Chapter 6
Potential of Carbonic Anhydrase Inhibitors in the Treatment of Oxidative Stress and Diabetes

Zafer Gurel and Nader Sheibani

Abstract Increased oxidative stress has been recognized as a major contributing factor to various pathological conditions including diabetes and its complications. Although, several mechanisms contribute to increased oxidative stress (OxS) in diabetes, increased levels of glucose and altered metabolic activities are major contributors to production of reactive oxygen species (ROS) and OxS. The identity of the target cells and metabolic pathways involved in ROS production provide a venue for treatment of the underlying causes and mitigation of diabetes complications including diabetic retinopathy. Diabetic retinopathy affects retinal neurovasculature, and loss of retinal vascular pericytes (PC) has been recognized as one of the early targets. We have shown that retinal PC are most sensitive to high glucose conditions in culture compared with retinal vascular endothelial cells (EC) and astrocytes (AC). We have proposed that retinal PC may differ in their metabolism of glucose compared with EC. Pericytes likely prefer oxidative metabolism for energy production, especially under high glucose conditions, generating excess ROS that drives their demise. This is mediated, in part, through activation of hexose biosynthetic pathway, enhanced O-GlcNAc modification and stabilization of P53, and attenuation of the Warburg effect. In support of this hypothesis, we recently showed retinal PC, but not EC, generate more superoxide under high glucose conditions. We also

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showed inhibition of carbonic anhydrases (CA) protects PC from adverse effects of high glucose. The inhibition of CA, especially those in the mitochondria (mCA), limits the production of bicarbonate that is essential for the first step of oxidative metabolism in the mitochondria. Thus, targeting of the mCA may provide a unique opportunity for modulation of PC metabolism mitigating the development and progression of diabetic retinopathy and likely other complications of diabetes.

Keywords Mitochondrial carbonic anhydrases · Retinal pericytes · Diabetic retinopathy · Oxidative stress · Oxidative metabolism

Abbreviations

AC	Astrocytes
AGE	Advanced glycation end product
AGER	AGE receptor
CA	Carbonic anhydrases
EC	Endothelial cells
mCA	Mitochondrial CA
OxS	Oxidative stress
PC	Pericytes
RPE	Retinal pigment epithelium
ROS	Reactive oxygen species
SMC	Smooth muscle cells
STZ	Streptozotocin
8OHdG	8-Hydroxy-2'-deoxyguanosine

6.1 Introduction

Diabetes is a chronic disease which affects a large portion of the working age population worldwide. The prevalence of diabetes has significantly increased in the past couple of decades and is continuing to rise at an alarming rate (Antonetti et al. 2012; Duh et al. 2017; Solomon et al. 2017; Shin et al. 2014a). Chronic exposure to high concentrations of glucose has adverse effects on the cellular metabolic activity affecting various vascular and tissue functions (Rask-Madsen and King 2013; Aghdam and Sheibani 2013; Bahtiyar et al. 2016; Campesi et al. 2017; Moran et al. 2013). High glucose levels is a major contributing factor to the development and progression of the disease, and epidemiological studies have demonstrated control of glucose levels is most effective in improvement of diabetes adverse health effects (Agardh et al. 1997; Sun et al. 2011; The Diabetes Control and Complications Trial Research G 1993). Chronic exposure to high glucose is associated with a

variety of pathologies including diabetic retinopathy, diabetic neuropathy, diabetic nephropathy, and cardiovascular and motor neuron dysfunctions. Over the years many studies have focused on delineating the underlying mechanisms that contribute to these pathologies. These studies have identified inflammation and oxidative stress (OxS) as key early mediators of diabetes adverse pathologies (Jha et al. 2018; King 2008; Mohamed et al. 2012; Nguyen et al. 2012; Roy et al. 2013; Scott and King 2004; Su and Xiao 2015; Reyk et al. 2003; Duarte et al. 2013). How diabetes leads to inflammation and OxS in various organs has been extensively studied. We know a great deal about the potential pathways that are impacted by high glucose conditions (Aboualizadeh et al. 2017; Bae et al. 2013; Chen et al. 2007; Cogan et al. 1984; Costa and Soares 2013; Ding et al. 2017; Du et al. 2002, 2013; Dugan et al. 2013; Frey and Antonetti 2011; Ibrahim et al. 2011; Stitt et al. 2005; Tang et al. 2013; Wang et al. 2009). These include the NADPH-oxidase pathway, the hexosebiosynthetic pathway, the pentose phosphate pathway, the polyol pathway, and the advanced-glycation end product pathway, all of which contribute to redox homeostasis. Increased OxS and activation of inflammatory pathways are considered as key contributors to the development and progression of diabetes complications. Thus, identification of the key pathways and major players involved may provide suitable targets for early intervention and attenuation of disease progression. Here we will focus on the impact of diabetes on ocular redox homeostasis and development and progression of diabetic retinopathy. We will also discuss the important role of mitochondrial carbonic anhydrases (mCA) in oxidative metabolism of glucose and their potential contribution to pathogenesis of diabetic retinopathy. We propose that inhibition of mCA may provide a novel intervention mechanism for elevation of OxS in retinal vasculature as a way to protect loss and dysfunction of retinal PC and retinal neurovasculature degeneration during diabetes.

