Chapter 5
Local RAS

5.1 Definition of the Local RAS

The concept of a circulating RAS is well established and known to play an endocrine role in the regulation of fluid homeostasis (see Section 4.1, Chapter 4). However, it is more appropriate to view the RAS in the contemporary notion as an "angiotensin-generating system", which consists of angiotensinogen, angiotensin-generating enzymes, and angiotensins, as well as their receptors. Some RASs can be termed as "complete", having renin and ACE involved in the biosynthesis of angiotensin II peptide, i.e. in a renin and/or ACE-dependent manner which is exemplified in the circulating RAS. On the other hand, some RAS can be termed as "partial", having alternate enzymes to renin and ACE, such as chymase and ACE2 (see Section 4.3, Chapter 4) available for the generation of angiotensin II and other bioactive angiotensin peptides in the biosynthetic cascade, i.e. in a renin and/or ACE-independent manner. Complete vs. partial RASs can be exemplified in the so-called intrinsic angiotensin-generating system or local RAS; for example, a local and functional RAS with renin and ACE-dependent but a renin-independent pathway have been indentified in the pancreas (Leung, 2007) and carotid body (Lam & Leung, 2002), respectively. In the past two decades, local RASs have gained increasing recognition especially with regards to their clinical importance. Distinct from the circulating RAS, these functional local RASs exist in such diverse tissues and organs as the pancreas, liver, intestine, heart, kidney, vasculature, carotid body, and adipose, as well as the nervous, reproductive, and digestive systems (Paul et al., 2006). Taken into previous findings from our laboratory and others together, Table 5.1 is a summary of some recently identified local RASs in various levels of tissues and organs.

Local RASs can operate in an autocrine, paracrine and/or intracrine manner, and exhibit multiple physiological effects at the cellular and tissue levels in addition to and distinct from those of the circulating RAS (Lavoie & Sigmund, 2003). In addition to hemodynamic actions, local RASs have multiple, novel functions including regulation of cell growth, differentiation, proliferation and apoptosis, reactive oxygen species (ROS) generation, tissue inflammation and fibrosis, and hormonal secretion (Leung, 2004). Such a diversity of roles makes local RASs attractive

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Table 5.1 Some examples of recently identified intrinsic angiotensin-generating systems in different tissues and organs

Tissues and organs	Components of local angiotensin-generating system
Heart	Renin, ACE, Chymase, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Vasculature	Renin, ACE, ACE2, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Brain	Renin, ACE, Tonin, Cathepsin, Chymase, Angiotensinogen, Ang I, Ang II, Ang IV, Ang (1–7), AT_1, AT_2 & AT_4 receptors
Ovary	Renin, ACE, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Uterus	Renin, ACE, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Testis	Renin, ACE, Angiotensinogen, Ang I, Ang II & AT_1 receptors
Epididymis	Renin, ACE, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Liver	Renin, ACE, Angiotensinogen, AT_1 & AT_2 receptors
Pancreas	Renin, ACE, Angiotensinogen, Ang I, Ang II, AT_1 & AT_2 receptors
Intestine	Renin, ACE, ACE2, Angiotensinogen, Ang II, AT_1 & AT_2 receptors
Adipose	Renin, ACE, Angiotensinogen, Ang I, Ang II & AT_1 receptors
Carotid body	ACE, Angiotensinogen, Ang II, AT_1, AT_2 & AT_4 receptors

therapeutic targets in diverse disease states. In the following sections, three of these functional local RASs, which have been characterized and investigated in my laboratory, will be presented for discussion and critical appraisal of their physiological roles and clinical relevances.

5.2 Local RAS in Carotid Body

The carotid body is a highly vascularised network of chemoreceptor cells, located at the bifurcation of the internal and external carotid arteries from the common carotid artery. Carotid body chemoreceptors, called glomus (or type 1, catechol-secreting) cells, release dopamine in response to rapid changes in arterial oxygen pressure (PO_2), carbon dioxide pressure (PCO_2), and pH, thus increasing carotid body sinus nerve activity and provoking centrally-mediated cardiopulmonary responses (Gonzalez et al., 1994). In this context, the carotid body is critical for physiological responses to acute, chronic and intermittent hypoxia. Hypoxia activation of the RAS might be associated with chronic cardiopulmonary diseases and sleep apnea. Of great interest in this context is emerging evidence for the existence of a local carotid angiotensin-generating system, which is upregulated by chronic hypoxia to alter carotid chemoreceptor sensitivity.

5.2.1 Expression and Function of Carotid Body RAS

As mentioned, newly discovered functions of intrinsic angiotensin-generating systems have been identified in many tissues and organs and are of particular physiological and pathophysiological interest. Such systems have recently been proposed to exist in the carotid body, where key RAS components including angiotensinogen, ACE, AT_1 and AT_2 receptors as well as AT_4 receptors (Table 5.1) are localized and expressed in the absence of renin, thus suggesting the existence of a renin-independent biosynthetic pathway for angiotensin II in the carotid body (Lam & Leung, 2002). The local RAS in carotid body plays a key role in modulating the carotid body response to hypoxia. Angiotensin II itself increases carotid body efferent activity, presumably via activation of the AT_1 receptor (Allen, 1998). Meanwhile, chronic hypoxia upregulates several key RAS components in the carotid body, including time-course dependent ACE activity (Lam & Leung 2003; Lam et al., 2004). Specifically, AT_1 receptor expression on glomus cells is enhanced, consistent with increased transcription and translation of the AT_1 receptor (Leung et al., 2000). Chronic hypoxia thus enhances AT_1 receptor-mediated efferent activity (Leung et al., 2000). In addition, AT_1 receptor activation increases intracellular calcium levels in dissociated glomus cells, an effect which is enhanced threefold by chronic hypoxaemia (Fung et al., 2001). Interestingly, postnatal hypoxemia is associated with an increased sensitivity of peripheral chemoreceptors in response to angiotensin II; in this condition, there is an upregulation of AT_{1a} receptor-mediated intracellular calcium activity, but a down-regulation of AT_{1b} receptor, of the chemoreceptors. These data may be important for adaptation of carotid body functions in the hypoxic ventilatory response and in electrolyte and water balance during perinatal and postnatal hypoxia (Fung et al., 2002).

