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INACTIVATION OF ANGIOTENSIN II RECEPTORS IN BOVINE ADRENAL CORTEX BY DITHIOTHREITOL

FURTHER EVIDENCE FOR THE ESSENTIAL NATURE OF DISULFIDE BONDS

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Abstract—Dithiothreitol (DTT), a disulfide reducing agent, significantly decreased specific [^{3}H]angiotensin II binding to membrane-bound and solubilized bovine adrenal cortical receptors. Scatchard analysis indicated a reduction in the maximum number of membrane-bound binding sites without change in affinity. The effect of DTT on membrane-bound receptors was readily reversed by the sulfhydryl oxidizing agent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), indicating that its action upon specific [^{3}H]angiotensin II binding is mediated via reduction of disulfide bonds rather than other properties of this agent. Preincubation of the membranes with unlabeled angiotensin II, but not its biologically inactive precursor angiotensin I, protected the receptor from deactivation by DTT. The data provide evidence that disulfide bonds essential for [^{3}H]angiotensin II receptor binding in bovine adrenal cortex are located at, or near, the active sites of the receptor.

Dithiothreitol (DTT)†, a disulfide-reducing agent [1], has been shown to affect the properties of several hormone and neurotransmitter receptors among which are included the nicotinic acetylcholine [2], β-adrenergic [3], gonadotropin [4] and insulin receptors [5]. Since DTT also specifically inhibits contractile responses of isolated aortic strips to angiotensin II, it has been suggested that disulfide bonds are necessary for the functional integrity of the vascular angiotensin II receptor [6]. The recent observation that DTT reduces angiotensin II receptor binding in rat mesenteric arteries is consistent with this view [7]. However, whether DTT affects angiotensin II vascular contractile responses and receptor binding as a result of its ability to reduce disulfide bonds or by another mechanism, as for example, its well established ion-chelating effect, has not been investigated. Moreover, since physiological responses to angiotensin II are recognized to vary from tissue to tissue [8], it is conceivable that angiotensin II receptors in different tissues may have different sensitivities to DTT. In the present study, DTT was found to markedly reduce angiotensin II receptor binding in bovine adrenal glands. The latter effect of DTT was related to its ability to reduce disulfide bonds at, or near, the active sites of angiotensin II binding to its receptors.

MATERIALS AND METHODS

Membrane preparation and treatment with DTT. Bovine adrenal membranes were prepared as described by Chang and Lotti [9]. Membranes corresponding to 0.25 g tissue were resuspended in 10 ml of buffer B (10 mM phosphate, 120 mM NaCl, 0.1 mM PMSF and 5 mM EDTA, pH 7.4) containing DTT (0.1, 1.0 or 5 mM) and incubated for 20 min at 30° or 37°. The reaction was terminated by centrifugation at 50,000 *g* for 10 min at 4°. After removal of the supernatant fraction, the pellets were resuspended in the binding assay buffer (buffer B containing 0.2% heat-denatured bovine serum albumin and 0.1 mM bacitracin) and used for [^{3}H]angiotensin II receptor binding as described below.

Binding assays for angiotensin II receptors. Binding of [^{3}H]angiotensin II to bovine adrenal membranes was performed as described by Bennett and Snyder [10] with the omission of DTT. [^{3}H]Angiotensin II binding to digitonin-solubilized receptor was assayed according to Chang and Lotti [9]. The assay mixtures contained 450 μl of receptor preparations (membrane-bound or digitonin-solubilized receptors), 25 μl of buffer (for total binding) or 25 μl of unlabeled angiotensin II (1 μM final concentration for nonspecific binding), and 25 μl of [^{3}H]angiotensin II to give a final concentration of 2.5 nM. [^{3}H]Angiotensin II was prepared in buffer B containing bacitracin (2.8 mg/ml) and bovine serum albumin (heat-denatured, 20 mg/ml). The reaction mixtures were incubated at 30° for 30 min. To separate free and bound angiotensin II from membrane-bound receptors, the incubation mixtures were filtered through Whatman GF/B filters under reducing pressure, and the filters were immediately washed with 4 × 4 ml of Tris buffer (50 mM, pH 7.4, containing 0.1 M NaCl). The filters were soaked overnight in 10 ml of Formula 947 (NEN), and radioactivity was determined.

