Dynamics of GRK2 in the kidney: a putative mechanism for sepsis-associated kidney injury

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Abstract

Renal vascular reactivity to vasoconstrictors is preserved in sepsis in opposition to what happens in the systemic circulation. We studied whether this distinct behavior was related to α1 adrenergic receptor density, G protein-coupled receptor kinase 2 (GRK2) and the putative role of nitric oxide (NO). Sepsis was induced in female mice by cecal ligation and puncture (CLP). Wild-type mice were treated with prazosin 12 hours after CLP or NOS-2 inhibitor, 30 min before and 6 and 12 hours after CLP. In vivo experiments and biochemistry assays were performed 24 hours after CLP. Sepsis decreased the systemic mean arterial pressure and the vascular reactivity to phenylephrine. Sepsis also reduced basal renal blood flow which was normalized by treatment with prazosin. Sepsis led to a substantial decreased in GRK2 level associated to an increase in α1 adrenergic receptor density in the kidney. The disappearance of renal GRK2 was prevented in NOS-2-KO mice or mice treated with 1400W. Treatment of non-septic mice with a NO donor reduced GRK2 content in the kidney. Therefore, our results show that a NO-dependent reduction in GRK2 level in the kidney leads to the maintenance of a normal α1 adrenergic receptor density, probably. The preservation of the density and/or functionality of this receptor in the kidney together with a higher vasoconstrictor tonus in sepsis lead to vasoconstriction. Thus, the increased concentration of vasoconstrictor mediators together with the preservation (and even increase) of the response to them may help to explain sepsis-induced acute kidney injury.

Keywords: G protein-coupled receptor kinase 2, adrenergic receptor, nitric oxide, sepsis, acute kidney injury
Introduction

Sepsis is defined as a life-threatening organ dysfunction caused by an exaggerated immune response of the host to infection, and it is the leading cause of death in intensive care units worldwide (1,2). Sepsis clinical findings may vary and are dependent on the etiological agent, the initial location of the infection, the previous health condition of the host (1,3). Sepsis high mortality rate and the associated costs remains a challenge in clinical medicine (4,5). Despite many attempts to introduce novel therapeutic molecules, the therapeutic approach to sepsis treatment still consists mainly of antibiotics, fluid, and vasopressors agents (6,7).

The cardiovascular dysfunction is one of the most important complication of sepsis and is determinant for both prognosis and outcome. This dysfunction involves myocardial depression, vasodilatation, decrease in vascular reactivity towards vasoconstrictors and increased vascular permeability, all of which may lead to hypovolemia and severe and untreatable hypotension (8,9). Cardiovascular dysfunction directly impacts on sepsis pathophysiology due to the reduction in oxygen delivery to tissues, resulting in anaerobic metabolism and hyperlactatemia, and it is an important contributor to organ failure (10).

One of the organs most affected by sepsis is the kidney, which may result in sepsis-associated acute kidney injury (S-AKI) which, in turn, worsens the prognosis and increases sepsis mortality (11,12). S-AKI pathophysiology is not well understood but decreased global renal blood flow and secondary tubular epithelial cell death are believed to be of importance (13). The microcirculatory dysfunction is characterized by the establishment of heterogeneous microcirculatory flow, with fewer capillaries with continuous flow and an increased number of capillaries with intermittent and stopped flow (13). Despite the organ loss of function, histological changes are discrete,
suggesting that the kidney failure should be more related to a dysfunction than to tissue lesion (14–16).

Sepsis induces hypotension and reduces the response to vasoconstrictors in several vascular beds (17,18). The cardiovascular failure triggers the activation of compensatory responses such as the adrenergic and the renin-angiotensin systems to raise blood pressure, which may be helped by exogenous infusion of vasoconstrictors (1,19). Interestingly, the vascular tonus in the kidney is maintained or even increased during sepsis. This is in sharp contrast to the systemic vasodilatation and impaired vascular reactivity observed in most of other vascular beds (17,20,21). Thus, the kidney (and the lung) keeps its ability to respond to the wave of endogenous vasoconstrictors released due to sepsis-induced hypotension and as such, presents a paradoxical vasoconstriction during sepsis (20,22).

A key element in sepsis is nitric oxide (NO) which has a relevant role in cardiovascular dysfunction (23). NO production in sepsis is increased by the expression of the enzyme nitric oxide synthase isoform 2 (iNOS; NOS-2) in response to inflammatory cytokines (24). Several studies have shown that inhibition of NO production or biological activity is beneficial in restoring the vascular response during sepsis (25–27).

