Erhöhte Phosphaturie, Kalziurie und Katecholamin-Freisetzung bei Mäusen mit einer gentechnisch veränderten, PKB-SGK-resistenten Glykogen-Synthase 3

PKB/SGK-Resistant GSK3 Enhances Phosphaturia, Calciuria, and Catecholamine Release

DISSERTATION

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat)

vorgelegt von
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Tübingen
2012

Tag der mündlichen Prüfung:	16.10.2012
Dekan:	Prof. Dr. Wolfgang Rosenstiel
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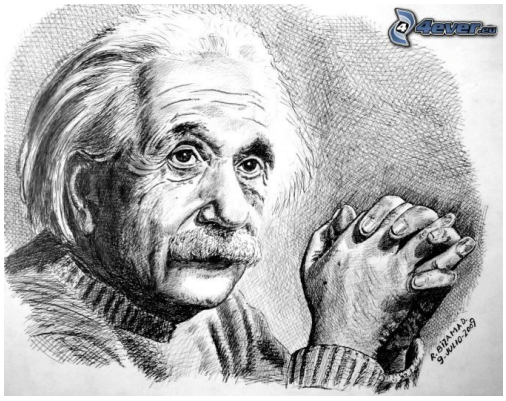
"Creating a new theory is not like destroying an old barn and erecting a skyscraper in its place. It is rather like climbing a mountain, gaining new and wider views, discovering unexpected connections between our starting points and its rich environment. But the point from which we started out still exists and can be seen, although it appears smaller and forms a tiny part of our broad view gained by the mastery of the obstacles on our adventurous way up."



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-Share in publications done in team work-

Name: Balasaheb D. Siraskar

List of Publications

1. *Enhanced catecholamine release in mice expressing PKB/SGK-resistant GSK3.* Pflugers Arch. 2011 Dec;462(6):811-9. Epub 2011 Sep 16.
2. *PKB/SGK-resistant GSK3 enhances phosphaturia and calciuria. J Am Soc Nephrology (JASN). 2011 May; 22(5):873-80. Epub 2011 Apr 14.*

Nr.	**Accepted for publication yes/no**	**Number of all authors**	Position of the candidate in list of authors	Scientific ideas of candidate (%)	Data generation by candidate (%)	Analysis and Interpretation by candidate (%)	Paper writing by candidate (%)
1	Yes	10	1st	45-50	45-50	45-50	10-15
2	Yes	17	5th	5-10	5-10	5-10	5-10

I certify that the above statement is correct.

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1. ABSTRACT

Glycogen synthase kinase 3 (GSK3) plays a decisive role in the regulation of multiple functions and acts as a downstream regulatory switch for various signaling pathways, including cellular responses to WNT, Growth Factors, Insulin, RTK (Receptor Tyrosine Kinases), Hedgehog pathways, and GPCR (G-Protein-Coupled Receptors) and is involved in a numerous signal transduction cascades involving cellular processes, ranging from glycogen metabolism, cell development, gene transcription, protein translation to cytoskeletal organization, cell cycle regulation, proliferation and apoptosis.

GSK3 is phosphorylated and its activity is inhibited by protein kinase B (PKB/Akt) and serum & glucocorticoid inducible kinase (SGK) isoforms, which are in turn activated by growth factors through phosphoinositide (PI) 3 kinase signalling.

In addition, abnormally high activities of GSK-3 protein (overexpression) have been implicated in several pathological disorders which include type 2 diabetes, neuron degenerative and affective disorders. This led to the development of new generations of inhibitors with specific clinical implications to treat these diseases.

PI3/PKB/Akt/SGK-dependent inhibition of GSK3 is disrupted in gene targeted knockin mice with mutated and thus PKB/SGK-resistant GSK3α,ß (*gsk3*^{KI}) where the serine of the PKB/SGK phosphorylation site has been replaced by alanine. Thus our GSK3^{KI} mouse is with overexpressed GSK3 activity which in turns resistant to PI3/PKB/Akt/SGK signalling pathway.

Recent experiments revealed that blood pressure is significantly higher in these mice (*gsk3*^{KI}) than in wild type mice (*gsk3*^{WT}). So to find out the underlying cause of elevated blood pressure was our first aim of this study. As discussed earlier that Insulin and IGF1-dependent signaling activates protein kinase B and serum-glucocorticoid kinase (PKB/SGK), which together phosphorylate and inactivate glycogen synthase kinase GSK3. Since insulin and IGF1 increase renal tubular calcium and phosphorus reabsorption and GSK3 plays important role in intestinal electrogenic phosphate transport. Our second aim of the study was to determine whether overexpression of GSK3 and the phosphate transporter NaPi-IIa in *Xenopus* oocytes decrease electrogenic phosphate transport and decrease renal tubular calcium and phosphorus reabsorption.

Looking forward to the first aim, we determined blood pressure with the tail cuff method, heart rate by ECG-measurements, catecholamine concentrations by ELISA, and

vanilylmandelic acid by HPLC. As a result, blood pressure and heart rate were significantly higher in *gsk3*^{KI} than in *gsk3*^{WT}mice. The α-adrenergic blocker prazosine (1 μg/g b.w.) and the ganglion blocker hexamethonium (40 μg/g b.w.) decreased blood pressure to a larger extent in *gsk3*^{KI} than in *gsk3*^{WT}mice and virtually abrogated the difference between genotypes. Similarly, the β-adrenergic blocker atenolol (5 μg/g b.w.) decreased the heart rate to a larger extent in *gsk3*^{KI} than in *gsk3*^{WT}mice and again dissipated the difference of heart rate between genotypes. Plasma epinephrine and norepinephrine concentrations, as well as urinary excretion of vanilylmandelic acid were significantly higher in *gsk3*^{KI} than in *gsk3*^{WT}mice. Thus, the observations reveal a completely novel function of PKB/Akt/SGK-dependent GSK3 signaling, i.e. regulation of catecholamine release.

Looking forward to second aim, to determine creatinine clearance, urinary flow rate, and urinary excretion of $Ca^{2+}$and phosphate, the mice were placed individually in metabolic cages for 24- our urine collection. The phosphate concentration was determined colorimetrically using commercial diagnostic kits. The urinary concentration of Ca^{2+} was measured by flame photometry or by a photometric method according to the manufacturer's instructions. The creatinine concentration in urine was determined using the Jaffe reaction, creatinine and calcium concentration in plasma were measured using a photometric method. The plasma intact parathormone concentration was measured using an ELISA kit. A radioimmunassay kit was used to determine the concentration of 1,25-(OH)2D3 in plasma. The bone density was determined by high-resolution microCAT-II small animal computed tomography (CT) scanner. Compared with wildtype animals, *gsk3*^{KI} animals exhibited greater urinary phosphate and calcium clearances with higher excretion rates and lower plasma concentrations. Isolated brush border membranes from *gsk3*^{KI} mice showed less sodium-dependent phosphate transport and Na-phosphate co-transporter expression. Parathyroid hormone, 1,25-OH vitamin D levels, and bone mineral density decreased in *gsk3*^{KI} mice, suggesting a global dysregulation of bone mineral metabolism. Overexpression of GSK3 and the phosphate transporter NaPi-IIa in *Xenopus* oocytes decreased electrogenic phosphate transport compared with NaPi-IIa–expressing oocytes. Taken together, PKB/SGK phosphorylation of GSK3 increases phosphate transporter activity and reduces renal calcium and phosphate loss.

Coming to the conclusion of this study, we demonstrate that PKB/Akt/SGKdependent phosphorylation of GSK3 participates in the control of catecholamine release and thus in blood pressure regulation. Disruption of the inhibitory effect of PKB/Akt/SGK on GSK3

leads to enhanced catecholamine release and increase of blood pressure. We also demonstrate that Akt/PKB-dependent regulation of GSK3 participates in the control of renal tubular phosphate transport. Loss of Akt/PKB-dependent inhibition of GSK3αβ leads to renal phosphate wasting, which presumably contributes to or even accounts for the decrease of PTH release with resulting calciuria, decreased formation of 1,25-(OH)2 D3, and decreased mineralization of bone. These observations thus show a novel, powerful element in the regulation of mineral metabolism.

2. ABSTRAKT

Die Glykogen-Synthase 3 (GSK3) spielt eine wichtige Rolle bei der Regulation zahlreicher Stoffwechselvorgänge und ist stromabwärts in verschiedenen intrazelluären Signalkaskaden anzutreffen (Insulin, Wachstumshormon, WNT, Tyrosinkinase-Rezeptor, G-Protein-Gekoppelte Proteine). Dabei werden verschiedene zelluläre Prozesse wie Glykogenstoffwechsel, Zellentwicklung, Gentranskription, Proteintranslation, Organisation des Zytoskeletts, Beeinflussung des Zellzyklus, Proliferation und Apoptose reguliert. Die Glykogen-Synthase 3 wird inhibiert, indem sie durch die übergeordnete Protein kinase B (PK B/Akt) oder durch die Serum-und Glucocorticoid-Induzierbare Kinasen (SGK) phosphoryliert wird, die ihrerseits durch Wachstumsfaktoren unter Beteiligung der Phosphoinositide 3-Kinase (PI3K) aktiviert werden. Eine abnorm gesteigerte GSK3-Proteinexpression wird mit Krankheiten wie dem Diabetes mellitus 2, neurodegenerativen und affektiven Störungen in Verbindung gebracht, was zur Entwicklung pharmakologischer Hemmstoffe der GSK3 führte. Durch gezielte Mutation und Austausch der Serin-Phosphorylierungsstelle der GSK3 durch Alanin konnte ein genetisches Mausmodell geschaffen werden, bei dem die Inhibition der GSK3 durch die PI3K, PKB oder SGK aufgehoben ist (PKB/SGK-resistente GSK3, *gsk3^{KI})*. Dies ermöglicht es, die physiologische und pathophysiologische Relevanz einer erhöhten GSK3-Aktivität zu untersuchen.

Erste Erkenntnisse an *gsk3^{KI}*- Mäusen zeigten einen im Vergleich zu Wildtyp-Mäusen signifikant höheren Blutdruck. Das erste Ziel der vorliegenden Arbeit bestand darin, die Ursache dafür herauszufinden. In weiteren Experimenten sollte zum anderen die Rolle der GSK3 bei der Regulation des tubulären Phosphattransports untersucht werden, da letzterer durch Insulin oder Insulin-like Growth Factor 1 stimuliert wird, die ihrerseits die GSK3 phosphorylieren und sie dadurch hemmen.

Der Blutdruck wurde mit der Schwanzplethysmographie ermittelt, die Herzfrequenz mittels EKG, die Plasma-Katecholamin-Konzentration mittels ELISA und die Vanillinmandelsäure als Abbauprodukt der Katecholamine aus dem Urin mittels HPLC. Der erhöhte Blutdruck der *gsk3^{KI}*- Mäusen ging mit einer erhöhten Herzfrequenz einher und wurde durch den alpha-Blocker Prazosin (1µg/g KG) und dem Gangleinblocker Hexamethonium (40 µg/g KG) stärker gesenkt als in Wildtyp-Mäusen. Analog dazu senkte der Beta-blocker Atenolol (5 µg/g KG) die Herzfrequenz stärker in *gsk3^{KI}*- Mäusen als in Wildtyp-Mäusen. Durch diese Behandlungen wurde der Unterschied zwischen *gsk3^{KI}*- Mäusen und Wildtyp-Mäusen

hinsichtlich des Blutdrucks und der Heruzfrequenz aufgehoben. Die Plasma-Adrenalin- und –Noradrenalin-Konzentration sowie die Ausscheidung des Vanillinmandelsäure im Urin waren in *gsk3*^{KI}- Mäusen signifikant höher als in Wildtyp-Mäusen. Die Befunde weisen zum ersten Mal auf eine neuartige Funktion der PKB/SGK-abhängige GSK3-Signalübertragung hin, nämlich der Katecholamin-Sekretion.

Um die Rolle der GSK3 bei der Regulation des Phosphattransports zu erforschen, wurden *gsk3*^{KI}- Mäuse in Stoffwechselkäfigen untersucht und die renale Phosphat-Ausscheidung im 24 – Stunden-Sammelurin bestimmt. Daneben wurden wichtige Regulatoren des Phosphat-Haushalts wie Parathormon oder 1,25-Dihydroxy-Vitamin D aus dem Plasma bestimmt. Zusätzlich wurde die Knochendichte mit einem hochauflösenden CT-Gerät für kleine Labortiere gemessen. Verglichen mit Wildtyp-Mäusen wiesen *gsk3*^{KI}- Mäuse eine höhere Phosphat- und Kalzium-Ausscheidung im Sammelurin auf und hatten eine niedrigere Phosphat- und Kalzium-Konzentration im Plasma. Aus den Nieren isolierte Bürstensaum-Membranen wiesen eine geringere Expression des Na-gekoppelten Phosphat-Transporters auf, was mit einer geringeren Transportrate einherging. Die Konzentration von Parathormon und 1,25-Dihydroxy-Vitamin D sowie die Knochendichte waren in *gsk3*^{KI}- Mäusen vermindert. In vitro-Versuche mit Oozyten, die den Na-gekoppelten Phosphat-Transporter exprimierten mit oder ohne gleichzeitige Expression von GSK3, zeigten einen reduzierten Phosphat-Transport bei Überexpression der GSK3. Die Ergebnisse zeigen, dass die Phosphorylierung der GSK3 durch die PKB/SGK den renalen Phosphattransport stimuliert und vor einer verminderten Knochendichte schützt.

Schlussfolgernd zeigt diese Arbeit, dass die PKB/SGK-abhängige Phopshorylierung der GSK3 in einem Mausmodell in vivo eine wichtige Rolle bei der Regulation des Blutdrucks und der Katecholamin-Freisetzung spielt und auch Knochen- und Mineral-Haushalt beeinflusst.

3. ACKNOWLEDGEMENTS

It is with immense pleasure, I record my humble gratitude to my research guide Prof. Dr. Florian Lang, for his expert guidance and critical review throughout the work. My sincere and heartfelt thanks for his abundant encouragement.

I am very grateful to Dr. Jakob Voelkl for his timely help, support, thought provoking suggestions and stimulating discussions during the progress of the work. I would like to thank Prof. Dr. Peter Ruth for giving me an opportunity to present the dissertation at the Faculty of Pharmacy and Chemistry, Eberhard Karls Universität Tübingen, Germany.

I am very grateful to Dr. Michael Föller for his encouragement and timely help during the progress of the work.

I am very thankful to my colleagues and friends Dan Yang Huang, Dr. Ferruh Artunc, Dr. Rexhep Rexhepaj, Dr. Daniela Kempe, Dr. Teresa Ackermann, Dr. Annia Umbach, Mohamed Siyabeldin E. Ahmed, Dr. Suchen Gu, Christina Leibrock, Undine E. Lang, Ioana Alesutan, Mentor Sopjani, Gerti Stange, Martin S. Judenhofer, Dr. Krishna M. Boini, Dr. Madhuri Bhandaru, Bernd J. Pichler, Juerg Biber, Carsten A. Wagner, Mr. Ganesh Pathare and Mr Diwakar Bobbala.

I also thanks to Tanja Loch, Lejla Subasic and Mr. Farooq for their help and support.

Finally, I would like to thank all other colleagues from the Institute of Physiology for their valuable help and support.

This task never completes without the mention of my beloved parents, brothers and my wife and son who blessed me in every aspect of my life.

4. CONTENTS

5. ABBREVATIONS

°C	Degree Celcius
µg	Microgram
µm	Micrometer
1,25-$(OH)_2D_3$	1,25-dihydroxy vitamin D3
AA	Amino Acids
ADP	Aadenosine Diphosphate
AIDA	Advanced Image Data Analyzer
ANNOVA	Analysis of variance
APC	Adenomatous Polyposis Coli
arb	Arbitrary Unit
Arg96	Argenine 96
ATP	Adenosine Triphosphate
B.W.	Body Weight
BBMV	Brush Border Membrane Vesicles
BP	Blood Pressure
BPM	Beats Per Minute
C/EBPα	CCAAT/Enhancer-Binding Protein Alpha
C/EBPβ	CCAAT/Enhancer-Binding Protein Beta
Ca^{2+}	Calcium Ion
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CK2	Casein kinase 2
CREB	Cyclase Response Element Binding Protein
cRNA	Complementary RNA
CYP27B1	Cytochrome P450, Family 27, Subfamily B, Polypeptide 1
DKK	Dickkopf
DM	Diabetes Melitus
DNA	Deoxyribonucleic Acid
DRAQ-5	1,5-bis{[2-(di-methylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9,10-dione,
DVL	Dishevelled
ECG	Electrocardiogram
EGF	Endothelial Growth Factor
eIF2B	Eukaryotic Protein Synthesis Initiation Factor 2B
ELISA	Enzyme Linked Immuno Sorbant Assay
eNOS	endothelial nitric oxide synthase
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
F_A	Factor A
FGF-23	Fibroblast Growth Factor 23
Fig	Figure
FITC	Flourescein Isothiocyanate
FRAT	Frequently Rearranged in Advanced T-Cell Lymphomas
FZD	Frizzled
g	Gram
G6P	Glucose-6-Phosphate
Gly	Glycine

5. ABBREVATIONS

Gly-rich	Glycine Rich
GPCR	G-Protein-Coupled Receptors
GS	Glycogen Synthase
GSK-3	Glycogen Synthase Kinase-3
$gsk3^{KI}$	Glycogen Synthase Kinase-3 Knockin
$gsk3^{WT}$	Glycogen Synthase Kinase-3 Wild Type
GSK3α	Glycogen Synthase Kinase-3 alpha
GSK3β	Glycogen Synthase Kinase-3 beta
H_2O	Water
HCl	Hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HPLC	High Pressure (or High Performance) Liquid Chromatography
hr	Hour
HSF-1	Heat Shock Protein-1
Hz	Hertz
I	Current
i.p.	Intraperitoneal
IGF-1	Insulin-Like Growth Factor-1
IP3	Inositol Trisphosphate
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
IRS-1	Insulin Receptor Substrate-1
K_2HPO_4	Dipotassium hydrogenphosphate
KCl	Potassium Chloride
kD	Kilo Dalton
LEF1	Lymphoid Enhancing Factor-1
Li+	Lithium Ion
LRP5/6	Lipoprotein Receptor-Related Protein 5/6
MAPK	Mitogen-Activated Protein Kinase
MAPKAP-K1	MAPK-Activated Protein Kinase-1 also called RSK
Mg^{2+}	Magnessium Ion
min	Minute
MITF	Microphthalmia-Associated Transcription Factor
ml	Mili Litter
mM	Mili Mole
mm^2	Square Millimeter
mmHg	Millimeter of Mercury
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
myc	Myelocytomatosis oncogene cellular homolog
nA	Nanoampere
Na^+	Sodium Ion
NaCl	Sodium Chloride
NaPi-IIa	Type IIa Na/Pi cotransporter
NaPi-IIc	Type IIc Na/Pi cotransporter
ND96	ND96 buffer [96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, titrated with NaOH to pH 7.4]

5. ABBREVATIONS

NFATc	Nuclear Factor of Activated T Cells
NF-kB	Nuclear factor κ B
ng	Nanogram
nl	Nanolitter
p70S6K	p70 S6 Kinase
p90RSK	p90 Ribosomal S6 Kinase
PBS	Phosphate Buffered Saline
PDK	3-phosphoinositide-dependent protein kinase
PFA	Paraformaldehyde
pg	Picogram
PGF	Platelet Growth Factor
pH	Potential of Hydrogen
Pi	Phosphate Ion
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphotidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol 3,4,5-Trisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B, also called Akt
PKC	Protein Kinase C
pmol	Picomol
PP1	Protein Phosphatase1
PTH	Parathyroid Hormone
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinases
s	Second
S.D.	Standard Deviation
S.E.M.	Standard Error of The Mean
S6	Small Subunit Ribosomal Protein S6
Ser	Serine
Ser21	Serine 21
Ser9	Serine 9
sFRP	Soluble Frizzled-Related Protein
SGK	Serum and Glucocorticoid-Inducible Kinase
SGK1	Serum and Glucocorticoid-Inducible Kinase 1
SGK3	Serum and Glucocorticoid-Inducible Kinase 3
TCFs	T cell factors
Thr	Threonine
TRPV5	Transient Receptor Potential Cation Channel V5
Tyr	Tyrosine
UDP-glucose	Uridine Diphosphate-Glucose
VMA	Vanilyl Mandelic Acid

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7. INTRODUCTION

Discovery and characterization of glycogen synthase kinase-3 (GSK3)

The glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed, highly conserved multifunctional serine/threonine protein kinase found in all eukaryotes, which mediates the addition of phosphate molecules onto serine and threonine amino acid residues.

It is also called Factor A (F_A) for its ability to activate the MgATP-dependent form of the protein phosphatase PP1 called F_C (1-4).

It was first discovered about 32 years ago as one of several protein kinases that phosphorylated and inactivated glycogen synthase (Embi, N. et al., 1980), the enzyme which is involved in converting glucose to glycogen (glycogen biosynthesis).

GSK3 acts as a downstream regulatory switch for various signaling pathways, including cellular responses to WNT, Growth Factors, Insulin, RTK (Receptor Tyrosine Kinases), Hedgehog pathways, and GPCR (G-Protein-Coupled Receptors) and is involved in a numerous signal transduction cascades involving cellular processes, ranging from glycogen metabolism, cell development, gene transcription, protein translation to cytoskeletal organization, cell cycle regulation, proliferation and apoptosis (Forde J.E., et al., 2007). GSK3 dysregulation has been implicated, in numerous diseases such as diabetes, inflammation, cancer, Alzheimer's and bipolar disorder (Doble B.W. et al., 2003). GSK3 negatively regulates insulin-mediated glycogen found in type II diabetics and obese animal models. Thus, its involvement in glucose metabolism increased scientific interest and from then, it has been extensively studied especially in the context of metabolic actions of insulin (Woodgett, J. R. et al., 2001).

The glycogen synthase kinase-3 (GSK-3) was subsequently purified to homogeneity from skeletal muscle (Woodgett, J. R. et al., 1984) and from the molecular cloning study it is confirmed that there were two closely related isoforms, GSK3α (molecular mass B51 kDa) and GSK3β (molecular mass B47 kDa), which are expressed ubiquitously in mammalian tissues (Woodgett, J. R. et al., 1990 and Woodgett, J. R. et al., 1991).