To separate free and bound angiotensin II from solubilized receptors, aliquots (0.4 ml) were placed

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† Abbreviations: DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); and PMSF, phenylmethane sulfonyl fluoride.

on the surface of 4-ml Sephadex G-50 (medium) columns (equilibrated with buffer B containing 0.25% digitonin). The eluates corresponding to the void volume (1.3 to 2.2 ml), determined using blue dextran, were collected. Fifteen ml of Formula 947 was added, and the radioactivity was counted by liquid scintillation.

DTNB reversal of DTT inhibition of [^{3}H]*angiotensin II binding.* Adrenal cortical membranes were pretreated with 5 mM DTT for 20 min at 30° and were centrifuged as described above. The pellets were then resuspended in the binding assay buffer containing 0.05, 0.15 or 0.5 mM DTNB and were assayed for [^{3}H]angiotensin II binding as described below. [^{3}H]Angiotensin II binding in membranes treated neither with DTT nor DTNB, only DTT (5 mM), or only DTNB, was determined concurrently and served as controls.

Protection from DTT inhibition of [^{3}H]*angiotensin II binding by preincubation with unlabeled angiotensin II.* Adrenal cortical membranes were first preincubated for 20 min at 30° in the presence of unlabeled angiotensin I or II (10, 30 and 100 nM). DTT (5 mM) was then added, and the membranes were incubated for an additional 20 min at 37° and centrifuged as described above. The pellets were then rewashed and centrifuged two additional times, using 40 vol. of buffer B, and assayed for [^{3}H]angiotensin II binding as described above. [^{3}H]Angiotensin II binding to membranes either treated or not treated with DTT (5 mM) and in the absence of unlabeled angiotensin I and II was determined concurrently and served as controls.

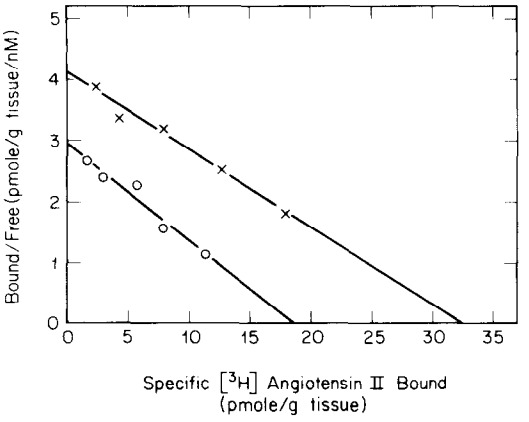

Fig. 1. Scatchard plots of specific [^{3}H]angiotensin II receptor binding in a typical experiment. Adrenal cortical membranes were treated (○——○) with DTT (5 mM, 30° for 20 min) in 10 mM phosphate buffer, 5 mM EDTA, 120 mM NaCl and 0.1 mM PMSF (pH 7.4). Control membranes (×——×) were treated concurrently without DTT. The membranes were used for specific [^{3}H]angiotensin II receptor binding assay after removing DTT by centrifugation. The concentrations of [^{3}H]angiotensin II used were 0.625 to 10 nM. The experiment, which was performed in triplicate, was replicated three times and the means ± S.E. of the maximum numbers of binding sites and binding affinities are given in the text. Specific binding is the difference between total binding and nonspecific binding (in the presence of 1 μM angiotensin II). Lines are fitted by linear regressions ($r = 0.97$ and 0.99 for treated and control respectively).

RESULTS

Effect of DTT concentration and reaction temperature on [^{3}H]*angiotensin II receptor binding in bovine adrenal membranes.* Preincubation with DTT for 20 min at 30° clearly reduced specific [^{3}H]angiotensin II binding to bovine adrenal membranes in a concentration-dependent manner. A 6, 21 and 55% reduction in [^{3}H]angiotensin II binding was observed at 0.1, 1 and 5 mM DTT respectively (Table 1). The reduction of [^{3}H]angiotensin II binding produced by DTT also appeared to be temperature dependent in that 77% compared to 55% inhibition ($P < 0.05$) of [^{3}H]angiotensin II binding occurred at 37° and 30° respectively (Table 1).

