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Stevioside and related compounds: Therapeutic benefits beyond sweetness

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ABSTRACT

Stevioside, an abundant component of *Stevia rebaudiana* leaf, has become well-known for its intense sweetness (250–300 times sweeter than sucrose) and is used as a non-caloric sweetener in several countries. A number of studies have suggested that, beside sweetness, stevioside along with related compounds, which include rebaudioside A (second most abundant component of *S. rebaudiana* leaf), steviol and isosteviol (metabolic components of stevioside) may also offer therapeutic benefits, as they have anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory actions. It is of interest to note that their effects on plasma glucose level and blood pressure are only observed when these parameters are higher than normal. As steviol can interact with drug transporters, its role as a drug modulator is proposed. This review summarizes the current knowledge of the pharmacological actions, therapeutic applications, pharmacokinetics and safety of stevioside and related compounds. Although much progress has been made concerning their biological and pharmacological effects, questions regarding chemical purity and safety remain unsolved. These issues are discussed to help guide future research directions.

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1. Introduction

1.1. Natural source

Natural sweeteners that can substitute for sucrose have caught great attention due to the growing incidence of obesity and diabetes. Much attention has been placed on stevioside, a sweet glycoside extracted from *Stevia rebaudiana* Bertoni. This plant is a small shrub originally grown in South America, particularly in Brazil and Paraguay where it is known as stevia or honey leaf, Kaa-he-e (Hanson & De Oliveira, 1993). Native peoples in South America have been using stevia extract as sweetener and traditional medicine for several hundred years (Kinghorn & Soejarto, 2002). Due to the sweetness and supposed therapeutic properties of its leaf, *S. rebaudiana* Bertoni has attracted economic and scientific interests. Japan was the first country in Asia to market stevioside as a sweetener in food and drug industry. Since then, cultivation of this plant has expanded to several countries in Asia, including China, Malaysia, Singapore, South Korea, Taiwan, and Thailand. It has also been successfully grown in the United States of America, Canada, and Europe (Brandle et al., 2000). Use of these sweetening compounds has increased dramatically due to health concerns related to sucrose usage, such as dental caries, obesity and diabetes.

1.2. Chemical structure and sweetness property

Stevioside is a diterpenoid glycoside, comprising an aglycone (steviol) and three molecules of glucose. In addition to stevioside,

several other sweet compounds such as steviobioside, rebaudioside A, B, C, D, E and ducoside A were isolated from *S. rebaudiana* Bertoni leaf. All of these isolated diterpenoid glycosides have the same chemical backbone structure (steviol) but differ in the residues of carbohydrate at positions C13 and C19 (Shibata et al., 1995). The major components of the leaf are stevioside (5–10% of total dry weight), rebaudioside A (2–4%), rebaudioside C (1–2%) and dulcoside A (0.4–0.7%) (Wood et al., 1955). The chemical structures of stevioside and its related compounds which include steviol, rebaudioside A, isosteviol and dihydroisosteviol are shown in Fig. 1.

The (fold) sweetness of these glycosides compared to sucrose is dulcoside A 50–120, rebaudioside A 250–450, rebaudioside B 300–350, rebaudioside C 50–120, rebaudioside D 250–450, rebaudioside E 150–300, steviobioside 100–125, and stevioside 300 (Crammer & Ikan, 1986). Stevioside is hydrolyzed by bacteria in the gastrointestinal tract to yield steviol and glucose (Wingard et al., 1980; Hutapea et al., 1997; Koyama et al., 2003a). Along with sweetness, stevioside possesses some bitterness and undesirable aftertaste (Jakinovich et al., 1990). However, this problem can be solved by enzymatic modification of stevioside by pullanase, isomaltase (Lobov et al., 1991), β-galactosidase (Kitahata et al., 1989), or dextrin saccharase (Yamamoto et al., 1994).

Stevioside is considered to be a sugar substitute and commercial sweetener, both in the form of stevioside and stevia extract (Kinghorn & Soejarto, 1985; Brandle & Rosa, 1998). They are used in variety of foods and products, such as pickled vegetables, dried seafood, soy sauce, beverages, candies, chewing gum, yogurt and ice cream, as well as in toothpaste and mouth wash. Stevia extract and stevioside are
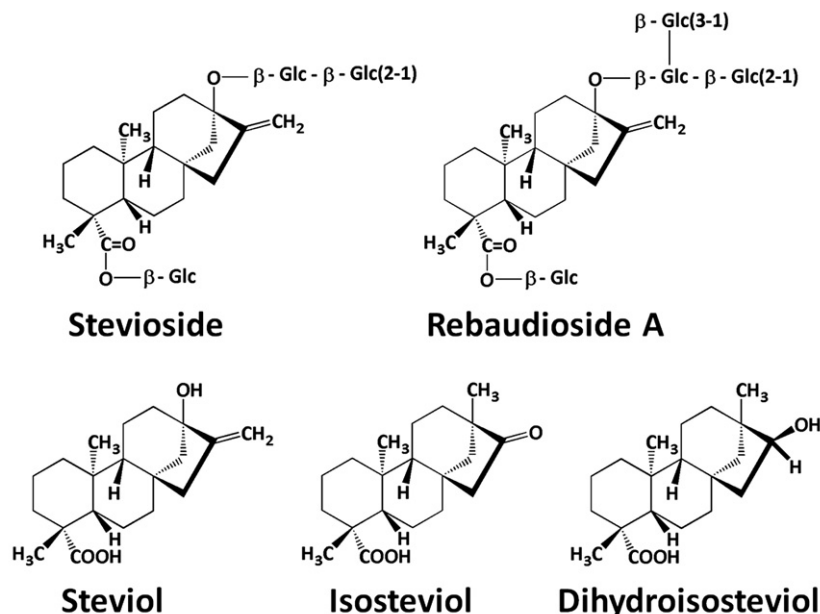

Fig. 1. Chemical structures of stevioside and its related compounds.

officially approved as food additives in Brazil, Korea and Japan (Choi et al., 2002; Mizutani & Tanaka, 2002) and in the United States, they are permitted as a dietary supplement. They have not yet been approved by the European Commission due to safety concern. In 2006, the meeting of the Joint FAO/WHO Expert Committee on Food Additive (JECFA) to evaluate certain food additives and ingredients, flavoring agents, and natural constituent of food announced a temporary accepted daily intake (ADI) of stevioside of up to 5.0 mg/kg body weight (BW) (JECFA, 2006).

In addition to their sweetness, stevia extract and stevioside have been used as a traditional medicine by local people in South America for hundreds of years (Kinghorn & Soejarto, 2002). Therapeutic benefits of these compounds will be discussed in the following sections.

1.3. Extraction and purification

Procedures for isolation of stevioside from *S.rebaudiana* leaves on a pilot scale mostly involve liquid extraction with such solvents as chloroform-methane, glycerol, and propylene glycol, followed by refinement involving extraction into a polar organic solvent, decolorization, coagulation, ion-exchange chromatography and crystallization (Kinghorn and Soejarto, 1985; Pasquel et al., 2000). Purification of stevioside can also be performed by ion-exchange chromatography, which has been accepted to being the best technique. Then the aqueous extract are reprecipitated and filtered to yield 90% pure stevioside. Recently, Pol et al. (2007) have developed a pressurized hot water extraction method for stevioside from *S. rebaudiana* Bertoni thereby establishing a "green" method of isolation.

2. Pharmacokinetics of stevioside

Pharmacokinetics describing the fate of stevioside administered to living organisms in terms of absorption, distribution, metabolism and excretion (ADME) is of particular importance as it provides an understanding of the degree and kinetics of systemic exposure to stevioside or its metabolic products. These data are necessary for safety assessment of stevioside.

2.1. Absorption

Stevioside, being a hydrophilic diterpenoid glycoside with a relatively high molecular weight (804.9) is unlikely to be absorbed in the intestine. In addition, gastric juice and digestive enzymes from animals and humans have failed to degrade stevioside (Wingard et al., 1980; Hutapea et al., 1997; Koyama et al., 2003a). However, bacterial intestinal flora of rats (Wingard et al., 1980), mice (Hutapea et al., 1997), pigs (Geuns et al., 2003a) and humans (Gardana et al., 2003; Koyama et al., 2003a) are able to convert stevioside into its aglycone, steviol. *Bacteroides* sp. is responsible for this conversion in the lower gastrointestinal tract of both rat and human (Gardana et al., 2003). Studies in human volunteers showed that after 3 days following a consumption of stevioside (750 mg/day), no measurable amount of stevioside was detected in the feces of all subjects, whereas free steviol was present (Geuns, 2007). Experimental studies using everted rat intestinal sac demonstrated that absorptive transport of steviol is much higher than that of stevioside (Geuns et al., 2003a). Similarly, an oral administration of steviol to rats resulted in a rapid increase in concentration of steviol in portal venous blood with time to peak concentration of about 15 min (Koyama et al., 2003b). Investigations of the transport properties of stevioside and steviol in monolayers of human intestinal Caco-2 cell line, a standard model for drug absorption study, indicated that transport of stevioside is very low (permeability coefficient, P_{app}, of 0.16×10^{-6} cm/s), whereas that of steviol transport is much faster and in favor of an absorptive direction

(P_{app} of 44.5×10^{-6} cm/s for absorption and P_{app} of 7.93×10^{-6} cm/s for secretion) (Geuns et al., 2003a). In addition, the mechanism of steviol absorption involves both passive diffusion and carrier-mediated transport through a monocarboxylic transporter. Taken together, these data indicate that in an oral ingestion of stevioside, it is steviol that is taken up by the intestine into the blood.