High-affinity binding sites for angiotensin IV, known as AT_4 receptors (also called insulin-regulated membrane aminopeptidase, IRAP) were found to be localized and expressed in various tissues and organs (Albiston et al., 2001). As discussed in Chapter 4, angiotensin IV, a pentapeptide containing a 3–8 peptide fragment from an angiotensin II metabolite, is a biologically active peptide of the RAS which exerts its action via mediation of AT_4 receptor. Interestingly, AT_4 receptors are expressed and localized in the rat carotid body and, more importantly, play a prominent role following its upregulation in chronic hypoxia (Fung et al., 2007). This observation is consistent with the fact that the expression and function of local RAS components, notably AT_1 receptors and ACE in the carotid body, are upregulated by chronic hypoxia (Leung et al., 2000; Lam & Leung 2003; Lam et al., 2004). In addition, activation of AT_4 receptors can increase local production of angiotensin II in the carotid body, which in turn elevates angiotensin IV and thus activates an intracellular signalling cascade leading to increased intracellular calcium concentrations. It is plausible to speculate that the activation of AT_4 receptors in the carotid body may serve as an additional pathway enhancing the angiotensin II action in the activation of the chemoreflex in the physiological response to chronic hypoxia (Fung et al., 2007). Taken together, all of these available data suggest that chronic hypoxia is a major factor in increasing major RAS component expression and function in the

carotid chemoreceptor, with likely changes in cardiopulmonary function. Thus the excitatory action of angiotensin II on the glomus cells may increase the chemosensitivity of the carotid body and may counteract the blunting effect of chronic hypoxia. Notwithstanding this involvement of AT_4 receptor-mediated carotid body function, details of the molecular and cellular mechanisms underlying the modulation of this receptor require extensive study.

5.2.2 Carotid Body RAS and Congestive Heart Failure

In a recent report using an animal model of congestive heart failure (CHF), a role for local glomus angiotensin II/AT_1 receptor signaling in increasing the sensitivity of Kv channels to hypoxia has been shown (Li & Schultz, 2006). In this study, high concentration of angiotensin II (>1 nM) directly inhibits Kv currents (I_k) while changes in Kv channel protein expression may contribute to the suppression of I_k and enhanced sensitivity of I_k to hypoxia in a CHF state. These findings have two significant implications in term of clinical setting: angiotensin II can enhance the oxygen sensitivity of Kv channels via the AT_1 receptor, and the cellular angiotensin

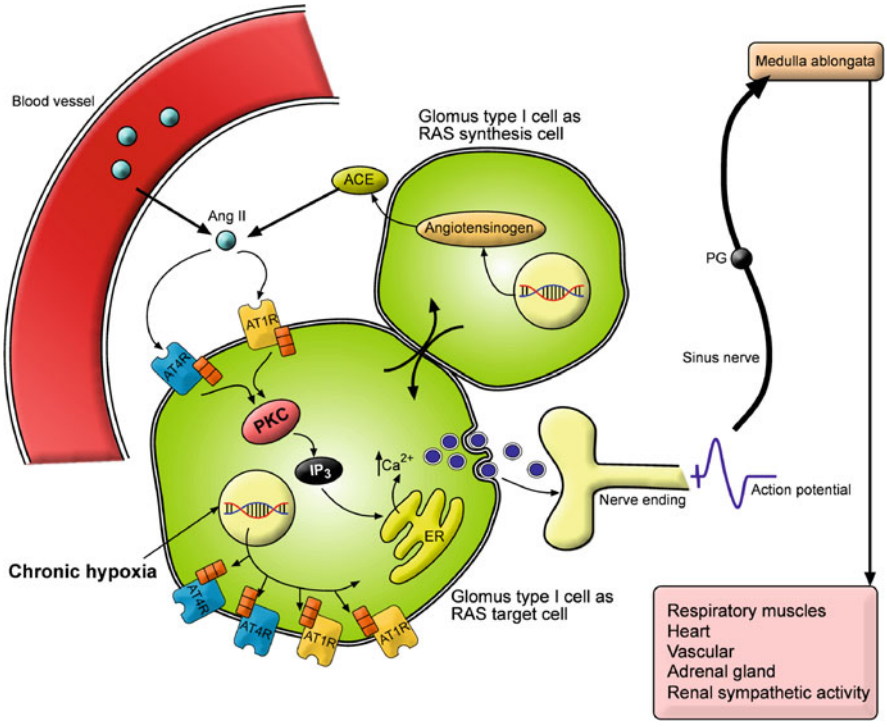

Fig. 5.1 Representation of the relationship of circulating RAS and carotid RAS involved in the regulation of cardiopulmonary function (modified from Leung et al., 2003)

II/AT_1 receptor pathway is functional in carotid glomus cells of CHF, but not normal animals. In spite of this solid evidence, the precise mechanism by which CHF upregulates the angiotensin II/AT_1 receptor and its function in the carotid body have yet to be resolved. In this regard, chronically impaired cardiac output resulting from CHF might be sufficient to render a prolonged deprivation of oxygen delivery to carotid glomus cells, a state akin to chronic hypoxia, thus leading to activation of its cellular RAS pathway. These data suggest that angiotensin II may have a paracrine and/or autocrine role in the modulation of enhanced carotid chemoreceptor sensitivity to chronic hypoxia characterized in CHF (Leung, 2006).

