The Effects of Ciglitazone on Enzyme Activities of Carbonic Anhydrase II and Glucose-6-Phosphate Dehydrogenase

Ciglitazonun Karbonik Anhidraz II ve Glukoz-6-Fosfat Dehidrogenaz Enzim Aktiviteleri Üzerine Etkileri

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ABSTRACT

Ciglitazone is a compound belonging to the thiazolidinedione (TZD) class and has hypoglycemic effects. Although ciglitazone is not used as a drug, it has been used as a template for the development of other TZD group drugs. Like many TZD group drugs, ciglitazone causes anemia. In this study, the effects of ciglitazone on the enzyme activities of Glucose-6-Phosphate Dehydrogenase (G6PD) and Carbonic Anhydrase II (CA II), which have important functions in maintaining erythrocyte functions and integrity, were investigated *in vitro*. Pure human erythrocyte CA II and G6PD enzymes were used for activity measurements. Both esterase and hydratase activities were measured for CA II. The result of activity measurements, it was observed that ciglitazone inhibited CA II (IC_{50} =0.0063 mM for hydratase activity, and IC_{50} =0.047 mM for esterase activity) and G6PD (IC_{50} = 0.067 mM) activities. As a result, it was concluded that these enzyme inhibitions may be important in the anemia-causing mechanisms of cigitazone.

Keywords: Anemia, Carbonic Anhydrase II, Ciglitazone, Glucose-6-Phosphate Dehydrogenase, PPARγ

ÖZ

Ciglitazone thiazolidinedione (TZD) sınıfına ait bir bileşiktir ve hipoglisemik etkilere sahiptir. Ciglitazone bir ilaç olarak kullanılmamasına rağmen, diğer TZD grubu ilaçların gelişmesi için bir kalıp olarak kullanılmıştır. Pek çok TZD grubu ilaç gibi ciglitazone da anemiye sebep olmaktadır. Bu çalışmada eritrosit fonksiyonlarının ve bütünlüğünün sürdürülmesinde önemli fonksiyonlara sahip olan Glukoz-6-Fosfat Dehidrogenaz (G6PD) ve Karbonik Anhidraz II (CA II) enzim aktiviteleri üzerine ciglitazonun etkileri *in vitro* olarak incelendi. Aktivite ölçümlerini için saf insan eritrosit CA II ve G6PD enzimleri kullanıldı. CAII için hem esteraz hem de hidrataz aktiviteleri ölçüldü. Aktivite ölçümleri sonucunda, ciglitazone'un CA II aktivitesini (hidrataz aktivitesi için IC_{50} =0.0063 mM ve esteraz aktivitesi için IC_{50} =0.047 mM) ve G6PD (IC50= 0.067 mM) aktivitesini inhibe ettiği gözlendi. Sonuç olarak bu enzim inhibisyonlarının, ciglitazone'un anemi oluşturma mekanizmalarında önemli olabileceği kanaatine varıldı.

Anahtar Kelimeler: Anemi, Ciglitazone, Glukoz-6-Fosfat Dehidrogenaz, Karbonik Anhidraz II, PPARγ

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INTRODUCTION

Carbonic anhydrases (CA, EC 4.2.1.1) are metalloenzyme with zinc ion in its active site and sixteen separate isoenzymes of CA have been found in mammals. Tissue distribution and intracellular location of the isoenzymes shows remarkable differences. CAs are responsible the reversible hydration of CO_2 to bicarbonate and proton.[1] Along with this reaction; CAs play important role in CO_2 transport, pH homeostasis, electrolyte secretion in tissues and organs, and many biosynthesis reactions (gluconeogenesis, lipogenesis, urea cycle, etc.).[2] Due to their critical involvement in the regulation of mentioned physiological processes, CAs played a major role in the pathophysiology of various diseases like glaucoma, renal tubular acidosis, osteoporosis, neuropathic pain, colorectal cancer and hemolytic anemia.[3]

CA I and II are cytosolic enzymes and those are the most abundant proteins in erythrocytes after hemoglobin. Although CA II has the highest catalytic activity among all other isoenzymes, both of these isoforms play a crucial role in maintaining the physiological pH of human blood by producing the bicarbonate ion. A defect in these enzymes makes it difficult for erythrocytes to maintain their integrity and functions.[4]

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) is control enzyme of the pentose phosphate pathway, catalyses reaction in which $NADP^+$ is reduced to NADPH. G6PD is mostly found in the cytoplasm, also in various organelles such as peroxisome, endoplasmic reticulum, lysosome, chloroplast, and mitochondria.[5] NADPH is vital for protecting the cell from oxidative stress caused by free radicals on many molecules, such as nucleic acids, proteins, and membrane lipids. In erythrocytes, the source of NADPH is only pentose phosphate pathway. This molecule has a vital role in reducing glutathione, which is crucial in antioxidant defense in red blood cells.[6]