2.2. Distribution

The distribution profile of steviol in specific organs allows prediction of its systemic effects and toxicological concerns of stevioside consumption. Distribution of steviol in such vital organs as brain and heart would be considered unfavorable as steviol or its metabolites may induce adverse reactions in those organs. For therapeutic purposes, preferential accumulation of steviol in the target organ is regarded as being desirable.

Nakayama et al. (1986) were the first to determine the pharmacokinetics of stevioside after a single dose of oral administration of ^{3}H-stevioside (125 mg/kg BW) into male Wistar rats. Maximal radioactivity level in blood (4.8 μg/ml) was found at 8 h after stevioside administration with an elimination half-life of 24 h. Analysis of radioactivity levels in key organs and tissues demonstrated preferential accumulation in small and large bowels. Following intravenous injection of radioactive ^{131}I-stevioside into Wistar male rats, radioactivity in heart, stomach, testes and muscle is less than 1.8% of the injected dose during the entire 24 h experiment (Cardoso et al., 1996). However, a significant amount of radioactivity was detected in liver, intestine and kidney. The accumulation in the liver is highest after 10 min of injection. Significant radioactivity is present in bile after 120 min, with an accumulative amount of 52% of the injected dose. Its presence is maximal in small and large intestines after 120 and 240 min following stevioside injection, respectively. High performance liquid chromatography (HPLC) analysis of bile showed steviol as the major metabolite. Whereas, urine analysis revealed the presence of stevioside as a major component with a much less amount of an unknown metabolite that was also found in the bile. These results indicate that metabolic conversion of stevioside to steviol in rats occurs in liver and there are two routes of stevioside excretion, via bile and urine. In addition, steviol may undergo enterohepatic circulation where steviol is excreted into bile and reabsorbed back to circulation. However, the presence of steviol in portal venous system was not documented in the study.

2.3. Metabolism

Steviol appears to be the major metabolite of stevioside appearing into the blood circulation following oral ingestion. As a consequence, much attention has been paid to its metabolism in liver. After incubating steviol with hepatic microsomes from rat and human, oxidation (monohydroxy and dihydroxy) metabolites of steviol are formed (Koyama et al., 2003b). This process requires NADPH-generating system, suggesting a phase I metabolism of steviol by cytochrome P450. However, as earlier in vivo studies in rat found unknown steviol conjugates in bile (Nakayama et al., 1986), further conjugation (phase II metabolism) of steviol metabolites may occur. Recently, Roberts and Renwick (2008) have reported that stevioside and rebaudioside A are metabolized to steviol glucuronide in rat.

Earlier Geuns et al. (2006) found steviol glucuronide in human urine after stevioside administration. In further studies in human subjects receiving stevioside orally at a dose of 750 mg/day for 3 days, steviol glucuronide was the only metabolite found in blood and urine, whereas no stevioside or steviol was detected (Geuns et al., 2007). Analysis of feces found only free steviol and no stevioside or steviol glucuronide, suggesting an additional route of steviol elimination. In a recent randomized double-blind, crossover study, pharmacokinetics of steviol glucuronide and free steviol in 8 healthy adult male subjects

receiving a single oral dose of rebaudioside A (5 mg/kg BW) or stevioside (4.2 mg/kg BW) showed plasma steviol level of 121 ng/ml after 6 h of administration (Wheeler et al., 2008). Steviol glucuronide is present in plasma of all subjects following oral administration of stevioside, with the median time to peak concentration of 8 h and mean maximum concentration of 1.89 μg/ml. In urine, the majority of steviol was found in the form of glucuronide conjugates (62% of dose) with a much lower amount of free steviol (0.04% of dose). Thus, it appears that, in addition to phase I metabolism, steviol undergoes phase II metabolism in which the greater part of steviol is conjugated with glucuronide before being eliminated in urine. This study also suggests the involvement of a similar metabolic pathway for rebaudioside A.

2.4. Excretion

An understanding of the excretion by which stevioside and its metabolic products are eliminated from the body is crucial for toxicological considerations of stevioside consumption. It determines the duration and amount of stevioside or its metabolites remaining in the body after ingestion. Thus, excretory mechanisms of stevioside in both humans and rats have been explored.

Steviol glucuronide is the common major metabolite found in circulation of both humans and rats. Biliary and urinary tracts appear to be the major routes for steviol glucuronide excretion. However, the extents to which this metabolite is excreted via these two routes are different between humans and rats. In the latter, the principal route is via biliary excretion of steviol glucuronide in feces (Wingard et al., 1980; Nakayama et al., 1986; Roberts & Renwick, 2008). In humans, urinary excretion seems to play a predominant role in steviol glucuronide excretion (Cardoso et al., 1996; Geuns et al., 2006, 2007; Wheeler et al., 2008). A recent metabolic study in humans showed that at 72 h after oral stevioside ingestion, steviol glucuronide excreted in urine and free steviol in feces accounts for 62% and 5.2% of the total dose of stevioside administered respectively (Wheeler et al., 2008). This difference between humans and rats in the pathway of steviol glucuronide excretion is thought to be due to the different molecular weight threshold for human and rat biliary excretion of organic anions (e.g. glucuronides). Organic anions with a molecular mass of more than 600 Da in humans and of 325 Da in rats are excreted in bile instead of through a glomerular filtration process in kidney (Renwick, 2008a). Thus, steviol glucuronide, with a molecular weight of 512.9 Da, is preferentially eliminated via biliary excretion in feces in rats.

Taken together, steviol glucuronide is the major metabolite of stevioside consumption and in humans urinary excretion is responsible for disposal of the greater part of this metabolite from the body. The excretory process probably involves renal organic anion transporters (Srimaroeng et al., 2005a).

3. Biological effects and potential therapeutic applications of stevioside and related compounds

3.1. Anti-hyperglycemic effect

At present, there is a sharp increase in incidence of type 2 diabetes mellitus and obesity as a result of aging, dietary habits and decreasing physical activities. These metabolic syndromes have become major public health problems in industrialized and developing countries. Type 2 diabetes mellitus is a chronic metabolic disorder resulting from defects in both insulin secretion from β-cells of islets and insulin action (DeFronzo, 1988). In addition to insulin abnormalities, pancreatic α-cell dysfunction and relative glucagon excess are involved (Unger, 1997). Postprandial hyperglycemia observed in type 2 diabetes is usually due to an increase in basal hepatic glucose production and a decrease in peripheral glucose

disposal. Therefore, correction of this imbalance at either the entry or exit step of plasma glucose should help to correct this pathological condition.

Currently, there is a popular use of herbal and alternative medicine for the treatment of diabetes. Indeed, extract from *S. rebaudiana* has long been used for the treatment of diabetes in South America (Kinghorn & Soejarto, 2002). In addition, stevioside, the major component of the extract, has a high sweetness with no calorie and only a small amount is needed for sweetening purposes. Thus, it should be a good alternative to sugar for diabetic patients. An early study showed that 0.5 g % of stevioside and 10 g % of powdered stevia leaves in both high-carbohydrate and high-fat diets given to rats caused a significant reduction in blood glucose level following 4 weeks of treatment (Susuki et al., 1977). Subsequently, the effect of aqueous extract of stevia leaves on glucose tolerance test was investigated in humans. Following intake of aqueous extract of stevia leaves, 5 g % at 6 h intervals for 3 days, there was a significant decrease in plasma glucose level during glucose tolerance test and after overnight fasting in all healthy subjects (Curi et al., 1986). These observations support the earlier notion that stevioside and stevia extract can be used to treat diabetic condition.