6.2 Oxidative Stress and Diabetes

The majority of cells in various tissues are equipped with different machineries to maintain cellular redox homeostasis. Generally, OxS is caused when the stress levels surpass the capacity of cellular protective mechanisms, which help to overcome this stress. As eluded to earlier, multiple pathways contribute to OxS during diabetes, and various tissues and cells utilize different means to overcome this stress. These differences in the capacity of different cells and/or tissues to overcome OxS, may in part, contribute to their selective sensitivity to adverse effects of hyperglycemia, including retinal neurovasculature and retinal vascular cells (Scott and King 2004; Reyk et al. 2003; Aboualizadeh et al. 2017; Du et al. 2013; Shah et al. 2013a; Hayden et al. 2010; Patrick et al. 2015). Hyperglycemia causes OxS in tissues whose glucose uptake is insulin independent, including the eye and brain microvascular cells (Balasubramanyam et al. 2002). Pericytes, in close contact with EC in the capillary beds of the retina (with the highest PC density of any tissue), are vital to microvessel integrity and are especially susceptible to OxS (Caldwell et al. 2005; Qaum et al.

2001). Pericyte death leads to EC dysfunction and death, thus altering production of factors essential for retinal vascular homeostasis (Aiello et al. 1995; Boeri et al. 2001; Caldwell et al. 2003; Joussen et al. 2004; Kowluru and Odenbach 2004a; Chan et al. 2010). Rupture-prone microaneurysms and acellular capillaries (Hammes et al. 2011; Giacco and Brownlee 2010) then arise leading to retinal ischemia, growth of leaky new blood vessels toward the vitreous, and retinal detachment and vision loss.

Diabetes is associated with chronic hyperglycemia and diabetic retinopathy (DR), a leading cause of blindness in the working age population (Bhavsar 2006). The disease affects PC vital for stabilization and function of retinal blood vessels as well as for proliferation and differentiation of the single layer of EC that line the vessels (Cogan et al. 1961; Haefliger et al. 1994; Kuwabara et al. 1961). Pericyte loss, an early event in DR (Kowluru and Abbas 2003; Li et al. 1999; Miller et al. 2006; Mizutani et al. 1996; Podesta et al. 2000; Zhang et al. 2008), leads to inflammation, vascular dysfunction and degeneration, and altered production of regulatory factors essential for retinal vascular homeostasis (Aiello et al. 1995; Boeri et al. 2001; Caldwell et al. 2003; Joussen et al. 2004; Chan et al. 2010; Kowluru and Odenbach 2004b). This enables fragile, rupture-prone new capillaries to grow toward the vitreous. Left untreated, the result is retinal detachment and vision loss. The key mechanism for PC glucose sensitivity in early diabetes remains elusive but may be linked to respiration (mitochondrial oxidative metabolism of glucose), their preferential source of energetics and OxS.

The role retinal PC loss has in DR is well documented (Hammes et al. 2011; Miller et al. 2006; Mizutani et al. 1996). Pericytes are sensitive to hyperglycemia-induced OxS, which is likely caused by excess superoxide generated during respiration (Du et al. 2003; Nishikawa et al. 2000; Wallace 1992) (Fig. 6.1). Superoxide is the precursor to all ROS (Turrens 2003). Kowluru et al. (2006) reported that overexpression of mitochondrial superoxide dismutase, an enzyme that neutralizes superoxide, prevents diabetic retinal damage. ROS produced during respiration triggers other pathogenic pathways, such as the polyol pathway (Das Evcimen et al. 2004; Nishimura et al. 1997), advanced glycation end product (AGE) formation (Du et al. 2000; Hammes et al. 1991; Nakamura et al. 1997), protein kinase C activation (Koya and King 1998; Xia et al. 1994) and the hexosamine pathway (Nerlich et al. 1998; Schleicher and Weigert 2000), all of which propagate even more ROS and OxS. Nishikawa et al. (Nishikawa et al. 2000) reported that normalized superoxide blocked these three pathways of hyperglycemic damage. Du et al. (2000) showed that superoxide overproduction activates the hexosamine pathway. We recently demonstrated this pathway is highly activated in PC, but not EC and AC, under high glucose conditions, and promotes *O*-GlcNAc modification, stabilization of P53 protein, and likely the death of retinal PC (Gurel et al. 2013; Gurcel et al. 2008).

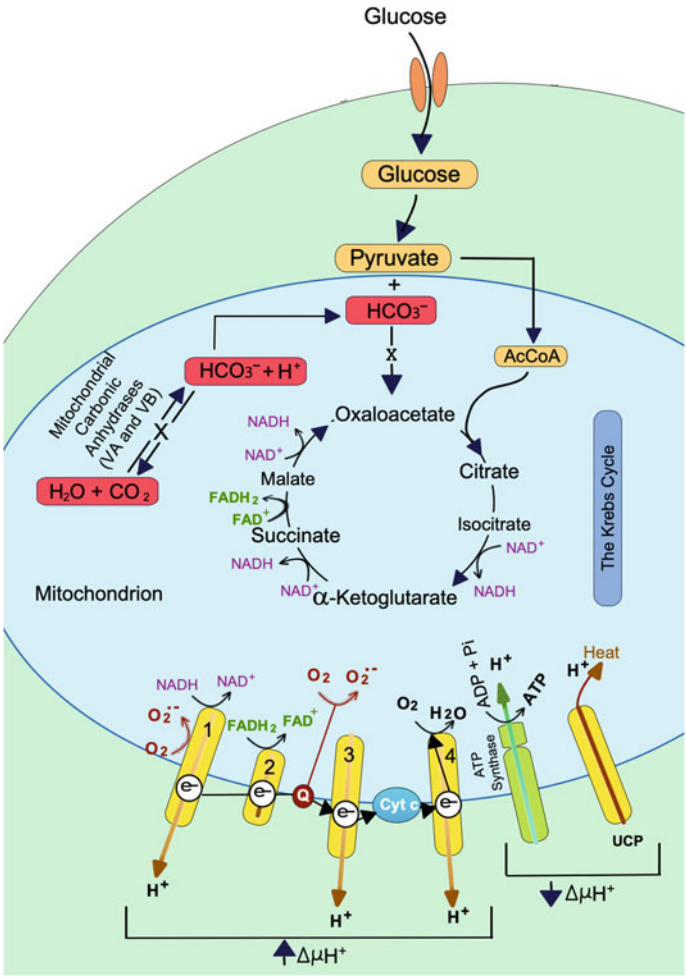

Fig. 6.1 Role of mitochondrial carbonic anhydrases (mCA) in oxidative metabolism of glucose and superoxide ($O_2^{\cdot -}$) production

