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EFFECTS OF TIMOLOL TREATMENT ON PANCREATIC ANTIOXIDANT ENZYMES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS: AN EXPERIMENTAL AND COMPUTATIONAL STUDY

EFEKTI TRETMANA TIMOLOLOM NA ANTIOKSIDANSNE ENZIME PANKREASA PACOVA SA DIJABETESOM IZAZVANIM PUTEM STREPTOZOTOCINA: EKSPERIMENTALNA I RAČUNSKA STUDIJA

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Summary

Background: The study aimed to investigate whether timolol-treatment has a beneficial effect on pentose phosphate pathway enzyme activities such as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGDH) enzyme activities and cAMP level in streptozotocin-induced diabetic rats in pancreatic tissues
Methods: Diabetes was induced by streptozotocin (STZ) in 3-month old male Wistar rats. The diabetic rats were treated with timolol (5 mg/kg body weight, for 12 weeks) while the control group received saline. Enzyme activities were determined in pancreas tissue. To support our results, we performed *in silico* calculations, using Protein Data Bank structures.
Results: Timolol treatment of STZ-induced diabetic rats had no noteworthy effect on high blood-glucose levels. However, this treatment induced activities of G6PD and 6PGDH in diabetic rats. Timolol treatment significantly increased cAMP level in diabetic pancreatic tissue. We found that timolol cannot bind strongly to either G6PD or 6PGD, but there is a relatively higher binding affinity to adenylyl cyclase, responsible for cAMP production, serving as a regulatory signal via specific cAMP-binding proteins.

Kratak sadržaj

Uvod: Cilj istraživanja je bio da se utvrdi da li tretman timololom ima pozitivan efekat na aktivnosti enzima pentoze fosfata, kao što su aktivnosti glukoze-6-fosfat dehidrogenaze (G6PD), enzimske aktivnosti 6-fosfoglukonat dehidrogenaze i cAMP nivo u tkivu pankreasa kod pacova kojima je dijabetes izazvan streptozotocionom.
Metode: Dijabetes je izazvan streptozotocionom (STZ) kod tromesečnih mužjaka vistar pacova. Pacovi sa dijabetesom su tretirani timololom (5 mg/kg telesne težine tokom 12 nedelja), dok je kontrolna grupa primila fiziološki rastvor. Enzimske aktivnosti su utvrđivane u tkivu pankreasa. Da bismo potkrepili naše rezultate, sproveli smo *in silico* računanja koristeći strukture Proteinske baze podataka.
Rezultati: Tretman timololom na pacovima kojima je dijabetes izazvan putem STZ-a nije imao značajan uticaj na visoke nivoe glukoze u krvi. Međutim, kod takvih pacova ovaj tretman je indukovao aktivnosti G6PD i 6PGDH. Lečenje timololom značajno je povećalo nivo cAMP-a u dijabetičnom tkivu pankreasa. Utvrdili smo da se timolol ne može snažno vezati ni za G6PD, ni za 6PGD, ali da postoji relativno veći afinitet vezivanja za adenilil ciklazu, odgovornu za proizvodnju cAMP, koja služi kao regulatorni signal putem određenih cAMP vezivnih proteina.

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List of abbreviations: G6PD, Glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; AC, Adenylyl cyclase; STZ, Streptozotocin; β-AR, Beta-adrenergic receptor; cAMP, Cyclic adenosine monophosphate.

Conclusions: Our data point out that timolol treatment has beneficial effects on the antioxidant defence mechanism enzymes in the pancreas of STZ-induced diabetic rats.

Keywords: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, cAMP, diabetes, timolol

Zaključak: Naši podaci ukazuju da tretman timololom ima pozitivne efekte na antioksidantne enzime odbrambenog sistema u pankreasu pacova sa dijebetesom izazvanim putem STZ-a.

Ključne reči: glukoza-6-fosfat dehidrogenaza, 6-fosfoglukonat dehidrogenaza, cAMP, dijabetes, timolol

Introduction

Diabetes mellitus (DM), characterised by hyperglycemia, is the most common life-threatening disease among the group of chronic metabolic disorders. There is undoubtedly a tight interconnection between carbohydrate, lipid, and protein metabolism in diabetes (1, 2). High glucose plays a major role in the initiation of various physiological and cellular-structural and metabolic alterations, including the production of glycation end products, abnormalities of signalling cascades, overproduction of endothelial growth factors, chronic inflammation and elevated production of reactive oxygen species (ROS). The decrease in antioxidant defence mechanisms causes abnormally high levels of free radicals, which causes damage to cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance (3, 4). The mechanisms of progressive decline of function and loss of β-cells in type 2 diabetes are not entirely understood (5).