Taken together, the aforementioned findings suggest that chronic hypoxia is a major factor that increases major RAS component expression in the carotid body and activity of the carotid chemoreceptors, likely accompanied by changes in cardiopulmonary function. Thus the excitatory action of angiotensin II and its actions via AT_1 and AT_4 receptors on the glomus cells may increase the chemosensitivity of the carotid body while counteracting the blunting effect of chronic hypoxia. Notwithstanding the evidence of a functional angiotensin-generating system in the carotid body, details of the molecular and cellular mechanism(s) underlying the modulation require extensive further investigations. The potential interactions between circulating and local RAS-mediated effects of the carotid body in regulating cardiopulmonary function during normoxia and hypoxia are briefly summarized in Fig. 5.1.

5.3 Local RAS in Liver

The liver is a metabolically active, highly aerobic organ. It receives approximately 30% of the total blood flow and extracts 20% of the oxygen used by the human body (Hardikar & Suchy, 2003). Liver functions include, but are not limited to: (1) filtration and storage of blood; (2) metabolism of carbohydrates, proteins, fats, hormones, and foreign chemicals; (3) formation of bile; (4) storage of vitamins and iron; (5) formation of coagulation factors; (6) synthesis of hormone precursors, such as angiotensinogen; (7) excretion and degradation of hormones, drugs and toxins; and (8) exocrine bile secretion. The liver is composed mainly of hepatocytes, or liver cells, which represent about 60% of the total cell population. In addition to the hepatic cells, the venous sinusoids are lined by three other types of cells, namely the typical endothelial cells, large Kupffer cells, and hepatic stellate cells. Kupffer cells are a type of macrophage capable of phagocytizing bacteria and other foreign matter in the hepatic sinus blood (Alison, 1986). Hepatic stellate cells represent a small and versatile population cell type in the liver. Its major function of resting or quiescent hepatic cell is to store vitamin A whilst, during activation by various stimuli (e.g. injured hepatocytes, activation of kupffer cells and inflammatory cytokine and reactive oxygen species), hepatic stellate cells acquire a myofibroblast-like phenotype which plays a key role in the pathogenesis of liver fibrosis, including collagen deposition and abnormal extracellular matrix remodelling (Atzori et al., 2009).

5.3.1 RAS and Liver Function

Angiotensinogen is best known to be synthesized in the liver and it is the obligatory component of the circulating and local RAS as discussed in previous sections. Systemic and local RASs regulate liver function and liver disease. This role is particularly true for the local hepatic RAS, which remains largely ambiguous in the liver. In this regard, our recent findings have demonstrated the expression of several RAS components in the liver. Some, but not all, of the RAS components, including renin, ACE, and, in particular, the two AT_1 receptor subtypes (AT_{1a} and AT_{1b}), are expressed in Kupffer cells. The precursor angiotensinogen, the mandatory component for a locally generating-angiotensin system, however, is *not* present in Kupffer cells. While both renin and ACE are present, the AT_2 receptor appears not to exist in Kupffer cells (Table 5.2). These data lend support for the existence of an angiotensin-generating system in the liver and a potential role of angiotensin II in hepatic Kupffer cells (Leung et al., 2003). Interestingly, our observations further suggest that angiotensin II could upregulate the expression of TGF-β and fibronectin, and that a local hepatic RAS acting via AT_1 receptors located on Kupffer cells may play a role in the regulation of a fibrogenic action. Using a D-galactosamine-induced rat model of liver failure, AT_1 receptor blockade was demonstrated to prevent the progression of acute liver injury, as evidenced by an improvement in survival, reduction of liver enzymes, hepatic histopathology, and reduction in fibrogenic tissue-specific inhibitor of metalloproteinsase protein (Chan et al., 2007). These findings further indicate that hepatic AT_1 receptors may mediate liver inflammatory responses in liver injury. On the other hand, the RAS is also known to play an important role in chronic liver diseases, including liver cirrhosis and non-alcoholic steatohepatitis (NASH), the latter being the most frequent cause of chronic liver impairment (Lubel et al., 2008). In fact, the RAS is closely involved in liver fibrosis through activation of hepatic stellate cells, which mediates their fibrogenic actions by over-expression of profibrogenic factors in the liver. In an animal model of NASH, it has been shown that inhibition of the AT_1 receptor attenuates aspartate aminotransferase levels, activation of hepatic stellate cells, oxidative stress, expression of transforming growth factor-β1, expression of collagen genes, and liver fibrosis (Hirose et al., 2007).

Table 5.2 Comparison of the presence and absence of major RAS component expression in the liver and in the Kupffer cells

RAS component	Kupffer cells	Liver
Angiotensinogen	–	+++
AT_{1a} receptor	+++	++
AT_{1b} receptor	+	++
AT_2 receptor	–	++
Renin	++	++
ACE	+	+

+++, Very strong expression; ++, strong expression; +, weak expression; –, no expression.

In addition to activation of the AT_1 receptor, several RAS components are also upregulated in an experimental model of liver fibrosis, including angiotensin (1–7) and ACE2 (Herath et al., 2007). Indeed ACE2 expression is elevated in injured liver, in both humans and rats, which in turn regulates RAS activity in liver fibrosis (Paizis et al., 2005). In a rat model of cirrhosis with bile duct ligation, it has been recently demonstrated that angiotensin (1–7) is highly upregulated in human liver disease and has anti-fibrotic action, further supporting the notion that the ACE2-angiotensin (1–7)-mas receptor axis represents a potential target for anti-fibrotic therapy observed in liver cirrhosis (Lubel et al., 2009). In addition, administration of angiotensin II increases portal pressure in experimental and human cirrhosis, whereas AT_1 receptor antagonism abrogates the angiotensin II effect. These data suggest that angiotensin II may be involved in the pathogenesis of portal hypertension in cirrhosis (Rockey & Weisiger, 1996, Schneider et al., 1999). Angiotensin II has also been shown to exert its contractive action on the postsinusoidal venules (Arroyo et al., 1981), and the localization of AT_1 receptors in hepatic stellate cells gives new insight into the direction of RAS research with respect to regulation of liver fibrosis (Bataller et al., 2000). Recent studies have also shown that angiotensin II can induce contraction and proliferation of hepatic stellate cells via the AT_1 receptor. Taken together, these data indicate that angiotensin II plays a pivotal role in the development of liver fibrosis through the activation of hepatic stellate cells (Yoshiji et al., 2001). On the other hand, Kupffer cells have been known to get actively involved in fibrotic processes of the liver (Casini, 2000). Indeed, Kupffer cells constitute 90% of the sessile macrophages of the reticuloendothelial system and they are a major source of cytokines (Poli, 2000). Cytokines are believed to activate hepatic stellate cells, induce the proliferation and differentiation of stellate cells, and stimulate the production of extracellular matrix components of hepatic stellate cells, such as TGF-β and fibronectin. Despite of the above convergent evidence, the potential role of the RAS and its clinical relevance in liver disease are yet to be determined; this uncertainty is particularly true for the local hepatic RAS, which remains largely ambiguous and to be investigated.

