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Affinity to Some Plant Growth Regulators on Human Erythrocytes Cytosolic Carbonic Anhydrase I and II

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Abstract

The purpose of this study was to investigate the in vitro effects of four commonly used plant growth regulators (PGRs): indole-3-acetic acid, indole-3-butyric acid, gibberellic acid and kinetin on human carbonic anhydrase I and II. Carbonic anhydrase I and II from human erythrocytes was purified by Sepharose-4B-L-tyrosine-1-sulfonamide affinity gel. IC_{50} values of the chemicals that caused inhibition were determined by means of activity percentage diagrams. The plant growth regulators used in this study effected the CA activity from human to various degrees. The concentrations of indole-3-acetic acid, indole-3-butyric acid and kinetin that inhibited 50% of the hCA I were 78.13 μM, 54.48 μM and 62.89 μM respectively. The concentrations of indole-3-acetic acid and indole-3-butyric acid that inhibited 50% of the hCA II were 75.47 μM and 38.05 μM, respectively. Conversely, the enzyme activity was increased by gibberellic acid and kinetin.

INTRODUCTION

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyzes a very simple but critically important physiological reaction: the involvement of the carbonic anhydrase (CA) enzyme family, which catalyzes the physiological hydration of CO_2 to yield bicarbonate and a proton, in many physiological/ pathological processes open up widespread opportunities for the development of diverse, specific inhibitors for clinical application [1-3].

CAs catalyse a simple physiological reaction, the conversion of CO_2 to the bicarbonate ion and protons. The active site of most CAs contains a zinc ion (Zn^{2+}), which is essential for catalysis. The CA reaction is involved in many physiological and pathological processes, including respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis; bone resorption; calcification; and tumorigenicity [4-13].

Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis [14-19]. Given the

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physiological importance of the CA, the metabolic impact of chemicals for crop production should receive greater study. However, there isn't much inhibition study available on CA activity.

Many chemicals are currently used in agriculture, and plant growth regulators (PGRs) are among those widely used. The amount and variety of PGRs which include indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, and kinetin have increased tremendously in recent years. Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are naturally occurring plant growth regulators extensively studied by phytochemists (Figure 1a,b). IAA and IBA are major plant growth hormone of the auxin class, affecting cell enlargement, division, and differentiation [20]. It is metabolized by two different pathways; conjugation with a variety of amino acids, peptides, and sugars forming non-reactive conjugates and oxidation by peroxidases leading to produce a toxic series of intermediates which could be used as the basis of a novel cancer therapy [21-23] Gibberellic acid (GA_3) (Figure 1c) plays important roles in many cellular processes including promotes stem elongation, overcomes dormancy in seed and buds, involved in parthenocarpic fruit development, flowering, mobilization of food reserves in grass seed germination, juvenility, and sex expression [24]. The amounts of these substances placed into the environment may soon exceed those of insecticides [25]. Kinetin (Kn) (Figure 1d) was isolated 50 years ago for the first time as a plant hormone. So this hormone was used for a long time [26,27].