Insulin deficiency causes a range of complications, including a significant global increase in chronic pancreatic disorders. Numerous studies demonstrate that hyperglycemia generates more ROS and attenuates antioxidative mechanisms and that hyperglycemia-induced oxidative stress plays a major role in the extracellular matrix expansion. Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) is the principal source of the major intracellular reductant – NADPH which is crucial for cellular processes such as redox regulation. G6PD is directly regulated by various negative regulators: cAMP, p53, TNFα, AMP kinase and positive insulin, phospholipase C-γ, EGF cellular signals (6–9). An up-regulation in G6PD is involved in insulin resistance via NADPH due to imbalanced energy metabolism and oxidative stress (5, 6, 10, 11). Another principal source of NADPH is 6-phosphogluconic acid dehydrogenase (6PGDH) (EC 1.1.1.44), which generates ribose-5-phosphate (R5P) and this molecule has important roles in the physiological regulation of the AMP-activated protein kinase pathway. Additionally, it is known that AMPK is phosphorylated and activated under certain cellular stress conditions, such as energy deprivation, low nutrient-condition modulation to adaptive changes and maintained metabolic homeostasis (12, 13).

Several animal studies focused on the target organs and tried to establish the specific organ damage and antioxidant enzyme activities organs. These pieces of research have demonstrated that there is a correlation between diabetes and the elevation of oxidative stress. This may be due to increased production of oxidants and decreased G6PD, 6PGD and other antioxidant enzyme activities in streptozotocin-induced diabetic rats (14–16). Adverse effects of diabetes on G6PD and 6PGDH have been widely studied in various laboratory animals. Stanton and his colleagues have shown that G6PD and NADPH play central roles in β-cell survival and the decreased level of NADPH is an important and a key cause of the increased oxidative stress. The mechanism behind diabetes and elevated ROS levels in different tissues may lead to various diseases and other complication (17). Another intracellular signalling pathway, also associated partly with intracellular oxidative stress, is β-adrenoceptor (β-AR) signalling, which includes adenylyl cyclases (ACs), an enzyme with key regulatory roles in essentially all cells and it is one of the key molecules in glucose metabolism. ACs catalyse the synthesis of the signalling molecule cAMP, which is an important second messenger and plays a prominent role in insulin secretion from β-cells of pancreatic islets and is implicated as a therapeutic target for diabetes (18–20) as well. In our previous studies, we showed that a nonspecific β-AR blocker timolol treatment of diabetic rats presented a marked protective action in cardiovascular system disorders and kidney damage, in part, due to the prevention of endoplasmic reticulum stress, similar to the action of known antioxidants (21, 22).

Timolol is used as a novel drug to treat various health problems such as antihypertensive, antiarrhythmic, antianginal, and antiglaucoma agent while no significant side effects are documented on mam-

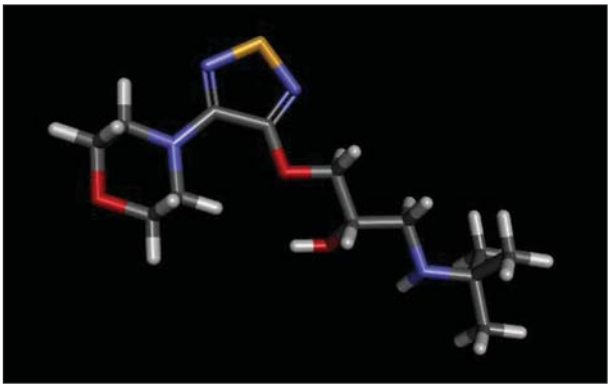

Figure 1 Scheme of timolol (from ref 21).

malian pancreas till now. Even in early studies, timolol was used in treatment after myocardial infarction in diabetic patients, migraine and disorders such as tremor. Its chemical structure is given in *Figure 1* (20–25). Taken into consideration its multifunctional actions, herein we aimed to investigate first the effects of timolol treatment on pentose phosphate pathway enzyme activities together with the cAMP level in STZ-induced diabetic rat pancreas tissue. Second, in order to demonstrate possible underlying mechanisms associated with its actions in tissues, we also performed an *in silico* analysis of timolol-binding to G6PD, 6PGD and AC, which is an enzyme responsible for cAMP production serving as a regulatory signal via specific cAMP-binding proteins, due to the process of cAMP on its controlling role in many biochemical and physiological processes.

Materials and Methods

Chemicals

Glucose 6-phosphate (G6P), 6-phosphogluconic acid (6PGD), nicotinamide adenine dinucleotide phosphate ($NADP^+$), magnesium chloride ($MgCl_2$), Tris [Tris (hydroxymethyl) aminomethane], BCA reagents were obtained from Sigma Chemical (St. Louis, MO, USA). For cAMP, we used Cayman Cyclic AMP Select ELISA Kit Item No: 501040. All other chemicals were of analytical grade and obtained from Sigma, USA.

Induction of diabetes in rats

All animal care and experimental procedure were performed by following Ankara University ethics guidelines (No: 108-403). The experimental diabetic animal procedures, including timolol-treatment, were performed as described previously. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (50 mg/kg body weight and dissolved in 0.1 mol/L citrate buffer, pH 4.5) in 3-month-old adult male Wistar rats (200–250 g body wt) (22). Rats with a blood glucose level >3 fold of controls were kept as diabetics (DM group) and tail vein fasting blood glucose levels were measured using a glucose analyser (Glucotrend, Roche). STZ-induced diabetic rats were divided into two groups: the untreated diabetic rats and the diabetic rats treated with timolol (DM + TIM). Timolol dissolved in tap water and administered intragastrically each morning for 12 weeks (5 mg/kg body weight). We used 10 rats in each group. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996). The protocol was approved by The University Ankara (AU.11-38). For biochemical analysis, samples of pancreas tissue were stored at -80 °C till use.

