Enhanced Glucose Tolerance and Pancreatic Beta Cell Function by Low Dose Aspirin in Hyperglycemic Insulin-Resistant Type 2 Diabetic Goto-Kakizaki (GK) Rats

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Key Words
Goto-Kakizaki rats • Serum • Pancreas • Insulin • Hyperglycemia • Oxidative stress

Abstract
Background/Aim: Type 2 diabetes is the most common metabolic disorder, characterized by insulin resistance and pancreatic islet beta-cell failure. The most common complications associated with type 2 diabetes are hyperinsulinemia, hyperglycemia, hyperlipidemia, increased inflammatory and reduced insulin response. Aspirin (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with the prevention of diabetes, obesity and related cardiovascular disorders. Aspirin has been used in many clinical and experimental trials for the prevention of diabetes and associated complications. Methods: In this study, five month-old Goto-Kakizaki (GK) rats, which showed signs of mild hyperglycemia (fasting blood glucose 80-95 mg/dl vs 55-60 mg/dl Wistar control rats) were used. Two subgroups of GK and Wistar control rats were injected intraperitoneally with 100 mg aspirin/kg body weight/day for 5 weeks. Animals were sacrificed and blood and tissues were collected after performing glucose tolerance (2 h post 2g IP glucose ingestion) tests in experimental and control groups. Results: Aspirin caused a moderate decrease in hyperglycemia. However, we observed a significant improvement in glucose tolerance after ASA treatment in GK rats compared to the nondiabetic Wistar rats. Also, the ASA treated GK rats exhibited a significant decrease in insulinemia. ASA treatment also caused a marked reduction in the pro-inflammatory prostaglandin, PGE2, which was significantly higher in GK rats. On the other hand, no significant organ toxicity was observed after ASA treatment at this dose and time period. However, the total cholesterol and lipoprotein levels were significantly increased in GK rats, which decreased after ASA treatment. Immunofluorescence staining for insulin/gluca gon secreting pancreatic cells showed improved beta-cell structural and functional integrity in ASA-treated rats which was also confirmed by SDS-PAGE and Western blot analysis. Conclusion: The improved
glucose tolerance in ASA-treated GK rats may be associated with increased insulin responses due to the anti-inflammatory properties of ASA and enhanced nitric oxide (NO) level which facilitated insulin signaling and energy utilization in target tissues. These results may have implications in determining the therapeutic use of ASA in insulin-resistant type 2 diabetes.

Introduction

Studies by the International Diabetes Federation (IDF) estimated that 382 million people had diabetes in 2013 and this number is expected to rise to 592 million by 2035 [1]. This increase is attributed to rapid economic development and changes in life style that have led to reduced physical activity, increased intake of refined high calorie diet and a rise in obesity [1, 2]. In 2012, the American Diabetes Association (ADA) had predicted the number of Americans with type 2 diabetes to be 23.3 million, with a projected cost of $306 billion [3]. Type 2 diabetes (T2D) is a multifactorial metabolic syndrome associated with progressive impaired insulin response and insulin resistance and characterized by compromised energy metabolism, hyperglycemia, hyperlipidemia, and cardiovascular abnormalities. However, the precise molecular mechanisms of disease progression and complications are still unclear. The Goto-Kakizaki (GK) rat is a spontaneous non-obese animal experimental model of type 2 diabetes. This strain was developed by selective breeding of glucose intolerant Wistar rats over many generations. The GK rats exhibit decreased β-cell numbers and function which is accompanied by mild hyperglycemia, impaired glucose-induced insulin secretion, marked glucose intolerance, peripheral insulin resistance and chronic inflammation [4-6]. Despite the mild hyperglycemia several manifestations of diabetes complications have been demonstrated in this model [7-9]. Growing evidence has linked T2D with low-grade systemic inflammation. Thus, the GK rat represents a good animal model for studying human T2D pathophysiology and the effects of therapeutic options such as the use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs). It is possible that existing low-grade systemic inflammation can be suppressed by experimental strategies aimed at blocking the production or action of proinflammatory signaling pathways, thereby treating T2D. The salicylate class of drugs, which are routinely used to reduce inflammation, was first used as an anti-diabetic drug by Ebstein, in 1876 [10]. There are several studies on the glycemic effects of salicylates and aspirin (acetylated salicylic acid) in the literature, but the mechanism of their antidiabetic effects is still unclear [11-12]. Recently, sodium salicylate and aspirin have been shown to inhibit activation of the transcription factor NF-κB [13] by preventing degradation of IKK-β, which regulates the inflammatory responses, and might therefore ameliorate insulin resistance and improve glucose tolerance in at least some patients with T2D. However, the exact mechanism and effects of aspirin on treatment of T2D have not been completely determined.

