



Activation of the AMP-related kinase (AMPK) induces renal vasodilatation and downregulates Nox-derived reactive oxygen species (ROS) generation



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ABSTRACT

AMP-activated protein kinase (AMPK) is a cellular energy sensor activated during energy stress to stimulate ATP production pathways and restore homeostasis. AMPK is widely expressed in the kidney and involved in mitochondrial protection and biogenesis upon acute renal ischemia, AMPK activity being blunted in metabolic disease-associated kidney disease. Since little is known about AMPK in the regulation of renal blood flow, the present study aimed to assess the role of AMPK in renal vascular function. Functional responses to the selective AMPK activator A769662 were assessed in intrarenal small arteries isolated from the kidney of renal tumour patients and Wistar rats and mounted in microvascular myographs to perform simultaneous measurements of intracellular calcium [Ca²⁺]_i and tension. Superoxide (O₂^{•-}) and hydrogen peroxide (H₂O₂) production were measured by chemiluminescence and fluorescence and protein expression by Western blot. Activation of AMPK with A769662 increased AMPK_α phosphorylation at Thr-172 and induced potent relaxations compared to AICAR in isolated human and rat intrarenal arteries, through both endothelium-dependent mechanisms involving nitric oxide (NO) and intermediate-conductance calcium-activated potassium (IK_{Ca}) channels, as well as activation of ATP-sensitive (K_{ATP}) channels and sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) in vascular smooth muscle (VSM). Furthermore, AMPK activator reduced NADPH oxidase 4 (Nox4) and Nox2-derived reactive oxygen species (ROS) production. These results demonstrate that A769662 has potent vasodilator and antioxidant effects in intrarenal arteries. The benefits of AMPK activation in rat kidney are reproduced in human arteries and therefore vascular AMPK activation might be a therapeutic target in the treatment of metabolic disease-associated kidney injury.

1. Introduction

AMPK is an ubiquitous heterotrimeric kinase considered as a master energy sensor/metabolic switch that is activated during energy stress in response to ATP depletion, to preserve cell survival under low-caloric conditions [1–4]. As the ratio of AMP/ATP increases, activation of AMPK turns on ATP-generating pathways (e.g. glucose uptake, glycolysis, fatty acid oxidation) and reduces ATP-consuming pathways (free fatty acid –FFA–, triglyceride and protein synthesis) [1]. AMPK is made up of a catalytic α -subunit (α_1 and α_2), a structural β -subunit (β_1 and β_2), and the AMP-binding site containing γ -subunit (γ_1 , γ_2 and γ_3). AMPK activation requires phosphorylation of the α -subunit and occurs

in response to increased AMP/ATP ratio, as well as through phosphorylation by upstream kinases such as the tumor suppressor liver kinases B1 (LKB1) or the Ca²⁺/calmodulin-dependent protein kinase β (CAMKK β) [2].

AMPK is not only involved in metabolism but also in the regulation of physiological processes such as mitochondria biogenesis, cell growth and proliferation, inflammation, oxidative stress and cell autophagy. AMPK also plays a key role in cardiovascular function, in part through phosphorylation and activation of endothelial nitric oxide synthase (eNOS) in endothelial cells and cardiomyocytes [5–9]. By stimulating nitric oxide (NO) production, the AMPK-eNOS endothelial signalling pathways is involved in endothelium-dependent vasodilatation [10] in

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Abbreviations

AMPK	AMP-activated protein kinase	K _{ATP}	ATP-sensitive potassium channels
ACh	acetylcholine	NADPH	nicotinamide adenine dinucleotide phosphate
BK _{Ca} channel	large-conductance calcium-activated K ⁺ channel	NO	nitric oxide
COX	cyclooxygenase	NOS	nitric oxide synthase
EC	endothelial cell	Nox	NADPH oxidase enzymes
EDH	endothelium-derived-hyperpolarization	O ₂ ^{·-}	superoxide
eNOS	endothelial nitric oxide synthase	Phe	phenylephrine
H ₂ O ₂	hydrogen peroxide	PSS	physiological saline solution
IK _{Ca} channel	intermediate-conductance calcium-activated K ⁺ channel	ROS	reactive oxygen species
		SERCA	sarcoplasmic reticulum Ca ²⁺ -ATP _{ase}
		VSM	vascular smooth muscle

response to physiological stimuli such as shear stress [7,11,12] or adipokines acting via Gq receptors like adiponectin and leptin [1].

The kidney is an organ with one of the highest energy consumption rates in the body, used for the regulation of fluids and electrolytes homeostasis and for waste excretion, and renal blood flow represents about 25% of cardiac output. AMPK is highly expressed in the kidney and regulates activity of various ion and creatinine transporters in renal tubular cells and reduces podocyte permeability and albuminuria upon kinase activation with adiponectin [13,14]. Acute renal ischemia is a potent activator of AMPK which induces cell survival mechanisms including mitochondrial protection and biogenesis [15], suppression of extracellular matrix proteins [16] and induction of autophagy [17]. Accordingly, AMPK activators have been shown to be protective and attenuate ischemia-reperfusion injury in canine models of renal transplantation and in rat models of kidney ischemia-reperfusion injury [18–20]. On the other hand, AMPK has been reported to exert antioxidant effects in the cardiovascular system by inhibiting the expression and activity of NADPH oxidase (Nox) subunits, suggestive of potential crosstalk between AMPK and Nox, despite how AMPK suppresses Nox remains to be clarified [21].

Reduced renal AMPK activity has been associated to impaired renal function and inflammation in the kidney of diabetic patients [22], in obesity-associated nephropathy [23] and in experimental models of chronic kidney disease [24,25], and is involved in renal inflammation and fibrosis [23]. Therefore, AMPK activation has recently been proposed as a therapeutic target of metabolic disease-associated nephropathy and experimental chronic kidney disease. Cardiovascular-protective effects of AMPK are well characterized [26] and many therapeutic agents used for the treatment of diabetes and atherosclerosis, including metformin, thiazolidinediones and statins may exert their cardiovascular protective effects by activation of AMPK [27–29]. However, despite the role of AMPK in kidney function and dysfunction is well established, little is known about its role in the regulation of renal vascular function.

The hypothesis of the present study is that activation of AMPK may regulate renal vascular function thus coupling renal blood flow to kidney metabolism. Therefore, we sought to investigate AMPK vasodilator effects in human renal arteries and to identify AMPK targets in kidney arterial tissue. For this, we assessed the contribution of the eNOS-NO endothelium-dependent pathway, as well as vascular smooth muscle (VSM) relaxant mechanisms involving K⁺ channels and the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA). Moreover, the interaction between AMPK and Nox-mediating mechanisms generating reactive oxygen species (ROS) in kidney vascular tissues were also investigated.

2. Methods

2.1. Animal model

Male Wistar rats were housed and maintained on standard chow and

water *ad libitum* at the Pharmacy School animal care facility. They were killed by decapitation and exsanguination at 12–14 weeks. The kidneys, heart and mesentery were quickly removed and placed in cold (4 °C) physiological saline solution (PSS) of the following composition (mM): NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.17, MgSO₄ 1.18, CaCl₂ 1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5% CO₂/95% O₂ to maintain pH at 7.4. Animal care and experimental protocols conformed to the European Union Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes and were approved by the Animal Care and Use Committee of Complutense University of Madrid. All animal experiments are reported in compliance with the ARRIVE Guidelines.

2.2. Patients

Intrarenal arteries and cortex samples were obtained from the kidney of renal tumour patients who underwent nephrectomy as earlier reported [30]. The investigation with human tissue conformed to the principles outlined in the Declaration of Helsinki. Permission was obtained from the Ethics Committee of the University Hospital Puerta de Hierro-Majadahonda, Spain (Reg. no 5.16) and patients gave their informed consent.

