Influence of annexin A7 on Insulin Sensitivity

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Luo Dong

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Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr.F.Lang

2. Berichterstatter: Professor Dr.B.Gallwitz

Statement of Authorship

I hereby declare that this thesis is my own original work and it has been written by me in its entirety. I have faithfully and properly cited all sources used in the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institute.

Dong Luo

Abstract

Background: Insulin sensitivity is impaired by PGE_2, a major product of cyclooxygenase-2 (COX-2), which thus interferes with cellular glucose uptake following a glucose load. As shown in erythrocytes, PGE_2 formation is inhibited by annexin A7. The present study defined the role of annexin A7 in glucose metabolism.

Methods: Gene-targeted mice lacking annexin A7 (*annexinA7*$^{-/-}$) were compared to wild type mice (*annexinA7*$^{+/+}$). 6-Keto-prostaglandin-$F_1\alpha$ (6-Keto-$PGF_1\alpha$) was determined by ELISA to estimate COX-2 activity in serum. Hepatic COX activity was determined by an ELISA assay. Glucose and insulin serum concentrations were determined following an intraperitoneal glucose load and glucose serum levels after intraperitoneal injection of insulin. Experiments were done without and with pretreatment of the mice with COX inhibitor aspirin.

Results: The serum 6-Keto-PGF1α level and hepatic COX activity were significantly higher in *annexinA7*$^{-/-}$ than in *annexinA7*$^{+/+}$ mice. The intraperitoneal glucose tolerance test revealed decreased glucose tolerance in *annexinA7*$^{-/-}$ mice compared to *annexinA7*$^{+/+}$ mice. Intraperitoneal insulin injection decreased the serum glucose level in both genotypes, an effect significantly less pronounced in *annexinA7*$^{-/-}$ than in *annexinA7*$^{+/+}$ mice. Intraperitoneal glucose administration resulted in higher serum insulin concentrations in *annexinA7*$^{-/-}$ than in *annexinA7*$^{+/+}$ mice. Aspirin pretreatment lowered the increase in insulin concentration following glucose injection in both genotypes and virtually abrogated the differences in serum insulin between the genotypes. Moreover, aspirin pretreatment improved glucose tolerance in *annexinA7*$^{-/-}$ mice.

Conclusions: Annexin A7 influences insulin sensitivity of cellular glucose uptake and thus glucose tolerance. These effects depend on COX activity.

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List of Abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AKT	Serine/threonine-specific protein kinase
b.w.	Body weight
COX	Cyclooxygenase
DAGs	Diglycerides
ELISA	Enzyme-linked immunosorbent assay
ENaC	Amiloride-sensitive epithelial sodium channel
eNOS	Endothelial NO synthase
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EPR	Prostaglandin E receptor
FPR	Formyl peptide receptor
GBM	Glioblatoma multiforme
GLUT4	Glucose transporter type 4
GS	Glycogen synthase
GSK-3	Phsophroylates glycogen synthase kinase-3
JNK	Jun N-terminal kinase
IGF	Insulin like growth factor
Ig	Immuno Globulin
IL	Interleukin
IRS	Insulin receptor substrate

MAPK	Mitogen activated protein kinase
NO	Nitric Oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OSM	Oncostatin M
PDK-1	3-phosphoinositide-dependent protein kinase-1
PGs	Prostaglandins
PGE_2	Prostaglandin E2
PGES	Prostaglandin E2 synthase
PGI_2	Prostaglandin I2
PGH_2	Prostaglandin H2
PKC	Novel protein kinases C
PI3K	Phosphatidylinositol-3-kinase
SEM	Standard error of the mean
SGLT	Sodium-dependent glucose cotransporter
SOCS-3	Suppressor of cytokine signaling 3
Shc	Adaptor protein p66
TBS	Tris-buffered saline
TAGs	Triglycerides
TNF-α	Tumor necrosis factor
TXA	Thromboxane
6-Keto-$PGF_1\alpha$	6-Keto-prostaglandin-$F_1\alpha$

1.Introduction

Diabetes mellitus is one of the major diseases in modern time, which affects around 6 million people in Germany according to the survey of the Robert Koch Institute in 2009 (http://www.rki.de). International Diabetes Federation (IDF) estimates that the number of people suffered from diabetes will rise to 552 million by 2030 globally (http://www.idf.org). Diabetes mellitus has been seen as a serious health threat in the worldwide, therefore early interference is urgently required. Type 2 diabetes mellitus is characterized by insulin resistance or relative insulin deficiency, which is a more prevalent form and comprises of 90% of people with diabetes around the world[1] . It is strongly associated with obesity, hypertension and dyslipidemia[2] . However, the precise mechanisms triggering the insulin resistance remain unclear.

1.1 Annexin and annexin A7

Annexins are a family of calcium dependent phospholipids binding proteins, which are found in almost all tissues and cell types. There are more than 160 unique annexin proteins from fungi and protists to plants and vertebrates. But only twelve annexin subfamilies have been discovered in humans (annexin A1-annexin A11 and annexin 13)[3]. These proteins are mainly distributed in the inner surface of the plasma membrane related to cytoskeleton and cellular structures.

In structure, there are four segments or annexin repeats, which form highly α-helical and tightly entwined bands, in an annexin core (Figure 1). The segments form two main convex-concave sides. Each annexin shares a common C-terminal protein core that contains the calcium binding sites (yellow ball in Figure 1). The more concave side is variable N-terminal region. It has ligand binding sites that can bind some proteins or signal molecules in cytoplasm and play its function. Therefore, the latter is seen as the deciding factor of individual annexin function [4]. Several members of annexin have been

shown to bind the S100 calcium-binding proteins, actin and formyl peptide receptor (FPR), which can regulate a variety of cellular activities such as differentiation and apoptosis[5,6].

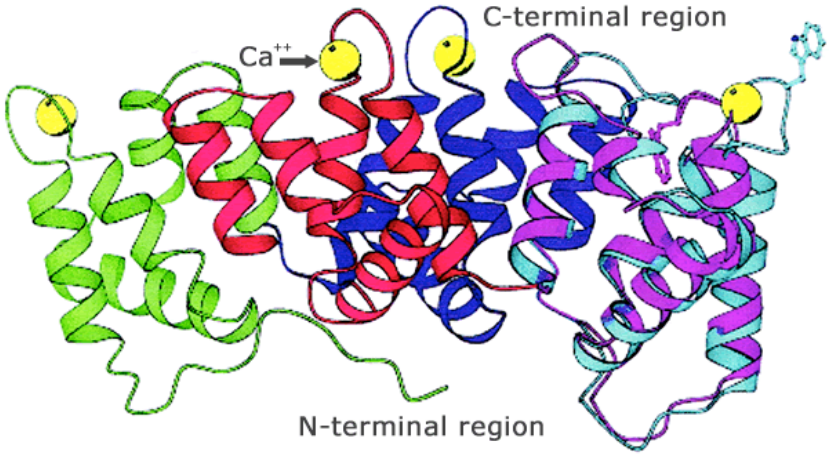

Figure 1. The three-dimensional crystal structure of human annexin A5. The four annexin repeats that are given in green (repeat I), blue (repeat II), red (repeat III), and violet/cyan (repeat IV). The yellow ball indicates Ca^{2+}. Modified from Gerke V and Moss SE (2002) [7].

The conservation of multiple annexins in many cell types suggests its fundamental role in cell biology. The development of knockout mice has provided great insight into the functions of annexins A1, A2, A5, A6，A7 and A8. Annexin A1 can cause changes in the inflammatory response and effects glucocorticoids regulation[8]. Annexin A1 deficient mice increase adipose tissue, suggesting annexin A1 as an important modulator in adiposity[9]. Annexin A2 is expressed at the surface of vascular vessels and related to blood clotting[10]. Annexin A2 deficient mice have defects in fibrin homeostasis, effecting the neovascularization[11]. Annexin A5 which is an anticoagulation may be linked to the development of obesity[12]. Annexin A6 expression, which is associated with tumor, suppress the cancer proliferation by inhibition of Ras /MAPK signaling pathway[13,14]. Annexin A6 also can regulate the mitochondrial morphogenesis, affecting the cellular energy supply by inhibiting Drp1 activity[15]. Annexin A8 affects the recruitment of leukocyte *via* stimulating the surface delivery CD63 in endothelial cells [16].

Annexin A7 (synexin) was the first annexin protein to be isolated and described[17]. It was

isolated as a reagent that mediated aggregation of chromaffin granules and fusion of phospholipids membranes in the presence of Ca^{2+}. Annexin A7 differs from other annexin proteins in that it possesses extraordinary long amino terminus[17]. This amino terminus carries an exon of 22 amino acids generated by alternative splicing leading to two isoforms: 47 and 51 kDa. Both of them widely expressed in murine and human tissues, but in the skeletal muscle, myocardium only 51 kDa isoforms is expressed [18], in platelets only 47 kDa expressed[19].

Annexin A7 influences many functions and metabolic processes. In cardiovascular research, annexin A7 deficiency may disturb calcium normal operation and conduction, which leads to severe electrical instability in the murine heart [20]. Lack of annexin A7 enhances eryptosis of erythrocytes, due to calcium overload, shorten its life of red blood cells and lead to anemia [21]. In tumors, high annexin A7 expression is associated with high aggressive potential in hormone-relative tumors such as malignancies of liver cancer, gastric cancer, nasopharyngeal carcinoma, colorectal cancer and breast cancer[22]. However, annexin A7 also has a tumor-suppression role in glioblastoma, glioblastoma multiforme, melanoma and prostate cancer. Annexin A7 is seen as a potential target in clinical diagnosis and treatment of certain tumors[22]. Further, annexin A7 takes part in the regulation of gastric acid secretion. Gastric acid cannot be upregulated by the glucocorticoid dexamethasone without annexin A7[23]. The isoform of annexin A7 inhibits PGE_2 formation and contributes to the regulation of secretion. According to a malaria study in annexin A7 knockout mice, parasites enhance PGE_2 formation and calcium permeability, while the wide type group significantly decreases it[24]. Annexin A7 is also abundantly expressed in some inflammatory diseases, such as inflammatory myopathies[25].

1.2 COX and prostaglandin

Cyclooxygenase (COX) is a central and rate-limiting enzyme responsible for the production of prostaglandins. There are main three known isoforms of COX: COX-1, COX-2 and COX-3. COX-1 is expressed in various of tissues, particularly in gastric, bowel mucosa and kidney [26].COX-1 is related to the production of prostaglandins (PGs) which are important lipid compounds for regulating normal physiological function, such as stomach mucous production, regulation of gastric acid and normal platelets mediation [27]. The expression of COX-2, as a part of immune response, is much lower than COX-1 in normal physiology. COX-2 can be stimulated by inflammatory cytokines, growth factors, carcinogens etc.. Aberrant COX-2 activation and PGs production are associated with infection and cancers (Figure 2) [28]. COX-3 is a new isoform and seen as a COX-1 variant. Similar with COX-1 function, COX-3 cannot be induced by acute inflammatory stimulation[29].