6.3 Retinal Pericytes and Metabolic Activity

Our lab has developed a novel method for culturing vascular cells, including EC, PC, and AC from mouse retina (Scheef et al. 2005, 2009; Su et al. 2003). Using these cells, we have determined the impact of high glucose on various cellular functions. High glucose stimulated EC migration (Huang and Sheibani 2008), and increased OxS and inflammatory cytokines in AC, impacting their proliferation, adhesion and migration (Shin et al. 2014b). High glucose induced significant apoptosis in PC, but not in EC or AC (Huang and Sheibani 2008; Shin et al. 2014b, c). This finding is supported by the fact that EC have a relatively low mitochondrial content (Groschner

et al. 2012) and rely primarily on glycolysis with in vitro glycolysis rates comparable to or even greater than cancer cells, and exceeding glucose and fatty acid flux by >200-fold (Dagher et al. 2001; Bock et al. 2013; Ghesquiere et al. 2014; Mertens et al. 1990). The advantages of glycolysis to EC include lower oxidative phosphorylation-generated ROS, maximum preservation of oxygen for transfer to perivascular supporting cells (SMC/PC), adaptation of EC to the hypoxic surroundings they will grow into, and production of lactate as a proangiogenic signaling molecule with free radical scavenging and anti-oxidant activity (Groussard et al. 2000). However, whether glycolysis is a predominant bioenergetics pathway for retinal EC needed further confirmation.

The enhanced pro-migratory activity of retinal EC in high glucose (Huang and Sheibani 2008) is consistent with glycolysis as their preferred bioenergetics (Eelen et al. 2013). This is further confirmed by gene expression profile of glucose metabolizing enzymes in these cells under various glucose conditions (our unpublished data). In contrast little is known about the bioenergetics pathway of SMC/PC. However, clear metabolic differences between EC and SMC/PC in the brain have been demonstrated (Spatz et al. 1986). Our preliminary investigations indicate that retinal EC and PC possess a distinct mechanism for controlling glucose uptake and perhaps its metabolism. Thus, elucidating retinal microvascular cell metabolic preferences is not only important for understanding normal vascular function but also for comprehending vascular diseases such as DR.

We have detected that glucose uptake and phosphorylation by PC increases under higher glucose conditions. However, EC, AC and retinal pigment epithelial (RPE) cells had tighter control of glucose uptake and phosphorylation under high glucose conditions. These results are consistent with enhanced glucose transport reported in bovine retinal PC compared with EC (Mandarino et al. 1994), and attenuation of PC migration and increased apoptosis under high glucose conditions (Gurel et al. 2013, 2014). Potential differences in glucose uptake and metabolism have been examined in SMC/PC and EC (Spatz et al. 1986; Betz et al. 1983; Kaiser et al. 1993; Li et al. 1985). These studies have defined “facilitative-diffusion mechanisms” for glucose transport in endothelial and perivascular supporting cells, including retinal EC and PC (Betz et al. 1983; Li et al. 1985). Pericytes and SMC have about a 3- to fivefold greater glucose transport than EC (Kaiser et al. 1993; Li et al. 1985). In addition, EC have more lactate and intracellular glucose (Mandarino et al. 1994; Kaiser et al. 1993). Glucose transport differences were attributed to changes in expression of glucose transporters, mainly Glut 1, in these cells (Mandarino et al. 1994). Our preliminary results are consistent with increased transport of glucose and phosphorylation in PC and different metabolic pathways used by EC compared to PC. However, no significant changes were observed in the expression of various glucose transport proteins in these cells. We propose that preferential glucose transport and respiration is responsible for increased OxS and loss of retinal PC under high glucose conditions. Our recent unpublished observations that EC have higher levels of citrate and succinate compared to PC, which further increases in high glucose conditions, give credence to this hypothesis. The levels of succinate decreased in PC under high glucose conditions.

The increased succinate levels in EC is consistent with increased levels of citrate and its conversion to itaconic acid, a blocker of mitochondrial complex II, resulting in accumulation of succinate and decreased respiration (Sapieha et al. 2008). Succinate acting through its receptor GPR91 enhances Hif-1α expression promoting PKM2 activity and glycolysis driving angiogenesis (Corcoran and O'Neill 2016). Gene expression analyses with an RT^2 Profiler™ PCR Array Mouse Glucose Metabolism, also showed upregulation of genes involved in glycolysis, in retinal EC exposed to high glucose conditions (our unpublished data). For example, the expression of pyruvate dehydrogenase kinase isozyme 4 (Pdk4, a major suppressor of mitochondrial activity) increased by twofold. On the other hand, the expression of genes that promote pyruvate generation and its utilization in TCA cycle were significantly upregulated in PC under high glucose conditions. There was a 21-, 5-, 4-, and 2.5-fold increase in Glucose-6-Phosphatase catalytic subunit (G6pc), Phosphoribosyl pyrophosphate synthetase 1-Like 1(Prps1l1), Phosphoglycerate kinase 2 (Pgk2), and Phosphorylase b kinase gamma catalytic chain 1 (Phkg1), respectively. Also, a twofold increase was observed both in Fructose-Bisphosphatase 1 (Fbp1) and Hexokinase 3 (Hk3) gene expression.