2.3. Dissection and mounting of microvessels

Renal interlobar or arcuate arteries, 2nd-4th order branches of the main renal artery were carefully dissected by removing the surrounding connective tissue from tumour-free parts of the kidney of human patients, or kidney of Wistar rats. For some experiments, mesenteric resistance arteries and coronary arteries of Wistar rats were dissected. Small samples of both renal arteries and cortex were also dissected out for ROS measurements, as described earlier [30]. Arterial segments were mounted in microvascular myographs (Danish Myotechnology, Denmark) by inserting two 40 µm tungsten wires into the vessel lumen and were equilibrated for 30 min in PSS maintained at 37 °C. The relationship between passive wall tension and internal circumference was determined for each individual artery and from this, the internal circumference, L₁₀₀, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ was calculated. The arteries were set to an internal diameter I₁ (I₁ = 0.9 × L₁₀₀) at which tension development is maximal.

2.4. Experimental procedures for the functional experiments

At the beginning of each experiment, arteries were challenged twice with 120 mM K⁺ (KPSS) in order to test vessel viability. Concentration-responses curves to the endothelial agonist acetylcholine (ACh) and to the β-adrenergic agonist isoprenaline were performed to assess arterial function on arteries precontracted with phenylephrine (Phe) (0.1–0.5 µM). The effects of the AMPK selective activator A769662 (Tocris Cookson, Bristol, UK) was assessed in intrarenal, coronary and

mesenteric arteries precontracted with Phe and responses to A769662 were added in a second concentration-curve in order to test whether the relaxations were reproducible (Fig. S1). The relaxant responses of A769662 were compared to those elicited by the AMPK activator (AICAR) (Tocris Cookson, Bristol, UK) by adding cumulative concentrations of these agents on arteries precontracted with Phe. The AMPK inhibitor (compound C, 0.5 μM) (Tocris Cookson, Bristol, UK) was tested on the relaxations to the AMPK activators and was added to the myograph chamber 30 min before a second concentration-response curve was performed. To evaluate whether the relaxant effect of AMPK was endothelium-dependent, endothelium-intact and endothelium-denuded rat and human renal arterial segments were pre-contracted with Phe and treated with A769662. The endothelium was mechanically removed by inserting a human hair in the vessel lumen and guiding it back and forwards several times. The absence of functional endothelium was confirmed by lack of the relaxation to ACh (10 μM), as earlier described [31]. Functional responses to A769662 were further obtained in the absence and presence of NOS synthase inhibitor (L-NOARG, 100 μM) (Sigma-Aldrich, Spain), inhibitors of intermediate-conductance K_{Ca} channels (IK_{Ca} , TRAM-34 1 μM) (Tocris Cookson, Bristol, UK), large-conductance K_{Ca} channels (BK_{Ca} , iberiotoxin 0.3 μM) (Tocris Cookson, Bristol, UK), Kir6 ATP-sensitive K channels (K_{ATP} , glibenclamide 1 μM) (Tocris Cookson, Bristol, UK) or the inhibitor of SERCA ATPase, cyclopiazonic acid (CPA, 10 μM) (Tocris Cookson, Bristol, UK) in rat and human renal arteries.

2.5. Measurements of VSM intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

Simultaneous measurements of VSM $[\text{Ca}^{2+}]_i$ and tension in intact renal interlobar arteries were performed as described earlier [31]. Arterial segments were mounted in a microvascular myograph placed on an inverted microscope (Zeiss Axiovert S100 TV) equipped for dual excitation wavelength microfluorimetry. Arteries were incubated in the dark in PSS with 8 μM Fura 2-acetoxymethyl ester (Fura 2-AM) (Thermo Fisher Scientific, Life Technologies SA, Madrid, Spain) and 0.05% Cremophor EL. After Fura-2-AM loading, arteries were washed for 30 min in PSS and were illuminated with alternating 340 nm and 380 nm light using a monochromator-based system (Deltascan, PTI). Simultaneous measurements of $[\text{Ca}^{2+}]_i$ and tension by Fura2-AM fluorescence were performed in rat interlobar arteries to further study AMPK-mediated calcium-dependent mechanisms in VSM. The ratio (R) F340/F380 was taken as a measure of $[\text{Ca}^{2+}]_i$. Time-response curves to 10 μM of the AMPK selective activator A769662 were performed in renal arteries with intact endothelium pre-contracted with Phe or with 30 mM K^+ in the absence and presence of inhibitor of IK_{Ca} TRAM-34 (1 μM). In order to restrict Ca^{2+} measurements to VSM when assessing the role of VSM K^+ channels or SERCA ATPase, experiments were performed in renal endothelium-denuded arteries pre-contracted with Phe. Then time-response curves to 10 μM A769662 were performed in the absence and presence of inhibitors of BK_{Ca} channels (iberiotoxin, 0.3 nM), K_{ATP} channel (glibenclamide, 1 μM) or SERCA ATPase (CPA, 10 μM).

2.6. Measurement of superoxide production by chemiluminescence

Changes in basal and NADPH-stimulated levels of superoxide ($\text{O}_2^{\cdot-}$) were measured by lucigenin chemiluminescence in renal arteries and cortex of wistar rats and human samples, as earlier reported [30,31]. Cortex samples and segments of the renal interlobar arteries were dissected and equilibrated in PSS for 30 min at room temperature and then incubated in the absence (basal) and presence of AMPK selective activator A769662 (30 μM) and the NADPH oxidase inhibitor Nox2ds-tat (1 μM) (AnaSpec Seraing, Belgium) for 30 min at 37 $^{\circ}\text{C}$. Samples were then transferred to microtiter plate wells containing 5 μM bis-N-methylacridinium nitrate (lucigenin) in the absence and presence of different ROS sources inhibitors and then stimulated with NADPH

(100 μM) which was added prior to ROS measurements. In another set of experiments, the protein kinase C activator phorbol 12,13-dibutyrate (PDBu, 10 μM) (Tocris Cookson, Bristol, UK) was used instead of NADPH in order to indirectly stimulate the Nox2-derived superoxide production. Chemiluminescence was measured in a luminometer (BMG Fluostar Optima), and for calculation baseline values were subtracted from the counting values under the different experimental conditions and superoxide production was normalized to dry tissue weight.

2.7. Measurement of hydrogen peroxide by Amplex Red

Hydrogen peroxide (H_2O_2) production was measured by Amplex Red assay Kit (Thermo Fisher Scientific, Life Technologies SA, Madrid, Spain) in renal arteries and cortex of wistar rats [30,31]. Samples were equilibrated in HEPES-physiological saline solution (PSS) for 30 min at room temperature and then incubated in the absence (basal) and presence of AMPK selective activator A769662 (30 μM) for 30 min at 37 $^{\circ}\text{C}$. Arteries and cortex samples were then transferred to microtiter plate black wells containing 10 mM final concentration (Amplex Red) and 10 U/ml final concentration (horseradish peroxidase) and some samples were stimulated with NADPH (100 μM) just prior to determination. Fluorescence was measured in a fluorimeter (BMG Fluostar Optima), using an excitation filter of 544 nm and an emission filter of 590 nm. Background fluorescence was subtracted from the counting values under the different experimental conditions and H_2O_2 production was normalized to dry tissue weight.

2.8. Western blot analysis

Renal arterial tissue incubated 30 min with A769662 or vehicle were snap frozen in liquid nitrogen, homogenized and lysed in buffer containing Tris-HCl (pH 7.5) 50 mmol/L, EGTA 1 mmol/L, EDTA 1 mmol/L, Triton X-100 1% vol/vol, sodium orthovanadate 0.1 mmol/L, sodium fluoride 50 mmol/L, sodium pyrophosphate 5 mmol/L, sucrose 0.27 mol/L and protease inhibitor cocktail. The protein concentration was determined by Bradford assay (Bio-Rad Laboratories, S.A, Madrid, Spain). Protein lysates (7 μg) were subjected to 6,5% SDS-PAGE, electrotransferred on a polyvinylidene fluoride membrane and probed with the following primary antibodies: pACC Ser⁷⁹ (1:1000; ref 3661), pAMPK α Thr¹⁷² (1:1000; ref 2535), AMPK $\alpha_{1/2}$ (1:1000; ref 2532) from Cell Signaling Technology (Leiden, The Netherlands); NADPH oxidase 4 (1:500; ref ab154244) from Abcam (Cambridge, UK); β -actin (1:5000; ref. A5316) from Sigma (St. Louis, MO, USA) [4,32]. The blots were visualized using enhanced chemiluminescence and quantified by densitometry with ImageJ free software. Values were expressed in relation to β -actin protein levels.