Tissue homogenization

The pancreas samples were homogenised with an Ultraturrax basic IKA T18 homogeniser (22,000 rpm/min with an S18N-10G probe for approximately 3 min with 3 volumes of 10 mmol/L Tris/HCl buffer containing 1 mmol/L EDTA and 1 mmol/L 2-mercaptoethanol at pH 7.6. After homogenization, samples were centrifuged in an Eppendorf centrifuge (5417 R) with 20,800 rcf for 50 min at 4 °C. Final supernatants were used for the measurement of enzyme activities and protein concentrations. Enzyme activities were determined spectrophotometrically using a UV-VIS Spectrophotometer (Ultraspec 2100 Pro Amersham Biosciences).

Protein determination

The protein concentrations indicated in this study were determined using a bicinchoninic acid protein assay (catalogue no. B9643, Sigma) with BSA (as a standard). We determined protein concentrations by comparison of the absorbance of the unknown samples to the standard curve prepared using the BSA protein standards. (26).

Glucose-6-phosphate dehydrogenase assay

G6PD activity was determined by monitoring NADPH production at 340 nm and 37 °C (27). The assay mixture contained 10 mmol/L $MgCl_2$, 0.2 mmol/L $NADP^+$ and 0.6 mmol/L G6P in 100 mmol/L Tris/HCl buffer, pH 8.0. Assays were performed in duplicate and activities were followed for 60 s. The reaction was linear during that time. One unit (U) of activity is the amount of G6PD required to reduce one μmol of $NADP^+$ per min under the assay conditions.

6-Phosphogluconate dehydrogenase assay

6-Phosphogluconate dehydrogenase activity was measured by substituting 0.6 mmol/L 6-PGA as a substrate in the assay mixture given above for G6PD measurement (28). One unit (U) of activity is the amount of 6PGDH required to reduce one μmol of $NADP^+$ per min under the assay conditions. Specific activity is defined as units per mg of protein.

cAMP assay

cAMP contents of tissue samples determined with Cayman Cyclic AMP Select ELISA Kit, Item No 501040. 1 volume of the supernatant was diluted with 1 volume of %5 trichloroacetic acid (TCA), and then the procedures were applied according to manufacturer's protocol.

Data analysis and statistics

Data are presented as mean ± SEM unless otherwise stated. Differences were determined by using Student's t-test with Bonferroni correction for multiple comparisons as required, using GraphPad Prism 6.0. P-values < 0.05 were considered as statistically significant.

Results

General parameters of rats

During experiments, we did not have animal mortality either in the streptozotocin (STZ) treated (DM group) or in timolol treated one (DM+TIM group). STZ-injected rats displayed hyperglycemia as indicated by significant increases in blood glucose levels compared with age-matched controls (21.1 ± 0.8 mmol/L vs 5 ± 0.6 mmol/L). Timolol treatment had no significant effect on high-blood glucose level (1.137 ± 0.06 mmol/L). However, it improved some STZ-induced diabetic symptoms, including a reduction in weight loss, to a small but a statistically significant level ($p<0.05$), although the weight of rats remained less than those of the controls (21, 22).

The effect of timolol treatment on the pentose phosphate pathway in pancreatic tissue of STZ-induced diabetic rats

We examined the effect of timolol treatment on G6PD and 6PGDH. As can be seen from the bar graphs given in F*igures 2A* and *3A*, STZ-induced diabetes caused significant decreases in the activities of these two enzymes in pancreatic tissue. Our data showed that G6PD and 6PGDH levels decreased in the diabetic animal groups compared to the control group as it can be seen from the bar graphs in *Figures 2 A* and *3A*. We have also compared our results with the ones of the diabetic rats treated with timolol. The significance levels: *P<0.001 vs control group, ¡P<0.01 vs diabetic group. We have demonstrated that timolol treatment of STZ-induced diabetic rats induced depressed activities of G6PD and 6PGDH in pancreatic tissue from STZ-induced diabetic rats. The generation of NADPH G6PD and 6PGDH in pancreatic tissue from STZ-induced diabetic rats is essential for protection against oxidative stress.