Salsalates have also been shown to enhance metabolic rate and energy expenditure in GK rats [14]. Recent studies on experimental rodent models of diabetes and clinical trials have demonstrated beneficial and deleterious effects of aspirin on diabetes-associated complications and insulin sensitivity depending upon the dose of aspirin and experimental model used [12, 15-18]. Our recent studies in diabetic rats have demonstrated that diabetes-associated hyperglycemia caused tissue-specific increase in oxidative stress and mitochondrial dysfunction [19-22]. The aim of the present study was to evaluate the potential role of aspirin and the biochemical mechanism involved in improving glucose tolerance and insulin sensitivity in type 2 mildly diabetic GK rats. We have demonstrated that low dose ASA treatment for 5 weeks reduced hyperglycemia with improved glucose tolerance and pancreatic insulin secretory function in these rats.
Materials and Methods

Chemicals
Aspirin, bovine serum albumin, picric acid, paraformaldehyde, trisodium citrate, potassium phosphate were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Polyclonal antibodies against insulin and glucagon were purchased from Dako, Denmark. FITC and TRITC conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Groove, Pa, USA). Kits for insulin were purchased from EMD Millipore Corporation (Billerica, MA, USA), PGE2 from Arbor Assays (Michigan, USA), total nitrate/nitrite from R & D Systems, Inc., (Minneapolis, MN, USA), SOD from Trevigen, Inc., (Gaithersburg, MD, USA). Reagents for SDS-PAGE and Western blot analyses were purchased from Gibco BRL (Grand Island, NY, USA) and Bio Rad Laboratories (Richmond, CA, USA).

Animals
Ten male GK rats (weighing 100-120 g) were procured from Taconic (Germantown, NY, USA) at five weeks of age and ten male Wistar rats of similar age and weight were procured from the Animal house facility of the College of Medicine & Health Sciences, United Arab Emirates University and were used as the non-diabetic controls to evaluate the progression of diabetes in GK rats. All animals were maintained under standard laboratory animal conditions including a 12-hour light/dark cycle with free access to food and water ad libitum. Approval for this project was obtained from the Animal Ethics Committee, College of Medicine & Health Sciences; United Arab Emirates University and all the animals were used according to the safe practice for animals in research guidelines as stipulated by NIH, USA. Body weights and blood glucose levels (using the OneTouch Ultra glucometer and test strips, LifeScan Inc., U.S.A) were regularly monitored.

Experimental protocol
After 4 months, the GK rats (avg. body wt. 319g) showed signs of hyperglycemia (fasting blood glucose 80-90mg/dl) while the control Wistar rats (avg. body wt. 323g) had a fasting blood glucose level of 50-60mg/dl. The animals were then divided into four subgroups, each containing 5 animals. Two subgroups of GK and Wistar control rats were injected intraperitoneally with 100mg ASA/kg body weight/ day for 5 weeks. This low experimental dose and time points with minimum side effects of ASA were selected based on the published reports in previous studies, using diabetic models including GK rats [12-18]. Towards the end of the experiment, the animals were subjected to a glucose tolerance test. Briefly, after an overnight fasting, animals were injected with glucose (2g glucose/kg body weight) intraperitoneally. Blood samples were collected from the tail vein at time 0 (prior to the glucose load), 60 and 120 minutes after the glucose challenge and glucose levels tested using the OneTouch Ultra glucometer and test strips.