2.9. Data presentation and statistical analysis

Results are expressed as either absolute values (units of R (F340/F380) or Nm^{-1} of tension) or as a percent of the response to either Phe or KPSS in each artery, as means \pm SEM of 6–10 arteries from 4 to 12 rats (1–2 artery from each animal) for the functional experiments. For the measurement of $\text{O}_2^{\cdot-}$ or H_2O_2 production, results are expressed in counts per minute (cpm) per mg of tissue and relative fluorescence units (RFU) per mg of tissue in arterial segments and cortex samples, respectively, as means \pm SEM of 5–7 patients or means \pm SEM of 4–10 rats.

Statistical significance was determined by using paired or unpaired Student's t-test where appropriate, or one-way ANOVA followed by Bonferroni's post hoc test for comparisons involving more than two groups. $P < 0.05$ were considered statistically significant. Calculations were made using a standard software package (GraphPad Prism 5.0, GraphPad Software, Inc., San Diego, CA, United States).

3. Results

3.1. AMPK activation relaxes intrarenal arteries

Normalized internal lumen diameters, l_i , vasomotor responses and endothelium-dependent relaxations of human renal arteries are shown in Table 1. Since vascular AMPK has recently been identified as a potent vasodilator in resistance arteries [33], the effects of the AMPK selective activator A769662 (3 μ M and 10 μ M) were assessed in interlobar arteries from human and rat kidney, as well as in rat mesenteric and coronary arteries, in order to compare the renal vascular effects of A769662 to that in small arteries from systemic and coronary vascular beds. AMPK activation produced potent relaxations of both human and rat intrarenal arteries (Fig. 1 a and c) and also in mesenteric resistance arteries (Fig. 1e). Notably, A769662 did not have significant relaxant effects in precontracted coronary arteries (Fig. 1g). The relaxations elicited by AMPK activation were compared to those evoked by the endothelium-dependent agonist ACh or the β -adrenoceptor agonist isoprenaline in each arterial bed (Fig. 1b, d, f and h). Relaxant responses to A769662 were reproducible in rat and human renal arterial segments (Supplementary Fig. S1).

Pharmacological characterization of AMPK activation in intrarenal arteries was performed by constructing concentration-response curves to two different AMPK activators: A769662 that selectively binds the AMPK β_1 subunit and AICAR, an unspecific and allosteric AMPK activator (Fig. 2a). A769662-elicited relaxations were about 2.5 orders of magnitude more potent than those produced by AICAR (Table 2), and therefore A769662 was used in the subsequent experiments. Treatment for 30 min with the AMPK inhibitor (compound C, 0.5 μ M) inhibited the relaxant responses elicited by both A769662 and AICAR in a second concentration-response curve (Fig. 2b and c; Table 2).

Western blot analysis confirmed that activity of AMPK was augmented by A769662 in intrarenal arteries as depicted by the 3-fold enhancement of AMPK α phosphorylation at Thr-172 after 20 min incubation with A769662 (Fig. 2d and e). In addition, A769662 increased phosphorylation of the AMPK's downstream target acetyl-CoA carboxylase (ACC) in renal arteries (Fig. 2f).

3.2. A769662 induces relaxations by endothelial NO release

AMPK plays a role in cardiovascular function in part through phosphorylation and activation of eNOS in endothelial cells and cardiomyocytes [8]. In order to assess the contribution of the endothelium to the relaxant responses of AMPK, human and rat renal arterial segments with intact endothelium or endothelium-denuded pre-contracted with Phe were stimulated with A769662 (3 and 10 μ M). Both mechanical endothelium removal and (Fig. 3 a and c) and inhibition of NOS with L-NOARG (Fig. 3 b and d) markedly reduced A769662-evoked relaxations thus suggesting that the vasodilator mechanism of AMPK is in part dependent on the vascular endothelium and involves the eNOS-NO pathway.

3.3. A769662 reduces VSM $[Ca^{2+}]_i$ and activates endothelial IK_{Ca} channels and VSM K_{ATP} sensitive channels to induce relaxation

Simultaneous measurements of VSM $[Ca^{2+}]_i$ and tension were performed in rat interlobar arteries to further investigate the role of AMPK in calcium-dependent mechanisms of human renal vasodilation. Time-response curves to 10 μ M of the AMPK selective activator A769662 produced relaxations that were accompanied by simultaneous decreases in VSM $[Ca^{2+}]_i$ (Fig. 4a). However, relaxations elicited by A769662 were larger than the corresponding decreases in VSM $[Ca^{2+}]_i$, thus suggesting the involvement of Ca^{2+} desensitization mechanisms (Fig. 4b), as confirmed by tension- $[Ca^{2+}]_i$ relationships for A769662 (Fig. 4c).

A769662-induced relaxations and $[Ca^{2+}]_i$ decreases were abolished

when raising the extracellular K^+ concentration (Fig. 4d and e) which suggests that K^+ efflux and hyperpolarization mechanisms are in part responsible for the AMPK relaxant effects in renal arteries. Since K_{Ca} channels have recently been involved in AMPK relaxant mechanisms in small arteries [33], the effects of selective K_{Ca} inhibitors were assessed in the AMPK responses of endothelium-intact renal arteries. Treatment with the inhibitor of IK_{Ca} channels TRAM-34 significantly reduced A769662-evoked relaxations in human renal arteries (Fig. 5a and b), and this inhibition was confirmed in rat intrarenal arteries as shown in Fig. 5e. Moreover, IK_{Ca} channel blockade caused a significant reversion of the decreases in $[Ca^{2+}]_i$ evoked by A769662 (Fig. 5c and d). In order to determine whether VSM BK_{Ca} channels may be involved in the relaxant mechanism of A-769662, as recently reported for small mesenteric arteries [33], the selective inhibitor of BK_{Ca} channels iberiotoxin was used in endothelium-denuded rat intrarenal arteries. A769662-elicited relaxations and $[Ca^{2+}]_i$ -decreasing effects were not sensitive to iberiotoxin (Fig. 5f and g). Therefore, the fact that A-769662-induced relaxations were sensitive to TRAM-34 in endothelium intact arteries but not to iberiotoxin in endothelium-denuded microvessels, suggests that AMPK activation might have a major effect on IK_{Ca} channels expressed in endothelial cells.

In the kidney, ATP-sensitive potassium channels (K_{ATP}) are localized in tubular epithelial cells, glomerular mesangial cells and VSM of renal blood vessels [34]. Activation of arterial smooth muscle K_{ATP} channels produces smooth muscle hyperpolarization leading to a reduction in $[Ca^{2+}]_i$ and vasodilatation [35]. Therefore, we assessed whether K_{ATP} channels might be involved in the relaxant responses elicited by AMPK activation in renal small arteries. Treatment with the selective K_{ATP} channel inhibitor glibenclamide markedly reduced A769662-evoked relaxations in human renal arteries (Fig. 6a and b) and significantly inhibited decreases in $[Ca^{2+}]_i$ induced by A-769662 in rat renal arteries (Fig. 6c and d).

3.4. Activation of SERCA is involved in the relaxations of A-769662

SERCA is an important and potent modulator of smooth muscle $[Ca^{2+}]_i$ and therefore the effects of the selective SERCA inhibitor cyclopiazonic acid (CPA, 10 μ M) on changes in VSM $[Ca^{2+}]_i$ and relaxation produced by AMPK activation were further explored in endothelium-denuded renal arteries, as shown in Fig. 7. Treatment with CPA induced a marked inhibition of the relaxations of A769662 in renal microvessels (Fig. 7a and b), suggesting that AMPK activates SERCA in VSM to produce vasodilation of human intrarenal arteries. This relaxant mechanism was confirmed in rat intrarenal arteries since treatment with CPA significantly reduced the VSM $[Ca^{2+}]_i$ -decreasing effects of A769662 (Fig. 7c and d).