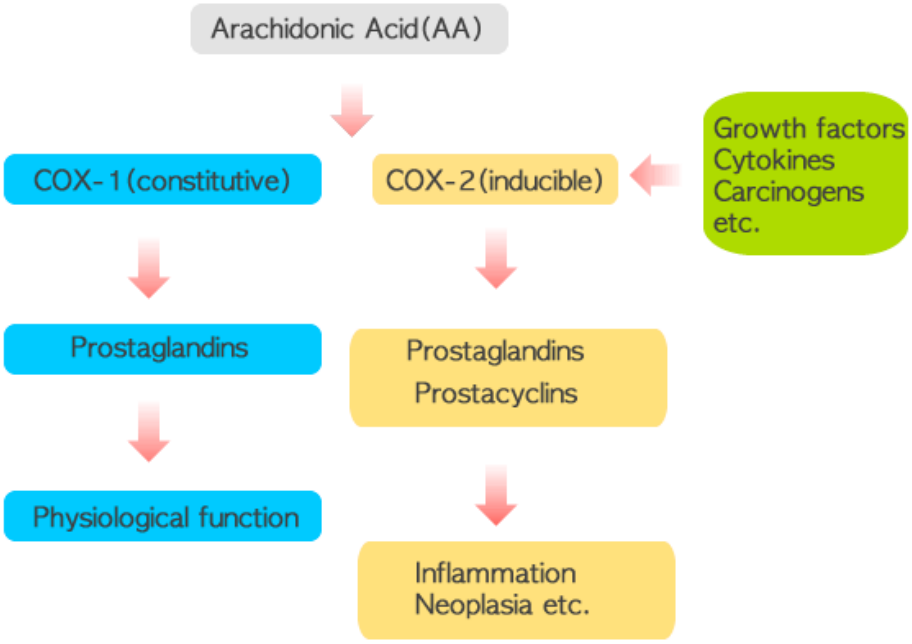

Figure 2. COX-1 and COX-2 pathways. COX-1 is constitutive and regulates normal physiological function. COX-2 is inducible by cytokines etc. and related to inflammation and cancers.

Prostanoids are a group of fatty acid derivatives including PGs, prostacyclin and thromboxane (TXA). COX enzyme converts arachidonic acid (AA) to prostaglandin H_2 (PGH_2), through peroxidase activity. PGH_2 is a precursor of the series-2 prostanoids and acted upon by one of a group of synthases that can modify the cyclopentane and produce different kinds of prostaglandins. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, can reduce the PGs formation by inhibiting COX enzyme, which directly impacts the quantity of PGs production (Figure 3).

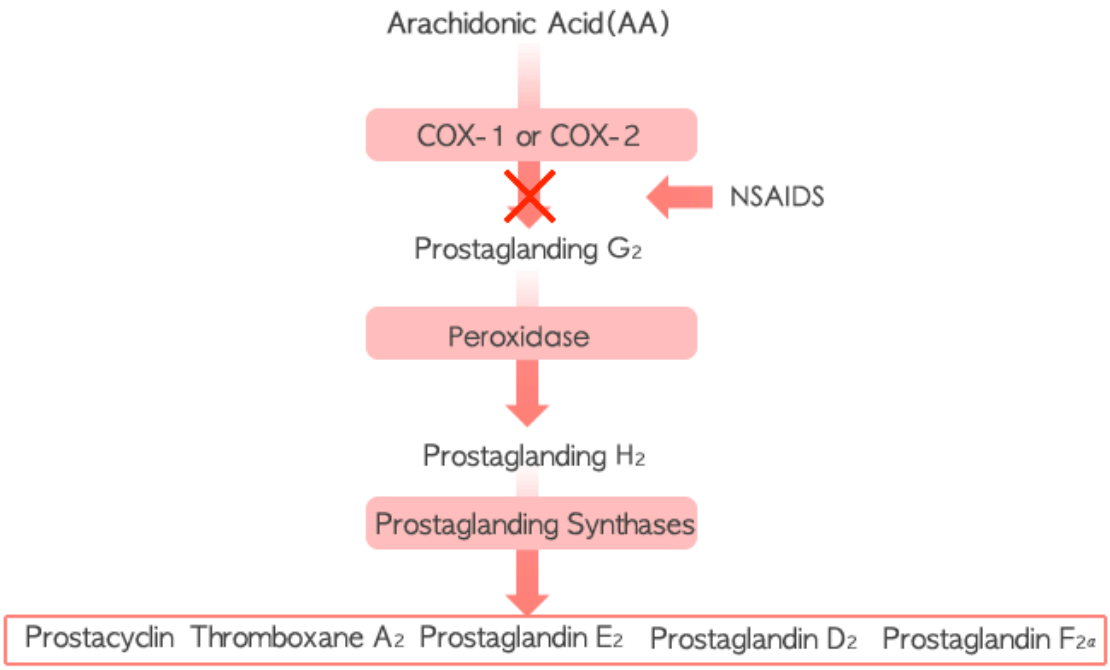

Figure 3. Prostaglandin biosynthetic pathways. Arachidonic acid is catalyzed by COX-1 or COX-2 resulting in synthesis of PGH_2. PGH_2 is converted to one of prostaglandins, dependent upon the end synthase. NSAIDS reduce the PGs through COX enzyme inhibiting.

1.3 PGE_2 and its effect in glucose metabolism

PGE_2 is an important mediator of immune-inflammatory responses inducing vasodilation by smooth muscle relaxation, stimulating fever by acting on the hypothalamus, and causing pain by sensitizing peripheral nociceptors[30]. PGE_2 is synthesized by the action of prostaglandin E2 synthase (PGES) which acts on COX derived PGH_2 and released by macrophages [31].

PGE_2 can conduct the signal through four different G-protein coupled receptors, termed E series prostaglandin receptors (EP1R-EP4R) [32]. Each receptor is encoded on a separate gene and differs in structure and function. EP3R holds prominent position and plays regulatory role in β-islet cells [33].

Recent evidence suggests PGE_2 has a significant role in obesity and diabetes mellitus. PGE_2 can increase leptin release from adipose tissue and inhibit lipolysis in white adipose tissue, predicting that PGE_2 signaling is important in the regulation of body weight and metabolic homeostasis[34]. Mice lacking EP3R can develop obesity and have a significantly higher body weight than wild type group when aged 10 weeks [35]. High level of PGE_2 and up-regulation of COX-2 in human adipocytes have been observed *in vitro* [36]. Mice lacking phospholipase A2, an essential component in PGE_2 biosynthesis, have a reduced PGE_2 concentration in adipose tissue and lowered weight gain [37]. Moreover, PGE_2 may simulate oncostatin M (OSM) which is produced by Kupffer cells, thereby resulting in insulin resistance in hepatocytes [38]. EP1R in the kidney play a critical role in the development of diabetic nephropathy[39]. The synthesis of PGE_2 and EP3R in β-cells can be up regulated in type 2 diabetes mellitus and result in β-cell dysfunction[40]. The higher levels of PGE_2 is also observed in patients with diabetic retinopathy suggesting that PGE_2 may have a pathogenic role in diabetic retinopathy[41].

Hypertension is a part of insulin resistance syndrome [42]. PGE_2 can affect and regulate the peripheral vascular tone by EPR. EP1R and EP3R, which are mainly expressed in pulmonary vascular vessels and nervous system, are functionally associated with vasoconstriction. Knocking out EP1R or EP3R causes the hypotension in mice [43,44]. In the kidney, COX-2 is an important mediator of the renin-angiotensin system. PGE_2 and PGI_2 from renal medullary play a critical role in regulating the secretion of renal rennin and the reabsorption of sodium and water in the medullary thick ascending limb and collecting duct. Mice with COX-2 deficiency or blocking COX-2 can decrease renal rennin production and secretion and induce mild to moderate elevation of blood pressure [45].

1.4 Insulin action and signaling

Insulin is an important peptide hormone in the metabolism of glucose and fat. It can stimulate cells in liver, skeletal muscle and adipose to absorb and utilize glucose from the blood and keep glycemic level homeostasis within the body. In the liver, insulin promotes glycogen synthesis through stimulating glycogen synthase (GS) and inhibiting glycogen phosphorylase. In muscle and adipose tissue, insulin induces an uptake of glucose by increasing glucose transporter type 4 (GLUT4) expression on the cell surface and then forms glycogen in muscle or converts the glucose to fatty acids in fat[46]. Hence, loss of insulin secretion or resistance to its actions can lead to high blood glucose concentration and result in diabetes mellitus.

There are two major signal pathways that conduct to effectors after insulin receptor substrate (IRS) binding with insulin. One pathway is metabolism signaling through IRS and phosphatidylinositol-3-kinase (PI3K) pathway, which regulates carbohydrate, fat, and protein metabolism or increases endothelial NO synthase (eNOS). The other is mitogenic pathway through Shc/RAS/MAPK that regulates gene transcription and cell proliferation. Any deficiency or mutation of the genes in this pathway or effected by other factors may lead to the disorder of the metabolic action of insulin.

Insulin receptors are widely expressed at the cell surface in insulin responsive tissues. It consists four parts (Figure 4), two extracellular insulin-binding sites (α- peptides chains, yellow color) and two transmembrane sites (β- peptides chains, pink color). The β-peptides chains are stimulated by autophoshorylation when α- peptides chains bind to insulin. It provides specific sites for binding the IRS protein and some downstream signaling molecules e.g. Shc protein[47]. IRS is activated through a phosphotyprosine process when binding to the insulin receptor.

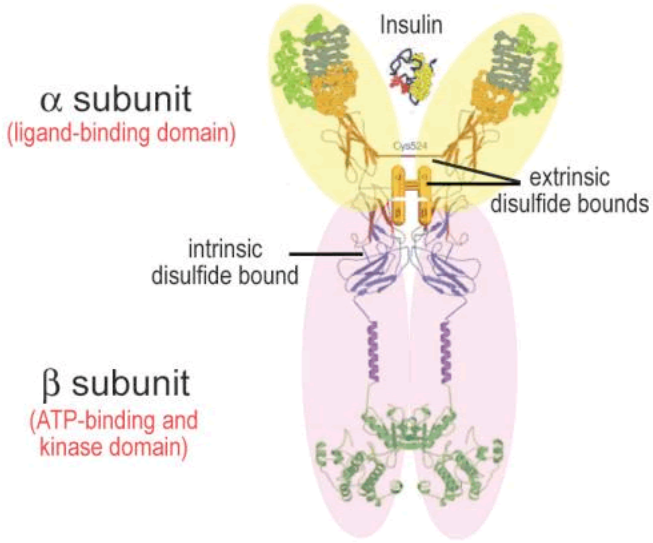

Figure 4. Insulin receptor structure. Insulin receptor monomer composed of α (yellow) and β subunit (purple). They are connected by disulfide bounds to form a functional receptor[48].

There are two main types of insulin receptor substrates: IRS-1 and IRS-2. Both of them are expressed in most insulin sensing-tissues. IRS-1 is related to GLUT4, which is an important glucose transporter in liver and adipose tissue. The main effect of IRS-1 is to promote skeletal muscle or adipose cells to utilize glucose whereas the IRS-2 regulates the function of β-islet cell and promote glycogen synthase (GS) in hepatocytes. Phosphorylated IRS activates PI3K and then results in serine/threonine-specific protein kinase (AKT) and 3-phosphoinositide-dependent protein kinase-1 (PDK-1) phosphorylation. AKT is a central intermediate for many metabolism processes in cells. There are three isoforms of AKT: AKT1, AKT2 and AKT3. AKT1 and AKT2 play the dominant action in insulin response in skeletal muscle and adipose tissue, whereas AKT3 can be activated in the liver[49,50]. AKT phosphorylates AS160, a protein that is required for GLUT4 translocation in cell[51]. AKT was also shown to be one of the insulin mediated kinases that phosphorylates glycogen synthase kinase-3 (GSK-3)[52]. This intracellular pathway promotes glycogen synthesis in liver and muscle *via* glycogen synthase activation and glucose uptake in adipose tissue by translocation GLUT4 to the cell surface membrane (Figure 5)[53].