5.3.2 Interaction Between Hepatic RAS and Vitamin D in T2DM

As just mentioned in the early beginning of Section 5.3, the liver is a vital organ containing several cell types, namely hepatocytes, stellate and Kupffer cells, with a central role in glucose metabolism and it is a key target for insulin action. As such, it is unsurprising that defects in liver cell function should increase the risks of type 2 diabetes mellitus (T2DM). Meanwhile, vitamin D is known best for its calcium and bone homeostasis but hypovitaminosis D, or vitamin D deficiency, also increases insulin resistance, impairs insulin secretion and is associated with, and predicts, all metabolic syndrome components, T2DM and also non-alcoholic fatty disease and fibrosis of the liver (NAFLD, a recognised part of the metabolic syndrome). In keeping with these, hypovitaminosis D is linked with enhanced risks of NAFLD (Targher et al., 2007) and hepatic fibrosis with cirrhosis developing in 10% of such

patients (Harrison, 2006). Hypovitaminosis D is associated with increased risks of each component of metabolic syndrome: central obesity, glycemia, blood pressure, adverse lipid profiles, insulin resistance, T2DM, and cardiovascular disease (see review by Boucher, 1998). Mechanisms explaining vitamin D's effects on insulin sensitivity are not fully elucidated but, as for islet cell function, may include increasing intracellular ionised calcium or modulation of the many genes with vitamin D receptor response elements in their promoter regions (Wang et al., 2005). NAFLD increases hepatic insulin resistance, independent of general obesity in humans while vitamin D inhibits adipogenesis via vitamin D receptor (VDR)-mediated inhibition of PPARγ activity (Woo et al., 2008). Vitamin D is both 25-hydroxylated and activated in liver (Hollis, 1990) by local 1α-hydroxylase and there are fully functional VDRs in Kupffer, stellate and endothelial cells as well as in the hepatocytes (Gascon-Barre et al., 2003); thus vitamin D could be expected to exert direct effects on hepatic insulin signalling pathway genes.

Concurrently, several clinical studies have reported inverse relationships between circulating concentrations of activated vitamin D and blood pressure in patients with high-renin activity hypertension (Lind et al., 1995; Burgess et al., 1990). VDR-null mice have been shown to have a defect with increases in renin expression consistent with the suggestion that vitamin D is a negative regulator of the RAS (Li et al., 2002). A potential mechanism for this effect has subsequently been demonstrated, showing that the angiotensin II feedback suppression of renin expression was different in VDR-null mice compared with wild type mice (Kong & Li, 2003). In light of these background findings, it is plausible to suggest that vitamin D, via direct actions and by modulation of RAS activity, could exert effects on hepatic insulin sensitivity, NAFLD and metabolic syndrome such as T2DM. As a first step to test our hypothesis, we first examine the localization, expression and regulation of major RAS components in an animal model of obesity-induced db/db diabetic mouse. Protein levels of AT_1 receptor, ACE and renin in obese diabetic mice are significantly upregulated while AT_2 receptor is down-regulated when compared with their respective control livers, as evidenced by Western blot analysis (Fig. 5.2). By means of double immunostaining, AT_1 receptors are specifically localized in hepatic stellate cells, hepatocytes and Kupffer cells with immunoreactivity being much intense in the livers from obese diabetic mice when compared with their respective control livers (Fig. 5.3). With these preliminary data available, we will further address our hypothesis that vitamin D specifically reduces hepatic insulin resistance through modulation of the local hepatic RAS. To address this issue, we will study the effects of hormonal vitamin D on hepatic RAS activity, on hepatic insulin receptors and on post-insulin receptor signalling pathways relevant to insulin sensitivity in vitro and in vivo. We will also determine whether human hepatic insulin sensitivity, specifically, increases with adequate supplementation in hypovitaminosis D and whether this is associated with circulating RAS activity reduction. Insulin resistance contributes to cardiovascular diseases, metabolic syndrome and T2DM, increasingly common world-wide problems presenting huge demand on healthcare resources. Thus, these findings may provide novel approaches to the prevention and management of NAFLD as well as of metabolic syndrome and its sequelae.

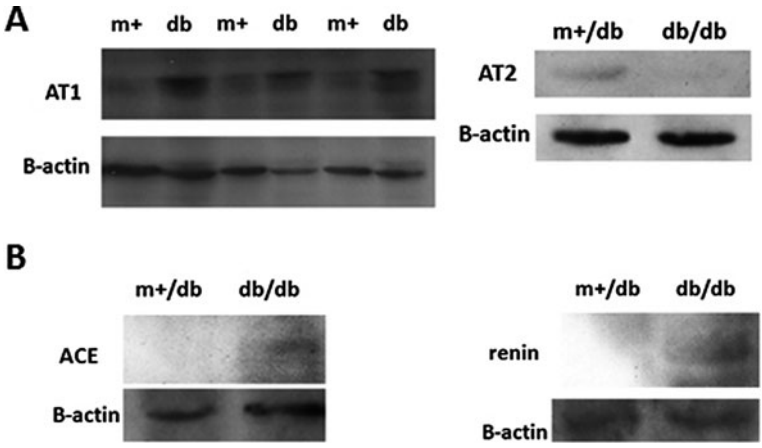

Fig. 5.2 Western blot analysis of some major RAS component expression in the liver of lean m+/db (m+) and diabetic db/db (db) mice. **a** Expression of AT1R protein. Expression of AT2R protein. **b** Expression of ACE protein. Expression of renin protein. The expression levels of AT1R, ACE and renin were upregulated in db/db mouse liver while AT2R had a reduced protein expression when compared with their respective controls