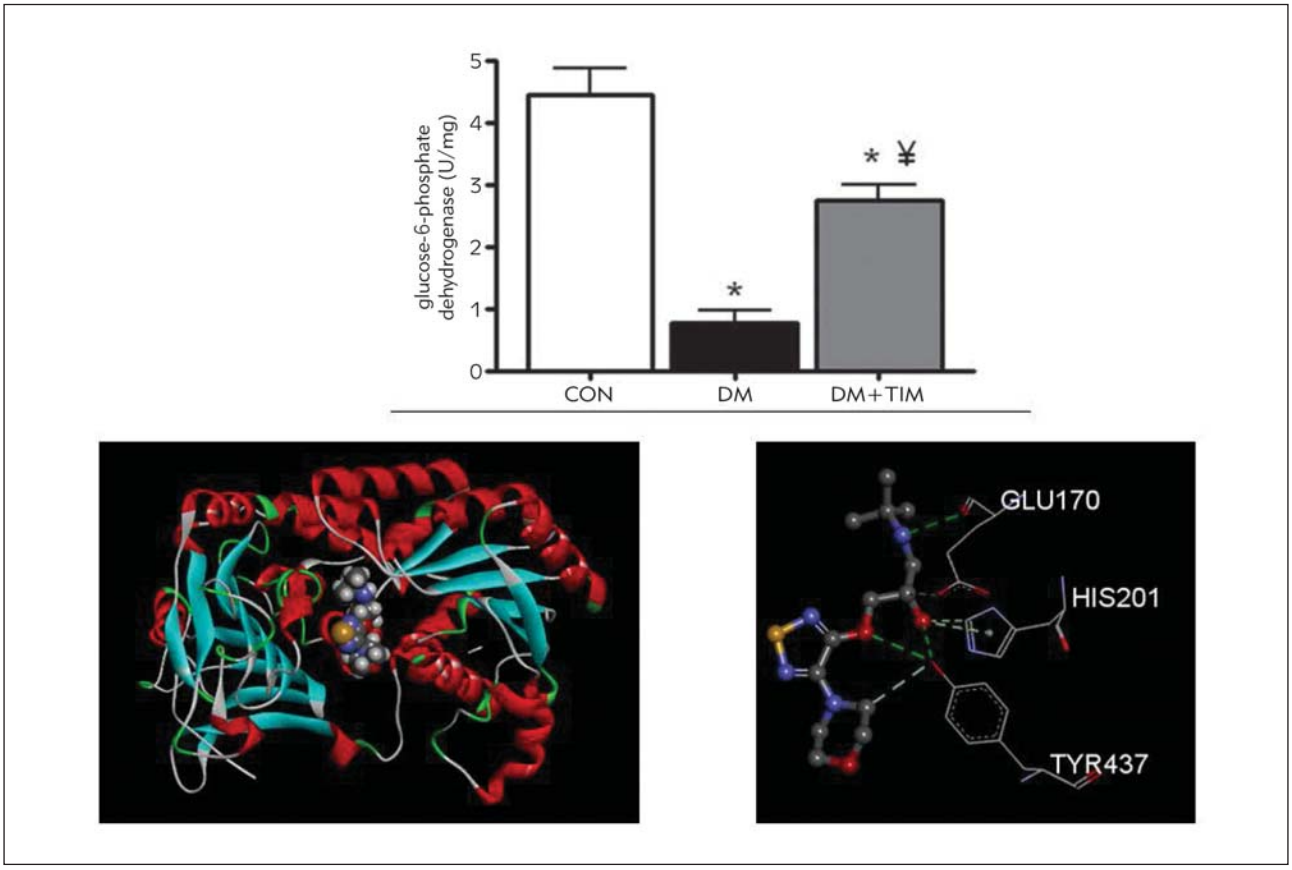

Figure 2 (A) Enzyme activity of G6PD measured in control (CON), STZ-diabetic (DM) and timolol treated diabetic (DM+TIM) rat pancreases. The bars are given as mean (±SEM). The significance levels; *P<0.001 vs CON, ¡P<0.01vs DM. (B) The best pose of timolol bound to G6PD. (C) Hydrogen bonds of timolol with GLU170, HIS201 and TYR437 of G6PD. 1QKI.pdb from ref. 25.

