Inhibition of Carbonic Anhydrase Results in Blood Lactate Accumulation (BLA) in STZ Induced Diabetic Rats

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Abstract

Carbonic anhydrase is a pH regulatory enzyme, whose inhibition leads to metabolic acidosis. We investigated whether carbonic anhydrase inhibition may be associated with blood lactate accumulation in Streptozotocin (STZ) induced diabetic rats. The study aimed to provide a new marker to assist in identifying diabetic individuals at a high risk of developing lactic acidosis. Erythrocyte carbonic anhydrase activity with 4-nitrophenyl acetate as substrates was investigated in (STZ) induced diabetic rats. STZ induced diabetic rats showed significant increase in erythrocyte carbonic anhydrase (CA) activity of 9.7 ± 0.7 μMol/min/μL and blood lactate level of 6.2 ± 1.2 mMol/L when compared with Non Diabetic group CA enzyme activity of 5.5 ± 0.6 μMol/min/μL and blood lactate level of 3.9 ± 0.5 mMol/L. Inhibition of erythrocyte carbonic anhydrase activity with Acetazolamide results in 6.9 fold increase in blood lactate level when compared with Non diabetic group. Therefore inhibition of Carbonic anhydrase in STZ induced diabetic rats' result in blood lactate accumulation and reduced blood glucose concentration.

Keywords: Carbonic anhydrase; Lactate; Diabetes

Introduction

Compared with non-diabetics, diabetics produce increased amounts of lactic acid which is the end product of glycolysis particularly in muscle cell and red blood cells. Some studies revealed that Lactate does not only increase in the early stages of diabetes but has also been shown to predict its occurrence in the future [1,2]. Lactic acidosis is one of the causes of metabolic acidosis which is an alarming metabolic signal of many pathological states. Metabolic acidosis is the most common serious acid-base disorder complicating diabetes mellitus which poses considerable cellular stress, as alterations in pH affect the structure and activity of many enzymes, which affects cell signaling, transport and metabolic function. Metabolic acidosis is associated with increased mortality [3]. Carbonic anhydrase manage metabolic acid production by facilitating H^+ excretion via the renal tubules and into the urine. Thus assist with intracellular buffering of acidic units. Carbonic anhydrase facilitate H^+ in conjunction with lactate removal through monocarboxylate transporters (MCT), which allows continued glycolytic ATP production and thus contributes to pH regulation. It has been reported that MCT dependent lactate-H^- flux is facilitated by bicarbonate transporters and carbonic anhydrase (CA) activity in various cells and tissues [4-7]. It has been reported that inhibition of Carbonic anhydrase was found to impair proton secretion into the proximal tubule lumen. At the same time, inhibition of Carbonic anhydrase also decreased the rate of acidification of urine, producing alkaline urine and eventually metabolic acidosis [8]. Carbonic anhydrase activity and lactic acidosis in diabetes mellitus have not yet been fully elucidated. Based on the association of carbonic anhydrase with metabolic acidosis, and the accumulating evidence linking carbonic anhydrase with lactate flux across MCT, the study aimed to discover the association of carbonic anhydrase with lactic acid accumulation in diabetes mellitus, thus we hypothesized that inhibition of carbonic anhydrase, may lead to blood lactate accumulation (BLA).

Material and Methods

Animal experiments

Male Wistar albino rats weighing 180-220 g were used in the study. They were kept in the Central Animal House of the Faculty of Pharmaceutical Science, (Ahmadu Bello University, Zaria) in colony cages at an ambient temperature of $25 \pm 2°C$ and relative humidity of 45–55% with 12 h light/dark cycles. They had free access to standard diet (Vitafeeds Nig. Ltd. Nigeria) and water *ad libitum*.

STZ induced diabetes (SID)

Overnight fasted rats, received 60 mg streptozotocin (STZ) per kg body weight, dissolved in ice cold citrate buffer (0.1 M, pH 4.5) intraperitoneally. 72 hours after induction, rats with marked hyperglycemia (blood glucose level>200 mg/dl) were selected and used for the study. 72 hours after STZ administration, animals were treated by oral gavage with 250 mg/kg/day Acetazolamide for 28 days.

