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Effect of Cortisol on Periosteal and Nonperiosteal Collagen and DNA Synthesis in Cultured Rat Calvariae

Ernesto Canalis

Endocrine Section, Department of Medicine and the Research Laboratory, Saint Francis Hospital and Medical Center, Hartford, Connecticut; and the University of Connecticut Health Center, Farmington, Connecticut

Summary. The effects of cortisol on bone formation are complex and may be modulated by the presence of periosteal cells or by factors released by the periosteal tissue. To test these possibilities, cortisol was examined for its effects on the incorporation of ^{3}H-proline into collagenase-digestible protein (CDP) and noncollagen protein (NCP), on DNA synthesis and on alkaline phosphatase activity in intact and in the periosteum and nonperiosteal bone of dissected calvariae from 21-day-old fetal rats. After 24 h of treatment, cortisol increased the incorporation of ^{3}H-proline into CDP in intact bones and in the nonperiosteal bone of calvariae dissected after the culture. Cortisol inhibited the incorporation of ^{3}H-thymidine into calvarial DNA but it caused a small increase in nonperiosteal DNA content. Cortisol did not affect the incorporation of ^{3}H-proline into CDP in calvariae dissected prior to the culture if the periosteum and nonperiosteal central bone were incubated separately; the stimulatory effect was observed only if the two tissues were cultured in the same vial and were in contact. In contrast, cortisol stimulated alkaline phosphatase activity in the central nonperiosteal bone of calvariae dissected before or after the culture. After 72–96 h of treatment, cortisol inhibited the labeling of CDP, NCP, and DNA and the DNA content in intact bones and in both periosteal and nonperiosteal central bone of calvariae dissected after the culture. In contrast, when the periosteum was removed before the incubation, these inhibitory effects were observed in the periosteum and not in the nonperiosteal bone. Cortisol inhibited alkaline phosphatase activity in intact bones treated for 96 h, but removal of the periosteum resulted in a stimulatory effect in the nonperiosteal central bone. These studies indicate that 24 h treatment with cortisol stimulates collagen synthesis in nonperiosteal bone, an effect that requires the presence of periosteal tissue. Exposure to cortisol for 72–96 h inhibits collagen, noncollagen protein, and DNA synthesis, an effect that is secondary to an inhibition of periosteal cell replication. Cortisol does not cause a direct inhibition of osteoblastic function.

Key words: Cortisol — Collagen synthesis — DNA synthesis — Alkaline phosphatase activity — Periosteum and nonperiosteal bone.

Cortisol is known to inhibit protein synthesis in isolated nonperiosteal bone cells but it has a dual effect on collagen synthesis in intact calvariae [1–6]. After short-term treatment, physiological concentrations of cortisol have a stimulatory effect whereas after long-term treatment cortisol causes an inhibitory effect.

The periosteum contains mostly fibroblasts and precursor cells [7–9] and investigators frequently remove it to enrich the osteoblastic cell population. However, the periosteal tissue may have a significant role in bone formation and it may be responsible for the differences between the cortisol effects observed in intact calvariae and in isolated nonperiosteal bone cells. The periosteum may affect bone formation by providing precursor cells, needed to achieve a normal osteoblastic cell population, or by providing bone growth factors, known to be released by cultured fibroblasts and intact bones [10–14]. The long-term inhibitory effect of cortisol on bone collagen is secondary to a decrease in cell pop-

Send off reprint requests to Dr. Ernesto Canalis, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105.

ulation, whereas the short-term stimulatory effect could be related to locally released growth factors [6, 12].

The present investigations were undertaken to determine if the short-term stimulatory and the long-term inhibitory effects of cortisol on calvarial collagen synthesis were dependent upon the presence of periosteal tissue or periosteal factors. For this purpose, cortisol was studied for its effects on collagen and DNA synthesis in intact calvariae and in the periosteum and periosteum-free bone of dissected calvariae.

Materials and Methods

Culture Technique

Half calvariae from 21-day-old fetal rats were cultured in sterile 25 ml vials containing 2 ml of modified BGJ_b medium supplemented with bovine serum albumin (4 mg/ml; Reheis Chemical Company; Phoenix, AZ) and 1 mM unlabeled proline [15, 16]. In the experiments used to study DNA synthesis, the medium was also supplemented with 0.1 mM thymidine. The vials were gassed with 5% CO_2 in air for 1 min, sealed with rubber stoppers, and placed in a continuously shaking water bath (40 oscillations/min) at 37°C for periods of 24 or 96 h. In the 96 h incubations, the culture medium was replaced daily with fresh BGJ medium.

