Biochemical Pharmacology, Vol. 32, No. 2, pp. 343–346, 1983.
Printed in Great Britain.

0006-2952/83/020343-04 $03.00/0

EVALUATION OF (+)-CATECHIN ACTION ON LYSYL OXIDASE ACTIVITY IN AORTIC TISSUE

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(*Received* 3 *May* 1982; *accepted* 23 *June* 1982)

Abstract—Small amounts of (+)-catechin (5 mg/kg body wt) administered intramuscularly to 8-day-old chicks raised the lysyl oxidase activity in aorta about 20%. (+)-Catechin had no effect on chicks that were copper-deficient. In the deficient chicks, (+)-catechin treatment prompted a substantially stronger increase in lysyl oxidase activity in response to $CuSO_4$. The observed increments in lysyl oxidase activity *in vivo* were sensitive to inhibition by β-aminopropionitrile (BAPN), suggesting that (+)-catechin was affecting the enzyme. (+)-Catechin in the assay medium partially inhibited lysyl oxidase activity. With excess copper ions present, (+)-catechin catalyzed a very strong release of volatile tritium from the substrate proteins. The release of tritium, indicative of lysyl oxidase activity, was not blocked by BAPN, suggesting that the activity *in vitro* was not enzyme catalyzed.

(+)-Catechin belongs to a family of naturally occurring bioflavonoids that have been shown to exert strong pharmacological action on the vascular system and connective tissue in general [1]. The compound blocks collagen biosynthesis by inhibiting prolyl and lysyl hydroxylases [2, 3] as well as rendering collagen more resistant to mammalian collagenases *in vitro* [4]. Interest in (+)-catechin as a pharmacological agent has come from studies which have shown that the compound, when administered to afflicted animals and cells in culture, reverses the symptoms of adjuvant arthritis [5, 6], juvenile diabetes [7], Ehlers–Danlos Syndrome Type V [8, 9], and osteolathyrism [10]. The latter two afflictions are believed to involve an impairment in the functioning of lysyl oxidase†, a copper-dependent enzyme found almost exclusively in connective tissues.

Speculation has been raised that enzymatic lysyl oxidase activity is stimulated by the (+)-catechin treatment [10]. Another possibility is that (+)-catechin in combination with copper may be capable of mimicking the oxidative activity of lysyl oxidase. Supporting these hypotheses are a series of studies that suggest that (+)-catechin augments collagen cross-linking *in vivo* [10, 11] and catalyzes the oxidative deamination of peptidyl lysyl residues in reconstituted collagen fibers *in vitro* [12]. Both of these actions are similar to known actions of the enzyme [13], and both are dependent on copper [12, 14]. No one has yet shown changes in lysyl oxidase activity *in vivo* in response to (+)-catechin administration.

In the present study, we have examined the effects of (+)-catechin on lysyl oxidase activity in chick aorta, noting its action both *in vivo* and *in vitro*. The studies *in vivo* included chicks that were deficient in copper in order to assess whether copper status had any influence on the (+)-catechin responses. Ceruloplasmin levels were also monitored to determine if the effects of (+)-catechin may be mediated through this serum copper protein. Lysyl oxidase levels in the chick appear to correlate positively with fluctuations in the ceruloplasmin levels of the serum [15]. As an added precaution, each study used β-aminopropionitrile (BAPN), a potent and select inhibitor of lysyl oxidase [16], to distinguish enzyme-catalyzed activity from non-specific chemical catalysis.

