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ASCORBIC ACID INHIBITION OF ALPHA-ADRENERGIC RECEPTOR BINDING

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Abstract—Relatively low concentrations of ascorbic acid inhibited the binding of the alpha-1 adrenergic antagonist [^{125}I]HEAT (DL-[β(3-iodo-4-hydroxyphenyl)-ethyl-aminomethyl]-tetralone) in rat submandibular gland and rat aorta. However, no inhibition was observed with this ligand in several other tissues, nor with several other ligands in these tissues. The inhibition observed was dependent on the concentration of both the ascorbic acid and the tissue. Maximal inhibition of [^{125}I]HEAT occurred in submandibular gland at 10 μM ascorbic acid with B_{max} values reduced 65% and no change in affinity. Ascorbic acid had a greater effect in assays in which less tissue was used, causing a 22% decrease in binding at 46 μg/ml, but a 48% decrease in binding at a tissue concentration of 12 μg/ml. EDTA prevented the loss of binding normally seen with ascorbic acid at a tissue concentration of 17 μg/ml. We suggest that, if an antioxidant is thought to be necessary in an assay system, its effects be carefully examined before routine use.

Ascorbic acid is often included in adrenergic radioligand binding incubations as a precautionary measure with the intent of retarding or preventing the oxidation of compounds such as epinephrine or norepinephrine. However, it rarely is demonstrated that the oxidation of the drugs actually is reduced [1]. In addition, several studies indicate that ascorbic acid alters the characteristics of the receptor binding sites when included in the binding assays for dopaminergic [2], opioid [3], serotonergic [4] and beta-adrenergic [5] receptors. The effect of ascorbic acid in these systems appears to be a decrease in the number of binding sites due to an ascorbic acid induced lipid peroxidation. However, we have not found any reports of an effect of ascorbic acid on alpha adrenergic receptor binding.

Our recent observations of an inhibitory effect of ascorbic acid in binding studies using an alpha-1 antagonist, [^{125}I]HEAT (DL-[β(3-iodo-4 hydroxyphenyl)-ethyl-aminomethyl)-tetralone), led us to examine several adrenergic ligands and tissues in an effort to better understand the ascorbic acid effect. We report here that ascorbic acid selectivity affects the [^{125}I]HEAT binding assay in a tissue-dependent manner.

MATERIALS AND METHODS

Sprague–Dawley rats of either sex were decapitated and the following tissues were removed: submandibular gland (SMG), lung, aorta and cerebral cortex. The tissues were frozen at −20° until the date of the assay except rat aorta which was assayed immediately. Dog aorta was removed from anesthesized dogs, freed of extraneous tissue, and chopped with a McIllwain chopper in the cold. The tissue was then frozen for up to 3 months until assayed.

On the day of the assay, aorta, SMG or lung was thawed and homogenized with a Brinkmann Polytron at setting 7 for two 20-sec bursts in ice-cold 50 mM Tris–HCl buffer (pH 8.0 at 25°). The homogenate was centrifuged at 49,000 *g* for 10 min. The pellet was rehomogenized in a similar manner, filtered through 53 μm nylon mesh and centrifuged as before. Rat cortex was thawed and homogenized with a Tekmar Tissumizer at setting 80 for 30 sec in ice-cold Tris–HCl buffer. The homogenate was centrifuged at 49,000 *g* for 10 min, and the homogenization process was repeated. The resulting pellets were resuspended as indicated in Table 1 and utilized in saturation and inhibition binding experiments.

Outdated human platelets were obtained from a local blood bank. Residual red blood cells were removed by centrifugation at 275 *g* for 10 min followed by centrifugation at 49,000 *g* for 10 min to collect the platelets. The platelet pellet was homogenized with lysing buffer (5 mM Tris, 5 mM EDTA, pH 7.5) for 20 sec with a Tekmar Tissumizer at setting 80. After centrifugation at 49,000 *g* for 10 min, the crude particulate membrane pellet was frozen at −20° for later use. On the date of the assay, the pellet was thawed, homogenized in lysing buffer, centrifuged, and resuspended.