3.1.1. Effect on glucose absorption

The effects of stevioside and steviol on glucose absorption have been investigated using in vitro jejunal ring tissue and everted sac (Toskulkao et al., 1995a). Stevioside at a high dose (5 mM) has no inhibitory effect on glucose absorption. However, 1 mM steviol inhibits glucose absorption by about 40%. Steviol causes a decrease in glucose accumulation in intestinal ring tissue, thereby possibly acting on the brush border membrane, similar to the action of phlorizin. Furthermore, steviol alters the morphology of intestinal absorptive cells. These results suggest that the possible site of inhibitory action of steviol may be on the mucosal side and/or at the intracellular organelles of intestinal absorptive cells (Toskulkao et al., 1995a). Studies from the same group of researchers (Toskulkao et al., 1995b) demonstrated that stevioside at doses of 1 and 5 mM does not inhibit intestinal glucose absorption in hamster jejunum, whereas 1 mM steviol inhibits glucose absorption by about 30% but does not affect the activity of intestinal Na^+-K^+-ATPase. There are reductions in intestinal mucosal ATP content and absorptive surface area. The investigators suggested that the inhibition of glucose absorption by steviol is due to a reduction in intestinal mucosal ATP content, which is a consequence of a decrease in intestinal mitochondrial enzyme activities at the level of phosphorylation and morphological alterations of intestinal absorptive cells. In fact, inhibition by steviol of glucose absorption in intestinal cells would result in a lowering of plasma glucose level, which may be undesirable in healthy individuals. However, the acceptable daily intake (ADI) of stevioside (5 mg/kg BW/day) would yield a maximum plasma concentration of steviol of approximately 20 μM if stevioside is completely converted to steviol (JECFA, 2006). This concentration of steviol is far below than that reported to inhibit intestinal glucose absorption. Therefore, more studies should be conducted using ADI amount to reevaluate the effect of steviol on glucose absorption. However, it is worth pointing out that stevioside does not interfere with glucose absorption.

3.1.2. Effect on glucose synthesis

The effects of stevioside on glucose synthesis have been studied in two types of diabetic rats, type 1 (insulin dependent) and type 2 (insulin independent) (Chen et al., 2005). Type 1 diabetes in rat was induced using streptozotocin (STZ) and type 2 diabetes was induced by giving food containing a high amount (60%) of fructose for 2 weeks. Both conditions were confirmed by measurement of fasting blood glucose levels. Tolbutamide (10 mg/kg BW; intraperitoneal injection) was used to confirm that rats had developed type 2 diabetes (Shennan et al., 1987). Stevioside lowers the high blood glucose levels in both

type 1 and type 2 diabetic rats. The hypoglycemic effect of stevioside on STZ-induced diabetic rat following oral intake of stevioside (1, 2 or 10 mg/kg BW/day for 15 days) is mediated via its effect on phosphoenol pyruvate carboxy kinase (PEPCK), a rate-limiting enzyme for gluconeogenesis controlling glucose production in the liver (Chen et al., 2005). In STZ-induced diabetic rat with very low insulin level PEPCK gene is over expressed (Giffin et al., 1993; Chan et al., 2003) and stevioside decreases PEPCK mRNA and protein concentrations in a dose-dependent manner (Chen et al., 2005). Thus, it seems likely that stevioside slows down gluconeogenesis in the liver via suppression of PEPCK gene expression leading to a decrease in plasma glucose level in diabetic rat.

Interestingly, a recent study has compared the effects of *S. rebaudiana* leaves and stevioside on glycemia and hepatic gluconeogenesis in normal rat (Ferreira et al., 2006). Oral administration to fasted male Wistar rats of stevioside/rebaudiana mixture at 5.5 mg/kg BW/day for 15 days has no effect, whereas stevia powder/leaves at a dose of 20 mg/kg BW/day lowers plasma glucose concentration by decreasing activities of pyruvate carboxylase and PEPCK. The explanation for this observation is not known. As stevia leaves contain a variety of steviol glycosides, the active compound(s) exhibiting the effect remains to be identified. However, it is worth pointing out that stevioside has no effect in lowering plasma glucose under normal condition in this study.

3.1.3. Effect on insulin secretion and sensitivity

The direct effects of stevioside and steviol on insulin release have been elucidated using normal mouse islets and INS-1 pancreatic β-cell line (Jeppesen et al., 2000). In the presence of 16.7 mM glucose both stevioside and steviol enhance insulin secretion from incubated islets in a dose-dependent manner (1 nM to 1 mM). Even though both stevioside and steviol possess an insulinotropic/anti-hyperglycemic effect, steviol is more potent than stevioside (Jeppesen et al., 2000). To determine whether the effect of stevioside observed in vitro occurs in the whole animal, the same group of researchers conducted glucose tolerance test in Goto-Kakizaki (GK) (a non-obese animal model of type 2 diabetes) and normal rats in the presence or absence of stevioside (Jeppesen et al., 2002). Bolus injection of stevioside (0.2 g/kg BW) along with glucose (2.0 g/kg BW) induces insulin secretion, suppresses glucagon level in the plasma and reduces blood glucose response to glucose tolerance test in anaesthetized GK rat. These results support the notion that stevioside possesses anti-hyperglycemic, insulinotropic and glucagonostatic effects in diabetic GK rats (Jeppesen et al., 2002).

The majority of diabetes incidence worldwide is type 2 diabetes mellitus (Kolterman et al., 1980). Under this condition, reduction of insulin sensitivity in peripheral tissues decreases glucose utilization efficiency leading to the development of hyperglycemia. Therefore, the role of stevioside in increasing insulin sensitivity has been studied in fructose-rich chow-fed rats (Chang et al., 2005). Insulin resistance in rat fed a diet containing 60% fructose has served as a useful animal model for inducing insulin resistance (Elliott et al., 2002). The ability of insulin to stimulate glucose disposal is markedly impaired in these rats, indicating a decline in insulin sensitivity in peripheral tissues associated with insulin resistance. This rat model represents the development of obesity and accompanying insulin resistance syndrome. Stevioside (5.0 mg/kg BW) given orally in fructose-rich chow fed rats markedly improves insulin sensitivity as indicated by glucose-insulin index. Repeated stevioside treatment 3 times daily delays development of insulin resistance as indicated by response to tolbutamide. The effect of stevioside on insulin sensitivity was further examined in insulin-sensitive (lean) and insulin-resistant obese Zucker rats (Lailerd et al., 2004). Acute oral intake of stevioside (500 mg/kg BW) in diabetic rats increases whole body insulin sensitivity, as determined by glucose-insulin index value, which indicates insulin sensitivity or insulin action on glucose disposal rate

following glucose loading. In addition, the direct effect of stevioside on glucose transport activity in skeletal muscle, the major site of glucose disposal, was examined in vitro. It was revealed that low concentrations of stevioside (0.01 to 0.1 mM) could improve insulin action on skeletal muscle glucose transport in both lean and obese Zucker rats, suggesting that a potential site of stevioside action is at the skeletal muscle glucose transport system.

Thus, the effects of stevioside in stimulating insulin secretion (Jeppesen et al., 2000) as well as in increasing insulin sensitivity (Lailerd et al., 2004) underscore its beneficial effects on glucose metabolism. Indeed, a reduction in plasma glucose and glucagons along with an increase in insulin secretion occur in type 2 diabetic Goto-Kakizaki rats following repeated oral intake of stevioside (25 mg/kg BW/day) for 6 weeks (Jeppesen et al., 2003). However, studies on longer duration of administration of stevioside as well as its metabolite, steviol, need to be further examined. Taken together, these findings further strengthen the application of stevioside in reversing insulin resistance or in improving insulin sensitivity in type 2 diabetes mellitus.

However, these results raise a concern of whether ingestion of stevioside in the fasting state could induce hypoglycemia, as in the situation of sulphonylurea, which stimulates insulin release and thereby constituting a potential threat for diabetic subjects. To answer this question, stevioside was given to fasting conscious GK and Wistar rats, at normal and low plasma glucose concentration (3.3 mM or less), and an insulinotropic action of stevioside was not found (Jeppesen et al., 2002). Indeed, stevioside stimulates insulin release in isolated mouse islets only at high glucose concentration (>8.3 mM) (Jeppesen et al., 2000). Hence, it is likely that stevioside elicits its beneficial effect by stimulating insulin release only in the diabetic state. However, more information regarding efficacy, nonglycemic benefits, safety profile and its long-term effects under these situations is required.

A limited number of experimental studies have been performed to evaluate the effect of stevia extract and stevioside on blood glucose level in humans. A standard test meal supplemented with 1 g of stevioside (1 g of starch was used as control) given to 12 type 2 diabetic subjects is able to reduce postprandial blood glucose levels by about 18% (Gregersen et al., 2004). A slight increase in circulating insulin and decreased glucagons levels were observed. The glucose-insulinogenic index indicating insulin secretion increases by 40% after stevioside treatment. As urinary glucose loss by stevioside intake was not observed, this implies that stevioside may have a direct effect on peripheral glucose disposal induced by insulin responsible for decreased postprandial blood glucose level. This may include increasing liver glycogen storage (Hubler et al., 1994). In rats, stevioside markedly increases hepatic glycogen synthesis after 24 h when 2.0 mM is given in drinking water and at 48 h with 1.0 mM, whereas steviol has no effect (Hubler et al., 1994). This action of stevioside would enhance glucose removal from plasma. However, no mechanism of action was reported in the study.