MATERIALS AND METHODS

Chicks were obtained from a local hatchery and were 8 days old at the time of the trials. Semi-purified diets with and without supplements of $CuSO_4$ were as previously described [17]. The (+)-catechin was purchased from the Sigma Chemical Co., St. Louis, MO, and was used without further purification. The compound was dissolved in propylene glycol and administered intramuscularly at a dose of 5 mg/kg body wt. Control animals received solvent. $CuSO_4$ in 0.15 M NaCl was administered intraperitoneally at a dose of 1 mg/kg body wt. Animals were killed 24 hr after the treatments. Aortic tissue was isolated, cleaned, and stored frozen (−20°) until assayed for lysyl oxidase activity. The assay of lysyl oxidase followed standard procedures used in this laboratory [18]. Substrate proteins for the assays were prepared from 8-day-old chick aortas that had been incubated for 24 hr in lysine-free Waymouth medium supplemented with 20 μCi/ml of L-[4,5-^{3}H]lysine (New England Nuclear Corp., Boston, MA; 35 Ci/mmole). The labeled aortas were homogenized, washed three times in 0.15 M NaCl with centrifugation, and boiled 8 min to destroy residual lysyl oxidase activity. Approximately 500,000 cpm of labeled proteins were

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† No Enzyme Commission (EC) number has been assigned to this enzyme.

used in each assay. Studies testing (+)-catechin *in vitro* used these same protein substrates. The $CuSO_4$ was added first (final concentration of 0.08 mM) followed by (+)-catechin (0.5 mM) of an equivalent volume of buffer (0.015 M potassium phosphate, 0.12 M NaCl, pH 7.6). After a brief incubation the substrate proteins were added and the mixture was incubated for 4 hr at 40°. Lysyl oxidase activity has been expressed as cpm tritium released per hr/g of aortic tissue (wet wt). All data are means ± S.D. Measurements of ceruloplasmin (ferroxidase, EC 1.12.3.1) in chick serum used *p*-phenylenedamine (PPD) as described previously [15]. Freshly collected pooled blood obtained by decapitation was allowed to clot for 60 min at room temperature. Serum was obtained by centrifugation (800 *g*, 10 min) and exactly 0.2 ml was used in the analysis. The values reported for ceruloplasmin and lysyl oxidase are the average of N determinations taken by combining four samples of sera or aortas for each single determination.

RESULTS

A single injection of (+)-catechin (5 mg/kg) into normal chicks raised the lysyl oxidase activity in the aorta by 17% in a period of 24 hr (Table 1). Effects attributed to the (+)-catechin were sensitive to inhibition by BAPN. The same treatment, repeated with chicks that had been raised for a 7-day pretrial period on diets lacking copper, resulted in no apparent stimulation of lysyl oxidase activity by (+)-catechin. $CuSO_4$, as expected, restored partial enzyme function (Table 1). However, when the deficient chicks were given (+)-catechin first and then a single intraperitoneal injection of $CuSO_4$ 3 hr later, a very strong response of lysyl activity to (+)-catechin was seen. In Table 1 this is seen as an enhancement in the lysyl oxidase activity in the catechin–$CuSO_4$-treated animals over those that had received only $CuSO_4$, a difference of about 1.5-fold. Again, all activity at 24 hr was BAPN sensitive, suggesting that (+)-catechin effects were directed at the enzyme. The dependence of copper status on (+)-catechin action is clearly seen in these results.

Effects of (+)-catechin on lysyl oxidase activity *in vitro* were evaluated next. Table 2 summarizes those results. Lysyl oxidase activity was measured in dialyzed extracts from aortic tissue. The release of tritium from aortic protein labeled biosynthetically with L-[4,5-^{3}H]lysine was measured quantitatively. In Table 2 it is seen that the extracts from control

Table 1. Catechin-induced activation of aortic lysyl oxidase *in vivo*: effect of copper status

	N	Lysyl oxidase activity −BAPN	Lysyl oxidase activity +BAPN*
Control	4	88.3 ± 4.9	0.1 ± 0.1
+ Solvent	4	84.8 ± 5.4	0.1 ± 0.1
+(+)-Catechin (5 mg/kg)	4	100.9 ± 8.8†	0.1 ± 0.1
Copper-deficient			
+ Solvent	4	4.2 ± 2.0	‡
+(+)-Catechin	4	5.1 ± 0.4	‡
+ $CuSO_4$	4	49.6 ± 7.3	0.1 ± 0.1
+(+)-Catechin, $CuSO_4$	4	75.3 ± 5.2	0.1 ± 0.1

* BAPN fumarate, 0.1 mM.
† Significantly different from solvent-treated at $P < 0.02$.
‡ No activity was detectable.