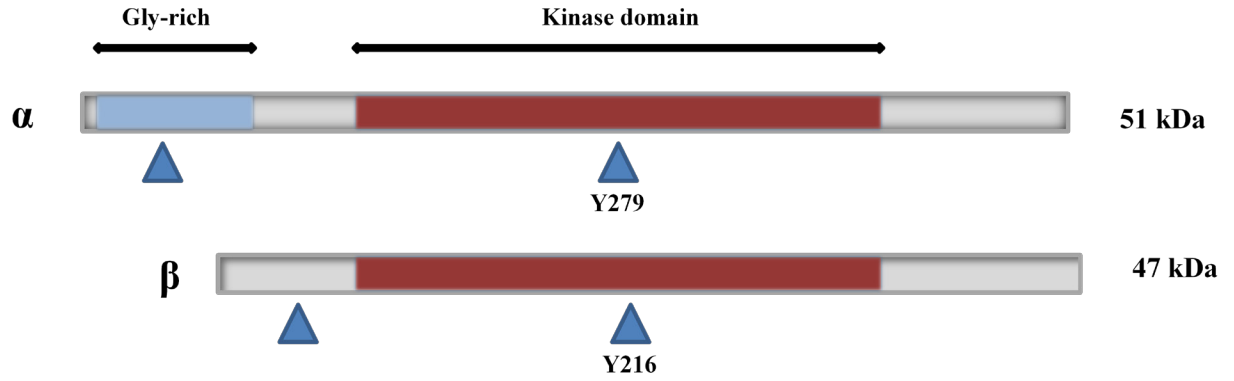

***Figure 7.1**: Schematic representations of GSK-3α and GSK-3β isoforms. Blue arrow-heads indicate sites of serine and tyrosine phosphorylation. A glycine-rich N terminal domain of GSK-3α and a conserved kinase domain from both GSK-3α/β are highlighted*

These isoforms, GSK-3α and GSK-3β are encoded by two distinct genes which are located on chromosomes 19q13.1-2 and 3q13.3-q21. The catalytic domains of GSK3 (of both GSK-3α and GSK-3β isoforms) exhibit a high degree of sequence similarity, but the isoforms diverge in their N- and C-terminal sequences (Ciaraldi T.P., et al 2007; Markou T., et al. 2008). Using chromosomal mapping the exact cytological locations of these isoforms have been identified, i.e. GSK-3α is located at 19q13.2 and GSK-3β maps to 3q13.3 (Shaw P.C., et al. 1998). They exhibit about 98% homology in their kinase domains, but they share only about 36% identity in the last 76 C-terminal amino-acid residues (figure 7.1) (Woodgett J.R., 1990).

Molecular cloning of GSK-3 cDNAs allowed investigations of species representation of GSK-3 genes. A genomic Southem blot of DNA isolated from yeast to humans probed with rat GSK-3 demonstrated that the kinase is ubiquitous throughout the animal kingdom. Indeed, homologues of GSK-3 have been cloned from a number of eucaryotic species (see Table 1.1). GSK-3 proteins share a remarkable degree of identity within the catalytic domain. In vertebrates, the identity over this region is greater than 90 %. In invertebrates, there appears to be only one form of the enzyme which is most closely related to GSK3P. The Drosophila GSK-3 homologue, termed Zeste-white 3shagg~ shares 76 % overall identity with the rat GSK3P, and can be replaced functionally by this clone. GSK-3 family members have also been identified in a variety of plants (Arabidopsis, *Petunin,* alfalfa and *Nicotinna)* (see Table 7.1). The high degree of conservation

between GSK-3 proteins, as well as their ubiquitous representation, indicates a fundamental role of this kinase family in regulaiion of cellular processes

Table7.1 Species representation of GSK-3 homologues

Species	**Name**	**Identity in the catalytic domain to rat GSK3β**
human	GSK-3α, β	98 (Stambolic, V., and J. R. Woodgett., 1994)
rat	GSK-3α, β	100 (Woodgett, J. R. 1991)
rabbit	GSK-3β	100 (Wang, Q. M., et al., 1994)
chicken	GSK-3β	98
Xenopus laevis	Xgs k-3	92 (Pierce, S. B., and D. Kimelman., 1995)
Drosophila melanogaster	Zeste-white 3^{shaggy}	88 (Siegfried, E., et al., 1992)
Caenorhabditis elegans		78
Dictyostelium discoideum	GSK-3α	71 (Harwood, A. J., et al., 1995)
Saccharomyces cerevisiae	MCK1, MDS1, MRK1, ScGSK3	44 -58 (Bianchi, M. W., et al., 1993)
Schizosaccharomyces pombe	Skp1	65 (Plyte, S. E., et al., 1996)
Arabidopsis thaliana	ASK α, γ, ATK1	68-70 (Bianchi, M. W., et al., 1994 and Jonak, C., et al., 1995)
Petunia hybrida	PSK4,6	70 (Decroocq-Ferrant, V., et al., 1995)
alfalfa	MSK1,2,3	65-70 (Pay, A., et al., 1993)
Nicotiana tabaccum	Ntk1	56 (Einzenberger, E., et al., 1995)

Although these isoforms, GSK-3α and GSK-3β are structurally similar, their expression patterns, substrate preferences and cellular functions are different (Woodgett J.R., 2001; Doble and Woodgett J.R., 2003). It has been reported by Hoeflich K.P., et al. 2000 that, mice after carrying homozygous deletions of exon 2 of the GSK-3β isoform suffer from major liver degenerations which was due to extensive hepatocyte apoptosis, leading to death of the embryo at day 16. Thus GSK-3α isoform was unable to compensate for the loss of GSK-3β. From this Hoeflich K.P., et al., concluded that the degenerative liver phenotype develops specifically from the loss of GSK-3β isoform (Doble and Woodgett 2003).

Regulation of gsk3 by insulin and growth factors

GSK-3 is, unlike most other protein kinases, active in the resting state and deactivated by phosphorylation. The most well defined mechanism of GSK-3 regulation is the phosphorylation of Serine 9 (Ser9) in GSK-3β and Ser21 in the GSK-3α isoform. This is in response to hormones such as insulin, endothelial growth factor and platelet growth factor.

It was first suggested in 1978 that the inhibition of GSK3 might underlie the insulin-induced dephosphorylation and thereby activation of glycogen synthase (Cohen, P., et al., 1978). This was confirmed and reported that insulin stimulates the dephosphorylation of glycogen synthase at the sites where it was phosphorylated by GSK3 (Parker, P. J., et al., 1983) and subsequently that insulin acutely inhibits GSK3 (Welsh, G. I. et al., 1993 and Welsh, G. I. et al., 1998). The inhibition of GSK3 by insulin was shown to result from its phosphorylation at an N-terminal serine residue (Ser^{21} in GSK3α and Ser^{9} in GSK3β) and that this is catalysed by protein kinase B (PKB; also called Akt) (Cross, D. A., et al., 1995).

Thus, insulin stimulates glycogen synthesis by inhibiting GSK-3 and dephosphorylation and thereby activation of glycogen synthase (figure 7.2b). PKB/Akt (PI3K pathway), some isoforms of protein kinase C (PKC), PKA, p90RSK and p70S6 kinase may all be involved in phosphorylation of GSK-3, thus inactivating it (Cohen, P., et al., 1997 and Cohen, P. et al., 1999).

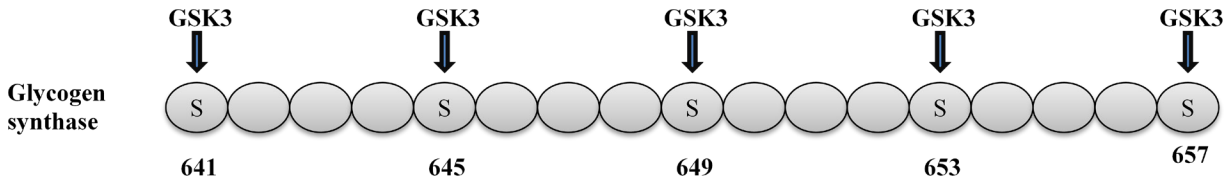

Figure 7.2a

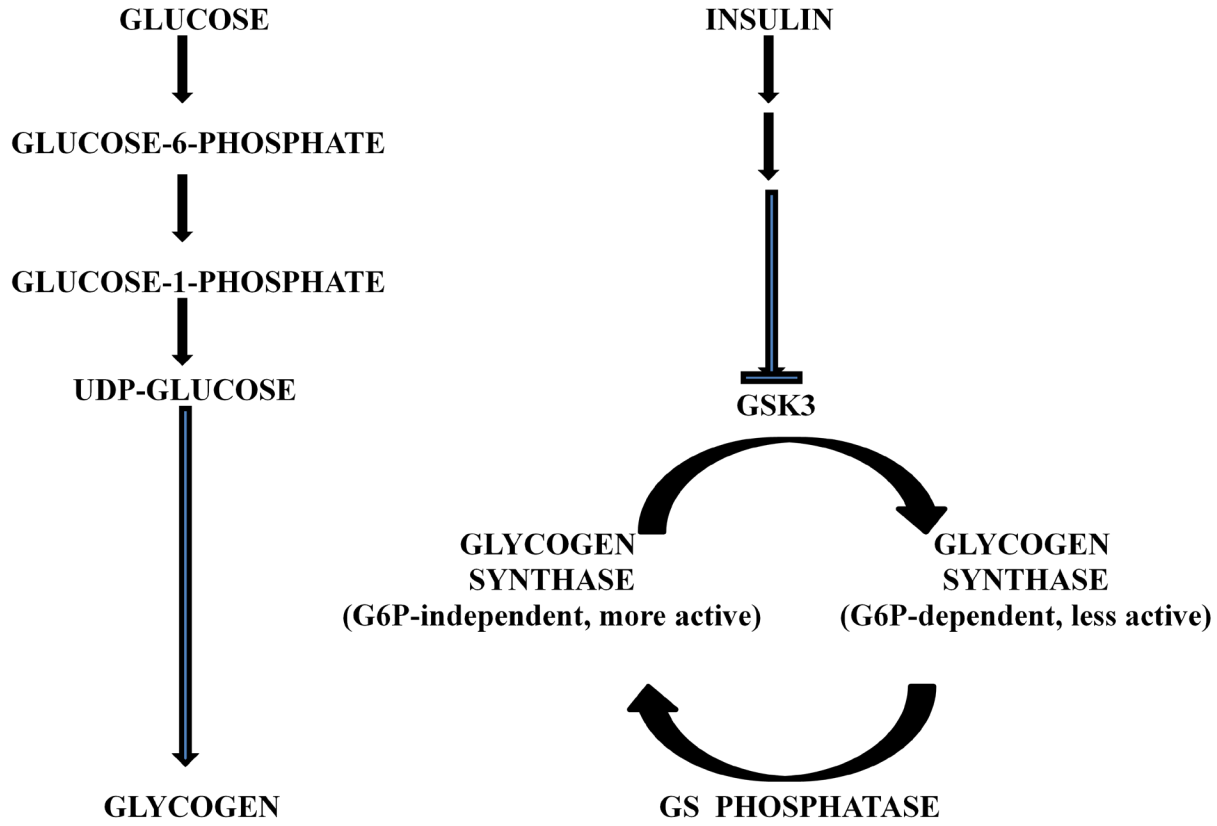

Figure 7.2b

Figure 7.2 Regulation of glycogen metabolism by glycogen synthase kinase 3 (GSK3).

(a) Casein kinase 2 (CK2) phosphorylates a priming Ser residue in the C terminus of glycogen synthase (Ser657 in the muscle isoform of Homo sapiens) to initiate a relay of GSK3-catalysed phosphorylations, as described in the text.

(b) Glycogen synthase activity is regulated by a phosphorylation–dephosphorylation cycle. In the preprandial state, plasma insulin concentrations are low and GSK3 is active. GSK3 phosphorylates glycogen synthase and converts it to the less-active (glucose-6-phosphate (G6P) dependent) form, thus inhibiting glycogen synthesis. When plasma insulin concentrations rise after feeding, insulin promotes phosphorylation of GSK3 and inhibits GSK3 activity. Dephosphorylation of glycogen synthase by protein phosphatases (GS phosphatase) subsequently increases the activity of glycogen synthase and promotes glycogen synthesis.

The eukaryotic protein synthesis initiation factor 2B (eIF2B), play important role in synthesis of eukaryotic protein. GSK3 catalyses the phosphorylation and inhibition of eIF2B with subsequent inhibition of protein synthesis. Thus insulin by inhibiting GSK3, stimulates the dephosphorylation and activation of eIF2B (Welsh, G. I. et al., 1993 and Welsh, G. I., et al., 1998). Insulin also suppose to stimulates protein phosphatase(s) that dephosphorylate(s) and thereby activates eIF2B, contributing to increased rate of protein synthesis.

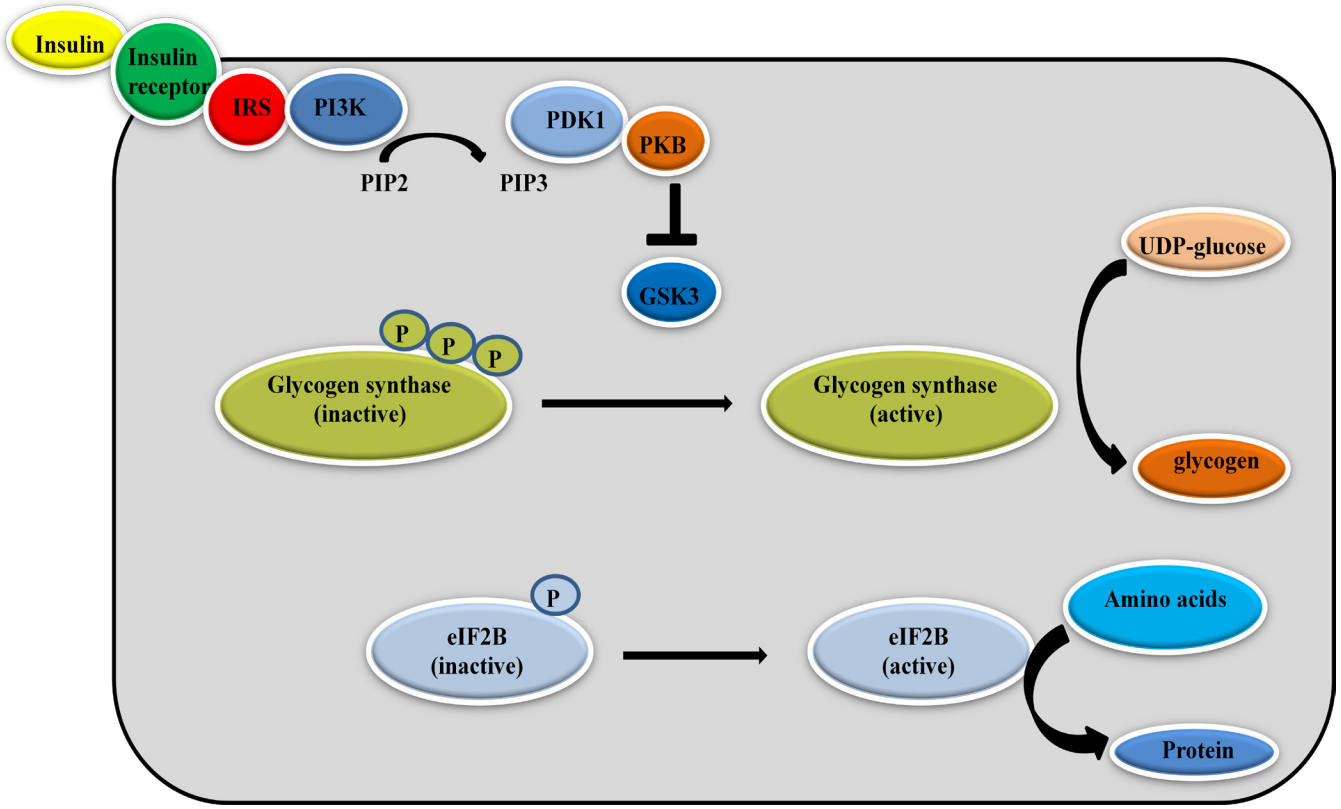

Figure 7.3 Insulin stimulates glycogen and protein synthesis via the inhibition of GSK3

The binding of insulin (yellow) to its receptor (green) in liver, adipose tissue and muscle, triggers the phosphorylation of IRS proteins (red) and their recruitment to the plasma membrane. The IRS proteins in turn become tyrosine-phosphorylated, recruiting PI 3-kinase (PI3K, purple) to the membrane, where it produces the second messenger PtdIns(3,4,5)P3 (PIP3). This molecule binds to PDK1 (dark blue) and PKB (pink), co-localizing them at the plasma membrane and allowing the former to activate the latter. Active PKB th inhibits GSK3 (dull blue) by phosphorylating Ser^{21} (GSK3α) and Ser^{9} (GSK3β). As a result, the residues on glycogen synthase (pale yellow) and the e-subunit of eukaryotic initiation factor 2B (eIF2B, lilac) that are targeted by GSK3 undergo a partial dephosphorylation, thereby increasing their activity and hence stimulating glycogen (orange) and protein (blue, bottom right) synthesis.

In addition, a downstream kinase of the classical mitogen-activated protein kinase (MAPK) cascade, known as MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK), phosphorylates GSK3 at Ser^{9}/Ser^{21} and thereby inhibit it. Thus growth factors and other signals activating the MAPK pathway, can also inhibits GSK3.

Thus in brief,

1) Insulin inhibits GSK3 via the PI 3-kinase-dependent/PKB pathway.
2) Signals such as tumour-promoting phorbol esters inhibit GSK3 via the classical MAPK cascade.
3) Other growth factors, such as epidermal growth factor (EGF), can inhibit GSK3 by both pathways (figure 7.3) (Shaw, M. et al., 1999).

The protein kinase such as p70 ribosomal S6 kinase-1 (S6K1) phosphorylates GSK3 at Ser^{9} / Ser^{21} and thereby inhibits it (Cross, D. A., et al., 1994). The amino acids thus activates S6K1 via mTOR, hence immunosuppressant drug such as rapamycin, which inactivates mammalian target of rapamycin (mTOR), a protein kinase required for the activation of S6K1, suppresses the inhibition of GSK3 induced by amino acids(figure 7.4). The phosphorylation of GSK3 at Ser^{9} / Ser^{21} also suppose to be induced by incubating cells with cAMP-elevating agents or cell-permeant cAMP analogues (Fang, X., et al., 2000 and Li, M., et al 2000).

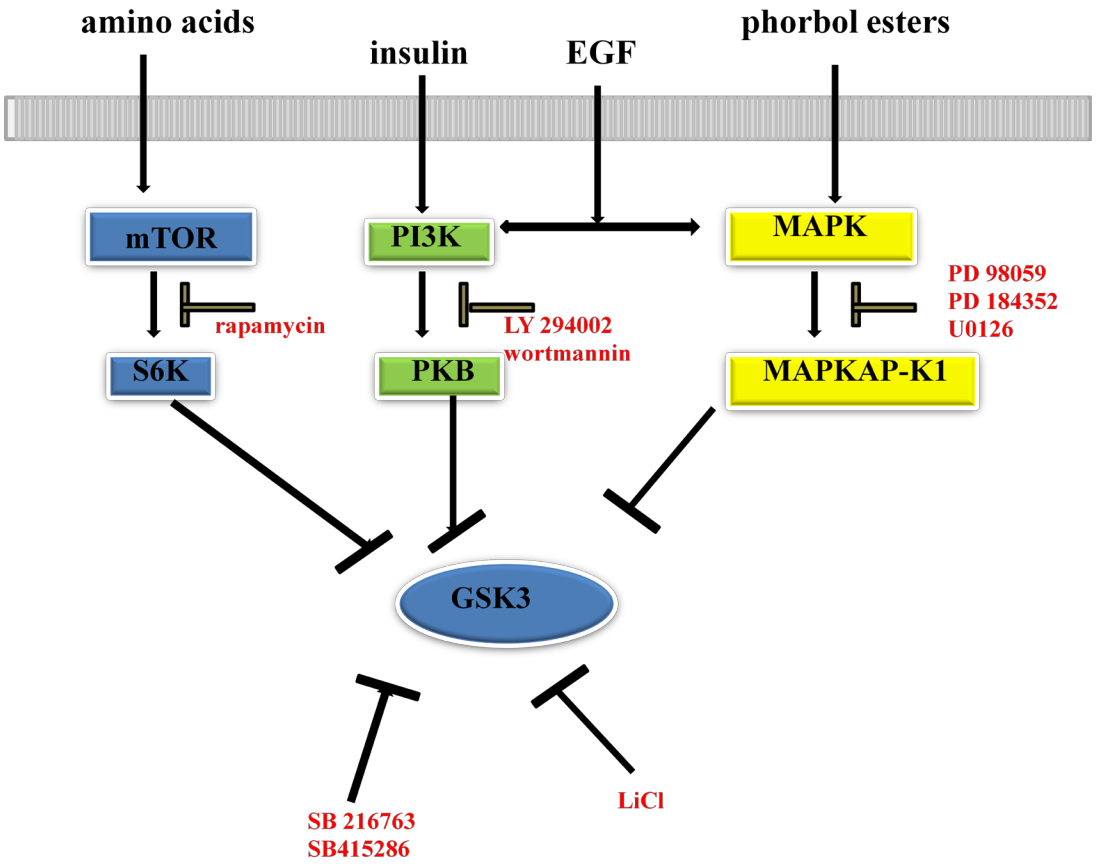

Figure 7.4 Several signalling pathways inhibit GSK3

GSK3 can be inhibited via several signal-transduction pathways, namely the PI 3-kinase - PKB pathway (green), the classical MAPK (yellow) cascade, where MAPKAP-K1 (also called RSK) inhibits GSK3, and the mTOR pathway, where p70 S6 kinase (S6K, lilac) inhibits GSK3 (purple). Insulin exerts its effects via the PI3K-PKB pathway, tumour-promoting phorbol esters via the classical MAPK cascade and amino acids via mTOR. However, some signals, such as EGF, can use more than one pathway to inhibit GSK3. The cell-permeant inhibitors indicated in the figure block one pathway selectively and have been critical in establishing the roles of these pathways in the regulation of GSK3 activity. Li+ ions, which inhibit GSK3 relatively selectively in vitro, have been useful in identifying potential roles for GSK3 in cells. More potent cell-permeant inhibitors of GSK3, such as SB 216763 and SB 415286, have been developed recently.

GSK3 as a key component of the W_{NT} signaling Pathway

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules. They were initially characterized for their roles in regulating embryonic development and tumorigenesis. Studies in the past decade have implicated Wnt signaling in a diverse range of physiological and pathophysiological processes, including bone development, angiogenesis, vasculature remodeling, myogenesis, adipogenesis, stem cell renewal and differentiation, and lipid and glucose metabolism.