In contrast to the above findings, Barriocanal et al. (2008) have recently reported that long term stevioside (>92% purity) consumption of 250 mg 3 times/day for 3 months, an amount similar to that be used as sweetener, has no pharmacological effect in type 1 and type 2 diabetic subjects as well as in normotensive and hypotensive individuals. It does not lower blood glucose or blood pressure. An explanation for this lack of stevioside effect is not known, but it is to be noted that stevioside seems to have the ability to lower plasma glucose and blood pressure only when these parameters are abnormally elevated. These results are consistent with those obtained earlier from short term effects in normal healthy subjects, in which stevioside (97% pure) given orally at a dose of 750 mg/day for 3 days has no significant effect on blood glucose and insulin concentrations (Geuns et al., 2007).

Stevioside has been shown to have a direct effect on glucagon secretion as well (Hong et al., 2006). Exposure to fatty acids of clonal

α-TC1-6 cells derived from an adenoma in transgenic mice results in glucagon hypersecretion and triglyceride accumulation. Stevioside (10 nM to 1 μM) is able to reduce the release of glucagon, possibly by enhancing mRNA expressions of carnitine palmitoyltransferase, peroxisome proliferator-activated receptor gamma (PPARγ) and stearoyl-CoA desaturase.

The second most abundant component of stevia leaf is rebaudioside A, which differs from stevioside by having one additional glucose moiety. This raises the possibility that rebaudioside A might possess hypoglycemic effects similar to those of stevioside and stevia extract. Indeed, rebaudioside A markedly stimulates insulin release from mouse islets in the presence of high glucose concentration (>6.6 mM) in a dose-dependent manner (Abudula et al., 2004). This action requires extracellular Ca^{2+}. Unexpectedly, in vivo study in long-term treatment of GK rats with rebaudioside A failed to stimulate insulin secretion (Dyrskog et al., 2005). Similarly, long-term consumption of rebaudioside A for 16 weeks by type 2 diabetes mellitus subjects has no effect on glucose homeostasis, lipid profile or blood pressure (Maki et al., 2008). The dosage of rebaudioside A (1000 mg/day) used in this study is about 7 times higher than the daily intake of sweetener for diabetic adults. The investigators concluded that rebaudioside A is well-tolerated and lacks pharmacological effect on glucose homeostasis in vivo.

Due to the important role of ATP-sensitive potassium (K_{ATP}) channels in pancreatic β-cell in coupling changes in plasma glucose concentration with insulin secretion, these K_{ATP} channels have been the targets of a number of different classes of therapeutic drugs for the treatment of type 2 diabetes (Ashcroft et al., 1994; Tucker & Ashcroft, 1998). Sulphonylurea is a commonly used drug for the treatment of hyperglycemia (Proks et al., 2002). Its binding to the high affinity subunit of K_{ATP} causes inhibition of the channels, leading to membrane depolarization, which then activates Ca^{2+} channels (Gribble & Reimann, 2002; Panten et al., 1996). The subsequent rise in cytosolic Ca^{2+} concentration stimulates insulin release from pancreatic β-cell. The drawback of this drug is that it can also induce hypoglycemia as it is able to stimulate insulin release at normal and low plasma glucose levels. Thus, it would be desirable to have drugs that stimulate insulin only at high plasma glucose levels. As rebaudioside A is able to markedly stimulate insulin secretion from isolated mouse islets in a glucose- and Ca^{2+}-dependent manner (Abudula et al., 2004), its mechanism of action was further determined using whole-cell patch clamp technique to evaluate the effect on K_{ATP} from β-cells isolated from mouse islets (Abudula et al., 2008). Indeed, rebaudioside A stimulates insulin secretion from pancreatic β-cells via inhibition of K_{ATP}, thereby allowing β-cells to depolarize and activate Ca^{2+} channels. This inhibition of K_{ATP} requires the presence of high glucose level underscoring the glucose dependency of rebaudioside A action. However, the signaling pathway by which high plasma glucose turns on the action of rebaudioside A is not known. This result is in contrast with previous in vivo study in GK rats (Dyrskog et al., 2005), but this may be due an impairment of rebaudioside A intestinal uptake. Thus, further studies on pharmacokinetics and pharmacodynamics of rebaudioside A are required to clarify this issue.

In addition to stevioside and steviol, isosteviol, a metabolic compound of stevioside, improves lipid profile and upregulates expression of key β-cell genes, including insulin regulatory transcription factors, thereby improving glucose homeostasis, increasing insulin sensitivity, lowering plasma triglyceride and lowering weight of diabetic KKAy mice (Nordentoft et al., 2008).

Chronic type 2 diabetes is normally accompanied by hypertension and dyslipidemia (UKPDS Group, 1998a, 1998b). Thus the ideal pharmacologic intervention in type 2 diabetes should also aim to lower blood pressure, lipid and glucose concentration in the plasma. As stevioside possesses blood pressure lowering and hypoglycemic effects (Jeppesen et al., 2003), it has a high potential to be used clinically for the treatment of these patients. Soy proteins decrease serum cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides (TG) (Anderson et al., 1995; Clarkson, 2002) and these beneficial effects are also observed on LDL-cholesterol in type 2 diabetes subjects. Indeed, long term treatment of type 2 diabetes and metabolic syndrome Zucker rats with soy protein alone was able to decrease total serum cholesterol by 19%, free fatty acid by 15% and triglyceride by 47% (Jeppesen et al., 2006). A synergistic effect on these parameters was observed when stevioside was added. In addition, the combined treatment also improves first-phase insulin response, suppresses glucagon level and has anti-hyperglycemic effects in diabetic GK rat. Whether these promising results can translate to new treatment or means of prevention remain to be determined in long-term clinical study with type 2 diabetic subjects.

Based on studies in cell, whole animal and human, stevioside and related compounds (steviol and rebaudioside A) affect plasma glucose via modulation of insulin secretion and sensitivity, which enhance glucose removal from the plasma. They also inhibit intestinal glucose absorption and glucose generation from the liver by altering the activities of a number of key enzymes involved in glucose synthesis, thereby reducing plasma glucose input. The possible anti-hyperglycemic actions of stevioside and related compounds are shown in Fig. 2. It is of interest to note that the effect of stevioside is dependent largely on the plasma glucose level, being observed only when plasma glucose level is elevated. Hence, it seems to be safe for normal healthy individuals. However, the mechanism of this effect is not known. Therefore, important issues, such as search for the active compound(s) in vivo following stevioside intake and determination of the mechanism of its action in humans, require immediate attention before stevioside can be developed for use as an affordable drug in the treatment of the growing population of diabetes patients in developing countries.

3.2. Anti-hypertensive effect

Mean arterial blood pressure (mABP) varies directly with tone of systemic vasculature or total peripheral resistance (TPR) and blood volume. Changes in any of these parameters affect mABP. In pathological state, arterial hypertension is a result of an inappropriate relationship between vasculature capacity/resistance and blood volume.

Early studies both in animals and humans demonstrated that stevioside and stevia extract decrease mABP by inducing vasodilation (decreased TPR) and diuresis as well as natriuresis, which leads to decreased plasma volume (Melis, 1995; Melis & Sainati, 1991a, 1991b). The anti-hypertensive effect of crude stevia extract (2.67 g of dry leaf/day) taken orally is time-dependent and requires prolonged administration. This is indicated by the observation that following oral administration of extract to normal and hypertensive rat, there is no significant change in blood pressure for the first 20 days. Indeed, the hypotensive effect of the extract was observed 40 and 60 days following administration (Melis, 1996). Similarly, reduced blood pressure occurs in rats following repeated oral dose of stevioside at 25 mg/kg BW/day for 6 weeks (Jeppesen et al., 2003). On the other hand, intravenous infusion of stevioside reduces blood pressure in both normal and hypertensive rats without any delay (Melis, 1992c). Furthermore, intravenous injection of stevioside in conscious spontaneously hypertensive rats produces a reduction in both systolic and diastolic blood pressure in a dose-dependent manner (Chan et al., 1998). Thus, acute hypotensive effect of stevioside can be seen only when given directly into systemic circulation.

