed by isolation and measurement of radioactive metabolites released by calvaria prelabelled with [³H]AA. The average uptake of labelled arachidonic acid in two separate experiments, each with one control and one copper-treated group of calvaria, was 17.5±1.2%. During the subsequent 48 h, 10.5% of this radioactivity was released into the medium from control bones and 10.9% was released from bones cultured with 5 µM Cu²⁺. Separation of the prostanooids in media using HPLC (see 'Methods') showed peaks of radioactive labelled compounds corresponding to 6-keto-PGF_{1α}, TxB₂, PGF_{2α}, and PGE₂ as identified by means of authentic [³H]-labelled compounds. There were no major unidentified peaks of radioactive labelled compounds present. There was evidence for small amounts of radioactive

Table 1 Prostaglandin production from calvaria in the presence of copper

	PGF (pg/bone)	PGE ₂ (pg/bone)	6-keto-PGF _{1α} (pg/bone)	% active resorption (⁴⁵ Ca)
Control	506±34	760±108	995±145	25.5±0.5
Cu ²⁺	696±65**	1132±119**	1393±97*	13.7±0.6**

Calvaria were precultured 24 h, then cultured in fresh medium with or without Cu²⁺ (5 µM) for 48 h. Results are shown as mean ± SEM. Ten calvaria in each treatment (from two litters). Significantly different from control; **p*<0.05, ***p*<0.02.

Table 2 Prostaglandin production from calvaria incubated with arachidonic acid and copper

	PGF (pg/bone)	PGE ₂ (pg/bone)	% active resorption (⁴⁵ Ca)
Untreated	440±64	859±72	20.9±0.9
Arachidonic acid (10 ⁻⁵ M)	1189±56*	5526±530	28.2±1.2
Arachidonic acid (10 ⁻⁵ M) plus Cu ²⁺	1596±80*	4711±358	14.4±0.7

Calvaria were precultured for 24 h, then cultured with or without arachidonic acid and Cu²⁺ (5 µM) for 48 h. Results are shown as mean ± SEM. Eleven calvaria in each treatment (from two litters). Significantly different from each other: **p*<0.001.

Table 3 [^3H]-Labelled arachidonic acid metabolites produced by calvaria incubated with or without copper

	Control dpm	% of total dpm	Copper dpm	% of total dpm	Ratio $\frac{\text{copper}}{\text{control}}$
<i>Experiment 1</i>					
6-keto-PGF _{1α}	12938	48.07	18172	48.71	1.40
TxB ₂	1841	6.84	2341	6.27	1.27
PGF _{2α}	2149	7.98	3025	8.11	1.41
PGE ₂	9985	37.10	13765	36.90	1.38
n	10		10		
<i>Experiment 2</i>					
6-keto-PGF _{1α}	18670	50.65	25799	50.37	1.38
TxB ₂	3775	10.24	5168	10.09	1.37
PGF _{2α}	2799	7.59	3988	7.78	1.42
PGE ₂	11614	31.51	16259	31.74	1.40
n	8		8		

Calvaria were prelabelled with [^3H]-AA and cultured with or without Cu $^{2+}$ (5 μM) as outlined in 'Methods'. Labelled prostanoids were separated by HPLC as outlined in 'Methods'.
dpm=Disintegrations per minute.

Table 4 Effect on active resorption of increasing concentrations of PGE₂ in the presence of copper

Control	PGE ₂ 10 $^{-8}$ M	Δ	Copper	PGE ₂ 10 $^{-8}$ M + copper	Δ
19.1 $\pm$ 0.5	23.7 $\pm$ 0.9	4.6	10.7 $\pm$ 1.3	11.8 $\pm$ 1.2	1.1 p<0.005
Control	PGE ₂ 10 $^{-7}$ M	Δ	Copper	PGE ₂ 10 $^{-7}$ M + copper	Δ
19.1 $\pm$ 0.5	27.3 $\pm$ 0.8	8.2	10.7 $\pm$ 1.3	15.6 $\pm$ 0.5	4.9 p<0.001
Control	PGE ₂ 10 $^{-6}$ M	Δ	Copper	PGE ₂ 10 $^{-6}$ M + copper	Δ
19.1 $\pm$ 0.5	31.4 $\pm$ 1.7	12.3	10.7 $\pm$ 1.3	18.5 $\pm$ 1.6	7.8 p<0.005

Calvaria were precultured for 24 h, the medium changed, and prostaglandins added with or without Cu $^{2+}$ (5 μM). Percentage active resorption (^{45}Ca) is expressed as mean $\pm$ SEM.
Significant differences between each treatment and its appropriate control are shown on right hand side.
Calvaria from two litters were pooled (n=5 each treatment).
 Δ =Difference.