It has been suggested by others and by us that the decrease in vascular reactivity to vasoconstrictors and in the cardiac response to inotropes observed in sepsis results from desensitization of adrenergic receptors (28–30). The density of adrenergic receptors (which belongs to the GPCR family) is an important factor in the response of the cell or organ to the sympathomimetic amines. Thus, the modulation of the function of these receptors is necessary for cellular and tissue homeostasis. To attenuate or cease signal transduction and to protect the cell from excessive
stimulation, receptor desensitization occurs. One of the main mechanisms involved in
the desensitization process is receptor phosphorylation and internalization. Although
GPCR phosphorylation can be carried out by different kinases, G protein-coupled
receptor kinases (GRK) are the main kinases in this regard (31,32). GRK constitutes a
family of seven serine/threonine protein kinases that specifically recognize and
phosphorylate agonist activated GPCRs, which results in their labeling for
internalization (33). Due to their ability to interfere with the density of adrenergic
receptors, GRK2 is involved in the regulation of vascular tone and is a relevant isoform
in the cardiovascular system (34,35). Recently, we have demonstrated that during
sepsis, NOS-2-derived NO activates GRK2 in the heart, leading to the decrease in β1
adrenergic receptor density, which, in turn, is associated with the decrease of cardiac
reactivity (28).

Therefore, considering the distinct vascular status between the kidney and the
systemic circulation during sepsis, the present report aimed to evaluate the role of
GRK2 and adrenergic receptor density as well the putative role of NO in renal response
to sepsis induced by cecal ligation and puncture in mice.

Materials and methods

Animals

All procedures used in this report have been approved by Universidade Federal de
Santa Catarina Institutional Committee for Animal Use in Research (Protocol
8443190617) and are in accordance with the Brazilian Government Guidelines for
Animal Use in Research (CONCEA) and the National Institutes of Health Guide for the
Care and Use of Laboratory animals (NIH, USA). Female Swiss mice (weighing 35–40 g)
provided by the University stock colony and female Black C57BL/6 NOS-2-KO and wild-
type mice weighing 30–35 g (kindly provided by Dr. Fernando de Queiroz Cunha, Department of Pharmacology, Medical Faculty, Universidade de São Paulo at Ribeirão Preto) were used. Animals were kept under standard laboratory conditions in ventilated racks (5 animals per cage) in a temperature (23 ± 2 °C) and light-controlled room (12-h light/dark cycle; lights on at 7 AM), and free access to filtered water and commercial rodent chow. For the sake of less manipulation and suffering of animals, we used naïve animals as controls instead of sham-operated mice throughout the experiments.

**Cecal ligation and puncture model**

Sepsis was induced by cecal ligation and puncture (CLP) as previously described (36). Briefly, animals were given tramadol hydrochloride (10 mg/kg) for analgesia and accommodated in a breathing chamber for induction of anesthesia by oxygen-isoflurane (5%) inhalation. Anesthesia was maintained by oxygen-isoflurane (3%) delivered by a face mask. Body temperature was maintained at 37 ± 1 °C by means of a heating pad. After laparotomy, the cecum was ligated distal to the ileocecal valve with surgical thread, and a transfixing puncture with a 20-gauge needle was made. A small amount of cecal content was squeezed through the puncture. The surgical planes were closed with thread and animals were kept in a heated environment to recover from anesthesia (60 to 120 min) and were given tramadol hydrochloride (5 mg/kg, s.c.) every 12 h for analgesia. All animals received 30 mL/kg of sterile warm Dulbecco’s phosphate-buffered saline (PBS; in mM, 137 NaCl, 2.7 KCl, 1.5 KH₂PO₄ and 8.1 NaHPO₄; pH 7.4) subcutaneously immediately after the surgery.