Studies in humans have also shown the effect of stevioside on cardiovascular system. Stevioside causes bradycardia and hypotension (Humboldt & Boech, 1977). Similarly, a slight hypotensive effect was observed in human subjects who received a tea prepared from *S. rebaudiana* (stevia extract) daily for 30 days (Boeckh & Humboldt, 1981). In these studies, stevioside was also suggested to have inotropic effect by shortening systole duration. This would reduce stroke
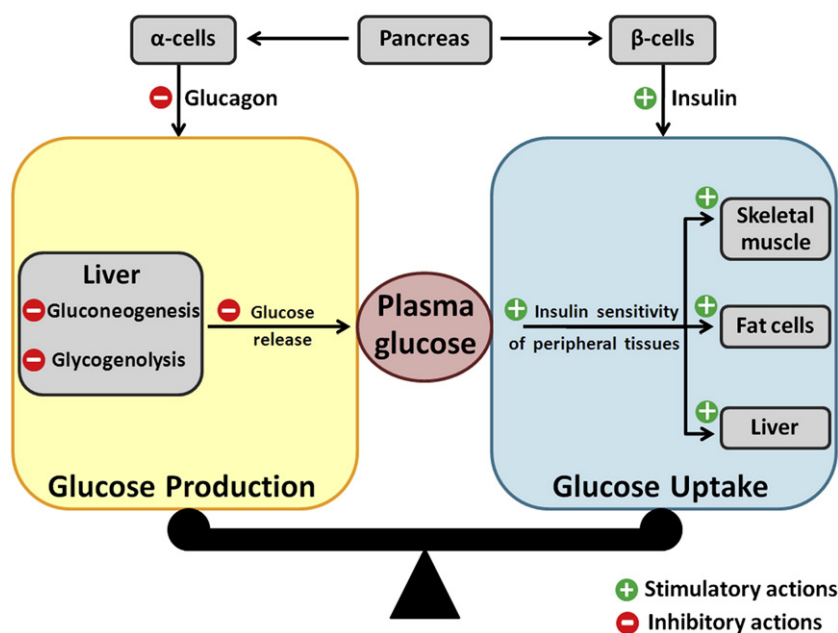
Fig. 2. The possible anti-hyperglycemic actions of stevioside and related compounds. Stevioside inhibits glucose production (minus sign) by 1) inhibiting glucagon secretion from α cell of pancreas which affects both gluconeogenesis and glycogenolysis, and 2) direct suppression of phosphoenol pyruvate carboxy kinase (PEPCK) activity, a rate limiting enzyme in gluconeogenesis in the liver. All of which causes a reduction of glucose release from the liver. On the other hand, stevioside, steviol and rebaudioside A stimulate glucose uptake (plus sign) by increasing insulin secretion from β cell of pancreas and enhancing insulin sensitivity of peripheral tissues promoting glucose uptake. Therefore, they exhibit anti-hyperglycemic action by reducing glucose production while increasing glucose uptake to maintain plasma glucose balance.

volume and then reduce mABP. Thus, both stevioside and stevia extracts seem to have anti-hypertensive effect.

Results from long-term clinical trials in humans with mild to moderate hypertension demonstrated that continued consumption of stevioside (750 mg/day) for one year reduces both systolic and diastolic blood pressure, whereas no significant side effect or alteration on lipid or fasting glucose was observed (Chan et al., 2000). Subsequent studies for up to 2 years with an increased dose of stevioside (1500 mg/day) showed that stevioside significantly decreases both systolic and diastolic blood pressure without any significant changes in body mass index, blood biochemistry values and left ventricular mass index (Hsieh et al., 2003). In addition, it was noted that the overall quality of life is significantly improved with stevioside treatment compared with placebo. These results further strengthen the hypotensive role of stevioside in humans. The investigators concluded that long-term oral stevioside intake is well-tolerated and may be considered as an alternative or supplementary therapy for hypertensive patients.

However, a lack of measurable effect of stevioside on mABP has recently been reported (Ferri et al., 2006). Oral administration for 6 weeks of a crude mixture of stevioside/rebaudioside A (15 mg/kg BW/day) to patients with mild essential hypertension produced no significant changes in blood pressure. This may be a consequence of the lower basal blood pressure values in these subjects along with differences in frequency of daily intake and duration of intake of stevioside compared to previous studies (Chan et al., 2000). Another possibility might be the purity or composition of the crude stevioside/rebaudioside A mixture, which may interfere with the effect of stevioside. Nevertheless, no major adverse clinical effects were observed. Geuns et al. (2007) also demonstrated that oral administration of stevioside (750 mg/day) for 3 days does not cause any significantly change in mABP in 9 subjects with normal blood pressure. These findings indicate that oral intake of crude stevioside is safe and supports the well-established tolerability during long-term used of stevioside as a sweetener. Similarly, consumption of 1000 mg of rebaudioside A for 4 weeks does not significantly alter mABP and heart rate in healthy adults with normal and low-normal blood pressure (Maki et al.,

2008). In fact, the intake amount of rebaudioside A in this study is about 10 times higher than that would be consumed as a sweetener (Renwick, 2008b).

Intracellular Ca^{2+} is important for myocardial contraction and vasoconstriction, which determines peripheral vascular resistance. Melis and Sainati (1991a) found that intravenous infusion of stevioside produces a marked hypotensive effect in a dose-dependent manner, which is likely to occur via a vasodilating effect acting through Ca^{2+} pathways. Verapamil, a known Ca^{2+} channel blocker of cardiac and vascular smooth muscles, enhances the systemic effect of stevioside, whereas $CaCl_2$ infusion induces a reduction in vasodilating response of stevioside (Melis, 1992a; Melis & Sainati, 1991a). Results using isolated aortic rings supported the notion that stevioside causes vasorelaxation via inhibition of Ca^{2+} influx into the vascular smooth muscle and is not effective in inhibiting intracellular Ca^{2+} release (Lee et al., 2001). This phenomenon was observed in the absence of endothelium (denuded vessel) showing that the vasorelaxation effect of stevioside is not related to nitric oxide.

However, the precise mechanism of stevioside anti-hypertensive action remains unclear. Indomethacin, a potent prostaglandin inhibitor, is able to abolish stevioside action on blood pressure (Melis & Sainati, 1991b), implying that the mechanism also involves prostaglandin activity. In addition, stevioside anti-hypertensive action occurs without changes in serum dopamine, norepinephrine and epinephrine levels, ruling out changes of sympathetic tone (Chan et al., 1998).

The anti-hypertensive effects of stevioside and stevia extract could be partly due to their effects on plasma volume. Intravenous infusion of stevioside in rats induces natriuresis, diuresis and increased renal plasma flow (RPF), but does not affect glomerular filtration rate (GFR) (Melis et al., 1986). As these phenomena are abolished by indomethacin it was suggested that stevioside may cause vasodilation of both afferent and efferent arterioles leading to increased RPF with no change in GFR (Melis & Sainati, 1991b). The increased urine flow rate or diuresis might have been due to decreased fluid and sodium reabsorption in the proximal tubule. This is supported by the findings of an increased glucose clearance following administration of stevioside in rat, indicating a drop in glucose reabsorption by proximal renal

tubular cells (Melis, 1992b). A diuretic effect of crude extract of *S. rebaudiana* has also been observed following oral administration in rat (Melis, 1995, 1996). Furthermore, steviol induces diuresis and natriuresis with no significant changes in renal plasma flow (RPF) and GFR after intravenous injection into rat (Melis, 1997). Thus, both chronic oral intake and acute intravenous administration of stevioside and steviol to rats produce diuresis and natriuresis leading to decreased plasma volume. However, those studies did not allow discrimination of the systemic effect from the direct effect on kidney function. Chatsudthipong and Thongouppakarn (1995) infused stevioside directly into renal artery of rat and observed diuresis, which occurs as a consequence of decreased proximal tubular reabsorption as indicated by lithium clearance. This result indicated that the target of stevioside is at the proximal tubule.

Taken together, stevioside reduces mABP by affecting both plasma volume and vascular resistance as shown in Fig. 3. However, stevioside appears not to have any significant impact on blood pressure in humans with normal and low-normal resting blood pressure. Stevioside blood pressure lowering-capacity is only observed in hypertensive subjects. Thus, there is a relatively low risk of development of hypotension in normal healthy human subjects at the amounts of stevioside encountered in the diet.

3.3. Anti-inflammatory and anticancer effects

Inflammation is an innate immune reaction of vascular tissues to noxious stimuli such as pathogen, injured cell or irritant. A satellite of cell types such as immune, epithelial and endothelial cells interactively participates in this process to remove the harmful stimuli and initiate the healing process. However, inflammation is also associated with a variety of disorders, such as autoimmune diseases (Atassi & Casali, 2008), inflammatory bowel disease (Bamias & Cominelli, 2007; Cho, 2008), atherosclerosis (Niessner et al., 2007) and cancer (Niessner et al., 2007). Furthermore, in some pathological circumstances, the consequences of inflammation can be devastating and are associated with functional deterioration of the affected organs. For example, in the case of inflammatory bowel disease, intestinal inflammation can lead to mucosal or even whole wall damage of the intestine, resulting in bloody diarrhea and malabsorption (Kelly, 1999). Any substance with anti-inflammatory effect will therefore be useful in preventing these undesirable inflammation-associated events.

There are ample evidences showing that stevioside has an anti-inflammatory effect both in vitro and in vivo. Initially, it was shown that skin inflammation induced locally by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was inhibited by steviol glycosides including stevioside (Yasukawa et al., 2002). In addition, the anti-tumor effect of stevioside was investigated as TPA is also known to induce cancer

formation in mammalian cells (Nakamura et al., 1995). Stevioside inhibits TPA-induced tumor promotion in a skin cancer model of two-stage carcinogenesis in mice. Mizushina et al. (2005) showed that isosteviol inhibits DNA polymerases and human DNA topoisomerase II, cellular targets for pharmacotherapy of cancer as well as inflammatory diseases. Moreover, isosteviol also retards growth of three different types of human cancer cells and inhibits inflammation induced by TPA.