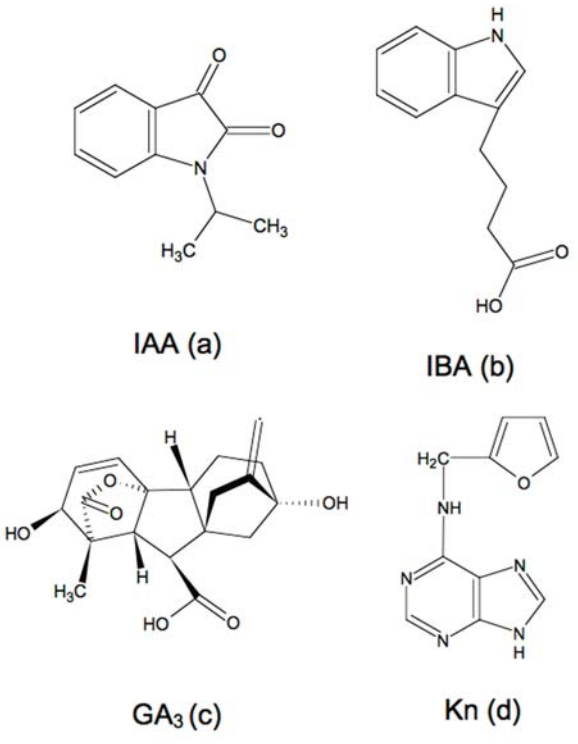

Figure 1. Structure of IAA (a), IBA (b), GA_3 (c), Kn (d).

In the literature, it is reported that IAA might induce the neuronal apoptosis in the S phase and lead to microencephaly [28]. Also, de Melo et al. determined that incubation for 24 h in the presence of IAA (1 mM) showed increase in the activities of SOD, CAT, and glutathione peroxidase [29]. John et al. observed that IAA possesses teratogenic effects in mice and rats [30]. In addition, it is found that, gibberellin A_3 induced liver neoplasm in Egyptian toads, and they suggested that the tumors could be diagnosed as hepatocellular carcinomas [31]. Ozmen et al. observed that abscisic acid and gibberellic acid affect on sexual differentiation and some physiological parameters of laboratory mice [32]. The effects of IAA and Kn were also investigated on human serum enzymes in vitro. IAA was found to inhibit aspartate aminotransferase and activate amylase, creatine phosphokinase and lactate dehydrogenase. Kn inhibited muscle creatine kinase while it activated aspartate aminotransferase and alanine aminotransferase [33]. Also, it was found that while the levels of LDH and CPK were increased significantly by IBA (indole butiric acid), the levels of AST, LDH, and CPK were increased significantly by IAA. In addition, the levels of AST, LDH, and CPK were increased significantly by kinetin [34]. Hsiao results suggest that kinetin has effective free radical-scavenging activity in vitro and antithrombotic activity in vivo [35]. On the other hand, some PGRs have been shown to affect the carbonic anhydrase isoenzymes of erythrocytes in humans and bovines [22].

Although PGRs are used for pest control and giving rise product on a wide variety of crops, little is known about the biochemical or physiological effects in mammalian organisms. Therefore, in this study in vitro inhibition of some important PGRs (indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, kinetin) hCA I and hCA II enzymes were evaluated in human.

MATERIALS AND METHODS

Materials

Sepharose 4B, L-tyrosine, sulfonamide, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck. Plant growth regulators were provided by the local pharmacy.

Preparation of Hemolysate

Blood samples were anticoagulated with ACD (Acid-citrate-dextrose) and centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was removed. The packed red cells were washed with NaCI (0.9%) three times and the erythrocytes were hemolysed with cold water. The ghost and intact cells were removed by centrifugation at 15000 rpm for 30 min at 4°C and the pH of the hemolysate was adjusted to 8.5 with solid Tris-base.

Carbonic Anhydrase Enzyme Assay

Carbonic anhydrase activity was measured by the Maren method which is based on determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO_2 hydration. Phenol red was added to the assay medium as the pH indicator, and the buffer was 0.5 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0). One unit of CA activity is defined as the amount of the enzyme that reduces by 50% the time of CO_2 hydration measured in the absence of enzyme. In the inhibition studies, the CO_2 concentration was 70 mM and five different inhibitor concentrations were used. IC_{50} values were calculated using computer regression analysis [36].

Total Protein Determination

The absorbance at 280 nm was used to monitor the protein in the column effluents. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [37] with bovine serum albumin as a standard.

Purification of Carbonic Anhydrase I and II by Affinity Chromatography

Human erythrocytes was isolated from 50 ml fresh human blood and put into anticoagulant tubes. The hemolysate was applied to an affinity column containing Sepharose-4B-L-tyrosine-sulfonamide [38,39] equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with 50 mL of 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.5). The human CA (hCA) isozymes were then eluted with 0.1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), which recovered hCA-I and hCA-II respectively. Fractions of 3 mL were collected and their absorbances measured at 280 nm.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed in order to verify the purified enzyme. It was carried out in 12% and 3% acrylamide concentrations for the running and stacking gel respectively, containing 0.1% SDS according to Laemmli et al. [40].