Shc/Ras/MAPK signaling pathway is another way to regulate the insulin effects on cells. It can transfer the signal from a receptor at the surface to the inside or nucleus of the cell. It mainly includes three ways ERK1/2, JNK and p38. ERK1/2 signal pathway is known as the most classic in MAPK pathways, which can be activated by phosphorylated Ras or some small molecules such as insulin like growth factor (IGF) and prostaglandins. ERK1/2 can cross through the membrane of cells or nucleus and plays an important role in cell proliferation and gene expression[54].

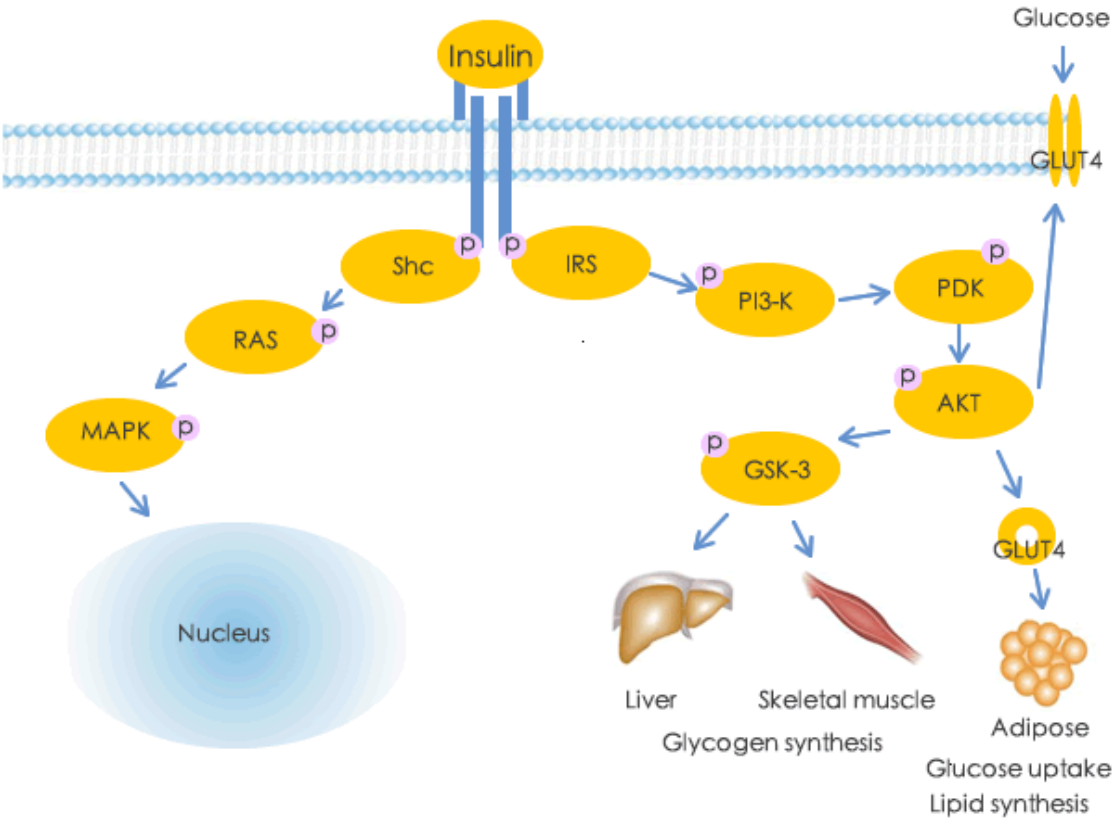

Figure 5. Insulin signaling through its metabolic and mitogenic pathways. Insulin induces glucose uptake in liver, muscle and adipose tissue through PI3K-AKT pathway and regulates gene function through Shc/Ras/MAPK pathway. Modified from Chiu and Cline (2010)[48].

1.5 Insulin signaling defects in insulin resistance

Insulin resistance is the common pathophysiologic basis of type 2 diabetes mellitus and the exact mechanism remains unclear. However, it has been demonstrated that insulin resistance is influenced by both environmental and genetic factors[55].

The first step of insulin action is initiated by the binding and activation of its receptors, which are expressed on the cell surface. It has been observed that IRS-1 low expression in skeletal muscle and adipocytes from patients with type 2 diabetes mellitus [56,57]. Several studies showed the insulin receptor unaltered or impaired in the type 2 diabetes mellitus [58]. The mutation in exon 20 of the insulin receptor gene may be an important reason for genetic insulin resistance [59]. As described above, IRS family has critical physiologic functions on insulin. IRS-1 knockout mice can develop a mild insulin resistance [60]. However, disruption of IRS-2 genes causes a severe insulin resistance and develops to type 2 diabetes mellitus eventually[61]. Researchers found impaired PI3K activity in the liver or skeletal muscle from Type2 diabetes both *in vivo* and *vitro*[62-64]. AKT is also an essential protein in the normal glucose metabolism. AKT2 deficient mice develop insulin resistance and diabetes-like syndrome[65]. Over expression of GLUT4 in adipose tissue enhances insulin sensitivity and glucose tolerance. Adipose tissue loss of GLUT4 results in a degree of insulin resistance[66].

Furthermore, some studies reveal that insulin signaling can be affected by other elements such as the inflammatory and hormonal factors, endoplasmic reticulum (ER) stress and metabolic 'overload' in insulin-sensing tissues[67]. TNF-α and IL-6 are multi functional cytokines. Increased TNF-α expression has been discovered in adipose tissues of obesity people[68]. Inhibiting phosphorylation of IRS-1 and disrupting the translocation of GLUT4 is the main effect of TNF-α in insulin resistance [69,70]. IL-6 can inhibit insulin receptor transduction *via* inducing the suppressors' cytokine signaling-3 (SOCS-3) in hepatocytes. SOCS-3 is a direct inhibitor of insulin receptor autophoshorylation in the cell [71,72].

Obesity or over-lipid accumulation is associated with mechanical stress in the cell. ER is a critical player in cell stress and ER stress can significantly decrease insulin-stimulated tyrosine phosphorylation of IRS-1 in hepatocytes and hypoactive JNK pathway [73]. Ingesting high-fat for a long period or infusing the lipid directly in rodents can lead to the accumulation of lipids, including triglycerides (TAGs), diglycerides (DAGs), and ceramides, and inducing insulin resistance[74,75]. Lipids over accumulation within liver cells and insulin resistance are strongly coupled[76], which triggers activation of novel protein kinases C (PKC) , relate to serine phosphorylation of IRS1, and alter the insulin signaling cascade[77,78]. Experimental work with PKC knockout mice demonstrates that it can protect mice from fat-induced insulin resistance [79].

1.6 Aims of the study

It has been previously shown that annexin A7 affects COX activity. These two key molecules participate in various metabolic processes. Furthermore, recent data indicates that insulin resistance has a strong association with COX activity and its metabolite PGE_2. However the role of annexin A7 in glucose homeostasis remains unclear. Therefore, we pursued to investigate the role of annexin A7 in glucose regulation. Our hypothesis states that annexin A7 modifies COX activity and prostaglandin formation thereby impacting insulin sensitivity and glucose regulation.

The aim of this study is to:

- Explore the possible mechanism of annexin A7 on the regulation of serum glucose concentration
- Describe the basic pathway leading to the effect of annexin A7 on glucose metabolism.
- The role of ENaC and SGLT1 from colon and jejunum *in vivo* using A7 knockout mice

2. Materials and Methods.

2.1 Materials

2.1.1 Animals

Experiments were performed in gene-targeted mice lacking annexin A7 (*annexinA7*$^{-/-}$) and in corresponding wild type mice (*annexinA7*$^{+/+}$). Generation, properties and genotyping of annexinA7$^{-/-}$ mice were described earlier [80]. Male age-matched mice (9-15 week-old) were used for the experiments. All animal experiments were conducted according to the German law for the care and use of animals and were approved by the responsible authority of the state of Baden-Württemberg (Regierungspräsidium Tübingen). Mice had free access to a standard chow diet (SSniff, Soest, Germany) and a tap water.

2.1.2 Blood and Serum Collection

Equipment and Chemicals

Names	Manufacturer and country of origin
Diethylether	Roth, Karlsruhe ,Germany
Na-Heparin capillaries	HirschmannLaborgeräte, Eberstadt, Germany
SST Tubes	BD Microtainer, Franklin Lakes, USA
Centrifuge 5417 R	Eppendorf, Hamburg, Germany

2.1.3 Tolerance test

Chemicals

Names	Manufacturer and country of origin
D-Glucose	Sigma, Schnelldorf, Germany
Saline	Fresenius Kabi, Bad Homburg, Germany
Phosphate buffered saline (PBS)	GIBCO, Carsbad, Germany
Long-acting Insulin	Lantus, Frankfurt, Germany

Equipment

Names	Manufacturer and country of origin
Timer	Roth, Karlsruhe, Germany
Syringe-driven Filter Unit	Millex Merk , Darmstadt, Germany
Needles (0.55mm×25mm)	BD, Drogheda, Ireland
Syringes (1ml)	Braun, Bad Arolsen, Germany
Accu-Chek Performa	Roche, Mannheim, Germany
Glucose Meter	Roche, Mannheim, Germany
Balance	Sartorius, Göttingen, Germany
Scalpel	Mediware, Wesel, Germany

2.1.4 ELISA Measurement

Equipment

Names	Manufacturer and country of origin
Eppendorf pipettes 1ml, 200µL，100µL,10µL	Eppendorf AG, Hamburg, Germany
Eppendorf tubes	Eppendorf AG, Hamburg, Germany
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Stripette 5,10,25ml	Coring Incorporated, Corning NY, USA
Vortex Genie	Scientific Indusreies, Bohemia NY, USA
Powerwaver XS2	BioTek Instruments, Inc. Winooski,USA
Utratip	Greiner bio-one, Frickenhausen, Germany
Shaker VIBRAX VXR	IKA, Staufen, Germany
MultiChanelPipet	Eppendorf, Hamburg, Germany

ELISA Kits and Chemicals

Names	Manufacturer and country of origin
Ultra Sensitive Mouse Insulin ELISA kit	Crystal Chem INC, Downers Grove, IL, USA
6-Keto-$PGF_{1\alpha}$ Immunoassay Kit	Biotrend, Köln, Germany
COX activity assay Kit	Cayman, Ann Arbor,USA
PGE_2 Immunoassay Kit	Demeditec, Kiel, Germay
Plasma Leptin ELISA Kit	EMD Millipore, Missouri, USA
Ampuwa	Fresenius Kabi, Bad Homburg, Germany

Software

Names	Manufacturer and country of origin
Sigma plot version 7.0	Systat Software Inc., Erkrath, Germany
GraphPad Instat version 3.05	GraphPad Software Inc., San Diego, USA

2.1.5 Ussing chamber measurement

Equipment

Names	Manufacturer and country of origin
Maclab/4e	AD Instruments, Dunedin, New Zealand
Pulse generator 5.1	Custom made
Mini Chamber	Custom made
Microscope	Carl Zeiss, Oberkochen, Germany
Waterbath	Labortechnik, Seelbach, Germany