Boonkaewwan et al. (2006) measured the release by a human monocytic THP1 cell line of proinflammatory cytokines (TNF-α and IL-1β) and nitric oxide, all known to participate in the development of a number of inflammatory disorders, and found that stevioside (1 mM) moderately stimulates their release in unstimulated THP1 cells by interacting with toll-like receptor-4, a principal receptor for lipopolysaccharide (LPS) on gram-negative bacteria. At this level of monocyte stimulation, stevioside could be beneficial in healthy individuals as a result of its effect in enhancing innate immunity. On the other hand, in LPS-stimulated THP1 cells, the same concentration of stevioside suppresses the release of TNF-α, IL-1β and NO by interfering with the signaling pathway of NF-κB, a transcription factor that controls the expression of inflammatory cytokines in these immune cells. Steviol has no effect on the release of these proinflammatory cytokines in both unstimulated and LPS-stimulated THP1 cells. Thus, in the case of an infected host, stevioside may be useful due to its ability to prevent undesirable effects of inflammatory response, and in healthy individual, it may offer an immune-related benefit as it can boost monocyte activity.

Stevioside and steviol exert anti-inflammatory effects on colonic epithelial cells. Under physiological regulation, colonocytes not only function to form a barrier across which fluid and electrolyte are transported but also serve as an innate immune sensor of microbial pathogen and commensal organisms (Sartor, 2008). The roles of colonocytes in inflammatory reactions are largely based on specific interaction between toll-like receptors on colonocytes and pathogen-derived antigens. This host–pathogen interaction leads to activation of NF-κB signaling pathway and subsequent induction of expression of proinflammatory cytokines, with IL-8, a neutrophil chemo attractant, being the major one. Recently, Boonkaewwan et al. (2008) evaluated the effects of stevioside and steviol on IL-8 release from a human colonic cell line. As maximal IL-8 release induced by TNF-α requires a shorter period of time than that induced by LPS, TNF-α was used as a stimulator. Using non-toxic doses of stevioside (<2 mM) and steviol (<0.2 mM), TNF-α-induced IL-8 release is unaffected by stevioside, but is suppressed by steviol. Immunoblot analysis showed that steviol reduces expression of NF-κB. As stevioside is completely degraded by resident microflora into steviol (Wingard et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Geuns 2003), it will be of particular
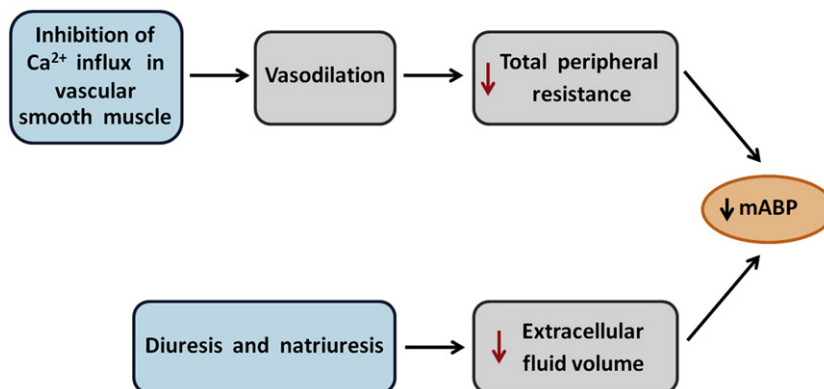
Fig. 3. Anti-hypertensive actions of stevioside. Stevioside inhibits Ca^{2+} influx in vascular smooth muscle, which causes vasodilation and consequentially reduction of total peripheral resistance. Stevioside also produces diuresis and natriuresis resulting in reduction of extracellular fluid volume. Thus, the underlining mechanism of anti-hypertensive action of stevioside is via its ability to reduce both factors that determine mean arterial blood pressure (mABP).

interest to evaluate the ability of oral stevioside to reduce inflammation or cancer transformation in an animal model of colitis.

Immunomodulatory activities in vivo of stevioside have recently been demonstrated (Sehar et al., 2008). Stevioside (6.25, 12.5 and 25 mg/kg BW) fed to mice promote phagocytic functions as indicated by an increased phagocytic index in carbon clearance test and increased humoral immune response as measured by an increase in antibody titre to test antigen. In vitro experiments also demonstrated the stimulatory effects of stevioside on phagocytic activity and on B and T cell proliferation stimulated by LPS and concanavalin A, respectively. These results further support the supposition that the oral consumption of stevioside may be useful in promoting immunity against infection by microorganisms.

3.4. Potential applications as antidiarrheal therapeutics

Diarrhea is defined as an increase in watery content or frequency of stool, typically more than 3 times/day (Binder, 1990). The most common cause of this pathological condition is intestinal infections with bacteria and viruses (Petri et al., 2008). These pathogens can cause either direct invasive damage to the intestine or deranged intestinal functions, resulting in diarrheal symptoms. Diarrhea can be classified into 4 types, namely, secretory, osmotic, motility-related and exudative diarrhea (Binder, 1990). Currently, the mainstay of diarrheal treatment is rehydration therapy and antibiotic treatment. However, the former is mainly supportive and the latter may not be effective in situations where antibiotic resistance exists. Thus, there is still a need to discover novel specific pharmacotherapy for diarrheal diseases. Identification of potential antidiarrheal drugs from traditional medicines represents an attractive strategy as their efficacy and safety have been supported by human experiences.

Potential application of stevioside in the treatment of diarrhea was originally suggested from observations of bactericidal and anti-rotavirus activities of hot water extract of S. rebaudiana. Tomita et al. (1997) were the first to report a bactericidal effect of this extract against a broad range of food-borne pathogenic bacteria, including enterohemorrhagic Esherichia coli, known to cause severe hemorrhagic/exudative diarrhea. This stevia extract also inhibits the growth of rotavirus, an RNA virus whose infection causes gastroenteritis in children, by interfering with rotavirus binding to host cells (Takahashi et al., 2001). A large polysaccharide-containing compound, molecular weight of 9800 Da, was isolated and suggested to be responsible for the viral inhibitory effect. However, other fractions of S. rebaudiana extract were also found to produce similar anti-rotavirus properties. As stevioside is a major constituent in this stevia extract, it is therefore possible that stevioside or other related glycosides contributes to the anti-diarrhea effects.

Stevioside has an inhibitory effect on intestinal smooth muscle contraction, stimulation of which results in hypermotility-associated diarrhea (Shiozaki et al., 2006). At a concentration of 1 mM, stevioside inhibits $CaCl_2$ (10 mM)-induced contraction of isolated guinea pig ileum by 40%. The mechanism was thought to be related to its inhibitory effect on Ca^{2+} influx into muscle cells. Thus, stevioside may be useful in the treatment of diarrhea resulting from intestinal hypermotility, such as irritable bowel syndrome and inflammatory bowel disease. Further studies in whole animal models are needed to demonstrate in vivo anti-diarrheal efficacy of stevia hot water extract in hemorrhagic diarrhea and stevioside in hypermotility diarrhea. In addition, these studies indicate low potency of stevioside and further optimization will be required to obtain more potent stevioside-derived compounds.

A therapeutic potential of steviol and its analogs (dihydroisosteviol) in the treatment of secretory diarrhea, which primarily results from excessive intestinal fluid secretion, has recently been demonstrated (Pariwat et al., 2008). In general, fluid secretion into intestine is driven by osmotic force generated by active secretion of anions, especially chloride, into intestinal lumen (Field, 2003). Bacterial enterotoxin is capable of stimulating active chloride secretion, followed by paracellular transport of sodium and water (Field, 1979). This enterotoxin-mediated hypersecretory response leads to a massive intestinal fluid loss and dehydration in secretory diarrhea (Field, 2003). Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel, provides the principal route for chloride secretion in most types of secretory diarrhea (Thiagarajah & Verkman, 2003). In a human intestinal T84 cell line, steviol but not stevioside inhibits cAMP-activated chloride secretion with an IC_{50} of 100 μM (Pariwat et al., 2008). Synthesis of steviol analogs has led to the identification of series of more active compounds, with the most potent being dihydroisosteviol, which reversibly inhibits cAMP-activated chloride secretion in T84 cells with an IC_{50} of 10 μM (Pariwat et al., 2008). Electrophysiological and enzymatic analysis indicated that dihydroisosteviol directly targets CFTR. This compound is nontoxic and specific to chloride secretion stimulated by cAMP, but not by calcium. In vivo mouse model of cholera showed that intestinal fluid secretion stimulated by Vibrio cholera enterotoxin is markedly reduced by dihydroisosteviol. Also, intraperitoneal administration of steviol is effective in reducing intestinal fluid secretion in this mouse model (Muanprasat, C., unpublished observation). These results are consistent with previous pharmacokinetics studies suggesting enterohepatic circulation of steviol (Nakayama et al., 1986; Cardoso et al., 1996). Steviol-related compounds compare favorably with other CFTR inhibitors under development (Ma et al., 2002; Muanprasat et al., 2004; Sonawane et al., 2005; Sonawane et al., 2006; Muanprasat et al., 2007; Sonawane et al., 2007; Sonawane et al., 2008), and therefore represent a novel option in the development of new antidiarrheal CFTR inhibitors. Further optimization of steviol analogs needs to be performed, including mechanistic studies, toxicity evaluation and in vivo efficacy in large animal models.