**Mean arterial pressure and organ blood flow**

Mice were anesthetized with ketamine and xylazine (90 and 15 mg/kg, respectively, supplemented at 15 min intervals) and body temperature was maintained at 37°C by
means of a heating pad. Heparinized PE-10 polyethylene catheter was inserted into the femoral vein for drug injections and a catheter 24 GA (BD Angiocath, Becton Dickinson, Juiz de Fora, MG, Brazil) was introduced in the right carotid artery for recording of mean arterial pressure (MAP). After stabilization for 10 min, animals received three consecutive doses of phenylephrine (2, 6 and 20 nmol/Kg) diluted in 20 µL of PBS to evaluate vascular reactivity. MAP data were recorded with a catheter pressure transducer (Mikro-Tip, Millar Instruments, Inc., Houston, TX, USA) coupled to a Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). Results were expressed as mean ± S.E.M. of the peak changes in MAP (as mmHg) relative to baseline and recorded following the administration of phenylephrine. Blood flow was determined in kidney and caudal vein using a Laser Doppler Blood Flow Assessment moorVMS-LDF (Moor instruments, Axminster, United Kingdom) with the optical probes VP1T and VP4s, respectively. The left kidney was exposed by an incision (1.5 cm) at the upper retroperitoneal region, the peritoneal fluid and blood were dried with a swab and the probe VP1T was carefully positioned touching the surface of the kidney approximately in the direction of renal artery entrance and covering a round area of approximately 0.2 cm². The probe VP4s was placed over the caudal vein, at 2 cm above the tail end. For infusion with vasoconstrictor, a heparinized PE-10 polyethylene catheter was inserted into the femoral vein for drug infusion (infusion pump Insight, São Paulo, Brazil). Phenylephrine was infused (40 nmol/kg/min; 50 µL/min) for 5 minutes and blood flow were recorded simultaneously in kidney and caudal vein for 15 min. Results were expressed as mean ± S.E. of the values obtained at 30 sec intervals.

Creatinine and urea assays
Levels of urea and creatinine in plasma were measured according to the manufacturer’s instructions, using commercially available clinical assay kits (Labtest, Lagoa Santa, MG, Brazil). Values were calculated based on a calibration factor and expressed in mg/dL.

**Nitrite and nitrate (NOx) determination**

Nitrite and nitrate NO metabolites (NOx) were determined as previously described (37). Zinc sulfate-deproteinized plasma samples were subjected to nitrate conversion to nitrite using *Escherichia coli* nitrate reductase for 3 h at 37 °C. After that, samples were centrifuged to remove bacteria, mixed with Griess reagent (1% sulfanilamide in 10% phosphoric acid/0.1% naphthylethylenediamine in Milli-Q water, 1:1) in a 96-well plate and read at 540 nm in a plate reader. NOx concentration was calculated by comparison with standard solution of sodium nitrate prepared in water and values are expressed as μM.

**Western blot analysis**

Kidney and heart were collected and quickly frozen in liquid nitrogen. Samples were homogenized in ice-cold lysis buffer (T-PER 78510, Thermo Fisher Scientific Inc., Rockford, IL, USA), protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA), sodium orthovanadate (1 mM), and phenylmethanesulfonyl fluoride (1 mM) centrifuged at 10,000 g for 20 min at 4 °C and the supernatant was collected for protein concentration determination and preparation for electrophoresis. Samples in Laemmli sample buffer (68.8 mM Tris-HCl, 26.3% glycerol, 2.1% SDS, 0.01% bromophenol blue; pH 6.8) were heat-denatured and equal amounts of protein extract (80 μg) were loaded per lane and electrophoretically separated using denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Afterward, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot Cell apparatus connected to a PowerPac™ HC power supply (both from Bio-Rad Laboratories, CA, USA) following the manufacturer’s
protocol. Next, membranes were blocked with 5% BSA in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h at room temperature and then immunoblotted with primary antibodies anti-β-actin (A3854, 1:40,000, Sigma-Aldrich, St. Louis, MO, USA), anti-GRK2 (sc-13143, 1:500, Santa Cruz, CA, USA) and anti-NOS-2 (N9657, 1:1000, Sigma-Aldrich, St. Louis, MO, USA) in blocking buffer at 4°C overnight. Following washing, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:5,000, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature and washed again. The membranes were exposed to HRP substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and immune complexes were visualized by chemiluminescence using Chemidoc MP System (Bio-Rad Laboratories, CA, USA). Bands were quantified by optical density using the software from the manufacturer, expressed as the ratio to β-actin and expressed as arbitrary units.

**RNA extraction and quantitative real-time polymerase chain reaction analysis (RT-PCR)**

Kidney and heart were collected 24 h after sepsis induction and RNA were extracted using a ReliaPrep® kit (Promega, Madison, WI, USA) for kidney and PureLink® kit or with TRIzol® reagent (both from Invitrogen, Carlsbad, CA, USA) for heart samples. cDNA was synthesized from 700 ng (kidney) and 1000 ng (heart) of DNase-treated RNA (DNA-free, Promega, Madison, WI, USA) using a High-Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantification of specific products was performed by qPCR using a GoTaq® qPCR Master Mix (Promega, Madison). Double-stranded products were amplified using specific primers for Grk2 variant 1 (forward 5’-TGCCAGAGCCCAGCATC-3’, reverse 5’-CGGAAAAGCAGGTACCTAAC-3’) with StepOnePlus Real-Time PCR System equipment (Applied Biosystems). Relative amounts of target gene were calculated by normalization to the expression of glyceraldehyde 3-
phosphate dehydrogenase (Gapdh) (forward 5’-CATCAGTGAGCTCTCCGAGACG-3’, reverse 5’-ATGCCAGTGAGCTCTCCGAGACG-3’) reference gene. RNA levels were compared using the 2−ΔΔCT method (38). Primer specificity in all samples was confirmed by single peak performances of qPCR products in melt curve analysis.