Chemicals

Names	Manufacturer and country of origin
NaCl	VWR Chemicals, Darmstadt, Germany
KH_2PO_4	Roth, Karlsruhe ,Germany
$K_2HPO_4 \times 3H_2O$	Merk, Darmstadt, Germany
KCL	Roth, Karlsruhe ,Germany
$MgCl_2 \times 6H_2O$	Merk, Darmstadt, Germany
Pyruvic Acid	Sigma, Schnelldorf, Germany
$CaCl_2 \times 2H_2O$	Sigma, Schnelldorf, Germany
$NaHCO_3$	Sigma, Schnelldorf, Germany
Calcium Gluconate	Sigma, Schnelldorf, Germany
Amiloride	Sigma, Schnelldorf, Germany
DMSO	Sigma, Schnelldorf, Germany
Carbogen	Westfalen AG, Muenster, Germany

Software

Names	Manufacturer and country of origin
Chart version.4.2	Axon Instruments, USA

2.1.6 Urine glucose Measurement

Equipment and Chemicals

Names	Manufacturer and country of origin
Eppendorf tubes	Eppendorf AG, Hamburg, Germany
Balance	Sartorius, Göttingen, Germany
Metabolic cages	Techniplast, Hohenpeissenberg, Germany
Microtest 96wells	Falcon, Frankin Lakes, USA
Vortex	Labnet Abimed, Langenfeld, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Eppendorf pipettes	Eppendorf AG, Hamburg, Germany
Ampuwa	Fresenius Kabi, Bad Homburg, Germany
Glucose Kit	Roche Diagnostics, Mannheim, Germany

2.2. Methods

2.2.1 Serum Collection

To obtain blood specimens, animals were lightly anesthetized with diethylether and approximately 130 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. After collection of the blood in SSD tubes, then centrifuged at 6,000-8,000 g for 3-8 minutes in a refrigerated centrifuge. The resulting supernatant is designated serum.

2.2.2 Animals Treatment

For some experiments, mice were pretreated with aspirin in drinking water at a concentration of 600 mg/l for 1-2 weeks consecutively.

2.2.3 Glucose Tolerance Test

In order to detect disturbances in glucose metabolism, mice are fasted overnight and glucose (2g/kg b.w.) was injected intraperitoneally (i.p.). The blood glucose

concentration is determined before and at 15th, 30th, 45th, 60th, 90th, and 120th minute after the injection. The glucose concentration was measured in a drop of tail blood utilizing a glucometer.

2.2.4 Insulin Tolerance Test

For determine the insulin resistance in body, mice are fasted for 4 h, and long-acting insulin was injected i.p. (0.5 U/kg b.w.). The blood glucose concentration is determined before and at 15th, 30th, 45th, 60th, 90th, and 120th minute after the injection. The glucose concentration was measured in a drop of tail blood utilizing a glucometer.

2.2.5 Determination of insulin, leptin and 6-Keto-$PGF_{1\alpha}$ in plasma

The serum insulin, leptin and 6-Keto-$PGF_{1\alpha}$ concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions. Results were derived using the standard curve method.

2.2.6 Determination of PGE_2 and COX activity in liver tissue

The PGE_2 concentration and COX activity in liver were determined with an ELISA method according to the manufacturer's instructions. Results were derived using the standard curve method.

2.2.7 Ussing Chamber Measurement

For the analysis of electrogenic intestinal glucose transport, distal jejunal segments were taken from the intestine, and the muscle layers were carefully separated from the luminal layer under a microscope. The luminal tissue was then mounted into a custom-made mini-Ussing chamber with an opening of 0.00769 cm^2. Under control conditions, the serosal and luminal perfusate for jejunal experiments contained (in mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 1.25 $CaCl_2$, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 Na pyruvic acid, 25 $NaHCO_3$,

20 mannitol (pH 7.4, NaOH). The solutions were gassed with 5% CO_2 prior to the Ussing chamber experiment, and the temperature was maintained at 37°C. Where indicated, glucose (20 mM) was added to the luminal perfusate at the expense of mannitol.

ENaC activity was estimated from the amiloride-sensitive potential difference and current across the colonic epithelium. After removing the outer serosal and the muscular layer of late distal colon under a microscope, tissues were mounted onto a custom-made mini-Ussing chamber with an opening area of 0.00769 cm^2. The serosal and luminal perfusate contained (in mM): 145 NaCl, 1 $MgCl_2$, 2.6 Ca-gluconate, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 glucose. To assess ENaC mediated transport, 50 μM amiloride (in DMSO) was added to the luminal perfusate. All experiments were carried out at a temperature of 37°C.

In all Ussing chamber experiments, the transepithelial potential difference (V_{te}) was determined continuously and the transepithelial resistance (R_{te}) was estimated from the voltage deflections (ΔV_{te}) elicited by imposing test currents (I_t) of 1 μA. The resulting R_{te} and I_{sc} were calculated according to Ohm's law. [81].

2.2.8 Urine Collection and Urinary glucose Measurement

For evaluation of the urinary glucose levels, mice were placed individually in metabolic cages for 24-hour urine and feces collection with free access to fluid and food. They were allowed a 3-day habituation period when food & water intake, urinary flow, urinary excretion of salt, fecal excretion and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently 24h collection of urine was performed for three consecutive days in order to obtain the urinary parameters. To assure quantitative urine collection, metabolic cages were siliconized and urine was collected under water-saturated oil. Urinary glucose levels were determined by the Glucose kit according to the manufacturer's instructions.

2.3 Statistical Analysis

Data are provided as arithmetic means ± SEM, n represents the number of mice studied. Differences were tested for significance using *Student´s unpaired two-tailed t-test*, and only results with $p < 0.05$ were considered statistically significant. Microsoft Excel 2011 and Graph pad Prism 6 were used for the calculation.

3. Results

3.1 Higher serum 6-Keto-PGF$_1\alpha$ levels in *annexinA7$^{-/-}$* mice

A previous study has reported higher levels of PGE_2 in erythrocytes from mice lacking annexin A7 (*annexinA7$^{-/-}$* mice)[82]. In the present study, due to the short half-life time (30s) of PGE_2, it is difficult to determine accurately *in vitro*. Instead, the stable and end product of PGI_2 metabolism 6-Keto-PGF$_1\alpha$ was used to estimate serum COX-2 activity. We measured 6-Keto-PGF$_1\alpha$ in serum from *annexinA7$^{-/-}$* and *annexinA7$^{+/+}$* mice. Figure 6 illustrates that the serum 6-Keto-PGF$_1\alpha$ concentrations was significantly (*, $p<0.05$) higher in *annexinA7$^{-/-}$* mice than in *annexinA7$^{+/+}$* mice pointing to higher COX-2 activity in mice lacking annexin A7.

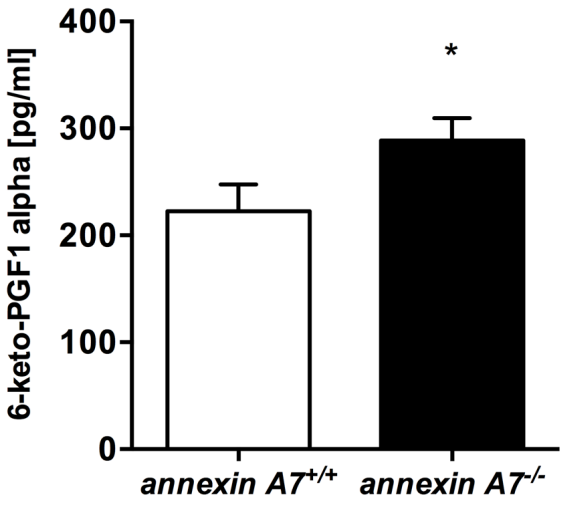

Figure 6. Serum 6-Keto-PGF$_1\alpha$ concentration in *annexinA7$^{+/+}$* and *annexinA7$^{-/-}$* mice.
Arithmetic means ± SEM (n = 6) of serum 6-Keto-PGF$_1\alpha$ concentration from *annexinA7$^{+/+}$* (white bar) and *annexinA7$^{-/-}$* mice (black bar).
* indicates significant difference using *Student's t-test*($p<0.05$).

3.2 Higher COX activity in *annexinA7*$^{-/-}$ mice liver

The liver is the major organ involved in glucose metabolism. The COX activity was determined in liver tissue. As illustrated in Figure 7, the total COX activity levels of the liver in *annexinA7*$^{-/-}$ mice was higher than *annexinA7*$^{+/+}$mice. The difference reached statistical significance (*, $p<0.05$). COX-1 activity in liver of *annexinA7*$^{-/-}$ mice was higher, but was not statically difference.

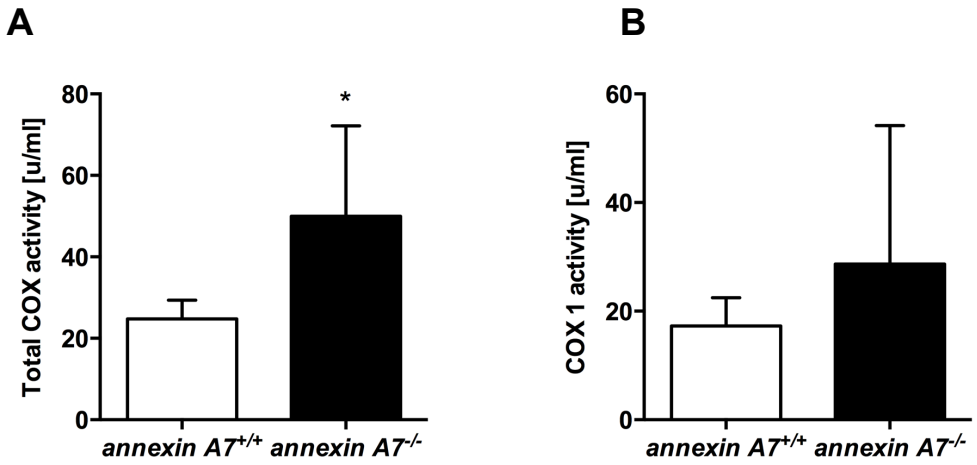

Figure 7. Liver total COX activity and COX-1 activity in *annexin A7*$^{+/+}$ and *annexin A7*$^{-/-}$ mice.

A. Arithmetic means ± SEM (n = 6) of the liver total COX activity of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

B. Arithmetic means ± SEM (n = 6) of the liver COX-1 activity of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

* indicates significant difference using *Student's t-test* ($p<0.05$).

PGE_2 concentration and COX-2 activity in liver tissue were also determined. In normal liver, COX-2 activity is either low or not detectable, but annexin A7 may modify the activity of COX-2. As shown in figure 8, PGE_2 concentration and COX-2 activity in liver tissue were higher in *annexinA7*$^{-/-}$ mice, however, not reaching statistical significance.