HT-29 human colonic carcinoma cells were grown in Dulbecco's Modified Eagles (DME) medium supplemented with 5% fetal calf serum and 5% newborn calf serum at 5% CO_2 in a humidified chamber at 37°. On the day of the assay, the confluent cells were scraped from 150 mm dishes and washed in phosphate-buffered saline and homogenized in ice-cold Tris–HCl buffer with a Tekmar Tissumizer at setting 80 for 20 sec. Centrifugation at 49,000 *g* was followed by homogenization of the pellet and

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Table 1. Assay conditions for saturation and competitive inhibition experiments

Radioligand	Tissue	Assay buffer	Assay volume
[^{125}I]HEAT	Rat SMG*, aorta, lung, cortex Dog aorta	25 mM Glycylglycine, pH 7.6	1 ml
[^{3}H]Prazosin	Rat SMG, aorta, lung, cortex	25 mM Glycylglycine, pH 7.6	2 ml
[^{3}H]Yohimbine	Human platelet, HT29 cells	25 mM Glycylglycine, pH 7.6	1 ml
[^{3}H]UK-14,304-18	HT29 cells	25 mM Glycylglycine, pH 7.6	1 ml
[^{3}H]Dihydroalprenolol	Rat SMG, cortex	50 mM Tris–HCl, pH 7.6	1 ml

* Submandibular gland.

another centrifugation at 49,000 *g*. The crude membrane particulate preparation was resuspended in 25 mM glycylglycine for binding experiments.

Saturation and inhibition experiments. For saturation binding studies, 970 μl of tissue was incubated with 20 μl of increasing concentrations of [^{125}I]-HEAT ligand with or without the stated concentration of ascorbic acid. A second set of similar tubes included 100 μM (−)-norepinephrine to determine nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. After a 45-min incubation at 23°, the incubations were terminated by rapid filtration with a Brandel Cell Harvester (Gaithersburg, MD) through GF/B filters previously soaked in 0.1% polyethylenimine. The filters were rinsed with 10 ml of Tris–HCl buffer and counted in a Tracor gamma counter with an efficiency of 77%. Data were plotted by the method of Rosenthal [6]. Protein concentration was estimated by the method of Lowry *et al.* [7], using bovine serum albumin as the standard.

For inhibition experiments, the appropriate volume of tissue was incubated with increasing concentrations of (−)-norepinephrine with or without 57 μM (0.001%) ascorbic acid and a fixed concentration of radiolabeled drug. Ascorbic acid up to 1 mM did not alter the pH of the buffer. The final assay volume and appropriate buffers are listed in Table 1. The incubations were terminated as before except that the GF/B filters were presoaked in polyethylenimine only for the alpha-1 adrenergic receptor assays. The radioactivity on the filters for tritiated ligands was determined by standard scintillation techniques with an efficiency of 41%.

Drugs. [^{125}I]HEAT (2200 Ci/mmole), [^{3}H]prazosin (80.9 Ci/mmole), [^{3}H]yohimbine (82.7 Ci/mmole), and [^{3}H]dihydroproprenolol (102.7 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). [^{3}H]UK-14,304-18 (84.0 Ci/mmole) was donated by New England Nuclear. (−)-Norepinephrine and ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

The results of inhibition binding experiments using norepinephrine as the competing ligand for alpha and beta adrenergic receptors indicated a tissue and ligand specific effect of 57 μM (0.001%) ascorbic acid (Table 2). In rat SMG and aorta, (−)-norepinephrine was 4-fold more potent (lower IC_{50}) in the presence of ascorbate as compared to its absence in inhibiting the binding of the alpha-1 antagonist [^{125}I]HEAT. A similar effect of ascorbic acid was not seen with the alpha-1 antagonist [^{3}H]prazosin in these same tissues. Non-significant effects of 20–30% of the IC_{50} were noted with the other three tissues with [^{125}I]-HEAT and in most tissues with [^{3}H]prazosin. In addition to the effect on the IC_{50} of norepinephrine, ascorbic acid also decreased the binding of alpha-1 radioligands in the absence of norepinephrine (B_o) in some tissues (Table 2). [^{125}I]HEAT binding was decreased about 45% in rat SMG and aorta and 29% in rat cerebral cortex. [^{3}H]Prazosin binding was decreased 28% in rat cortex. For several other ligands and tissues, no effect of 57 μM ascorbic acid was observed (Table 2). These include the alpha-2 antagonist [^{3}H]yohimbine in the human platelet and the human colon carcinoma cell line HT29, the alpha-2 agonist [^{3}H]UK-14,304-18 in HT29 cell membranes and the beta adrenergic antagonist [^{3}H]dihydroalprenolol in rat cortex and SMG.