To study the effects of cortisol on periosteal and nonperiosteal central bone, the periosteum was removed from the superior and inferior surfaces of the calvariae with a scalpel blade at a 40-fold magnification. The efficacy of the dissection was determined by bone histology. The dissected tissues were fixed in neutral formalin, embedded in paraffin and the histological sections stained with hematoxylin-eosin. The periosteum was rich in elongated fibroblastic cells and did not contain round osteoblastic-type cells which were the predominant cells observed in the periosteum-free bone. The dissection was performed either before starting or after completing the incubation. When the dissection was performed prior to the culture, the periosteum and central nonperiosteal bone were cultured in three different ways: (1) Periosteum and nonperiosteal center were cultured in separate culture vials. (2) Periosteum and nonperiosteal center were cultured in the same vial but physical contact was prevented by the use of an organ culture grid. The grid maintained the two tissues separate but permitted free flow of culture medium from one tissue to the other. (3) Periosteum and nonperiosteal center were cultured in the same vial and allowed to come in contact, resulting in a visible attachment of the periosteum to the nonperiosteal tissue. This was confirmed on histological sections where the attachment of the periosteum was observed along one side of the periosteum-free bone. It was not possible to determine if migration of cells between the two tissues had occurred during the culture period but contact was sufficient to allow for it. When the dissection was performed after the culture, intact calvariae were incubated, labeled with radioactive tracers, and then dissected.

Cortisol (Sigma Chemical Company; St. Louis, MO) was dissolved in absolute alcohol and diluted 1:10,000 or greater in BGJ. Cortisol was present in the culture medium throughout the incubation period except when specified otherwise. To study the short-term stimulatory effects of cortisol, calvariae were continuously exposed to cortisol during 24 h cultures or were exposed to the steroid for the last 24 h of a 96 h culture. In one 96 h culture, only half of the medium was replaced daily, so that the calvarial tissues were continuously exposed to conditioned medium prior to and during the 24 h treatment with cortisol, which was given at the end of the incubation. To study the long-term inhibitory effects of cortisol, calvariae were continuously exposed to the steroid for the last 72 h of a 96 h incubation or throughout a 96 h culture period. The length of the cortisol exposure required to observe the stimulatory or inhibitory effects on bone collagen synthesis was based on previous observations using the same culture system [5, 6].

Collagen and Noncollagen Protein Synthesis

Labeled proline (2,3^{3}H, 5 μCi/ml; specific activity 20–30 Ci/mmol; New England Nuclear; Boston, MA) was added for the last 2 h of the culture period. The intact and dissected calvariae were extracted with 5% trichloroacetic acid (TCA), acetone, and ether, homogenized, and an aliquot was incubated with repurified bacterial collagenase (Worthington Biochemical Corporation; Freehold NJ). The enzyme preparation used was repurified on a column of Sephadex G-200 and did not contain any proteolytic activity on noncollagen substrates (Tryptophan-labeled bones). The labeled proline incorporated into CDP and NCP was measured according to the method of Peterkofsky and Diegelmann [17]. Percent collagen synthesis was calculated after multiplying NCP by 5.4 to correct for the relative abundance of proline in collagen and noncollagen protein. This was based on previous observations by Peterkofsky [18].

DNA Synthesis

DNA synthesis was studied by examining the incorporation of ^{3}H-thymidine into acid insoluble residues and total DNA content. To study effects on DNA labeling, calvariae were incubated and thymidine methyl-^{3}H (5 μCi/ml; specific activity 65–80 Ci/mmol; ICN Chemical and Radioisotope Division; Irvine, CA) was added during the last 60 min of the culture period. At the end of the incubation, intact and dissected calvariae were washed with cold 5% TCA, acetone, and ether and the radioactivity incorporated was determined after digesting them with NCS tissue solubilizer (Amersham Corporation; Arlington Heights, IL). To measure DNA content, calvariae were homogenized, extracted with 5% TCA and 10 mM potassium acetate and dried. The DNA was measured by fluorometry according to a modification of the method of Kissane and Robbins [19].

Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured by the hydrolysis of *p*-nitrophenyl phosphate [20]. Calvariae were homogenized in a NaCl (9g/lt) solution with 0.1% Triton X-100 (Sigma Chemical Company; St. Louis, MO) and sonicated. An aliquot was incubated with 8 mM *p*-nitrophenyl phosphate in 0.5 M 2-amino 2-methyl 1-propanol buffer for 30 min at 37°C and the *p*-nitrophenol liberated was measured in a spectrophotometer at a wavelength of 410 nm.