**Immunofluorescence assays**

Kidneys and heart were removed and fixed in 4% PFA for 24 h at room temperature then incubated in 30% sucrose for 24 h, 4 °C. Samples were then frozen in Tissue-Tek (Sakura Finetek Inc., Torrance, CA, USA) at -80 °C. Seven μm cryostat sections were obtained (Leica CM 1850 UV Biosystems; Leica Microsystems, Wetzlar, Germany) and placed on poly-lysine (Sigma-Aldrich, St Louis, MO, USA) coated slides. Cold acetone (-20 °C) fixation was performed on slides for 5 minutes. After that, sections were blocked with 5% BSA in PBS for 1 h at room temperature and then incubated with primary antibody anti-GRK2 (1:50) in blocking buffer at 4 °C overnight. Following washing, secondary antibody donkey anti-mouse Alexa fluor® 594 (A31623, 1:1000, Molecular Probes, Eugene, OR, USA) and 4,6-diamidino-2-phenylindole (DAPI, 1 µg/mL, Thermo Scientific Rockford, IL, USA) were incubated for 1 h at room temperature. Finally, sections were washed with PBS and mounted with coverslips using aqueous mounting media Gel Mount® (Sigma-Aldrich, St Louis, MO, USA). A fluorescence confocal microscope (Nikon A1RSiMP) and Imaging Software Nis Elements 4.20 (both from Nikon, Tokyo, Japan) were used to capture images. Images were obtained from at least 3 different regions from 2 different slices of each animal per group. Relative fluorescence intensity was quantified using the ImageJ® software (version 1.36b; NIH, Bethesda, MD, USA). Values of fluorescence intensity from negative control slices and background signal were subtracted from all images. All images were acquired with the same microscope settings.
**Alpha-1 adrenergic receptor labeling assay**

To study α1-adrenoceptor density, we used a fluorescent labeling with an antagonist probe. Tissues were collected and prepared as above. Following fixation, sections were incubated with α1-adrenoceptor ligand BODIPY™ FL Prazosin (QAPB; Molecular Probes, Eugene, OR, USA), 250 nM for 1 h at room temperature. Nuclei were stained with DAPI (1 µg/mL). Non-fluorescent prazosin (NFP) was used as a competition molecule for QAPB binding sites in tissue sections from control animals. Once QAPB equilibrium was established (1 h), slices were incubated with NFP (250 µM, 1 h, room temperature). The incubation with NFP reduced the fluorescence by more than 75% confirming the specificity of the labeling of QAPB towards the α1-adrenoceptor. After washing, all sections were mounted with coverslips using aqueous mounting media Gel Mount®. Images were acquired using a fluorescence confocal microscope (Leica DMI6000B®) and LAS AF Lite® software (both from, Leica Microsystems, Wetzlar, HE, Germany). Image analyses were performed as described in immunofluorescence section and all images were acquired with the same microscope settings.

**Experimental protocols**

Animals were randomly assigned and distributed in equal numbers to septic and non-septic groups. Swiss mice were treated with prazosin, an α1 adrenergic receptor antagonist (1 mg/Kg, s.c., Sigma-Aldrich), 12 h after CLP or with a selective NOS-2 inhibitor, 1400W (1 mg/Kg, s.c., Sigma-Aldrich) or PBS (vehicle), 30 min before and 6 and 12 h after sepsis induction. Animals were euthanized by anesthetics overdose 24 h after surgery or prepared to mean arterial pressure and blow flow measurements. A group of normal Swiss mice received a NO donor, SNAP (45 nmol/Kg, s.c.) or PBS (vehicle), were euthanized after 4 h by anesthetics overdose and the kidney was harvested and prepared for analysis. Black C57BL/6 NOS-2-KO and wild type controls
(WT) were submitted to CLP-induced sepsis and after 24 h were euthanized by anesthetics overdose and the kidney was harvested and prepared for analysis.