We next investigated the effect of various concentrations of ascorbic acid on the binding of a single concentration of either [^{125}I]HEAT or [^{3}H]prazosin in the SMG (Fig. 1). Ascorbic acid decreased [^{125}I]-HEAT binding in a dose-dependent manner but did not decrease [^{3}H]prazosin binding except at the highest concentration (1 mM). The IC_{50} of ascorbic acid in decreasing [^{125}I]HEAT binding was approximately 2 μM. The addition of 1 mM EDTA to [^{125}I]-HEAT assays prevented the loss of binding caused by ascorbic acid (Fig. 1).

To determine if the decrease in [^{125}I]HEAT binding in SMG was due to a decrease in the number of binding sites (B_{max}) or a decrease in the affinity (increase in K_D), saturation experiments were done in the presence of various concentrations of ascorbic acid. The results of these experiments indicated that ascorbic acid caused a dose-related decrease in measurable alpha-1 adrenergic receptor number with no change in affinity (Table 3, Fig. 2). The maximum decrease was 65% at 10 and 100 μM ascorbic acid.

One of the differences between the [^{125}I]HEAT and [^{3}H]prazosin experiments was that lower protein (tissue) concentrations were used with [^{125}I]HEAT

Table 2. Inhibition of radioligand binding by norepinephrine in the absence and presence of 57 μM ascorbic acid in various tissues

Radioligand	Receptor	Tissue	IC_{50} − Ascorbic acid (nM)	n_H	IC_{50} + Ascorbic acid (nM)	n_H	B_o + Ascorbic acid / − Ascorbic acid	N
[^{125}I]HEAT	α-1	Rat SMG*	139 ± 31	0.86 ± 0.02	44 ± 28		59 ± 9	3
	α-1	Rat aorta	188 ± 27	0.66 ± 0.03	43 ± 31		53 ± 4	2
	α-1	Rat lung	460 ± 53	0.87 ± 0.11	340 ± 40	0.70 ± 0.09	96 ± 2	3
	α-1	Rat cerebral cortex	658 ± 42	0.87 ± 0.07	463 ± 38	0.62 ± 0.03	71 ± 6	3
	α-1	Dog aorta	795 ± 25	0.89 ± 0.04	590 ± 0	0.85 ± 0	97 ± 1	2
[^{3}H]Prazosin	α-1	Rat SMG	305 ± 81	0.86 ± 0.03	230 ± 52	0.97 ± 0.03	96 ± 1	3
	α-1	Rat aorta	140 ± 13	0.67 ± 0.06	130	0.65 ± 0	91	1
	α-1	Rat lung	520 ± 53	1.03 ± 0.04	377 ± 27	0.82 ± 0.09	100 ± 2	3
	α-1	Rat cerebral cortex	663 ± 19	0.89 ± 0.05	540 ± 124	0.89 ± 0.05	72 ± 3	3
[^{3}H]Yohimbine	α-2	Human platelet	385 ± 50	0.83 ± 0.05	348 ± 76	0.79 ± 0.08	99 ± 5	3
	α-2	HT29 cells	41 ± 9	0.44 ± 0.03	45 ± 12	0.46 ± 0.02	94 ± 1	3
[^{3}H]UK-14,304-18	α-2	HT29 cells	6.6 ± 1.7	0.83 ± 0.01	7.9 ± 3.1	0.72 ± 0.05	104 ± 8	3
[^{3}H]Dihydroalprenolol	β	Rat cerebral cortex	300 ± 113	0.56 ± 0.02	332 ± 94	0.61 ± 0.03	98 ± 5	3
		Rat SMG	198 ± 32	0.69 ± 0.02	139 ± 48	0.73 ± 0.08	99 ± 4	3

Inhibition experiments were performed by incubating aliquots of the tissue preparation in the presence of increasing concentrations of (−)-norepinephrine with and without 0.06 mM ascorbic acid. Values are mean ± S.E., and n_H is the Hill coefficient. B_o is specific binding in the absence of inhibitor. SMG = submandibular gland.