In Vitro Inhibition Kinetic Studies

For the inhibition studies of indole-3-acetic acid, indole-3-butyric acid, kinetin and gibberellic acid different concentrations of plant growth regulators were added to the enzyme activity. The Maren method [41] is based on determination of the time required for the pH of a standard solution to decrease from 10.0 to 7.4 due to CO_2 hydration.

The assay solution was 0.5 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0) and Phenol Red was added as the pH indicator. One unit of CA activity is defined as that amount of the enzyme that reduces by 50 % the time of CO_2 hydration measured in the absence of enzyme.

Activity % values of carbonic anhydrase for different concentrations of each plant growth regulators were determined by regression analysis using Microsoft Office 2000 Excel. Carbonic anhydrase enzyme activity without a plant growth regulator was accepted as 100% activity. For the regulators having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs.

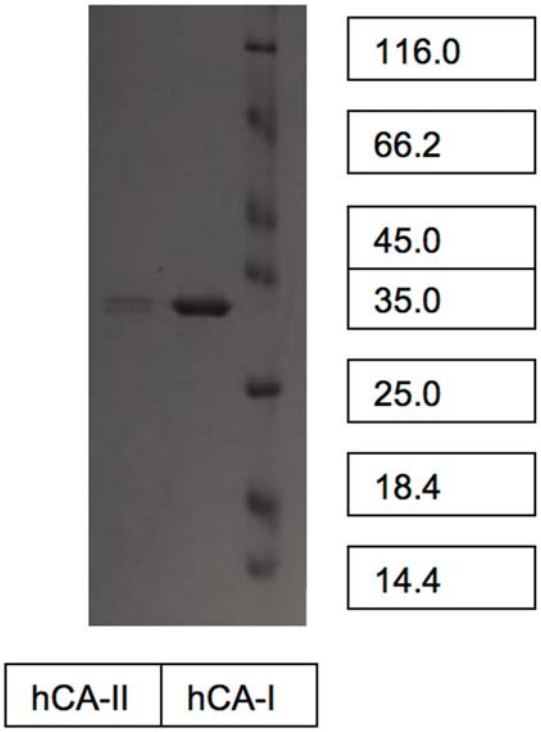

Figure 2. SDS-PAGE of carbonic anhydrase. The poled fractions from an affinity chromatography (Sepharose-4B-L-tyrosine-sulfonamide) was analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 contained 5 µl of various molecular mass standards: β-galactoxidase, (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase, (35.0), restriction endonuclease (25.0), β-lactoglobulin (18.4), lysozyme (14.4).

RESULTS

The inhibitory effects of some commonly used PGRs namely, indole-3-acetic acid, indole-3-butyric acid, gibberellic acid, and kinetin on human cytosolic carbonic anhydrase I and II activity were investigated. Cytosolic carbonic anhydrase I and II was purified by using the affinity gel [42]. The purity of the enyzmes was confirmed with SDS gel electrophoresis (Figure 2). Carbonic anhydrase activity was measrued by the Maren method [41].

Inhibition graphs, using the PGRs with concentrations as described in Section 2, is shown in Figure 3. Different inhibition effects of the applied PGRs were obtained. IC_{50} values of the inhibited by indole-3-acetic acid, indole-3-butyric acid and kinetin was found to be 78.13 µM, 54.48 µM, 62.89 µM for hCAI, respectively. IC_{50} values of the inhibited by indole-3-acetic acid and indole-3-butyric acid was found to be 75.47 µM, 38.05 µM for hCAII, respectively. The auxin hormone indole-3- butyric acid has been shown to be the strongest inhibitor against the hCAI and hCAII activity (Figure 3b). Conversely, kinetin was considerably stimulated the hCA II activity but hCAI was inhibited by this PGR at the applied concentrations (Figure 3c). Gibberellic acid considerably stimulated the hCAI and hCA II activity (Figure 3d). hCAI and hCAII were inhibited by Indole-3-acetic acid at the applied concentrations too (Figure 3a).