Experimental design

For the short term investigation (14 Days): 10 rats were divided into two groups of five animals each: group I (Normal control); group II (Diabetic control) rats. For the long term investigation (28 Days): 18 rats were divided into three groups of six animals each: group I (Normal control); group II (Diabetic control) without treatment and group III (Diabetic treated with Acetazolamide).

Metabolic assays

Blood samples were obtained from the tail tip and Blood glucose, lactate, cholesterol and triglycerides measured using (Accutrend GCT Meter, Roche, Germany with Cobas® test strips).

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Carbonic anhydrase activity

Carbonic anhydrase activity was determined as mentioned by Verpoorte et al. [9] with some modification described by Parui et al. [10] using spectrophotometer. In this assay, the esterase activity of carbonic anhydrase was determined from the hydrolysis rate of 3mM p-nitrophenyl acetate to p-nitrophenol. The protocol consisted of 100 µL hemolysate placed in 1 cm spectrometric cell containing 1.4 ml 0.05 M Tris- HCl, pH: 7.4 and 1.5 ml p-nitrophenyl acetate. The change of absorbance was measured over the period of 3 min at 348 nm before and after adding the hemolysate. The absorbance was measured by a UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer). One unit of enzyme activity was expressed as µmol of p-nitrophenol relased/min/µL from hemolysate at room temperature (25°C) [10,11]

Statistical analysis

Results were presented as mean ± standard deviation between groups, comparisons was performed by the analysis of variance (ANOVA) (using SPSS 20.0 for windows Computer Software Package). Significant correlation was compared by Pearson's correlation and Duncan's new Multiple Range test; a probability level of less than 5% ($P<0.05$) was considered significant [12].

Results

Effect of (14 days) STZ-induced diabetes on erythrocyte carbonic anhydrase

We investigated whether STZ induced diabetes exhibited an effect on erythrocyte carbonic anhydrase activity in vivo. We first started by investigating changes in erythrocyte carbonic anhydrase activity in non-diabetic random and non-diabetic fasting wistar rats. We observed that carbonic anhydrase activity slightly increases in non-diabetic fasting state (Table 1). We also observe a decrease in glucose level and increase in lactate, cholesterol and triglyceride level in the non-diabetic fasted rats compared to non-diabetic random. Similarly STZ induced diabetic rats show a significant (p<0.05) increase in carbonic anhydrase activity, with significant increase in glucose, lactate, cholesterol and triglycerides compared to non-diabetic control (Table 1).

Effect of (28 days) STZ-induced diabetes on erythrocyte carbonic anhydrase

We assessed whether long term uncontrolled diabetes will have effect on erythrocyte carbonic anhydrase. We observed that there was significant (p<0.05) reduction in carbonic anhydrase activity and significant increase in both glucose and lactate compared with the non-diabetic control (Table 2).

Carbonic anhydrase Inhibition (with Acetazolamide) increases lactate level in STZ induced diabetic rat

Acetazolamide had hypoglycemic properties in wistar rats with STZ-induced diabetes, a model of insulin-deficient diabetes. There was significant increase in lactate level in STZ-induced diabetic rats treated with Acetazolamide (250 mg/kg/day) for 28 days (14.6 ± 3.8 mMol/L) compared to normal control (2.1 ± 0.6 mMol/L). In contrast, Acetazolamide enhanced the lactate accumulation in the STZ-induced diabetic rats by 6.9-fold. A significant (p<0.05) reduction in cholesterol and increase in triglycerides was observed in rats with STZ-induced diabetes, treated with Acetazolamide (250 mg/kg/day) for 28 days (Table 2).