3.5. Interaction with renal organic ion transporters

A dose of 1.5 g/kg BW of stevioside given subcutaneously and 4.1 g/kg BW given via intragastric tube to rat and hamster respectively causes nephrotoxicity as indicated by an increase in blood urea nitrogen and plasma creatinine levels (Panichkul et al., 1988; Toskulkao et al., 1994a, 1994b). Histopathological changes were found mainly in proximal tubules, suggesting that this might be a major target site of stevioside (Toskulkao et al., 1994a). Renal proximal tubules serve an important function in eliminating various compounds and xenobiotics via the organic anion and cation secretory systems (Pritchard & Miller, 1993). Thus, inhibition or interference of these secretory transport systems reduces clearance of xenobiotics and therapeutic drugs, potentially leading to altered therapeutic efficacy of the latter or even to increased toxic side-effects.

Stevioside and steviol inhibit uptake in rat renal cortical slices of p-aminohippurate (PAH), the prototypical organic anion, suggesting that stevioside and steviol are affecting handling of such compounds by organic anion secretory system of the kidney (Toskulkao et al., 1994b). However, at a pharmacological concentration (0.7 mM) and that maximally dissolved in buffer, stevioside has a small and reversible inhibitory effect on rabbit renal proximal tubular transport of PAH, and does not affect cellular ATP content and Na^+/K^+-ATPase activity (Jutabha et al., 2000). On the other hand, steviol at a lower concentration (10 μM) demonstrates a significant inhibition of PAH transepithelial transport. Although the precise mechanism by which stevioside and steviol inhibit PAH transepithelial transport still remains unclear, the findings indicate a role in inhibiting or interfering with basolateral organic anion transporters (OATs) (Chatsudthipong & Jutabha, 2001; Jutabha et al., 2000).

Several OAT isoforms have been cloned and characterized. In humans, hOAT1, hOAT2 and hOAT3 are expressed at the basolateral

membrane (Hosoyamada et al., 1999; Cha et al., 2001; Motohashi et al., 2002), whereas hOAT4 and hOAT5 are present at the apical membrane of proximal tubule (Babu et al., 2002; Cha et al., 2000; Youngblood & Sweet, 2004; Anzai et al., 2005). Methotrexate transport via hOAT1, hOAT3, and hOAT4 is inhibited by probenecid, penicillin G and nonsteroidal inflammatory drugs (Takeda et al., 2002), whereas hOAT2-mediated tetracycline transport is inhibited by diverse structural compounds, including bromosulphophthalein (BSP), diclofenac, erythromycin, ibuprofen, bumetanide and furosamide (Burckhardt & Burckhardt, 2003; Kobayashi et al., 2005). However, no function has yet been assigned for hOAT5.

To determine which specific basolateral organic anion transporter in the renal proximal tubule is affected by stevioside and steviol, the functions of organic anion transporters (hOAT1, hOAT2, hOAT3 and hOAT4) singly expressed in *Xenopus laevis* oocytes and renal S2 cells were evaluated (Srimaroeng et al., 2005a, 2005b). No hOATs are inhibited by stevioside (50 μM–1 mM). Likewise, electrophysiological analysis showed that stevioside does not produce membrane currents in oocytes expressing winter flounder OAT1 (Srimaroeng et al., 2005a). Furthermore, stevioside shows no inhibitory effect on uptake of PAH and estrone sulfate, specific substrate of OAT1 and OAT3 respectively, in mouse renal cortical slices (Srimaroeng et al., 2005b), indicating that stevioside does not interact with and is not transported by either basolateral OAT1 or OAT3.

The lack of interaction of stevioside with basolateral membrane OATs could be due to its large size, as it is composed of one molecule of steviol and three molecules of glucose, and neutrality at physiological pH would make it unlikely to access the substrate binding site. Although the results obtained in the cloned transporters were clear for human and mouse OATs (Srimaroeng et al., 2005a, 2005b), there is a small and reversible inhibitory effect of stevioside on PAH transport of rabbit renal proximal tubule (Jutabha et al., 2000). This discrepancy might be attributed to a sufficient amount of inhibitory stevioside derivatives present at this high concentration of stevioside (0.7 mM) used in the rabbit study. Moreover, it is possible that these findings might reflect species differences. For instance, the affinity of hOAT1 for PAH transport is higher than that of other species (Burckhardt et al., 2001), and likewise, mouse OAT2 had a higher affinity for PGE_2 than that of hOAT2 and rat OAT2 (Dantzler & Wright, 2003).

However, these findings are in contrast with earlier in vivo studies that found urinary stevioside clearance to be greater than that of inulin, indicating tubular secretion (Melis, 1992b; Cardoso et al., 1996). Possible explanations include that the stevioside used in the in vivo studies might contain other stevioside derivatives, or that some metabolism to stevioside occurs after its injection. As stevioside does not appear to interact with renal transporters, this implies that it should not be secreted intact. Stevioside intake in human has been shown to be excreted in urine as steviol conjugated with glucuronide (Geuns et al., 2006). Alternatively, another transport process may be responsible for the in vivo findings. Recently, a novel organic anion transporter from human kidney (OATP4C1) has been characterized (Mikkaichi et al., 2004). This basolateral transporter handles organic anions as well as digoxin and ouabain, large neutral cardiac glycosides, and thus OATP4C1 may possibly be responsible for transport of stevioside.

Similar to stevioside, steviol inhibits transepithelial transport of PAH in isolated perfused rabbit renal proximal tubule (Chatsudthipong & Jutabha, 2001). However, steviol is an effective inhibitor of hOAT1- and hOAT3-mediated transport expressed in both *Xenopus* oocytes and renal S2 cells (Chatsudthipong & Jutabha, 2001; Srimaroeng et al., 2005a, 2005b). Steviol also inhibits PAH and ES transport in mouse renal cortical slices (IC_{50} of 12.8 ± 3.0 and 67.6 ± 1.8 μM, respectively) (Chatsudthipong & Jutabha, 2001; Srimaroeng et al., 2005a, 2005b).

The interactions of stevioside and steviol with organic cation transporters (OCTs) have been investigated in Chinese hamster ovary

(CHO-K1) cells stably expressing rabbit (rb)OCT1 and rbOCT2 and in isolated S2 segments of rabbit proximal tubules (Chatsudthipong et al., 2003). In CHO-K1 cells, steviol but not stevioside inhibits organic cation uptake via these transporters in a dose-dependent manner. RbOCT1 exhibits a higher affinity for steviol than rbOCT2. In intact renal tubules, both stevioside and steviol are able to inhibit substrate uptake, but the inhibitory effect of steviol is greater than that of stevioside. These data indicate that steviol interacts with rabbit OCTs in both systems, but the interaction of stevioside is limited to the intact renal tubules.

Experimental data collected to date indicate that steviol and possibly stevioside to a lesser extent interact with both organic anion and cation transport systems. Steviol may have a profound effect on these systems if it is present at 10 μM in plasma. The ADI of stevioside (5 mg/kg BW/day) would yield a maximum plasma concentration of steviol of approximately 20 μM if stevioside is completely converted to steviol (JECFA, 2006). A recent study showed that following a single oral dose of stevioside (4.2 mg/kg administered to healthy adult male subjects) plasma steviol was detected at a level of 121 ng/ml (0.38 μM) (Wheeler et al., 2008). The majority of steviol is present in a conjugated form. At this low plasma concentration following consumption with ADI amount of stevioside, steviol should not have any effect on the renal transport systems. On the other hand, steviol may be developed for therapeutic purposes. It could help delay clearance of drugs leading to enhanced efficacy. Therefore, it is important that more research be conducted to evaluate the effect of stevioside consumption on therapeutic efficacy of drugs.

4. Safety aspects: toxicity, carcinogenicity and teratogenicity

Due to their popular use as non-caloric sugar substitutes and remedies, toxicological properties of stevioside and steviol have been extensively studied in both in vitro setting and in experimental animals. In addition, carcinogenic and teratogenic potential as well as reproductive effects of stevioside and steviol have also been evaluated.

4.1. Toxicological studies

In vitro studies involving a number of different cell lines from kidney and intestine indicated that stevioside at a concentration as high as 2 mM needs to be exposed to cells to affect cell viability, but 0.2 mM steviol significantly reduce cell viability (Srimaroeng et al., 2005b; Boonkaewwan et al., 2008). This toxic effect might have been a consequence of an interruption in mitochondrial metabolism as suggested from earlier studies (Toskulkao et al., 1997; Srimaroeng et al., 2005b).