Statistical analysis

Statistical analysis was done using Student $t$-test to evaluate the significance of differences between two groups or one-way or two-way analysis of variance (ANOVA) to evaluate differences among multiple groups, using Bonferroni post hoc test as a multiple comparison method. Graphs and statistical analysis were performed using GraphPad Prism® version 8.4.2 software (San Diego, CA, USA) and $p$ values $< 0.05$ were considered significant.

Results

Blood pressure, vascular reactivity, and blood flow during sepsis

It has been well demonstrated that sepsis induces a significant reduction of vascular reactivity, decreases systemic vascular resistance, and induces hypotension. As shown in the typical trace recording (Figure 1A), 24 h after CLP surgery the basal blood pressure of septic animals was 20% lower compared to control animals. In addition, the vascular reactivity to phenylephrine was severely impaired in septic animals compared to control group. Quantification of the data reveals that the response to phenylephrine was reduced by ~80% in sepsis (Figure 1B). The basal blood flow in the kidney of septic animals was reduced and prazosin (1 mg/kg, injected s.c. 12 h after CLP surgery) rescued the kidney blood flow to control levels (Fig. 1C). The ability of phenylephrine to cause vasoconstriction and reduction in the blood flow is preserved in the tail of normal animals but lost during CLP. On the other hand, the flow to the kidney of septic mice is unchanged by the infusion of the phenylephrine (Figure 1D). It is important to note that both measurements were done in the same animals.
Renal markers

As seen in Figure 2, plasma levels of creatinine (Figure 2A) and urea (Figure 2B) were elevated during sepsis. These findings indicated that the kidney is already failing.

Alpha1 adrenergic receptor density in the kidney and in the heart

To explore the α1 adrenergic receptor density in kidney and heart during sepsis, we performed a binding assay with the fluorescent α1-adrenoceptor ligand QAPB. Twenty-four hours after sepsis induction, the α1 adrenergic receptor density was increased by three-fold in the kidney when compared to control group (Figures 3A and 3C). Although glomeruli appear to express the receptor, the intensity of the labeling is greater in renal tubules and vessels. In contrast, the α1 adrenoceptor receptor density did not change in septic heart (Figures 3B and D).

GRK2 in septic kidney and heart

Since the density of α1 adrenergic receptor was higher in the septic kidney but not in the heart, we sought to study the GRK2 levels in both organs (Figure 4). In a distribution like the α1 adrenergic receptor, GRK2 was present in the glomeruli but its content was greater in tubules and vessels. Sepsis substantially reduced GRK2 content in all structures (Figures 4A and 4C). As for the heart, GRK2 seems to be evenly distributed in the cardiac tissue and sepsis increased its content (Figures 4B and 4D).

As shown by Western blot analysis, GRK2 levels were reduced to almost nil in the septic kidney (Figure 5A) whereas it was even increased in the heart (Figure 5B). Grk2 mRNA levels were significantly decreased in kidney (Figure 5E) but unchanged in the heart (Figure 5F).

NO pathway and GRK2 dynamics in kidney during sepsis

Considering the prominent role of NO in vascular dysfunction of sepsis, we went to investigate the putative role of NO in renal GRK2 levels. We first evaluated NOS-2...
expression in kidney and NO metabolites (nitrate and nitrite; NOx) in plasma. Sepsis induced an increase in NOS-2 expression in kidney (Figures 6A and 6B). To confirm that the increased expression of NOS-2 in the kidney (and presumably in other organs and tissues) indeed produced NO, results show that plasma levels of NO stable metabolites (nitrate + nitrite) increased 5-fold 24 hours after CLP surgery (Figure 6C).

To study the role of NO in the renal GRK2 dynamics during sepsis, septic mice were treated with 1400W, a selective inhibitor of NOS-2, 30 min before and 6 and 12 h after sepsis induction. The treatment with 1400W prevented the loss of GRK2 content in septic kidney (Figures 7A and 7B). Confirming that NOS-2 was the main source of NO affecting GRK2 in the septic kidney, NOS-2 KO mice were submitted to sepsis and again the loss in GRK2 content induced by sepsis was prevented (Figures 7C and 7D). An interesting result was obtained when we treated normal, non-septic mice with SNAP, a nitric oxide donor and measured the renal content of GRK2. The treatment with the NO donor reduced GRK2 content in the kidney by 20% (Figures 7E and 7F).