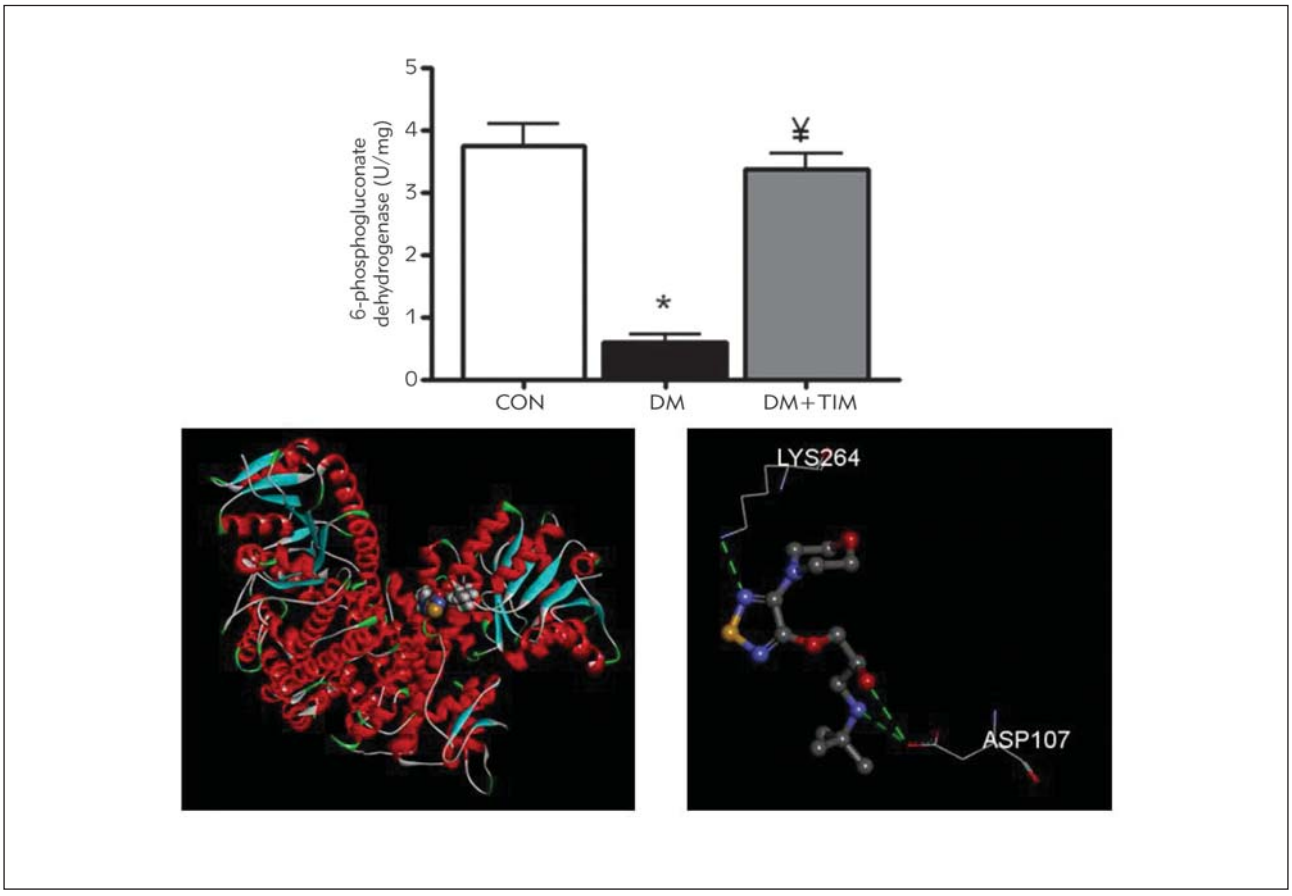

Figure 3 Enzyme activity of 6PGDH measured in control (CON), STZ-diabetic (DM) and timolol treated diabetic (DM+TIM) rat pancreases. The bars are given as mean (±SEM). The significance levels; *P<0.001 vs CON, ¦P<0.01vs DM. (B) The best pose of timolol bound to 6PGDH. (C) Hydrogen bonds of timolol with GLU170, HIS201 and TYR437 of 6PGDH. 3FWN. pdb from ref. 26.

Pancreatic cAMP level

Figure 4A shows the pancreatic cAMP level in the STZ-induced diabetic group compared to the control one. The bar graph representation of mean (±SEM) values for cAMP levels shows that there is no significant difference between these two groups. However, timolol treatment of STZ-induced diabetic group rats induced a significant increase in cAMP level.

In silico binding calculations for timolol to both G6PD and 6PGDH and Adenylyl Cyclase

In order to estimate the structural features of the possible binding of timolol to proteins such as G6PD, 6PGDH and AC and its binding strength to these proteins, we performed *in silico* binding calculations. Timolol was docked to each of the three proteins using the commercial software GOLD (Gold Suite V5.2.2). The structure of timolol was downloaded from PubMed. The Protein Data Bank codes for the three proteins are as follows: 1QKI.pdb for G6PD, 2ZYD for 6PGDH and 4CLF for AC. The surface of each protein was fully scanned by GOLD to find the best docking pocket, followed by detailed docking to the sites (29–31).

The calculated best binding Gibbs free energy for G6PD is found as -24.4 kJ/mol, which corresponds to an IC50 of 55 μmol/L. The best pose for timolol in G6DP is presented in *Figure 2B*. In this pose, timolol makes 4 hydrogen bonds with the residues GLU170, HIS201 and TYR437 of 6GDP as it can be seen in *Figure 2C*.

The Protein Data Bank structure 2ZYD.pdb is dimeric and complexed with glucose. Glucose was removed from the structure before docking simulations. The calculated best binding Gibbs free energy for 6PGDH is found as -24 kJ/mol which corresponds to an IC50 of 65 μmol/L. The best pose for timolol in 6PGDH is presented in *Figure 3B*. In this pose, timolol makes 2 hydrogen bonds with the residues LYS264 and ASP107 (*Figure 3C*).

We examined the effect of timolol treatment on the cAMP level as it can be seen in *Figure 4A*. The cAMP level measured in control (CON), STZ-diabetic

