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Biochimica et Biophysica Acta 1192 (1994) 286–288

BBA Biochimica et Biophysica Acta

Rapid Report

Effect of dithiothreitol on angiotensin II receptor type II in rat ovarian cultured granulosa cells

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(Received 10 March 1994)

Abstract

Dithiothreitol markedly increased the ligand binding affinity of angiotensin II (AII) receptor type II (AT_2) without affecting its antagonist selectivity in cultured ovarian granulosa cells, demonstrating that this AT_2 is of the dithiothreitol-sensitive type. Dithiothreitol is useful for specifically detecting low levels of the AT_2 in the ovary, where it plays roles that are probably related to atresia.

Key words: Granulosa cell; Angiotensin II; Receptor; Antagonist

Angiotensin II (AII), a biologically active peptide in the renin-angiotensin system, elicits a variety of biological actions, related mainly to the regulation of blood pressure and fluid osmolarity [1,2]. Recent pharmacological and molecular biological studies have identified two distinct subpopulations of AII receptors, designated AT_1 and AT_2 [3–7]. All of the known physiological functions of AII are mediated by the AT_1 receptor. In contrast, the functional roles of the AT_2 receptor have not yet become apparent. In 1988 we identified novel AII receptors in the bovine ovary, corresponding to the AT_2 receptor according to the present nomenclature, based upon their sensitivity to dithiothreitol (DTT), which was distinct from that of AII receptors previously identified in other tissues [8]. That is, DTT stimulated the AII binding capacity of ovarian membranes by increasing the ligand binding affinity, whereas the agent reduced the AII binding capacity of adrenal membranes in which the AT_1 receptor predominates. Granulosa cells in ovarian follicles provide a target for studying the molecular events associated with atresia, which features the fragmented nucleosomal DNA characteristic of apoptosis [9–13]. The AT_2 receptor is localized in atretic follicles but not in healthy follicles [14], indicating the involvement of this receptor in the process of atresia. Pucell et al. [14] have found the existence of the AT_2 receptor in rat granulosa cells in culture by means of competitive binding studies using the subtype-selective antagonists such as Dup753 for the AT_1 and PD123319 for the AT_2 receptors, but correlation with the responses to DTT was not examined. On the other hand, the presence of AT_2 receptor subpopulations was indicated by their sensitivity to sulfhydryl reducing agents in the rat brain [15,16]. Therefore, we characterized the AT_2 receptor in rat granulosa cells in culture, focussing upon the effect of DTT on the receptor.

In this study, granulosa cells were isolated from ovaries of diethylstilbestrol (Sigma)-treated Sprague-Dawley rats (CLEA, Japan) according to the method of Knecht et al. [17]. Briefly, immature 21-day-old rats were implanted with diethylstilbestrol (Sigma) for five days to stimulate granulosa cell proliferation, then granulosa cells were harvested by follicular puncture. Viable cells ($5 \cdot 10^5$) were pipetted into 12×75-mm polystyrene tubes and cultured in 1 ml McCoy's 5A medium (GIBCO) in a humidified 95% air, 5% CO_2 atmosphere at 37°C for 48 h. Thereafter, the cells were

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SSDI 0005-2736(94)00093-5

incubated with or without 4 mM DTT at 22°C for 15 min, then with a final concentration of 50 pM ^{125}I-[Sar^1,Ile^8]AII (Amersham), a non-selective AII antagonist, in 200 μl binding buffer consisting of Hanks'-balanced salt solution (Sigma) containing 0.1% crystallized bovine serum albumin, 1 mM PMSF, 50 μg/ml leupeptin, and 25 μg/ml antipain at 4°C for 2 h. For equilibrium binding studies, cells exposed or not to DTT were incubated with increasing concentrations of ^{125}I-[Sar^1,Ile^8]AII in the presence or absence of 10^{-5} M unlabeled [Sar^1,Ile^8]AII. Free and bound radioligands were separated by centrifugation as described by Feng et al. [18].

In a first series of experiments, we performed a competitive binding study in the presence and absence of DTT using ^{125}I-[Sar^1,Ile^8]AII as the ligand. As shown in Fig. 1, unlabeled [Sar^1,Ile^8]AII inhibited the radioligand binding dose-dependently under both conditions. However, the IC_{50} value ($4 \cdot 10^{-10}$ M) in the cells exposed to DTT was approximately 7.5-fold lower than that ($3 \cdot 10^{-9}$ M) in the untreated cells. The agent also increased the specific binding of ^{125}I-[Sar^1,Ile^8]AII up to 3.0-fold over the control level measured in the absence of the competitor. These data suggested that exposing of cells to DTT increased the affinity of AII receptors for the ligand [Sar^1,Ile^8]AII.

