EMD638683, a Novel SGK Inhibitor with Antihypertensive Potency

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Key Words

Metabolic syndrome • Type II diabetes • Hyperinsulinism • Blood pressure • Salt sensitivity

Abstract

The serum- and glucocorticoid-inducible kinase 1 (SGK1) is transcriptionally upregulated by mineralocorticoids and activated by insulin. The kinase enhances renal tubular Na⁺-reabsorption and accounts for blood pressure increase following high salt diet in mice made hyperinsulinemic by dietary fructose or fat. The present study describes the *in vitro* and *in vivo* efficacy of a novel SGK1 inhibitor (EMD638683). EMD638683 was tested *in vitro* by determination of SGK1-dependent phosphorylation of NDRG1 (N-Myc downstream-regulated gene 1) in human cervical carcinoma HeLa-cells. *In vivo* EMD638683 (4460 ppm in chow, i.e. approx. 600 mg/kg/day) was administered to mice drinking tap water or isotonic saline containing 10% fructose. Blood pressure was determined by the tail cuff method, and urinary electrolyte (flame photometry) concentrations determined in metabolic cages. *In vitro* testing disclosed EMD638683 as a SGK1 inhibitor with an IC₅₀ of 3 µM. Within 24 hours *in vivo* EMD638683 treatment

significantly decreased blood pressure in fructose/saline-treated mice but not in control animals or in SGK1 knockout mice. EMD638683 failed to alter the blood pressure in SGK1 knockout mice. Following chronic (4 weeks) fructose/high salt treatment, additional EMD638683 treatment again decreased blood pressure. EMD638683 thus abrogates the salt sensitivity of blood pressure in hyperinsulinism without appreciably affecting blood pressure in the absence of hyperinsulinism. EMD638683 tended to increase fluid intake and urinary excretion of Na⁺, significantly increased urinary flow rate and significantly decreased body weight. Conclusion: EMD638683 could serve as a template for drugs counteracting hypertension in individuals with type II diabetes and metabolic syndrome.

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Introduction

Insulin is antinatriuretic in rats, dogs and humans, and presumably accounts for the development of salt-sensitive hypertension in type II diabetes [1-3]. The antinatriuretic effect of insulin appears to result from direct action on tubular transport in distinct nephron seg-

ments [4-7]. Insulin stimulates the epithelial Na⁺ channel (ENaC) and may thus lead to renal retention of NaCl [7-9], which in turn favours the development of hypertension [10, 11]. The effect of insulin on ENaC requires activation of the phosphatidylinositol-3-kinase (PI3K) [12-14].

The regulation of ENaC activity by insulin involves the serum- and glucocorticoid-inducible kinase (SGK1) [15, 16]. Expression of SGK1 has previously been shown to be stimulated by mineralocorticoids [17-19]. SGK1 is activated by insulin through a signalling cascade involving PI3K and phosphoinositide-dependent kinase (PDK1) [15]. Targets of SGK1 action include the epithelial Na⁺ channel (ENaC) [17, 20-22]. SGK1 is expressed in the aldosterone-sensitive distal nephron [18], and presumably contributes to the effect of aldosterone, insulin like growth factor (IGF-1) and insulin on ENaC activity and thus renal salt reabsorption [13, 23-25]. Moreover, SGK1 has previously been shown to be critically important for glucocorticoid-induced hypertension [26].

SGK1 stimulates renal tubular salt reabsorption in addition by upregulation of the NaCl cotransporter [27, 28]. Beyond that, SGK1 stimulates other renal transport molecules including K⁺ channels, the Na⁺/K⁺ATPase, the Na⁺, K⁺, 2Cl⁻ cotransporter NKCC2, several Ca²⁺ channels, the Na⁺/H⁺ exchanger NHE3, and a variety of organic substrate transporters, e.g. Na⁺-coupled glucose transporter SGLT1, peptide and amino acid transporters [29-33].