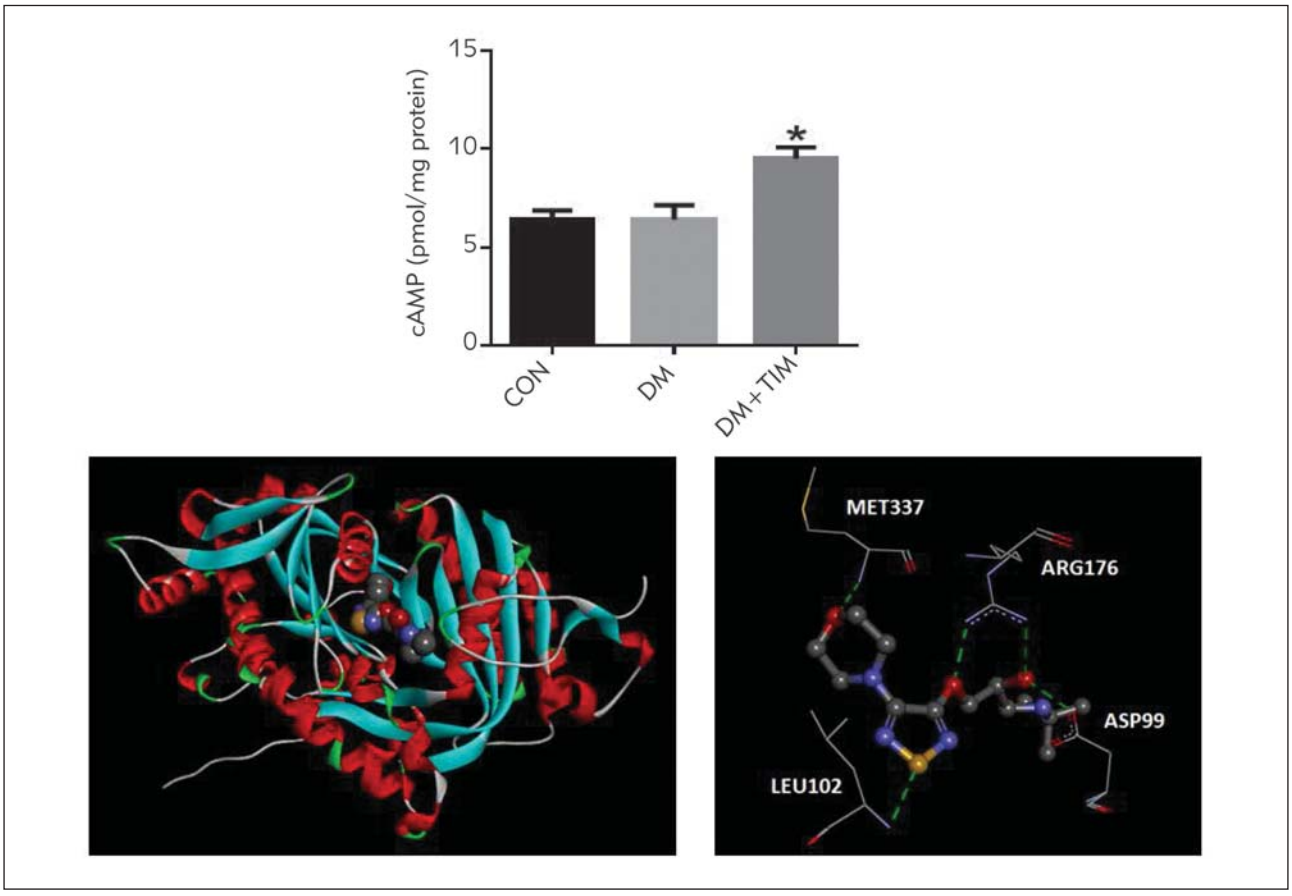

Figure 4 (A) cAMP level measured in control (CON), STZ-diabetic (DM) and timolol treated diabetic (DM+TIM) rat pancreases. The bars are given as mean (±SEM). *P<0.05 vs CON or DM. (B) The best pose of timolol bound to adenylyl cyclase. (C) Hydrogen bonds of timolol with MET337, ASP99, ARG176 and LEU102. 4CLL pdb from Ref. 7.

(DM) and timolol treated diabetic (DM+TIM) rat pancreases. The bars are given as mean (±SEM). *P<0.05 vs CON or DM (*Figure 4A*). The calculated best binding Gibbs free energy for AC is found as -32.2 kJ/mol, which corresponds to an IC50 of 2.4 μmol/L. The best pose for timolol in AC is presented in *Figure 4B*. In this pose, timolol makes four hydrogen bonds with the residues with MET337, ASP99, ARG176 and LEU102 (*Figure 4C*).

Discussion

In this research, we present a brief molecular docking approach for structure-based timolol and G6PD, 6PGD and adenylyl cyclase enzymes and we have calculated binding affinity to these enzymes. Considering the limitation of computer resources we have also done experimental studies showing the interaction between timolol and the enzymes G6PD and 6PGDH. We have studied the effects of timolol-treatment in streptozotocin-induced diabetic rats in pancreatic tissue enzymes because antioxidant enzyme activities must be a key place in pancreatic beta cell toxicity and diabetic condition, resulting from STZ induction. It can be seen from the graphs that, STZ-induced diabetes caused significant decreases in the activities of G6PD and 6PGDH enzyme activities in pancreatic tissue. However, we have demonstrated that timolol treatment of STZ-induced diabetic rats induced depressed activities of G6PD and 6PGDH in pancreatic tissue. The graph 2 and 3 show the increased activities of G6PD and 6PGDH enzyme activities after timolol treatment. Several different factors may be involved in the rate of the enzymatic reactions such as temperature, pH, enzyme concentration, substrate concentration. However, all of these variables were constant in our experimental design so that only timolol treatment induces the enzyme activities. We thought about the factors and/or signals regulating the enzyme activities. We thought that timolol might act upon negative regulators. Aldosterone, cAMP, cAMP-dependent PKA, CREM-cyclic AMP response element modulator, arachidonic acid, p38 MAP kinase, p53, TNFα, AMP kinase are the negative regulators of G6PD enzyme (8). However, we did not have too much chance to have a look into all of these parameters. We have cho-

sen the cAMP activity because pancreas tissue is too small to look into all of these activities. We have thought that timolol may inhibit adenylyl cyclase enzyme activity. Therefore we have had to measure the cAMP concentration. *Figure 4A* shows the pancreatic cAMP level in the STZ-induced diabetic group compared to the control. There is no significant difference between STZ-diabetic (DM) and timolol treated diabetic (DM+TIM) rat pancreases. However, timolol treatment of the STZ-induced diabetic group rats induced a significant increase in the cAMP level. The findings from this study raise many questions, and we need more research to better understand the timolol treatment on diabetes, and it has become evident that timolol plays an interesting role in the modulation of a variety of enzyme activities.

