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INHIBITION OF BONE RESORPTION IN CULTURE BY (+)-CATECHIN

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Abstract—A pretreatment with (+)-catechin renders embryonic mouse calvaria in culture resistant to the action of bone resorbing agents, either parathyroid hormone (PTH), prostaglandin E_2 or retinoic acid, and inhibits in a parallel way the enhanced excretion of *N*-acetyl-β-glucosaminidase, a reference lysosomal enzyme, induced by these agents; it has, however, no effect on the small spontaneous leakage of lactate dehydrogenase from the explants. Moreover, the resorption induced in calvaria by a pretreatment with PTH or retinoic acid is inhibited by a further culture with catechin. This inhibition of bone resorption is discussed in relation with the collagen-stabilizing properties of (+)-catechin.

(+)-Catechin [or (+)-cyanidanol-3] belongs to the family of the flavonoids, a class of natural products of high pharmacological potency widely distributed in the plant kingdom [1]. It has been shown to stabilize collagen molecules, rendering them less soluble [2] and less susceptible to degradation by collagenase [3–5]. Collagen is the main organic constituent of the extracellular matrix of bone and its removal is necessary for bone resorption. Therefore it appeared that (+)-catechin, by acting upon bone collagen, could well render bone tissue less prone to resorption. This hypothesis was evaluated in a tissue culture system where bone explants are led to resorb under the action of either PTH, retinoic acid or PGE_2.*

EXPERIMENTAL

Bone resorption and enzyme release were monitored in cultures of calvaria from 19-day-old NMRI mouse embryos [6]. The calvaria were cultured in medium 199 (2 calvaria/2 ml medium) according to two experimental protocols, as indicated in the legends of the table and figure. Following the first protocol, the calvaria were precultured for 18 hr with or without (+)-catechin or (+)-catechin chlorhydrolysinate (a gift from Dr P. Niebes, Continental Pharma, Mont-Saint-Guibert, Belgium), rinsed thoroughly with medium 199 and further cultured for 2 or 3 days with either PTH, retinoic acid or PGE_2 (obtained from Sigma, St Louis, MO) with daily renewal of medium. Following the second protocol, the calvaria were precultured for 8 hr with PTH or retinoic acid; after rinsing, they were then cultured for the next 18 hr with or without (+)-catechin, rinsed again and cultured for a further 72 hr without any additive with daily renewal of medium. In both series of experiments, control calvaria were cultured either without additive or, to prevent any "spontaneous" resorption, with both indomethacin and salmon calcitonin (from Sigma). The solvents used for PGE_2, retinoic acid and indomethacin (respectively ethanol, dimethylsulfoxide and dimethylformamide) had no effect on bone resorption at their final concentrations (0.02–0.05%) in the culture fluids. Bone resorption was evaluated [7] by visual assessment of resorption lacunae using a scale from 0 to 5 and by measuring the amounts of hydroxyproline left in the explants at the end of the cultures and those of calcium accumulated in the medium. NAβGase [6] and LDH [7] released into the medium were assayed as described; it was verified that the addition of 1 mM (+)-catechin to calvaria-conditioned culture media containing these enzymes had no effect on their activities in these assays. The NAβGase content of the calvaria was evaluated in tissue homogenates as done previously [8]. Unless otherwise indicated, chemicals and media used were from suppliers previously mentioned [6].

RESULTS AND DISCUSSION

Calvaria precultured for 18 hr in the absence of (+)-catechin exhibited typical resorption lacunae and lost 25–50% of their initial content in hydroxyproline and calcium during their subsequent cultivation in the presence of either PTH (Fig. 1), retinoic acid or PGE_2 (Table 1). The development of the PTH-induced resorption was inhibited in a dose-dependent manner when the bones were precultured with 0.1–1 mM (+)-catechin or (+)-catechin chlorhydrolysinate, both forms of catechin providing similar results (Fig. 1); the resorption was almost abolished by a preculture with 1 mM (+)-catechin. Simultaneously, the excretion of NAβGase, a reference lysosomal enzyme whose excretion is enhanced during bone resorption [8], was inhibited in a closely parallel manner while the activity of this enzyme in the tissue at the end of the cultures was slightly increased. The pretreatment

* Abbreviations used: LDH, lactate dehydrogenase; NAβGase, *N*-acetyl-β-glucosaminidase; PGE_2, prostaglandin E_2; PTH, parathyroid hormone.