5.4 Local RAS in Intestine

The two primary physiologically roles of intestine are the digestion of food and absorption of electrolytes and nutrients such as glucose. Glucose is a crucial metabolic substrate for most cell types. To achieve glucose uptake by different cell types in our body, there are two categories of glucose transporters, one is the secondary active transporters, i.e. SGLTs and the other is the facilitated transporters, i.e. GLUTs (Table 5.3). The absorption of glucose involves an entry-and-exit mechanism mediated by two separate protein carriers located in the brush border membrane (BBM) and basolateral membrane (BLM) of enterocytes (Wright et al., 1994). This mechanism is energized by a BBM bound ATP-dependent Na^+ pump (SGLT1), an active glucose transporter, that maintains a Na^+ gradient favouring the entry of Na^+ with the concomitant co-transport of glucose or galactose into the enterocytes. SGLT proteins are expressed in many tissues, particularly SGLT1 in the epithelial cells of the small intestine (Wright et al., 2004, 2006) while SGLT2 and SGLT1 are found in the kidney (Pajor et al., 1992; Kanai et al., 1994). A second carrier (GLUT2) is located in the BLM then facilitates glucose expulsion from the cell. The GLUT family of transporters consists of 14 members, but the most commonly expressed isoforms involved in glucose uptake by renal and intestinal epithelia are GLUT1 and GLUT2 (Kellett et al., 2008). At the enterocyte BBM, SGLT1 is a high-affinity, low-capacity transporter that allows active glucose uptake from the intestinal lumen. SGLT1-mediated glucose uptake, in turn, promotes the insertion of GLUT2 into the apical membrane, which provides an additional high capacity pathway for glucose transport that is crucial for absorbing the high level of

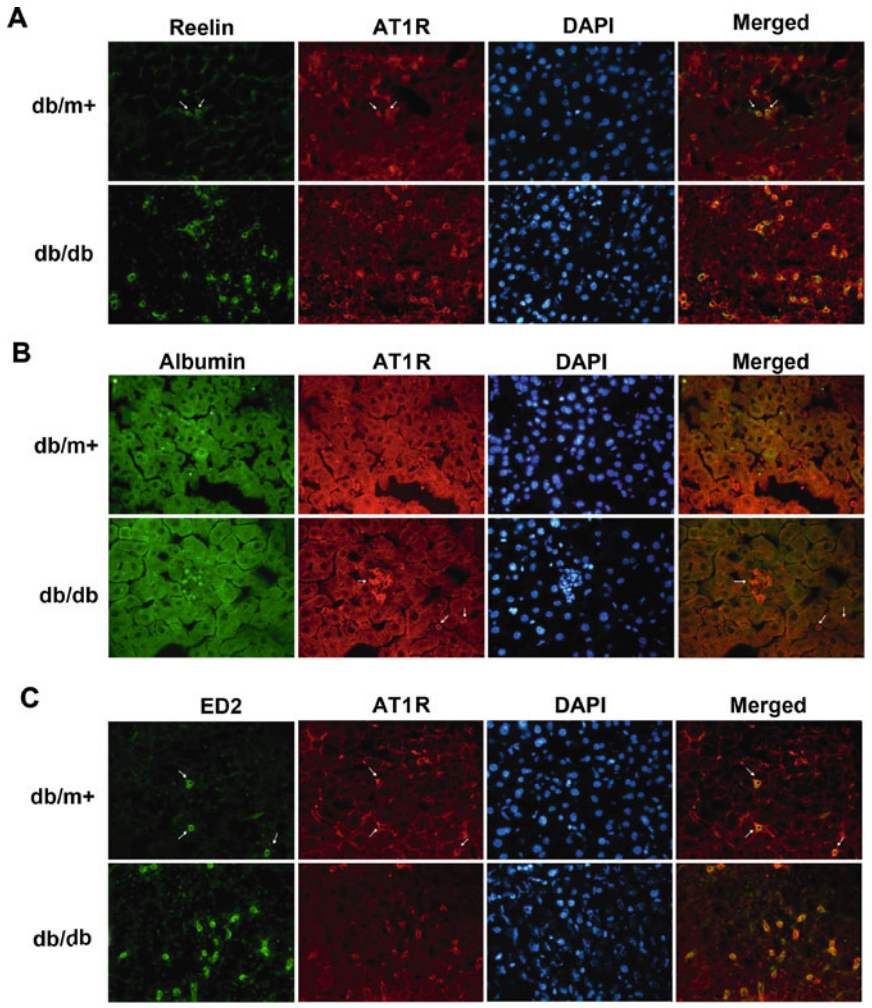

Fig. 5.3 The expression and localization of AT1R in stellate cells (Reelin as a marker) and hepatocytes (albumin marker) and Kupffer cells (ED2 as a marker) from the liver of obese diabetic db/db mice and control lean db/m+ mice. Immunoreactivity of AT1R was immunolabeled with specific liver cell markers of stellate cells, Reelin (**a**), of hepatocytes, Albumin (**b**) and of Kupffer cells, ED2 (**c**). AT1R immunoreactivity was stained with red; Reelin, albumin and ED2 immunoreactivity was stained with green (see *arrows*). Overlay of the immunofluorescence labeling of db/db livers showing more intense immunostaining for AT1R was observed in both stellate cells and Kupffer cells but not in hepatocytes (*merged images*), when compared with their respective control livers. DAPI was used as a marker for cell nuclei (*blue*). Original magnification, ×630 (For interpretation of the references to colour in this figure legend, please be referred to the online version)

luminal glucose generated at peak carbohydrate digestive activity. Indeed, the transport capability of GLUT2 at the BBM can be up to three times greater than that of SGLT1 (Kellett, 2001; Kellett & Brot-Laroche, 2005). Glucose exits the enterocyte