Discussion

The result of Table 1 suggests that blood lactate might have increased due to increased anaerobic oxidation of glucose in STZ induced diabetic rats. Because diabetes results in a situation in which glucose homeostasis is impaired and that it may account for the excess conversion of glucose to lactate, due to decreased rate of oxidative metabolism of glucose, including reduced rates of muscle glycogen synthesis, as suggested by decreased muscle glycogen content in the postabsorptive state [13], and a lack of higher rates of glucose oxidation to carbon dioxide [14]. Red blood cells produce lactic acid as a byproduct of the regeneration of ATP during anaerobic glycolysis but cannot use lactic acid. Nguyen and Bonano, [15] found evidence supporting the notion that lactate-H+ cotransport via monocarboxylate transporters (MCT) 1,2 and 4 is facilitated by HCO_3^-, Carbonic anhydrase (CA) activity, Na^+ /H^+ exchange, and $1Na^+ : 2HCO_3^-$ co transport. Thus the increase in carbonic anhydrase activity seen in STZ induced diabetic rats (Table 1) may come as an adaptive response to increase the facilitated efflux of lactate from the glycolytic cells leading to increased blood lactate concentration. It has been reported that MCT dependent lactate-H+ flux is facilitated by bicarbonate transporters and carbonic anhydrase (CA) activity in various cells and tissues [4-7].

We may therefore suggest that the increased lactate efflux from erythrocyte and other glycolytic tissues in the STZ induced diabetic rats may reflect the mass-action effect of higher intracellular lactate concentration. This mass action of lactate accounts for the increased activity of carbonic anhydrase that facilitate lactate efflux and result in increased blood lactate concentration.

A recent study by Crawford et al. [1] and Nicky et al. [2] showed that lactate is not only increased in the early stages of diabetes but has also been shown to predict its occurrence in the future. Forbath et al. [16] and DeMeutter and Shreeve [17]; had also reported that there is an increase in lactate production, in diabetic dogs and humans. The

Table 1: Effect of streptozotocin (STZ) induced diabetes on erythrocyte anhydrase and metabolic parameters *In vivo*.

Parameters/Groups	Non Diabetic Random	Non Diabetic Fasting	Diabetic (STZ)
Carbonic anhydrase (µMol/min/µL)	5.5 ± 0.6	6.3 ± 0.2	9.1 ± 0.7[a]
Glucose (mMol/L)	4.2 ± 1.1	3.5 ± 0.2	12.7 ± 3.9[a]
Lactate (mMol/L)	3.9 ± 0.5	4.2 ± 0.9	6.2 ± 1.2[a]
Cholesterol (mMol/L)	3.8 ± 0.1	3.9 ± 0.07	4.3 ± 0.02[a]
Triglyceride (mMol/L)	1.0 ± 0.2	1.5 ± 0.5	2.5 ± 0.6[a]

The effect of STZ induced diabetes on erythrocyte carbonic anhydrase activity and other metabolic parameters. [a]P<0.05 vs Non Diabetic Random group; (n=5).

Table 2: Effect of Acetazolamide Treatment on streptozotocin (STZ) Induced Diabetic Rats.

Parameters/Groups	Non-Diabetic	Diabetic (STZ)	Diabetic (STZ) ± Acetazolamide
Carbonic Anhydrase (µMol/min/µL)	4.9 ± 0.2	2.8 ± 0.3[a]	1.4 ± 0.4[b]
Glucose (mMol/L)	5.5 ± 0.8	17.1 ± 4.1[a]	5.1 ± 1.9[b]
Lactate (mMol/L)	2.1 ± 0.6	4.8 ± 0.6[a]	14.6 ± 3.8[b]
Cholesterol (mMol/L)	4.0 ± 0.0	4.5 ± 0.0	1.0 ± 0.1[b]
Triglyceride (mMol/L)	1.1 ± 0.2	1.16 ± 0.2	4.1 ± 0.1[b]

The effect of the inhibition of erythrocyte carbonic anhydrase activity with acetazolamide at 250mg/kg/day for 28days on blood lactate level and other metabolic parameters in STZ induced diabetic rat. [b]P<0.05 vs Diabetic (STZ) group; [a]P<0.05 vs Non Diabetic group (n=6).

present study which resulted in 2.2 fold increase (Table 2) in blood lactate level in STZ induced diabetic rats when compared with normal control is therefore consistent with previous studies.