Urinary Na⁺ excretion is normal in SGK1 knockout mice under normal salt intake, but cannot be adequately decreased following exposure to salt deficient diet [34]. Thus, dietary salt deficiency leads to marked decrease of glomerular filtration rate and blood pressure in those mice [34]. Conversely, enhanced SGK1 activity may be expected to increase blood pressure by stimulation of both renal Na⁺ reabsorption [34] and salt appetite [35]. As a matter of fact, a certain variant of the SGK1 gene has been associated with increased blood pressure in humans [36].

SGK1 apparently plays a decisive role in the hypertensive effect of hyperglycemia and hyperinsulinemia [15]. Hyperglycemia upregulates SGK1, which in turn participates in the upregulation of ENaC. Both, high fat diet [37] and fructose loading [38], lead to hyperglycemia, hyperinsulinism and increase in blood pressure [4, 37, 39-46]. In normoinsulinemic mice, the exposure to high salt diet leads to an only slight increase of blood pressure, which is similar in gene-targeted mice lacking functional SGK1 (*sgk1*^{-/-}) and wild type mice (*sgk1*^{+/+}) [37, 38]. Following induction of hyperinsulinemia by high fat diet [37] or

Fig. 1. Formula of the SGK-inhibitor EMD638683

high fructose intake [38], however, the intake of salt leads to a sharp increase of blood pressure in *sgk1*^{+/+} mice, an effect completely lacking in *sgk1*^{-/-} mice [37, 38]. According to those observations, the hypertension following hyperinsulinism fully depends on the presence of SGK1. Along those lines, the SGK1 gene variant previously shown to be associated with increased blood pressure [36, 47] was later observed to be associated with enhanced insulin sensitivity of blood pressure [48].

In view of those observations, a SGK1-inhibitor was expected to lower blood pressure in patients with hyperglycemia and salt excess as well as in animals under fructose & high salt intake. Thus, an inhibitor against SGK1 was developed (EMD638683, Fig. 1) and the effect of this inhibitor on blood pressure in animals pretreated with fructose and high salt intake was determined. To further explore the role of SGK1 during EMD638683, experiments have been performed in SGK1 knockout mice (*sgk1*^{-/-}) and wild type mice (*sgk1*^{+/+}).

Materials and Methods

In vitro assays

In a first series of experiments, the inhibitory effect of EMD638683 on SGK1 was compared to that on a panel of other kinases (Table 1a). The methods employed have been described in detail previously [49]. In short, most kinases tested were of human origin and full length. The AMPK (AMP-activated protein kinase) complex was purified from rat liver. The other kinases were either expressed as GST (glutathione transferase) fusion proteins in *Escherichia coli* or as hexahistidine (His6)-tagged proteins in Sf21 (*Spodoptera frugiperda* 21) insect cells. The methods of kinase activation have been described previously [49]. Determinations of protein kinase activities were carried out robotically at room temperature (21 °C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, U.S.A.) in a 96-well format. The substrates used for the individual protein kinases were described previously [49]. The assays were initiated with MgATP and stopped by the addition of 5 µl of 0.5 M orthophosphoric acid and spotted

on to P81 filter plates using a unifilter harvester (PerkinElmer, Boston, MA, U.S.A.). In a series of single experiments the activities of the kinases in the presence of 1 μ M EMD638683 were compared to that in the absence of EMD638683.

In a second series of experiments, IC₅₀ values of EMD638683 were determined after carrying out assays at ten different concentrations of EMD638683 for each kinase listed in Table 1b.

In a third series of experiments, the inhibitory effect of EMD638683 was determined in HeLa-cells. To this end, phosphorylation of N-Myc downstream-regulated gene 1 (NDRG1) protein, which has previously been shown to be a specific SGK1 target [50], was determined in human cervical carcinoma (HeLa) cells, which express NDRG1 but none of the other isoforms [50]. Results of this series are provided in Fig. 2. The cells were plated in 6-wells MTPs (Costar Corning, # 3506) at a density of 10 – 20 $\times 10^3$ cells/cm² in Dulbecco's DMEM medium, supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine and 1 mM sodium pyruvate. After 24 hrs at 37 °C and 5 % CO₂ in a cell incubator, each well was further supplemented with 25 μ l of a 100X dimethyl sulfoxide (DMSO) solution of the compound; the solution was diluted 100-fold in the supernatant of the cell culture, thus resulting in the anticipated SGK1-inhibitor concentration at a 1 % DMSO concentration. DMSO was routinely using in these experiments in order to be able to compare the effect of EMD638683 to the effect of other, less soluble compounds, which are not reported in this paper. The cells were then incubated for another 24 hrs under the same conditions.