The Wnt proteins are defined based on sequence homology to the original Wnt members: mouse WNT1 (originally called int-1) and *Drosophila melanogaster* Wingless (Wg). Mutations in Wnt genes exhibit a variety of intrinsing phenotypes in the mouse, *Caenorhabditis elegans*, and *Drosophila*, indicating that Wnt proteins function in diverse developmental processes. Numerous studies have established a canonical signaling pathway that leads to β-catenin stabilization, via Wnt signalling (Fig. 1.5). In the absence of Wnt, a number of proteins, including AXIN, adenomatous polyposis coli (APC), GSK3, casein kinase Iα (CKIα)), and β-catenin, form a complex (referred to as the β-catenin destruction complex), in which β-catenin is phosphorylated by CKIα) and GSK3. This phosphorylation event targets β-catenin for proteasome-mediated proteolytic degradation. When Wnt proteins bind cell-surface receptors frizzled (FZD) and lipoprotein receptor-related protein (LRP) 5/6, GSK3-dependent β-catenin phosphorylation is suppressed through a mechanism that requires the scaffold protein dishevelled (DVL), resulting in stabilization of β-catenin (Figure 7.5). Stabilized β-catenin enters the nucleus and interacts with transcriptional regulators, including lymphoid enhancing factor-1 (LEF1) and T cell factors (TCFs), to activate gene transcription. Mammals express 19 Wnt genes as well as many Wnt antagonists.

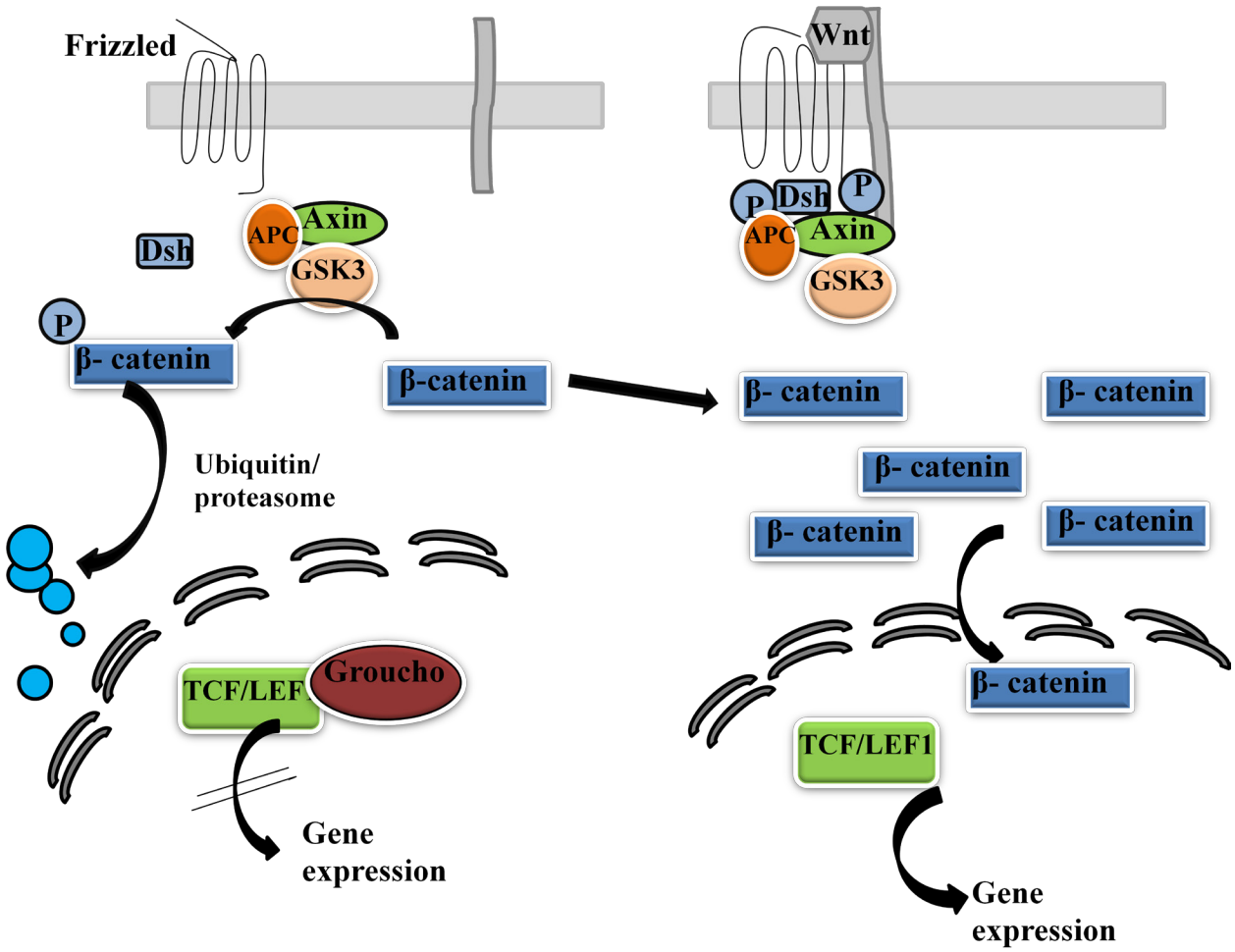

Figure 7.5 Schematic representation of simplified canonical Wnt signaling pathways

There are generally two pools of β-catenin in cells. One pool is associated with cadherins, whereas the other is degraded in the absence of Wnt by the β-catenin destruction complex. Wnt binds two cell surface receptors (LRP5/6 and FZD) and leads to phosphorylation at least of Thr-1479 by CKIγ, Ser-1490 by GSK3, and Thr-1493 by yet to be identified CKs on LRP6. These phosphorylation events are required for AXIN recruitment and β-catenin stabilization. Stabilized β-catenin enters the nucleus and activates gene transcription activation. Two of the Wnt antagonists, Dickkopf (DKK) and soluble frizzled-related protein (sFRP), are also shown.

Substrates of GSK3: general points

GSK3 preferentially phosphorylates substrates on Ser/Thr residues in a 'relay' fashion. A 'priming' phosphorylation on a Ser/Thr residue is catalysed by a protein kinase distinct from GSK3. This increases the rate of phosphorylation by GSK3 at a Ser/Thr residue -4 to the priming phosphorylation (that is, the fourth residue lying N-terminal to the priming phosphorylation, for example, Ser-Xaa-Xaa-Xaa-Ser(P)). The first GSK3-mediated phosphorylation may serve to prime a second phosphorylation at any Ser/Thr residue lying -4 relative to this. The extent of such relays may be limited and consist of only two residues as in the transcription factor c-Jun (residues 239 and 243 for H. sapiens c-Jun, the former being the GSK3 site and the latter being the priming site;(Cohen and Goedert, 2004). In contrast, a relay system of up to five Ser residues between residues 641 and 657 (the latter representing the priming site; Figure 7.2a) potentially operates in the N terminus of H. sapiens muscle glycogen synthase. Individual priming phosphorylations are catalysed by one of a number of different protein kinases, including casein kinases (for example, casein kinase 2 catalyses the priming phosphorylation in glycogen synthase), PKA, cyclin-dependent protein kinases, MSKs and dual specificity tyrosine phosphorylated and regulated kinases (Cohen and Goedert, 2004). The crystal structure of GSK3b accounts for the requirement for a priming phosphorylation and for the inhibitory effect of phosphorylation of Ser9 (Dajani et al., 2001; Frame et al., 2001). Thus, GSK3b (Arg96) interacts with the priming phosphate in a substrate, orientating it for phosphorylation at the -4 position. Phosphorylation of GSK3b(Ser9) causes an intramolecular interaction with Arg96, and the N terminus of the enzyme then serves as a pseudosubstrate to block both the binding site for the priming phosphate and access of any substrate to the catalytic pocket.

Criteria needed to establish that a protein is a Physiological substrate for gsk3

Following are the criteria that need to be met before a protein can truly be regarded as a physiological substrate for GSK3 (figure 7.6):

1. The substrate should be phosphorylated by GSK3 *in vitro* at the same residue(s) as that occurred *in vivo.*

2. Phosphorylation should get abolished by mutagenesis of this (these) site(s) to (a) non-phosphorylatable residue(s).

3. The signals known to inhibit GSK3 should decrease the phosphorylation of the endogenous protein in cells.

4. Upon incubation of cells with cell permeant inhibitors of GSK3, dephosphorylation of endogenous proteins at the relevant site(s) should occur.

5. Phosphorylation of the residues targeted by GSK3 should affect the function of the protein in a manner consistent with physiological effects of the agonist(s) that regulate(s) GSK3 activity.

6. Phosphorylation of the protein at the GSK3 site(s) should not occur in cells that carry targeted disruptions of the genes encoding GSK3, and be restored when GSK3 is replaced.

7. Dephosphorylation of the protein at the GSK3 site(s) should not occur in response to the appropriate signal in cells that do not express one of the protein kinases that lies upstream of GSK3; for example, in PDK1−/− embryonic stem cells, PKB, MAPKAP -K1 and p70 S6 kinase are not activated, and GSK3 is not inhibited, in response to IGF-1 or tumourpromoting phorbol esters (Williams, M. R., et al., 2000).

Table 7.2 Proteins reported to be substrates of GSK3 (Sheelagh Frame and Phillip Cohen, 2001)

Putative substrate	**Biological process**	**Function proposed for phosphorylation**
Glycogen synthase	Glycogen synthesis	Inactivates
eIF2B	Protein synthesis	Inactivates
ATP citrate lyase	Fatty acid synthesis	None
Axin	Wnt signalling	Stabilizes protein and recruits b-catenin
b-Catenin	Wnt signalling/adhesion	Targets for degradation
APC	Wnt signalling	Facilitates binding of b-catenin to APC and decreases binding of APC to microtubules
MUC1/DF3	Glycoprotein	Decreases affinity of MUC1 for b-catenin
Cyclin D1	Cell division cycle	Nuclear export and targets for degradation
Jun	Transcription factor	Inhibits DNA binding and transactivation
Myc	Transcription factor	Targets for degradation
NFATc	Transcription factor	Promotes nuclear export, inhibits DNA binding
C/EBPa	Transcription factor	None
C/EBPb	Transcription factor	Inhibits DNA binding and transactivation
CREB	Transcription factor	Enhances transcriptional activity
MITF	Transcription factor	Enhances binding to the tyrosinase promoter
HSF-1	Transcription factor	Inhibits DNA binding and transactivation
Tau	Microtubule-binding protein	Inhibits binding to microtubules
MAP1B	Microtubule-binding protein	Maintains microtubular instability
Presenilin-1	Transmembrane protein	Disposal of excess C-terminal fragments of presenilin, generated by endoproteolytic processing
Insulin receptor substrate 1	Insulin signalling	Inhibits insulin receptor signalling

Table 7.2 shows how well these criteria have been met for proposed proteins to act as GSK3 substrates, few of them met first criterion. There is sufficient evidence stating that GSK3 which phosphorylate particular proteins in cells is based on the finding that Li^+ affect the function of the protein in a manner consistent with dephosphorylation having occurred, but this is not applicable to all cases. On the contrary, Li^+ being a relatively specific inhibitor of GSK3, it can also inhibit a few other protein kinases (Davies, S. P., et al., 2000) too. It is however important to shown that the effects of the drug disappear when a drug resistant mutant is overexpressed or replaces the endogenous wild-type enzyme (Brown, E. J.,et al., 1995, and Eyers, P. A., et al., 1998). But none of the substrates meet the sixth or seventh criteria.

Proposed substrates of GSK3

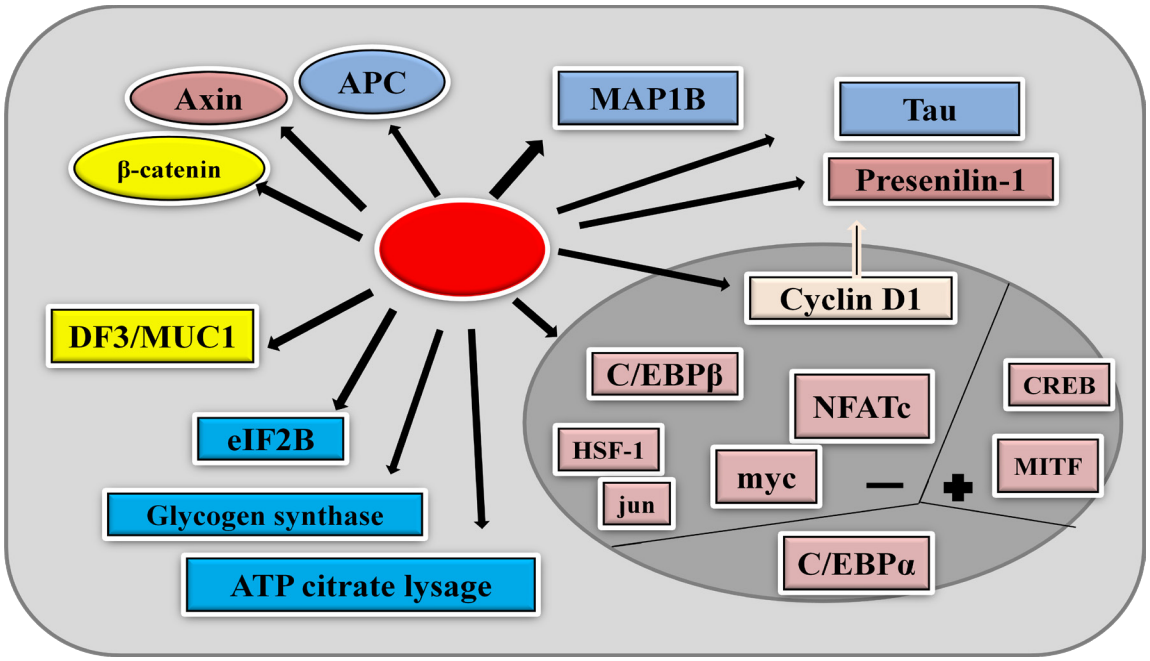

Figure 7.6 Proposed substrates of GSK3

Putative substrates are colour-coded according to their proposed function in the cell ; transcription factors (mauve), enzymes that regulate metabolism (blue), proteins bound to microtubules (turquoise), scaffold proteins (orange), or components of the cell division cycle machinery (pink) or involved in cell adhesion (yellow). The evidence that these proteins are substrates of GSK3 is summarized in Table 7.2. Transcription factors are subdivided into those that are inhibited (®), activated (-) or unaffected by the phosphorylation by GSK3. A complex consisting of GSK3, Axin, APC and b-catenin (depicted by ellipses) is critical for regulating embryogenesis. The phosphorylation of cyclin D1 by GSK3, which is thought to occur in the nucleus of the cell, promotes its nuclear export and degradation. Presenilin-1 is reported to act as a scaffold, which facilitates the phosphorylation of tau by GSK3.

Preclinical efficiency of GSK-3 inhibitors

It was nearly 50 years after the discovery of the unique properties of lithium in manic-depression illness (bipolar affective disorder) that GSK-3 was identified as one of its main targets (Klein, P.S., et al., 1996, Phiel, C.J. and Klein, P.S., 2001, and Jope, R.S., 2003). Since then, lithium has been used widely as a pharmacological inhibitor of GSK-3, despite the millimolar concentrations that are required to affect GSK-3 in living cells. The definitive mood-stabilizing properties of lithium, the insulin-mimetic properties of GSK-3 inhibition and the GSK-3-dependent abnormal phosphorylation of Tau and production of amyloid-b in Alzheimer's disease have stimulated the search for potent, selective inhibitors of GSK-3. At present≥30 inhibitors have been described, some with IC_{50} values in the nanomolar range. These GSK-3 inhibitors have been found beneficial in various diseases and health conditions (Table 7.3).

Table 7.3 GSK3 inhibitors (Bradley W.Doble and James R. Woodgett, 2003)

Drug type	**Specific drug examples**	**Mode of action**	**Clinical effects/effects in cell culture**
Aloisines	Aloisine A, Aloisine B	ATP-competitive	Inhibits cell proliferation
Beryllium	N/A	Competes for Mg2+ and ATP	No data
Bivalent zinc	N/A	Undetermined; does not compete for substrate	Insulin-mimetic
Hymenialdisine	Dibromo-hymenialdisine	ATP-competitive	Suppresses inflammation; inhibits tau phosphorylation
Indirubins	5,5¢-dibromo-indirubin	ATP-competitive	Anti-mitotic; anti-tumoural; inhibits tau phosphorylation
Lithium	N/A	Competes for Mg2+	Mood stabilization; prevents polyglutamine toxicity in Huntington's disease
Maleimides	Ro 31-8220, SB-216763, SB-415286	ATP-competitive	Insulin-mimetic; prevention of death in culture neuronal
Muscarinic agonists	AF102B, AF150	Unclear	Inhibits tau phosphorylation

The role of GSK3ß in cardiovascular system

Insulin signaling includes stimulation of phosphoinositide (PI) 3 kinase with subsequent activation of protein kinase B (PKB/Akt) and the serum and glucocorticoid inducible kinase (SGK) isoforms [12, 16]. PKB/SGK regulate a wide variety of target proteins including the glycogen synthase kinase GSK, which is phosphorylated and thus inhibited by PKB (Cross HR,et al., 1995 and Shaw M, et al., 1997) and SGK .

In order to elucidate the physiological impact of PKB/SGK-dependent regulation of GSK3, gene targeted mice were generated, in which the serine within the PKB phosphorylation site was replaced by alanine ($GSK3\alpha^{21A/21A}$, $GSK3ß^{9A/9A}$) thus yielding resistance of GSK3 to inactivation by PKB/SGK (McManus EJ, et al., 2005). Expectedly, the knockin mice carrying these mutations ($gsk3^{KI}$) are resistant to the effect of insulin on muscle glycogen synthase (McManus EJ, et al., 2005). Furthermore, the mice are resistant to isoproterenol induced heart failure (Webb IG, et al., 2010). Recent physiological evaluation of those mice revealed an increase in blood pressure (Boini KM, et al., 2008). The enhanced blood pressure could be lowered by the α-adrenergic antagonist prazosine (Boini KM, et al., 2009), pointing to a role of the sympathetic nerve system. It was also shown, that gsk3ki mice exibit a hyperactive behaviour (Ackermann TF, et al., 2010).

The present study explored whether the differences of blood pressure between $gsk3^{KI}$ mice and wild type mice ($gsk3^{WT}$) were dependent on adrenergic stimulation and paralleled by differences in catecholamine release. To this end, blood pressure was determined prior to and following administration of the α-adrenergic antagonist prazosine (Kobinger W., 1984) or the ganglion blocker hexamethonium (Randall WC,et al., 1998), as well as blood pressure and cardiac rate prior to and following administration of the ß-blocker atenolol (Ram CV., 2010). Moreover, catecholamine plasma levels as well as urinary excretion of the catecholamine metabolite vanilylmandelic acid (Goldstein DS, et al., 2003) were determined in $gsk3^{KI}$ and in $gsk3^{WT}$ mice.

The role of GSK3ß in renal tubular calcium and phosphate transport

The kidneys play a central role in the regulation of mineral homeostasis by mediating excretion or reabsorption, respectively, of phosphate, calcium, and magnesium. Phosphate

reabsorption occurs in the proximal tubule and is mediated by at least three distinct sodium-dependent phosphate cotransporters, namely NaPi-IIa (SLC34A1), NaPi-IIc (SLC34A3), and Pit-2 (SLC20A2), located in the apical brush border membrane (Biber J, et al., 2009; Murer H,et al., 2004 and Villa-Bellosta R, et al., 2009).

Renal phosphate reabsorption is regulated by various factors including dietary phosphate intake, acid-base status, and various hormones, such as parathyroid hormone (PTH), 1,25-(OH)2 vitamin D3, FGF-23, insulin, and IGF1(Allon M., 1992; DeFronzo RA, et al., 1976; Feld S, et al., 1996; Jehle AW, et al., 1998; Murer H, et al., 2000; Nowik M, et al., 2008 and Picard N, et al., 2010). Active calcium reabsorption is mediated by the TRPV5 calcium channel expressed in the luminal membrane of the distal convoluted tubule and connecting tubule (Boros S,et al., 2009; Hoenderop JG, et al., 20005 and Woudenberg-Vrenken TE, et al., 2009). The expression and activity of TRPV5 is regulated by several factors similarly regulating renal phosphate transport, such as dietary calcium intake, PTH, klotho, acid-base status, and 1,25-$(OH)_2$ vitamin D3 (Boros S,et al., 2009; Hoenderop JG, et al., 20005 and Woudenberg-Vrenken TE, et al., 2009). The role of insulin and IGF1 has remained controversial (DeFronzo RA, et al., 1975 and Hoskins B, et al., 1984).

The intracellular signaling cascades mediating the effects of these hormones on renal phosphate transporters are still incompletely understood. Signaling of the phosphaturic hormone PTH involves the protein kinases A and C and ERK1/2, (Bacic D, et al., 2003) leading to the internalization and degradation of the NaPi-IIa cotransporter in the mouse and rat kidney (Bacic D, et al., 2006). Signaling mediating the stimulating effect of insulin and IGF1 on renal phosphate reabsorption (Allon M., 1992; DeFronzo RA, et al., 1976 and Feld S, et al., 1996) has remained ill-defined. Signaling of insulin includes stimulation of the PI3 kinase pathway with subsequent activation of protein kinase B (PKB/Akt) and the serum- and glucocorticoid-inducible kinase (SGK) isoforms (Hawkins PT, et al., 2006 and Lang F, et al., 2006). Both, PKB (Cross HR, et al., 1995 and Shaw M, et al., 1997) and SGK (Sakoda H, et al., 2003 and Wyatt AW, et al., 2006) isoforms are known to phosphorylate and thus to inhibit the glycogen synthase kinase GSK3. However, nothing is known about the regulation of epithelial phosphate and Ca^{2+} transport by GSK3.

This study aimed to define the role of PKB/SGK-dependent regulation of GSK3 in the control of renal tubular calcium and phosphate transport. To this end, renal mineral excretion was analyzed in gene-targeted *gsk3*^{KI} mice. In those mice (*gsk3*^{KI}), GSKα and GSK3β are resistant against inactivation by PKB/SGK (McManus EJ, et al., 2005). As shown before, *gsk3*^{KI} mice are resistant to the effect of insulin on muscle glycogen synthase (McManus EJ, et al., 2005).

8. AIMS OF THE STUDY

The aims of the present study were to determine:

1. Whether, the over expression of GSK3 (as in GSK3 ^{KI} mouse model) is the underlying cause of elevated blood pressure. If so, then whether this elevated blood pressure is dependent on adrenergic stimulation and paralleled by enhanced catecholamine release.