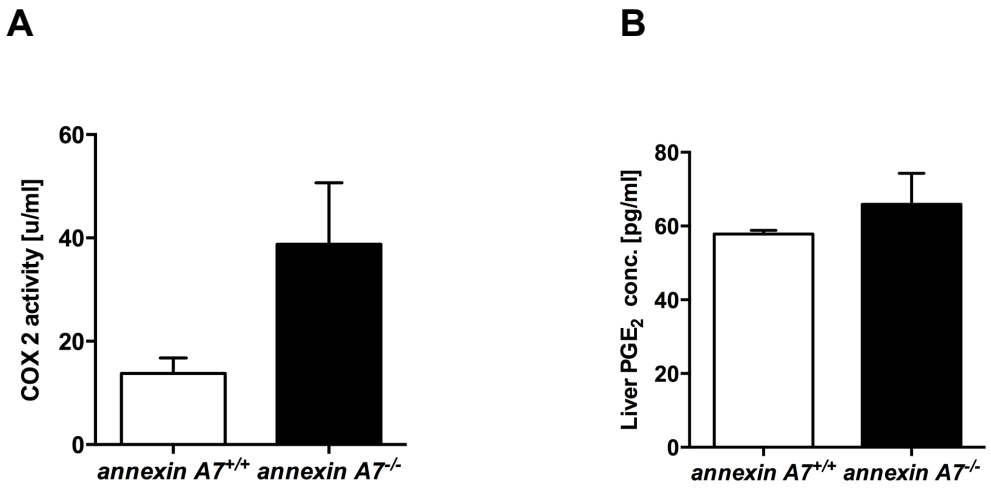

Figure 8. Liver COX-2 activity and PGE_2 concentration in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

A. Arithmetic means ± SEM (n = 6) of the liver COX-2 activity of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

B. Arithmetic means ± SEM (n = 4) of the PGE_2 concentration in liver of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

3.3 Decreased glucose tolerance in *annexinA7*$^{-/-}$ mice

Since COX activity and prostaglandins have been shown to negatively influence hepatic glucose tolerance, the fasted glucose levels were analyzed in *annexinA7*$^{-/-}$ mice and *annexinA7*$^{+/+}$ mice. Following an overnight fasting period the blood glucose concentration was not significantly different in *annexinA7*$^{-/-}$ mice (73 ± 6 mg/dl, n =5) and *annexinA7*$^{+/+}$ mice (66 ± 3 mg/dl, n =7) (Figure 9A).

To test further for differences between *annexinA7*$^{-/-}$ mice and *annexinA7*$^{+/+}$ mice in glucose metabolism, glucose tolerance was determined. After overnight fasting, an intraperitoneal glucose load (2 g/kg b.w.) was followed by a rapid increase in the blood glucose concentration (Figure 9B), which was more pronounced in *annexinA7*$^{-/-}$ mice than in *annexinA7*$^{+/+}$ mice (Figure 9B). The difference of blood glucose concentration reached statistical significance before, and 15, 30, 45, and 60 min after the glucose load. These results suggest compromised glucose tolerance in *annexinA7*$^{-/-}$ mice.

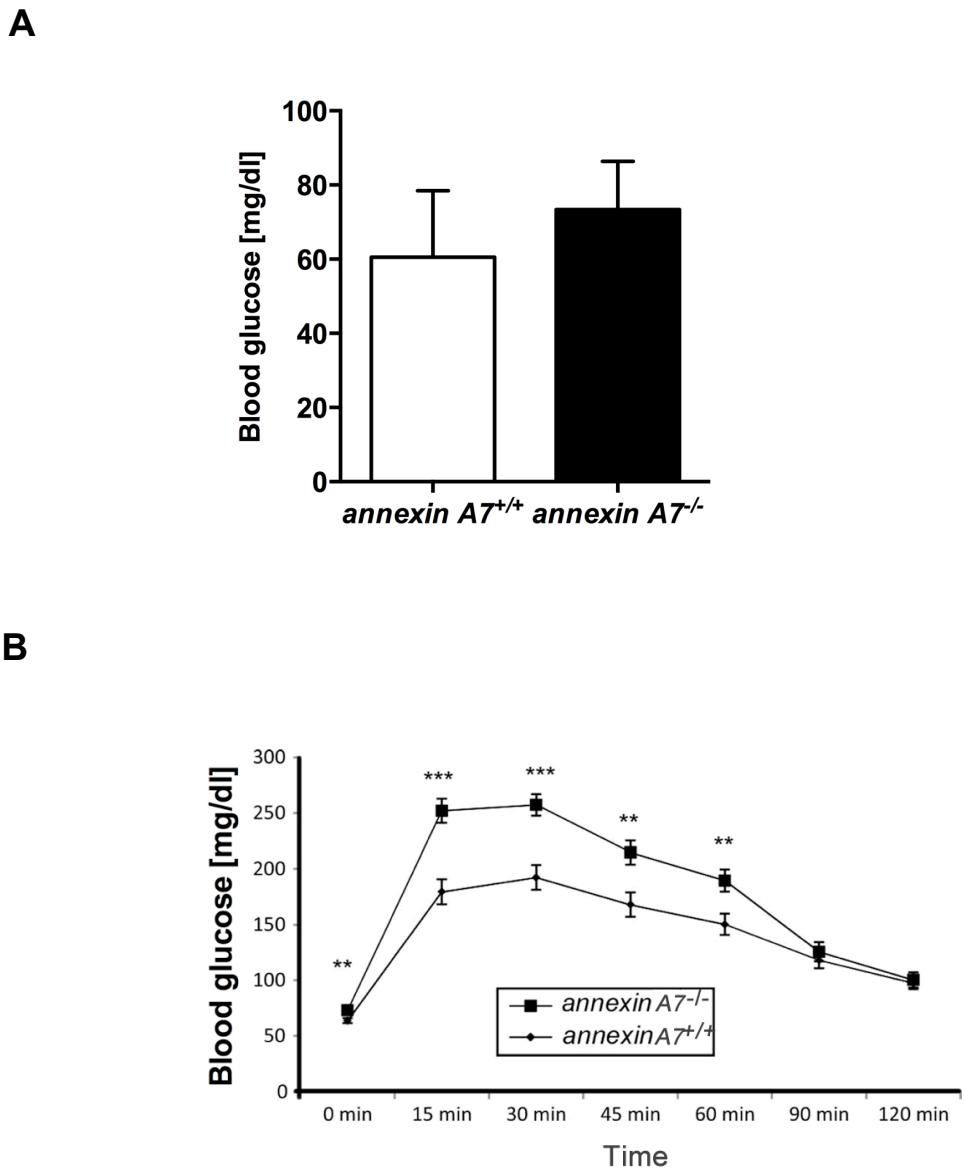

Figure 9. Blood glucose concentration following intraperitoneal glucose injection in *annexin A7*$^{+/+}$ and *annexin A7*$^{-/-}$ mice.

A. Arithmetic means ± SEM (n = 5-7) of the overnight fast blood glucose concentration of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

B. Arithmetic means ± SEM (n = 17-18) of the blood glucose concentration following intraperitoneal injection of glucose (2 g/kg body weight) into *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

, * indicate significant difference using *Student's t-test* ($p<0.01$, $p<0.001$).

3.4 Lower insulin sensitivity of *annexinA7*$^{-/-}$ mice

Decreased glucose tolerance in *annexinA7*$^{-/-}$ mice may be the consequence of reduced insulin sensitivity of peripheral glucose uptake or impaired insulin secretion. The next series of experiments therefore explored glucose-induced insulin secretion in *annexin A7*$^{-/-}$ and *annexinA7*$^{+/+}$ mice. To this end, mice were fasted overnight, and glucose (2 g/kg b.w.) was injected intraperitoneally. After 30 min, blood was drawn and serum insulin determined. Figure 10 illustrates that the serum insulin level was significantly higher in *annexinA7*$^{-/-}$ mice than in *annexinA7*$^{+/+}$ mice following the glucose load. This result demonstrates intact insulin secretion in *annexinA7*$^{-/-}$ mice and suggests that rather reduced insulin sensitivity accounts for their decreased glucose tolerance.

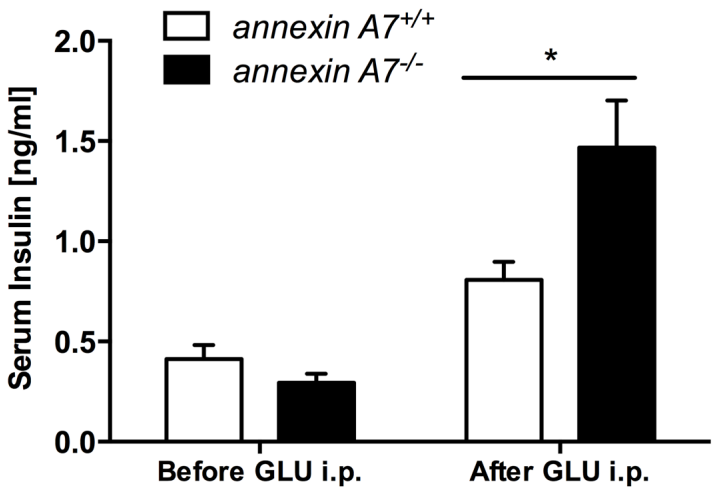

Figure 10. Insulin serum concentration before and after intraperitoneal glucose injection (GLU i.p.) in *annexin A7*$^{+/+}$ and *annexin A7*$^{-/-}$ mice.

Arithmetic means ± SEM (n = 12-14) of the serum insulin level 30 min after intraperitoneal injection of glucose (2 g/kg b.w.) into *annexinA7*$^{+/+}$(white bar) and *annexinA7*$^{-/-}$ (black bar) mice.

* indicates significant difference using *Student's t-test* ($p<0.05$).

Hence, further experiments explored an impact of annexin A7 deficiency on the insulin sensitivity of peripheral glucose uptake. To this end, insulin was injected intraperitoneally (0.5 U/kg b.w.) after 4 hours of fasting and blood glucose concentration measured every 15 minutes. As illustrated in Figure 11, the insulin administration was followed by a decrease of the blood glucose concentration, which was significantly less pronounced in *annexinA7*$^{-/-}$ mice than in *annexinA7*$^{+/+}$ mice pointing to decreased peripheral insulin sensitivity in *annexinA7*$^{-/-}$ mice.

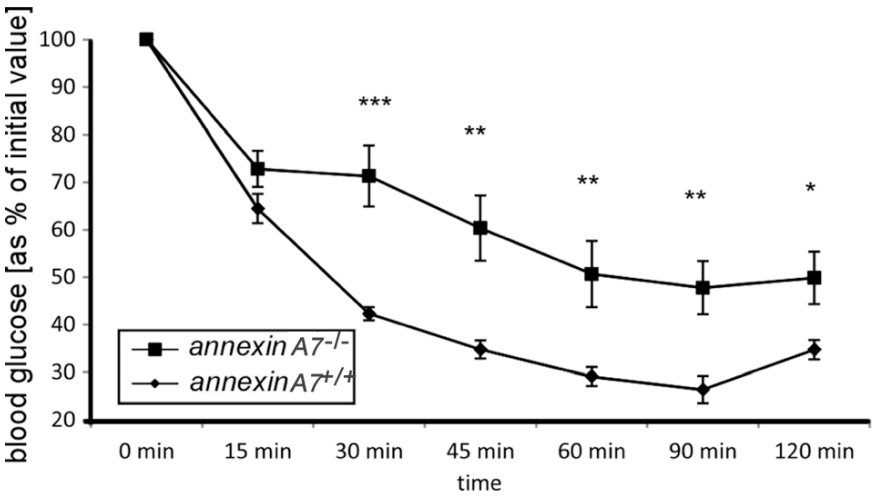

Figure 11. Blood glucose concentration following intraperitoneal insulin injection in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

Arithmetic means ± SEM (n = 13) of the blood glucose concentration following intraperitoneal injection of insulin (0. 5 U/kg b.w.) into *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice. The values are expressed as percentage of the blood glucose level at t = 0 min.