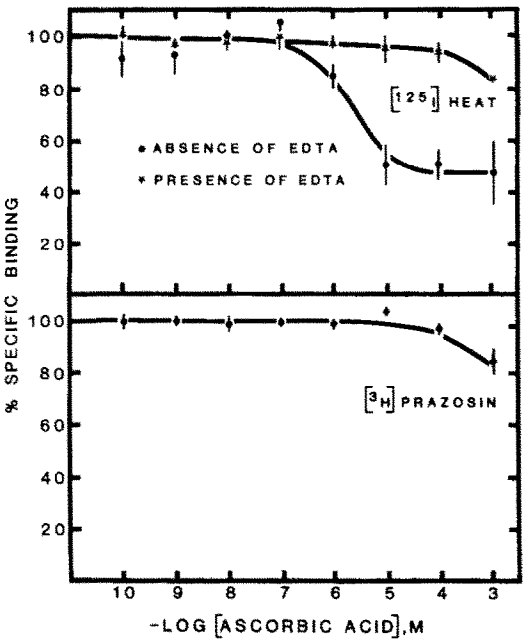

Fig. 1. Ascorbic acid effect on the binding of a single concentration of [^{125}I]HEAT and [^{3}H]prazosin in the rat submandibular gland. Aliquots of the tissue preparation were incubated with increasing amounts of ascorbic acid and 10 pM [^{125}I]HEAT or 100 pM [^{3}H]prazosin with or without 1 mM EDTA.

due to its higher specific activity. Thus, we examined the effect of different tissue concentrations on the ascorbic acid effect in the SMG. The magnitude of the ascorbic acid effect was clearly tissue dependent (Fig. 3). An ascorbic acid concentration of 100 μM caused a 22% decrease in binding at a tissue concentration of 46 μg/ml, but a 48% decrease at 12 μg/ml (Fig. 3). The presence of EDTA at a tissue concentration of 17 μg/ml prevented the loss of binding normally seen with ascorbic acid.

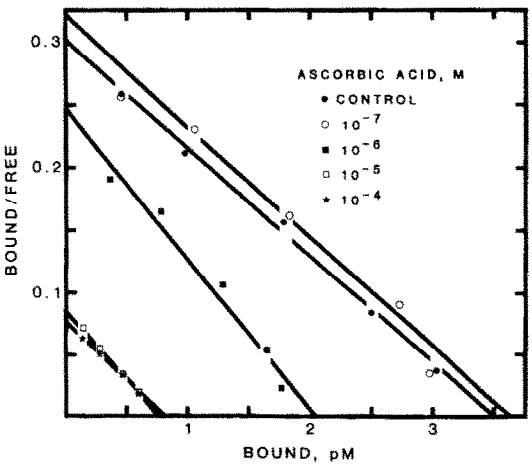

Fig. 2. Saturation curves from a typical experiment in which various concentrations of ascorbic acid were included. Aliquots of the tissue preparation were incubated with increasing concentrations of [^{125}I]HEAT (2–135 pM) in 25 mM glycylglycine buffer. The K_D and B_{max} values were derived from computer-derived Rosenthal plots.

Table 3. Ascorbic acid effect in rat submandibular gland binding of [^{125}I]HEAT

	K_D (pM)	B_{max} (fmoles/mg protein)
Control	11.4 ± 0.9	205 ± 22
0.1 μM Ascorbic acid	12.5 ± 0.8	218 ± 25
1.0 μM Ascorbic acid	10.5 ± 1.0	155 ± 25
10.0 μM Ascorbic acid	10.6 ± 1.3	72 ± 21
100.0 μM Ascorbic acid	9.8 ± 0.5	73 ± 30

Saturation curves were obtained by incubating a crude particulate membrane preparation (20 μg/ml) with increasing concentrations of [^{125}I]HEAT (2–135 pM) in 25 mM glycylglycine buffer with the indicated concentration of ascorbic acid. The K_D and B_{max} were derived from computer-derived Rosenthal plots. Values are mean ± S.E., N = 4.

DISCUSSION

We examined the norepinephrine inhibition of alpha-1, alpha-2, and beta radioligands in several tissues in the presence and absence of ascorbic acid. The IC_{50} values of norepinephrine were not substantially different in the presence and the absence of a relatively low ascorbic acid concentration (57 μM) except in rat aorta and SMG with the alpha-1 antagonist [^{125}I]HEAT. Saturation experiments in rat submandibular gland in the presence of ascorbic acid indicated a dose-dependent decrease in B_{max} as determined by [^{125}I]HEAT with no change in affinity. The effect of ascorbic acid was maximal at 10 μM.

Other investigators have reported decreases in receptor binding due to ascorbic acid. Dunlap *et al.* [3] reported maximum decreases in opiate binding at 1 mM ascorbic acid in rat brain and implicated lipid peroxidation as a probable cause. Heikkila *et al.* [8] reported a dose-dependent decrease in dopamine agonist binding in rat corpus striatum and a U-shaped dose–response curve for antagonist with a maximal effect of approximately 1 mM. Similarly, the maximal effect on [^{3}H]dihydroalprenolol binding (beta-adrenergic receptors) was at 0.5 mM [5]. Thus, the inhibitory effects on [^{125}I]HEAT binding occur at much lower ascorbic acid concentration than those previously reported for other ligands.