Hyperglycemia results in higher glucose levels in tissues whose glucose uptake is insulin independent such as the brain, eye, and kidney (Balasubramanyam et al. 2002), which could not down regulate glucose transport into the cells in the face of chronic high glucose in the surrounding medium (Kaiser et al. 1993). Increased respiration has been implicated in hyperglycemia-induced OxS (Brownlee 2001, 2005), mitochondrial dysfunction, and mitophagy (Singh et al. 2017; Devi et al. 2013). However, actual respiration changes were not reported until we showed that PC challenged with high glucose exhibit significant increases in respiration and mitochondrial ROS production (Shah et al. 2013a). We also showed OxS and apoptosis in PC in response to high glucose (Shah et al. 2013b). Thus, it is important to determine how altered respiration rates affect ROS, OxS, mitophagy flux, and apoptosis in retinal vascular cells. Our published results strongly suggest that these approaches will provide a new therapeutic target for tackling the adverse effects of diabetes on retinal vasculature. These studies can lead to projects with drug developers, clinical endocrinologists and ophthalmologists, shifting the paradigm of treating diabetic complications in the retina and possibly in other tissues such as brain and kidney.

6.4 Metabolic Activity, Oxidative Stress, and Diabetic Retinopathy

Although mitochondria play an important role in OxS associated with diabetes the target cells involved remain unknown. Previous studies have shown that OxS, resulting from hyperglycemia, contributes to the early retinal PC dysfunction during diabetes (Hammes et al. 2002; Ejaz et al. 2008) and initiation of neuroinflammation through MCP-1 production (Kowluru et al. 2010). We showed that these adverse

effects of high glucose on PC were attenuated by topiramate treatment, a sulfamate substituted monosaccharide, which inhibits carbonic anhydrases (CA). We proposed that superoxide production is likely impacted by CA expressed in the mitochondria (mCA) (Shah et al. 2000).

Glucose is metabolized to pyruvate by phosphorylation of phosphoenolpyruvate in the last step of glycolysis, which is catalyzed by pyruvate kinase (PK), a multi-subunit enzyme that exists in several isoforms (Mazurek 2011). The PKM2 isoform predominantly expressed in muscle is also expressed in the retina (Morohoshi et al. 2012), and its activity depends on its tetramerization (Lincet and Icard 2015). PK is regulated by phosphofructokinase (PFK) and its phosphorylation status, and was recently shown to be a substrate of protein tyrosine phosphatase 1B (PTP1B) (Bettaieb et al. 2013), consistent with a role of PTP1B in obesity and diabetes (Panzhinskiy et al. 2013). The resulting pyruvate then enters the mitochondria, where it is metabolized to acetyl CoA and oxaloacetate. The carboxylation of pyruvate to oxaloacetate requires bicarbonate (HCO_3^-), which is provided by mCA by catalyzing the reversible hydration of carbon dioxide. HCO_3^- must be produced in mitochondria and cannot be imported from the cytosol, as mitochondrial membranes are impermeant to HCO_3^-. Oxaloacetate condenses with acetyl CoA to yield citrate. Citrate is oxidatively decarboxylated to succinate via α-ketoglutarate. Finally, oxaloacetate is regenerated from succinate to complete the cycle (Fig. 6.1).

The elevated succinate level could promote angiogenesis in hypoxia and ischemia through interaction with its receptor GPR91, expressed on retinal ganglion (Sapieha et al. 2008) and pigment epithelial cells (Favret et al. 2013). In addition, alterations in succinate levels during diabetes may contribute to the development and progression of DR perhaps through its interaction with GPR91 (Li et al. 2014). Thus, a careful temporal evaluation of changes in succinate levels with diabetes, and identification of cellular targets is essential.

The electron donors, NADH and $FADH_2$, generated in the Krebs cycle, enter the electron transport chain reaction where ATP is generated, and superoxide is a byproduct. Small fluctuations in the steady state concentration of superoxide may actually play a role in intracellular signaling (Droge 2002). Uncontrolled increases lead to free radical mediated chain reactions, which indiscriminately target proteins (Stadtman and Levine 2000), lipids (Rubbo et al. 1994), polysaccharides (Kaur and Halliwell 1994), and DNA (LeDoux et al. 1999; Richter et al. 1988). In diabetes, excess glucose in tissues whose glucose uptake is insulin independent such as retina (Balasubramanyam et al. 2002) leads to excess superoxide. The ROS triggered by superoxide cause oxidative inactivation of –SH containing enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PK, thus compromising glycolysis, a major bioenergetic pathway in the retina (Hegde et al. 2010).