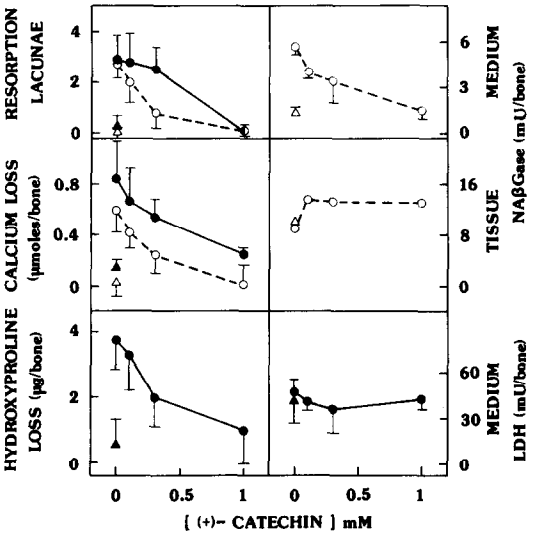

Fig. 1. Inhibition of parathyroid hormone-induced bone resorption by a pretreatment with (+)-catechin. Calvaria were first cultured for 18 hr with the indicated concentrations of (+)-catechin (Experiment 1: solid lines, ▲, ●) or (+)-catechin chlorhydrolysinate (Experiment 2: broken lines, △, ○). They were then rinsed and further cultured for 2 days without added catechin but with (●, ○) or, for the controls, without (▲, △) PTH, 0.3 units/ml. Bone resorption, LDH or NAβGase released into the medium over the whole culture period and the NAβGase content of the explants were then evaluated. Each point is the mean ± SD of 4 cultures of 2 calvaria each except for the activity of NAβGase in the tissue, which was determined in homogenates prepared from 8 calvaria at the end of their cultures.

with (+)-catechin had no effect on the release of LDH (Fig. 1), a cytoplasmic enzyme that leaks out of cells when they are damaged, suggesting that it was not cytotoxic. Moreover, the action of (+)-catechin was not restricted to PTH-induced bone resorption as the resorption induced by structurally different agents, retinoic acid or PGE_2, was inhibited as well (Table 1). Furthermore, the action of (+)-catechin in this culture model was not due to a direct prevention of the action of the bone resorption-inducing agents on bone cells. Indeed, (+)-catechin did also inhibit the development of bone resorption when it was induced in the explants by a brief exposure to PTH or to retinoic acid prior to the addition of the inhibitor to the cultures (Table 2). Thus the action of (+)-catechin is likely to be due to an inhibition of the bone resorption processes as such.

The mechanism of that inhibition is, however, unknown. According to present views, bone resorption is the result of a complex chain of cellular and biochemical events. Indeed, PTH and many other bone resorption-inducing agents act initially on osteoblasts or on related bone lining cells to induce a response that will then allow "resting" osteoclasts to be recruited and to have access to the mineralized bone matrix where they may become "activated" [9–12]. The nature of this osteoblast response is still uncertain. We have established that both tissue collagenase [13] and cysteine-proteinases [6] are required for the development of bone resorption. Osteoblasts may respond to bone resorption-inducing agents by secreting latent procollagenase [14, 15] and plasminogen activator [16]: the latter may generate plasmin, which is a good activator of procollagenase [17, 18], and the resulting active collagenase could possibly expose bone mineral to osteoclastic contact by digestion of a thin layer of unmineralized collagen that apparently separates surface cells from the underlying mineralized tissue [10]. Moreover, bone-resorbing agents may induce

Table 1. Effect of pretreatment with (+)-catechin on the resorption induced in cultured bones by retinoic acid or PGE_2

	Resorption lacunae	Hyp in tissue (μg/calv)	Ca release (μmole/calv)	NaβGase release (mU/calv)	LDH release (mU/calv)
Exp. A					
Non-cultivated calvaria	0 ± 0	14.8 ± 1.5	—	—	—
Calvaria cultivated with					
(1) no additive	0 ± 0	15.4 ± 1	0.12 ± 0.13	1.19 ± 0.28	25 ± 3
(2) catechin, then no additive	0 ± 0	14.6 ± 1.6	−0.08 ± 0.08	0.60 ± 0.20	28 ± 2
(3) no additive, then retinoic acid	3 ± 0.7	8.7 ± 1.1	0.54 ± 0.17	1.96 ± 0.15	36 ± 5
(4) catechin, then retinoic acid	0 ± 0*	14.1 ± 2.1*	−0.06 ± 0.06*	0.93 ± 0.22*	42 ± 2
Exp. B					
Non-cultivated calvaria	0 ± 0	13 ± 1.3	—	—	—
Calvaria cultivated with					
(1) calcitonin + indomethacin	0 ± 0	12.9 ± 0.9	−0.08 ± 0.17	0.75 ± 0.19	57 ± 8
(2) no additive, then PGE_2	1.5 ± 0.5	10.7 ± 1.5	0.65 ± 0.23	2.45 ± 0.58	50 ± 14
(3) catechin, then PGE_2	0 ± 0*	14.3 ± 2†	0.22 ± 0.19†	0.56 ± 0.23*	40 ± 7