At the end of 5 weeks, animals were sacrificed by decapitation and blood was collected from the jugular vein. Pancreas from the animals was quickly excised and a portion of it was fixed in Zamboni’s solution and processed for immunofluorescence studies. The rest of the pancreatic samples were homogenized (25% w/v) in isotonic 100mM potassium phosphate buffer (pH 7.4) containing 1mM EDTA and 0.1mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 600g for 10 min and the supernatant was used for Western blot analysis. Protein concentration in the homogenate and serum was measured using Bio-Rad reagent as described before [19-22].

Serum analysis
Blood samples were centrifuged at 3000g for 10-15 min and the serum was used to assay key organ function tests, cholesterol and lipoproteins, glucose levels and uric acid on the COBAS® INTEGRA 400 plus auto-analyzer (Roche, Basel, Switzerland). Insulin, PGE2, total NO and SOD assays were also performed in the serum samples using appropriate kits as suggested in vendor’s protocols and as described before [20, 23].

Immunofluorescence studies
Localization of insulin and glucagon in pancreatic islets of control Wistar and GK rats was performed by a double-labeled immunofluorescence method as described by Adeghate et al. [24]. Briefly, pancreatic tissue pieces were fixed overnight in Zamboni’s solution, dehydrated in graded concentration of ethanol, cleared using xylene and embedded in paraffin wax at 55°C. Sections of 5 µm thickness were cut on a microtome.
Amiri et al.: Hypoglycemic Effects of Aspirin

(Shandon AS325, Pittsburg, PA, USA), deparaffinized with sequential changes in xylene, and rehydrated with descending concentrations of ethanol. This was followed by antigen retrieval, conducted by boiling the slides in citrate buffer followed by gradual cooling. The sections were then treated with a blocking agent (0.5% bovine serum albumin) for 45 minutes at room temperature after washing in PBS and then incubated overnight at 4°C with insulin antibody (1:1000 dilution) followed by the corresponding anti-guinea pig FITC-conjugated secondary antibody after washing in PBS. The sections were then incubated with glucagon antibody (prediluted) overnight at 4°C followed by anti-rabbit TRITC-conjugated secondary antibody and then mounted using Immunomount® (Thermo electron Corporation, USA) and viewed with the Carl Zeiss fluorescent microscope.

**Analysis of insulin-and glucagon-immunoreactive cells in the pancreatic cells**

To assess the effect of ASA treatment on the size of the islets and the number of insulin- and glucagon-immunoreactive cells, the total number of positive cells and the size of the islets of Langerhans from control and GK rats treated with/without ASA were measured using Axiovision® 3.0 Image Analysis System (Zeiss, Gottingen, Germany) attached to the Carl Zeiss fluorescent microscope. The ratio of insulin positive to glucagon positive cells was then calculated. The number of insulin-immunoreactive cells divided by the total number of cells gave the percentage of insulin secreting cells. Insulin- or glucagon-positive cells were counted in visible sections at X40 magnification. A total of 10 random islets were analyzed from a total of 5 slides in each group.

**SDS-PAGE and Western blot analysis**

Pancreatic homogenates (50µg) and serum samples (10µg) were electrophoretically separated by 15% SDS-PAGE and transferred onto nitrocellulose paper using previously described techniques [19-23]. Briefly, the nitrocellulose membranes were blocked with 5% non-fat milk, after which they were immunoblotted with mouse monoclonal insulin antibody. The blots were then incubated with goat anti-mouse antibody and developed using the Pierce Western blot substrate as described before [19-23]. The expression of insulin was assessed by immunoreaction with specific antibodies by Western blot analysis. Densitometric analysis of the protein bands was performed using the Typhoon FLA 9500, GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and expressed as percentage protein compared to the control group.