Thereafter, the supernatants were removed (by suction) and the cells washed 1 x with 1 ml /well ice cold phosphate-buffered saline (PBS) solution. To each well, 250 μ l of the ice-cold lysis-buffer (50 mM Tris/HCl, 1 mM ethylene glycol tetraacetic acid (EDTA), 0.5 mM activated Na₃VO₄, 10 mM glycerophosphate, 50 mM NaF, 5 mM Na-pyrophosphate, 1 % Triton X100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 1 μ M microcystin and 1 μ g/ml each of aprotinin, pepstatin and leupeptin) were added. The cells were scraped off the bottom of the well and the cell suspension was sucked up several times with an Eppendorf pipette to lyse and homogenize the cells. The cell lysates (250 μ l/vial) were transferred into pre-chilled Eppendorf vials (at – 24 °C). The cell suspensions were sonified for 1-2 sec; the cell lysates were shock frozen with liquid nitrogen and stored at – 24 °C.

16 μ l aliquots of the cell lysates were transferred to 6 μ l of the 4 X NuPage[®] lithium dodecyl sulfate LDS-sample buffer plus 1 μ l β -mercaptoethanol and heated for 10 min at 70 °C. For the detection of the P-NDRG1- and NDRG1-levels, 20 μ l aliquots of the samples were loaded onto a NuPage[®] -SDS-gel (4 – 12 % Bis-Tris-Gel [for P-NDRG1-detection] or 7% Bis-Tris-Gel [for the detection of NDRG1]) and separated according to the protein size. The protein bands were electrophoretically transferred onto 0.2 μ m nitrocellulose membranes and immunoblotted using NDRG1 or NDRG1 phospho Thr x 3 antisera, respectively, at a concentration of 1 μ g/ml. Both antisera were obtained from Prof. Sir Philip Cohen, Division of Signal Transduction Therapy, University of Dundee, Scotland. For the detection of P-NDRG1,

10 μ g/ml of the not phosphorylated nona-peptide RSRSHSEGE was added to the incubation buffer.

Binding of the primary antibody was detected using rabbit peroxidase conjugated antishoop IgG antibody (1 : 5000 dilution, Calbiochem) followed by enhanced chemiluminescence (SuperSignal West Dura Extended Duration, Pierce). The P-NDRG1-level was expressed normalized to the NDRG1-level in the samples, which were determined after stripping the Nitrocellulose membranes using the RestoreTM Western Blot Stripping Buffer and Procedure from Pierce.

When using the P-NDRG1-antiserum, a decrease in the phosphorylation level of the NDRG1 protein could be detected easily. The decrease in the intensity of the band on the Western blot, using the P-NDRG1- antiserum was determined, plotted on a semi-logarithmic plot against the cell culture medium concentration of the SGK1-inhibitor and used for the assessment of the cellular inhibitory potency (IC₅₀-value) of the SGK1-inhibitor, which was obtained after a 24 hours incubation of the cells in cell medium plus the respective concentrations of EMD638683. At this time point, the extra- and intracellular concentrations were considered to be in equilibrium.

In vivo experiments

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

Mice deficient in SGK1 (*sgk1*^{-/-}) were generated, bred and genotyped as previously described [34, 51]. The experiments were performed in mice on original SV129 background backcrossed for 4 generations to C57BL/6J. SGK1 wild type (*sgk1*^{+/+}) mice (3 males and 3 females, age 4 months) were fed a control diet (diet 1314, 0.2% Na⁺, 0.7% K⁺, Altromin, Lage, Germany) and had free access to tap drinking water before the experiments. To develop hyperinsulinemia in mice, tap drinking water was replaced by 10 % fructose for 3 weeks. Subsequently, isotonic saline was added to the fructose containing drinking water for further 14 days.