Table 1. Effect of cortisol, 30 nM, on the incorporation of ^{3}H-proline into CDP and NCP, on the incorporation of ^{3}H-thymidine into acid insoluble residues, and on DNA content in intact calvariae and in periosteum and nonperiosteal central bone of calvariae dissected after the completion of a 24 hr culture

Treatment	CDP (dpm/bone)	NCP (dpm/bone)	% Collagen synthesis	^{3}H-Thymidine incorporation (dpm/bone)	DNA (μg/bone)
Intact calvariae					
Control	14,875 ± 1,103	25,762 ± 2,339	9.8 ± 1.1	6,578 ± 608	11.1 ± 0.7
Cortisol	30,590 ± 2,464[a]	25,860 ± 2,421	18.0 ± 2.1[a]	4,237 ± 637[a]	12.6 ± 0.7
Dissected calvariae					
Periosteum					
Control	8,729 ± 1,239	17,997 ± 1,687	9.2 ± 1.9	5,495 ± 461	8.9 ± 0.3
Cortisol	6,280 ± 623	10,715 ± 1,080[a]	9.8 ± 0.8	2,848 ± 165[a]	7.9 ± 0.6
Nonperiosteal bone					
Control	6,385 ± 918	4,119 ± 628	22.5 ± 1.6	1,068 ± 60	3.3 ± 0.2
Cortisol	18,198 ± 1,805[a]	6,726 ± 468[a]	33.1 ± 2.9[a]	944 ± 96	4.3 ± 0.3[a]

Values are means ± SE for 6 half calvariae cultured for 24 h in the continuous presence or absence of cortisol, 30 nm, and pulsed with ^{3}H-proline (CDP and NCP) for the last 2 h or with ^{3}H-thymidine for the last 1 h of the culture period. The periosteum was removed by dissection after the incubation. Percent collagen was corrected for the relative abundance of proline in CDP and NCP

[a] Significantly different from control, $P < 0.02$

Statistical Methods

Data are expressed as mean and standard error (SE) of dpm/half calvaria and percent collagen synthesis. The DNA content is expressed as μg/half calvaria and alkaline phosphatase activity as nanomoles of *p*-nitrophenol released/half calvaria/30 min. Statistical differences were analyzed using Student's *t* test.

Results

Effects of Short-Term (24 h) Treatment with Cortisol

The continuous treatment with cortisol, 1–100 nM, for 24 h increased the incorporation of ^{3}H-proline into CDP in intact calvariae (Fig. 1, Table 1). Cortisol did not affect the labeling of NCP; thus, the percent collagen synthesized was increased. Cortisol inhibited the incorporation of ^{3}H-thymidine into acid insoluble residues but it had no effect on DNA content. When calvariae were dissected at the end of the 24 h culture, the stimulatory effect of cortisol on the incorporation of ^{3}H-proline into CDP was observed only in the central nonperiosteal bone (Table 1). The inhibitory effect on the incorporation of ^{3}H-thymidine was observed exclusively in the periosteal layers. In contrast, cortisol caused a 30% increase in DNA content in the nonperiosteal bone without affecting the periosteal DNA content. This effect was observed in two additional experiments where calvariae had been dissected after the culture, but it was not consistently observed if the dissection occurred before the culture (not shown). Histological sections of bones treated with cortisol, 30 nM, revealed a small increase in cellularity limited to the nonperiosteal bone.

There was a decrease in the labeling of CDP in control calvariae dissected prior to the incubation compared with intact bones. Further, cortisol at 0.1–1,000 nM did not affect the incorporation of ^{3}H-proline into CDP in either the periosteum or nonperiosteal central bone when the two tissues were incubated separately (Fig. 1). Treatment with 30 nM cortisol for 24 h did not alter CDP labeling in the periosteum or in the nonperiosteal central bone of calvariae dissected prior to the incubation if the two tissues were cultured in different vials or in the same vial, but separated by an organ culture grid. However, if the periosteum and nonperiosteal central bone of dissected calvariae were incubated in the same vial, they came in contact and were attached during the incubation, and cortisol increased the labeling of CDP by 52% (Fig. 2). This effect was similar to that observed in the same experiment in intact calvariae in which cortisol increased CDP labeling from a control value of (mean ± SE; n = 6) 24943 ± 1110 to 36015 ± 447 dpm/half calvaria ($P < 0.05$).