**Discussion**

The main findings of the present report are: i) sepsis induced decreases in the mean arterial pressure, in vascular reactivity to phenylephrine and in the renal blood flow in mice; ii) sepsis increased α1 adrenergic receptor density in the kidney but not in the heart; iii) sepsis-induced reduction in renal blood flow was blocked by prazosin treatment; iv) renal GRK2 levels were significantly reduced whereas cardiac GRK2 levels increased in septic animals; v) NOS-2 protein expression in kidney and plasma NOx levels increased in septic animals; vi) the disappearance of renal GRK2 was prevented in NOS-2-KO mice or normal mice treated with 1400W; and vii) the treatment of normal mice with the NO donor SNAP reduced GRK2 content in the kidney.
As for the model of sepsis we used, the findings that basal blood pressure of septic mice was lower than that from control animals and that their response to phenylephrine were severely reduced, indicate that mice have developed septic shock.

When the blood pressure and tissue perfusion fall below certain limits powerful regulatory systems are activated to counteract the hypotension/low tissue perfusion to restore the physiological condition. Two important systems in this regard are the adrenergic and renin-angiotensin systems. High levels of norepinephrine (39,40) and renin (41) have been found during sepsis, indicating activation of both systems.

However, the attempt to restore blood pressure by releasing vasoconstrictors may be hampered by the loss of vessel responsiveness in the general circulation. Therefore, and despite activation of these systems, most of the vascular beds of the host remain vasodilated thus contributing to organ dysfunction and eventually death. The reasons for this loss in the vessel response are not fully understood but they may include receptor desensitization, receptor heterodimerization, shifts in transduction cascades, exhaustion and uncoupling of transduction mechanisms, etc (30,42–46).

The response of the kidney to sepsis is different from most other vascular beds. Whereas many of them (such as the splanchnic bed) are severely vasodilated (hence the hypotension), the kidney is paradoxically vasoconstricted during sepsis (20). Our results confirm that picture. Kidney basal blood flow is reduced when compared to another vascular bed, the tail in the present work. The “anomalous” condition of the kidney is more evident when a vasoconstrictor was infused in septic animals. The tail blood flow of control mice sharply decreases following phenylephrine infusion whereas no such response was seen in septic mice, confirming the hyporesponsiveness to vasoconstrictors. The kidney of septic mice in contrast, does not present alterations in its flow during phenylephrine infusion. Together with the reduced basal flow, this lack
of response indicates that the kidney is already vasoconstricted. It should be kept in the mind that overall reduction in blood flow may not necessarily leads to S-AKI. In a model of ovine hyperdynamic sepsis with renal failure, the total renal blood flow even increases, but the redistribution of intra-renal blood flow leading to medullary tissue hypoxia may be one of the factors leading to S-AKI (47). Notwithstanding the mechanism involved (vasoconstriction, flow redistribution, other), our results confirm that kidney dysfunction is taking place as both markers of organ damage are elevated. When kidney function is compromised during sepsis and S-AKI ensue, the condition is much aggravated (48).

It has been demonstrated that one mechanism of impaired vascular and cardiac reactivity observed in sepsis results from desensitization of adrenergic receptors (29,49). We then posed the question of why the kidney behaves differently from other vascular beds in sepsis. Initially we thought that α1 adrenergic receptor may be involved. This idea was forwarded by our previous results showing that cardiac β-adrenergic receptor density, essential for the adrenergic effect in the heart, was negatively modulated during sepsis (28). The results shown in the present report indicate that α1 adrenergic receptor density is indeed increased in the kidney but not in the heart, suggesting that the increased α1 receptor density might well be the reason for the “anomalous” renal vasoconstriction, in contrast to what happens in other vascular beds. Supporting this conclusion was the finding that prazosin, a selective α1 receptor blocker, increased kidney blood flow in septic animals. One interesting aspect is that the increased density of α1 receptor during sepsis was found in both renal vessels and tubules. We did not explore this finding further but α1 adrenergic receptors are abundant in kidney tubules affecting sodium reabsorption, for example. Several reports on this matter indicate that the α1 receptor subtypes present in tubules are α1B and
\( \alpha_1A \), whereas vessels in general have the \( \alpha_1D \) subtype. Although we do not have evidence on how and whether tubular \( \alpha \) adrenergic receptors would directly interfere in renal vascular tone, indirect effects due to its activation such as renin production/release, sodium handling, prostanoid secretion, potassium channel activation/inhibition and others, are conceivably putative mechanisms linking tubular \( \alpha \) receptors and regulation of kidney tone (50-52).

GRK2 is a relevant kinase involved in adrenergic receptor desensitization. Upon activation, GPCR can be phosphorylated by GRK and thus labelled to mobilize arrestin, leading to receptor internalization (33). This mechanism is operative for the heart \( \beta \)-adrenergic receptor during sepsis upon GRK2 phosphorylation, as we have previously shown (28). Therefore, we set out to examine GRK2 profile in the kidney of septic mice.