*, **, *** indicate significant difference using *Student's t-test* ($p<0.05$, $p<0.01$, $p<0.001$).

3.5 Reduced glucose tolerance in *annexinA7*$^{-/-}$ mice depends on COX activity

Further experiments were performed to elucidate whether the reduced glucose tolerance of *annexinA7*$^{-/-}$ mice was secondary to enhanced COX activity. Mice were treated with COX inhibitor aspirin and the glucose tolerance test was repeated. It is shown in Figure 12 that in aspirin-treated animals the intraperitoneal glucose load (2 g/kg b.w.) was followed by an increase in the blood glucose concentration, which was less pronounced in *annexinA7*$^{-/-}$ mice than in *annexinA7*$^{+/+}$ mice.

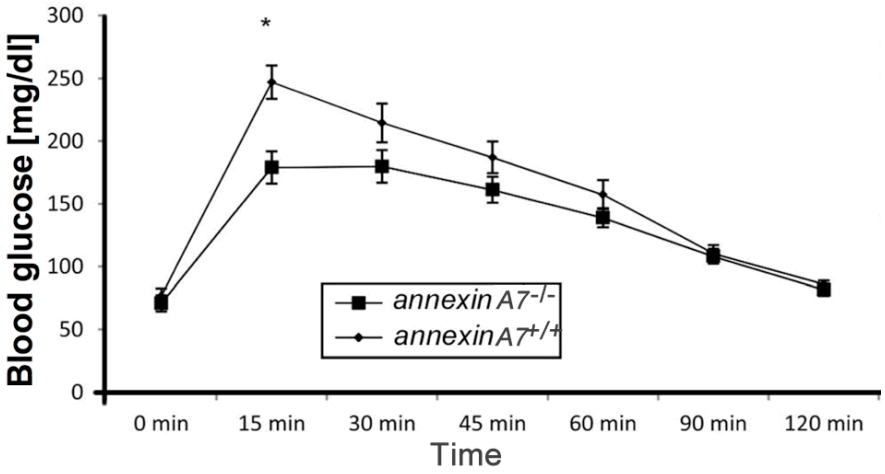

Figure 12. Glucose tolerance in aspirin-treated *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.
Arithmetic means ± SEM (n = 10-12) of the blood glucose concentration following intraperitoneal injection of glucose (2 g/kg body weight) into *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice treated with aspirin (600 mg/l in drinking water) for 1-2 weeks.
* indicates significant difference using *Student's t-test* ($p<0.05$).

Since aspirin treatment markedly improved glucose tolerance in *annexinA7*$^{-/-}$ mice, insulin was again determined in aspirin-treated animals after a glucose load. In contrast to untreated mice, glucose-induced insulin secretion was lower in aspirin-treated *annexinA7*$^{-/-}$ mice and not significantly different from *annexinA7*$^{+/+}$ mice (Figure 13). This result suggests an improvement of insulin resistance of aspirin-treated *annexinA7*$^{-/-}$ mice.

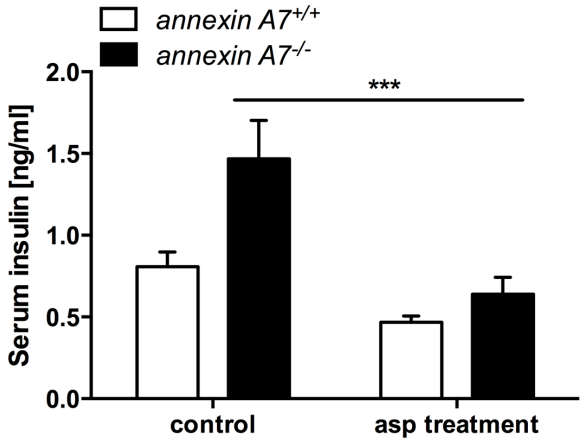

Figure 13. Glucose-induced insulin secretion in aspirin-treated *annexinA7*$^{+/+}$ and *annexin A7*$^{-/-}$ mice.

Arithmetic means ± SEM (n = 6-7) of the serum insulin level 30 min after intraperitoneal injection of glucose (2 g/kg b.w.) into *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ (black bar) mice treated with aspirin (600 mg/l in drinking water) for 2 weeks.

*** indicate significant difference using *Student's t-test* ($p<0.001$).

3.6 Leptin levels in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

Leptin secretion by white adipose tissue and plays a critical role in the regulation of food intake and maintenance the normal body weight. Leptin can regulate COX activity and stimulate PGE_2 and $PGF_{2\alpha}$ secretion by induce COX-2 activation[83,84]. To assess whether annexin A7 modifies the leptin level hereby affecting the COX activity, serum leptin concentration was measured.

The results show the body weight of both genotypes were similar (22.73±0.67g and 21.62±0.32g) in 12 week-old-mice (Figure 14A), the serum leptin concentration in *annexinA7*$^{-/-}$ mice was lower than *annexinA7*$^{+/+}$ mice but without statistical significance (Figure 14B).

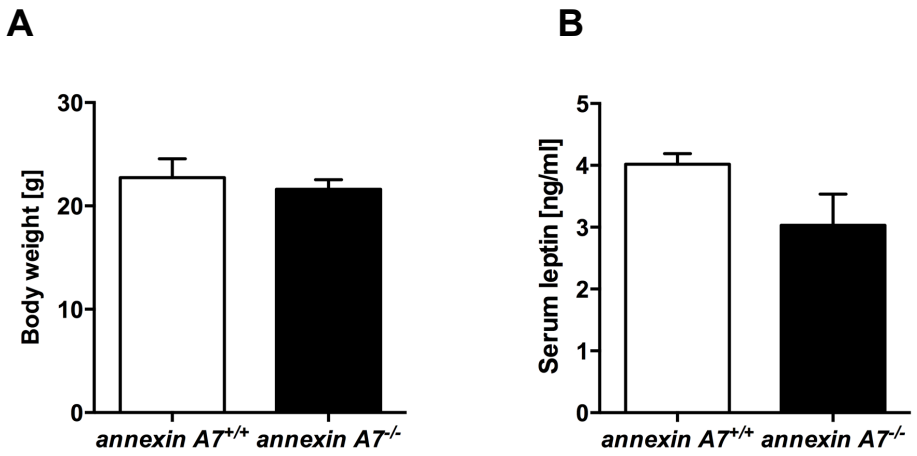

Figure 14. Body weight and serum leptin concentration in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

A. Arithmetic means ± SEM (n = 8, 12 weeks old) of the body weight of *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

B. Arithmetic means ± SEM (n = 4) of the serum leptin concentration in *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ mice (black bar).

3.7 Lower SGLT1 activity in jejunum of *annexinA7*$^{-/-}$ mice.

Annexin A7 is expressed in the alimentary tract and related to gastric and colonic neoplasm[22]. The intestinal glucose absorption is mediated by SGLT1 and the jejunum is the major organ for the digestion and glucose absorption. To elucidate the influence of annexin A7 on the sodium-dependent glucose cotransporter-1 (SGLT1) activity and thus effects the blood glucose level, distal segments of jejunum from *annexinA7*$^{-/-}$ mice and *annexin A7*$^{+/+}$ were mounted into mini-Ussing chambers and subjected to electrophysiological analysis. The transepithelial resistance (R_t) approached $5.8 \pm 0.4\ \Omega\ cm^2$ (n = 5) in *annexinA7*$^{+/+}$ mice and $6.6 \pm 0.4\ \Omega\ cm^2$ (n = 5) in *annexinA7*$^{-/-}$ mice (Figure 14A). Neither the transepithelial potential difference nor the transepithelial resistance was significantly different between *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$.

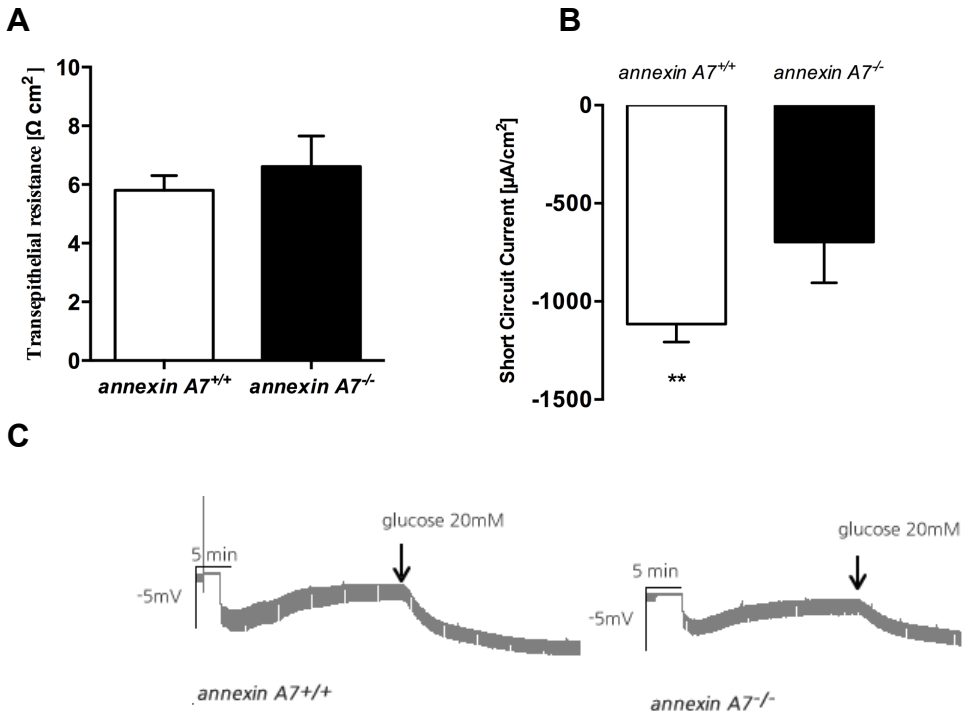

Figure 15. Glucose-induced current in jejunum of *annexinA7*$^{+/+}$ and annexin*A7*$^{-/-}$ mice.

A. Arithmetic means means ± SEM (n = 5) resistance of intestine epithelium from *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ (black bar) mice.

B. Arithmetic means ± SEM (n = 5-11) of glucose-induced current in distal jejunum from *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ (black bar) mice.

C. Original tracings from typical experiments illustrating the effect of 20 mM glucose (addition indicated by black arrows) on the transepithelial potential difference in *annexinA7*$^{+/+}$ (left panels) and *annexinA7*$^{-/-}$ (right panels) mice.

** indicates significant difference using *Student's t-test* ($p<0.01$).

The partial isosmotic replacement of mannitol by glucose created a lumen-negative shift of the transepithelial potential difference (ΔVg) without significantly altering the transepithelial resistance. ΔV_g and R_t allowed the calculation of the glucose-induced current, which was significantly lower in *annexinA7*$^{-/-}$ than in *annexinA7*$^{+/+}$ mice (**, $P<0.01$) (Figure 15). Accordingly, the SGLT1 activity was significantly lower in *annexinA7*$^{-/-}$ than in *annexinA7*$^{-/-}$ mice. However, we found higher glucose concentration in *annexinA7*$^{+/+}$ mice. This result suggests that the activity of SGLT1 has no directly correlation with blood glucose level between *annexinA7*$^{-/-}$ and *annexinA7*$^{+/+}$ mice.