In another experiment, calvariae were dissected prior to a 96 h culture and the periosteum and nonperiosteal central bone were cultured in control BGJ medium either in different vials, in the same vial separated by an organ culture grid, or in the same vial and allowed to come in contact during the incubation. Half of the BGJ medium was replaced daily, so that it was conditioned by the presence of

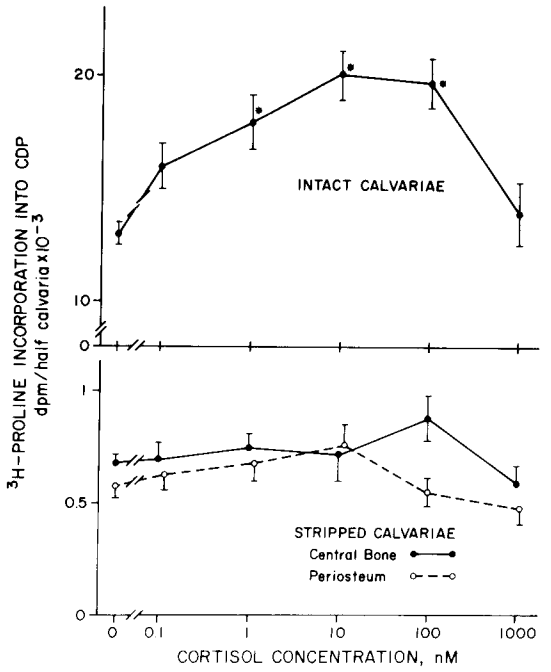

Fig. 1. Effect of cortisol on the incorporation of ^{3}H-proline into CDP in intact bones and in calvariae dissected prior to a 24 h culture. *Bars* represent mean values and *vertical lines* SE for 6 half calvariae cultured for 24 h in the continuous presence or absence of cortisol and pulsed with ^{3}H-proline for the last 2 h. The periosteum was removed from the central bone by dissection before the incubation and periosteum and nonperiosteal central bone were cultured in separate vials. *Significantly different from control $P < 0.05$.

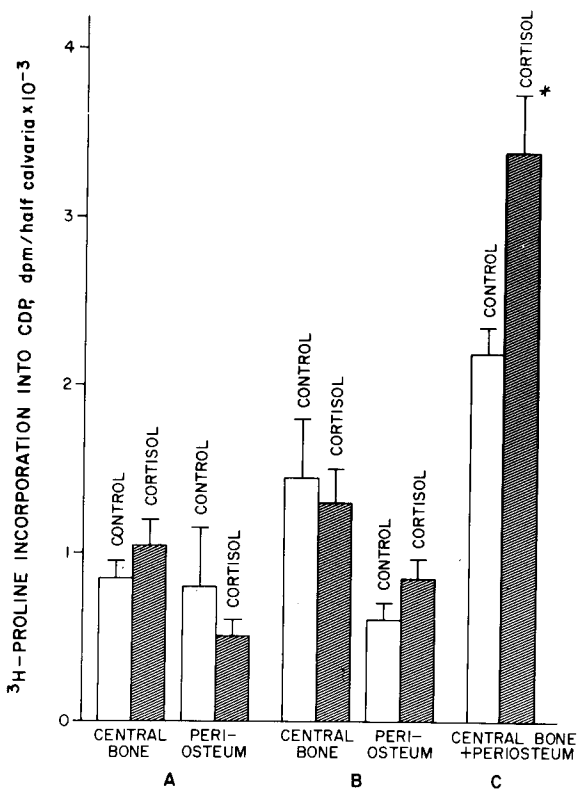

Fig. 2. Effect of cortisol, 30 nM, on the 2 h incorporation of ^{3}H-proline into CDP in periosteal and nonperiosteal central calvaria from 21-day-old fetal rats. The periosteum was removed from the central bone by dissection before the incubation and calvariae were cultured in three different ways: (A) Periosteum and central bone were cultured in separate vials. (B) Periosteum and central bone were cultured in the same vial but physical contact was prevented by maintaining the central bone separated from the periosteum. (C) After the dissection, periosteum and central bone were cultured in the same vial and allowed to come in contact and attach. Bars represent mean values and vertical lines SE for 6 half calvariae cultured in the continuous presence or absence of cortisol for 24 h. *Significantly different from control, $P < 0.01$.

one or both of the two cultured tissues. The incorporation of ^{3}H-proline into CDP and NCP in the central nonperiosteal bone of untreated calvariae was 3 to 4 times higher when the periosteum and central bone were cultured in the same vial, separated by an organ culture grid, than when they were incubated in individual vials (Table 2). However, the addition of 30 nM cortisol for the last 24 h of the 96 h culture period increased the incorporation of ^{3}H-proline into CDP only when the periosteum and nonperiosteal central bone were cultured in the same vial and came in contact and physically attached. This effect was similar to that observed in intact bones cultured in the same experiment, in which cortisol increased the labeling of CDP by 150%.

Continuous treatment with cortisol, 30 nM, for 24 h increased alkaline phosphatase activity in intact bones and in the nonperiosteal central bone of calvariae dissected before or after the culture, but cortisol had no effect on periosteal alkaline phosphatase activity (Table 3).