For evaluation of renal excretion, the mice were placed individually in metabolic cages (Techniplast Hohenpeissenberg, Germany) for 24 hours urine collection [52]. They were allowed a 3-day habituation period when food & water intake, urinary flow rate, urinary excretion of salt, and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 12 hours collection (7 pm to 7 am) of urine was performed to obtain the urinary parameters. As blood pressure measurements were made during the day, a 24 hours collection of urine was not possible in this series of experiments. To assure quantitative urine collection, metabolic cages were siliconized and urine was collected under water-saturated oil. In the following, the control food was replaced by placebo food for four days, and then the placebo food was replaced by food containing EMD638683 (4460 ppm in the chow, which resulted in a dose of approx. 600 mg/kg/day) for 4 days. Finally, the EMD638683 containing food was again replaced by placebo food. As a control series, placebo food and EMD638683 containing food was administered to *sgk1*^{+/+} and *sgk1*^{-/-} mice (n = 3-5 males and 2-3 females, age 4 months) that were fed control food and tap drinking water with-

Table 1a. Inhibition of kinases by EMD638683. Residual activities of the respective kinases at 1 μ M EMD638683 in % of the value in the absence of EMD638683. Results from single experiments. The percent values for those kinases, which exhibited a percent value of < 50 % (e.g.: MSK1, PRK2, and the SGK isoforms SGK2 and SGK3), are highlighted in bold.

MKK1	MAPK-integrating protein kinase 1	79
MAPK2	mitogen-activated protein kinase 2	91
JNK1- α -1	c-Jun N-terminal kinase-alpha-1	84
SAPK2- α	stress-activated protein kinase-alpha	132
SAPK2- β	stress-activated protein kinase-beta	94
SAPK3	stress-activated protein kinase 3	106
SAPK4	stress-activated protein kinase 4	69
MAPKAP-K1 α	MAPK-activated protein kinase-K1-alpha	90
MAPKAP-K2	MAPK-activated protein kinase-K2	88
MSK1	mitogen- and stress-activated protein kinase 1	32
PRAK	p38-regulated activated kinase	94
PKC- α	protein kinase C-alpha	83
PDK1	3-phosphoinositide-dependent protein kinase 1	104
PKB- α	protein kinase B (also called Akt)	95
SGK1	serum- and glucocorticoid-induced kinase 1	15
SGK2	serum- and glucocorticoid-induced kinase 2	29
SGK3	serum- and glucocorticoid-induced kinase 3	25
S6K1	S6 kinase 1	84
GSK3- β	glycogen synthase kinase 3	94
ROCK-II	Rho-dependent protein kinase II	83
AMPK	AMP-activated protein kinase	98
CHK1	checkpoint kinase 1	89
CK2	casein kinase 2	110
CSK	C-terminal Src kinase	105
CDK2/cyclin A	cyclin-dependent protein kinase 2	89
CK1- Δ	casein kinase-delta	99
DYRK1- α	dual-specificity, tyrosine-phosphorylated and regulated kinase-alpha	93
NEK6	NIMA (never in mitosis in <i>Aspergillus nidulans</i>)-related kinase 6	81
Nek2a	NIMA (never in mitosis in <i>Aspergillus nidulans</i>)-related kinase	96
LCK	lymphocyte cell-specific protein-tyrosine kinase	78
PKA	cAMP-dependent protein kinase	55
PBK	PDZ binding kinase	97
MAPKAP-K1 β	MAPK-activated protein kinase-K1-beta	62
IKK- β	inhibitory κ B kinase-beta	101
smMLCK	smooth-muscle myosin light-chain kinase	93
PRK2	protein kinase C-related kinase 2	49
MNK-2- α	MAPK-integrating protein kinase-alpha	76
CAMKI	calmodulin-dependent kinase	82
PIM-2	provirus integration site for Moloney murine leukemia virus-2	94
MAPK8	mitogen-activated protein kinase 8	92
MNK-1	MAPK-integrating protein kinase-1	89
SRPK1	serine-arginine protein kinase 1	89
PKB- β	protein kinase B-beta (also called Akt 2)	101
CHK2	checkpoint kinase 2	85
SRC	sarcoma kinase	100
EF2K	elongation-factor-2 kinase	83
MARK3	microtubule-affinity-regulating kinase 3	87
MST2	mammalian homologue Ste20-like kinase 2	87
PKD2	protein kinase D 2	89
PLK1	polo-like kinase 1	98
DYRK3	dual-specificity, tyrosine-phosphorylated and regulated kinase 3	86
HIPK2	homeodomain-interacting protein kinase 2	78
PAK4	p21-activated protein kinase 4	90
PAK5	p21-activated protein kinase 5	94
PAK6	p21-activated protein kinase 6	98
CAMKKb	CaMK kinase	113
Pim-1	provirus integration site for Moloney murine leukemia virus-1	80
Pim-3	provirus integration site for Moloney murine leukemia virus-3	84
BRSK2	brain-specific kinase 2	87
MELK	maternal embryonic leucine-zipper kinase	101
PKC- ζ	protein kinase C-zeta	95
MAPK1	mitogen-activated protein kinase 1	89
FGFR1	fibroblast-growth-factor receptor kinase 1	73
IRR	Insulin Receptor Related Receptor	102
EphA2	Ephrin A2 receptor	87
MST4	mammalian homologue Ste20-like kinase 4	107
Syk	Spleen Tyrosine Kinase	81
YES1	Yamaguchi sarcoma viral oncogene homologue 1	91
IKK- ϵ	inhibitory κ B kinase-epsilon	89
TBK1	TANK-binding kinase 1	95