3.8 Urinary glucose levels in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.

In the kidney, sodium-dependent glucose cotransporter-2 (SGLT2) plays a central role in glucose reabsorption. SGLT2 inhibitors have been applied in clinical treatment for diabetes mellitus[85]. In present study, urinary glucose was measured to estimate the activity of SGLT2. The result shown in figure 16, there was no significant difference between annexinA7$^{+/+}$ and annexinA7$^{-/-}$ mice, indicating that annexinA7 can not modify the SGLT2 activity then thus affects the blood glucose.

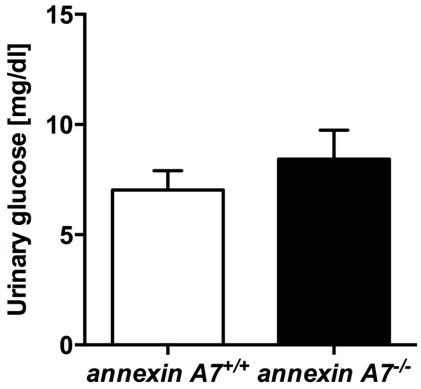

Figure 16. Urinary glucose concentration in *annexinA7*$^{+/+}$ and *annexinA7*$^{-/-}$ mice.
Arithmetic means ± SEM (n = 5) of the urinary glucose concentration of *annexinA7*$^{+/+}$ (white bar) and *annexin7*$^{-/-}$ mice (black bar).

3.9 Higher ENaC activity of colon in *annexinA7*$^{-/-}$ mice.

The electrogenic amiloride-sensitive epithelial sodium channels (ENaC) are abundantly expressed in the distal colon. The activity of ENaC, which can be regulated by insulin and PGs, is related to the sodium balance, the blood volume and the blood pressure[86-88]. To evaluate whether annexin A7 influence on the ENaC activity, the ENaC activity in colon was determined by Ussing Chamber.

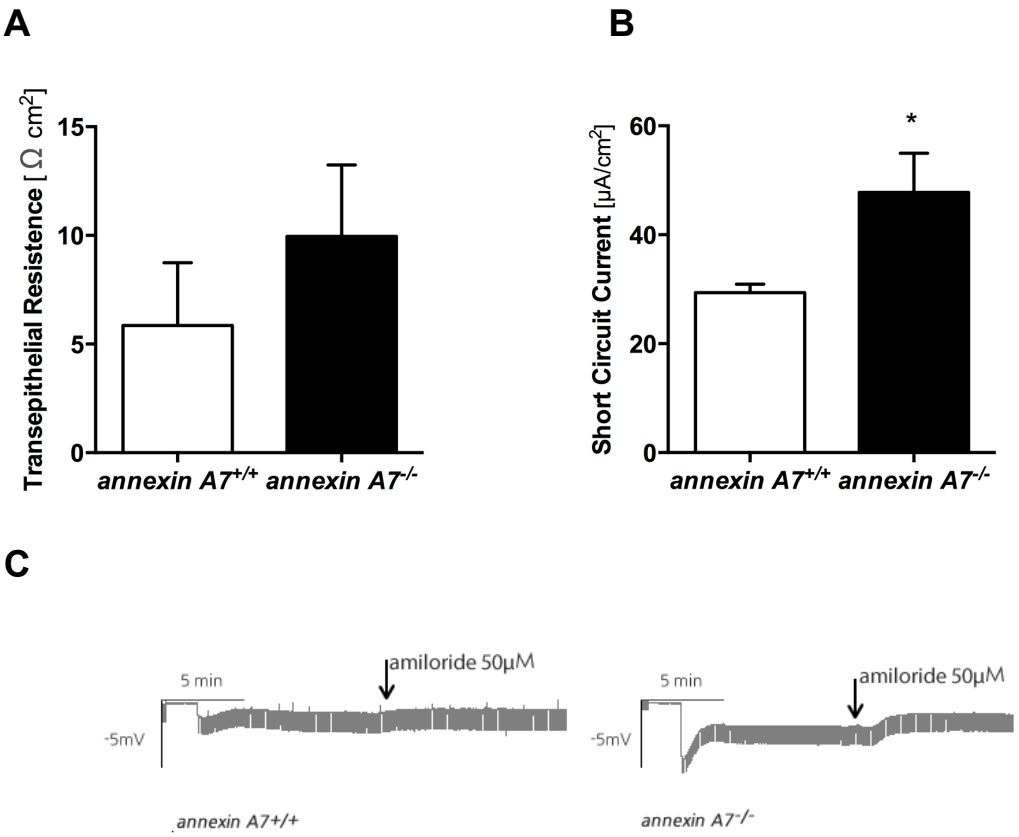

Figure 17. Amiloride-induced current in colon of *annexinA7*$^{+/+}$ and annexin*A7*$^{-/-}$ mice.

A. Arithmetic means means ± SEM (n = 4) resistance of colon epithelium from *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ (black bar) mice.

B. Arithmetic means ± SEM (n = 4) of amiloride-induced current in colon from *annexinA7*$^{+/+}$ (white bar) and *annexinA7*$^{-/-}$ (black bar) mice.

C. Original tracings from typical experiments illustrating the effect of 50µM amiloride (addition indicated by black arrows) on the transepithelial potential difference in *annexinA7*$^{+/+}$ (left panels) and *annexinA7*$^{-/-}$ (right panels) mice.

* indicates significant difference using *Student's t-test* ($p<0.05$).

As illustrated in Figure 17, the transepithelial resistance (Figure 17A) between *annexinA7*$^{+/+}$ and annexin*A7*$^{-/-}$ mice have no significantly difference, but the amiloride sensitive current was significantly increased in annexin*A7*$^{-/-}$ mice (*, $P<0.05$)(Figure 17B). This result suggests that annexin A7 downregluate ENaC activity and may affect the blood pressure.

4. Discussion

Annexin A7 is a widely conserved gene including fungi, protists, plants and vertebrates and has been shown to be involved in many different cellular processes, some of which implicate COX upregulation that occurs in a variety of pathophysiological conditions including diabetes mellitus. To assess whether annexin A7 was a negative effect on the glucose metabolism, gene-targeted mice lacking annexin A7 mice (*annexinA7*$^{-/-}$) were used to compare wild-type mice (*annexinA7*$^{+/+}$). Our studies reveal a novel function of annexin A7 in the regulation of cellular glucose uptake. Increase in serum glucose concentration following an intraperitoneal glucose load was significantly higher *annexinA7*$^{-/-}$ mice than in corresponding wild type mice. The observations point to impaired glucose uptake in annexin A7 deficiency. Glucose uptake is decreased in *annexinA7*$^{-/-}$ mice despite a sharper increase of insulin plasma levels following a glucose load in *annexinA7*$^{-/-}$ mice as compared to *annexinA7*$^{+/+}$ mice. Accordingly, the hyperglycemia reflects insulin resistance of cellular glucose uptake. The decline of plasma glucose concentration following injection of insulin was significantly blunted in annexin A7 deficient mice.

4.1 The effect of annexin A7 deficiency on glucose tolerance

The present observations found an increase of serum glucose concentration in *annexinA7*$^{-/-}$ mice was significantly higher than in *annexinA7*$^{+/+}$ mice after an

intraperitoneal glucose load. The maintenance of normal glucose homeostasis depends on a finely balanced dynamic interaction between insulin secretion and cellular sensitivity to insulin. Insulin production disorder can lead to type 1 diabetes mellitus. Reduced cellular sensitivity to insulin can lead to abnormal glucose metabolism and form type 2 diabetes mellitus. Type 2 diabetes mellitus pathogenesis is multifactorial process and the precise mechanisms remain unclear.

We postulate that impaired insulin secretion or impaired cellular glucose uptake in part due to annexin A7 deficiency. In order to verify this hypothesis, insulin concentration in mice serum was determined. Prior to an intraperitoneal glucose load, the basal concentration of serum insulin was lower in *annexinA7*$^{-/-}$ mice and *annexinA7*$^{+/+}$ mice, without statistical significance. However, after glucose loading, the concentration of serum insulin increased more in *annexinA7*$^{-/-}$ mice than *annexinA7*$^{+/+}$ mice within 30 minutes. This result demonstrates that impaired insulin production in *annexinA7*$^{-/-}$ does not account for their decreased glucose tolerance.

Insulin is the primary factor for cellular glucose uptake in normal metabolism. Insulin decreases the blood glucose levels mainly by promoting glucose uptake into cells, stimulating glycogen synthesis in liver and skeletal muscle. Insulin tolerance test was applied to assess the impact of annexin A7 deficiency on the insulin sensitivity of peripheral glucose uptake. The result revealed an impaired insulin effect on the blood glucose level in *annexinA7*$^{-/-}$ mice. This indicates that *annexinA7*$^{-/-}$ mice have peripheral insulin resistance. Interestingly, the heterozygote mouse model of annexin A7 deficiency (*annexinA7*$^{+/-}$mice) exhibited higher receptor-dependent glucose-induced insulin secretion [89].

Defective insulin signaling and action may cause the impairment of insulin action and lead to insulin resistance. Annexin A7 is related to the Ca^{2+} and phospholipid-binding protein in tissue and has a strong association with cellular protein function and defective intracellular calcium signaling[80]. Hence, annexin A7 deficiency can affect proteins, receptors or transporters that are related to the insulin function and thereby induce the insulin resistance. Further studies are required to confirm the effect of annexin A7 on those factors.

4.2 The mechanism of annexin A7 impacting on insulin sensitivity

The insulin resistance was at least partially due to enhanced COX activity in annexin A7-deficient mice. Enhanced formation of PGE_2, a by-product of COX-2 metabolism, in *annexinA7*$^{-/-}$ mice has already been observed in erythrocytes [90]. COX-2 is a critical mediator in inflammation and immune cell reaction. Inflammation, which is an important factor in the pathogenesis of type 2 diabetes mellitus, can induce insulin resistance[91]. Lately, research has suggested that chronic subclinical inflammation state may be an underlying mechanism for the insulin resistance[92]. Prostaglandins, which act as signaling intermediates, can also mediate the insulin resistance process. Overproduction of prostaglandin D_2 (PGD_2) can increase insulin resistance through enhance lipophilic molecules in fat [93]. Elevated spinal COX-2 protein expression and PGE_2 release were observed in diabetic rats[94]. As outlined in the introduction, glucose is taken up into muscle through insulin-sensitive GLUT4. It was suggested that COX-2 can regulate and decrease GLUT4 expression *in vitro*[95].

Our studies showed deranged glucose metabolism which was paralleled by elevated serum levels of 6-Keto-$PGF_{1\alpha}$, which is a stable end product of prostaglandin metabolism indicating COX-2 activity [96,97]. Moreover, a COX enzyme assay revealed higher hepatic COX activity in *annexinA7*$^{-/-}$ mice than *annexinA7*$^{+/+}$ mice. Recently, PGE_2 has been shown to cause insulin resistance in hepatocytes through affecting the function of insulin receptor substrate and preventing an insulin-dependent activation of AKT and glycogen synthesis[98]. Prostaglandins may therefore contribute to deranged glucose metabolism in type 2 diabetes mellitus [98]. Thus, we further provide evidence that hepatic COX activity is, at least in part, regulated by annexin A7.

In order to elucidate whether the impaired glucose tolerance of *annexinA7*$^{-/-}$ mice was related to the enhance COX activity, mice were treated with COX inhibitor aspirin. Aspirin can decrease prostaglandins production and reduce inflammation through binding and inhibiting COX enzyme. It has been suggested that aspirin can enhance the insulin sensitivity by keeping IRS away from other kinases that may reduce IRS activity[99]. The beneficial effect of COX inhibition on glucose intolerance has been reported earlier in a study showing that aspirin treatment ameliorates peripheral insulin resistance [100].