Fig. 2 shows saturation isotherms for ^{125}I-[Sar^1, Ile^8]AII binding to cultured granulosa cells. The data, expressed as a Scatchard plot, yielded an apparent straight line indicative of one class of binding sites in cultured granulosa cells exposed to DTT or not. Dithiothreitol caused little change in the B_{max} value but markedly decreased the K_d value by about 65%; $K_d = 2.0 \cdot 10^{-9}$ M, $B_{max} = 12\,600$ sites/cell without DTT and $K_d = 7.5 \cdot 10^{-10}$ M, $B_{max} = 11\,300$ sites/cell with DTT. These K_d values were consistent with the IC_{50} values determined above with and without DTT,

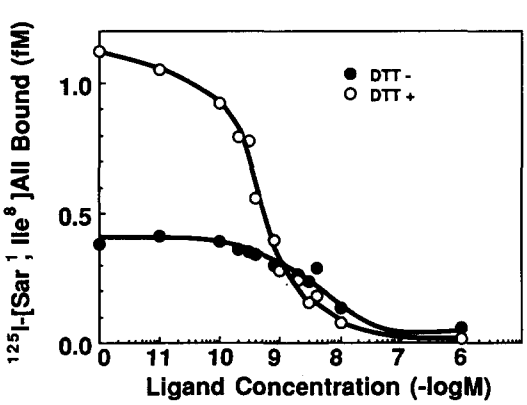

Fig. 1. Competitive inhibition curves of ^{125}I-[Sar^1,Ile^8]AII binding to cultured ovarian granulosa cells. Cells exposed (○) or not (●) to DTT were incubated with ^{125}I-[Sar^1,Ile^8]AII in the presence of the indicated concentrations of unlabeled [Sar^1,Ile^8]AII at 4°C for 2 h. The data represent the mean of two determinations.

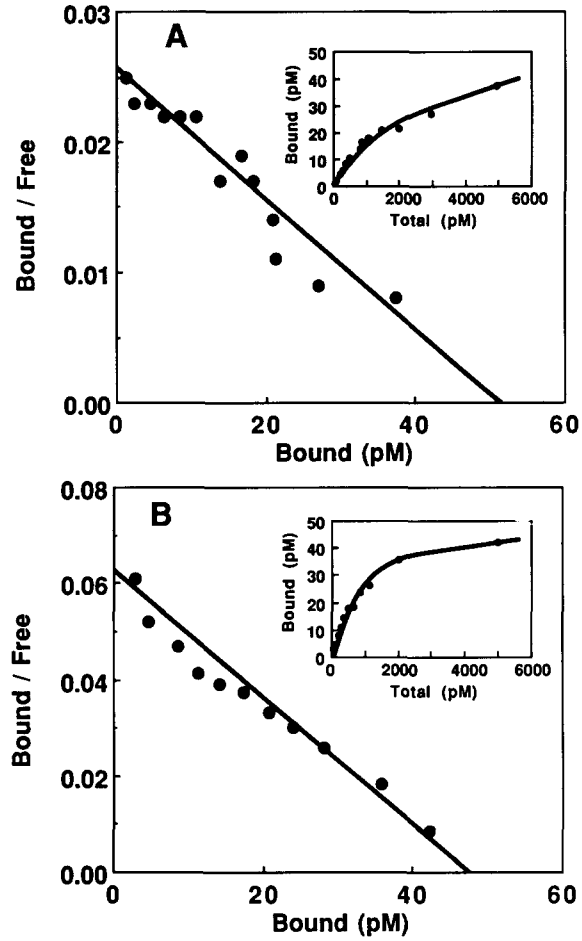

Fig. 2. Saturation isotherm and Scatchard plot analysis of ^{125}I-[Sar^1,Ile^8]AII binding to cultured ovarian granulosa cells. Cells exposed (B) or not exposed (A) to DTT were incubated with increasing concentrations of ^{125}I-[Sar^1,Ile^8]AII (50 pM–5 nM) in the presence or absence of 10^{-5} M unlabeled [Sar^1,Ile^8]AII. The specific binding of ^{125}I-[Sar^1,Ile^8]AII was shown in the insets. The saturation data were plotted according to Scatchard to determine the binding parameters.