In Exp. A, calvaria were cultured for 18 hr without additive (groups 1 and 3) or with 0.8 mM (+)-catechin chlorhydrolysinate (groups 2 and 4). They were then rinsed and cultured for a further 48 hr in fresh media containing either no additive (groups 1 and 2) or 10^{-6} M retinoic acid (groups 3 and 4). In Exp. B, calvaria of group (1) were cultured over the whole culture period with 14 μM indomethacin and 0.9 U of calcitonin/ml. Calvaria of groups (2) and (3) were cultured for 18 hr without additive (group 2) or with 0.8 mM (+)-catechin chlorhydrolysinate (group 3); they were then rinsed and cultured for a further 72 hr in fresh media containing 10^{-6} M PGE_2. The results are the means ± SD of 4 cultures of 2 calvaria each. The significance of the differences observed between groups 3 and 4 (Exp. A) or 2 and 3 (Exp. B) was evaluated with Student's t test: * $P < 0.0025$; † $P < 0.025$ (calv, calvarium; Hyp, hydroxyproline).

Table 2. Effect of (+)-catechin on the resorption of calvaria pretreated with PTH or retinoic acid

	Resorption lacunae	Hyp in tissue (μg/calv)	Ca release (μmole/calv)	NAβGase release (mU/calv)	LDH release (mU/calv)
Exp. A					
Non-cultivated calvaria	0 ± 0	14.9 ± 0.3	—	—	—
Calvaria cultivated with					
(1) PTH, then no additive	3 ± 1.1	11.3 ± 1.2	0.75 ± 0.38	2.58 ± 0.81	—
(2) PTH, then catechin, then no additive	0 ± 0*	14.5 ± 1.4*	0.03 ± 0.09*	1.09 ± 0.14*	
(3) calcitonin + indomethacin	0 ± 0	13.4 ± 1.7	−0.21 ± 0.09	0.62 ± 0.14	—
Exp. B					
Non-cultured calvaria	0 ± 0	13 ± 1.3	—	—	—
Calvaria cultured with					
(1) retinoic acid, then no additive	3.6 ± 0.7	8.4 ± 0.1	0.59 ± 0.18	1.69 ± 0.30	55 ± 5
(2) retinoic acid, then catechin, then no additive	0 ± 0*	13.6 ± 0.4*	0.20 ± 0.15*	1.34 ± 0.09†	57 ± 7
(3) no additive	0 ± 0	11.6 ± 0.7	0.25 ± 0.24	1.26 ± 0.33	55 ± 10
(4) calcitonin + indomethacin	0 ± 0	12.9 ± 0.9	−0.08 ± 0.17	0.75 ± 0.19	57 ± 8

Calvaria were cultured successively for 8 hr either with 2.3 U of PTH/ml (Exp. A, groups 1 and 2) or with 10^{-6} M retinoic acid (Exp. B, groups 1 and 2), then for the next 18 hr without (groups 1) or with (groups 2) 0.8 mM (+)-catechin chlorhydrolysinate, and for a further 72 hr without any additive (groups 1 and 2). Control calvaria were cultured either with 14 μM indomethacin and 0.9 U of calcitonin/ml or without any additive. The results are the means ± SD of 4 cultures of 2 calvaria each. The significance of the differences observed between groups 1 and 2 (in both Exp.) was evaluated with Student's *t*-test: * $P < 0.01$; † $P < 0.05$ (calv, calvarium; Hyp, hydroxyproline).

changes in the cell shape of osteoblasts, resulting in less tight packing of the cells so that osteoclasts can more easily insinuate themselves to come into contact with the mineralized bone matrix [19, 20]. Activated at this contact, the osteoclasts would then secrete protons to solubilize the mineral [8, 21] and lysosomal hydrolases [8], including cysteine-proteinases [6]. Cysteine-proteinases may either directly degrade collagen fibres [22] or activate latent procollagenase [18], possibly procollagenase of osteoblastic origin (osteoclasts do not seem able to produce collagenase [14]) that could be bound and stored on the collagen of the resorbing bone matrix [23], so as to allow the action of collagenase on the demineralized bone collagen.

The pharmacological inhibition of bone resorption could thus result from interactions occurring at many different levels. Like the other flavonoids [1, 24], (+)-catechin has a high chemical reactivity and is able to interact with many enzyme systems or biological structures. It is known from *in vitro* experiments to increase the resistance of collagen to the action of collagenase [3–5], an effect that is apparently not due to intact catechin but to some of its oxidation products [5]. Interestingly, this action has been advocated to explain the fact that (+)-catechin renders embryonic heart tissue more resistant to the invasion by tumour cells [25]. It may thus be speculated that in bone tissue, (+)-catechin or its derivatives may gain access and bind to the thin layer of unmineralized collagen that separates resting osteoclasts from the mineralized matrix and prevent its removal by osteoblast-secreted collagenase. This could be sufficient to inhibit the whole process of bone resorption by preventing osteoclast activation. The fact that (+)-catechin inhibits the enhanced excretion of lysosomal enzymes induced by bone resorbing agents is compatible with this interpretation.

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