In line with enhanced COX activity in *annexinA7*$^{-/-}$ mice, the difference in glucose tolerance between *annexinA7*$^{-/-}$ mice and *annexinA7*$^{+/+}$ mice was reversed by treating the mice with aspirin. Moreover, aspirin treatment of *annexinA7*$^{-/-}$ mice reduced glucose-induced insulin secretion.

Notably, the effect of annnexin A7 deficiency on parasitemia and clinical course of malaria and the blunted stimulation of gastric acid secretion by dexamethasone were similarly reversed by inhibition of cyclooxygenase with aspirin [90,23]. Accordingly, the phenotype of *annexinA7*$^{-/-}$ mice is at least partially due to inhibition of cyclooxygenase. This observation does, however, not rule out that further other annexin A7 sensitive mechanisms contribute to the regulation of glucose metabolism.

4.3 The effect of annexin A7 on SGLT1 and ENaC activity

SGLT1 is the primary intestinal glucose transporter. SGLT1 expression is under an indirect control by insulin and increased in type 2 diabetes mellitus[101,102]. The mice lacking SGLT1 or inhibition of SGLT1 activity can improve glucose tolerance[103]. Interestingly, the activity of SGLT1 was significantly lower in *annexinA7*$^{-/-}$ mice. Current research suggests that PGE_2 inhibits SGLT1 by reducing the cotransporter expression[104]. We report that COX activity and PGI_2 end product 6-Keto-$PGF_{1\alpha}$ were significantly higher in *annexinA7*$^{-/-}$ than *annexinA7*$^{+/+}$ mice. This data imply that SGLT1 activity in jejunum is regulated by annexin A7.

Hypertension is part of insulin resistance syndrome and ENaC is a key protein in regulation of blood volume and blood pressure[105]. It has been observed that PGE_2 induces ENaC upregulation in the mouse cortical collecting duct cells and in *Xenopus* lung epithelium[106,107]. Recent studies reveal the activation of ENaC can trigger the PGE_2 formation and release in embryo implantation[108], demonstrating the action of PGE_2 and ENaC may be mutual. Moreover, insulin can also enhance the ENaC activity by increasing its channel density[109]. We report that higher ENaC activity in

annexinA7$^{-/-}$ mice. Thereby, Annexin A7 may regulate the ENaC activity and affect the blood pressure.

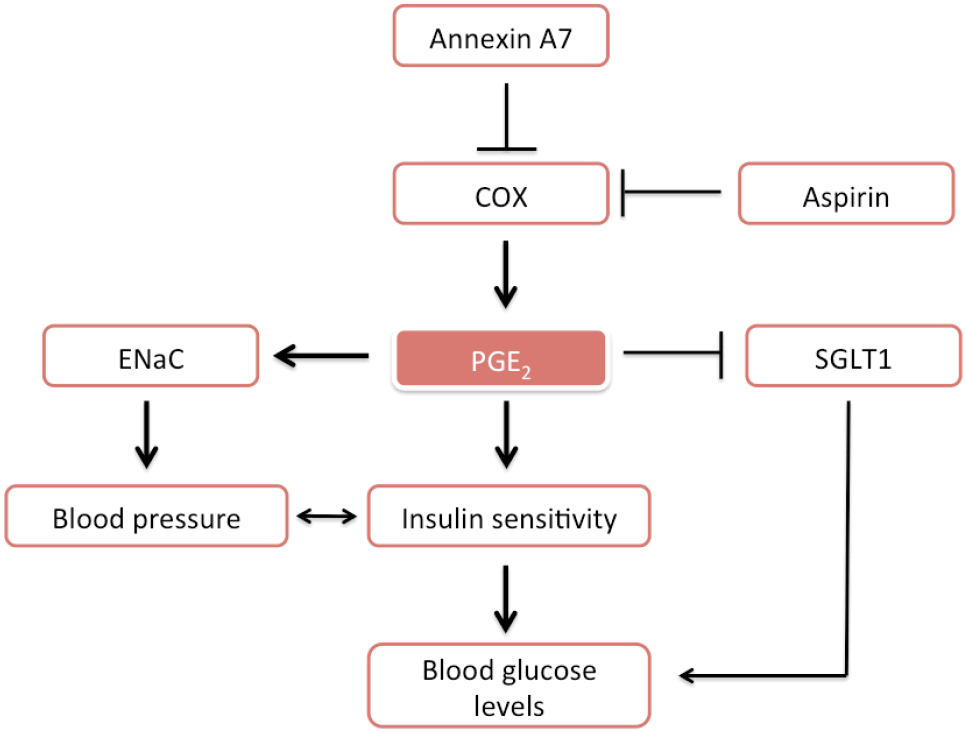

Figure 18. The mechanism of annexin A7 impacting on insulin sensitivity.

In summary (figure 18), annexin A7 inhibits COX activity then reducing PGE_2 synthesis. Insulin sensitivity is decreased by PGE_2. In annexin A7-deficient mice, we also found insulin sensitivity was attenuated through the treatment of aspirin. Therefore, annexin A7 enhances the insulin sensitivity through its COX inhibition. PGE_2 can reduce SGLT1 activity and decrease the glucose absorption in the jejunum. Due to the insulin resistance exist in annexin A7-deficient mice, however, blood glucose levels cannot reflect the difference of SGLT1 activity. Further studies will be required to evaluate the effect of annexin A7 on SGLT1 expression, cellular localization and activity. Annexin A7 can also decrease ENaC activity through reducing PGE_2 synthesis. Higher ENaC activity is associated with hypertension and

diabetes mellitus. Blood pressure in annexin A7-deficient mice and ENaC expression should be future investigated.

The clinical relevance of this finding is reinforced by the observation that the COX-2 higher expression in vascular endothelial cells, islets of langerhans and peripheral nerve of type 2 diabetes mellitus patients, when compared to non-diabetic individuals[110-112]. Inflammation, especially the chronic subclinical inflammation state, is an underlying mechanism for the insulin resistance in type 2 diabetic mellitus. Our findings have revealed that annexin A7 deficiency or mutation may aggravate insulin resistance in diabetes. Lack of samples from diabetic patients is a limitation of our study. Nevertheless, we found a significant correlation between annexin A7 and COX activity as well as insulin resistance. Annexin A7 may be a novel therapeutic target for clinical application for type 2 diabetes mellitus in the future.

Conclusion

Annexin A7 deficiency decreases the insulin sensitivity of cellular glucose uptake, an effect virtually abrogated by inhibition of cyclooxygenase with aspirin. Annexin A7 deficiency increases the ENaC activity in the colon possibly leading to high blood pressure. Annexin A7 deficiency decreases the Sglt1 activity in the jejunum lowering glucose absorption.

5. Zusammenfassung

Hintergrund: Die Insulinsensitivität wird durch $PGE_{2,}$ ein Hauptprodukt der Cyclooxygenase 2 (COX-2), gestört, welches nach Glukosegabe die zelluläre Glukoseaufnahme beeinflusst. Wie schon in Erythrozyten gezeigt werden konnte, wird die PGE2-Bildung durch Annexin A7 gehemmt. Die vorliegende Arbeit ermittelte die Rolle von Annexin A7 im Glukosemetabolismus.

Methoden: Mäuse mit gezielter Annexin A7 Genausschaltung wurden mit Wildtypmäusen verglichen. 6-Keto-prostaglandin-F1α wurde mit enzymgekoppeltem Immunadsorptionstest (ELISA) gemessen um die COX-2-Aktivität im Serum zu bestimmen. Die Hepatische COX-Aktivität wurde durch ELISA bestimmt. Die Serumkonzentrationen von Glukose und Insulin wurden nach intraperitonealer Gabe von Glukose und das Serum-Glukoselevel nach intraperitonealer Injektion von Insulin bestimmt. Die Experimente wurden jeweils mit und ohne Vorbehandlung der Mäuse mit dem COX-Inhibitor Aspirin durchgeführt.

Resultate: Das 6-Keto-prostaglandin-F1α-Level im Serum und die hepatische COX-Aktivität waren signifikant höher in Annexin A7 Knockout-Mäusen als in Wildtyp-Mäusen. Der intraperitoneale Glukosetoleranztest brachte eine erniedrigte Glukosetoleranz in Annexin A7 Knockout-Mäusen verglichen mit Wildtyp-Mäusen zum Vorschein. Das Serum-Glukoselevel wurde durch eine intraperitoneale Insulininjektion in beiden Genotypen erniedrigt, ein Effekt, welcher signifikant weniger ausgeprägt in Annexin A7 Knockout als in Wildtyp-Mäusen war. Die intraperitoneale Gabe von Glukose führte zu höheren Serum-Insulinkonzentrationen in Annexin A7 Knockout-Mäusen als in Wildtyp-Mäusen. Die Aspirinvorbehandlung verringerte die Erhöhung der Insulinkonzentration nach Glukoseinjektion in beiden Genotypen und hob den Unterschied der Insulinkonzentration im Serum nahezu auf. Zudem verbesserte die Aspirinvorbehandlung die Glukosetoleranz in Annexin A7 Knockout-Mäusen.

Schlussfolgerung: Annexin A7 beeinflusst die Insulinsensitivität bei Glukoseaufnahme und damit die Glukosetoleranz. Diese Effekte sind von der COX-Aktivität abhängig.

6.Publications

Dong Luo, Abul Fajol, Anja Umbach, Angelika A. Noegel, Stefan Laufer, Florian Lang, Michael Föller (2014) *Influence of Annexin A7 on insulin sensitivity of cellular glucose uptake*. Pfluegers Archiv - European Journal of Physiology. 2014 Jun; DOI 10.1007/s00424-014-1541-z. PMID: 24903239

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Umbach AT, Luo D, Bhavsar SK, Hosseinzadeh Z, Lang F (2013) *Intestinal Na^+ loss and volume depletion in JAK3-deficient mice.* Kidney Blood Press Res. 2013;37(4-5):514-20. PMID: 24281140

Warsi J, Hosseinzadeh Z, Dong L, Pakladok T, Umbach AT, Bhavsar SK, Shumilina E, Lang F (2013) *Effect of Janus kinase 3 on the peptide transporters PEPT1 and PEPT2.* J Membr Biol. 2013 Dec;246(12):885-92. PMID: 23934551

Hosseinzadeh Z, Dong L, Bhavsar SK, Warsi J, Almilaji A, Lang F (2014) *Upregulation of peptide transporters PEPT1 and PEPT2 by Janus kinase JAK2.* Cell Physiol Biochem. 2013;31(4-5):673-82. PMID: 23711493

7.Curriculum Vitae

Dong Luo

Date and place of birth: July 16, 1984. Linyi, China

Contact: luodong716@gmail.com

Education

Doctoral research (2011-2014)

Institute of Physiology I, Eberhard Karl University of Tuebingen, Germany

Master of Medicine (2008-2011)

Guangzhou Overseas Chinese Hospital, Jinan University, Guangdong, China

Bachelor of Medicine (2002-2008)

Clinical medicine college, Taishan Medical University, Shandong, China

Clinical medicine college, Binzhou Medical College, Shandong, China

Lab Skills

Animal experiment

Ussing chamber experiment

ELISA Measurement

Western-blot Analysis

Professional Experience

Served as a resident/teaching assistant in clinical medicine college (Jinan University, Guangzhou, China)

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