**Statistical Analysis**

Data analysis was performed using SPSS software, version 21. All results are expressed as means ± SEM of at least four determinations. Statistical significance of the data was assessed using analysis of variance followed by LSD’s post-hoc analysis. P values < 0.05 were considered statistically significant.

**Results**

**Effect of ASA on body weight and blood glucose and insulin levels**

Aspirin treatment had marginally lowered the body weight-both in control Wistar (325g vs 294g) and GK rats (343g vs 326g). The blood glucose level (Fig. 1A) in GK rats treated with ASA was moderately (17%) reduced (from 14.7mM to 12.5mM). This could be due more efficient glucose uptake in ASA-treated GK rats. Similarly, blood insulin level (Fig. 1B) which was elevated (> 2-fold) in GK rats was significantly reduced after ASA treatment. No significant changes in blood glucose or insulin levels were observed in control non-diabetic rats treated with ASA. These results may suggest increased energy expenditure in ASA-treated diabetic rats which is presumably due to increased insulin sensitivity and responses in peripheral tissues. Further studies on the expression of GLUT receptors and insulin responses in various tissues are in progress.

**Effect of ASA on glucose tolerance**

ASA treatment significantly (20-30%) improved glucose tolerance (2 h post I.P. administration of 2g glucose/ kg body weight) in diabetic rats compared to control rats (Fig. 2). Improved glucose tolerance in ASA-treated rats was observed within 30 minutes
Amiri et al.: Hypoglycemic Effects of Aspirin

Fig. 1. Blood glucose and insulin levels in control Wistar (C) and GK rats treated with/without ASA (n=5). Serum separated from the blood of the rats was subjected to analysis for glucose (A) on the COBAS® INTEGRA 400 plus auto-analyzer and insulin levels (B) were measured using the ELISA kit as described in the Materials and Methods. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. (**) indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK).

Fig. 2. Glucose tolerance test in control Wistar (C) and GK rats treated with/without ASA (n=5). After an overnight fast, animals were injected with glucose (2g glucose/kg body weight) intraperitoneally. Blood samples were collected from the tail vein at time 0 (prior to the glucose load), 60 and 120 minutes after the glucose challenge and glucose levels tested as described in the Materials and Methods. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference. (*) indicates p< 0.05 compared to control, (**) indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK).

of glucose ingestion and continued up to 2 h post glucose ingestion. On the other hand, no significant alterations in glucose tolerance were observed in control non-diabetic rats treated with ASA. These results suggest enhanced utilization of glucose by peripheral tissues after ASA treatment in the GK rats.

Effect of ASA on tissue toxicities

Low dose ASA treatment showed no significant alterations in the level of total serum proteins, uric acid, ALT, AST, LDH and CK (Fig. 3A-F) suggesting no significant drug toxicity. However, GK rats exhibited a marked increase in cholesterol and total lipoproteins which were significantly reduced after ASA treatment (Fig. 4A, B). An increase in alkaline phosphatase and conjugated bilirubin was also observed in GK rats (Fig. 4C and 4D). This appears to be associated with mild cholestasis due to hypercholesterolemia in GK rats. However, ASA treatment reduced the level of alkaline phosphatase enzyme almost to the control level while direct bilirubin concentration remained higher than that of control.

Effects of ASA on oxidative/nitrosative stress

A significant decrease in NO level was observed in GK diabetic rats (Fig. 5A). ASA treatment significantly increased the level of NO. A slight increase in NO production was also
observed in ASA treated control rats. Serum SOD level was significantly increased in ASA-treated GK diabetic rats but not in ASA-treated non-diabetic control rats (Fig. 5B).