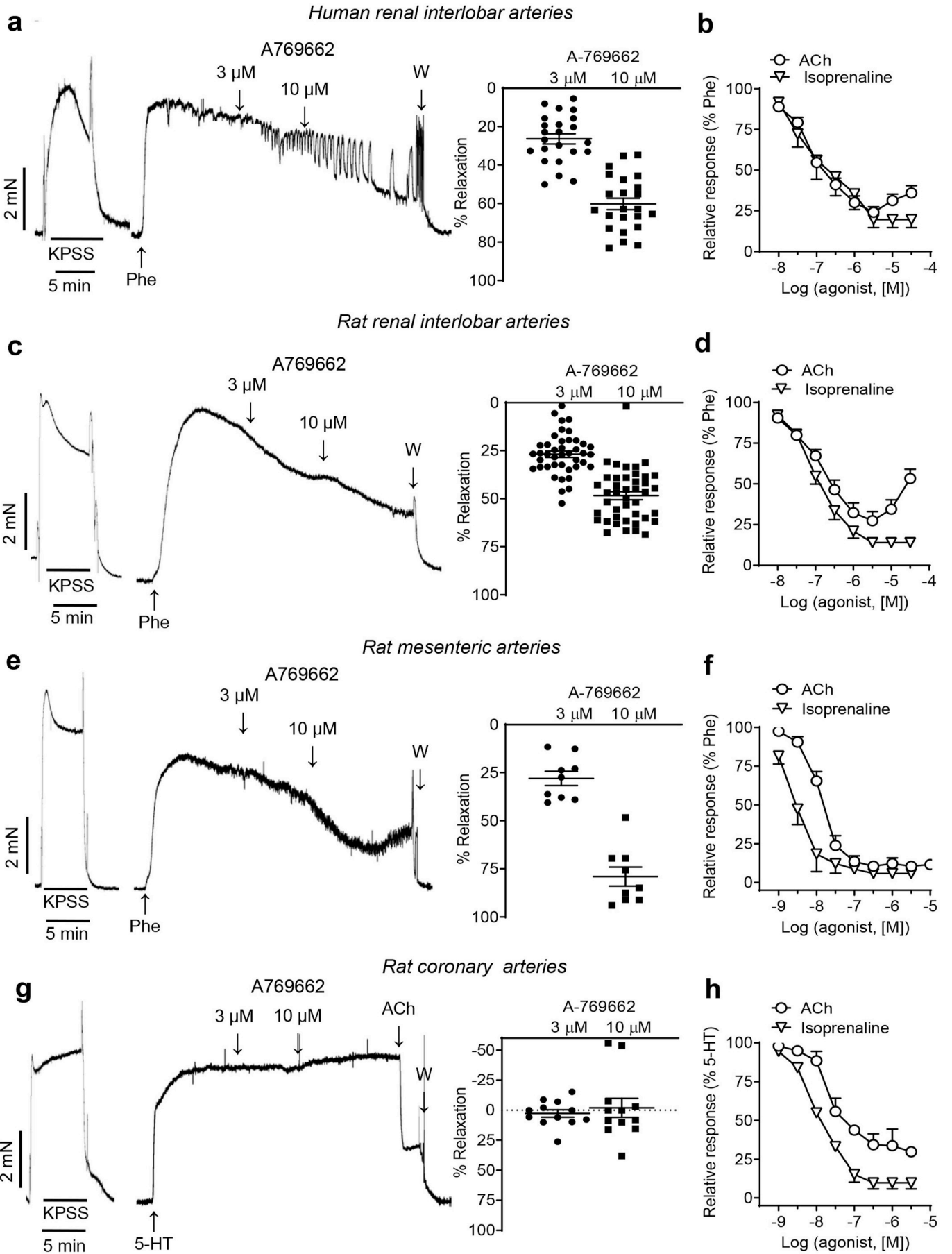
3.5. A769662 reduces ROS generation through inhibition of Nox2 and Nox4

AMPK is known to modulate reactive oxygen species (ROS) generation in the vascular endothelium [21] and therefore we explored whether AMPK activation plays a role in the regulation of ROS in the renal vascular wall. Basal $O_2^{\cdot-}$ levels were enhanced by NADPH addition and acute treatment with the AMPK activator A769662 induced a

Table 1
Vasomotor function of the human intrarenal arteries.

		n
l_i (μ m)	454 \pm 28	25
KPSS (Nm^{-1})	1.97 \pm 0.30	28
ACh (Relaxation %)	73.4 \pm 4	26

Values represent mean \pm S.E.M. of the number n of individual arteries, 3–4 from each patient (7 patients). l_i is the effective lumen diameter of the arteries.



(caption on next page)

Fig. 1. AMPK activation induces vasorelaxation of renal and mesenteric but not coronary arteries

Original recordings showing A769662-induced relaxations of intact renal interlobar arteries from human (a) and rat (c) kidney and rat mesenteric arteries (e), and the lack of effect of AMPK activator in coronary arteries from rat (g). Average vasorelaxant effects of A769662 (3 μ M and 10 μ M) (a,c,e,g) compared to those of the endothelial agonist acetylcholine (ACh) and the β -adrenoceptor isoprenaline (b,d,f,h) in rat renal, mesenteric and coronary arteries. Results are expressed as percentage of the increases in tension induced by phenylephrine (Phe) or serotonin (5-HT) for coronary arteries. Data are shown as the mean \pm SEM of 9–28 arteries from 6–14 rats (1–2 per animal) and as the mean \pm SEM of 20 arteries from 7 patients.

powerful inhibition of NADPH-derived $O_2^{\cdot-}$ production in samples of arteries and cortex from human and rat kidneys (Fig. 8a and b). Since Nox2 and Nox4 are involved in kidney physiological processes and are a source of vasodilator H_2O_2 in the renal endothelium [30], we next assessed whether these Nox subunits are targets of A769662 antioxidant action in renal arteries. Basal H_2O_2 production assessed by Amplex Red fluorescence assay was markedly increased by NADPH addition and blunted by the AMPK activator in both renal interlobar arteries and cortex from rats (Fig. 8c). Furthermore, Western blot analysis showed that protein levels of Nox4 were significantly reduced in renal arteries acutely treated with A769662 (Fig. 8d), thus suggesting that AMPK activation downregulates Nox4-derived arterial H_2O_2 production.

To assess the involvement of Nox2 in the antioxidant action of AMPK, the effects of A769662 on NADPH-stimulated $O_2^{\cdot-}$ production were measured in the absence and presence of the selective Nox2 inhibitor, Nox2ds-tat, that inhibits the assembly of the cytosolic subunit p47_{phox} with Nox2 thus blocking enzyme activity. NADPH-derived $O_2^{\cdot-}$ levels were reduced to a similar extent by both Nox2ds-tat and A769662, and combined treatment with the AMPK activator and the Nox2 inhibitor did not further inhibit NADPH-derived $O_2^{\cdot-}$ production

Table 2

Relaxant effects of the AMPK activators AICAR and A769662 and effect of the AMPK inhibitor compound C in rat renal interlobar arteries.

	pEC ₅₀ (%)	E _{max} (%)	n	I ₁ (μ m)
A769662	4.92 \pm 0.05	71.9 \pm 5.7	6	308 \pm 16
+ Compound C	3.89 \pm 0.15**	43.5 \pm 6**	6	312 \pm 16
AICAR	2.67 \pm 0.09†††	61.8 \pm 0.9	6	316 \pm 17
+ Compound C	1.79 \pm 0.1*	33.1 \pm 13	6	326 \pm 15

Values represent mean \pm S.E.M. of the number *n* of individual arteries, 1–2 per animal. pEC₅₀ is $-\log EC_{50}$, EC₅₀ being the agonist concentration giving half-maximal relaxation; E_{max} = maximal relaxation (% Phe). Significant differences were analyzed by ANOVA or paired or unpaired Student *t*-test. **P* < 0.05; ***P* < 0.01 versus control before treatment and †††*P* < 0.001 versus A769662.