We proposed that mCA inhibition, by virtue of its ability to reduce HCO_3^-, could slow DR onset and progression by three mechanisms: reducing superoxide production, altering Krebs cycle intermediate levels such as succinate with important role in DR, and increasing pyruvate. Pyruvate inhibits oxidative inactivation of GAPDH and PK by scavenging ROS. In addition, pyruvate provides NAD^+, a cofactor for GAPDH. NAD^+ is produced during reduction of pyruvate to lactate. The published

results showed that inhibiting CA with topiramate prevents OxS build up and PC loss in the diabetic mouse (Price et al. 2012, 2015). Our unpublished studies show that germline deletion of mCA reduces retinal OxS and topiramate prevents acellular capillary formation in diabetic mouse retina. Topiramate is a sulfamate substituted monosaccharide with impressive safety record (Leniger et al. 2004; Nishimori et al. 2005) in clinical use for other diseases (Deutsch et al. 2003; Liang et al. 2005; Roy Chengappa et al. 2001) and can be tested for prevention of DR. This is further encouraged with the low dose of topiramate needed (Price et al. 2015) and lack of potential systemic effect if given topically. No published reports explain how mCA inhibition affects PK isoforms or succinate and pyruvate pathways. Thus, how diabetes and mCA inhibition affect these pathways are of significant importance.

6.5 Carbonic Anhydrases

The carbonic anhydrases (CA) are a family of zinc metalloenzymes that catalyzes the hydration of CO_2 and dehydration of bicarbonate (Sly and Hu 1995) in the following reaction:

$CO_2 + H_20 \Leftrightarrow H^+ + HCO_3{}^-$. Twelve enzymatically active CA isozymes have been identified, including five cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), five membrane bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms (CA VA and CA VB), and a secreted CA isozyme (CA VI) (Sly and Hu 1995; Hewett-Emmett and Tashian 1996; Carter et al. 1990; Tureci et al. 1998; Mori et al. 1999). These isozymes differ widely in tissue specification, enzymatic kinetics, subcellular localization, participated pathways and in susceptibility to varied inhibitors (Hewett-Emmett and Tashian 1996; Carter et al. 1990; Tureci et al. 1998; Mori et al. 1999; Karler and Woodbury 1960). Three catalytically not active forms are also known, which are denominated CA-related proteins (CARP), CARP VIII, CARP X, and CARP XI (Hewett-Emmett and Tashian 1996). It was reported in the 1960s that mitochondria contain CA activity, which is classified as a separate isozyme, CA V (Karler and Woodbury 1960; Williams 1965; Chappell and Crofts 1965). It was later revealed that mCA are encoded by two separate genes, CA VA and CA VB (Nagao et al. 1993; Fujikawa-Adachi et al. 1999).

The human orthologue for the CA VA has been mapped to chromosome 16q24 (Nagao et al. 1995), while CA VB was mapped to chromosome Xp22.1 (Fujikawa-Adachi et al. 1999). The genes in mice are referred to as Car5A and Car5B and in humans as CA5A and CA5B by The Human Genome Organization Nomenclature Committee. Further studies reported that CA VB are much more highly conserved between mouse and human (95% identity) than the CA domains of mouse and human CA VA (78% identity) (Shah et al. 2000). Western blot analyses indicated that CA VA and CA VB represented in close molecular weights, 29 and 31 kDa, respectively. However, their tissue expressions are different, CA VA is only detectable in liver and skeletal muscle, whereas CA VB expression is detected in a wide range of tissues including heart, liver, lung, kidney, testes, muscle, and in most other tissues (Shah

et al. 2000). These differences in tissue-specific expression suggest that these two isoforms may replace each other's in different tissues. Furthermore, the difference in sequence conservation between CA VB and CA VA may indicates the functional variations in between these two isoforms.

CO_2 and HCO_3^- cycle plays an important role in mitochondrial metabolism and their regulation is crucial for mitochondrial functions (Dodgson et al. 1980). The enzymes of the tricarboxylic acid cycle that produce CO_2 are located within the mitochondrial matrix (Karler and Woodbury 1960), as are those enzymes that fix CO_2 in the pathways of gluconeogenesis and urea production. CO_2 freely penetrates into the mitochondria; however, mCA are required to convert CO_2 to HCO_3^- to support the metabolic needs for essential pathways. The synthesis of carbamoyl phosphate is the first irreversible step of ureagenesis. This reaction is catalyzed by carbamoyl phosphate synthetase-I, and it consumes HCO_3^- rather than CO_2 (Lusty 1978). Furthermore, carbamoyl phosphate is used as a co-substrate by ornithine transcarbamylase in the synthesis of citrulline, which is the first intermediate of the urea cycle (Cohen 1981). The use of CA inhibitors (e.g., acetazolamide) significantly reduced the synthesis of citrulline in isolated mitochondria (Dodgson et al. 1983) and hepatocytes (Dodgson and Forster 1986a). This confirmed that liver mCA plays its physiological role in urea synthesis by supplying the HCO_3^-.

Besides ureagenesis, mCA are involved in gluconeogenesis. The mammalian liver is the primary site of gluconeogenesis, and the carboxylation of pyruvate occurs exclusively in the mitochondria (Winter et al. 1982). Pyruvate carboxylase mediates the first reaction in gluconeogenesis from pyruvate, and HCO_3^- is required for this step (Dodgson and Forster 1986b). The treatment of hepatocytes with a membrane permeant sulfonamide, ethoxzolamide resulted in a reduction in the rate of pyruvate carboxylation in intact mitochondria (Dodgson and Forster 1986b). As such, when there is a requirement for bicarbonate as substrate, mCA are functionally important for gluconeogenesis in the male guinea pig liver (Dodgson and Forster 1986b). The importance of mCA in gluconeogenesis is supported by studies on other mammalians, such as mouse (Shah et al. 2013c) and rat tissues (Dodgson and Contino 1988). It has been shown that ethoxzolamide inhibits lipogenesis from pyruvate, similar to gluconeogenesis (Lynch et al. 1995). The effects of sulfonamides on hepatic de novo lipogenesis was initially reported as a result of the regulation of acetyl CoA carboxylase. However, later reported studies suggested that pyruvate carboxylase may be responsible for these effects (Lynch et al. 1995). A recent study indicated that ethoxzolamide and another sulfonamide, trifluormethanesulfonamide reduced de novo lipogenesis in 3T3-L1 adipocytes (Hazen et al. 1996). Furthermore, citrate concentration was reduced in ethoxzolamide treated adipocytes, which was possibly caused by a decrease in the export of mitochondrial citrate to the cytosol. This reduction of mitochondrial citrate appeared to be enough to cause the decrease in de novo lipogenesis (Hazen et al. 1996). This is consistent with the hypothesis that sulfonamide CA inhibition of de novo lipogenesis is through a decrease in substrate (i.e., bicarbonate) availability to pyruvate carboxylase, rather than acetyl CoA carboxylase inhibition alone causing an increase in citrate levels (Hazen et al. 1996).