Streptozotocin is an antibiotic that is toxic to pancreatic islet β-cells and is commonly used to produce DM, and streptozotocin injection has been shown to produce oxidative stress, lipid peroxidation, and biochemical alterations such as reduced glutathione level and reduced glutathione and oxidised glutathione ratio and glutathione redox cycle enzymes (22, 32–35). Therefore, researchers trying to find various extracts, drug-like molecules, antioxidant supplementations, vitamins, drug molecules to increase the enzyme activities which are responsible for improving GSH redox state, increasing reduced glutathione pool, and increase GSH/GSSG ratio and for cellular prevention against glucose toxicity, oxidative stress, enzyme activity normalizations in diabetic animal models (22, 35–39). In the present study, we have demonstrated that timolol treatment of STZ-induced diabetic rats had no noteworthy effect on high blood glucose level, while this treatment induced marked protection against hyperglycemia induced depressed activities of G6PD and 6PGDH in pancreatic tissue from STZ-induced diabetic rats. Our binding data, particularly associated with timolol high binding affinity to soluble AC show the importance of timolol action via AC in the pancreas. Our data on *in silico* calculations showed that timolol could bind tighter to the AC enzyme, then G6PD or 6PGD. Interestingly, timolol treatment of STZ-induced diabetic rats markedly prevented these changes, providing a perfect protection against hyperglycemia-induced tissue injury. These data may imply that timolol action may be due to its antioxidant-like action in mammalian tissue (22).

It is known that timolol competes with adrenergic neurotransmitters such as catecholamines for binding to β-ARs in heart and vascular smooth muscle (39). However, its action on the pancreas is not known yet. It is well accepted that hyperglycemia generates more ROS in mammalian tissues and decreases antioxidant defence mechanism (40). It is also known that hyperglycemia-induced oxidative stress played a major role in the pathogenesis of several organ dysfunctions in diabetes. The important key point is the imbalance between the production of ROS and the biological system's radical detoxification system and cells use enzymatic reactions to maintain protection against different ROS. The high or low ratio of [NADPH] / [$NADP^+$] concentration is one of the most significant factors in cellular ROS concentration which is mainly regulated by the first and the third pentose phosphate pathway enzymes G6PD and 6PGD. Drugs or various drug-like molecules will possibly have antioxidants act through several mechanisms to prevent oxidant-induced macromolecule or cell damages. They can decrease the generation of ROS, scavenge ROS, or interfere with ROS-induced alterations by enzymatic reactions which is an essential core function of protection from various radicals and protecting protein damage would seem to be an essential component in diabetes (40, 41). In this regard, we have previously shown that STZ-induced diabetes associated with kidney damage is mostly dependent on increased oxidative stress and timolol, having an antioxidant-like action which presented marked protection against hyperglycemia associated tissue damage (22).

Our present data showed that timolol preserved depressed activity levels of G6PD and 6PGD in STZ-induced diabetic rat pancreas, although results of *in silico* calculations demonstrated its not sufficiently strong binding to G6PD and 6PGD. From here, one can propose that the beneficial effect of timolol on the enzyme activities of the pentose-phosphate pathway seems related to its antioxidant-like action. Timolol binding affinity to soluble AC was relatively high compared to others, and it induced a significant increase in the cAMP level of pancreatic tissue from STZ-induced diabetic rats as well.

These normalised enzyme activities may be due to two possibilities: the first is that timolol directly binds to these enzymes; G6PD and 6PGDH as shown in *Figures 2* and *3* with hydrogen bonds, which in turn increases the enzyme activity and leads to their proper functioning in pancreases. Therefore, one can derive a conclusion that increased concentration of NADPH enables pancreatic cells to overcome the oxidative stress through the action of the glutathione detoxification system.

The second possibility: timolol binds to AC which in turn increases the enzyme activity. (42). Specific inhibition of G6PD alone led to decreased β-cell survival and decreased β-cell proliferation (7). It was proposed in 1973 that glucose-induced release of insulin from perfused rat islets is associated with the elevated islet cAMP then, it was clarified in 2001 by Rutter that many factors affecting cAMP concentration and recently it has been clarified that physiological and pharmacological AC and its products cAMP regulates a wide range of physiological processes in almost all organisms. Soluble adenylyl cyclase and transmembranous adenylyl cyclases can

be found all over the cytoplasm, inside the nucleus, in mitochondria. The generated cAMP have different key roles in metabolism, especially define diverse signalling cascades in glucose metabolism, gene expression, and energy metabolism and have distinct roles in different organs (43–45). These statements are strongly in line with our present data. Supporting these responses, it has been also demonstrated that decreased NADPH level is associated with diabetic kidney pathology, altered NO production, aldosterone-mediated endothelial dysfunction, and dialysis-associated anemia (46).