In acute and chronic toxicity evaluations of stevioside ingestion investigated in mouse, rat and hamster, stevioside intake as high as 15 g/kg BW produces no acute toxicity (Akashi & Yokoyama, 1975; Mitsuhashi, 1976; Medon et al., 1982; Xili et al., 1992; Toskulkao et al., 1997). However, oral administration of steviol is lethal with an LD_{50} of 5–20 g/kg BW, depending on animal species, with hamster being more susceptible to steviol toxicity than rat and mice (Toskulkao et al., 1997). Histological examination of steviol-treated hamster revealed proximal tubular cell degeneration, the extent of which is correlated with the rise in serum blood urea nitrogen and creatinine, indicators of deterioration of renal function. Thus, the possible cause of death in steviol-treated hamster is acute renal failure.

Ingestion of stevioside (750 mg/day for 3 month) by healthy individuals or those with underlying diseases such as diabetic mellitus and hypertension produced no adverse effects or abnormalities in liver and renal function tests and serum electrolytes (Hsieh et al., 2003; Barriocanal et al., 2008).

An ADI of 0–2 mg/kg of body weight for steviol, equivalent to 0–5 mg/kg BW of stevioside, as suggested by Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006), was estimated to yield a stevioside concentration in colon at about 0.05–0.2 mM, a

concentration at which no toxicity to intestinal cells is expected (Boonkaewwan et al., 2008). According to previous pharmacokinetic studies in humans, after oral administration of a single dose of 4.2 mg of stevioside/kg BW, mean maximal concentration of steviol glucuronide and free steviol in plasma is 1.89 μg/ml (3.7 μM) and 0.19 μg/ml (0.38 μM) respectively (Wheeler et al., 2008). This level of steviol should not be toxic to human cells, and steviol glucuronide appears not to be converted back to steviol.

4.2. Carcinogenicity evaluation

The carcinogenic potential of stevioside is of particular concern and a number of investigations using different experimental models have been conducted to evaluate the mutagenic effects of stevioside and steviol. Bacterial genetic analysis revealed that stevioside is not mutagenic (Pezzuto et al., 1985; Pezzuto et al., 1986; Suttajit et al., 1993; Matsui et al., 1996a; Klongpanichpak et al., 1997). However, from various mutagenic assays the genotoxic potential of steviol remains inconclusive. In mutation assays using *Salmonella typhimurium* TM677, steviol showed genetic toxicity after metabolic activation by liver homogenate (Pezzuto et al., 1985; Matsui et al., 1989; Matsui et al., 1996a; Temcharoen et al., 1998; Terai et al., 2002). Mutagenic mechanisms of metabolically activated steviol were shown to be through induction of transition, transversion, duplication and deletion of genetic material (Matsui et al., 1996b). Oxidative metabolites of steviol, such as 15-oxo-steviol, were suggested to account for this mutagenicity (Terai et al., 2002). In addition, studies using *E. coli* (Nunes et al., 2006) and cultured mammalian cells (Matsui et al., 1996a) suggested that steviol is able to produce genetic lesions that could lead to cancer formation. Nevertheless, other bacterial mutagenic assays, such as reverse mutation, Ames and rec assays failed to demonstrate steviol mutagenic activity (Matsui et al., 1996a; Klongpanichpak et al., 1997). Furthermore, cultured blood lymphocytes from human donors display no chromosomal alterations after exposure to stevioside and steviol (Suttajit et al., 1993; Temcharoen et al., 2000).

In vivo testing of stevioside carcinogenic potential has also been performed in both mouse and rat. Stevioside given orally does not increase the incidence of cancer in rat (Yamada et al., 1985; Hagiwara et al., 1984; Xili et al., 1992; Toyoda et al., 1997; Sekihashi et al., 2002). No evidence of carcinogenicity of stevioside was obtained from in vivo studies in mice (Matsui et al., 1996a; Yasukawa et al., 2002). Indeed, stevioside suppresses tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in mice skin (Yasukawa et al., 2002). However, Nunes et al. (2007), using a comet assay, reported that ingestion of stevioside causes DNA damage in cells of peripheral blood, liver, brain and spleen, with the most profound lesions observed in liver. However, these results need to be confirmed and it must be noted that a positive result in comet assay may not always associate with development of cancer (Sasaki et al., 2000). In 2006, JECFA stated that there was no evidence of stevioside-induced genotoxicity in vitro and in vivo. At dosages of up to 2.0 g/kg BW (males) and 2.4 g/kg BW (females), stevioside did not promote any incidence of cancer.

4.3. Fertility and teratogenicity evaluation

A concern about antifertility and teratogenic effects of stevioside was raised after it was shown that stevia decoctions decrease live birth rate in rats (Planas & Kuc, 1968), but these results could not be replicated (Shiotsu, 1996). A number of studies have demonstrated that oral stevioside has no effect on fertility in mice (Akashi & Yokoyama, 1975), rats (Mori et al., 1981; Xili et al., 1992) or hamsters (Yodyingyuad & Bunyawong, 1991). Rats fed with food containing up to 1% (w/w) stevioside have no changes in spermatogenesis or interstitial cell proliferation (Yamada et al., 1985). However, when fed an unusually high dose (2.6 g/day) of stevia extract for 2 months rats can manifest a decrease in fertility (Melis, 1999). However, this might

possibly have been due to the presence of minor components that are toxic at these high amounts of stevia extract.

As development of chicken embryo is sensitive to toxicants, teratogenicity of stevioside and steviol was tested in fertile broiler eggs (Geuns et al., 2003b). After injection of stevioside or steviol into eggs, embryonic development undergoes normally without any decrease in embryonic mortality and body weight of hatchling, or any structural deformation. Moreover, oral stevioside has no adverse effect on fertility, mating performance, pregnancy or condition of litters in rat and hamster (Mori et al., 1981; Oliveira-Filho et al., 1989; Yodyingyuad & Bunyawong, 1991). However, toxicity in pregnant hamsters and their embryos develops after oral administration of steviol at doses higher than 500 mg/kg BW (Wasuntarawat et al., 1998). This amount of steviol is equivalent to stevioside dosage of 1250 mg/kg BW, which is much higher than ADI (5 mg/kg BW) of stevioside or doses claimed to have a therapeutic efficacy in the treatment of diabetic mellitus or hypertension (as discussed above).

Taken together, most studies are in agreement demonstrating that oral stevioside, at an acceptable daily intake (5 mg/kg BW), is safe and not carcinogenic or teratogenic.

5. Future directions

A great deal has been learned about the biological and pharmacological effects of stevioside and related compounds. However, there are areas where important gaps in knowledge remain, preventing it from earning the "Generally Recognized as Safe (GRAS)" license from the Food and Drug Administration. Thus, even though it has been approved for use as food additive in several countries, its use in the United States is limited to a "dietary supplement". Its status in the European Union (EU) is similar. Before this situation can change the following issues should be considered.

1) Human studies — Most of the available studies of stevioside and related compounds (steviol and rebaudioside A) have been performed in cell cultures, isolated tissues, or experimental animals. Only few studies have been carried out in humans. Clearly, comprehensive clinical studies in humans are needed. Such information is a prerequisite to any rational design of pharmaceutics related to stevioside.

2) Chemical purity — Many of the existing studies have used different forms of stevioside (e.g., extract vs. pure compound). This makes it difficult to draw firm conclusions regarding the basis for the observed effects (Chen et al., 2005; Ferreira et al., 2006; Wong et al., 2006). Therefore, purity of the test compounds needs to be considered. Certainly ingested stevioside appears to be completely metabolized to steviol prior to its absorption. Thus, it would be appropriate to focus future work on steviol or its metabolites in order to identify active compound(s) responsible for the observed effects following intake of stevioside. Using this approach our own recent study has shown that steviol, but not stevioside, may prevent intestinal fluid loss in secretory diarrhea by inhibiting CFTR function (Pariwat et al., 2008). Similarly, we found that steviol, but not stevioside, is transported by the organic anion transporters in the renal tubule (Srimaroeng et al., 2005a). Taken together, these data indicate that steviol is the pharmacologically active compound. Thus, the full scope of steviol pharmacology should be assessed.

3) Route — As noted above, most stevioside studies have been conducted following oral administration. Intravenous infusion of stevioside or steviol needs to be examined over the range of pharmacological effects that have been reported. Even such basic issues as whether stevioside is converted to steviol in the plasma have not yet been addressed. Indeed, such studies using purified stevioside, steviol, rebaudioside A and their metabolites will go far towards clarifying the mechanism of their actions.

4) Safety — Importantly, remaining uncertainties about the safety of stevioside must be resolved, with particular attention to the reproductive effects that have given rise to FDA concern (FDA, 2007). Lacking this critical data, it will not be possible to proceed with development of stevioside and its derivatives as pharmaceutics or as non-caloric sweeteners for human foods.

Acknowledgments

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