Our results demonstrate that both GRK2 mRNA and protein levels were reduced in the kidney, in sharp contrast with the heart, where GRK2 mRNA was unchanged and protein levels were even increased, probably due to increased mRNA translation to produce the enzyme protein.

NO has been shown to be a relevant player in sepsis, mainly regarding the cardiovascular dysfunction (reviewed in 23). Our present results confirm that NO is indeed produced in sepsis, as NOS-2 expression in the kidney was elevated and plasma levels of its metabolites nitrate and nitrite were also elevated. We have previously shown that NO has a prominent role in the activation of cardiac GRK during sepsis (28).

The involvement of NO in the disappearance of kidney GRK2 was demonstrated both pharmacologically and genetically. NOS-2 selective inhibitor 1400W increased GRK2 levels in the septic kidney and GRK2 levels of NOS-2-KO mice were not reduced during sepsis. Interestingly, when normal animals were given the NO donor SNAP, the renal content of GRK2 was reduced after few hours, thus confirming that NO is indeed
involved in the kidney GRK2 disappearance. All these findings clearly show that NO effect on kidney GRK2 is distinct from the relationship between NO and GRK2 in other organs, such as the heart (reviewed in 53). For instance, expression NOS-2 is involved in GRK2 upregulation by a mechanism dependent on soluble guanylyl cyclase in septic neutrophils (54). Furthermore, NO can directly interact with GRK2 by S-nitrosylation and affect kinase functionality (55).

Our study has limitations. For instance, we do not know the mechanism whereby NO causes the reduction in GRK2 renal content and, at the same time, increases it in the heart. In the same line, we do not know whether the renal density of other relevant receptors (ß-adrenergic, AT1, etc) is altered during sepsis and what would be the putative role of GRK2 in this event. Since prazosin permeates cell membrane, it stains both cell membrane and intracellular α adrenergic receptors [56]. Finally, the role of the substantial density of alpha-adrenergic receptors in renal tubules and their role in kidney function and dysfunction merits further studies.

In conclusion, the results present herein suggest a new mechanism to help explain the occurrence of sepsis-associated acute kidney injury. NO in the kidney induces a substantial reduction in GRK2 level which may lead to a reduced phosphorylation and internalization of the α1 adrenergic receptor. The preservation of the density and/or functionality of this receptor in the kidney may lead to a vasoconstriction due to the higher vasoconstrictor tonus in sepsis, contributing to the development of S-AKI. As a corollary of this hypothesis, the administration of vasoconstrictors in the attempt to raise the blood pressure during septic shock may worsen S-AKI. If the findings presented here hold true for the clinical setting, GRK2 and α1 adrenergic receptor provides interesting targets to be studied to reduce sepsis-associated acute kidney injury.
**Clinical perspectives**

- Sepsis and septic shock still cause a high number of deaths worldwide. Sepsis cardiovascular dysfunction, leading to hypotension and hyporesponsiveness to vasoconstrictors, has a leading role in organ failure. Distinct from other vascular beds, the renal vascular response to vasoconstrictors is preserved. To counteract the hypotension, vasoconstrictor systems are activated. Although these systems fail to increase systemic blood pressure, they induce renal vasoconstriction.

- The present study shows in vivo and in vitro that during sepsis, G-protein receptor kinase (GRK2) almost disappears in the kidney, but it is increased in the heart. GRK2 reduction leads to an increase in α1 adrenergic receptor density in the kidney. Pharmacological and genetic ablation of NOS-2 prevents GRK2 disappearance.

- Our results show that a NO-dependent reduction in GRK2 level in the kidney may lead to a reduced phosphorylation and internalization of the α1 adrenergic receptor. The preservation of the density and/or functionality of this receptor in the kidney may lead to a vasoconstriction due to the higher vasoconstrictor tonus present in sepsis, thus contributing to the development of sepsis-associated acute kidney injury. Thus, GRK2 and α1 adrenergic receptor may be relevant targets to be studied to reduce sepsis-associated acute kidney injury.

**Data Availability**

The data used to support the findings of the present study are available from the Corresponding Author upon request.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.
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CRediT Author contributions

TOR: Conceptualization; Investigation; Writing Original Draft; Visualization; Formal analysis. GMN: Conceptualization; Investigation; Visualization. VVH: Investigation. MAF: Investigation. JA: Conceptualization; Investigation; Writing Original Draft; Visualization; Writing - Review & Editing; Supervision; Funding acquisition. The manuscript has been reviewed and approved by all authors.