**Effects of ASA on PGE2 level**

Figure 6 shows that prostaglandin E2 level was significantly (about 2-fold) increased in GK diabetic rats indicating an increased inflammatory response. ASA treatment drastically reduced the level of PGE2 both in GK diabetic and control rats.

**Effect of ASA on pancreatic insulin/glucagon secretory functions**

Figure 7 shows immunoreactivity of insulin- (beta) and glucagon- (alpha) secreting cells in the pancreatic islets. It is apparent that the hormone secreting cells have significantly degenerated in GK rats while ASA treatment has improved the integrity and size of pancreatic islets both in the control as well as GK rats treated with ASA (Fig. 8A). Quantitative analysis confirmed increased pancreatic islet size which may be associated with their increased ability to secrete insulin in ASA treated animals (Fig. 8B). As shown, the control animals treated with ASA also exhibited relative increase in insulin secretion which is statistically significant compared to the control untreated animals due to the heterogeneity of hormone secretion in these animals.
secreting cells (Fig. 8B). In addition, ASA treatment also enhanced the insulin/glucagon ratio which was significantly reduced in GK diabetic rats (Fig. 8C).

**Effect of ASA on the expression of insulin**

Insulin expression in the serum of GK and control rats was also studied by SDS-PAGE using an antibody against insulin. As shown in Fig. 9, an increased expression of insulin...
was observed in GK rats confirming the insulinemia in GK diabetic rats when compared to control non-diabetic rats (Fig. 9A). ASA treatment reduced the amount of insulin in the serum supporting the results obtained by ELISA. These results suggest alterations in glucose stimulated insulin after ASA treatment. An increased specific expression of insulin was also observed in the pancreas of GK diabetic rats after ASA treatment (Fig. 9B) suggesting the recovery of beta cells to produce insulin. Although statistically not significant, the level of insulin expression in GK rats treated with ASA appears to be slightly higher than that seen in the control rats. This might suggest an enhanced response of glucose induced insulin secretion in GK rats after ASA treatment.

Discussion

Low grade systemic inflammation is considered an integral part of T2D and plays an important role in glycemic control [25]. Thus GK rats are considered to be a useful model to evaluate the mechanism of effects of ASA on disease progression. Our results have shown that low dose ASA treatment ameliorated hyperglycemia, hyperinsulinemia and increased glucose utilization (tolerance) by peripheral tissues without causing any appreciable toxicity in target tissues.

Insulinemia, hypercholesterolemia and hyperlipoproteinemia, in GK rats may have caused insulin resistance resulting in hyperglycemia [25-27]. ASA treatment resulted
Amiri et al.: Hypoglycemic Effects of Aspirin

Cellular Physiology and Biochemistry

Fig. 8. Quantitation of insulin- and glucagon-immunoreactive cells in the pancreatic cells. Pancreatic sections from control Wistar (C) and GK rats treated with/without ASA (n=5) were stained by a double-labeled immunofluorescence method and visualized with a Carl Zeiss fluorescent microscope as described in the Materials and Methods. To assess the effect of aspirin treatment, the size of the islets (A) was measured using Axiovision® 3.0 Image Analysis System (Zeiss, Gottingen, Germany) attached to the Carl Zeiss fluorescent microscope. Insulin- or glucagon-positive cells were counted in visible sections at X40 magnification. The number of insulin-immunoreactive cells was divided by the total number of cells to give the percentage of insulin secreting cells (B). The ratio of insulin positive to glucagon positive cells were also calculated (C). A total of 10 random islets were analyzed from a total of 5 slides in each group. Results are expressed as mean ± S.E.M. and asterisks indicate significant difference. (* indicates p< 0.05 compared to control, ** indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK, ∆ ∆ indicates p< 0.001 compared to GK).