The therapeutic effects of timolol are in blocking β-adrenoceptors. However, Gomes et al. demonstrated that this molecule has beneficial effects which have been associated with the antioxidant properties such as NO and $ONOO^-$ and ROS scavenging activity (47). Diabetes-induced ROS are very important in the pathogenesis of diabetes and timolol has the beneficial effect on scavenging radicals (4). G6PD and 6PGDH enzymes are the key enzymes in the antioxidant system, and both of these enzymes have many essential biochemical, metabolic and physiological roles. Both of these enzymes' product, NADPH, is an essential molecule in various biochemical syntheses such as cell growth, proliferation, and detoxification in eukaryotic cells (8, 48). However, diabetes-induced damage in various tissues is dependent on protein glycosylation, oxidation and alterations in enzyme activities, which are the underlying causes of diabetes complications, and mainly depends on the generation of reactive oxygen species and various types (15, 22, 40, 49, 50). In this study, diabetes is produced in animals by the drug streptozotocin, and this drug produces active oxygen species, and in this way, it destructs pancreatic beta cells and mediates the occurrence of diabetes (50, 51). However, timolol has the property of scavenging activity of various types of radicals (47), and the possible mechanism is working on the activation of G6PD and 6PGDH enzymes (22). Because of this research, we have observed that timolol caused significant changes in the activities of G6PD and 6PGDH antioxidant enzyme activities in the pancreas while in our previous research we also observed that timolol-treatment of diabetic rats enhanced the depressed activities of G6PD, 6PGDH, glutathione-S-transferase and glutathione reductase in the kidney tissues (22).

Cyclic AMP is one of the key molecules in the regulation of metabolism, such as involvement in the release of insulin and it is also known that insulin deficiency stimulates the production of the cyclic AMP (52). In the present study, we have also demonstrated that timolol treatment of STZ-induced diabetic rats increased the cAMP level compared to the control group as can be seen from bar graphs (*Figure 4A*). However, STZ-induced diabetic rats did not affect the cAMP level compared to the control group as it can be seen from the bar graphs (*Figure 4*). There may be multiple possible interpretations from our data and our findings on timolol may open novel windows on diabetes therapy and may also provide new openings for future research on diabetes.

Here, we have shown the interaction between a timolol molecule and enzymes at the atomic level, which allows us to characterise the behaviour of timolol in the binding site of G6PD, 6PGD and adenylyl cyclase enzymes.

On the other side, in docking process, we have predicted the timolol position and orientation within these binding sites of the G6PD, 6PGDH and adenylyl cyclase enzymes and we have calculated binding affinity to these enzymes.

The Protein Data Bank structures are X-ray crystal structures of proteins. We used the following structures: 1QKI.pdb for G6PD, 2ZYD for 6PGDH and 4CLF for AC. The three-dimensional structural accuracy of these structures is known and is as follows: 1QKI.pdb-3.00Å, 2ZYD.pdb-1.50Å, and 4CLF.pdb-1.98Å. These are the structures downloaded from the Protein Data Base website and are used in our ligand binding calculations. The software GOLD uses these protein crystal structures as target structures and binds ligand timolol to appropriate regions on surfaces of a protein. The resulting timolol+pdb complex is the one that has the highest affinity of binding. The reported complexes in the paper have been obtained in this manner.

Based on our results, we suggest that the main timolol antioxidant mechanism may be working on increasing the activities of antioxidant enzymes and/or other unknown protein-protein interactions and/or drug-protein interactions. However, steps of the main mechanism are still largely unknown.

Conclusions

Hyperglycemia and oxidative stress result in pathological changes in diabetic subjects. In the present study, our results suggest that timolol affects antioxidant enzymes via enzyme cascade interaction in STZ-induced diabetic rats. G6PD, 6PGD and cAMP play essential roles, which are involved in diverse physiological processes such as metabolism, oxidative stress, cell cycle, and diabetes. Timolol treatment presented protective effects against STZ-induced diabetes-related oxidative stress by upregulation of G6PD and 6PGDH activities. More importantly, herein, for the first time, we demonstrated that timolol treatment of STZ-induced diabetic rats exerted beneficial effects against hyperglycemia associated pancreatic injury via upregulation of G6PD and 6PGDH activities. These data imply the important role of timolol – possibly its protection of β-cells against the cytotoxic effects of ROS in STZ-induced diabetes-induced oxidative stress. *In silico* calculations showed

that timolol could strongly bind adenylyl cyclase enzyme, and then G6PD and 6PGD. Timolol treatment induced activities G6PD and 6PGD in the diabetic rats and the generation of NADPH by these enzymes is essential for protection against oxidative stress in diabetes.

On the whole, we may say that the normal activities of these enzymes have protective and key effects on detoxification of ROS, which prevents hyperglycemia-induced pancreas damage by enhancing the depressed antioxidant defence in the pancreas. The normal level of these enzyme activities provides a benefit to the organism to combat oxidative stress-induced complications of STZ-induced diabetes.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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