out fructose and saline. The composition of the placebo and EMD638683 containing food was identical (diet 1310, 0.2% Na⁺, 0.9% K⁺, Altromin, Lage, Germany) and similar to the control diet (see above).

In another series of experiments *sgkl*^{+/+} mice (6 males and 2 females, age 7 to 10 months) were treated for 4 weeks with 10 % fructose containing isotonic saline, and received either placebo food or EMD638683 containing food. Urinary concen-

trations of Na⁺ and K⁺ were measured by flame photometry (AFM 5051, Eppendorf, Germany).

Systolic arterial blood pressure was determined by application of the tail-cuff method. As reviewed previously [53], the tail cuff approach to determine arterial blood pressure requires certain precautions to reduce the stress of the animals, including appropriate training of the mice over multiple days, prewarming to an ambient temperature of 29°C, measurement in a quiet and 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 10–12 am). All these precautions were taken in the present study. Tail-cuff measurements were performed according to the user's manual for the blood pressure analyzer (IITC Model 179, Hugo Sachs Elektronik, Germany). All recordings and data analysis were done using a computerized data acquisition system and software (PowerLab 400 and Chart4; AdInstruments). Five tail-cuff measurements were made in one session. The blood pressure for the session was accepted when the deviations of the five blood pressure readings were less than 5 mm Hg.

Data are provided as means ± standard deviation (SD) or standard error (SEM), *n* represents the number of independent experiments. All data were tested for significance using paired or unpaired Student *t*-test, and only results with *P* < 0.05 were considered statistically significant.

Results

Fig. 1 shows the formula of the SGK-inhibitor EMD638683. The substance is well soluble in water. The effect of EMD638683 has been screened towards a panel of 69 protein kinases using an assay described previously [49]. Table 1a shows the percentage of respective kinase activity in the presence of EMD638683 as compared with the kinase activity following incubations in the absence of the inhibitor. Among the total number of kinases tested those kinases have been selected for further IC₅₀ determinations, which either had shown a percent value of < 50 % (e.g.: mitogen- and stress-activated protein kinase 1 MSK1, serum- and glucocorticoid-inducible kinase SGK2 & SGK3, and protein kinase C-related kinase PRK2, highlighted in bold in Table 1a), or which are known homologous kinases belonging to the group of so-called “AGC”-kinases [54]. The results of this further analysis are shown in Table 1b.