Along with studies that used CA inhibitors, Shah and associates performed targeted disruption of the murine CA genes, Car5A and Car5B (Shah et al. 2013c). In this study, Car5A null mice were reported as poor breeders, with smaller litters as compared to wild-type littermates. However, their breeding normalized when their water was supplemented with sodium–potassium citrate. Their blood ammonia concentrations were significantly higher, yet their fasting blood sugars were normal (Shah et al. 2013c). Car5B null mice had normal growth, normal blood ammonia and fasting blood sugars levels. Car5A/B double-knockout (DKO) mice showed more severe growth problems and hyperammonemia than Car5A null mice. Supplementation with sodium–potassium water was not enough to normalize breeding. In addition, DKO mice survival rate and fasting blood glucose levels were markedly lower than wild type mice. This study indicated that both Car5A and Car5B contribute to ureagenesis and gluconeogenesis. Car5A plays a predominant role in ammonia detoxification, and while Car5B plays a role in both ureagenesis and gluconeogenesis, its role became evident only in a Car5A null background (Shah et al. 2013c).

Although CA are traditionally considered to be transport enzymes, especially mCA are involved in several biosynthetic pathways. Studies carried out with CA inhibitors, or with CA mutations, indicate that mCA are important for providing HCO_3^- to the initial steps in urea, fatty acid and glucose synthesis. Thus, modulation of their activity might have various therapeutic potential, such as reduced OxS and obesity.

6.6 Mitochondrial Carbonic Anhydrases and Oxidative Stress

Oxidative stress in diabetes is generated by excess ROS (Nishikawa et al. 2000; Du et al. 2000), which are normal byproducts of electron transport chain (ETC) reactions in the reduction of glucose to H_2O and CO_2 in the production of ATP (Chen et al. 2003; Liu et al. 2002). In diabetes, more glucose floods to the Krebs cycle, especially in insulin-insensitive tissues (Liu et al. 2002), thus increasing the rate of production of electron donors (reduced flavin adenine dinucleotide and reduced nicotinamide adenine dinucleotide). These electron donors create a proton gradient across the inner mitochondrial membrane during ETC reactions. Under higher electrochemical potential difference, the life time of superoxide-generating electron-transport intermediates are prolonged (Brownlee 2001). This causes a marked increase in the production of ROS, superoxide dismutase, and hydroxyl radicals and when it surpasses the threshold value generates OxS in tissues. Hyperglycemia induced OxS and mitochondrial dysfunction are involved in the pathogenesis of various complication of diabetes (Jha et al. 2018; Wu et al. 2018). Higher mitochondrial superoxide level has been detected in human aortic EC under high glucose conditions (Brownlee 2005). Mitochondrial CA regulate the oxidative metabolism of glucose, and thus, play important roles in the generation of ROS and OxS.

Recent studies indicated that using mCA inhibitors may decrease OxS in various tissues. The application of topiramate, a potent mCA inhibitor, prevented the OxS in the brain of diabetic mice (Price et al. 2012). Same study also reported a significant decline in cerebral PC numbers, at 12 weeks of diabetes that was also rescued by topiramate treatment (Price et al. 2012). This study has provided the first evidence that inhibition of CA activity reduces diabetes-induced OxS in the mouse brain and rescues cerebral PC loss. Similar results were obtained in an in vitro study using immortalized cerebral PC cultures (Shah et al. 2013b). This study indicated that both high glucose-induced OxS and apoptosis of PC could be rescued by pharmacological inhibition of CA (Shah et al. 2013b). Furthermore, the overexpression of mCA VA significantly increased intracellular ROS and apoptosis of PC (Patrick et al. 2015). In contrast, the genetic knockdown of mCA VA significantly reduced high glucose-induced ROS and apoptosis in PC (Price and Sheibani 1863).

6.7 Carbonic Anhydrase Inhibitors and Prevention of Diabetic Retinopathy

Currently available CA inhibitors are grouped under two main classes: the metal-chelating anions and the unsubstituted sulfonamides and their bioisosteres. These inhibitors target the Zn^{2+} ion, preventing its interaction with enzymes by substituting or changing its coordination (Boddy et al. 1989). Sulfonamides, which are the most important CA inhibitors, such as the clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide, bind to Zn^{2+} ion in the deprotonated state and form a slightly distorted tetrahedral adduct (Supuran and Scozzafava 2007). Carbonic anhydrase inhibitors are widely used for different clinical applications. They were first used as diuretics and are currently as anti-epileptic, anti-obesity, anticancer and antiglaucoma agents. The diversity of CA isoforms, their tissue diffusion, and their different biological functions have created some hurdles to overcome in the pursuit of the use of CA inhibitors as therapeutics. Conversely, these specific attributes of the CA isoforms can also provide extensive opportunities to create more specific drug-design approaches.