To ascertain whether the decrease in [^{3}H]angiotensin II binding by DTT was due to a change in the maximum number of binding sites or the affinity of the receptors, [^{3}H]angiotensin II receptor binding was performed at several ligand concentrations. Scatchard analysis indicated a reduction in the maximum number of binding sites (32.2 ± 0.1 vs 20.4 ± 1.6 pmoles/g tissue, $P < 0.025$) without change in affinity (7.7 ± 0.2 vs 6.8 ± 0.5 nM, $P > 0.1$) (Fig. 1).

Table 1. Effect of DTT concentration and reaction temperature on specific [^{3}H]angiotensin II receptor binding in adrenal cortical membranes*

DTT concn (mM)	Temperature (°)	[^{3}H]Angiotensin II binding (pmoles/g tissue ± S.E.)	%
0	30	9.6 ± 0.22	100
0.1	30	9.0 ± 0.09	94
1.0	30	7.6 ± 0.33†	79
5	30	4.3 ± 0.04‡	45
5	37	2.2 ± 0.01§	23

* Adrenal membranes were preincubated at the indicated temperature with various concentrations of DTT and then centrifuged to remove DTT. The pellets were then resuspended in assay buffer without DTT and used for [^{3}H]angiotensin II binding studies.

†,‡,§ The difference from control (without DTT) is significant at $P < 0.05$, $P < 0.005$ or $P < 0.001$ respectively.

Table 2. DTNB reversal of DTT-induced inhibition of [^{3}H]angiotensin II receptor binding*

DTNB concn (mM)	Specific [^{3}H]angiotensin II binding (pmoles/g tissue ± S.E.)
0	5.7 ± 0.16
0.05	7.5 ± 0.22†
0.15	9.6 ± 0.45†
0.50	10.6 ± 0.40†

* Bovine adrenal cortical membranes were incubated with 5 mM DTT (30° for 20 min) and centrifuged. The pellet was resuspended in the assay buffer containing the indicated concentration of DTNB and was assayed for [^{3}H]angiotensin II binding. Specific [^{3}H]angiotensin II binding determined in concurrent studies using neither DTNB nor DTT was 10.2 ± 0.07 pmoles/g tissue.

† The difference from control (no DTNB) is significant at $P < 0.005$.

DTNB reversal of DTT-induced inhibition of [^{3}H]angiotensin II receptor binding. DTNB, at concentrations ranging from 0.05 to 0.5 mM, reversed the effect of 5 mM DTT on specific [^{3}H]angiotensin II binding in a dose-dependent manner. A complete reversal of the effect of DTT occurred at concentrations of 0.15 to 0.5 mM (Table 2). The concentrations of DTNB employed (0.05 to 0.5 mM), by themselves, had no significant effect upon specific [^{3}H]angiotensin II binding.

Protection from DTT inhibition of [^{3}H]angiotensin II binding by preincubation with unlabeled angiotensin II. Membranes preincubated with unlabeled angiotensin II and DTT, and subsequently washed, exhibited significantly higher [^{3}H]angiotensin II binding than membranes similarly treated with DTT alone (Table 3). The increase in [^{3}H]angiotensin II binding in tissues preincubated with unlabeled angiotensin II (10, 30 and 100 nM) was 33, 57 and 100% respectively. Control studies verified that preincubation with unlabeled angiotensin II alone at these concentrations had no significant effect on subsequent [^{3}H]angiotensin II binding after washing.

Specific [^{3}H]angiotensin II binding in membranes similarly preincubated with unlabeled angiotensin I and subsequently washed was not significantly different from membranes treated with DTT alone (data not shown).