Effects of Long Term (72 to 96 h) Treatment with Cortisol

In contrast to the short-term effects, continuous treatment with cortisol for 96 h caused a dose-dependent inhibition of ^{3}H-proline incorporation into CDP and NCP in intact calvariae (Table 4, Fig. 3). The effect on CDP was greater than on NCP so that the percent of collagen synthesized was decreased. Cortisol at 100–1,000 nM also had a marked inhibitory effect on the incorporation of ^{3}H-thymidine into acid insoluble residues and decreased the DNA content by 24–41% (Table 4, Fig. 4). When cal-

Table 2. Effect of cortisol, 30 nM, on the incorporation of ^{3}H-proline into CDP and NCP in periosteum and nonperiosteal central bone of calvariae dissected prior to a 96 h culture and exposed to cortisol for the last 24 h

Treatment		CDP (dpm/bone)	NCP (dpm/bone)	% Collagen synthesis
Periosteum cultured apart from nonperiosteal bone				
Periosteum	Control	1,616 ± 339	3,508 ± 446	7.7 ± 1.1
	Cortisol	1,136 ± 126	3,171 ± 240	5.5 ± 0.7
Central bone	Control	490 ± 237	1,002 ± 469	6.1 ± 1.8
	Cortisol	798 ± 204	1,912 ± 613	6.5 ± 0.9
Periosteum and nonperiosteal bone cultured in same vial but not in contact				
Periosteum	Control	1,185 ± 217	4,108 ± 343	5.1 ± 0.7
	Cortisol	951 ± 127	3,787 ± 665	4.8 ± 0.5
Central bone	Control	2,128 ± 381	3,830 ± 428	9.2 ± 0.6
	Cortisol	1,698 ± 381	3,350 ± 504	8.0 ± 0.9
Periosteum and nonperiosteal bone cultured in same vial and in contact				
	Control	3,443 ± 635	8,076 ± 1,020	7.1 ± 0.6
	Cortisol	8,190 ± 1,268[a]	13,289 ± 1,864[a]	10.3 ± 0.9[a]

Values are means ± SE for 6 half calvariae cultured for 96 h in the continuous presence of cortisol, 100 nM, and pulsed with ^{3}H-proline of the culture medium was replaced daily so that bones were cultured in the continuous presence of conditioned medium. Calvariae were pulsed with ^{3}H-proline for the last 2 h of the incubation. The periosteum was removed from the central bone by dissection before the incubation and bones were cultured in three different ways: (1) Periosteum and nonperiosteal bone were cultured in separate vials. (2) Periosteum and nonperiosteal bone were cultured in the same vial but physical contact was prevented by placing the central bone inside and the periosteum outside an organ culture grid. (3) After dissection, periosteum and nonperiosteal bone were cultured in the same vial and allowed to come in contact and attach. Percent collagen was corrected for the relative abundance of proline in CDP and NCP

[a] Significantly different from control, $P < 0.01$

Table 3. Effect of cortisol, 30 nM, on alkaline phosphatase activity in intact calvariae and in periosteum and nonperiosteal central bone of calvariae dissected before and after a 24 h culture

Calvariae	Alkaline phosphatase activity/half calvaria (*p*-nitrophenol nmol/30 min)	
	Control	Cortisol
Intact	6510 ± 373	9006 ± 590[a]
Dissected after culture		
Periosteum	2853 ± 200	2704 ± 153
Nonperiosteal bone	4016 ± 378	5484 ± 338[a]
Dissected before culture		
Periosteum	1198 ± 122	1508 ± 176
Nonperiosteal bone	2092 ± 318	3285 ± 194[a]

Values are means ± SE for 6–18 half calvariae cultured for 24 h in the continuous presence or absence of cortisol. The periosteum was removed by dissection before or after the culture

[a] Significantly different from control, $P < 0.05$

variae were dissected after the completion of the 96 h incubation, cortisol inhibited the labeling of CDP, NCP, and DNA and the DNA content to a similar extent in the periosteum and in the nonperiosteal central bone. In addition, histological sections showed a generalized decrease in cellularity in calvariae treated with cortisol, 100 nM, for 96 h.

To determine if the effects of cortisol were dependent upon the periosteal cell population, the periosteum was removed prior to the incubation. The incorporation of ^{3}H-proline into CDP and of ^{3}H-thymidine into DNA was decreased in dissected, control, untreated calvariae when compared with control intact bones (Fig. 3 and 4). Continuous treatment with cortisol, 100–1,000 nM, for 96 h or for the last 72 h of a 96 h culture period, inhibited the labeling of CDP and DNA in the periosteum of dissected bones but it had no effect in the central nonperiosteal bone. Further, control nonperiosteal bones contained (mean ± SE; n = 6) 2.3 ± 0.2 μg and cortisol-treated (100–1,000 nM) contained 2.1 ± 0.26–2.8 ± 0.6 μg DNA/half calvaria. Histological sections of dissected calvariae showed that the cellularity of the nonperiosteal bone was the same in control and cortisol-treated (100 nM) bones. The effect of cortisol was not altered by culturing the periosteum and central nonperiosteal bone in the same vial and separating the two tissues with an organ culture grid. Exposure to 100 nM cortisol for the last 72 h of a 96 h culture inhibited the labeling of periosteal CDP from a control value of (mean ±