The comparison reveals that EMD638683 has also an inhibitory effect on cAMP-dependent protein kinase (PKA), mitogen- and stress-activated protein kinase 1 (MSK1), protein kinase C-related kinase 2 (PKR2), and the SGK isoforms SGK2 and SGK3. None of the other kinases tested are appreciably inhibited at the concentrations which are effective on SGK1. Furthermore, the efficacy of the substance was determined utilizing the phos-

Kinases		
MAPKAP-K1	MAPK-activated protein kinase-K1-alpha	2818
MSK1	mitogen- and stress-activated protein kinase;	37
SGK1	serum- and glucocorticoid-inducible kinase	1
p70S6K1	S6 kinase 1	2545
GSK3β	glycogen synthase kinase 3 beta	4364
ROCK2	Rho-dependent protein kinase 2	1091
AMPK	AMP-activated protein kinase	7909
PKA	cAMP-dependent protein kinase	27
PRK2	protein kinase C-related kinase	109
PKBβ	protein kinase B beta (also called Akt2)	3273
AURORA B	Aurora kinase B	9091
PKC ζ	protein kinase C zeta;	327

Table 1b. Selectivity ratios of EMD638683 towards other kinases (results from single experiments for each kinase). The relative inhibitory effects (or its selectivity ratios) of EMD638683 versus other kinases were calculated by dividing the IC₅₀-values for EMD638683 for the respective kinase by the IC₅₀-value of EMD638683 for the SGK1. Each IC₅₀ value of EMD638683 was determined after carrying out assays at ten different concentrations, with each data point determined in triplicate.

Fig. 2. Dose response curve for the inhibitory effect of EMD638683 on NDRG1 phosphorylation. Arithmetic means ± SD (*n* = 4) of phosphorylated NDRG1 abundance in HeLa cells as a function of EMD638683 (EMD) concentration.

phorylation of NDRG1, a specific target of SGK1. As illustrated in Fig. 2, EMD638683 inhibited the NDRG1 phosphorylation, an effect requiring $3.35 \pm 0.32 \mu\text{M}$ EMD638683 in the cell culture medium for half maximal effect (IC₅₀).

A second series of experiments aimed to define the *in vivo* efficacy of EMD638683. Replacement of drinking water with 10% fructose in isotonic saline increased blood pressure from $83 \pm 2 \text{ mmHg}$ to $109 \pm 6 \text{ mmHg}$ (*n* = 5). As illustrated in Fig. 3, the treatment of fructose/

	Placebo	EMD	Placebo
Body Weight (g)	23.2 ± 1.2	22.6 ± 1.1*	22.5 ± 1.1§
Food intake (mg/12h g BW)	102.4 ± 6.9	107.1 ± 7.9	117.3 ± 5.6#§
Fluid Intake (μl/12h g BW)	192.4 ± 14.6	232.7 ± 21.9	225.3 ± 20.9
Urinary flow rate (μl/12 h g BW)	33.1 ± 6.4	49.2 ± 10.8*	46.5 ± 11.4
Urine Na ⁺ (μmol/12h)	340.5 ± 40.8	388.3 ± 32.1	366.1 ± 34.2
Urine Na ⁺ (μmol/12h g BW)	14.6 ± 1.43	17.18 ± 1.04	22.52 ± 1.13#§
Urine K ⁺ (μmol/12h)	269.5 ± 69.8	280.0 ± 23.3	227.1 ± 14.4#
Urine K ⁺ (μmol/12h g BW)	11.1 ± 2.14	12.5 ± 1.08	10.1 ± 0.32#

Table 2. Functional parameters during four day treatment with EMD638683. Body weight, food and fluid intake, urinary flow rate, and renal excretions of Na⁺ and K⁺ in fructose plus saline treated *sgkl*^{+/+} mice prior to and following a four day treatment with EMD638683. Arithmetic means ± SEM (n=6 each group). * p<0.05 vs. respective value under placebo food prior to the EMD638683 treatment; # p<0.05 vs. respective value under EMD638683 treatment; § p<0.05 vs. respective value of placebo food prior to the EMD638683 treatment.

high salt drinking animals with EMD638683 containing food for four days led to normalization of systolic blood pressure (from 111 ± 4 to 87 ± 3 mmHg). Replacement of the EMD638683 containing food with placebo food was followed by a similarly fast re-increase of systolic blood pressure to values similar to those prior to EMD638683 treatment (106 ± 11 mmHg).