(Fig. 8e). To confirm that A769662 may have the same pharmacological target as Nox2ds-tat, the PKC activator PDBu was used, since PKC augments assembly of p47_{phox} with Nox2 thus stimulating Nox2 activity

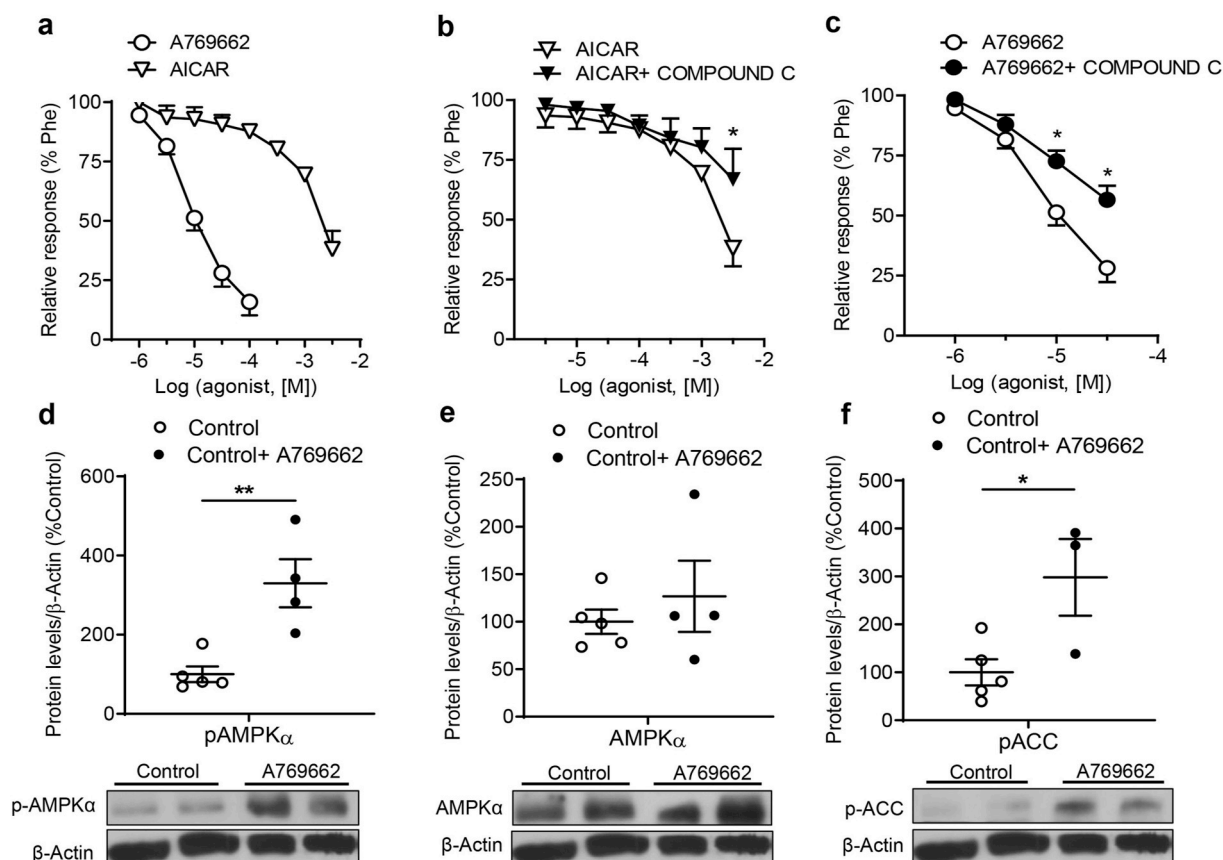
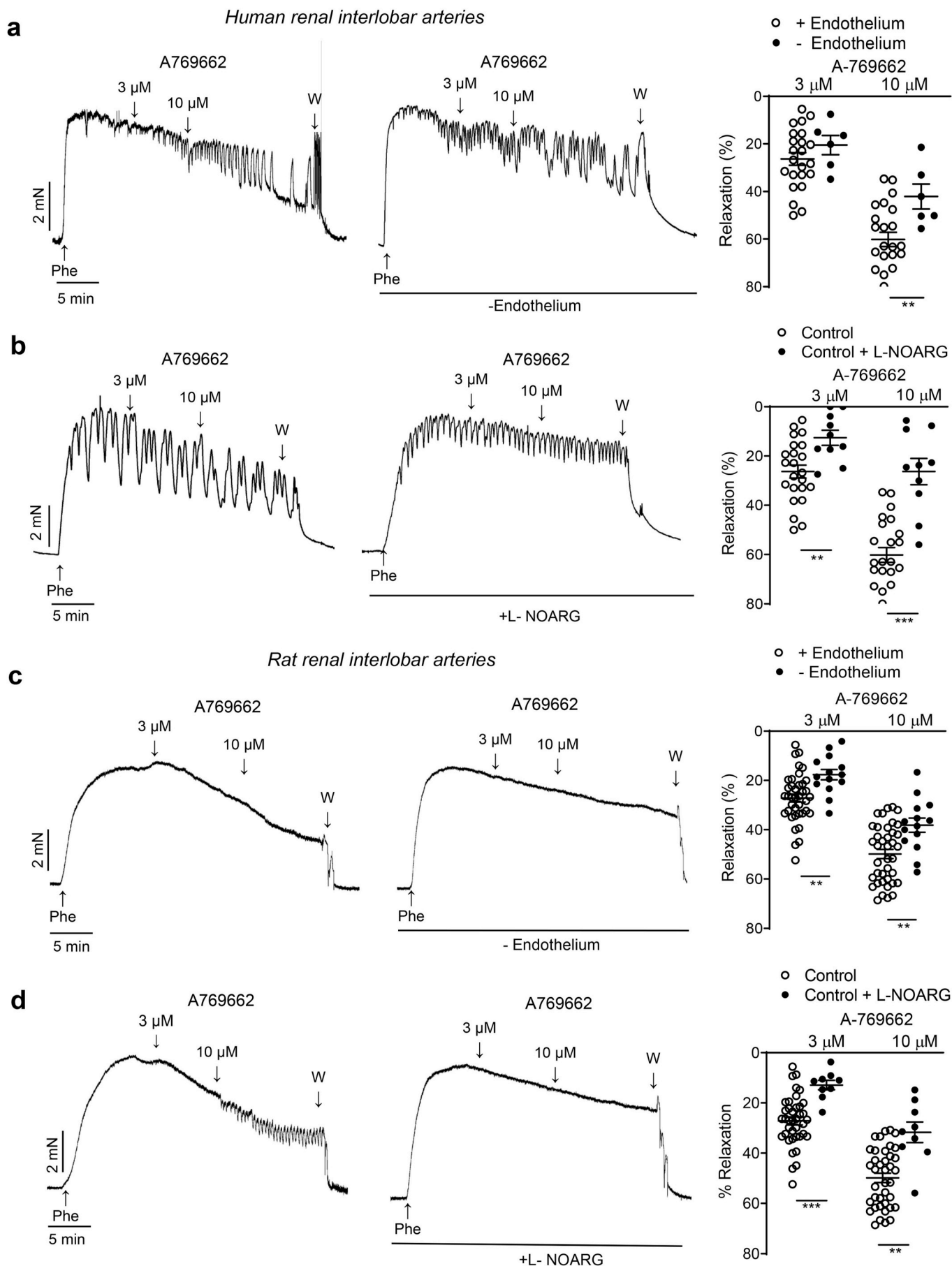


Fig. 2. A769662-induced potent relaxations compared to AICAR- and phosphorylates AMPK and ACC in renal arteries

Relaxant responses of AMPK activation with A769662 vs AICAR in renal arteries from rats (a) and inhibitory effect of compound C (0.5 μ M) (b,c). Data are shown as the mean \pm SEM of 6–8 arteries from 6 rats. Western blots analysis for pAMPK α (d), AMPK α (e) and pACC α (f) protein levels in samples of renal arteries from rats with or without acute treatment with A769662 (10 μ M). Results were quantified by densitometry and presented as a ratio of density of the protein band vs that of β -actin from the sample. Data are shown as the mean \pm SEM of 4–5 animals. Statistical significant differences between means were analyzed by using unpaired Student's *t*-test **P* < 0.05, ***P* < 0.01 vs control.