G6PD deficiency is one of the most common metabolic diseases in the world. In these patients, due to NADPH deficiency, over production of free radicals as consequence of certain drugs, food, or infection. Elevated free radicals in turn cause impaired erythrocyte membranes and oxygen delivery as well as aging of erythrocytes and cell all of which may lead to life-threatening hemolytic anemia.[7,8] Ciglitazone (5-[4-(1-methylcyclohexylmethoxy)benzyl]thiazolidine-2,4-dione) is an antidiabetic agent belonging to the thiazolidinediones (TZDs).[9] Ciglitazone is one of the agonists of peroxisome proliferator activated receptor gamma (PPARγ).[10] These agonists increase insulin sensitivity, thereby reducing blood glucose levels in patients with insulin resistance.[11] But side effects such as hepatotoxicity, fluid retention, haemodilution and increased cardiovascular disease risk have been limited the use of the compounds.[12] Another side effect of TZDs is anemia.[13] Although the exact mechanism is not elucidated, it has been suggested that eryptosis may be effective in the process.[14] Studies have focused on metabolic processes that TZDs can change via affecting gene expression, however, enzyme inhibition or activation specific to the chemical structures of these drugs have been conducted in limited number of studies to date.[15,16]

In this study, the effects of ciglitazone on activities of G6PD and CA II enzymes, which are important for the function and integrity of erythrocytes, have been investigated *in vitro*.

MATERIALS AND METHODS

Chemicals

Commercial human erythrocytes CA II was used from Sigma and G6PD enzyme was provided by the Chemistry Department of Atatürk University. Glucose-6-Phosphate (G6P), Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$), p-Nitrophenyl acetate, Vancomycin, Acetazolamide and Ciglitazone were purchased from Sigma.

CA II Hydratase Activity

Hydratase activity of carbonic anhydrase enzyme was measured by Wilbur-Anderson method.[17] CA II (0.1 mL) and 0.1 mL of 10% DMSO (since ciglitazone was also dissolved in DMSO) added to 2.6 mL HEPES buffer (25 mM, pH = 8.80). Then 2.2 mL of saturated CO_2 solution was added as substrate. For blank measurements the assay was repeated without enzyme. Different concentrations of ciglitazone (0.04, 0.02 and 0.01 mM) were used inhibition experimentals. The same procedures were repeated with acetazolamide, which is a potent CA II inhibitor. The decrease of pH from 8.20 to 7.00 in CO_2 hydration was followed by a pH meter and the elapsed time was measured. Activity measurements were performed in ice. Each measurement was repeated three times and average values were calculated. The enzyme reaction time (t_c) was subtracted from the enzyme-free CO_2 hydration time (t_0) and the result was divided by tc. Thereby enzyme unit was calculated. One enzyme unit (U) of CO_2 hydratase activity was calculated using the equation; $EU=(t_0-t_c)/t_c$

CA II Esterase Activity

CA esterase activity was measured according to the method developed by Armstrong.[18] One enzyme unit of CA II esterase activity is defined as hydrolysis of 1 μmol p-nitrophenyl acetate to p-nitrophenol and acetate in 1 min. In the method, carbonic anhydrase hydrolyzes p-nitrophenyl acetate to p-nitrophenol and acetate and the absorbance of p-nitrophenol is measured spectrophotometrically at 348 nm. For CA II activity, 1.2 mL of Tris-SO_4 buffer (0.05 M, pH=7.00), 0.3 mL of CA II and 0.3 mL of water were added to the test tubes and mixed thoroughly at room temperature. Then 1.2 mL of p-nitrophenyl acetate solution (3 mM) was added and the mixture was transferred to quartz cuvette. Absorbance was read at 348 nm for 3 minutes. The difference between the absorbances read at the beginning and at the end of 3 minutes was determined as the esterase activity. For blank measurements, this was repeated with different concentrations of ciglitazone (0.05 mM, 0.025 mM, and 0.0125 mM) and without enzyme. The same procedures were applied for acetazolamide

Glucose-6-Phosphate Dehydrogenase Activity

G6PD enzyme activity was measured according to the Beutler method.[19] The method is based on the conversion of G6P into 6-phosphoglucolactone in the presence of G6PD. It is based on the principle of following the absorbance change of the reduced $NADP^+$ at 340 nm, 37°C for a certain time (4 minutes). Briefly, 100 μL of Tris buffer (pH=7.4, 0.5 M), 100 μL of $MgCl_2$ (0.63 M), 100 μL of $NADP^+$ (3.8 mM), 50 μL of enzyme solution, 550 μL pure water and 100 μL G6P (33 mM) were mixed and incubated at 37°C for 10 min. Then absorbance was read at 340 nm. Different concentrations of ciglitazone (0.15 mM, 0.075 mM, and 0.0375 mM) were used inhibition experimentals. For blank measurements the assay was repeated without enzyme. The same procedures were performed with Vancomycin which is the G6PD inhibitor.