2. Whether, PKB/SGK phosphorylation of GSK3 increases phosphate transporter activity and reduces renal calcium and phosphate loss. Thus thereby, whether overexpression of GSK3 and the phosphate transporter NaPi-IIa in *Xenopus* oocytes decrease electrogenic phosphate transport and decrease renal tubular calcium and phosphorus reabsorption. Thus to show novel role of PI3/PKB/Akt/SGK-resistant GSK3 in the control of renal tubular calcium and phosphate transport.

9. MATERIALS AND METHODS

9.1 Materials

9.1.1 Chemicals

1,25-(OH)2D3 Kit (IDS, Boldon, UK)
Agarose-Gel-Elektrophorese-Chamber (Biorad, München, Germany)
Alkaline Phosphatase (Promega Gmbh., Mannheim, Germany)
Aqua Ad Injectabili (Ampuwa, Niefern, Germany)
Atenolol (Sigma-Aldrich, Hannover, Germany)
Beta-Mercaptoethanol (Sigma-Aldrich; Hannover, Germany)
Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA)
Catecholamine ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany)
Creatinine Kit Jaffe's Reaction (Sigma, St. Louis, MO)
Diethylether (Carl Roth, Karlsruhe, Germany)
DRAQ-5 Dye (1:1000, Biostatus, Leicestershire, UK).
Goat anti-Rabbit or Donkey anti-Mouse Antibodies (1:5000)
GSK3 Antibody (1:100, Cell Signaling, USA)
GSK3β cDNA(Deutsches Ressourcenzentrum fur Genomforschung, Berlin, Germany)
Haematoxylin and Eosin (Carl Roth, Karlsruhe, Germany)
Hexamethonium hzdrochloride (Sigma-Aldrich, Hannover, Germany)
Horseradish Peroxidase (GE Healthcare, Formerly Amersham Biosciences, US)
Hydrochloric acid (6 M HCl)
Isoflurane (Essex, Munich, Germany)
Mannitol (VWR International, BVBA, Belgium)
Milk powder 5% (Carl Roth, Karlsruhe, Germany)
Mouse Monoclonal anti-β-actin Antibody (42 kD; Sigma; 1:5000)
Nitrogen Liquide (Linde, Wiesbaden, Germany)
Normal Goat Serum 5%
Para formaldehyde (Carl Roth, Karlsruhe, Germany)
PBS tablets (Invitrogen corporation for Invitrolaboratory, UK)
Parafine (Merck, Darmstadt, Germany)
Parathormone ELISA Kit (Immunotopics, San Clemante, CA)
Phosphate Diagnostic Kit (Roche Diagnostics, Mannheim, Germany)
Polyacrylamide Gels 8% (Carl Roth, Karlsruhe, Germany)
Polyvinylidene Fluoride Membranes (Immobilon-P; Millipore, Bedford, MA)
Potassium chloride 3M (3MKCl) (Carl Roth, Karlsruhe, Germany)
Prazosine (Sigma-Aldrich, Hannover, Germany)
ProLong Gold Antifade Reagent (Invitrogen, UK)
Rabbit Polyclonal anti-NaPi-IIa (1:6000),
Rabbit polyclonal anti-NaPi-IIc (1:10,000)
Rabbit polyclonal anti-Pit-2 (1:3000)
Secondary FITC Goat Anti-Rabbit Antibody (1:1000, Invitrogen, UK).

Silicon Spray (Dr. Schumacher GmbH, Malsfed, Germany)
Sterilium (BODE, Chemie, GmbH, Hamburg, Germany)
Triton X-100 (Roche Diagnostics, Mannheim, Germany)
Ttrisodium PFA (6 mM)
Tween 20 (Böhringer Ingelheim, Manheim, Germany)
Diets (Altromin, Lage, Germany)
Low-Phosphate Diet C1047 [0.24% Na^+, 0.71% K^+, 0.95% $Ca2^+$, 131mg/kg phosphorus]
Standard Diet C1310/1314 [0.24% Na^+, 0.71% K^+, 0.95% $Ca2^+$]
Control Diet C1000 [0.24% Na^+, 0.71% K^+, 0.95% $Ca2^+$, 7523 mg/kg phosphorus]

9.1.2 Solutions

Phosphate Buffered Saline (PAA Laboratories GmbH., Germany)
RLT-Buffer (Qiagen, Basel, Switzerland)
Trisbuffer (pH 8.5) (Sigma-Aldrich, Hannover, Germany)

Phosphate Buffered Saline

PBS was prepared from ready soluble PBS pellets (PAA laboratories) according to manufactureres instruction.

4% Paraformaldehyde

4% PFA was prepared by dissolving PFA powder (Carl Roth, Karlsruhe, Germany) at 60^°C in PBS overnight. PFA was aliquted and stored at -20°C until use without refreezing.

9.1.3 Instruments/Equipments

Non-Invasive Blood Pressure System (NIBP) (IITC life sciences, Los Angeles, United States of America)
Power Labs Dual Bio Amps (ML135) (ADInstruments GmbH., Germany)
Power Labs Dual Bio Cable (Dual Bio to 5 Alligator, 1 m) (ADInstruments GmbH., Germany)
Small Animal Anesthesia Machine (Isoflurane Vaporizer) (Rothacher medical GmbH, Bern Switzerland)
Metabolic Cages (Techniplast, Hohenpeissenberg, Germany)
High Pressure Liquid Chromatography (HPLC) (Jasco Labor-u. Datentechnik GmbH., Germany)
Microtome (Thermo Scientific)
Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany)
Flame Photometry (ELEX 6361; Eppendorf)
Dri-Chem Clinical Chemistry Analyzer (FUJI FDC 3500i; Sysmex, Norsted, Germany)
Small Animal Computed Tomography (MicroCAT-II, Siemens Preclinical Solutions)
qRT-PCR - ABI PRISM 7700 Sequence Detection System (Applied Biosystems)
DIANA III-Chemiluminescence Detection System (Raytest, Straubenhardt, Germany)

9.1.4 Software

AD ECG Analysis Module (ADInstruments GmbH, Germany)
Advanced Image Data Analyzer AIDA, Raytest
Blood Pressure Computerized Data Acquisition Software (PowerLab 400 and Chart 4, Colorado Springs, Colorado Springs, USA)
Data Link Version 1.0.0 (Herbert &Scheneider Software & CAM, Siglingen, Germany)
GraphPad Instat Version 3.00 (GraphPad Software Inc., San Diego, USA)
Image J Software
Inveon Research Workplace software (Siemens Preclinical Solutions)
Microsoft Office 2007-Excel, Word, PowerPoint (Microsoft GmbH., Remscheid,Germany)
Microsoft Windows 95 (Microsoft GmbH., Remscheid, Germany)
Sigma Plot Version 7.0 (Systat Software Inc., Erkrath, Germany)
Zeiss Axiovision Software (Zeiss AG, Oberkochen, Germany)

9. MATERIALS AND METHODS

9.2 Methods

GSK3 and Catecholamine

All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities.

Mice were generated, in which the codon encoding Ser9 of GSK3ß gene was changed to encode nonphosphorylatable alanine (GSK3ß$^{9A/9A}$), and simultaneously the codon encoding Ser21 of GSK3α was changed to encode the nonphosphorylatable GSK3α$^{21A/21A}$ thus yielding the GSK3α/β$^{21A/21A/9A/9A}$ double knockin mouse (*gsk3*^{KI}) as described previously (McManus EJ et al., 2005). The mice were compared to corresponding wild type mice (*gsk3*^{WT}) at 4 months of age.

Mice were fed a control diet and had free access to tap drinking water. Where indicated, animals were treated with intraperitoneal injection of α-adrenoreceptor antagonist prazosine (1 µg/g b.w.), ganglion blocker hexamethonium (40 µg/g b.w.) or β-adrenergic receptor antagonist atenolol (5 µg/g b.w.). Drugs were administered at intervals of more than 48 hrs during the afternoon.

9.2.1 ECG recordings

Mice were sedated by isoflurane inhalation [Janssen et al., 2004]. Induction of isoflurane anaesthesia was facilitated by 5% isoflurane, which was maintained by 1-1.5% isoflurane. ECG recording were obtained by Bio Cable- (Dual Bio to 5 Alligator, 1 m with built-in Dual Bio Amps (ML135) at a sampling rate of 1 kHz, high Pass 10kHz, low pass 500 Hz) connected to PowerLabs. For electrical conduct, AgCl electrodes were bathed in 1M KCl solution on a 24 well plate suitbale to the position of the paws. After induction of anaesthesia, the mouse was positioned on the plate and the extremities bathed in the KCl solution.

The recordings were analysed by AD ECG Analysis Module (Adinstruments company). After baseline readings, atenolol (5 µg/g b.w.) was administered and heart rate was monitored for 10 minutes.

Recording of ECG in two derivations: I (right front paw – left front paw) and II (right front paw – left leg).

9.2.2 Blood pressure determinations

Systolic arterial blood pressure was determined by the tail-cuff method (IITC life sciences, Los Angeles, United States of America). As reviewed earlier (Meneton P., et al., 2000), the tail cuff approach to determine arterial blood pressure requires certain precautions to reduce the stress of the animals. Animals are placed in a clean plastic animal holder (IITC life sciences, Los Angeles, United States of America). The tail is positioned inside a tail cuff with a **photoelectric sensor** use light and photo sensors for pulse detection. The ambient temperature is kept constant at 29 °C. The animals were accustomed to the tail cuff procedure by simulating the measurements without recording of blood pressure. Mice were accustomed to the tail cuff procedure for 1 week. Provided precautions were taken to reduce the stress of the animals, including appropriate training of the mice over multiple days, prewarming to an ambient temperatu re of 29°C, measurement in a quiet, semi darkened and clean environment, and performance of the measurements by one person and during a defined day time, when blood pressure is stable (between 1-3 PM). Measurements were taken during 10 mins of repeated measurement of tail cuff systolic blood pressure. Blood pressure was then calculated as mean of three days measurements after sufficient training of the animals.

In a second study group blood pressure was measured by tail cuff method during isoflurane anaesthesia (1.5% isoflurane). Blood pressure was measured after induction of anaesthesia for 10 minutes followed by intraperitoneal injection of α-adrenoreceptor antagonist prazosine (1 µg/g b.w.), ganglion blocker hexamethonium (40 µg/g b.w.) or β-adrenergic receptor antagonist atenolol (5 µg/g b.w.). Drugs were administered at intervals of more than 48 hrs during the afternoon. All recordings and data analysis were done using a computerized data acquisition system and software (Power Lab 400 and Chart 4; AdInstruments).

9.2.3 *Measurement of Serum Epinephrine and Norepinephrine Concentrations*

To obtain blood specimens suitable for measurement of plasma catecholamines as described (Grouzmann E, et al., 2003), animals were lightly anesthetized with diethylether (Roth, Karlsruhe, Germany) or isoflurane (Essex, Munich, Germany) and about 200 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Plasma epinephrine and norepinephrine concentrations were measured using an ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany).

9.2.4 *Measurement of urinary vanilylmandelic acid and epinephrine concentrations*

For evaluation of renal excretion, both, *gsk3*^{WT} and *gsk3*^{KI} mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24 h urine collection as described previously (Vallon V., 2003). For measurement of urinary epinephrine, urine was collected following a 12hr light or dark period. To adapt to the new environment, the animals were allowed a 3 day habituation period. Subsequently, a small amount of 6 M HCl was added to the urine collecting tube placed in the cage, and the acidic urine was collected for the next 24 hours and urinary Vanilyl Mandelic Acid (VMA) concentrations were determined utilizing high pressure liquid chromatography (HPLC). Urine epinephrine concentrations were measured using an ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany). To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil.

9.2.5 Adrenal gland histology

Adrenal glands of both, *gsk3*^{WT} and *gsk3*^{KI} mice were removed after sacrificing the animal. Tissues were immediately fixed by 4% formaldehyde overnight and embedded in paraffin. Tissues were cut in the mid-section of the adrenal gland and after mounting on glass slides stained with hematoxylin and eosin (Roth, Karlsruhe, Germany). Slides were digitized on a Zeiss Axio Observer.A1 microscope utilizing Zeiss Axiovision software (Zeiss AG, Oberkochen, Germany). Images were evaluated with Image J software (http://rsbweb.nih.gov/ij/).

9.2.6 Immunofluorescence

To show the expression of GSK3, 5-µm-thick frozen tissue sections from the adrenal glands of *gsk3*^{WT} mice were fixed with 4% PFA for 15 min at room temperature. After washing twice with PBS the slides were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature. Then, the specimens were exposed overnight at 4°C to GSK3 antibody (1:100, Cell Signaling, USA). The slides were rinsed three times with PBS and incubated for 1.5 h at room temperature with secondary FITC goat anti-rabbit antibody (1:1000, Invitrogen, UK). After three washing steps the nuclei were stained for 10 min at room temperature with DRAQ-5 dye (1:1000, Biostatus, Leicestershire, UK). All the slides and coverslips were mounted with ProLong Gold antifade reagent (Invitrogen, UK). Images were taken on a Zeiss LSM 5

EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40_/1.3 NA DIC. Images were analyzed with the instrument's software.

9.2.7 *In Vitro* Expression

For generation of cRNA, constructs were used encoding wild-type human NaPi-IIa (Busch AE, et al., 1995) and human GSK3β. The cRNA was generated as described previously (Strutz-Seebohm N, et al., 2007). GSK3β cDNA was from the "Deutsches Ressourcenzentrum fu¨r Genomforschung," Berlin, Germany. For electrophysiology, *Xenopus* oocytes were prepared as described previously (Boehmer C, et al., 2008 and Boehmer C, et al., 2008). Ten nanograms of NaPi-IIa cRNA was injected on the first day and 7.5 ng of GSK3β cRNA was injected on the second day after preparation of *Xenopus* oocytes. All experiments were performed at room temperature 3 days after the second injection. Two electrode voltage-clamp recordings were performed at a holding potential of -50 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments). The control solution (superfusate/ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.4. Phosphate (2 mM) was added to induce NaPi-IIa– dependent currents. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 seconds. For the determination of NaPi-IIa surface expression, defolliculated oocytes were incubated with primary rabbit anti-NaPi-IIa antibody (diluted 1:500; Lifespan Biosciences, Seattle, WA) and secondary, peroxidase-conjugated goat antirabbit antibody (diluted 1:1000; Cell Signaling, Danvers, MA). Individual oocytes were placed in 96-well plates with 10 µl of Super Signal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The chemiluminescence of the oocytes was quantified in a luminometer (WalterWallac2 plate reader; Perkin Elmer, J gesheim, Germany) by integrating the signal over a period of 1 second. Results display normalized arbitrary light units that are proportional to the detector voltage.

9.2.8 *In Vivo Urine collection* Experiments and dietary treatment

The mice were compared with corresponding wild-type mice (*gsk3WT*). The mice were fed control diet (Altromin, Lage, Germany) containing 7523 mg/kg phosphorus or a phosphorus-

deficient diet containing 131 mg/kg phosphorus, as indicated. They had free access to tap drinking water. To determine creatinine clearance, urinary flow rate, and urinary excretion of Ca^{2+} and phosphate, the mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24-hour urine collection as described previously (Vallon V, 2003). They were allowed a 2-day habituation period, during which food and water intake, urinary flow rate, and excretion of electrolytes were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24-hour collection of urine was performed for 3 consecutive days to obtain the urinary parameters. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil. The phosphate concentration was determined colorimetrically using commercial diagnostic kits (Roche Diagnostics, Mannheim, Germany). The urinary concentration of Ca^{2+} was measured by flame photometry (ELEX 6361; Eppendorf) or by a photometric method according to the manufacturer's instructions (dri-chem clinical chemistry analyzer FUJI FDC 3500i; Sysmex, Norsted, Germany). The creatinine concentration in urine was determined using the Jaffe reaction (Sigma, St. Louis, MO); creatinine and calcium concentration in plasma were measured using a photometric method. The plasma intact parathormone concentration was measured using an ELISA kit (Immunotopics, San Clemante, CA). A radioimmunassay kit was used to determine the concentration of 1,25-(OH)2D3 (IDS, Boldon, UK) in plasma.

Bone density analysis

For the analysis of bone density, animals were killed, and legs were amputated and fixated in formalin. The samples were scanned with a high-resolution microCAT-II (Siemens Preclinical Solutions) small animal computed tomography (CT) scanner using a field of view of 3.1×3.1×4.8 cm3. The x-ray tube parameters were set at 80 kVp and 400 μA. The images were acquired with 720 angular projections (exposure time, 1200 ms per projection) over 360° and binned with a factor of two, yielding a spatial resolution of approximately 38 μm. The total scan time was 24 minutes. Reconstructed CT images were analyzed with the Inveon Research Workplace software (Siemens Preclinical Solutions) by drawing a standard-sized container around the femur and applying a region growth routine to segment the trabecular bone structure. For all samples, the same upper and lower density threshold was applied, and the relative numbers of trabecular bone density were compared.

Blood and urinary concentrations

To obtain blood specimens, animals were lightly anesthetized with diethyl ether (Roth, Karlsruhe, Germany) and approximately 150 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Plasma and urinary concentrations of Na+, K+ and Ca2+ were measured by flame photometry (AFM 5051, Eppendorf, Germany). Plasma and urinary creatinine concentrations were measured by using an enzymatic colorimetric method (Creatinine PAP, Labortechnik, Berlin, Germany).

9.2.9 RNA Extraction and Real-Time RT-PCR

Snap-frozen kidneys were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) and supplemented with ß-mercaptoethanol, resulting in a final concentration of 1%. Total RNA was extracted and reverse transcribed as described previously (Nowik M, et al., 2008). Quantitative real-time qRT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for all genes of interest were as described (Nowik M, et al., 2008). For analysis of the data, the threshold was set to 0.06 because this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of the gene of interest was calculated in relation to hypoxanthine guanine phosphoribosyl transferase. Relative expression ratios were calculated as $R=2^{[Ct(HPRT)\_Ct(test\ gene)]}$, where Ct represents the cycle number at the threshold 0.06.

9.2.10 BBMV Preparation and Phosphate Transport Assays

BBMVs were prepared from rat kidney cortex and outer medulla using the Mg^{2+} precipitation technique as described previously (Biber J, et al., 1981 and Biber J, et al., 2007). The phosphate transport rate into BBMVs was measured in freshly prepared BBMVs at 25°C in the presence of inward gradients of 100mM NaCl or 100mM KCl and 0.1mM K-phosphate. The substrate Pi was prepared with 0.125 mM K_2HPO_4 and (Brown AJ, et al., 2002) P (1 µCi/ml) to yield a final concentration of 0.1 mM, close to the expected apparent *K*mPi for Na^+ -dependent transport in renal BBMVs. The stop solution contained 100mM mannitol, 5mM Tris-HCl, pH7.4, 150mM NaCl, and 5mM Pi. The Na^+ dependence was established by incubating BBMVs in solutions in which KCl replaced NaCl equimolarly. Phosphate uptake was determined after 60 seconds, representing initial linear conditions, and after 120 minutes, to determine the equilibrium values.

To distinguish between Na^+ -dependent Pi uptake mediated by SLC34 family members (*e.g.,* NaPi-IIa and NaPi-IIc) and other Na^+ -dependent phosphate transporters such as SLC20 family members (*e.g.,* Pit-1 and Pit-2), we used trisodium PFA (final concentration, 6 mM) added to the same solution with 107 mM NaCl. PFA has previously been shown to have a higher selectivity for SLC34 than for SLC20 phosphate transporters at this concentration (Villa-Bellosta R, et al., 2009). The total protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). BBMVs were stored at −80°C until further use.

9.2.11 Western Blotting

After measurement of the protein concentration (Bio-Rad), 10 µg of renal brush border membrane proteins was solubilized in loading buffer containing dithiothreitol and separated on 8% polyacrylamide gels. For immunoblotting, the proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). After blocking with 5% milk powder in Trisbuffered saline/0.1% Tween-20 for 60 minutes, the blots were incubated with the primary antibodies: rabbit polyclonal anti-NaPi-IIa (1:6000),45 rabbit polyclonal anti-NaPi-IIc (1:10,000), (Nowik M, et al., 2009) rabbit polyclonal anti-Pit-2 (1:3000; kindly provided by Dr. V. Sorribas, University of Zaragoza, Spain) (Villa-Bellosta R, et al., 2009), and mouse monoclonal anti-β-actin antibody (42 kD; Sigma; 1:5000) either for 2 hours at room temperature or overnight at 4°C. Membranes were incubated for 1 hour at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies 1:5000 linked to alkaline phosphatase (Promega) or to horseradish peroxidase (Amersham). The protein signal was detected with the appropriate substrates (Millipore) using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using the software Advanced Image Data Analyzer AIDA, Raytest to calculate the protein of interest/β-actin ratio.

9.2.12 Statistical Analysis

Data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using paired or unpaired *t* tests or ANOVA. GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA) was used. Only results with $P < 0.05$ were considered statistically significant.

10.RESULTS

10.1 GSK3 and Catecholamine

Similar to what was reported earlier (Boini KM, et al., 2008), the blood pressure was significantly ($p<0.01$) higher in *gsk3*^{KI} mice (112 ± 3 mmHg, n = 6) than in *gsk3*^{WT} mice (96 ± 4 mmHg, n = 7; mean of 3 days measurement after 7 days training). Moreover, heart rate was significantly ($p<0.001$) higher in *gsk3*^{KI} mice (575 ± 15 bpm, n = 6) than in *gsk3*^{WT} mice (431 ± 13 bpm, n = 7). To explore whether the hypertension of *gsk3*^{KI} resulted from increased adrenergic stimulation, the effect of adrenergic receptor blockers on blood pressure of *gsk3*^{KI} and *gsk3*^{WT} mice was analysed. As a result, treatment with the α-adrenergic blocker prazosine (1 µg/g b.w.) resulted in a decrease of blood pressure to a larger extent in *gsk3*^{KI} than in *gsk3*^{WT} mice (Fig. 10.1.1). In the presence of prazosine, the blood pressure still tended to be slightly higher in *gsk3*^{KI} mice (95 ± 5 mmHg, n = 6) than in *gsk3*^{WT} mice (89 ± 4 mmHg, n = 7), a difference, however, not reaching statistical significance. Treatment with the ganglion blocker hexamethonium (40 µg/g b.w.) was followed by a more profound decrease of blood pressure in *gsk3*^{KI} mice (Fig. 10.1.1). In the presence of hexamethonium blood pressure was identical in *gsk3*^{KI} mice (81± 5 mmHg, n = 6) and in *gsk3*^{WT} mice (81 ± 6 mmHg, n = 7).