respectively. Thus, DTT caused a significant increase in the affinity of AII receptors for [Sar^1,Ile^8]AII. This is characteristic of the AT_2 receptor, demonstrating that the AT_2 receptor previously identified based upon its response to AII antagonists in rat granulosa cells is of the DTT-stimulated type. This receptor must be identical with the AT_2 receptor of which the cDNA has recently been cloned from rat fetal and PC12W cell cDNA libraries [6,7], because DTT increased the AII binding activity of the cloned receptor transiently expressed in COS-7 cells [6]. In contrast, Tsutsumi et al. [15,16] have suggested the existence of AT_2 receptor subclasses which are insensitive to DTT or decreased by this sulfhydryl reducing agent in the rat brain. For example, AII binding to AT_2 receptors in the inferior olive and the hypoglossal nucleus of young rats was not influenced by DTT even with 10 μM Dup753, whereas the binding to ventral and mediodorsal thalamic, and

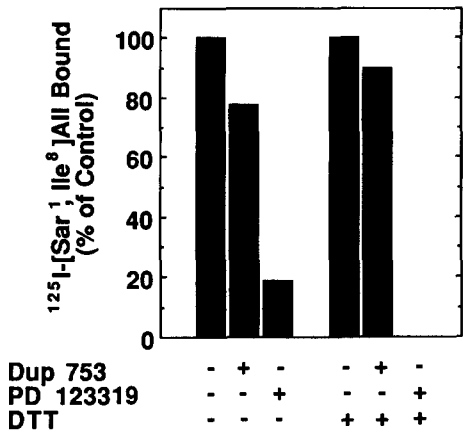

Fig. 3. Effect of subtype-selective AII antagonists on ^{125}I-[Sar^1,Ile^8]AII binding to cultured ovarian granulosa cells. Cells exposed or not to DTT were incubated with ^{125}I-[Sar^1,Ile^8]AII in the presence or absence of Dup753 (10^{-4} M) or PD123319 (10^{-4} M). Results are given as the percent of control binding measured in the absence of the competitors. Each data represents the mean of two independent experiments each determined in duplicate.

medial geniculate nuclei, and the cerebellar cortex was impaired in response to DTT. Since the cloned receptor possesses seven transmembrane domains, this study also shows that a disulfhydryl bond(s) affecting the ligand binding is located in extracellular domains of the receptor molecule.

We next compared the binding specificity of the AT_2 receptor for the AT_1- and AT_2-selective antagonists Dup753 and PD123319, respectively, between the cells exposed to DTT or not. As shown in Fig. 3, 10^{-4} M PD123319 caused an 82% inhibition in ^{125}I-[Sar^1,Ile^8]AII binding, whereas 10^{-4} M Dup753 decreased the binding by only 23% in the absence of DTT, again indicating the predominance of the AT_2 receptor in granulosa cells. Similar data were obtained from the DTT-treated cells. PD123319 at 10^{-4} M completely abolished the radioligand binding but there was little inhibition by 10^{-4} M Dup753. Therefore, the specificity of the AT_2 receptor for the two antagonists was the same, whether or not it was exposed to DTT. Together with the data from the competitive binding study, these results clearly showed that DTT increases the ligand binding activity of the AT_2 receptor without changing the selectivity for the subtype-specific antagonists.

The predominance of the AT_2 receptor in granulosa cells of atretic follicles in vivo [14] raised the possibility that AII might play an important role via this type of receptor in the development of atresia. However, the involvement of a small population of the AT_1 receptor cannot be excluded based upon our data since 10^{-4} M Dup753 induced a slight decrease in the ^{125}I-[Sar^1,Ile^8]AII binding in the absence of DTT. In fact, Currie et al. [19] have reported that 25% of granulosa cells prepared from rat ovarian follicles exhibit an increase in intracellular Ca^{2+} concentrations in response to AII, which is characteristic of the AT_1 receptor. The presence of the AT_1 receptor is also supported by the fact that the B_{max} value with DTT is a little lower than that with DTT. To elucidate the roles of the AT_2 receptor in the process of atresia, specific detection of the AT_2 receptor is essential. For this purpose, DTT, which markedly decreases the ligand binding activity of the AT_1 receptor and increases that of the AT_2 receptor without affecting the antagonist selectivity, will provide a useful method.

We would like to thank Ms. Norma Foster for her help in preparing the manuscript. This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan, and by a grant for a Special Research Project on Circulation Biosystem from the University of Tsukuba.

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