(caption on next page)

Fig. 3. Endothelial-derived NO is involved in the relaxations induced by AMPK activation in renal arteries

Original recordings showing the inhibitory effects of mechanical endothelium removal and NOS blockade with L-NOARG (100 μ M) on the relaxations induced by AMPK activator A7699662 in intrarenal arteries from human (a,b) and rat (c,d) kidney. Results are expressed as percentage of the increases in tension induced by phenylephrine (Phe). Data are shown as the mean \pm SEM of 5–15 rats (1–2 arteries per animal) and as the mean \pm SEM of 4–7 patients (2–3 arteries per patient). Statistical significant differences between means were analyzed by paired Student's *t*-test ***P* < 0.01, ****P* < 0.001.

and ROS production. Basal $O_2^{\cdot-}$ levels were significantly increased by PDBu addition as shown in Fig. 8f. PDBu-stimulated $O_2^{\cdot-}$ levels were significantly larger in cortex than in renal arteries and were blunted by Nox2ds-tat and A769662 in both arteries and cortex samples (Fig. 8f), thus suggesting overlapping mechanisms.

4. Discussion

Previous studies have shown that AMPK serves a role in renal

physiology by mediating kidney cell survival mechanisms upon activation by acute ischemia and by regulating activity of various tubular cell transporters [13–15,20]. Moreover, AMPK activation is protective in metabolic disease-associated nephropathy wherein AMPK activity is blunted [23]. The present findings demonstrate that AMPK might be involved in metabolic regulation of renal blood flow, since activation of this kinase causes vasodilatation of human renal arteries through both endothelium-dependent and direct VSM mechanisms; moreover, our data show that AMPK also modulates Nox-derived ROS production.

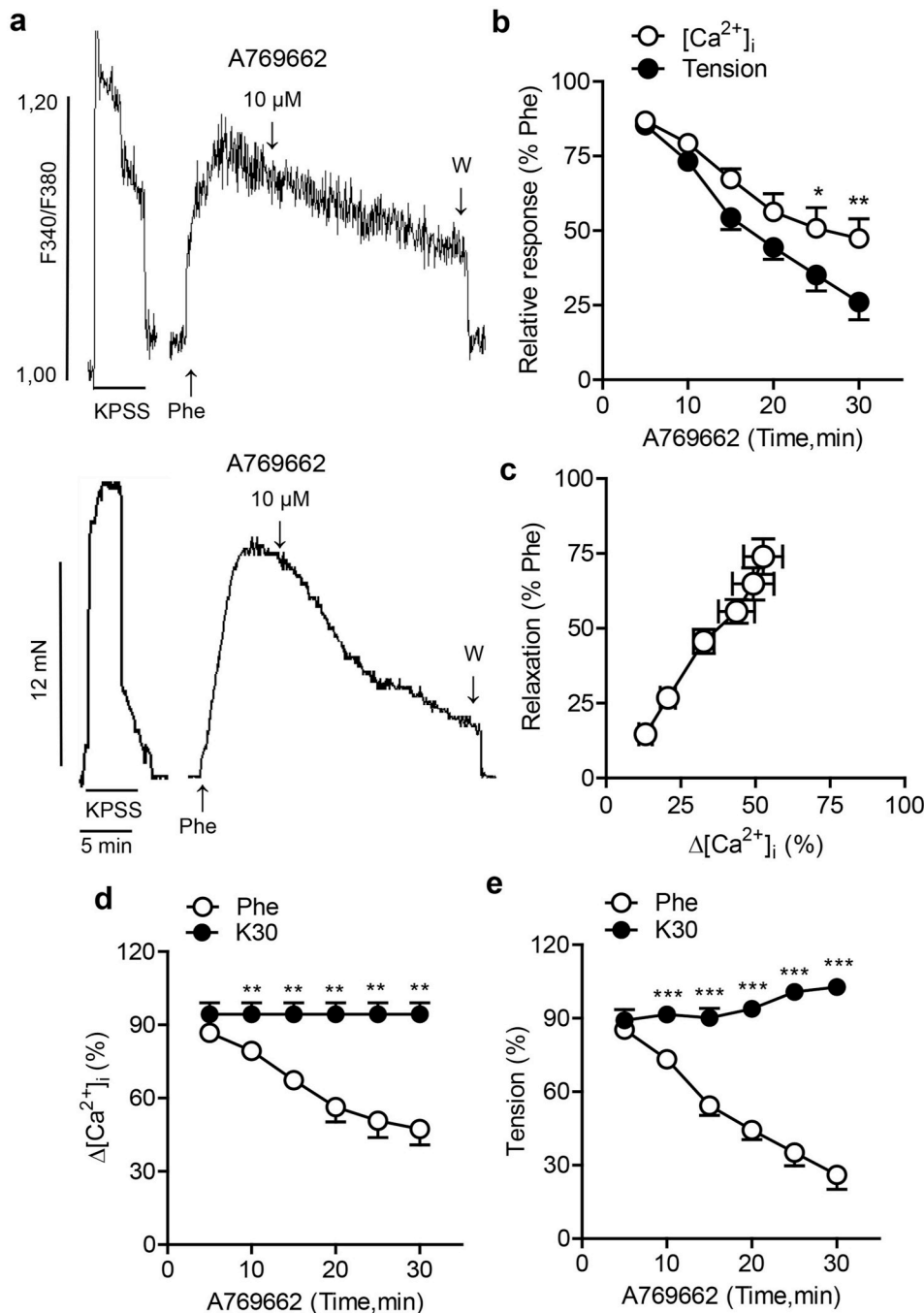


Fig. 4. AMPK activation relaxes renal arteries by reducing VSM $[Ca^{2+}]_i$ and by calcium-independent mechanisms.

Representative traces showing simultaneous recordings of VSM $[Ca^{2+}]_i$ and tension and the effect of the AMPK activator A7699662 in endothelium-intact rat renal arteries pre-contracted with phenylephrine (Phe). (a) Average concentration-dependent decreases in $[Ca^{2+}]_i$ (upper panel) and relaxations (lower panel) in response to A769662 (b) and $[Ca^{2+}]_i$ -tension relationships for the effect of A769662 in renal arteries. (c) AMPK activation-induced decreases in $[Ca^{2+}]_i$ and relaxations in arteries precontracted with Phe were abolished when raising extracellular K^+ in arteries precontracted with 30 mM K^+ (d, e) All results are expressed as percentage of the increases in $[Ca^{2+}]_i$ or tension induced by Phe or with 30 mM K^+ . Data are shown as the mean \pm SEM of 5 arteries, 1 per animal. Statistical significance was calculated by paired Student's *t*-test **P* < 0.05, ***P* < 0.01.