Additionaly, blood pressure was again determined in another study group of anaesthetized mice (1.5% isoflurane). Under isoflurane anesthesia, *gsk3*^{KI} mice showed a similar tendency for increased blood pressure (119 ± 5mmHg in *gsk3*^{KI} mice vs 101 ± mmHg in *gsk3*^{WT} mice, $p<0.01$; Fig. 10.1.2). Treatment with prazosin did produce a more pronounced decrease in blood pressure in *gsk3*^{KI} mice, which abrogated the differences in blood pressure between *gsk3*^{KI} mice (86 ± 2 mmHg, n = 6) and *gsk3*^{WT} mice (88 ± 1 mmHg n = 6). After similar baseline blood pressure measurements (*gsk3*^{KI} 126 ± 1 mmHg, *gsk3*^{WT} 107 ± 3 mmHg; $p<0.01$), treatment with the ganglion blocker hexamethonium (40 µg/g b.w.) again reduced blood pressure of *gsk3*^{KI} mice (96 ± 1 mmHg, n = 5) to similar levels as in *gsk3*^{WT} mice (97 ± 5 mmHg, n = 6; Fig. 10.2.2).

Treatment of *gsk3*^{KI} and *gsk3*^{WT} mice with the ß-blocker atenolol (5 µg/g b.w.) did not significantly decrease blood pressure, but significantly decreased heart rate (Fig. 10.1.3) in mice anaesthetized with 1.5% isoflurane. Atenolol decreased heart rate significantly more in *gsk3*^{KI} mice than in *gsk3*^{WT} mice. As a result, in the presence of atenolol the heart rate was not significantly different between *gsk3*^{KI} mice (371 ± 23 bpm, n = 6) and *gsk3*^{WT} mice (353± 21 bpm, n = 7).

The increased blood pressure could result from stimulation of sympathetic activity with enhanced catecholamine release. As illustrated in Fig.10.1.4A, the plasma epinephrine concentration was indeed significantly ($p<0.0001$) higher in $gsk3^{KI}$ mice than in $gsk3^{WT}$ mice. A similar difference could be observed while using a different anaesthetic regimen during the collection of the blood, namely isoflurane anaesthesia (Fig 10.1.4B). Concordantely, the norepinephrine plasma concentration was significantly ($p<0.01$) higher in $gsk3^{KI}$ mice than in $gsk3^{WT}$ mice (Fig. 10.1.4C).

The increased plasma catecholamine concentrations could have resulted from enhanced release or delayed degradation of catecholamines. To discriminate between those two possibilities, the urinary excretion of epinephrine and the catecholamine degradation product vanilyl mandelic acid was determined. As shown in Fig. 10.1.5A, the urinary excretion of vanilyl mandelic acid was significantly ($p<0.05$) higher in $gsk3^{KI}$ than in $gsk3^{WT}$ mice. Urinary epinephrine levels were increased in $gsk3^{KI}$ mice during a 12hr dark (Fig. 10.1.5B) as well asa 12 hr light period (5C, both $p<0.01$).

In theory, the increased production of catecholamines in $gsk3^{KI}$ mice could have been dependent on a pheochromocytoma or hypertrophy of the adrenal medulla. No abnormalities could be shown by histological evaluation of the adrenal gland (Fig 10.1.6). Furthermore, adrenal medulla size was not significantly different between $gsk3^{KI}$ mice (161 ± 22 mm², $n = 7$) and $gsk3^{WT}$ mice (155 ± 23 mm², $n=6$).

If gsk3 is involved in the production or release of catecholamines, it should be expressed in chromaffine cells of the adrenal medulla. As shown by immunostaining in Fig. 10.1.7, gsk3 is expression could be detected by confocal microscopy after immunostaining in the adrenal glands of $gsk3^{WT}$ mice.

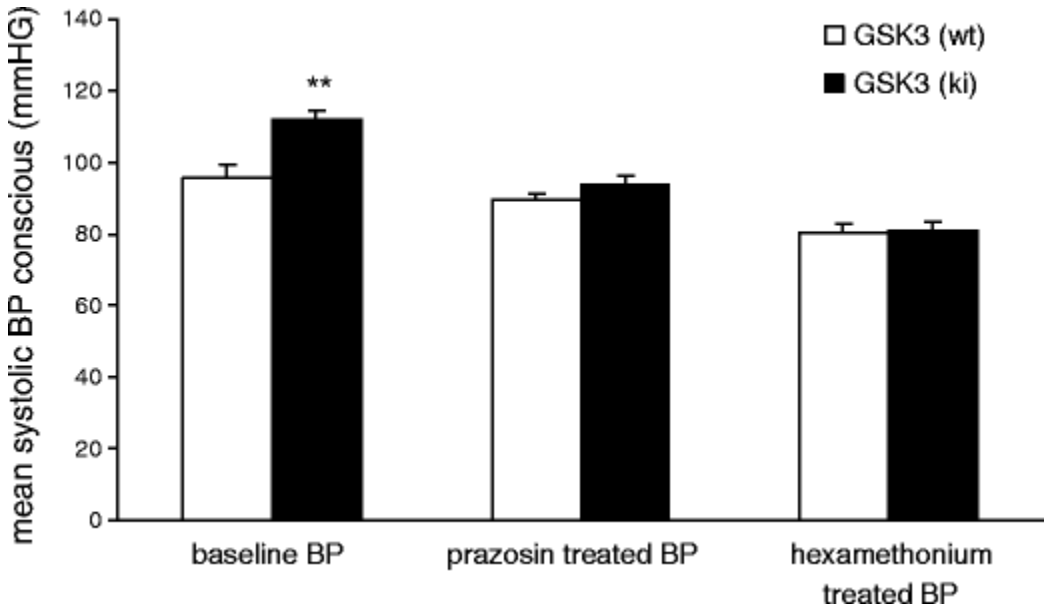

Fig. 10.1.1: Systolic blood pressure in *gsk3*^{KI} and *gsk3*^{WT} mice prior to and following administration of prazosine or hexamethonium in conscious mice

Arithmetic means ± SEM of systolic blood pressure (mmHg) in GSK3 knockin mice (*gsk3*^{KI}, closed bars; n=6) and wild type mice (*gsk3*^{WT}, open bars; n=7) at baseline (mean of three days measurement) and after administration of prazosin (1 µg/g b.w.) and hexamethonium chloride (40 µg/g b.w.); treatments performed on different non-consecutive days. ANOVA, ** indicates significant ($p<0.01$) difference from respective value of *gsk3*^{WT} mice.

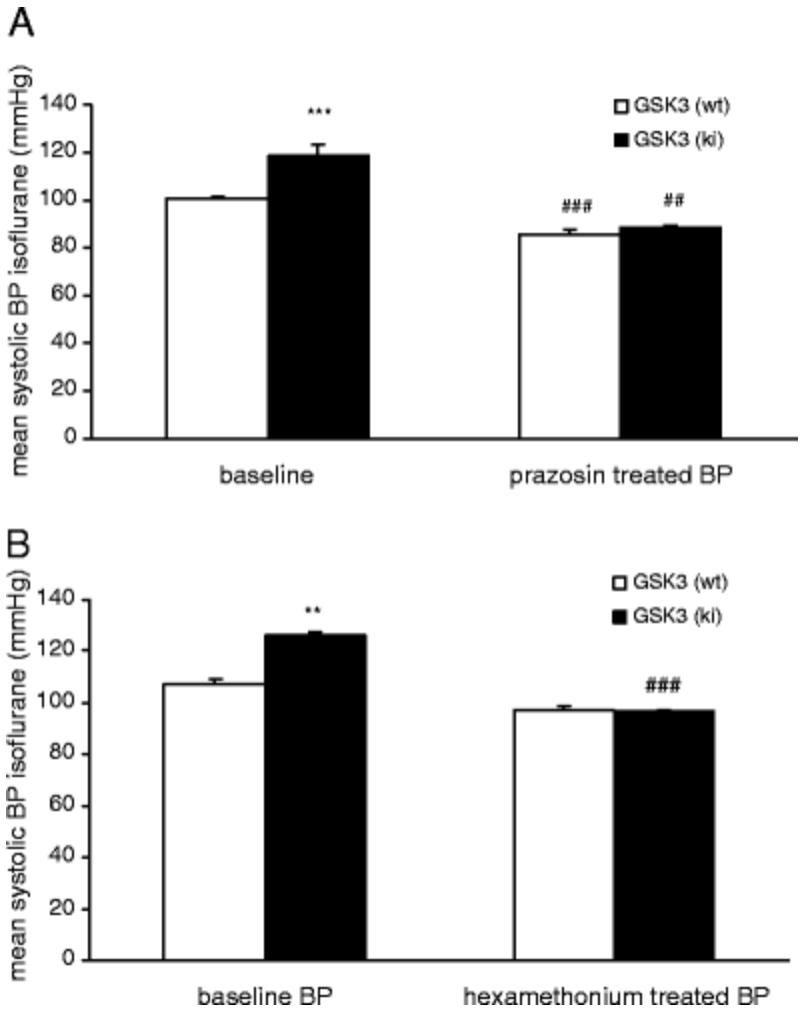

Fig. 10.1.2: Systolic blood pressure in *gsk3^{KI}* and *gsk3^{WT}* mice prior to and following administration of prazosine or hexamethonium under isoflurane anaesthesia

a. Arithmetic means ± SEM of systolic blood pressure (mmHg) in GSK3 knockin mice (*gsk3^{KI}*, closed bars; n=5-6) and wild type mice (*gsk3^{WT}*, open bars; n=6). Measurements were performed under 1.5% isoflurane anaesthesia at baseline and immediatley followed by administration of prazosin (1 µg/g b.w.). b. Arithmetic means ± SEM of systolic blood pressure (millimeters of mercury) in GSK3 knockin mice (gsk3^{KI}, closed bars; n=5) and wild type mice (gsk3^{WT}, open bars; n=6). Measurements were performed under 1.5% isoflurane anesthesia at baseline and immediately followed by administration of prazosin and hexamethonium chloride (40 µg/g b.w.). ANOVA; ** and *** indicates significant ($p<0.01$, $p<0.001$) difference from respective value of *gsk3^{WT}* mice; ## and ### indicates significant ($p<0.01$, $p<0.001$) difference from respective baseline value of the same genotype.

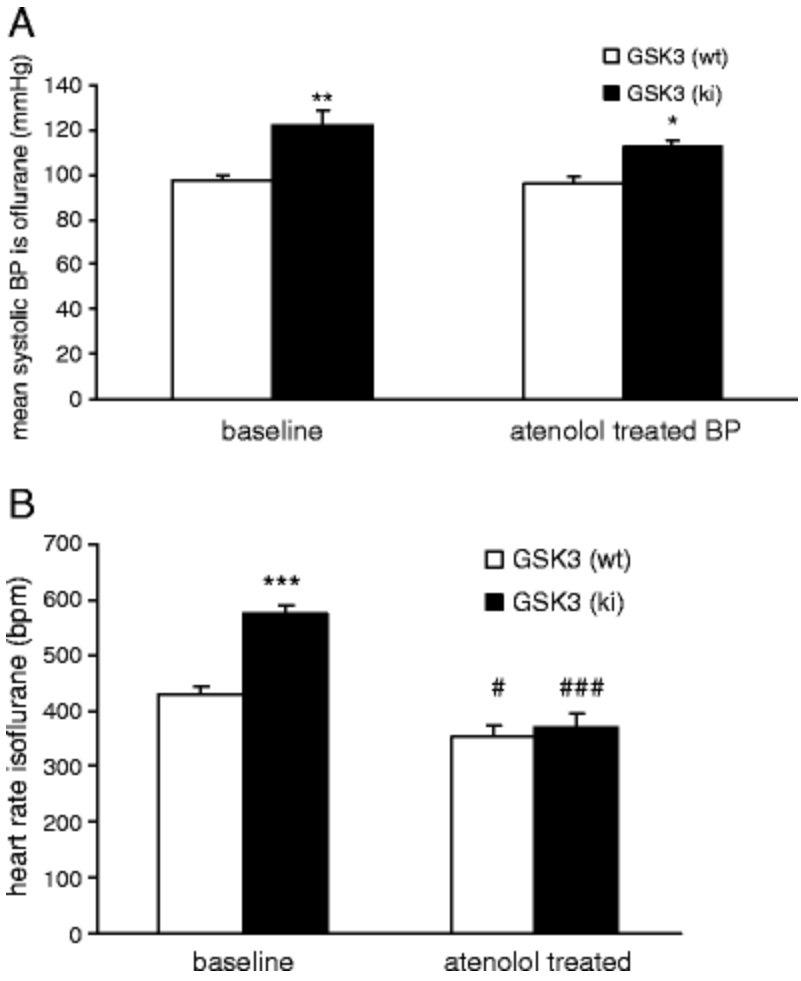

Fig.10.1.3: Systolic blood pressure and heart rate in *gsk3*^{KI} and *gsk3*^{WT} mice prior to and following administration of atenolol under isoflurane anaesthesia

A. Arithmetic means ± SEM of systolic blood pressure (mmHg) in GSK3 knockin mice (*gsk3*^{KI}, closed bars; n = 6) and wild type mice (*gsk3*^{WT}, open bars; n = 6) before and after atenolol treatment (5µg/g). ANOVA, *,** and *** indicates significant ($p<0.05$, $p<0.01$, $p<0.001$) difference from respective value of *gsk3*^{WT} mice.

B. Arithmetic means ± SEM of heart rate (beats/min) in GSK3 knockin mice (*gsk3*^{KI}, closed bars; n = 6) and wild type mice (*gsk3*^{WT}, open bars; n = 7) before and after atenolol treatment (5 µg/g). ANOVA, *** indicates significant ($p<0.001$) difference from respective value of *gsk3*^{WT} mice; #, ### indicates significant ($p<0.05$, $p<0.001$) difference from respective baseline value of the same genotype. .

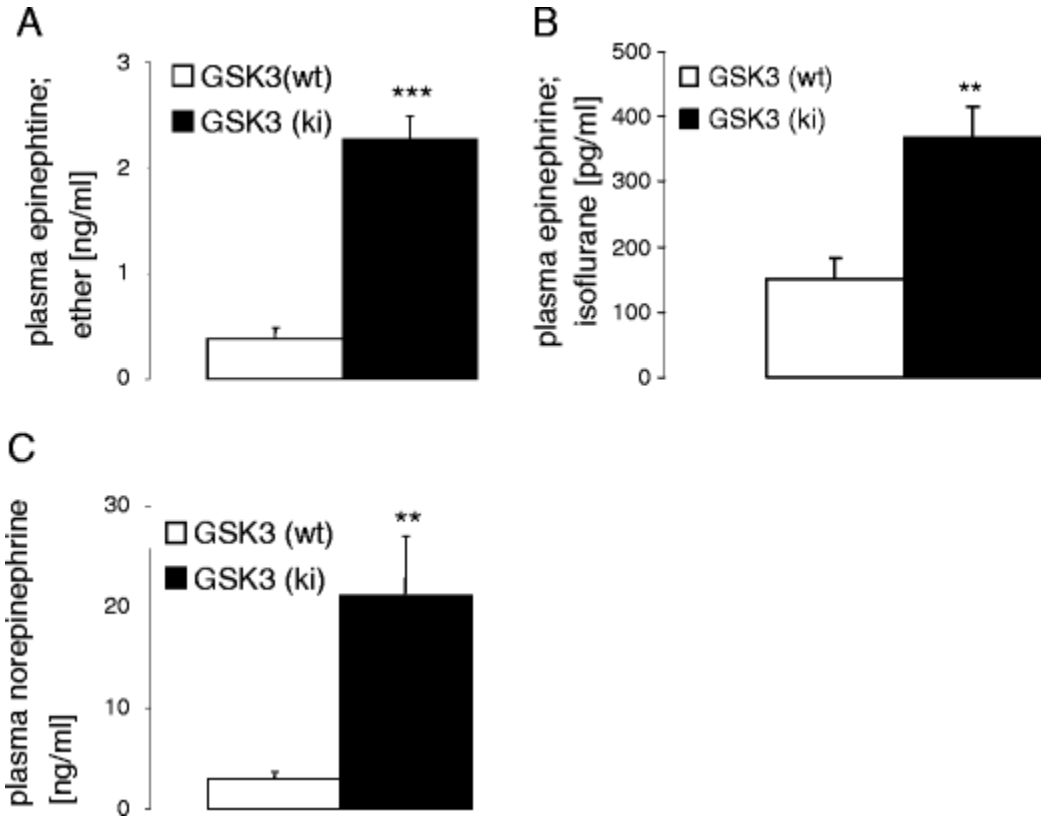

Fig. 10.1.4: Plasma epinephrine and norepinephrine concentrations in *gsk3*^{KI} and *gsk3*^{WT} mice

Arithmetic means ± SEM of plasma epinephrine under ether (**A**) or isoflurane anaesthesia (b) and norepinephrine (**C**) concentrations in GSK3 knockin mice (*gsk3*^{KI}, closed bars; n = 6) and wild type mice (*gsk3*^{WT}, open bars; n = 8). T-test, **, *** indicate significant ($p<0.01$, $p<0.001$) difference from respective value of *gsk3*^{WT} mice.

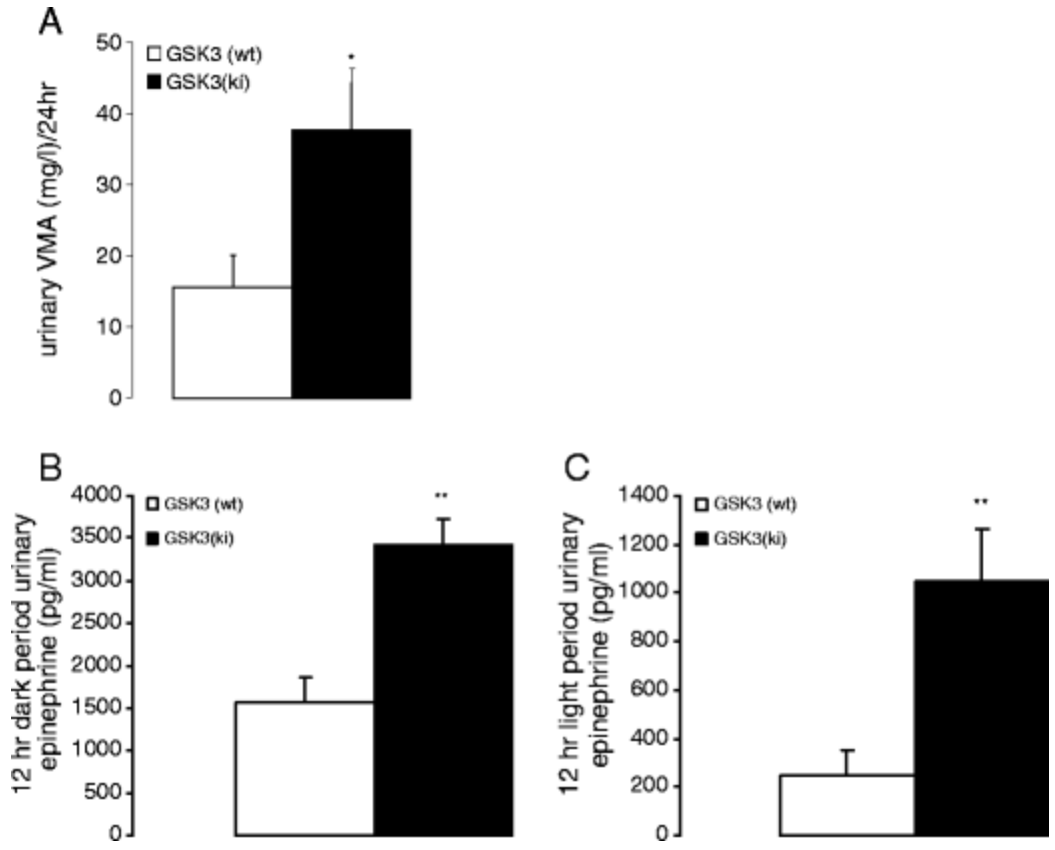

Fig. 10.1.5: Urinary vanilylmandelic acid and epinephrine concentrations in *gsk3^{KI}* and *gsk3^{WT}* mice

Arithmetic means ± SEM of urinary vanilylmandelic acid concentration (A) and epinephrine concentrations during 12hr dark (B) or light (C) period in GSK3 knockin mice (*gsk3^{KI}*, closed bars; n = 6) and wild type mice (*gsk3^{WT}*, open bars; n = 6). T-Test, *,** indicates significant ($p<0.05$, $p<0.01$) difference from respective value of *gsk3^{WT}* mice.

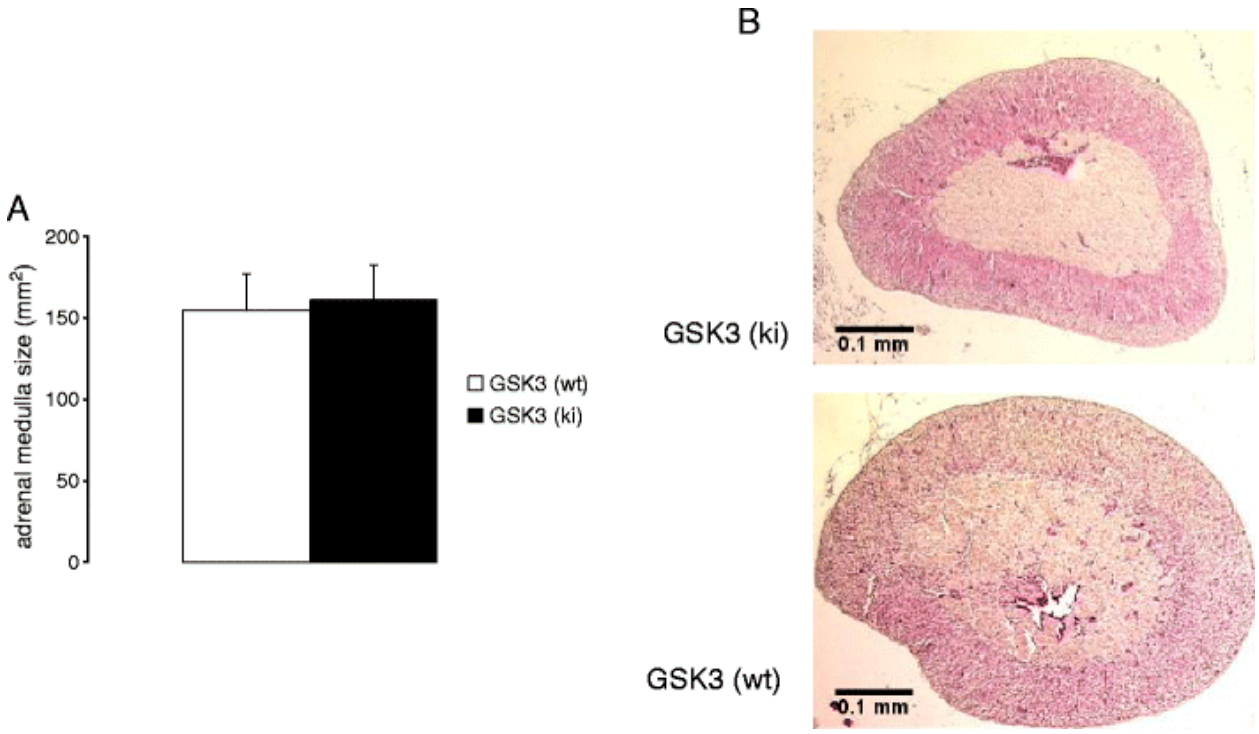

Fig. 10.1.6: Histological sections of adrenal glands of *gsk3*^{KI} and *gsk3*^{WT} mice

A. Arithmetic means ± SEM of midsectional area (mm²) of the adrenal medulla in GSK3 knockin mice (*gsk3*^{KI}, closed bars; n = 6) and wild type mice (*gsk3*^{WT}, open bars; n = 6).