Published studies have provided mixed results regarding the potential of carbonic anhydrase inhibitors to modulate blood glucose levels. Previously Acetazolamide and Ethoxyzolamide were found to inhibit gluconeogenesis in vitro or after acute administration in rats. [18-20] our studies indicate that Acetazolamide treatment in STZ induced diabetic rats' lowers glucose production and increases glycolytic production of lactate. We found (Table 2) 6.9 fold increase in blood lactate level when compared with normal control and 3.0 fold increases in blood lactate level when compared with untreated diabetic group.

Our findings are consistent with previous findings indicating that prolong untreated diabetes result in decreased activity of erythrocyte carbonic anhydrase. Gambhir et al. [21] observed decrease in carbonic anhydrase activity in type II diabetic patients when compared with Normal subjects. They therefore speculate that the change in the CA activity may be of fundamental importance in the regulation of intracellular; pH for the basic control of metabolism in diabetes mellitus. Some early evidences suggest that the changes in carbonic anhydrases activities in erythrocytes may be an initial step of altered metabolism in diabetes mellitus [11] Dodgson and Watford [22] examined previously the changes in the activity of CA-III in hepatocytes of acute diabetic rats. Diabetes resulted in 50% reduction in the activity of CA-III. They observed an approx. 98% reduction in CA-III content in the liver of chronic diabetes mellitus rats relative to controls. A 75% reduction in serum CA-III content relative to control values after the administration of streptozotocin was observed.

We may therefore suggest that, the low blood glucose concentration and increased blood lactate level may not come as a coincidence given lactate's association with glucose in diabetes and the accumulating evidence linking lactate flux with carbonic anhydrase as described previously that carbonic anhydrase facilitate lactate flux through MCTs.

One possible explanation in terms of potential mechanism for the increased blood lactate concentration may be due to reduction in carbonic anhydrase activity, which has been reported to facilitate lactate influx and efflux across the MCTs. Therefore, the MCTs may not facilitate lactate influx into the liver cells due to reduction of carbonic anhydrase. Klier et al. [23] showed that transport activity of MCT1 and MCT4 is enhanced by cytosolic carbonic anhydrase. They suggested that MCT4 is the main pathway to export lactate out of glycolytic cells, which may produce larger amount of lactate during metabolic demand, while MCT1 can both serve as a lactate importer and exporter. The question remains whether inhibition of carbonic anhydrase that facilitate lactate import across MCT1 into liver cells is responsible for blood lactate accumulation and decreased hepatic gluconeogenesis? Could it be the same reason why metformin reduces blood glucose by inhibiting hepatic gluconeogenesis while leading to blood lactate accumulation?

Hems et al. [24] showed that gluconeogenesis from lactate in the isolated perfused rat liver was markedly inhibited when the pH of the perfused was less than 7.1. Subsequently Lloyd et al. [25] showed that, under similar conditions of simulated metabolic acidosis in the perfused rat liver, lactate uptake was also inhibited and that this occurred when hepatic mean intracellular pH had fallen below 7.05. We may therefore propose that the increase release of lactate from muscle

and erythrocytes (the major lactate producers) and increase uptake by the liver may be responsible for sustained hyperglycemia in diabetes. Finally we may conclude that carbonic anhydrase plays a significant role in lactic acidosis and possibly in hepatic gluconeogenesis. Our identification of carbonic anhydrase as an endogenous marker for Blood lactate accumulation (BLA) puts one more piece of the complexity of diabetes and its treatment in place. It helps to answer one of critical questions about the causes of lactic acidosis in diabetes most especially due to metformin treatment.

Declaration of Interests

We declare no competing interests.

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