RESULTS AND DISCUSSION

Effect of Ciglitazone on CA II Hydratase Activity

The *in vitro* effect of ciglitazone and acetazolamide on CA II esterase activity was determined using different concentrations of ciglitazone and acetazolamide solution. Our results showed that ciglitazone inhibited hydratase activity. It was observed that ciglitazone caused a weaker inhibition compared to the acetazolamide used to test the accuracy of the experiments (IC_{50}= 0.0063 mM and 0.00044 mM, respectively). Inhibition graph and IC_{50} value are given in Figure 1 for ciglitazone.

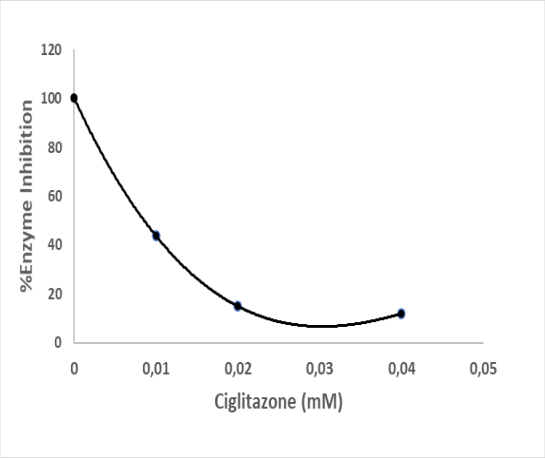

Figure 1. Inhibition rates of ciglitazone on CA II hydratase activity (IC_{50} = 0.0063 mM).

Effect of Ciglitazone on CA II Esterase Activity

The *in vitro* effect of ciglitazone and acetazolamide on CA II esterase activity was determined using different concentrations of inhibitor solution. It was observed that ciglitazone inhibited esterase activity less than hydratase activity (IC_{50} = 0.047 mM and 0.0063 mM, respectively) Inhibition graph and IC_{50} value are given in Figure 2 for ciglitazone.

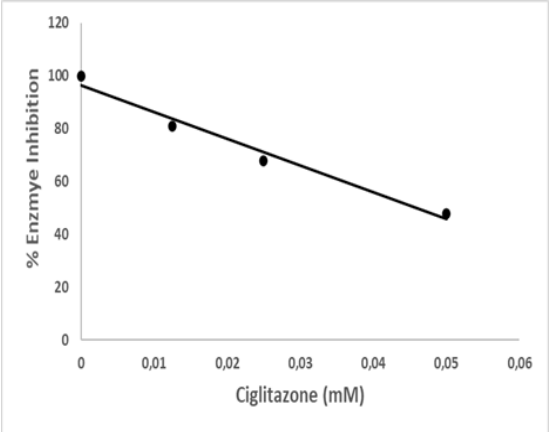

Figure 2. Inhibition rates of ciglitazone on CA II esterase activity (IC_{50} = 0.047 mM).

Effect of Ciglitazone Glucose-6-Phosphate Dehydrogenase Enzyme Activity

The *in vitro* effect of ciglitazone on G6PD enzyme activity was determined by adding different concentrations of ciglitazone solution to the experimental medium in certain volumes. Vancomycin, shown to inhibit the enzyme, was used as a control and to compare the efficacy of ciglitazone. From the results obtained, it was observed that ciglitazone produced a weaker inhibition than Vancomycin (IC_{50} = 0.067 mM and 0.037 mM, respectively). Inhibition graph and IC_{50} value are given in Figure 3 for ciglitazone.

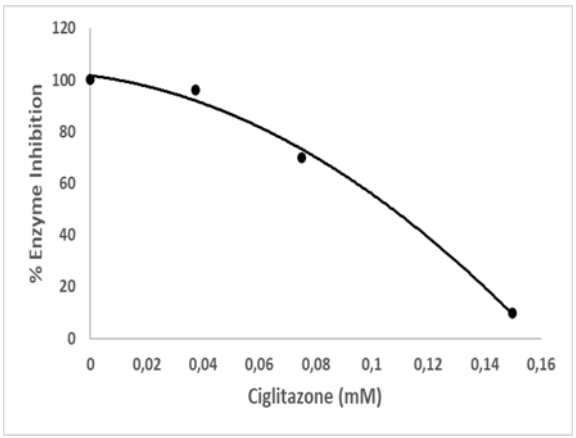

Figure 3. Inhibition rates of ciglitazone on G6PD enzyme activity (IC_{50} = 0.067 mM).