Table 2. Effects of (+)-catechin *in vitro* on the release of tritium from aortic proteins labelled with L-[4,5-^{3}H]lysine

Source of enzyme	Additions to medium	^3H_2O released* (cpm) −BAPN	^3H_2O released* (cpm) +BAPN†
Control	None	2519.6 ± 151.3	1.1 ± 0.5
	(+)-Catechin (0.5 mM)	1194.7 ± 40.1	1.3 ± 0.3
Copper-deficient	None	287.5 ± 18.8	4.1 ± 3.5
	(+)-Catechin	123.4 ± 21.0	4.4 ± 2.5
	$CuSO_4$ (0.08 mM)	539.2 ± 21.2	203.2 ± 11.1
	(+)-Catechin, $CuSO_4$	3915.4 ± 236.4	3788.6 ± 150.2
No enzyme added	(+)-Catechin	270.2 ± 41.6	198.4 ± 21.3
	$CuSO_4$	754.8 ± 49.2	405.8 ± 11.2
	(+)-Catechin, $CuSO_4$	9807.5 ± 969.4	9419.2 ± 351.1

* Each value is the mean of three assays ± S.D.
† BAPN fumarate, 0.1 mM.

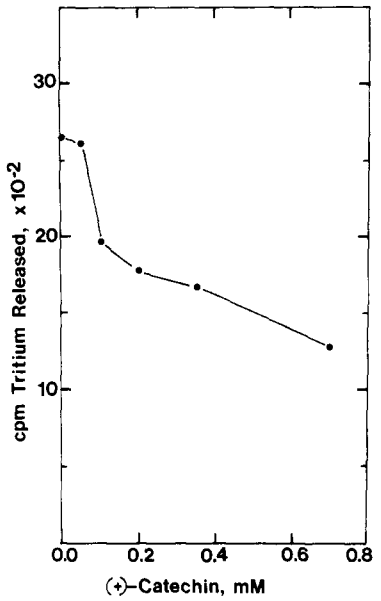

Fig. 1. Inhibition of lysyl oxidase activity *in vitro* with (+)-catechin. Lysyl oxidase activity was measured in 4 M urea extracts of aortic tissue. Extracts were dialyzed against 0.015 M phosphate, 0.12 M NaCl (pH 7.6) prior to the assay.

tissue released substantial amounts of tritium. With (+)-catechin present at 0.5 mM, the release of tritium was reduced by almost 50%. A follow-up study (Fig. 1) showed that graded increments of (+)-catechin inhibited lysyl oxidase activity in the crude extracts. Complete inhibition was not reached, however, even when (+)-catechin approached 1.0 mM. Extracts from the deficient tissue, as expected, showed only a marginal release of tritium (Table 2). That release was suppressed further with (+)-catechin present. Note that $CuSO_4$ brought about a slight increase in the release of tritium when added to the extracts. However, when both $CuSO_4$ and (+)-catechin were added together, a very substantial release of tritium was seen. That release was not sensitive to inhibition by BAPN (Table 2). In fact, the combination of (+)-catechin with $CuSO_4$ stimulated the release of tritium when no apparent source of lysyl oxidase was present in the medium. The apparent lysyl oxidase activity was completely insensitive to BAPN. Clearly such effects *in vitro* cannot be attributed to a stimulation of an enzyme-catalyzed reaction.

Table 3. Effects of (+)-catechin treatment on the ceruloplasmin oxidase activity in serum

Time (hr) after administration*	N	PPD oxidase activity† (I.U.)
0 (no injection)	4	1.14 ± 0.16
8	4	0.99 ± 0.12
16	4	1.10 ± 0.11
24	4	1.11 ± 0.11

* (+)-Catechin, 5 mg/kg.
† Micromoles of oxidized product per min/l of serum.