DISCUSSION

The amount and variety of PGRs used have increased tremendously in recent years. This increase caused a positive effect on crop production, however, certain regulators, their residues, metabolites and/or contaminants have created many unforeseen adverse effects on the environment. Under some conditions, PGRs may be present in very low concentrations which have no

immediate detectable effect. These small amounts of chemicals can cause sublethal damage to organisms and this is more insidious and difficult to define than ocute toxicity. Sublethal effects may be further enhanced by persistent PGRs which are accumulated in the organisms and magnified in the food chain. In this study, IAA, IBA, GA_3, and Kn were preferred because there is no information about its' side effects on hCAI and hCAII. Choosen which used in this study, PGRs are found in plants as endogen hormones and wide variety of biologically active compounds.

We found that activity of hCAI was inhibited by IAA, IBA, and Kn. Conversely, the activity of hCAI was stimulated by GA_3. Activity of hCAII was inhibited by IAA and IBA but it was stimulated by GA_3 and Kn. Many reports have been supported to other enzyme studies. For example, it is reported that, some serum enzymes namely aspartate aminotransferase, amilase, creatine phosphokinase, and lactate dehydrogenase were inhibited by IAA [19]. As similar *in vitro* data, it was found that glutathion peroxidase and catalase was strongly inhibited by IAA and kinetin [20]. In addition, *in vivo* reports showed that IAA has inhibition effects on glutathion reductase and glutathion peroxidase [21]. The CA reaction is involved in many physiological and pathological processes; particularly biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis; bone resorption; calcification; and tumorigenicity [4-13].

IAA and IBA are major plant growth hormones of the auxin class, affecting cell enlargement, division, and differentiation. Although this regulators indispencible for plant growth, its showed an inhibition effects on hCAI and hCAII which were an important physiological role of organism. Also Kn has inhibition effect on hCAI activity to other regulators. It is