Abbreviations

CLP, cecal ligation and puncture; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GRK2, G protein-coupled receptor kinase 2; MAP, mean
arterial pressure; NFP, Non-fluorescent prazosin; NO, nitric oxide; NOS-2, nitric oxide synthase 2; NOx, nitrite and nitrate; PFA, paraformaldehyde; PBS, Dulbecco's phosphate-buffered saline; QAPB, BODIPY™ FL Prazosin; S-AKI, sepsis-associated acute kidney injury; SNAP, S-nitroso-N-acetylpenicillamine; TBST, Tris-buffered saline with Tween®.

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Legends to Figures

Figure 1. Effect of phenylephrine on blood pressure and organ blood flow of septic mice

Mice were submitted to sepsis and after 24 h were prepared for mean blood pressure (MAP) and blood flow measurements in tail and kidney. Naïve animals served as controls. Panel A: Trace recording of a typical experiment showing the hypotension and the loss in phenylephrine response in septic mice. Panel B: Quantification of the vasoconstriction response to phenylephrine. Panel C: Basal blood flow in tail and kidney and the effect of prazosin (1 mg/kg, injected s.c. 12 h after CLP surgery). Panel D: Blood flow in tail and kidney during phenylephrine infusion (40 nmol/kg/min). For Panels B and C each symbol corresponds to one mouse and columns represent the mean ± S.E. *p < 0.05 compared to control using Student t-test (Panel B) and one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (Panel C). Panel D: n = 8 animals per curve; * p < 0.05 compared to naïve group, two-way analysis of variance (ANOVA).

Figure 2. Sepsis increases plasma creatinine and urea levels

Creatinine (Panel A) and urea (Panel B) in plasma. Each symbol corresponds to one mouse and columns represent the mean ± S.E. *p < 0.05 compared to the respective control using Student t-test.

Figure 3. Sepsis increases α1 adrenergic receptor labelling in kidney but not in the heart

Kidney (Panel A) and heart (Panel B). Representative photomicrographs of BODIPY™ FL Prazosin fluorescent ligand (QAPB; green) and DAPI (blue) in control and septic
FIGURE 4. SEPSIS REDUCES GRK2 CONTENT IN KIDNEY BUT NOT IN THE HEART

Kidney (Panel A) and heart (Panel B). Representative photomicrographs of GRK2 (red) and DAPI (blue) in control and septic mice. Horizontal bars mean 50 μm. Fluorescence intensity in kidney (Panel C) and heart (Panel D) was quantified and expressed in arbitrary units (A.U.) and normalized to picture area. At least 3 photomicrographs obtained from each animal were used for quantification. To allow for a better visualization, the brightness was increased by 10% in all Panels. Each symbol corresponds to one mouse and columns represent the mean ± S.E. * p < 0.05 compared to control group (Student t-test).

FIGURE 5. SEPSIS REDUCES GRK2 PROTEIN AND mRNA LEVELS IN THE KIDNEY BUT NOT IN THE HEART

Kidney (Panel A) and heart (Panel B) were harvested 24 after CLP surgery. Representative blots are shown in Panel C (kidney) and Panel D (heart). β-actin was used as a loading control. RT-PCR for Grk2 in kidney (Panel E) and heart (Panel F), normalized by Gapdh mRNA. Each symbol corresponds to one mouse and columns represent the mean ± S.E. * p < 0.05 compared to control group using Student t-test.
Figure 6. Sepsis induces NOS-2 expression in kidney and increases plasma NOx levels

Samples were taken 24 h after CLP surgery. Representative blot is shown in Panel B and the quantification is shown in Panel A. ß-actin was used as a loading control. Panel C: plasma levels of NO stable metabolites (nitrate + nitrite). Each symbol corresponds to one mouse and columns represent the mean ± S.E. * p < 0.05 compared to control group using Student t-test.

Figure 7. Pharmacological and genetic ablation of NOS-2 inhibits sepsis-induced reduction of GRK2 in the kidney.

For results shown in Panels A-D, kidney was harvested 24 h after CLP surgery. Mice were treated with 1400W (4 µmol/kg, s.c.) 30 min before and 6 and 12 h after sepsis induction (Panels A and B). NOS-2 knockout mice were submitted to CLP surgery the kidney was harvested and GRK2 content determined (Panel C and Panel D). Panels E and F: normal mice were injected with SNAP a NO donor (45 nmol/kg, s.c.), and the kidney was harvested after 4 h and assayed for GRK2 content. Each symbol corresponds to one mouse and columns represent the mean ± S.E. * p < 0.05 using Student t-test (Panel E) or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (Panels A and C).