Table 4. Effect of cortisol on the incorporation of ^{3}H-proline into CDP and NCP and on the incorporation of ^{3}H-thymidine into acid insoluble residues and on DNA content in intact calvariae and in periosteum and nonperiosteal central bone of calvariae dissected after the completion of a 96 h culture

Treatment	CDP (dpm/bone)	NCP (dpm/bone)	% Collagen synthesis	^{3}H-Thymidine incorporation (dpm/bone)	DNA (μg/bone)
Intact calvariae					
Control	44,376 ± 4,677	43,201 ± 3,894	15.9 ± 0.5	3,276 ± 534	11.1 ± 0.5
Cortisol	7,009 ± 1,031[a]	10,423 ± 1,341[a]	11.0 ± 0.4[a]	467 ± 40[a]	6.6 ± 0.1[a]
Dissected calvariae					
Periosteum					
Control	10,825 ± 790	20,278 ± 1,661	9.1 ± 0.6	2,369 ± 345	7.1 ± 0.5
Cortisol	1,542 ± 314[a]	4,942 ± 755[a]	5.3 ± 0.3[a]	319 ± 58[a]	5.1 ± 0.3[a]
Nonperiosteal bone					
Control	30,404 ± 2,242	14,561 ± 1,247	28.1 ± 1.5	1,130 ± 91	3.8 ± 0.3
Cortisol	4,988 ± 465[a]	3,867 ± 316[a]	19.2 ± 0.3[a]	228 ± 19[a]	2.5 ± 0.1[a]

Values are means ± SE for 6 half calvariae cultured for 96 h in the continuous presence of cortisol, 100 nM, and pulsed with ^{3}H-proline (CDP and NCP) for the last 2 h or with ^{3}H-thymidine for the last 1 h of the culture period or with cortisol, 1 μM, and examined for DNA content. The periosteum was removed by dissection after the incubation. Percent collagen was corrected for the relative abundance of proline in CDP and NCP

[a] Significantly different from control, $P < 0.01$.