Table 5 Effect of copper on active resorption (^{45}Ca) in the presence of promoters of resorption

Control	Arachidonic acid	Δ	Copper	Arachidonic acid + copper	Δ
22.6 $\pm$ 0.8 (n=15)	28.8 $\pm$ 1.3 (n=15)	+6.2	12.5 $\pm$ 0.6 (n=15)	16.1 $\pm$ 0.6 (n=15)	+3.6 p<0.005
Control	PGE ₂	Δ	Copper	PGE ₂ +copper	Δ
24.1 $\pm$ 0.8 (n=14)	42.7 $\pm$ 1.4 (n=14)	+18.6	13.2 $\pm$ 0.4 (n=14)	22.5 $\pm$ 0.9 (n=14)	+9.3 p<0.001
Copper	PGF _{2α}	Δ	Copper	PGF _{2α} +copper	Δ
17.9 $\pm$ 1.9 (n=9)	32.1 $\pm$ 1.3 (n=9)	+14.2	9.9 $\pm$ 1.1 (n=9)	17.3 $\pm$ 0.6 (n=9)	+7.4 p<0.001

Calvaria were precultured for 24 h, medium changed, and additions made as shown. (Arachidonic acid (10 $^{-5}$ M), PGE₂, or PGF_{2 α} (10 $^{-5}$ M) and Cu $^{2+}$ (5 μM)). Percentage active resorption (^{45}Ca) is expressed as mean $\pm$ SEM.
Significant differences between each treatment and its appropriate control are shown on right hand side. Ten calvaria in each treatment (from two litters).

material which may have corresponded to PGE₂ and PGF_{2α} metabolites, but these were not identified by authentic [³H]-labelled compounds. The results of these two separate experiments are shown in Table 3. In both experiments there was evidence that Cu²⁺ (5 μM) enhanced the production of each of the prostanoids identified. The relative amounts of 6-keto-PGF_{1α}, PGF_{2α}, PGE₂, and TxB₂ were unchanged by exposure to Cu²⁺ in the two experiments. The amount of [³H]-PGE₂ was probably underestimated, since experiments using chromatographically pure [³H]-PGE₂ showed that, under the culture conditions employed, 24.8% was converted to PGA₂.

The effect of copper in combination with prostaglandins was investigated by adding increasing amounts of PGE₂ (10⁻⁸ M to 10⁻⁶ M) to calvaria cultured with or without Cu²⁺ (5 μM). The results outlined in Table 4 compare the ability of PGE₂ at each concentration to increase resorption under these conditions. As can be seen, the reduced resorption in the presence of Cu²⁺ cannot be overcome by increasing concentrations of PGE₂. Arachidonic acid (10⁻⁵ M) or a higher concentration of PGE₂ (10⁻⁵ M) or PGF_{2α} (10⁻⁵ M) were also unable to reverse the effect of Cu²⁺ (Table 5).

Calvaria were cultured with or without Cu²⁺ with the addition of indomethacin at a concentration (7.0×10⁻⁷ M) sufficient to inhibit prostaglandin synthesis.¹⁷ Cu²⁺ decreased the ⁴⁵Ca resorption to 77.4% of that seen with indomethacin alone (p<0.05, n=5).

Discussion

A number of studies have confirmed that prostaglandins, particularly those of the E series and F series, and prostacyclin are potent stimulators of bone resorption *in vitro*.^{12 20} Mouse calvaria produce prostaglandins under appropriate culture conditions.^{13 14} Our own studies would indicate the involvement of these compounds in maintaining basal bone resorption *in vitro*, since non-toxic concentrations of indomethacin (≤7.0×10⁻⁷ M) decreased both bone resorption and prostaglandin production.¹⁷ Under normal culture conditions the relative amounts of [³H]-labelled prostanoids released by calvaria prelabelled with [³H]-arachidonic acid were in the order 6-keto-PGF_{1α}>PGE₂>PGF_{2α} and TxB₂. These results are generally in agreement with those of Voelkel *et al.*¹⁵ except that there was no evidence for the production of [³H]-6, 15-diketo-PGF_{1α}.