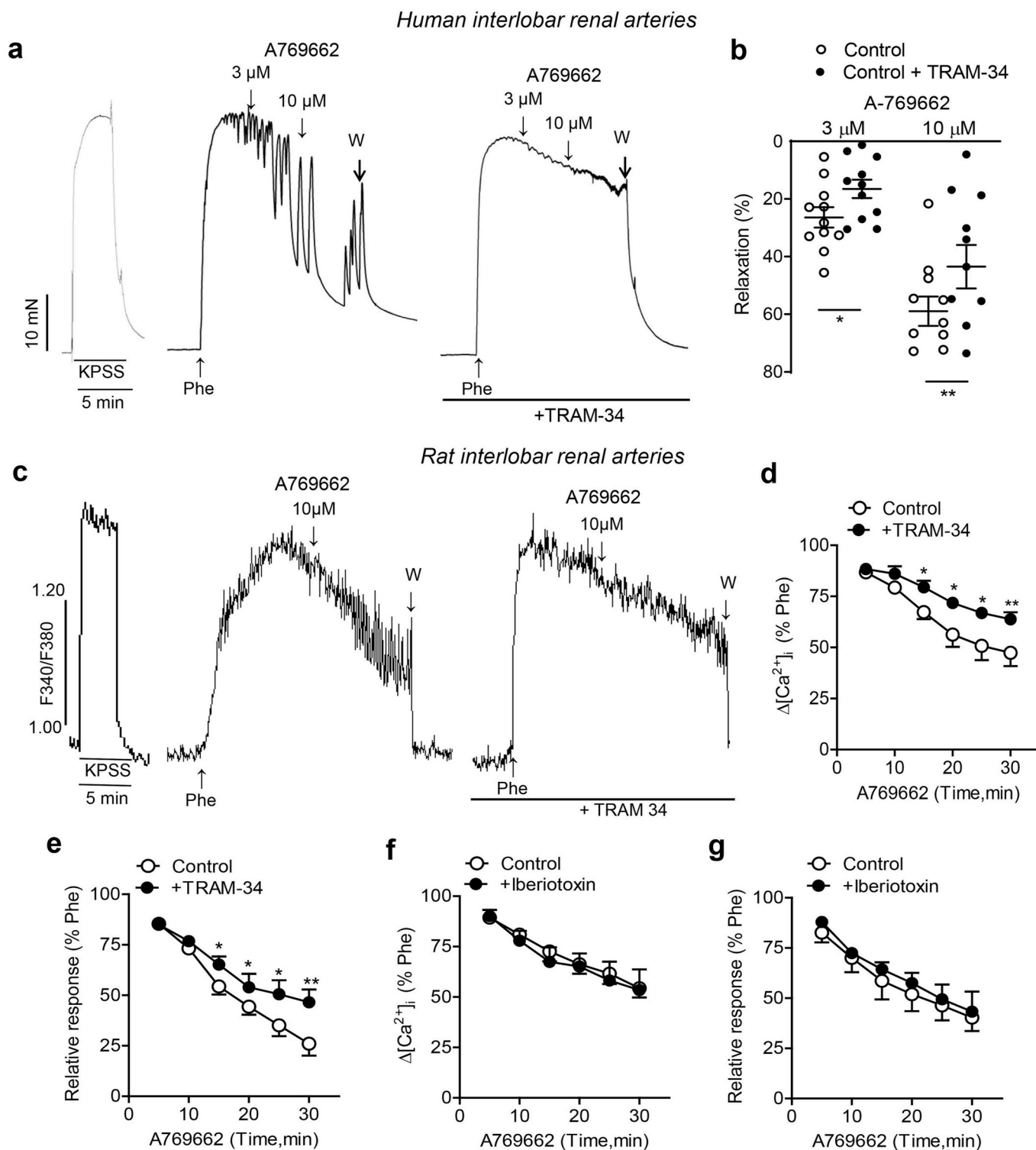


Fig. 5. Intermediate-conductance K_{Ca} (IK_{Ca}) channels are implicated in AMPK-induced vasodilation of human renal arteries.

Representative traces showing the effect of the inhibitor of intermediate-conductance K_{Ca} (IK_{Ca}) channels TRAM-34 (1 μ M) on the A769662-elicited vasodilation of human renal arteries (a) and the average inhibitory effect of TRAM-34 (b). Data are shown as the mean \pm SEM of 10 arteries from 4 patients. Original recordings showing A769662-induced decreases in VSM $[Ca^{2+}]_i$ (top) that were blunted by TRAM-34 (bottom) in intact renal interlobar arteries from rat. (c) Average inhibitory effect of TRAM-34 on the decreases in $[Ca^{2+}]_i$ (d) and the relaxations to A769662 (e) in renal interlobar arteries. Average inhibitory effect of the inhibitor of high-conductance K_{Ca} channels (BK_{Ca}), Iberiotoxin (0.03 μ M) on the decreases in $[Ca^{2+}]_i$ (f) and the relaxations to A769662 (g) in endothelium-denuded renal interlobar arteries. Data are shown as the mean \pm SEM of 4–5 rats (1 artery per animal). Statistical significance was calculated by paired Student's t-test * $P < 0.05$, ** $P < 0.01$.

Specific features of kidney AMPK-mediated arterial vasodilatation such as activation of endothelial IK_{Ca} channels and VSM K_{ATP} channels are first unveiled.

Pharmacological activation of AMPK with the thienopyridine derivative A769662 induced potent relaxations of arteries from the rat kidney. Notably, that effect is reproduced in a similar extend in human

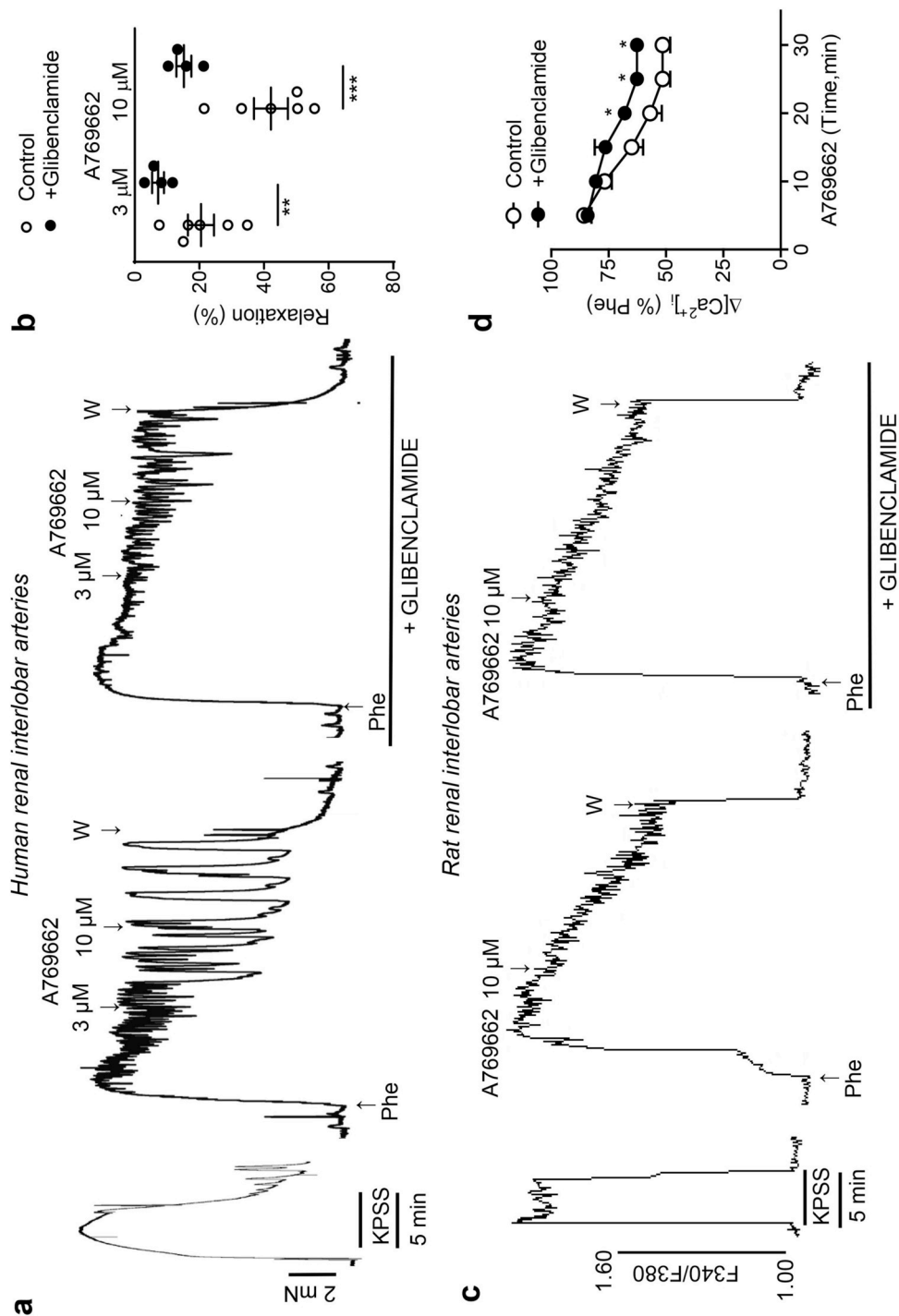


Fig. 6. A769662 activates ATP-sensitive K^+ (K_{ATP}) channels in VSM of human renal arteries

Representative traces illustrating the inhibition of both A769662-elicited vasorelaxation (top) in human renal arteries (a) and decreases in VSM $[Ca^{2+}]_i$ (bottom) in rat renal arteries from rat (c) induced by the K_{ATP} channel blocker glibenclamide (1 μ M). Arteries were endothelium-denuded. Summarized data showing the average changes in relaxation (b) and $[Ca^{2+}]_i$ (d) induced by A769662 in presence of glibenclamide. Values means \pm SEM of n = 4–5 rats (1 artery per animal) and the mean \pm SEM of from 4 patients (2 arteries per patient).