Table 5.3 Different forms of glucose transporters that are involved in active or sodium-dependent (SGLTs) and passive or facilitated (GLUTs) mechanisms. Their locations in tissues and respective function are indicated

Transporter	Tissue	Function
SGLT1	Mainly gut	Intestinal uptake of glucose
SGLT2	Mainly kidney	Renal uptake of glucose
GLUT1	All tissues; mainly red blood cell and brain	Basal uptake of glucose; transport across the blood-brain barrier
GLUT2	Beta-cell of the pancreas, liver, kidney and gut	Regulation of insulin release; other aspects of glucose homeostasis
GLUT3	Brain, kidney, placenta, and other tissues	Uptake into neurons and other tissues
GLUT4	Muscle and adipose tissues	Insulin-mediated uptake of glucose
GLUT5	Gut and kidney	Intestinal absorption of fructose

via GLUT2 or simple diffusion across the BLM. Apart from glucose and galactose, a third form of absorbable monosaccharides is fructose which is transported by another facilitated glucose transporter, GLUT5. In summary, Fig. 5.4a illustrates SGLT1-mediated glucose (as well as galactose) uptake by enterocyte under normal condition. Notwithstanding the involvement of this well-established secondary active transport for glucose uptake, the precise mechanisms by which SGLT1 and GLUT2 are mediated by some novel regulators, and consequently affect glucose uptake at the BBM of enterocytes, have yet to be fully elucidated.

5.4.1 Expression and Function of an Enterocyte RAS

There are diverse factors as well as ever-growing candidates that govern the glucose transporters located at the enterocytes for intestinal glucose uptake. In this regard, the rate of intestinal glucose transport is subject to regulation by both hormones and luminal peptides, which have stimulatory or inhibitory effects. Such stimulatory regulators include insulin (Pennington et al., 1994), pancreatic glucagon (Debnam & Sharp, 1993), gastric inhibitory peptide (Cheeseman & Tsang, 1996), glucagon-like peptide-2 (Cheeseman, 1997), and cholecystokinin (Hirsh & Cheeseman, 1998) as well as leptin (Ducroc et al., 2005) and angiotensin II (Wong et al., 2007; Leung, 2008), the latter two being inhibitory peptides. As far as angiotensin II is concerned, there are several local RASs with functional correlates that have been identified in the gastrointestinal system including, the salivary glands, stomach, intestine, colon, liver, and pancreas (Paul et al., 2006, Leung, 2007). Local RAS components, such as ACE and renin, have been detected in the small intestinal mucosa (Erickson et al., 1992; Seo et al., 1991). It has been proposed that mucosal ACE may function as a brush border membrane peptidase (Yoshioka et al., 1987). Despite the finding that ACE is present at the intestinal BBM, there is no information available on the transport actions of locally produced angiotensin II at this membrane (Stevens et al.,

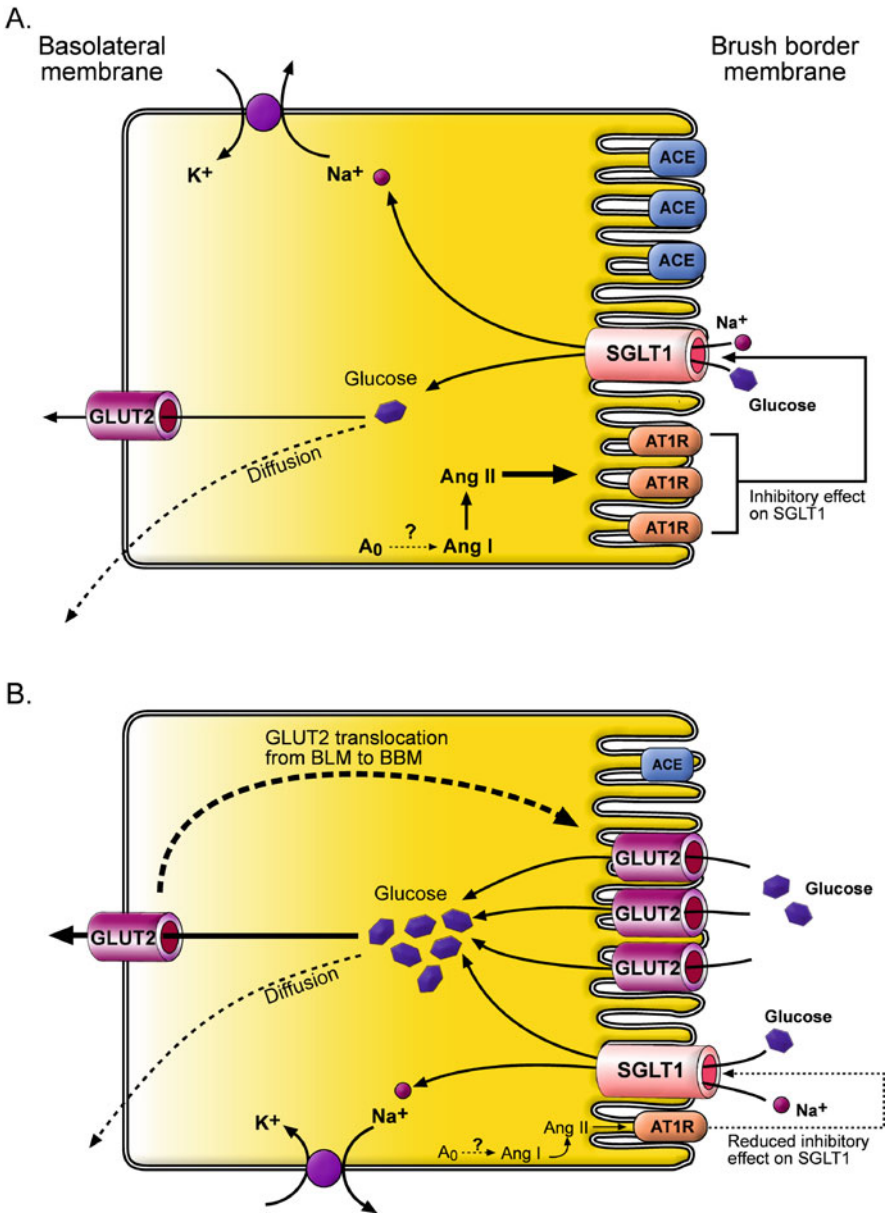

Fig. 5.4 A schematic diagram proposing the potential mechanism of an enterocyte RAS-mediated SGLT1 dependent glucose uptake at the BBM under normal (**a**) and diabetic condition (**b**)