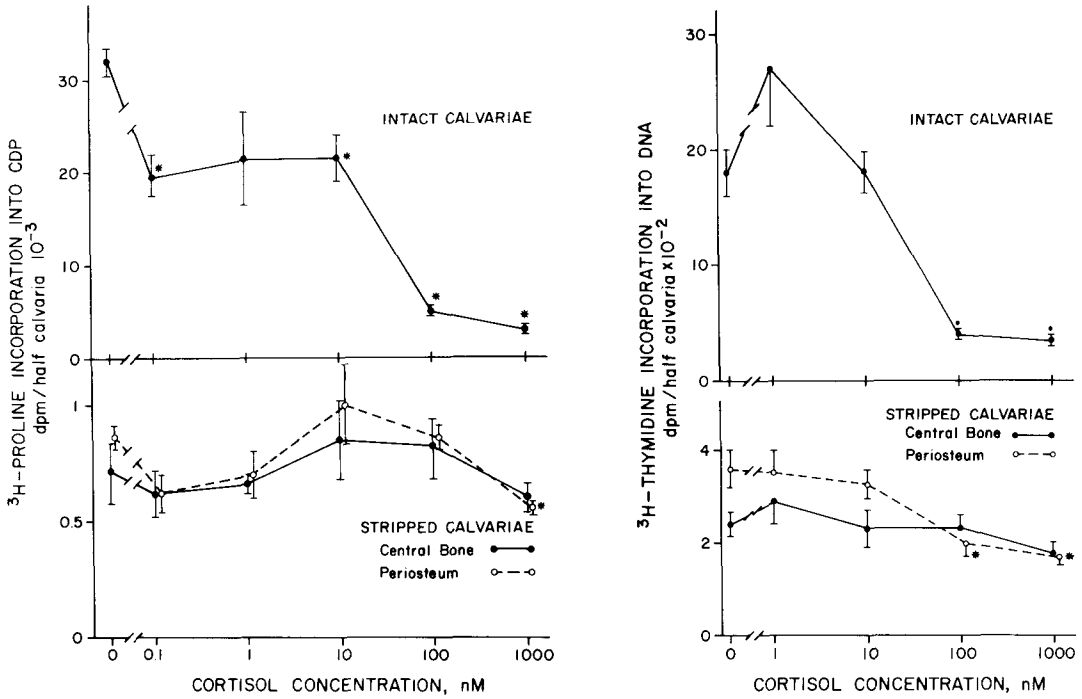

Fig. 3. Effect of cortisol on the incorporation of ^{3}H-proline into CDP in intact bones and in calvariae dissected prior to a 96 h culture. *Bars* and *vertical lines* represent means and SE for 6 half calvariae cultured for 96 h in the continuous presence or absence of cortisol and pulsed with ^{3}H-proline for the last 2 h. The periosteum was removed from the central bone by dissection before the incubation and periosteum and nonperiosteal central bone were cultured in separate vials. *Significantly different from control $P < 0.05$. **Fig. 4.** Effect of cortisol on the incorporation of ^{3}H-thymidine into acid insoluble residues (DNA) in intact bones and in calvariae dissected prior to a 96 h culture. *Bars* and *vertical lines* represent means and SE for 6 half calvariae cultured for 96 h in the continuous presence or absence of cortisol and pulsed with ^{3}H-thymidine for the last 1 h. The periosteum was removed from the central bone by dissection before the incubation and periosteum and nonperiosteal central bone were cultured in separate vials. *Significantly different from control, $P < 0.05$.

Table 5. Effect of cortisol on alkaline phosphatase activity in intact calvariae and in periosteum and nonperiosteal central bone of calvariae dissected before a 96 h culture

Treatment	Alkaline phosphatase activity/half calvaria (*p*-nitrophenol nmol/30 min)		
	Intact calvariae	Dissected calvariae	
		Periosteum	Nonperiosteal bone
Control	8349 ± 563	723 ± 65	1646 ± 89
Cortisol			
10 nM	6162 ± 163*	617 ± 78	1587 ± 273
100 nM	4615 ± 237*	873 ± 103	2789 ± 383*
1 μM	3720 ± 438*	772 ± 84	2564 ± 288*

Values are means ± SE for 5–20 half calvariae cultured for 96 h in the continuous presence or absence of cortisol. The periosteum was removed by dissection before the culture. Data are pooled from two experiments.
* Significantly different from control, $p < 0.05$.

SE; n = 12) 1155 ± 95 to 735 ± 133 dpm/half calvaria ($P < 0.01$) and the labeling of periosteal DNA from 311 ± 21 to 197 ± 10 dpm/half calvaria ($P < 0.01$) without affecting the nonperiosteal bone which was separated by an organ culture grid. Exposure to 100 nM cortisol (72–96 h) also inhibited the labeling of CDP and DNA in dissected calvariae if the periosteum and nonperiosteal bone were cultured in the same vial and came in contact. However, the magnitude of the effect was only equal to that observed in the periosteum cultured alone (not shown).

Cortisol treatment for 96 h at concentrations of 10 nM–1 μM inhibited alkaline phosphatase activity in intact calvariae. In contrast, when calvariae were dissected prior to the culture, cortisol had no effect in the periosteum, and stimulated alkaline phosphatase activity in the central nonperiosteal bone (Table 5).

Discussion

The present studies confirm that short-term exposure to cortisol stimulates calvarial collagen synthesis whereas long-term treatment has an inhibitory effect secondary to a decrease in cell population [5, 6]. When calvariae were dissected at the end of the incubation, the stimulatory effect of cortisol on collagen was limited to the central nonperiosteal bone. Since this tissue is relatively rich in osteoblasts [7–9], these results suggest that cortisol affects osteoblastic collagen synthesis. This is supported by demonstrating that short-term treatment with cortisol stimulates nonperiosteal alkaline phosphatase activity, and by previous work showing that it stimulates calvarial type I collagen synthesis, both parameters of osteoblastic function [6]. Cortisol also caused a small inhibition of ^{3}H-thymidine incorporation after 24 h of treatment which was not associated with a decrease in DNA content. Further, dissection of the calvariae revealed a small increase in DNA content in the nonperiosteal bone which could be responsible in part for the cortisol stimulation of bone collagen synthesis. However, the stimulatory effect on bone collagen was of much greater magnitude than that on DNA content indicating that another mechanism was also responsible for the effect on collagen synthesis.