Fig. 9. Expression of insulin in serum and pancreatic homogenates from control Wistar (C) and GK rats treated with/without ASA (n=5). Pancreatic homogenates (50µg) and serum samples (10µg) were electrophoretically transferred by 15% SDS-PAGE and Western blot analysis to visualize the expression of insulin (A). Beta-actin was used as the loading control. The histograms in Fig. 9B shows densitometric analysis of the protein bands and are expressed as percentage protein compared to the control group. Asterisks indicate significant difference. (**) indicates p< 0.001 compared to control, ∆ indicates p< 0.05 compared to GK). The figures shown are representative of 3-4 independent analyses.
in reducing the levels of cholesterol and lipoproteins. Our results have also indicated a marked increase in serum PGE2 level in GK rats which may have contributed to increased inflammation. ASA treatment, on the other hand, markedly reduced the production of PGE2. Increased PGE2 and E-prostanoid receptors (EP2/3) negatively regulate insulin secretion and signaling and has been linked to the pathophysiology of T2D [26-27]. Therefore, inhibition of PGE2 by ASA may play a significant role in PGE2/EP3 mediated modulation in beta-cell function and insulin signaling.

ASA has been reported to protect cells from oxidative damages by stimulation of endothelial NO production [28]. Low levels of H₂O₂ have also been shown to induce NO production. There are reports that NO synthesis is reduced in T2D and hypertension and that insulin regulates NO production [29-30]. We observed that NO level was low in GK rats and ASA treatment increased NO production, which may have stimulated glucose tolerance and increased insulin response. There are reports that ASA increases NO production in cells and tissues [28] and that NO plays an important role in increasing insulin sensitivity [28-30].

We observed that ASA treatment increased serum SOD activity. SOD converts toxic superoxides to H₂O₂ and thus may help in H₂O₂-dependent insulin signaling and increased glucose transport in target cells. Insulin signaling is reported to be positively and negatively affected by reactive oxygen species (ROS) production. At a low physiological level, H₂O₂ is involved in enhancing insulin signaling by activating insulin receptor-tyrosine kinase activity and engaging the IRS-1/P13k/AKT-dependent downward signaling to increase basal glucose transport activity [31]. At lower concentrations, H₂O₂ has been reported to mimic insulin signaling even in the absence or low levels of insulin and inhibits PTP1B activity to prolong insulin receptor tyrosine kinase activity. In contrast, in the presence of insulin, H₂O₂ inhibits insulin signaling by activating deleterious serine/threonine kinases developing insulin resistance [31-33]. Persistent hyperglycemia and insulinemia, systemic and islet inflammation and increased ROS are all involved in developing insulin resistance and beta cells structural and functional defects in T2D [34-37]. Clinical and experimental trials have validated the beneficial effects of anti-inflammatory therapies including ASA treatment [35, 25-27]. Emerging results suggest that both beta cell mass and abnormal secretory functions are implicated in T2D [36-37]. As shown in our immunofluorescence study, ASA treatment has improved both the structural and functional properties of beta islets. This may be associated with the anti-inflammatory property of ASA which helps in regenerating functional beta cells, glucose stimulated insulin secretion, insulin sensitivity, glucose transport and energy utilization in type 2 diabetes.

Conclusions

Low dose ASA treatment for 5 weeks has improved glucose tolerance and beta cells function in hyperglycemic diabetic GK rats. These results may have implications in designing strategies for therapeutic management of type 2 diabetes.

Abbreviations

GK (Goto-Kakizaki); ASA (aspirin); NSAIDs (non-steroidal anti-inflammatory drugs); T2D (type 2 diabetes); PGE2 (prostaglandin E2); SOD (superoxide dismutase); NO (nitric oxide); SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis).

Acknowledgements

The research is supported by funds from the Sheikh Hamdan Bin Rashid Al Maktoum Award for Medical Sciences, Terry Fox Cancer Research Fund and a grant from the Research
Committee, CMHS, UAE University. Part of this work will be used for the thesis completion of Ms. Layla Ishaq Amiri for Master of Medical Sciences, Biochemistry and Molecular Biology program.

Disclosure Statement

No conflict of interest to disclose for any of the authors.

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