The treatment with EMD638683 containing food for four days did not significantly modify fluid and food intake (Table 2) and did not significantly alter the urinary Na⁺ and K⁺ excretion, but significantly increased the urinary output and significantly decreased body weight (Table 2).

Additional studies were performed in SGK1 knockout (*sgkl*^{-/-}) mice, to explore whether an effect of EMD could be elicited in the absence of SGK1. As shown in Fig. 4, EMD638683 treatment did not influence the systolic blood pressure in tap water drinking SGK1 knockout mice (Fig. 4). EMD638683 treatment further did not influence systolic blood pressure of wild type mice, which were drinking plain tap water and were thus not hyperinsulinemic and not volume expanded (Fig. 4).

A second series of experiments explored whether chronic treatment with EMD638683 is similarly effective as short-term treatment with EMD638683. To this end, animals were fed 10% fructose in saline for four weeks. EMD638683 or placebo containing food was administered along with the fructose/high salt. Similar to what was observed in the short-term EMD638683 treatment, systolic blood pressure was significantly lower in animals receiving EMD638683 as compared to animals receiving placebo food (Fig. 5). Thus, chronic EMD638683 treatment prevented the rise in blood pressure caused by the fructose/high salt treatment.

Fig. 3. Systolic blood pressure in fructose plus saline drinking wild type mice prior to, during and after a four day treatment with EMD638683. Arithmetic means ± SEM (n = 5) of systolic blood pressure in wild type mice under placebo food (control) and EMD638683 containing food (EMD). (A) Arithmetic means of blood pressure values at individual days, (B) arithmetic means of control (open bar), EMD (closed bar) and recovery (open bar) period. ** p<0.01 vs. respective value under control diet.

Discussion

The present study describes the *in vitro* and *in vivo* efficacy of a novel SGK1 inhibitor, i.e. EMD638683. The substance exerts half maximal inhibition of SGK1 at 3.35 μM in a cellular assay and is clearly most effective

Fig. 4. Systolic blood pressure in tap water drinking wild type mice (*sgk1*^{+/+}) and SGK1 knockout mice (*sgk1*^{-/-}) prior to and during a four day treatment with EMD638683. Arithmetic means $\pm$ SEM (n = 5-8) of systolic blood pressure in tap water drinking wild type mice (*sgk1*^{+/+}, open bars) and SGK1 knockout mice (*sgk1*^{-/-}, closed bars) under placebo food (control) and EMD638683 containing food (EMD).

on SGK1 compared to the other kinases, tested in a panel of 69 kinases. For inhibition of MSK1, SGK2&3, and PRK2, moderately higher concentrations were needed than for SGK1 inhibition. For all other kinases, concentrations are needed at least one order of magnitude higher than required for inhibition of SGK1.

The present observations further reveal the efficacy of the inhibitor in SGK-dependent hypertension. As already shown in an earlier study [38], treatment with fructose and saline leads to a substantial increase of blood pressure in wild type mice. The effect of fructose/saline loading on blood pressure is completely lacking in SGK1 knockout mice [38]. Similar observations have been made with high fat diet and saline loading [37]. Those studies led to the conclusion that the hypertensive effect of hyperinsulinism is fully accounted for by SGK1. Notably, blood pressure is not different between *sgk1*^{-/-} and *sgk1*^{+/+} mice in the absence of hyperinsulinemia and salt excess (control conditions). Under those conditions, the lack of SGK1 is fully compensated by an increase of aldosterone plasma levels [34].