The short-term stimulatory effect of cortisol on collagen synthesis was not observed in calvariae dissected before the incubation if the periosteum and central nonperiosteal bone were cultured separately or if their contact was prevented by an organ culture grid that allowed free exchange of medium. In contrast, cortisol stimulated collagen synthesis when the two dissected tissues came in contact and were physically attached during the culture period. This indicates that physical proximity of the periosteum to the nonperiosteal bone is required to observe the cortisol stimulatory effect on bone collagen synthesis. The labeling of CDP, NCP, and DNA was decreased in control bones dissected prior to the culture, compared with control intact bones, indicating that cellular loss had occurred. However, the lack of a cortisol stimulatory effect on collagen synthesis could not be attributed to cellular death, since the effect was restored if the periosteum and central bone came in contact during the culture and cortisol stimulated alkaline phosphatase activity in dissected central bone. In addition, 24 h treatment with cortisol did not stimulate collagen synthesis in dissected bones that had been previously cultured in control medium for 72 h which should have allowed the tissues to recover from the trauma of the dissection. Further, insulin, parathyroid hormone, and epidermal growth factor were found to have the same effects whether the nonperiosteal bone was dissected before or after the culture [21]. Although the reason for the different effects of these agents and cortisol is unknown, it may be related to differences in the mechanism of action of peptide hormones and steroids.

The collagen synthesized by the untreated nonperiosteal bone was higher if the periosteum was cultured in the same vial separated by an organ culture grid than if the two tissues were cultured in different vials. This suggests that the periosteum

releases material with bone collagen stimulatory activity but this material did not modify the lack of a cortisol effect on nonperiosteal collagen synthesis. This might have been due to dilution of a soluble factor with the culture medium. However, partially purified conditioned medium from periosteal cultures stimulated collagen synthesis in intact calvariae but did not modify the lack of a cortisol effect in dissected calvariae (E. Canalis, unpublished observations). These results suggest that periosteal tissue and not factors are required to obtain a cortisol stimulatory effect on nonperiosteal collagen, or that gross interactions between the periosteum and nonperiosteal bone might permit higher concentrations of the putative periosteal factor to reach the nonperiosteal bone. Neither periosteal tissue or factors were required to observe a cortisol stimulatory effect on alkaline phosphatase activity which occurred in the osteoblastic rich nonperiosteal bone of dissected calvariae. This is in accord with studies showing a stimulatory effect of cortisol in cloned osteoblasticlike osteosarcoma cell lines [22].

In contrast to its short-term collagen stimulatory effect, cortisol had a marked inhibitory effect on collagen and noncollagen protein synthesis in intact calvariae treated for 72 and 96 h. The effect was oberved in the periosteum and in the central nonperiosteal bone of calvariae dissected after the culture, and it was concomitant to a marked and generalized inhibition of DNA synthesis. This confirms previous investigations indicating that the cortisol inhibition of collagen and noncollagen protein synthesis is secondary to a decrease in cell population [6]. When calvariae were dissected prior to the incubation, the inhibitory effect of cortisol on collagen and DNA synthesis was observed only in the periosteum, rich in fibroblasts and progenitor cells. The selective loss of a nonperiosteal cortisol inhibitory effect on collagen and DNA synthesis after the removal of the periosteum indicates that the generalized inhibitory effect of cortisol observed in intact cultured bones is secondary to an inhibition of periosteal preosteoblastic cells. Moreover, the removal of the periosteum resulted in a stimulatory effect of cortisol on alkaline phosphatase activity in nonperiosteal bone treated for 96 h. This stimulatory effect is in accordance with studies on osteoblasticlike osteosarcoma cell lines where short- and long-term treatment with cortisol stimulates alkaline phosphatase activity (M. Centrella and E. Canalis, unpublished). The loss of the stimulatory effect of cortisol on nonperiosteal collagen is probably related to the removal of the periosteum which needs to be present to obtain this effect.

Thus, it appears that cortisol does not have a direct inhibitory effect on osteoblastic cell function, and dissecting calvariae after the culture may generate misleading results. The loss of a nonperiosteal cortisol inhibitory effect on collagen and DNA synthesis does not seem to be secondary to the immediate trauma of the dissection since cortisol had a periosteal effect. Further, the results were not modified by culturing the dissected bones for 24 h in control medium prior to the cortisol treatment and the cortisol effect on alkaline phosphatase activity was maintained. Other authors have reported inhibitory effects of glucocorticoids on protein synthesis and cell replication in nonperiosteal bone cells but they have used gentle periosteal scraping followed by enzymatic digestions of the calvariae [1–4]. It is possible that a number of periosteal cells were preserved in the preparations used, which could be responsible for the effects observed. In the present studies, we dissected the periosteum with the use of a microscope and removed all of the periosteal tissue, which seems to account for the differences observed. Another possibility may be related to the concentrations of glucocorticoids tested, since studies showing an inhibitory effect of glucocorticoids on osteoblastlike cells frequently used higher concentrations than those tested in the present investigations [2, 23].

In conclusion, these studies indicate that short-term treatment with cortisol stimulates collagen synthesis in nonperiosteal bone, an effect that requires the presence of periosteal tissue. Long-term exposure to cortisol decreases periosteal DNA synthesis which results in a generalized inhibition of calvarial collagen synthesis but cortisol does not cause a direct inhibition of osteoblastic function.

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