Several studies have provided evidence that CA inhibitors may be effective against obesity (Picard et al. 2000; Gadde et al. 2003,2011; Allison et al. 2012). Topiramate and zonisamide are potent anticonvulsant agents and are currently in use as antiepileptic drugs (Ben-Menachem 1996; Kellett et al. 1999; Schmidt et al. 1993). Some studies indicate that topiramate and zonisamide also cause loss of body weight in obese animals (Picard et al. 2000) and patients (Supuran 2008; Scozzafava et al. 2013) via the disruption of the de novo lipogenesis. Topiramate inhibits several CA isozymes, such as CA II, VA, VB, VI, VII, XII and XIII. The mechanisms of CA inhibitor-induced weight loss are not well defined. However, the inhibition of CA VA and VB may play an important role due to the involvement of mCA VA and VB in various metabolic pathways, such as lipogenesis and glucogenesis (described

above). Indeed, molecular modeling and structural studies have indicated that topiramate and zonisamide have strong affinity for mCA VA and VB (Ki's for mCA VA and VB are of 63 and 30 nM, respectively) (Nishimori et al. 2005; Vitale et al. 2007; Dodgson et al. 2000). Another study suggested that the inhibition of mCA cause weight loss due to a reduction in the rate of pyruvate that passes through the pathway, and disruption in glycolysis, allowing for fatty acid oxidation to become the dominant pathway (Arechederra et al. 2013).

Indeed, mCA regulate the metabolism of pyruvate by accelerating the rate at which pyruvate carboxylase can convert pyruvate and bicarbonate into oxaloacetate (Jitrapakdee et al. 2008). The pyruvate is derived from glucose through glycolysis, and this pathway is in equilibrium with the fatty acid pathway. Therefore, mCA inhibitors may shift the flux of mitochondrial metabolism toward using fatty acids and slow the metabolism of pyruvate (Arechederra et al. 2013).

Recent studies indicate that the inhibition of mCA can be effective against diabetes complications, such as diabetic retinopathy. Initial studies reported that the use of CA inhibitors, such as acetazolamide is effective in treatment of macular edema when administered systemically (Gelisken et al. 1990). During the past two decades, emerging evidence has suggested that CA inhibitors may hold promise in the treatment of diabetic retinopathy (Weiwei and Hu 2009). A series of study by Shah and associates indicated that genetic knockout (Shah et al. 2013c) or pharmacological inhibition (Shah et al. 2013b; Price et al. 2012) of CA were able to reduce respiration, ROS, and PC apoptosis in brain. As one of the most metabolically active tissues in the body, the brain is notably vulnerable to OxS. Therefore, reducing OxS may protect the brain from the damage caused by hyperglycemia. One of the first reports about the effect of CA inhibitors against diabetes was from streptozotocin (STZ)-induced diabetic mouse model (Price et al. 2012). STZ treated mice have progressive hyperglycemia, OxS and decreased PC to EC ratio in the mouse brain, which can lead to deterioration of the blood–brain barrier (BBB). Price et al. reported that the inhibition of CA with topiramate prevented the OxS in the brain and restored normal cerebral PC to EC ratio (Price et al. 2012). Shah and Sheibani (2013) later showed that high glucose-induced intracellular OxS can cause brain PC death by apoptosis, and treatment with pharmacological inhibition of CA significantly reduced PC apoptosis (Shah et al. 2013b).

Increased ROS production is a major cause of OxS in diabetes. Cerebral PC generate ROS by oxidative metabolism of glucose (respiration) under high glucose conditions (Shah et al. 2013a). The rate of respiration and ROS production are reversed upon pharmacological inhibition of CA in high glucose challenged cerebral PC (Shah et al. 2013a). Furthermore, the overexpression of mCA VA significantly increased intracellular ROS and apoptosis of PC. Both ROS and the percent of apoptotic PC were significantly reduced upon inhibition of mCA VA (Patrick et al. 2015). Similarly, the genetic knockdown of mCA VA significantly reduced high glucose-induced ROS and apoptosis in brain PC (Price and Sheibani 1863). Animal studies supported in vitro studies; morphologic and permeability abnormalities detected in BBB of STZ-induced diabetic CD-1 mice. The CA inhibitor topiramate was able to prevent STZ-induced BBB damage in certain regions (Salameh et al. 2016). These

data demonstrated that mCA are important in the regulation of high glucose-induced ROS production and cerebral PC death, with CA inhibitors affording protection of cerebral PC during diabetes.

Besides cerebral PC, retinal PC are also highly sensitive to hyperglycemia, and loss of retinal PC are recognized as one of the earliest signs of DR (Cogan et al. 1961; Hammes et al. 2002). Retinal PC, but not retinal EC or AC, undergo significant apoptosis in response to chronic exposure to high glucose conditions (Gurel et al. 2014). High-glucose induced OxS detected in pig retinal PC by increased 8-OHdG (Kubo et al. 2009) and in bovine retinal PC by advanced glycation end products (AGEs) and receptor of AGE (RAGE) (Yamagishi et al. 1995). Recently, a time-lapse microscopy study by our group demonstrated that the rate of mitochondrial ROS production in mouse retinal PC was significantly higher under high glucose condition, whereas this rate remained unchanged in retinal EC. In this study, we used MitoSOX™ to indicate the production of superoxide in mitochondria. Therefore, this study provided direct evidence on mitochondria-related ROS generation in retinal PC under high glucose conditions (Ghanian et al. 2018). Furthermore, various studies reported that diabetes or high glucose-driven PC loss may be prevented by anti-oxidative agents such as the treatment with nicanartine (Hammes et al. 1997), Trolox (Ansari et al. 1998), pigment epithelium-derived factor (Yamagishi et al. 2002; Sheikpranbabu et al. 2011) and resveratrol (Kim et al. 2012).