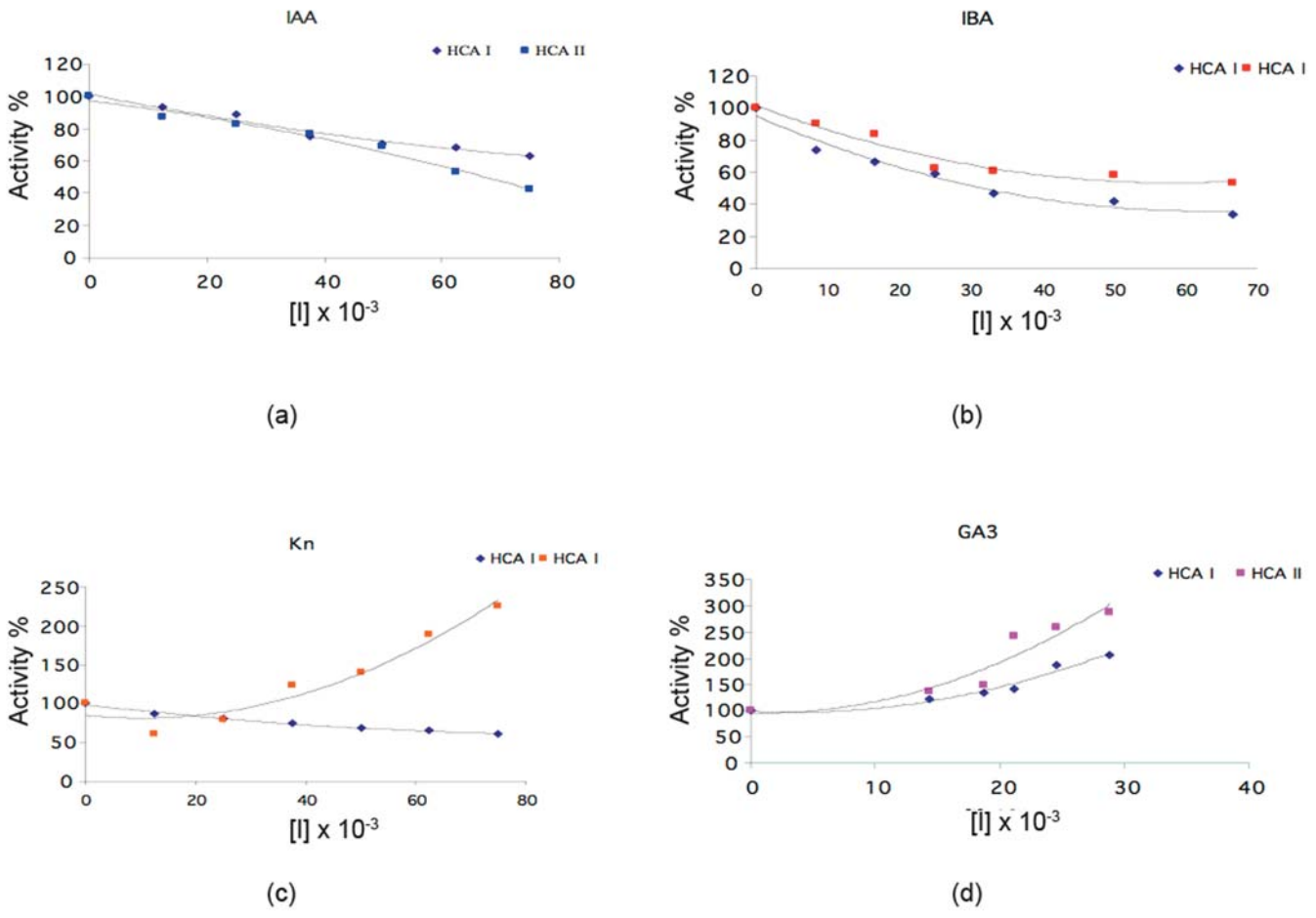

Figure 3. Activity (%) curves of hCAI and hCAII in different IAA,IBA, GA_3, Kn concentrations.

reported that, GA_3 significantly decreased serum aspartate aminotransferase, creatine phospho-kinase, and lactate dehydrogenase. Antioxidant enzyme activities such as superoxide dismutase significantly decreased in the erythrocyte, liver and brain tissue of rats with GA_3 [24]. We determined that, hCAI and hCAII activity were stimulated by GA_3.

As different from other PGRs, Kn is the first and best known regulators. We determined that, hCAII activity were stimulated by kinetin. Kn is an anti-oxidant both *in vitro* and *in vivo*. It protects DNA against oxidative damage to 8-oxo-dG mediated by the Fenton reaction [32]. Also Kn acts as a strong inhibitor of oxidative and glycoxidative protein-damage generated *in vitro* [13]. However, another study showed that, exposure Kn can result toxicological effects in vertebrates. It was found that, glutathion-S-transferase and catalase were inhibited by Kn in vivo [20]. In another study showed that, glutathion reductase, glutathion peroxidase, and adenosine deaminase were significantly reduced with Kn [21].

Conclusion, the aim of this study is to define the effects of these plant hormones on cytosolic carbonic anhydrase I and II and thus evaluate the environmental and toxicological effects of these compounds in vitro. Although, PGRs being a major component of the growing process in plants and caused a positive effect on crop production, its dramatically inhibit the hCAI and hCAII. These findings are important, because CA catalyzes critically important physiological reactions. Even though they are consumed in small amounts, these three plant growth regulators can still affect hCAI and hCAII. Concequently, the inappropriate use of PGRs is potentially a risk to human health and they are also magnified in the food chain.

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