1988; Naim, 1992). Vascular infusion of low concentrations of angiotensin II have been shown to stimulate intestinal fluid transport via the AT_2 receptor; however, higher levels of angiotensin II inhibit fluid transport via an AT_1 receptor-dependent

process (Jin et al., 1998). Studies using proximal tubule cells, where the glucose uptake process is very similar to that in enterocytes, imply that locally secreted angiotensin II affects sugar transport. For example, angiotensin II inhibits uptake of the glucose analogue, α-methyl-glucopyranoside into LLC-PK_1 cells and reduces expression of the sodium-dependent glucose transporter at the apical membrane (Kawano et al., 2002). Despite of these findings, direct involvement of a local RAS in the control of intestinal glucose transport, however, has yet to be conclusively demonstrated in the literature.

Against this background, we recently found compelling evidence for the existence of an enterocyte RAS that generates angiotensin II locally and leads to rapid inhibition of SGLT1-mediated intestinal glucose uptake (Wong et al., 2007). In this study, expression of key RAS components at the gene and protein levels were examined in jejunal and ileal enterocytes. Mucosal uptake of glucose by everted intestinal sleeves, before and after addition of angiotensin II to the mucosal buffer, was measured in the presence or absence of losartan, an AT_1 receptor antagonist. The results revealed that enterocytes express angiotensinogen, ACE, and AT_1 and AT_2 receptors; AT_1 receptors and angiotensinogen proteins were specifically localized to the BBM. Expression of angiotensinogen (a mandatory component of a local RAS) and AT_1 and AT_2 receptors, but not ACE, was greater in the ileum than the jejunum. Addition of angiotensin II to the mucosal buffer inhibited phlorizin-sensitive (SGLT1-dependent) jejunal glucose uptake in a rapid and dose-dependent manner, and reduced the expression of SGLT1 at the BBM. Losartan attenuated the inhibitory action of angiotensin II on glucose uptake. Despite this inhibitory effect on glucose uptake, angiotensin II did not affect jejunal uptake of L-leucine, suggesting a specific action on intestinal glucose absorption. The proposed mechanism by which the rapid inhibitory effects of enterocyte-derived angiotensin II is involved in the regulation of SGLT1-mediated intestinal glucose transport at the BBM under normal condition are depicted in Fig. 5.4a.

5.4.2 Enterocyte RAS and Diabetes Mellitus

In light of our enterocyte RAS findings above, we proceed to explore the clinical relevance of this enterocyte RAS in AT_1 receptor-mediated SGLT1-dependent glucose uptake in the intestine. Previous studies have shown that streptozotocin-induced diabetes mellitus promotes glucose transport across the rat intestinal BBM (Hopfer, 1975; Debnam et al., 1988; Burant et al., 1994) and that this is a likely consequence of increased BBM expression of SGLT1 and GLUT2 (Debnam et al., 1988, 1995; Kellett & Brot-Laroche, 2005). Nevertheless, the mechanisms of increased transport remain largely unexplored. Previous studies have shown that proximal tubule cells are able to synthesize and secrete angiotensin II into the luminal fluid, and that angiotensin II might be involved in the regulation of SGLT-mediated transport in these cells (Park & Han, 2001). Interestingly, exposure of proximal tubule cells to high glucose at a level similar to that seen in the plasma of streptozotocin-diabetic animals reduced angiotensin II binding of these cells (Park et al., 2002).

Against this background, we hypothesized that the expression of RAS components at the enterocyte BBM would be subject to regulation by diabetes mellitus and thus affect intestinal glucose uptake (Wong et al., 2009). Our preliminary results showed that streptozotocin-induced diabetes 2 weeks in duration was sufficient to cause a 5-fold increase in blood glucose level, reduce mRNA and protein expression of AT_1- and AT_2-receptors, and reduce ACE levels in isolated jejunal enterocytes. Angiotensinogen expression was, however, stimulated by diabetes whilst renin was not detected in either control or diabetic enterocytes. Considering these data, it is thus plausible to speculate that a local RAS, in the absence of renin, i.e. a renin-independent angiotensin-generating system, exists and is involved in angiotensin II production in the intestine.

On the other hand, streptozotocin-induced diabetes stimulated glucose uptake by 58% and increased the expression of SGLT1 and GLUT2 proteins in purified BBM by 25 and 135%, respectively. Our immunocytochemistry results confirmed that there was an increased BBM expression of GLUT2 in the diabetes condition, which was probably due to the rapid translocation or trafficking of GLUT2 from the BLM to the BBM. Addition of angiotensin II (5 μM), or its non-peptide analogue L-162313 (1 μM), to mucosal buffer decreased glucose uptake by diabetic jejunum by 18 and 24%, respectively. Accordingly, the potency of L-162313 is about 63 times greater than angiotensin II. Furthermore, this inhibitory action of angiotensin II was due to reduced phlorizin-sensitive (SGLT1-dependent) rather than phloretin-sensitive (GLUT2-dependent) transport, and was abolished by the AT_1 receptor antagonist losartan (1 μM). The decreased efficacy of angiotensin II on glucose uptake in diabetes compared to that noted previously in jejunum from normal animals (Wong et al., 2007) is likely to be due to altered RAS expression in diabetic enterocytes, together with disproportionately increased GLTU2 expression at the BBM. Since the rate of intestinal glucose transport affects insulin release and thus glycaemic control in humans, our work raises the issue of whether the enterocyte RAS may be a therapeutic target for reducing glucose transport. On the other hand, the higher potency of L-162313, compared to angiotensin II, in terms of affecting glucose transport, makes it possible that non-peptide AT_1 receptor agonists may be used as gut-directed treatments to reduce postprandial hyperglycaemia in diabetic patients (Wong et al., 2009). In conclusion, The proposed mechanisms by which the release of inhibitory effects of enterocyte-derived angiotensin II may be involved in the regulation of SGLT1-mediated and enhanced trafficking of GLUT2-mediated intestinal glucose transport at the BBM in diabetic intestine (Fig. 5.4b).