Table 3. Protection from DTT inhibition of [^{3}H]angiotensin II binding by preincubation with unlabeled angiotensin II*

Angiotensin II concn (nM)	Specific [^{3}H]angiotensin II binding (pmoles/g tissue ± S.E.)
0	2.1 ± 0.01
10	2.8 ± 0.16†
30	3.3 ± 0.13‡
100	4.2 ± 0.06§

* Bovine adrenal cortical membranes were first preincubated (20 min at 30°) with unlabeled angiotensin II at the indicated concentrations. DTT (5 mM) was then added and the membranes were reincubated (20 min at 37°), repeatedly centrifuged and washed three times, and assayed for [^{3}H]angiotensin II binding. Specific [^{3}H]angiotensin II binding determined in concurrent studies using neither unlabeled angiotensin II nor DTT was 8.82 ± 0.33 pmoles/g tissue.

†,‡,§ The difference from control (no unlabeled angiotensin II) is significant at $P < 0.025$, $P < 0.005$ or $P < 0.001$ respectively.

Effect of DTT on the specific [^{3}H]angiotensin II receptor binding to solubilized receptors. In the presence of 5 mM DTT, specific [^{3}H]angiotensin II receptor binding (assayed at 2 nM) to solubilized adrenal cortical receptors was decreased significantly to 27% of control (0.13 ± 0.01 vs 0.49 ± 0.03 pmole/g tissue, $P < 0.005$).

DISCUSSION

A major finding of the present study is that DTT pretreatment reduced specific [^{3}H]angiotensin II binding to bovine adrenal cortical membranes. This effect was able to be related to a reduction in the maximum number of binding sites for [^{3}H]angiotensin II rather than to a change in binding affinity. The fact that DTNB, a sulfhydryl-oxidizing agent, could completely reverse the DTT inhibition of [^{3}H]angiotensin II binding strongly suggests that the action of DTT was mediated via its ability to reduce disulfide bonds, rather than via other properties of the molecule such as ion chelation.

Previous investigators using different tissues have employed DTT in [^{3}H]angiotensin II receptor binding assays primarily for the purpose of preventing angiotensin II degradation by proteases [10–12]. In agreement with our studies, DTT was observed to reduce [^{3}H]angiotensin II binding in rat mesenteric artery preparation [7]. However, in contrast to the present studies, Glossmann *et al.* [11], also employing membranes prepared from bovine adrenal cortex, reported that DTT (5 mM) enhanced specific [^{3}H]angiotensin II binding. The reason for the discrepancy between their result and ours is not clear. However, differences in the compositions of incubation buffer employed might account for the differences observed. The buffer system used by Glossman *et al.* did not contain EDTA, whereas in our assay 5 mM EDTA was employed as an ion chelator and protease inhibitor in addition to PMSF, bacitracin and bovine serum albumin. Since EDTA was absent from the buffer used by Glossman *et al.*, it is conceivable that the DTT enhancement of binding was due to the ion-chelating effect of DTT rather than to disulfide bond reduction. In fact, this laboratory has since substituted EDTA for DTT in their latest publication [13]. This contention is supported by our unpublished observations that EDTA, an ion chelator without disulfide-reducing effect, increased specific [^{3}H]angiotensin II binding in the absence of DTT. Moreover, DTT did not decrease specific [^{3}H]angiotensin II binding in the absence of EDTA, presumably because of the apparent opposing effects of ion chelation and disulfide bond reduction on [^{3}H]angiotensin II binding.

The essential nature of receptor disulfide bonds in the biological activity of angiotensin II has been suggested previously on the basis that DTT specifically abolished contractions of the rabbit aorta induced by angiotensin II [6]. The inhibition of [^{3}H]angiotensin II binding to bovine adrenal membranes produced by DTT in the present study could

be reduced significantly by the presence of angiotensin II but not, under similar conditions, by its biologically inactive precursor, angiotensin I. The data are consistent with the view of a prominent role for disulfide bonds in angiotensin II action and further that these bonds are located at, or near, the active sites of [^{3}H]angiotensin II binding. Since DTT also inhibited [^{3}H]angiotensin II binding to digitonin-solubilized angiotensin II receptors, the results would suggest that it is unlikely that DTT produced some alteration of membrane structure which, in turn, affected the properties of the receptor.

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