B. Example images of adrenal glands from GSK3 knockin mice (*gsk3*^{KI}) and wild type mice (*gsk3*^{WT}). Scale bar indicates 100 µm

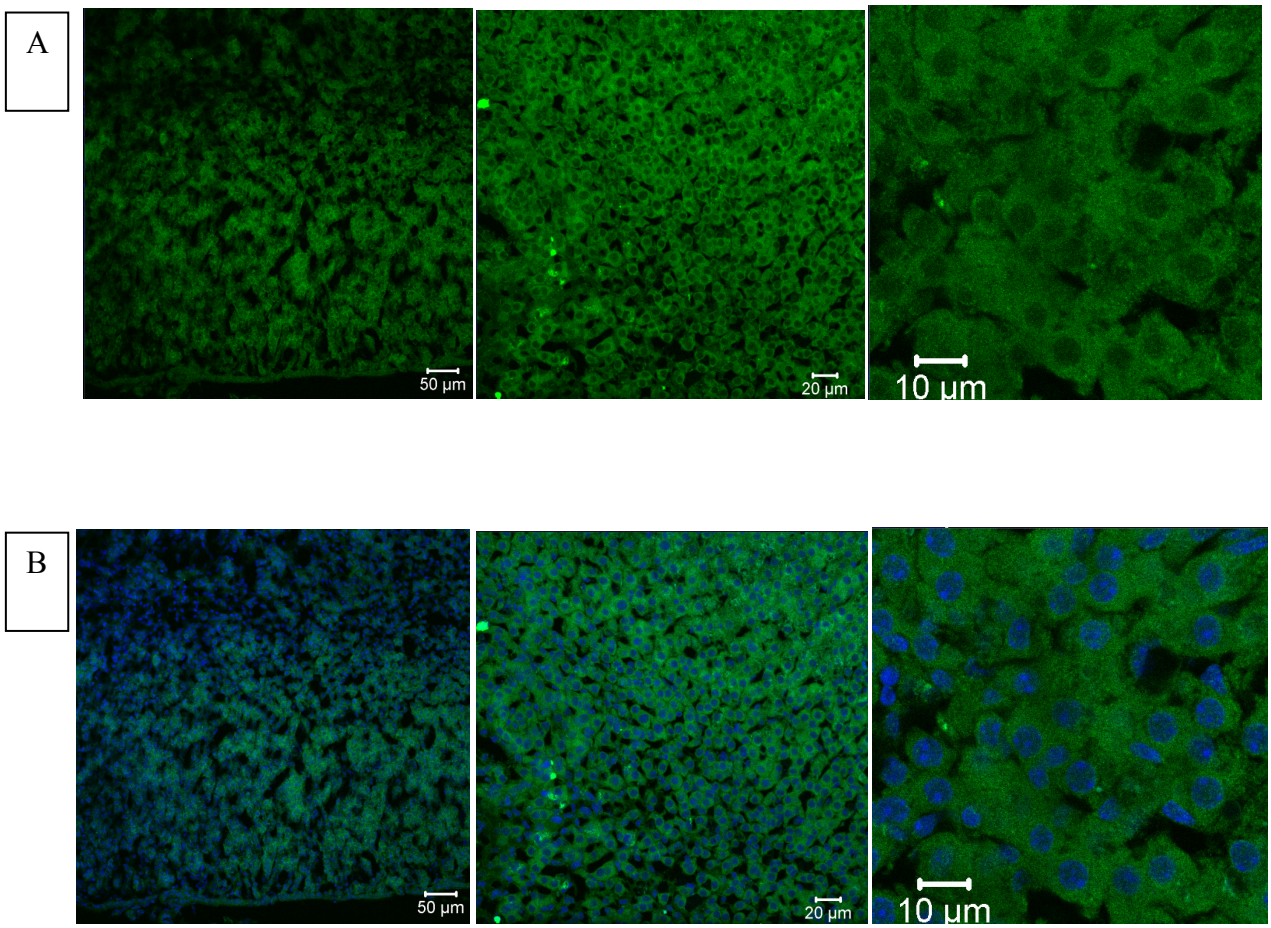

Fig. 10.1.7: GSK3 Expression in adrenal glands of *gsk3^{WT}* mice

Confocal laser scanning microscopic analysis of adrenal glands of *gsk3^{WT}* mice frozen sections stained with anti-GSK3 antibody, and anti-rabbit-FITC was used as secondary antibody. Pictures show specific FITC-related fluorescence at adrenal glands of *gsk3^{WT}* mice (a). Nuclei were visualized by DRAQ5™ staining and merged (b).

10.2 GSK3, Phosphaturia and Calciuria

The reabsorption of phosphate (Pi) from the proximal tubule is mainly determined by sodium-dependent transports expressed in the brush border membrane. Electrophysiological studies reveal that Pi transport via Na/pi cotransporters is electrogenic (Murer H and Biber J., 1996). Hence in the first series of our study, we analyzed the influence of GSK3β on Napi-IIa, a major renal tubular sodium-dependent phosphate transporter *in vitro*. We found that *Xenopus oocytes* injected with water, upon exposure to phosphate (2mM) in bath solution did not induce any significant current, confirming that these oocytes do not express any significant endogenous electrogenic phosphate transporter (Figure 10.2.1A). In the same set of experiment oocytes injected with cRNA encoding electrogenic phosphate transporter NaPi-IIa, and upon exposure to phosphate as previous, showed an inward current of 58 ± 10 nA (*n*=21 oocytes). Whereas coexpression of GSK3β with phosphate transporter NaPi-IIa in the *Xenopus oocytes* showed decreased an inward current of 27 ± 5 nA (*n* = 17 oocytes). To determine the *in vitro* influence of GSK3β on the membrane abundance of Napi-IIa, a chemiluminescence-based assay was performed. To this end we found significant reduction in surface expression of NaPi-IIa by coexpression of GSK3β (Figure 10.2.1B). The decrease of NaPiIIa activity is not simply caused by the expression of an additional protein. The phosphate induced current in NaPi-Iia–expressing oocytes is, for instance, similar with and without coexpression of mTOR, when the kinase is inhibited by rapamycin (Kempe DS, et al., 2010).

To explore further, the activity and expression of sodium-dependent electrogenic phosphate transporters of both family SLC34 (e.g., NaPi-IIa and NaPi-IIc) and SLC20 (*e.g.,* Pit-1 and Pit-2), in brush border membrane vesicles (BBMVs) from the kidney cortex and outer medulla of GSK3αβ knockin (ki) mice ($gsk3^{KI}$) and corresponding wild-type (wt) mice ($gsk3^{WT}$) was assessed. Both SLC34A1 and SLC34A3 are localized at the brush border membrane of proximal tubule cells and have not been detected in other segments of the nephron (Custer M, et al., 1994 and Segawa H., et al., 2002). In the absences of phosphonoformic acid (PFA, 6 mM) the sodium-dependent uptake of phosphate by both families of phosphate transporters (NaPi-IIa, NaPi-IIc, Pit-1 and Pit-2) into BBMVs was significantly reduced in $gsk3^{KI}$ mice (Figure 10.2.2). In presence of phosphonoformic acid (PFA, 6 mM), the sodium-dependent uptake of phosphate by SLC20 families (*e.g.,* Pit-1 and Pit-2) of phosphate transporters into BBMVs was significantly reduced in both genotypes (Figure 10.2.2). Upon addition of phosphonoformic acid

(PFA, 6 mM) $gsk3^{KI}$ mice still shows less residual sodium-dependent phosphate transport activity compared to corresponding wild-type (wt) mice ($gsk3^{WT}$).

In order to investigate the abundance of phosphate transporters (NaPi-IIa, NaPi-IIc, and Pit-2), additional experiment of immunoblotting of kidney cortex of BBMVs was performed. As shown in Figure 10.2.3, significantly reduced abundance of NaPi-IIa and of NaPi-IIc protein was found in $gsk3^{KI}$ mice compared with corresponding wild-type (wt) mice ($gsk3^{WT}$). Semiquantitative real-time PCR analysis showed significantly increased mRNA abundance of NaPi-IIa and Pit-2 in $gsk3^{KI}$ mice, suggesting that GSK3 acts partially upstream of the transcription of these transporters (all data expressed as mRNA expression normalized to HPRT mRNA abundance; see Concise Methods; NaPi-IIa: $gsk3^{WT}$: 29.0 ± 3.2, n = 6; $gsk3^{KI}$: 59.97 ± 11.12, n = 5; $P < 0.05$; NaPi-IIc: $gsk3^{WT}$: 0.22 ± 0.01, n = 6; $gsk3^{KI}$: 0.22 ± 0.03, n = 5; not significant; Pit-2: $gsk3^{WT}$: 0.16 ± 0.01, n = 6; $gsk3^{KI}$: 0.34 ± 0.04, n = 5; $P < 0.01$).

Similar to what was reported earlier (Boini KM, et al., 2008), *in vivo* metabolic cage experiments were performed in $gsk3^{KI}$ and $gsk3^{WT}$ mice. The body weight was found similar in both $gsk3^{KI}$ mice (29.1 ± 0.3 g, n = 10) and $gsk3^{WT}$ mice (28.9 ± 1.4 g, n = 10). The urinary flow rate was significantly higher in $gsk3^{KI}$ mice (95.2 ± 10.1µl/24 h per gram body weight, n = 6) compared with corresponding wild-type mice ($gsk3^{WT}$) (24.1 ± 4.8µl/24 h per gram body weight, n = 6). Additionally the creatinine clearance was significantly higher in $gsk3^{KI}$ mice (0.39 ± 0.06 ml/24 h, n = 6) than in $gsk3^{WT}$ mice (0.19 ± 0.02 ml/24 h, n = 6).

To determine renal handling of phosphate in both $gsk3^{KI}$ and $gsk3^{WT}$ mice fed on control diet and phosphorus-deficient diet, we perform metabolic cages experiments and determined urinary phosphate excretion and the fractional phosphate excretion. As shown in Figure 10.2.4 (top right panel), under normal diet the urinary phosphate excretion was significantly larger in $gsk3^{KI}$ than corresponding wild-type mice ($gsk3^{WT}$). The fractional phosphate excretion before starting phosphorus-deficient diet was found higher in $gsk3^{KI}$ (63.9 ± 13.6%) (n=6) than corresponding wild-type mice ($gsk3^{WT}$) 31.1 ± 6.9% (n = 5). The values were not significantly different. But on the 3rd day after phosphorus-deficient diet, the fractional phosphate excretion amounted to 1.4 ± 0.1% (n = 5) in $gsk3^{WT}$ mice and to 31.8 ± 11.1% (n = 6) in $gsk3^{KI}$ mice, values significantly ($P < 0.05$) different (Figure 10.2.4, bottom panel). Theoretically hyperphosphatemia (abnormally elevated level of phosphatein in blood) expected to cause excess excretion of urinary phosphate. However, $gsk3^{KI}$ mice showed significantly lower plasma

phosphate concentration compared to corresponding wild-type mice ($gsk3^{WT}$), as shown in Figure 10.2.4, top left panel. This could be due to decreased intestinal uptake of phosphate because plasma PTH and activated vitamin D (1,25-(OH)2D3) control its uptake (Brown AJ, et al., 2002). As our $gsk3^{KI}$ mice has lower plasma concentrations of PTH and activated vitamin D (1,25-(OH)2D3), naturally the uptake of phosphate in the intestine is less and hence plasma concentration is less.

To explore further reason of phosphaturia, as renal tubular reabsorption of phosphate is controlled by plasma PTH and 1,25-(OH)2 vitamin D3 (Murer H, et al., 2000), we determined plasma PTH and 1,25-(OH)2 vitamin D3 in both $gsk3^{KI}$ and wild-type mice ($gsk3^{WT}$). However, as shown in Figure 10.2.5A (left panel), the plasma concentration of PTH was significantly lower in $gsk3^{KI}$ than in corresponding wild-type mice ($gsk3^{WT}$). The plasma PTH stimulates the renal 1α-hydroxylase (Portale AA, et al., 2000), the rate-limiting enzyme of 1,25-(OH)2D3 formation (Portale AA, et al., 2000 and Kato S, 1999). So low plasma PTH levels are expected to decrease the plasma 1,25-(OH)2 D3 concentration. As shown in Figure 10.2.5A (right panel), the plasma concentration of 1,25- (OH)2 D3 was indeed significantly lower in $gsk3^{KI}$ than in corresponding wild-type mice ($gsk3^{WT}$). As 25-hydroxyvitamin D3 1α-hydroxylase catalyzes the last step in the synthesis of the active 1,25-(OH)2D3, we determined mRNA expression of CYP27B1 (25-hydroxyvitamin D3 1a-hydroxylase) in both $gsk3^{KI}$ and wild-type mice ($gsk3^{WT}$). We found higher transcript levels of CYP27B1 (25-hydroxyvitamin D3 1α-hydroxylase) in $gsk3^{KI}$ mice than in corresponding wild-type mice ($gsk3^{WT}$); this difference, however, did not reach statistical significance (Figure 10.2.5B).

As the PTH stimulates the absorption of calcium across the intestinal brush border membrane, passing through ion channels such as TRPV6 (is a member of *Transient receptor potential* selective for calcium ions) (Barley NF et al., 2001). Expression of TRPV6 is 1,25-(OH)2 D3 dependent. Indeed our $gsk3^{KI}$ mice has lower plasma Ca^{2+} concentration compared with corresponding wild-type mice ($gsk3^{WT}$); this difference, however, did not reach statistical significance (Figure 10.2.6, left panel). The plasma PTH and 1,25-(OH)2 vitamin D3 stimulates renal tubular reabsorption of $Ca^{2+\ +}$ via upregulation of the TRPV5 channels. Hence decreased plasma PTH and 1,25-(OH)2 vitamin D3 concentration is expected to blunt the renal tubular Ca^{2+} reabsorption. As shown in Figure 10.2.6 (right panel), the renal Ca^{2+} excretion were indeed larger in $gsk3^{KI}$ than in $gsk3^{WT}$ mice. Additional experiments in metabolic cages with normal

diet and a phosphorus-deficient diet for 3 days were performed and fractional excretion of calcium was determined. We found significantly higher values of fractional excretions of calcium in $gsk3^{KI}$ mice fed on normal diet (2.4 ± 0.2%, n = 5) compared with corresponding wild-type mice ($gsk3^{WT}$) (1.6 ± 0.2%, n = 4).. On 3rd day after starting phosphorus-deficient diet, fractional excretion of calcium were found to be 0.8 ± 0.1% (n = 4) in $gsk3^{WT}$ mice and that of 11.7 ± 5.4% (n = 5) in $gsk3^{KI}$ mice.

The bone mineralization controlled at three level, systemic hormones, bone cells (osteoblasts, osteoclasts and osoteocyts) and cell products, acting in a paracrine / autocrine manner. The mineral ions Ca^{2+} and Pi along with PTH and 1,25-(OH)2 vitamin D3 play a central role in controlling bone mineralization (Roney S. and Gregory L., 2011). Our gsk3KI mice shows excess renal loss of both Ca^{2+} and phosphate together with low PTH and 1,25-(OH)2 vitamin D3 levels is expected to compromise the mineralization of bone. Thus, bone density was determined. As shown in Figure 10.2.7, femur bone density was indeed significantly lower in $gsk3^{KI}$ than in $gsk3^{WT}$ mice.

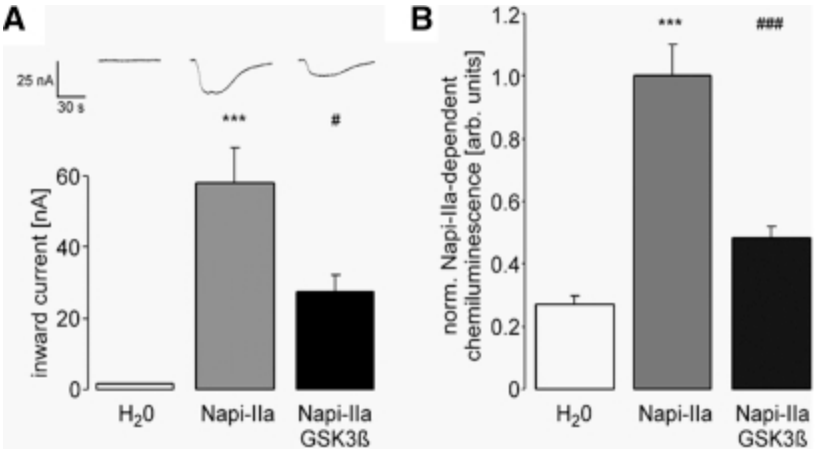

Fig.10.2.1. Coexpression of GSK3 inhibits electrogenic phosphate transport in NaPi-IIa–expressing *Xenopus* oocytes.

(A) Arithmetic means ± SEM (n = 13 to 21) of phosphate (2 mM)-induced inward currents (I_{Pi}) in *Xenopus* oocytes injected with water (left bar), Napi-IIa cRNA (middle bar), or Napi-IIa and GSK3β cRNA (right bar). ***Statistically significant difference from absence of Napi- IIa cRNA ($P < 0.001$). #Difference from absence of GSK3β cRNA ($P < 0.05$). (B) Arithmetic means ± SEM (n = 42 to 60) of the normalized chemiluminescence intensity of Napi-IIa expression in *Xenopus* oocytes injected with water (left bar), Napi-IIa cRNA (middle bar), or Napi-IIa and GSK3β cRNA (right bar). ***Statistically significant difference from absence of Napi-IIa cRNA ($P < 0.001$). ###Difference from absence of GSK3β cRNA ($P < 0.001$).

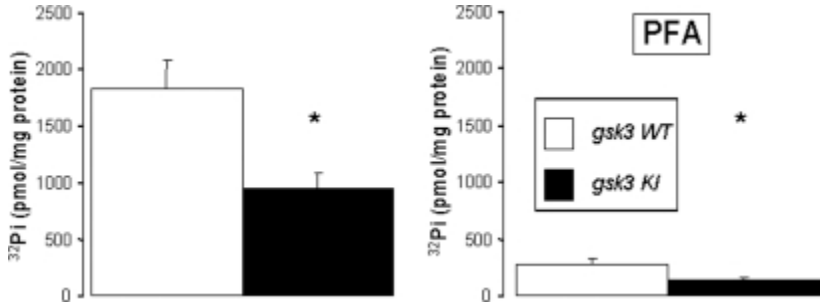

Fig.10.2.2. Sodium-dependent phosphate transport activity in BBMVs from *gsk3^{KI}* and *gsk3^{WT}* mice.

Arithmetic means ± SEM (n = 5 to 6 each group) of the sodium-dependent transport rates into isolated BBMVs after 1 minute in the absence (left bars) and presence (right bars) of 6 mM PFA to block phosphate transport mediated by SLC34 family members. *$P < 0.05$.

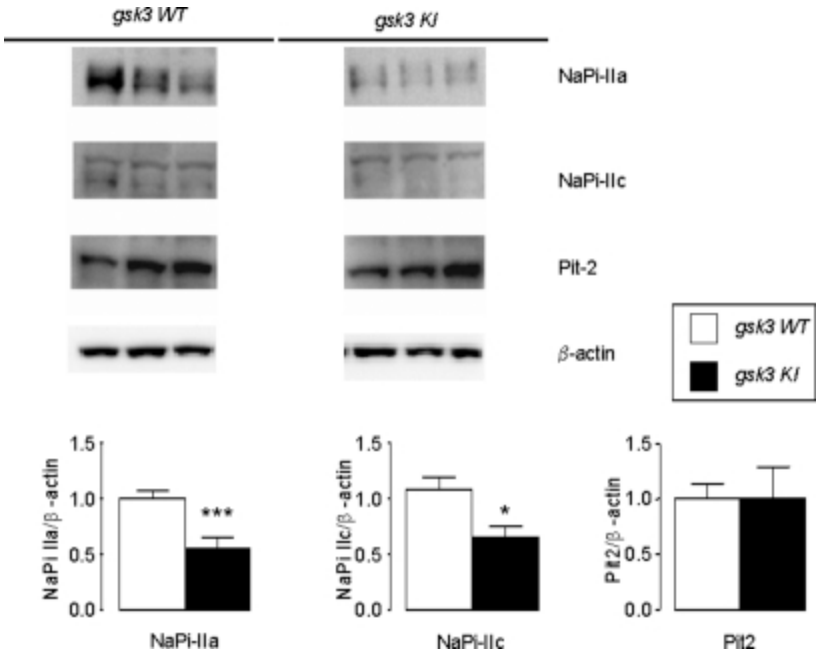

Fig.10.2.3. Protein abundance of renal sodium-dependent phosphate cotransporters in the brush border membrane in KIdneys from *gsk3*^{KI} and *gsk3*^{WT} mice.

Western blots for NaPi-IIa, NaPi-IIc, and Pit-2. All membranes were stripped and reprobed for β-actin to control for loading. Densitometry was performed and the ratio of the protein of interest over β-actin calculated. Bar graphs present data as arithmetic means ± SEM (*n* = 5 to 6 each group).