Retinal PC have different metabolic dynamics than their companion, retinal EC. Under high glucose conditions, glucose uptake increased significantly in retinal PC, however retinal EC demonstrated better control on glucose flow into the cell (our unpublished data). Thus, elevated glucose uptake and increased oxidative phosphorylation may explain increased ROS production and retinal PC loss during diabetes. Interestingly, mouse retinal PC had higher Car5a expression than other cells, including retinal ChEC, REC, RAC and RPE. For Car5b, both retinal PC and RAC had high expression compared to ChEC, REC, and RPE cells (Fig. 6.2). Currently, there is a lack of studies that indicate a protective effect of CA inhibitors on retinal PC under diabetic conditions. However, we propose that CA inhibitors could reduce OxS in retinal PC as occurs in cerebral PC. Thus, CA inhibitors have great potential for use against DR through anti-obesity and protective effects in cerebral and retinal PC.

6.8 Conclusions

Attenuation of oxidative metabolism by CA inhibitors provide protection in retinal PC under high glucose conditions. Additional studies of current CA inhibitors and discovery of new and more specific mCA inhibitors will be important in prevention and treatment of diabetic retinopathy. Topiramate is one of the most investigated agents among CA inhibitors. Zoinisamide has similar effects, and it is a more potent inhibitor of CA VA than CA II (Vitale et al. 2007; Simone et al. 2005). Furthermore, investigators have reported other CA inhibitors that are effective on mCA.

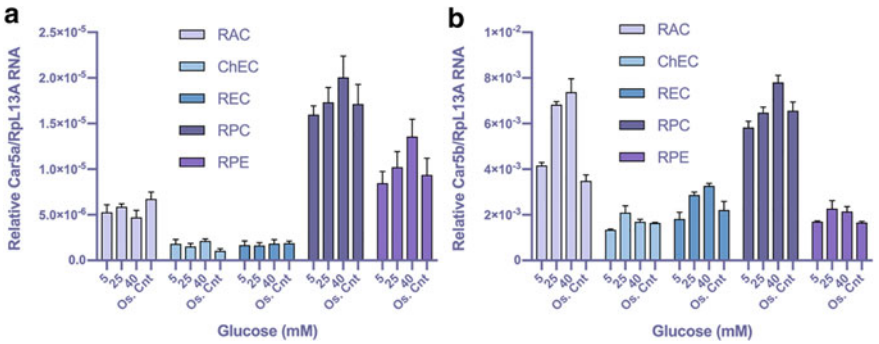

Fig. 6.2 RNA expression of Car5a and Car5b in retinal cells. Car5a (**a**) and Car5b (**b**) expression levels were determined by qPCR for each cell type grown under D-glucose (5, 25 or 40 mM) conditions for five days as well as an osmotic control (5 mM D-Glucose plus 35 mM L-Glucose; "Os. Cnt; Osmotic Control"). RNA expression for each gene was normalized to RpL13A. Cell types assessed included Retinal astrocyte (RAC), Choroidal endothelial cells (ChEC), Retinal endothelial cells (REC), Retinal pericytes (RPC), and Retinal pigment epithelial (RPE) cells. The qPCRs were performed with three biological replicates and in triplicates

Some of the investigated sulfonamides, such as the ureido benzenesulfonamides and the acylated sulfanilamides showed higher affinity for CA V than for the other isozymes, CA II included (Vullo et al. 2004). (*N*-(2-fluoro-4-sulfamoyl-phenyl)-2-(2-thienyl)acetamide (II) and *N*-(2-bromo-4-sulfamoyl-phenyl)-2-phenyl-acetamide (IV)) compounds are reported as highly selective for CA VA and CA VB over CA I and CA II (Guzel et al. 2009). Investigation of 4-(4-phenyltriazole-1-yl)-benzene sulfonamide derivatives (Poulsen et al. 2008), series of aromatic/heterocyclic sulfonamides (Winum et al. 2007) and a small series of 2-substituted-1,3,4-thiadiazole-5-sulfamides (Smaine et al. 2008) indicated that several of them form each group are selectivity effective for mCA VA and VB. Another report indicated that sulpiride and ethoxzolamide were almost two times more effective inhibitors of the mCA VB over the cytosolic isozymes (Nishimori et al. 2005). Moreover, testing a series of (*R*)-/(*S*)-10-camphorsulfonyl-substituted aromatic/heterocyclic sulfonamides demonstrated that all tested compounds are selective for mCA VA and VB over CA I, CA II with the best being thiadiazol sulfonamide X (Maresca and Supuran 2011).

Inhibiting mCA deprives the mitochondria of bicarbonate, thus reducing the respiration rate, OxS, PC loss, and retinal vascular degeneration. A key question that still needs further exploration is whether PC respiration rate is key to retinal microvasculature disruption and dysfunction in early diabetes. These findings would also establish mCA as possible therapeutic targets whose inhibition can delay the onset and/or slow down the progression of DR and neuroinflammation. Topiramate is in clinical use for other diseases, which makes the translational research potential of this work a distinct possibility. In addition, confirmation that topiramate protects the retina would spur novel studies to determine whether it protects from other microvascular complications of diabetes, including neuropathy and nephropathy. Therefore,

the selective inhibition of mCA may lead to the development of novel pharmacological applications for prevention and treatment of diabetes complications with limited potential systemic adverse effects.

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