5.4.3 ACE2-Angiotensin (1–7)-Mas Receptor Axis and Intestinal Glucose Uptake

Angiotensin converting enzyme 2 (ACE2), the first homologue of ACE2, is a newly identified member of RAS (Bindom & Lazartigues, 2009). Apart from being a cellular receptor for the severe acute respiratory syndrome-associated coronavirus, SARS-CoV (Ren et al., 2006), ACE2 is a membrane-bound mono-carboxypeptidase

capable of generating angiotensin (1–9) and angiotensin (1–7) by cleaving the terminal leucine and phenylalanine residue from angiotensin I and angiotensin II, respectively (Iwai & Horiuchi, 2009). As a result there are two separate pathways responsible for angiotensin (1–7) production (Bindom & Lazartigues, 2009). Angiotensin (1–7) is a bioactive peptide of the RAS which has been shown to have beneficial effects on renal and cardiovascular diseases, possibly via the mediation of counter-regulating ACE-angiotensin II-AT1R (Santos et al., 2008). The G-protein-coupled receptor, mas receptor, acts as receptor for angiotensin (1–7). It is found to be expressed in many tissues such as brain, heart, kidney and liver (Iwai & Horiuchi, 2009). In view of this fact, we propose the existence of an ACE2-angiotensin (1–7)-mas axis and its potential role in intestinal glucose uptake. We thus test this hypothesis using a cell model of Caco-2, (Sambuy et al., 2005) a human colon carcinoma cell line by examining the expression and regulation of ACE2-angiotensin (1–7)-mas receptor axis under high glucose exposure.

Our preliminary data shows that components of ACE2, angiotensin (1–7) and mas receptor are expressed in the Caco-2 cells (Fig. 5.5); ACE2 were markedly

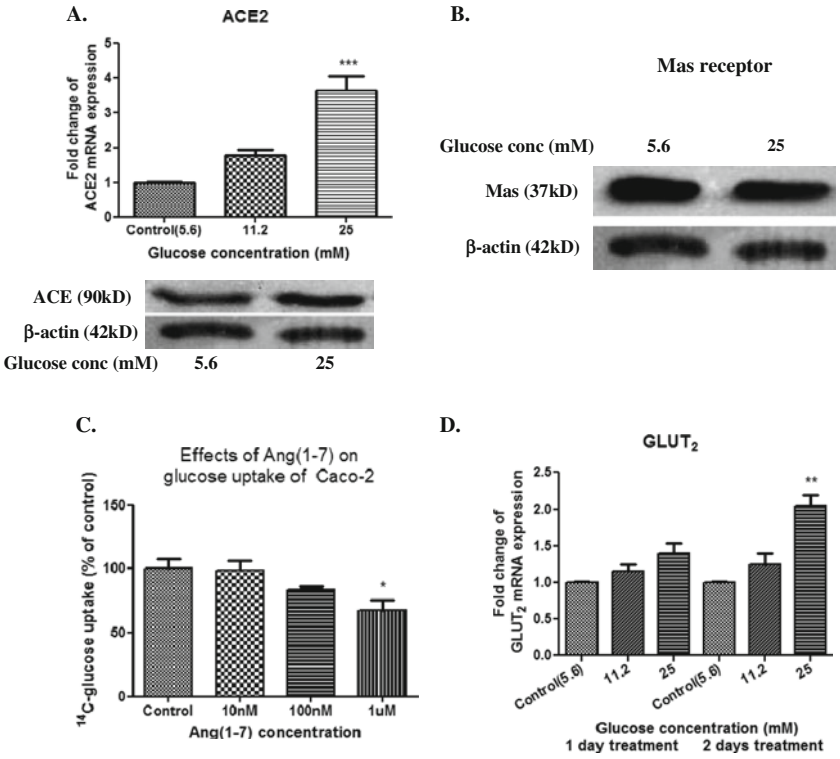

Fig. 5.5 Expression and regulation of ACE2-angiotensin (1–7)-mas receptor under high (25 mM) and normal (5.6 mM) in Caco-2 cell line. **a** ACE2 gene and protein expression. **b** Mas receptor protein expression. **c** ^{14}C-glucose uptake in response to angiotensin (1–7). **d** GLUT2 gene expression

upregulated while being no significant increase in mas receptor under exposure to high glucose when compared with normal concentrations, i.e. 5.6 mM vs. 25 mM (Fig. 5.5a,b). Interestingly, exogenous addition of angiotensin (1–7) appears to dose dependently inhibit glucose uptake by the cell line, as demonstrated by ^{14}C-glucose assay (Fig. 5.5c). On the other hand, GLUT2 gene expression is also dose-dependently upregulated in polarized Caco-2 cells after high glucose exposure. It is significantly upregulated after being exposed to 25 mM glucose for 2 days (Fig. 5.5d). Taken these findings together, our preliminary data indicate the existence of a functional ACE2-angiotensin(1–7)-Mas axis in Caco-2 cell line, which may provide an alternative mechanism for GLUT2 and/or SGLT1-mediated intestinal glucose uptake in normal and diabetic conditions.

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