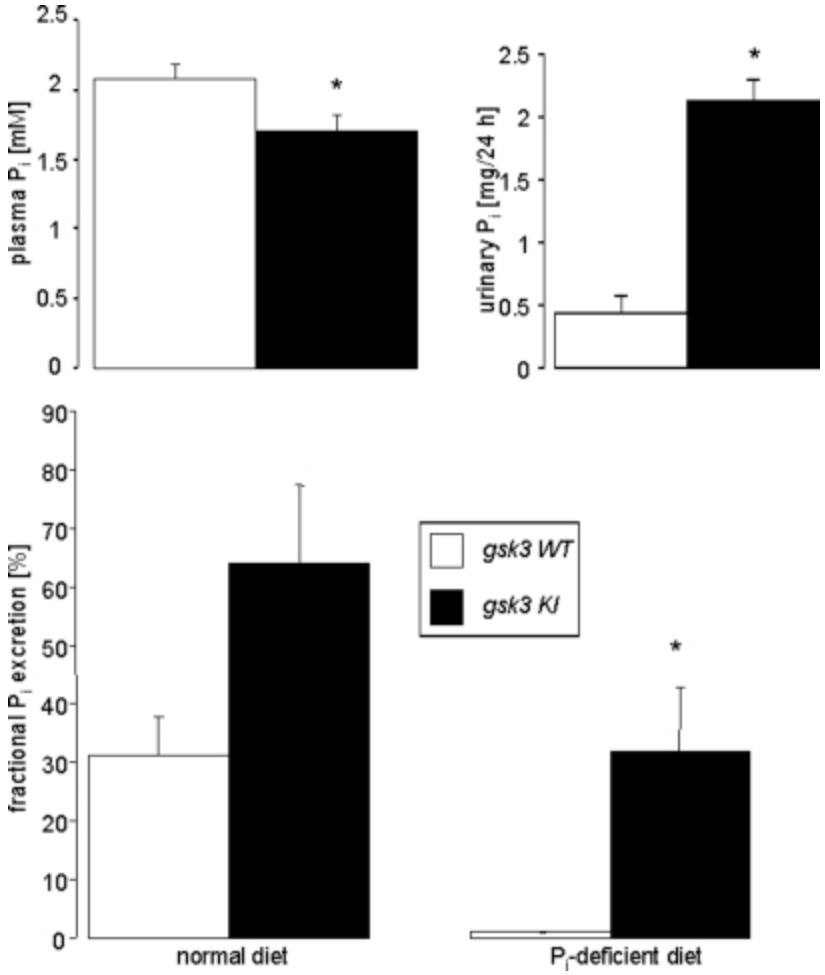

Fig.10.2.4. Plasma phosphate concentration, urinary phosphate excretion, and fractional phosphate excretion in *gsk3*^{KI} and *gsk3*^{WT} mice.

Arithmetic means ± SEM (n = 6 to 10) of plasma phosphate concentration (top left panel) and urinary phosphate excretion (top right panel) in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and corresponding wild-type mice (*gsk3*^{WT}, open bars). The bottom panel depicts the arithmetic means ± SEM (n = 5 to 6) of the fractional phosphate excretion of GSK3 knockin mice and wild type mice maintained under a normal diet (left bars) or maintained for 3 days under a phosphorus-deficient diet (right bars). *$P < 0.05$ *versus* respective value of *gsk3*^{WT} mice

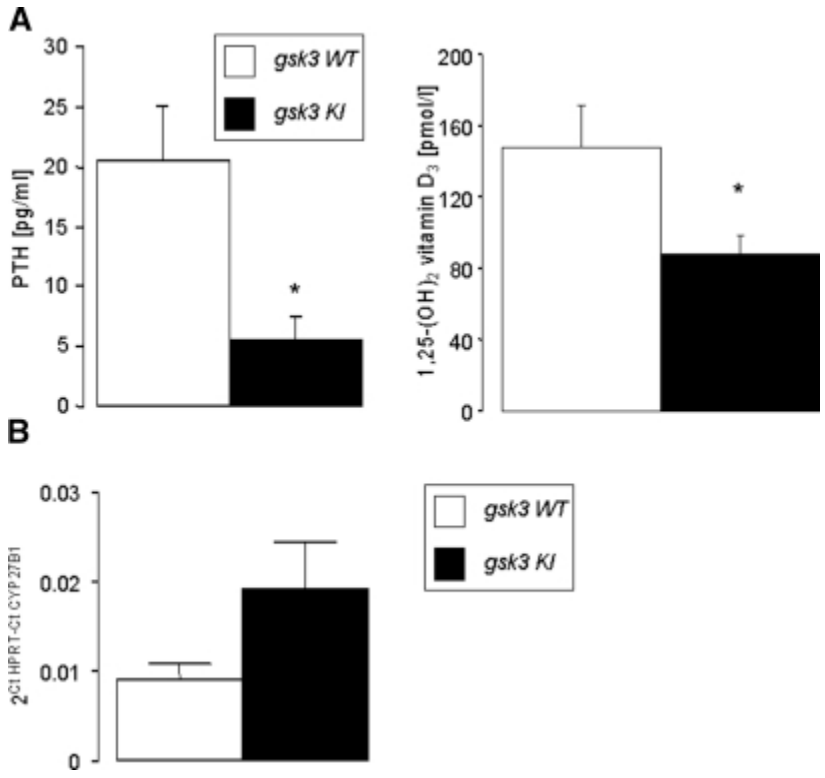

Fig.10.2.5. Plasma PTH and 1,25-(OH)2D3 concentration in *gsk3*^{KI} and *gsk3*^{WT} mice.

(A) Arithmetic means ± SEM of plasma PTH (left panel, n = 6) and 1,25-(OH)2D3 (right panel, n = 17 to 19) concentration in GSK3 knockin mice (*gsk3*^{KI}, closed bars) and corresponding wild-type mice (*gsk3*^{WT}, open bars). *$P < 0.05$ *versus* respective value of *gsk3*^{WT} mice. (B) mRNA expression of CYP27B1 (25-hydroxyvitamin D3 1a-hydroxylase). Arithmetic means ± SEM (n = 5 to 6).

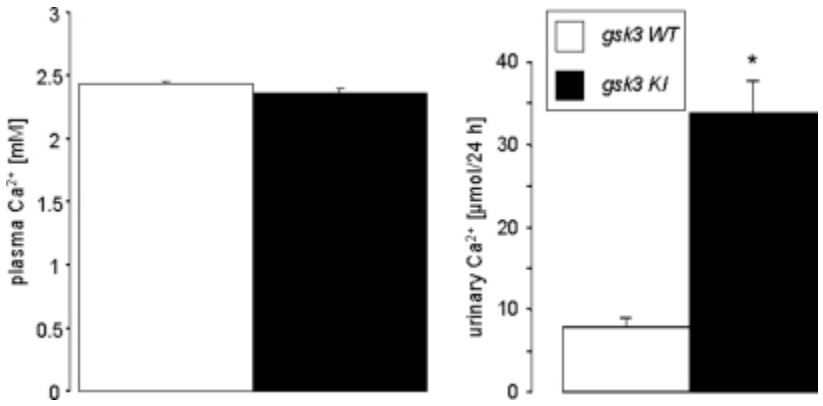

Fig.10.2.6. Plasma calcium concentration and urinary calcium excretion in *gsk3^{KI}* and *gsk3^{WT}* mice.

Arithmetic means ± SEM (n = 6 to 19) of plasma calcium concentration (left panel) and urinary calcium excretion (right panel) in GSK3 knockin mice (*gsk3^{KI}*, closed bars) and corresponding wild-type mice (*gsk3^{WT}*, open bars). *$P < 0.05$ *versus* respective value of *gsk3^{WT}* mice.

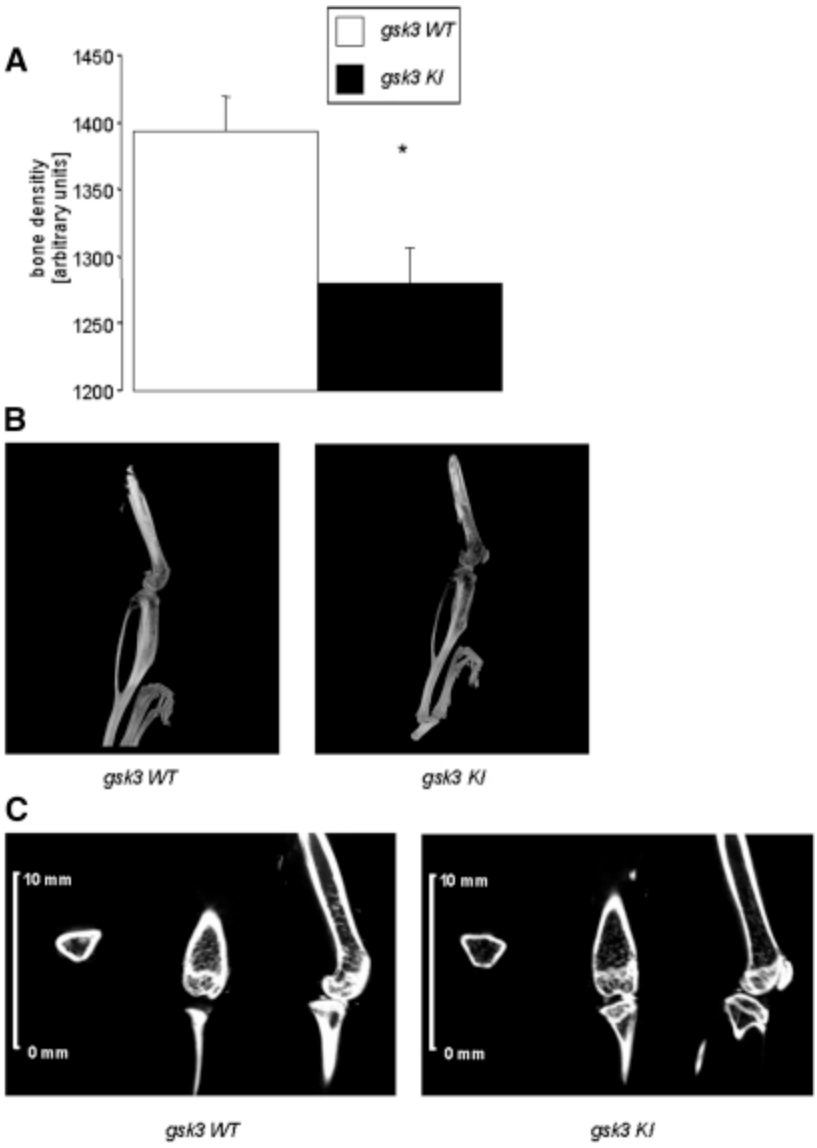

Fig.10.2.7. Bone density of *gsk3^{KI}* and *gsk3^{WT}* mice.

(A) Arithmetic means ± SEM (n = 4 each group) of bone density in GSK3 knockin mice (*gsk3^{KI}*, closed bar) and corresponding wild-type mice (*gsk3^{WT}*, open bar). *$P < 0.05$ *versus* respective value of *gsk3^{WT}* mice. (B) Original microCT image of the leg of a GSK3 knockin mice (*gsk3^{KI}*, right image) and of a corresponding wildtype mouse (*gsk3^{WT}*, left image). (C) Cuts from microCT images in tranaxial, coronal, and sagittal directions through the femur and knee. These high-resolution microCT images display the degradation of trabecular bone and compacta in GSK3 knockin mice (*gsk3^{KI}*, right image) and in wild-type mice (*gsk3^{WT}*, left image).

11. DISCUSSION & CONCLUSION

The primary aim of the current study was to determine the role of protein kinase B(PKB)/Serum and glucocorticoid inducible kinase (SGK) resistant glycogen synthase GSK3 in the handling of catecholamine release, renal calcium and phosphate excretion, and intestinal phosphate transport.

In doing so, as the generations of GSK3β knockout mouse was not possible due to their embryonic death. we generated mice carrying a mutation of GSK-3α in which the codon encoding Ser21 was changed to encode a nonphosphorylatable Ala residue (GSK-3α $^{21A/21A}$), and at the same time carrying a mutation of GSK-3β in which the codon encoding Ser9 was changed to encode a nonphosphorylatable Ala residue (GSK-3β $^{9A/9A}$), are expected to be resistant to PKB/SGK-dependent phosphorylation of GSK-3. PI3 kinase-dependent inhibition of GSK3 mediates the effect of insulin on glycogen synthase (Cohen P, et al., 2004 and McManus EJ, et al., 2005). Accordingly, the effect of insulin on muscle glycogen synthase is abrogated in *gsk3*^{KI} mice (McManus EJ, et al., 2005).

According to the present observations, mice carrying the PKB/SGK-resistant GSK-3 mutants (*gsk3*^{KI}) shows significantly higher blood pressure and heart rate compared to their corresponding wild-type mice (*gsk3*^{WT}). In addition to this our laboratory found increased urine flow in the (*gsk3*^{KI}) mice corresponding to their wild-type mice (*gsk3*^{WT}). With the further estimations of electrolytes from urine we come to known that, (*gsk3*^{KI}) mice has increased renal calcium and phosphate excretions. Further the results corresponding to above two aims have been discussed in detail.

GSK3 and Catecholamine

The present study discloses a novel function of phosphoinositide (PI) 3 kinase-dependent GSK3 regulation, i.e. the tuning of catecholamine release. As observed earlier (Boini KM, et al., 2008), the arterial blood pressure is enhanced in mice expressing protein kinase B(PKB)/Serum and glucocorticoid inducible kinase (SGK) resistant glycogen synthase GSK3. The GSK3 isoforms were made resistant to PKB/SGK by introducing a mutation of GSK3ß, in which the serine of the PKB phosphorylation site had been replaced by an alanine (GSK3ß$^{9A/9A}$) and a mutation in GSK3α, in which the serine of the PKB phosphorylation site had been replaced by an alanine (GSK3α$^{12A/12A}$). PI3 kinase-dependent inhibition of GSK3 mediates the effect of insulin on

glycogen synthase (Cohen P, et al., 2004 and McManus EJ, et al., 2005). Accordingly, the effect of insulin on muscle glycogen synthase is abrogated in *gsk3*^{KI} mice (McManus EJ, et al., 2005).

In the present study, we demonstrate that the increase in blood pressure is paralleled by an increase in heart rate under slight anaesthesia. The alterations of both parameters result from differences in catecholamine release, which was significantly higher in *gsk3*^{KI} mice than in *gsk3*^{WT} mice.

Accordingly, administration of the α-adrenergic receptor antagonist prazosine or the ganglion blocker hexamethonium decresased the blood pressure to a larger extent in *gsk3*^{KI} mice than in *gsk3*^{WT} mice and virtually abolished the differences in blood pressure between *gsk3*^{KI} and *gsk3*^{WT} mice. Similar to what has been reported previously for several mouse strains (Berthonneche C, et al., 2009), administration of the β-adrenergic antagonist atenolol failed to significantly modify blood pressure in *gsk3*^{KI} and *gsk3*^{WT} mice. However, atenolol treatment virtually abrogated the differences of heart rate between *gsk3*^{KI} and *gsk3*^{WT} mice.

The disappearance of the differences in heart rate following atenolol treatment indicates that the differences were not due to a direct regulation of intrinsic cardiac pacemaking activity by GSK3. Isolated hearts of *gsk3*^{KI} mice show a similar heart rate as *gsk3*^{WT} mice (Webb IG, et al., 2010). Moreover, cardiac function was similar in *gsk3*^{KI} mice and *gsk3*^{WT} mice under anaesthesia (Webb IG, et al., 2010).

The differences in blood pressure and heart rate were in large part due to altered plasma catecholamine levels. Interestingly, the plasma concentrations of epinephrine, and norepinephrine were both significantly enhanced in *gsk3*^{KI} mice. Of note, epinephrine plasma levels measured during isoflurane anaesthesia were lower than during ether anaesthesia. This could be due to different lots of the ELISA test or the increased epinephrine release due to diethylether exposure (Laborie C et al, Neurosc Letters 2005). Thus, GSK3 apparently influences the synthesis of the catecholamines at an early step. In theory, the catecholamine concentrations could have been increased due to slowed degradation. However, the increased urinary excretion of the catecholamine metabolite vanilyl mandelic acid clearly demonstrates that excessive formation and not delayed degradation accounts for the enhanced catecholamine plasma levels in *gsk3*^{KI} mice. Accordingly, increased catecholamine production presumably accounts for blood pressure increase and tachycardia of *gsk3*^{KI} mice.

The *gsk3*^{KI} mice show hyperactive behaviour, which could further contribute or even account for the increased sympathetic activity. The role of GSK3 in the regulation of the sympathetic nerve system is further underscored by the contribution of GSK3 to the action of the drug cocaine.

The present observations do not rule out further GSK3 sensitive mechanisms affecting blood pressure regulation. For instance, inhibition of GSK3 by lithium has been reported to upregulate endothelial nitric oxide synthase (eNOS) (Nakatani K, et al., 2004).

Accordingly, resistance of GSK3 to the inhibitory effect of PKB/SGK could, at least in theory, decrease eNOS activity, which may favor an increase in blood pressure in gsk3^{KI} mice.

The present observations strongly suggest, however, that the differences of blood pressure between gsk3^{KI} and gsk3^{WT} mice are in large part due to differences in adrenergic stimulation.

The present paper does not define the origin of the catecholamines. However, increased catecholamine release has previously been shown to parallel manic behavior (Fitzgerald PJ, et al., 2009; Ising M, et al., 2006; Maas JW, et al., 1994 and Swann AC, et al., 1991), which has previously been observed in the gsk3KI mice (Ackermann TF, et al., 2010).

The mice display increased curiosity, decreased anxiety, hyperactivity, and decreased immobility in the forced swimming test (Ackermann TF, et al., 2010). Along those lines, antimanic drugs, such as clozapine, olanzapine, lithium, and valproate, are considered to affect PKB-dependent regulation of GSK-3 (Aubry JM, et al., 2009; Gould TD, et al., 2005 and Klein PS, et al., 1996).

Hyperactivity is similarly observed in transgenic mice overexpressing GSK-3 (Prickaerts J, et al., 2006). GSK-3 overexpression upregulates PKB expression and downregulates expression of PPP2R3A, a regulatory subunit of the PKB-inactivating phosphatase PP2A, effects leading to eventual inhibition of GSK3 and thus mitigation of the phenotype (Prickaerts J, et al., 2006). In the gsk-3^{KI} mice, this negative feedback is disrupted, and thus the phenotype is more pronounced than in GSK3 overexpressing mice. The role of GSK3 in the regulation of the sympathetic nerve system is further underscored by the contribution of GSK3 to the action of the drug cocaine

(Miller JS, et al., 2009). Collectively, those observations suggest that the enhanced catecholamine release is secondary to excessive central stimulation of the sympathetic nerve system. Along those lines, the differences in blood pressure between $gsk3^{KI}$ and $gsk3^{WT}$ mice were abrogated in the presence of ganglion blocker hexamethonium.

The increased blood pressure of $gsk3^{KI}$ mice presumably contributes to proteinuria (Boini KM, et al., 2009) of those mice. Accordingly, the proteinuria of $gsk3^{KI}$ mice was abrogated by lowering of blood pressure with prazosin (Boini KM, et al., 2009). Renal injury following 18 days of mineralocorticoid excess was again more pronounced in $gsk3^{KI}$ than in $gsk3^{WT}$ mice (Boini KM, et al., 2009), a finding again likely to result from enhanced blood pressure in $gsk3^{KI}$ mice. Beyond its role in catecholamine release and blood pressure control, GSK3 plays a pivotal role in cardiac hypertrophy (Fiedler B, et al., 2004; Sugden PH, et al., 2008 and Trivedi CM, et al., 2007). Specifically, $gsk3^{KI}$ mice are protected against cardiac hypertrophy following adrenergic stimulation (Webb IG, et al., 2010), and gene-targeted mice lacking GSK3α develop progressive cardiomyocyte and cardiac hypertrophy and contractile dysfunction (Zhou J, et al., 2010).

In conclusion, we demonstrate that PKB/Akt/SGKdependent phosphorylation of GSK3 participates in the control of catecholamine release and thus in blood pressure regulation. Disruption of the inhibitory effect of PKB/Akt/SGK on GSK3 leads to enhanced catecholamine release and increase of blood pressure.

GSK3, Phosphaturia and Calciuria

In an attempt to second aim, we confirmed the previous observation (Boini KM, et al., 2008) that creatinine clearance and urinary flow rate are higher in mice expressing protein kinase B(PKB)/serum and glucocorticoid-inducible kinase (SGK)-resistant glycogen synthase kinase GSK3 ($gsk3^{KI}$). With further estimations of electrolytes we found that compared with wild-type mice ($gsk3^{WT}$), $gsk3^{KI}$ mice are both hypercalciuric (high excretion of calcium in urine) and hyperphosphaturic (high excretion of phosphate in urine).

The homeostasis of inorganic phosphate is primarily determined by kidneys. The filtered Pi reabsorption is controlled by expression and activity of Na^+ -dependent phosphate transporters in the brush border membrane of the proximal tubule. These sodium-dependent Pi cotransporters

(Na/Pi-cotransporters) belongs to the SLC 20 (SLC20A2) and 34 (SLC34A1, SLC34A3) families. Therefore renal excretion of Pi is controlled by a number of hormones, including phosphatonins, and metabolic factors but only very little is known about intracellular signaling events mediating the control of these transporters (Biber J, et al., 2009, Murer H, et al., 2000 and 2004 and Picard N, et al., 2010). Parathyroid hormone, dopamine, phosphatonins (FGF23, sFRP-4, MEPE), glucocorticoids, atrial natriuretic peptide, phosphate loading, metabolic acidosis, carbonic anhydrase inhibitors and estrogen inhibits proximal tubular Pi reabsorption whereas growth hormone, insulin-like growth factors , 1,25(OH)2D3, and phosphate depletion increased Pi reabsorption. Our results showed that GSK3 plays an important role as evident from the stimulation of NaPi-IIa activity in *Xenopus* oocytes by GSK3 and the decreased phosphate fluxes in isolated BBMVs from $gsk3^{KI}$ mice. Reduced activity of SLC34 transporters (NaPi-IIa and NaPi-IIc) and other PFA-resistant phosphate transporters was detected. The expression of Pit2, resistant to PFA, was, however, not altered, suggesting that either only its activity is affected by GSK3 or other unknown PFA-resistant phosphate transporters contribute to the residual activity in the presence of PFA.

As we know that phosphaturia may either primary (excess excretion of phosphate by kidney, as from primary renal dysfunction) or secondary (excess excretion of phosphate in urine due to hyperparathyroidism origin). The phosphaturia of $gsk3^{KI}$ mice cannot be explained by plasma phosphate concentration, which was actually significantly decreased.