Although non-toxic levels of Cu²⁺ (5 μM) lowered bone resorption (p<0.001), the synthesis of

PGE₂, PGF, or 6-keto-PGF_{1α} was not decreased (Table 1). Rather, the levels of these prostaglandins appeared to increase. Moreover, when [³H]-arachidonic acid was added to the culture medium in the presence of Cu²⁺, there was again evidence of an increase in the synthesis of all these prostaglandins and also in TxB₂ (Table 3). There was, however, no effect of Cu²⁺ on the relative amounts of these four prostanoids, in contrast to the findings of Maddox,¹¹ who found an increase in PGF_{2α} at the expense of PGE₂ in sheep seminal vesicle homogenates incubated with Cu²⁺ (100 μM). The increase in total prostanoid synthesis did not appear to be caused by an increase in substrate availability, since the mobilisation of [³H]-arachidonate from pre-labelled calvaria was almost identical in the presence and absence of Cu²⁺. These results may therefore be interpreted as suggesting that the influence of Cu²⁺ is to enhance the activity of cyclo-oxygenase in this *in-vitro* system.

The possibility that copper was affecting the action of prostaglandins on bone resorption was also considered. Arachidonic acid (10⁻⁵ M) increased both prostaglandin production and active resorption (Table 2). This arachidonic acid-stimulated resorption was significantly reduced in the presence of Cu²⁺ (Table 5) despite an increase in PGF production (Table 2), that is, the production of prostaglandins was increased, but their action was inhibited by Cu²⁺. This observation was confirmed when either PGE₂ or PGF_{2α} was added to Cu²⁺. Increasing concentrations of PGE₂ (10⁻⁸ M to 10⁻⁵ M) and high concentrations of PGF_{2α} (10⁻⁵ M) were unable to reverse the inhibition of resorption caused by Cu²⁺ (Tables 4 and 5).

These results suggest that Cu²⁺ may 'uncouple' the resorptive response to prostaglandins, but its exact mechanism of action cannot be deduced from these experiments. It is likely that Cu²⁺ has more than one locus of action. This was suggested by experiments where calvaria were cultured with indomethacin (7.0×10⁻⁷ M) with and without Cu²⁺ (5 μM). In these experiments PGE₂ release was lowered to <6.0% of the amounts in cultures without indomethacin.¹⁷ With the further addition of Cu²⁺ resorption was decreased below that seen with indomethacin alone, suggesting an inhibitory action independent of prostaglandin synthesis. This could possibly be due to a direct effect on the activity of β-glucuronidase and perhaps other lysosomal enzymes implicated in bone resorption.²¹ β-Glucuronidase activity released into the media from calvaria and measured by the method of Willcox²² was decreased to 86% control values in the presence of Cu²⁺ (p<0.001, n=24). The complexity of the resorption process was evident also

from experiments using parathormone stimulation of resorption. The parathormone-stimulated resorption was virtually unaffected by the presence of Cu^{2+} ($5 \mu\text{M}$) in contrast to the resorption stimulated by prostaglandins (results not shown).

This study does not suggest any simple interpretation of the action of copper as an anti-inflammatory agent in inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis, and periodontal disease. It gives some indication of the need for care in devising experimental models for these conditions, since the possibility of an anti-inflammatory (or antiresorptive activity) of copper in opposing prostaglandin action rather than synthesis has not always been considered.²³ Thus the high concentrations of both prostaglandins^{24, 25} and Cu^{2+} ²⁶ in rheumatoid arthritis synovial fluid cannot necessarily be interpreted as suggesting that Cu^{2+} has no anti-inflammatory activity.

In summary, the mechanism of action of Cu^{2+} in decreasing bone resorption appears complex. Evidence has been presented to show that the release of prostaglandins is not reduced, but rather enhanced, possibly due to a non-specific effect on the activity of cyclooxygenase. It would appear that Cu^{2+} not only impedes the action of exogenous prostaglandins on bone but may also inhibit resorption mediated by lysosomal enzymes or other as yet undefined mechanisms.

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