Discussions

Drugs belong to the TZDs are used in treatment insulin resistant and type 2 DM due to their hypoglycemic effects**.** Ciglitazone is a compound that incidentally found a hypoglycemic effect during hypolipidemic drug research by Takada Ltd in 1982. Although ciglitazone was the first TZDs to be discovered, it was not used commercially due to its side effects and its antidiabetic activity lower than the TZDs developed later. However, it has been used in many studies as a reference in the examination and evaluation of the effectiveness of TZDs.[20]

Studies investigated the anemia-causing properties of TZDs, especially the suppression of blood production in the bone marrow at the gene level. But no conclusion has been reached showing that these drugs have an effect on the bone marrow.[21] In a study examining the effects of ciglitazone and prostaglandin J on erythrocyte death (eryptosis), it was reported that the amount of Ca^{2+} in the erythrocyte increased in the ciglitazone applied group and triggered cell death. However, since these cells do not have nuclei, it has been suggested that some metabolic pathways important in its integrity are affected by ciglitazone independently from PPAR.[14]

Erythrocytes cannot respond to changes in their environment at the gene level due to the absence of nuclei. Compounds affecting enzyme activities cause problems in performing the functions of erythrocytes and maintaining cell integrity.[22] So the effects of ciglitazone on activities of G6PD and CA II enzymes, which are important for the function and integrity of erythrocytes, were investigated *in vitro*.

The effect of ciglitazone on CA II purified from human erythrocytes on both hydratase and esterase activities is shown in Figure 1 and 2. Ciglitazone inhibited the activities of both enzymes. The inhibition on hydratase activity (IC_{50} = 0.0063 mM) is stronger than the inhibition on the esterase activity (IC_{50} = 0.047 mM). In studies about CA inhibition, acetazolamide a potent CA inhibitor, is used generally. When the inhibitors effect of ciglitazone compared with acetazolamide, inhibition effect of ciglitazone on CA II hydratase acitiviy is 10-fold lower (IC_{50} = 0.00044 mM for acetazolamide) and on CA II esterase activity is 100-fold lower (IC_{50} for acetazolamide = 0.00064 mM) according to the acetazolamide. Therefore, the inhibition by ciglitazone may be important in evaluating the functions of CA II. In erythrocytes CO_2 is transformed and transported by HCO_3^- and excreted in the lungs by CA II. Besides combining with various membrane proteins, CA II regulates ion passage through the erythrocyte membranes and acid-base balance. Inhibition of these functions may impair the functioning of erythrocytes and cell integrity.[23,24] Although it has no physiological significance, measurement of esterase activity of CA, has been a method preferred by researchers in many kinetic studies because it is a spectrophotometric method and its high reproducibility.[25] In this study, it was observed that ciglitazone inhibits the esterase activity of CA II. However, the IC_{50} values obtained are approximately 10-fold different from each other (IC_{50} = 0.0063 mM for hydratase activity, IC_{50} = 0.047 mM for esterase activity). This result can be attributed to the different reaction mechanisms and substrate binding sites of both enzymes.

In erythrocytes G6PD is the only source of NADPH used as reducing power. As an antioxidant molecule, NADPH is very important in preserving the integrity of the erythrocyte membrane and its functions. The decrease in the amount of NADPH makes the erythrocytes more fragile and susceptible to anemia.[26] The effects of ciglitazone on the G6PD enzyme purified from human erythrocytes are given in Figure 3. According to these results, ciglitazone inhibited G6PD activity. Compared to vancomycin (IC_{50} = 0.037 mM), ciglitazone (IC_{50} = 0.067 mM) is a weaker inhibitor. G6PD deficiency is the most common enzyme deficiency in the world. One of the symptoms seen due to this enzyme deficiency is hemolytic anemia.

In most cases of G6PD deficiency, an absolute enzyme deficiency is replaced by a mutant enzyme that lacks a certain amount of activity. Enzyme deficiency is usually seen

when foreign materials taken from outside inhibit the enzyme. Especially with antibiotics, anti-malaria drugs, antipyretics, and ingestion of fava plant G6PD deficiency is manifested seriously.[27] Investigating the effects of drugs on G6PD is important for regulating the use of related drugs in people with this enzyme deficiency. Although ciglitazone is not used as a drug, inhibition of G6PD related anemia by ciglitazone is an important finding. These results are valuable in terms of revealing a target enzyme for anemia observed in the use of TZDs.

CONCLUSION AND RECOMMENDATIONS

In conclusion, ciglitazone has an inhibitory effect on CA II and G6PD which is responsible for erythrocyte function and integrity, and that the anemia observed in the use of TZDs may be due to the inhibition of these enzymes. The effects of other TZDs on these enzymes can be examined. The effect of ciglitazone under similar conditions can be investigated with the same enzymes *in vivo*.

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