Plasma PTH level is another major regulator of renal phosphate transport (Murer H, et al., 2000). PTH acts directly on proximal tubular cells and inhibits both apical and basolateral Na/pi cotransporters by mechanisms that involve the internalization of cell surface Npt2a protein (Kempson SA et al., 1995) and its subsequent lysosomal degradation (Pfister MK et al., 1998). PTH binding to receptors on the basolateral membrane activates protein kinase A(PKA) and/or protein kinase C (PKC) signaling pathways, whereas PTH binding to apical receptors activates PKC (Traebert M, et al., 2000). The PKA and PKC signaling pathways converge on the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway to internalize Npt2a protein (Bacic D et al., 2003). Although the downstream targets for ERK/MAPK-mediated phosphorylation remain unknown, changes in the phosphorylation state of Npt2a are not associated with its PTH-induced internalization (Jankowski M et al., 2001).

Rather, regulation of Na/Pi cotransport by PTH may be achieved by the phosphorylation of proteins that associate with Npt2a.

In brief PTH stimulates the internalization and subsequent degradation of NaPi-IIa (Murer H, et al., 2000 and Bacic D, et al., 2006). The hormone thus enhances renal phosphate excretion and decreases plasma phosphate concentration. A decreased plasma PTH concentration is thus expected to enhance renal phosphate reabsorption and to increase plasma phosphate concentration, which was the opposite of what we observed in *gsk3*^{ki} mice.

The release of PTH is mainly controlled by serum calcium concentration through negative feedback. Also partially decreased plasma phosphate concentration inhibits PTH release (Martin DR, et al., 2005). However, the decrease of the plasma phosphate level is very discrete and hardly accounts for the low plasma PTH levels.

As we know that plasma PTH stimulates the renal 1α-hydroxylase (Portale AA, et al., 2000), the rate-limiting enzyme of 1,25-(OH)2D3 formation (Portale AA, et al., 2000 and Kato S, 1999). Thus low plasma PTH levels are expected to decrease the plasma 1,25-(OH)2 D3 concentration. As expected, the *gsk3*^{KI} mice displayed decreased plasma 1,25-(OH)2 D3 concentration. On the other hand, formation of 1,25-(OH)2D3 is stimulated in a PTH-independent manner by cellular phosphate depletion (Perwad F, et al., 2005). The transcript levels of the 1α-hydroxylase tended to be enhanced in *gsk3*^{KI} mice. The possibility must be considered that GSK3 influences 1α-hydroxylase and 1,25-(OH)2D3 formation by further mechanisms in addition to its influence on phosphate balance and PTH plasma concentration.

However, PTH enhances the uptake of phosphate from the intestine into the blood (Brown AJ, et al., 2002). In the intestines, absorption of phosphate is mediated by an increase in activated vitamin D (1,25-(OH)2D3). Hence decreased 1,25-(OH)2D3 formation is expected to decrease intestinal phosphate absorption and that impaired intestinal phosphate uptake could contribute to the development of hypophosphatemia, which was found in *gsk3*^{KI} mice.

Moreover, PTH enhances the absorption of calcium across the intestinal brush border membrane, passing through ion channels such as TRPV6 (Barley NF et al., 2001). Calbindin is a vitamin D-dependent calcium-binding protein in intestinal epithelial cells which functions together with TRPV6 and calcium pumps (PMCA1) in the basal membrane to actively transport calcium into the blood. Thus *gsk3*^{ki} mice having low plasma PTH and 1,25-(OH)2D3 expected to absorb less calcium to cause hypocalcemia. ,which could be compensated by bone resorption in

osteoclasts. Since PTH and 1,25-(OH)2D3 stimulate renal tubular Ca^{2+} reabsorption via upregulation of the TRPV5 channel (Hoenderop JG, et al., 2005, Woudenberg-Vrenken TE, et al., 2009 and Friedman PA, 2000), the decreased plasma PTH and 1,25-(OH)2D3 concentration in *gsk3*^{KI} mice could thus contribute to the calciuria of *gsk3*^{KI} mice by affecting translation, trafficking, and/or activity of TRPV5 channels.

Despite the decreased PTH plasma concentration, the bone mass was significantly decreased in *gsk3*^{KI} mice. Thus, the renal Ca^{2+} and phosphate loss of *gsk3*^{KI} mice may impair mineralization of bone. Phosphate is known to inhibit the generation of new and to stimulate apoptosis of mature osteoclasts (Kanatani M, et al., 2003). Moreover, Akt/PKB-dependent regulation of GSK3 may more directly participate in the regulation of bone cells. Akt/PKB regulates the survival of both osteoblasts (Chaudhary LR, et al., 2001) and osteoclasts (Kwak HB, et al., 2008 and Lee SE, et al., 2002).

In conclusion, Akt/PKB-dependent regulation of GSK3 participates in the control of renal tubular phosphate transport. Loss of Akt/PKB-dependent inhibition of GSK3αβ leads to renal phosphate wasting, which presumably contributes to or even accounts for the decrease of PTH release with resulting calciuria, decreased formation of 1,25-(OH)2 D3, and decreased mineralization of bone.

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13. PUBLICATIONS OBTAINED DURING Ph.D. WORK

1. *Effects of Gum Arabic (Acacia senegal) on renal function in diabetic mice.*
   Omaima Nasir, Rexhep Rexhepaj, Teresa F. Ackermann, Madhuri Bhandaru, Ammar Ebrahim, Ferruh Artunc, Anja T. Umbach, Daniela S. Kempe, Goverdhan Puchchakayala, ***Balasaheb Siraskar****, Michael Föller, Amal Saeed, Florian Lang*
   Kidney & Blood Pressure Research Jan 2012.

2. *Enhanced catecholamine release in mice expressing PKB/SGK-resistant GSK3.*
   Balasaheb Siraskar*, Jakob Völkl, Mohamed Siyab el din Ahmed, Michae Hierlmeier, Shuchen Gu, Evi Schmid, Christina Leibrock, Michael Föller, Undine E. Lang, Florian Lang*
   Europian J of Physiology 2011.

3. *PKB/SGK-resistant GSK3 enhances phosphaturia and calciuria.*
   Föller M, Kempe DS, Boini KM, Pathare G, ***Siraskar B****, Capuano P, Alesutan I, Sopjani M, Stange G, Mohebbi N, Bhandaru M, Ackermann TF, Judenhofer MS, Pichler BJ, Biber J, Wagner CA, Lang F.*
   J Am Soc Nephrology (JASN). 2011 May; 22(5):873-80. Epub 2011 Apr 14.

4. *Decreased bone density and increased phosphaturia in gene-targeted mice lacking functional serum- and glucocorticoid-inducible kinase 3.*
   Bhandaru M, Kempe DS, Rotte A, Capuano P, Pathare G, Sopjani M, Alesutan I, Tyan L, Huang DY, ***Siraskar B****, Judenhofer MS, Stange G, Pichler BJ, Biber J, Quintanilla-Martinez L, Wagner CA, Pearce D, Föller M, Lang F.*
   Kidney Int.2011 Jul; 80(1):61-7. doi: 10.1038/ki.2011.67. Epub 2011 Mar 30.

5. *Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice.*
   Siraskar B*, Ballal A, Bobbala D, Föller M, Lang F.*
   Cell Physiol Biochem. 2010; 26(3):347-54. Epub 2010 Aug 24.

6. *Hyperaldosteronism in Klotho-deficient mice.*

   *Fischer SS, Kempe DS, Leibrock CB, Rexhepaj R, **Siraskar B**, Boini KM, Ackermann TF, Föller M, Hocher B, Rosenblatt KP, Kuro-O M, Lang F.*

   Am J Physiol Renal Physiol. 2010 Nov;299(5):F1171-7. Epub 2010 Aug 18.

7. *Responses to diuretic treatment in gene-targeted mice lacking serum- and glucocorticoid-inducible kinase.*

   *Artunc F, Ebrahim A, **Siraskar B**, Nasir O, Rexhepaj R, Amann K, Friedrich B, Risler T, Lang F.*

   Kidney Blood Press Res. 2009; 32(2):119-27. Epub 2009 Apr 25.

8.

14. POSTER PRESENTATIONS/CONFERENCES

1. ***Down-regulation of the renal outer medullary K+ channel ROMK by the AMP-activated protein kinase (AMPK).***
   Balasaheb Siraskar, Dan Yang Huang, Gulab Siraskar, Mentor Sopjani, Michael Föller, Florian Lang.

   91st Annual Meeting of the German Physiological Society.

   DPG 2012, March 22 – 25, 2012, in Dresden, Germany.

2. ***The role of AMP-activated protein kinase (AMPK) in renal function after ischemic/reperfusion injury in mice.***
   Balasaheb Siraskar, Dan Yang Huang, Huanhuan Gao, Shuchen Gu, Lang F.
   Institute of Physiology, Eberhard Karls Universität Tuebingen, Tuebingen, December 2011.

3. ***Enhanced catecholamine release in mice expressing PKB/SGK-resistant GSK3.***
   Balasaheb Siraskar, Jakob Völkl, Mohamed Siyab el din Ahmed, Michael Hierlmeier, Shuchen Gu, Evi Schmid, Christina Leibrock, Michael Föller, Florian Lang.
   Institute of Physiology, Eberhard Karls Universität Tuebingen, Tuebingen, Sept. 2011.

4. ***Elevated blood pressure in Gαi2 knockout mice.***
   Balasaheb Siraskar, Dan Yang Huang, Nürnberg B, Florian Lang.
   Department of Pharmacology and Toxicology, Eberhard Karls Universität Tuebingen, Tuebingen, Jun. 2010.

5. ***Renal function of PDK1 hypomorphic mice.***
   Balasaheb Siraskar, Kempe D.S., Boini K.M., Artunc F., Henke G., Sandulache D., Grahammer F., Nammi S., Lang F.

   88th Annual Meeting of the German Physiological Society.
   DPG 2009, March 22 – 25, 2009, in Giessen, Germany.

6. ***Responses to diuretic treatment in gene-targeted mice lacking serum- and glucocorticoid-inducible kinase 1.***

   Balasaheb Siraskar, Artunc F, Ebrahim A, Nasir O, Rexhepaj R, Amann K, Friedrich B, Risler T, Lang F.

   Nephrology Congress, Tuebingen, Germany, September 2008.

15. ACADEMIC TEACHERS

Curriculum Vitae

Mr. Siraskar B. D.

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Present Address:
Institute of Physiology,
University of Tuebingen,
Tuebingen Germany,
72076.

Permanent Address:
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Pune, 412207

Personal Details:

Date of Birth *: 06-06-1974*

Blood Group *: O^+*

Height *: 164 cm*

Weight *: 63 Kg*

Gender *: Male*

Marital Status *: Married*

Nationality *: Indian*

Languages Known:
English, Hindi & Marathi

E-Mail Address:
balasaheb.siraskar@student.uni-tuebingen.de
b_siraskar1975@yahoo.com

Career Objective

To pursue a Postdoctoral Fellowship to effectively utilize and enhance my knowledge and technical skills and to develop as an individual scientist

Educational background

Ph. D (Pharmacy)

Submitted thesis entitled, ***PKB/SGK-Resistant GSK3 Enhances Phosphaturia, Calciuria, and Catecholamine Release*** *for Ph.D. degree.*

M. Pharmacy (Pharmacology)

From Department of Pharmacology, Poona College of pharmacy, Pune University, Pune (MS), India securing overall ***First class*** *for the thesis in 2000.*

B. Pharmacy

From Bharati Vidhyapeeth's. Poona College of Pharmacy, Pune University, Pune (MS), India with ***first class*** *in 1998.*

H.S.C. (Chemistry, Physics, Mathematics & Biology)

From YCM, Nanded, Aurangabad Divisional Board, Aurangabad (MS) India ***first class*** *with* ***distinction*** *in 1994.*

S.S.C (General)

From Aurangabad Divisional Board, Aurangabad (MS) India ***first class*** *with* ***distinction*** *in 1991.*

Employment Details

1. *Working as a Doctoral student in the Institute of Physiology, Tuebingen, Germany from August 2008 to till today.*
2. *Worked as an Assistant Professor in pharmacology in Dr. D.Y. Patil Pratishthan's Dr. D.Y. Patil College of Pharmacy, Pimpri, Pune from 1st August 2005 to July 2008.*
3. *Worked as a Lecturer in pharmacology in Dr. D.Y. Patil Pratishthan's Dr. D.Y. Patil College of Pharmacy, Pimpri, and Pune from 1st September 2004 to 31st July 2005.*

Research Interest:
Renal and Cardiovascular System.

References:
Dr. Ferruh Artunc, MD
Unviersity of Tuebingen
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Otfried-Mueller-Str. 10
72076 Tuebingen
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ferruh.artunc@med.uni-tuebingen.de

Dr. (Mrs.) **Danyang Huang Ph.D.**
Institute of Pharmacology and Toxicology,
Wilhelmstr.56
72074, Tuebingen
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4. *Worked as a Lecturer in pharmacology in Bharati Vidyapeeth's College of Pharmacy, Kolhapur from 1st August 2000 to 31st August 2004*

Achievements

*Qualified GATE (Graduate Aptitude Test in Engineering) an exam conducted by the Indian Institute of Technology-**Powai**, in 1998 with 97 percentile Less than 5% students qualify this exam*

Publications (International Journals)

1. *Effects of Gum Arabic (Acacia senegal) on renal function in diabetic mice Kidney & Blood Pressure Research Fri, 6 Jan 2012*
   *Omaima Nasir, Rexhep Rexhepaj, Teresa F. Ackermann, Madhuri Bhandaru, Ammar Ebrahim, Ferruh Artunc, Anja T. Umbach, Daniela S. Kempe, Goverdhan Puchchakayala, **Balasaheb Siraskar**, Michael Föller, Amal Saeed, Florian Lang*
   Kidney & Blood Pressure Research Jan 2012.

2. *Enhanced catecholamine release in mice expressing PKB/SGK-resistant GSK3*
   Balasaheb Siraskar**, Jakob Völkl, Mohamed Siyab el din Ahmed, Michae Hierlmeier, Shuchen Gu, Evi Schmid, Christina Leibrock, Michael Föller, Undine E. Lang, Florian Lang*
   Europian J of Physiology 2011.

3. *PKB/SGK-resistant GSK3 enhances phosphaturia and calciuria.*
   *Föller M, Kempe DS, Boini KM, Pathare G, **Siraskar B**, Capuano P, Alesutan I, Sopjani M, Stange G, Mohebbi N, Bhandaru M, Ackermann TF, Judenhofer MS, Pichler BJ, Biber J, Wagner CA, Lang F.*
   J Am Soc Nephrology (JASN). 2011 May; 22(5):873-80. Epub 2011 Apr 14.

4. *Decreased bone density and increased phosphaturia in gene-targeted mice lacking functional serum- and glucocorticoid-inducible kinase 3.*
   *Bhandaru M, Kempe DS, Rotte A, Capuano P, Pathare G, Sopjani M, Alesutan I, Tyan L, Huang DY, **Siraskar B**, Judenhofer MS, Stange G, Pichler BJ, Biber J, Quintanilla-Martinez L, Wagner CA, Pearce D, Föller M, Lang F.*
   Kidney Int.2011 Jul; 80(1):61-7. doi: 10.1038/ki.2011.67. Epub 2011 Mar 30.

5. *Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice.*
   Siraskar B*, *Ballal A, Bobbala D, Föller M, Lang F.*
   Cell Physiol Biochem. 2010; 26(3):347-54. Epub 2010 Aug 24.

6. *Hyperaldosteronism in Klotho-deficient mice.*
   *Fischer SS, Kempe DS, Leibrock CB, Rexhepaj R, **Siraskar B**, Boini KM, Ackermann TF, Föller M, Hocher B, Rosenblatt KP, Kuro-O M, Lang F.*
   Am J Physiol Renal Physiol. 2010 Nov;299(5):F1171-7. Epub 2010 Aug 18.

7. *Responses to diuretic treatment in gene-targeted mice lacking serum- and glucocorticoid-inducible kinase.*
   *Artunc F, Ebrahim A, **Siraskar B**, Nasir O, Rexhepaj R, Amann K, Friedrich B, Risler T, Lang F.*
   Kidney Blood Press Res. 2009; 32(2):119-27. Epub 2009 Apr 25.

Papers Under Revision

1. *Down-regulation of the renal outer medullary K^+ channel ROMK by the AMP-activated protein kinase*
   Balasaheb Siraskar*, *Dan Yang Huang, Gulab Siraskar, Mentor Sopjani, Michael Föller, Florian Lang*
   Europian J of Physiology 2012.

Papers Written (By Lang F)

1. *G alpha i2-sensitive electrogenic intestinal glucose absorption.*
   Balasaheb Siraskar[1,2]*, *Dan Yang Huang[1], , Rexhep Rexhepaj[2], Shuchen Gu[2], Bernd Nürnberg[1], Florian Lang[2]*

Publications (Indian journals)

1. *A research paper entitled, "Anti-asthmatic Activities of Ethanolic Extract of Stem Bark Bauhinia variegate Linn", published in Advances in Pharmacology & Toxicology, Vol. 8, Issue3, Dec. 2007, 67-72.*

2. *A research paper entitled, "Study of Hydroalcoholic Extract of portulaca oleracea L. for Immunomodulatory Activity In Mice", published in International Journal of Green Pharmacy, Vol.1, Issue2, July-Sept. 2007, 45-50.*

3. *A research paper entitled, "Haematinic Activity of Ethanolic Extract of Stem Bark Bauhinia variegate Linn", published in International Journal of Green Pharmacy, Vol.1, Issue3, Oct.-Dec. 2007, 28-33.*

4. *A research paper entitled, "Study of Hydroalcoholic Extract of portulaca oleracea L. for Immunomodulatory Activity In Mice", published in Advances in Pharmacology & Toxicology, Vol. 8, Issue3, Dec. 2007, 109-116.*

5. *A research paper entitled, "Hypoglycemic and Antihyperglycemic activity of Nardostachys jatmansi Roots", for 'Nigerian Journal of Natural products and Medicine.*

Poster presentations

1. *Renal function of PDK1 hypomorphic mice (473) 88th Annual Meeting of the German Physiological Society, Giessen, Germany. Monday, March 23, 2009.*

2. *Evaluation of Antiulcer Effect of Raphaus sativus L. in Rats.*
   *Kothavade P.S.[a], Rade C.P.[b], Takarkhede S.[b] **Siraskar B.D.**[a] IPCA Tuesday, January 6, 2009*

3. *Responses to diuretic treatment in gene-targeted mice lacking serum- and glucocorticoid-inducible kinase 1.*
   *Artunc F, Ebrahim A, **Siraskar B**, Nasir O, Rexhepaj R, Amann K, Friedrich B, Risler T, Lang F. Nephrology Congress, Tuebingen, Germany, September 2008.*

Research Guidance for (M. Pharm Students)

1. *Evaluation of Immunomodulatory Activity of Few Psychopharmacological Drugs in Experimental Animals-Parikh Achint Bipinbhai (2004-05).*

2. *A Study of Potential Anti-Inflammatory Activity of a Herbal Drug- Nilesh E. Borkar (2004-05).*

3. *Pharmacological Evaluation of a Herbal Drug for Wound Healing Activity- Gauri Kulkarni (2004-05).*

4. *Evaluation of Immunomodulatory Activity of a Herbomineral Drug-Vaibhav V. Mandre (2004-05).*

5. *Pharmacological Evaluation of a Herbal Drug for Its Antidiabetic Activity And Diabetic Complications In Experimental Animals-Deepti Talera (2004-05).*

6. *Evaluation of Antiasthmatic Activity of a Herbal Drug- Rajesh R. Patil (2004-05).*

7. *Study of Antiasthmatic Activity of a Herbal Drug- Anand Bokare (2004-05).*

8. *Study of Hepatoprotective and Hepatoregenerative Activity of A Polyherbal Mixture In Laboratory Animals- Vikram Nimbalkar (2005-06).*

9. *Protective Effect of Trigonella Foenum Graecum Linn. In Doxorubicin Induced Toxicities In Laboratory Animals. - Nilesh S. Badgujar (2005-06).*

10. *To Study Immunomodulatory Activity of Few H2-Antagonist And B2 Agonist In Experimental Animals. - Atit A. Seth. (2005-06).*

11. *Anti-asthmatic Activity of Ethanolic Extract of Stem bark of Bauhinia variegata Linn. - Dhonde Satish M. (200-07)*

12. *Study of Hydroalcoholic Extracts of Portulaca oleracea L. for Immunomodulatory Activity in Mice. - Kulkarni Ashish V (200-07)*

13. *Study of Hydroalcoholic Extract of Portulaca oleracea L. for Hepatoprotective Activity. - Kulkarni Abhijeet S. (200-07)*

14. *Evaluation of Hepatoprotective Activity of Methanolic Extract of Leaves and Stems of Derris trifoliate Lour. - Bingi Shivaji S. (200-07).*

Sponsored research Activities

1. *Recipient of Rs.3, 00,000/-from Pune University (2006-2008) for project: Study of Herbal Mixtures Consisting Various Indigenous Plants for Their Hepataprotective And or Hepatoregenerative Study on Laboratory Animals.*

2. *Recipient of Rs.20, 000/-from Sharangdhar Pharmaceuticals Ltd., Pune (1998-2000) for project: Evaluation of Novel Herbal Drug for its Hepatoprotective Activity.*

Membership under Govt. / Professional Bodies

1. *Registered as a Pharmacist under Maharashtra State Pharmacy Council (MSPC) Reg.No.49469.*

2. *Registered as a Life member under Association of Pharmacy Teachers of India (APTI) Reg. No. MA/LM-617.*

3. *Registered as a Life member under Indian Pharmacological Society (IPS). Reg. No. LS/500.*

Fellowship of Academic Bodies

Qualified GATE-98 with 97.11 percentile and obtained fellowship from AICTE.

Conferences, Short Term Courses/Participated, Attended

1. Completed training workshop on 'Techniques of Animal Tissue Culture' conducted at Centre For Genetic Diagnosis, Deenanath Mangeshkar Hospital & Research Centre, Pune.

2. Attended one day seminar on 'Proteomics & Genomics sponsored by University of Pune on 1st Dec. 2007 at MMM's College of Pharmacy, Pune.

3. Attended '54th Indian Pharmaceutical Congress 2002' at Bharati Vidyapeeth's Poona College of Pharmacy, Pune.

4. Participated one day seminar on 'Rheololgy of Pharmaceutical and Cosmetic Products' organized on 18th August 2003, at Bharati Vidyapeeth's Poona College of Pharmacy, Pune.

5. Attended 'Refresher course Cum Workshop' for Pharmacist sponsored by PCI conducted at Bharati Vidyapeeth's Poona College of Pharmacy, Pune on 4th and 5th March 2000.

Examiner/Paper setter/Moderator

Appointed as Examiner, paper setter and moderator in Pune University, Shivaji University & SRTM University for undergraduate examination.

Declaration

The information given in these curriculum vitae is correct.
Date: 26-07-2012

Place: Tuebingen, Germany ***Balasaheb Siraskar***