SGK1 has previously been shown to be inhibited by GSK650394 [55, 56]. The inhibitor is more than 30 fold selective for SGK1 than for IGF1R, Rho-associated protein kinase (ROCK), yanus kinase isoforms (YAK1, YAK3), protein kinase B isoforms (AKT1, AKT2, AKT3), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A) and PDK1 and less than 10 fold selective for SGK1 than for Aurora and c-Jun N-terminal kinase

Fig. 5. Systolic blood pressure of fructose plus saline drinking wild type mice treated with either placebo or EMD638683 for 4 weeks. Arithmetic means $\pm$ SEM (n = 5-8) of systolic blood pressure in animals at the end of a four week treatment with 10 % fructose in isotonic saline as drinking water and either placebo food (control, open bars) or EMD638683 containing food (EMD, closed bars). * p<0.05 vs. respective value under placebo food.

isoforms (JNK1, JNK3) [56]. The inhibitor does apparently not discriminate between SGK1 and SGK2 [56]. Comparison of those data with the data compiled in Tables 1a and 1b suggests that EMD638683 is a more selective SGK1 inhibitor than GSK650394. However, the experimental conditions and the kinases tested were not identical and thus, additional experiments are required to compare the selectivity of the two SGK1 inhibitors.

The present study reveals that EMD638683 is effective *in vivo*. To the best of our knowledge, no *in vivo* efficacy of GSK650394 has been published previously.

If SGK1 plays a critical role in the blood pressure enhancing effect of hyperinsulinemia, then the blockade of SGK1 should reverse the increase of blood pressure in hyperinsulinemic mice. The present study demonstrated that this is indeed the case for EMD638683. Notably, EMD638683 was completely ineffective in SGK1 knockout mice or in wild type mice without fructose/saline treatment. Thus, EMD638683 reverses the hypertension caused by hyperinsulinism/salt excess, but does not affect the blood pressure of normoinsulinemic mice or mice without salt excess.

Obviously, SGK1 plays a similarly important role in humans [48]. The SGK1 gene variant associated with increased blood pressure [36] has a particularly strong effect on blood pressure in hyperinsulinemia [48]. Thus, similar to what has been observed in mice [37, 38], hyperinsulinism sensitizes the blood pressure to salt intake, an effect involving SGK1. The human gene variant affects

some 5 % of the common population [36]. A gain of function gene variant of SGK1 could simultaneously increase blood pressure [36] and body mass index [57]. Moreover, the gene variant is associated with enhanced risk to develop diabetes [58]. Thus, SGK1 may indeed be one of the signalling molecules contributing to metabolic syndrome or syndrome X, a condition characterized by the coincidence of several disorders including hypertension, obesity, insulin resistance and hyperinsulinemia [59]. Metabolic syndrome shares several attributes of Cushing's syndrome, but does not require increased plasma cortisol levels [60]. Instead, the disorder may be caused by inappropriate activity of downstream signaling elements which could well include the serum- and glucocorticoid-inducible kinase (SGK1). According to the present study, increased blood pressure and presumably other SGK1-dependent aspects of metabolic syndrome could be favourably influenced by EMD638683 or related drugs.

In the present experiments, EMD638683 treatment had little effect on other parameters (Table 2). The alterations of urinary flow rate and decrease of body weight were expected in view of the role of SGK1 in the regulation of ENaC. Renal K⁺ excretion tended to be higher in EMD638683 treated animals. As shown earlier [51], renal K⁺ excretion is impaired in *sgk1*^{-/-} mice, an

effect presumably due to both, deranged K⁺ channel regulation [61, 62] and decreased electrical driving force resulting from decreased ENaC activity [51].

As shown in the present study, EMD638683 is effective on both, human and murine SGK1. SGK1 is a highly conserved molecule with high similarity between mammalian and shark sequence [63]. The specificity of the inhibitor in mice is documented by the lack of effect in *sgk1*^{-/-} mice.

In conclusion, inhibition of SGK1 with EMD638683 leads to a decrease of blood pressure in hyperinsulinemic, but not in normoinsulinemic wild type mice and SGK1 knockout mice. In view of the similar role of SGK1 in humans [48], SGK-inhibitors such as EMD638683 or related drugs may prove to be a first choice for the treatment of hypertension in diabetic patients.

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