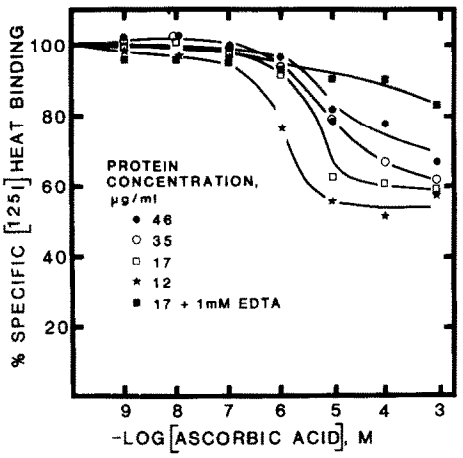

Fig. 3. Effect of tissue concentration on the ascorbic acid effect of [^{125}I]HEAT binding in rat submandibular gland. The indicated tissue concentrations were incubated with increasing concentrations of ascorbic acid and 15 pM [^{125}I]-HEAT with 100 μM (−)-norepinephrine indicating nonspecific binding in 25 mM glycylglycine buffer.

Heikkila *et al.* [9] examined lipid peroxidation of tissue along with antagonist binding at dopamine receptors and found a strong correlation between these two events. The effects of ascorbic acid were reversed in this study by EDTA and other presumed inhibitors of lipid peroxidation. Bacopoulos [10] in similar experiments saw no effect of ascorbic acid, but the incubation buffer in these experiments contained 5 mM EDTA which would adequately block effects of ascorbic acid. In a study of neuroleptic binding to canine corpus striatum, Chan *et al.* [11] found decreased binding to membrane receptors, but no decrease in binding to solubilized receptors, indicating an action of ascorbate on the membrane matrix of the receptor. Again, EDTA and also Mn^{2+} prevented the decrease in membrane receptor binding. In rat cortex, Heikkila [5] found a modest loss of [^{3}H]dihydroalprenolol binding in the presence of ascorbic acid which was correlated with lipid peroxidation. Addition of low levels of iron greatly potentiated the effects of ascorbic acid. These studies strongly suggest that ascorbic acid induced lipid peroxidation is a probable cause of the decreased membrane receptor binding. Our study, because of the reversal of inhibition by EDTA, also implicated lipid peroxidation as a possible mechanism for the decreased receptor density.

However, the decrease in binding which we observed was not universal as it appears to be in some other receptor systems [12, 13]. In the rat SMG, cortex and aorta assays with [^{125}I]HEAT, considerably less tissue was used than in the other assays because of factors such as the specific activity of the radioligand, the receptor density and tissue availability. This may, in part, explain why we saw effects of ascorbic acid in these assays but not the other assays. The tissue concentration experiments in the SMG support this hypothesis.

It seems clear from the number of studies which have shown negative effects of ascorbic acid that one should be cautious about its use in *in vitro* studies. In addition, in many assay systems, an antioxidant may not be needed. For example, Heikkila and Cabbat [1] compared the effects of ascorbic acid on [^{3}H]dopamine decomposition in the presence and

absence of tissue. They found that tissue was important in preventing decompsition of the [^{3}H]dopamine and that this decomposition was not further prevented by the presence of ascorbic acid.

The question arises about the possible effects of ascorbic acid *in vivo*. Both [^{3}H]prazosin and [^{3}H]-clonidine binding of rat cortex membrane were decreased when ascorbic acid was included as an antioxidant for 6-hydroxydopamine injection into rats in denervation studies [14]. The mechanism of action of ascorbic acid *in vivo* is probably not by lipid peroxidation. It seems likely that ascorbic acid may reduce components associated with the plasma membrane and affect the steric molecular configuration and electrical charge of macromolecules in the membrane [15, 16]. These changes in the membrane could induce ionic interactions that could modify a number of events including binding to the membranes *in vitro*.

We have found that low concentrations of ascorbic acid significantly decreased receptor binding of the alpha-1 antagonist [^{125}I]HEAT in the rat SMG and aorta. The magnitude of the inhibition was dependent on both the ascorbic acid and tissue concentrations. With several other tissues and ligands, no such effect was seen. We suggest that, if an antioxidant is thought to be necessary in an assay system, its effects be examined carefully before routine use. If ascorbic acid is used, it may be useful to also include EDTA in the incubation.

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