In a further characterization of (+)-catechin action *in vivo*, the oxidase activity of ceruloplasmin was measured after the treatment. In Table 3 it can be seen that control chicks maintained a constant level of ceruloplasmin for at least 24 hr after the (+)-catechin administration. This would suggest that the dose of (+)-catechin that elevated lysyl oxidase activity (5 mg/kg) was more targeted to aorta and not to oxidase activity in general.

DISCUSSION

(+)-Catechin is potentially a potent pharmacological agent capable of influencing the metabolism of connective tissue. The scope and mechanism of (+)-catechin action, however, remain obscure. The present study has provided evidence that lysyl oxidase activity in connective tissue may be a biochemical target of the flavonoid *in vivo*. At the same time one is cautioned against a simple interpretation of the data. (+)-Catechin showed stimulatory activity only when the copper status of the animal was adequate (Table 1). A normal well-fed animal would certainly be expected to respond to (+)-catechin in the manner described in this study. It should be pointed out that the (+)-catechin administered to the chicks was about 5% of the dose used in experiments with rats [5, 10, 11]. This is one reason for concluding that the effects of (+)-catechin on lysyl oxidase activity are quite potent. A dose–response curve would better define the lower limits of (+)-catechin action.

The studies *in vitro* also point to a complex mechanism of action for (+)-catechin. At 0.5 mM and with no free copper ions present, the flavonoid inhibited lysyl oxidase activity. Inhibition of the crude enzyme did not become progressively severe with increasing concentrations of (+)-catechin, however. One reason may be that chick aorta contains multiple forms of lysyl oxidase [19], and according to Kagan *et al.* [20] each lysyl oxidase isozyme in aorta (bovine aorta) responds differently to specific inhibitors of the enzyme. As yet we have not tested (+)-catechin with a single molecular species of lysyl oxidase. Nonetheless, the data suggest that lysyl oxidase is another enzyme in collagen and elastin biosynthesis whose activity is reduced by (+)-catechin *in vitro* and which perhaps would be added to the list of enzymes known to be inhibited by bioflavonoids [21].

In combination with free copper ions, (+)-catechin catalyzed a very strong release of tritium from lysyl oxidase substrates. This activity was not inhibited by BAPN. Cetta *et al.* [14] reported a similar "pseudo" lysyl oxidase activity associated with a (+)-catechin–copper complex that they prepared. Such complexes have been shown to catalyze the oxidative deamination of peptidyl lysyl residues leading to the formation of hydroxylysinonorleucine and lysinonorleucine, the cross-linking compounds of collagen [12]. It now seems clear that (+)-catechin–copper complexes must exert a direct chemical effect on the labeled protein substrates apart from any stimulation of latent lysyl oxidase activity. There is no way of

telling whether such non-enzymatic effects of (+)-catechin carry over to its action *in vivo*.

Presently the mechanism by which (+)-catechin stimulates lysyl oxidase activity *in vivo* is unknown. Had the flavonoid affected copper absorption, one would have expected to see an increase in the ceruloplasmin levels in the serum after (+)-catechin administration. Ceruloplasmin, a serum copper protein, has been linked to the activation of lysyl oxidase in chicks recovering from copper deficiency [15, 22]. Copper is the major regulator of lysyl oxidase in copper-deficient animals [23]. Thus, (+)-catechin must make more of the copper that is absorbed available to the enzyme as suggested by the large increase in copper-induced activation of lysyl oxidase in (+)-catechin-treated chicks.

It is now apparent from several studies that copper complexes are quite effective in arresting inflammatory disorders of connective tissue from a variety of causes [24–26]. Since bioflavonoids are relatively safe natural products that, according to the present study, can be used at very low levels to exert an effect, our results support the use of (+)-catechin–copper complexes as potential anti-inflammatory drugs for connective tissue.

Acknowledgements—This research was supported by U.S. Public Health Service Grant AM-26604. We also acknowledge excellent technical assistance by Ms. Shivaun Schulte.

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