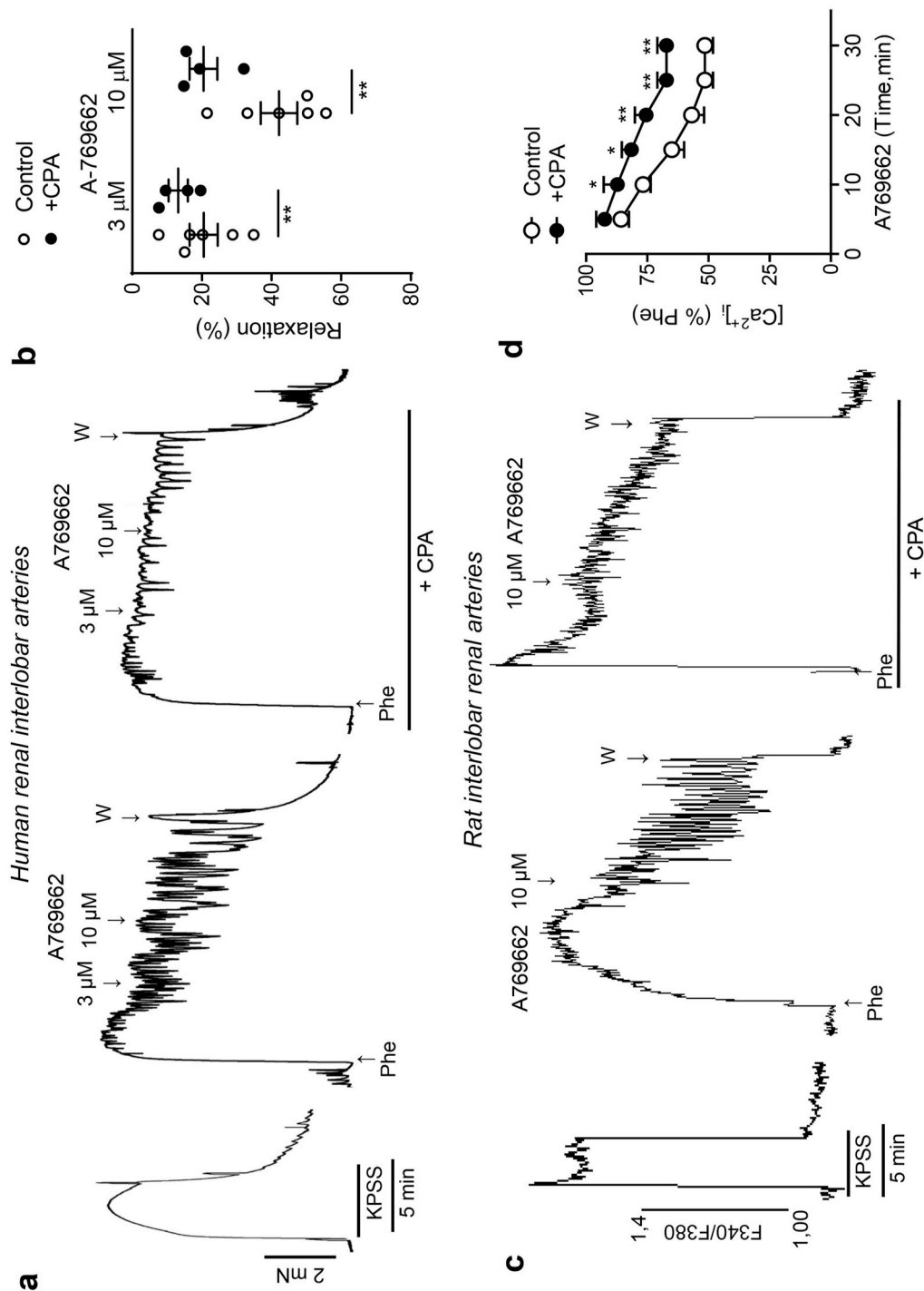
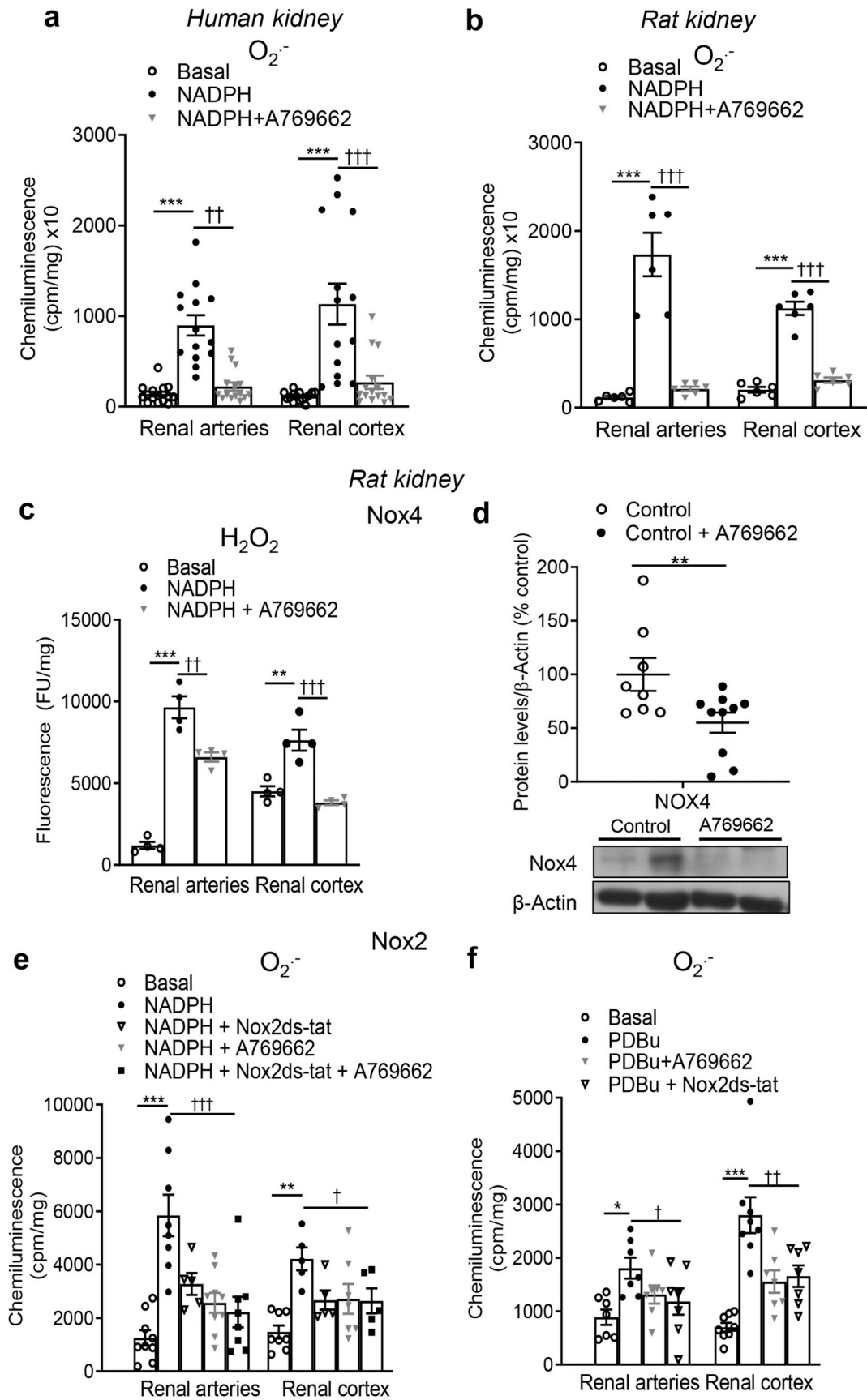


Fig. 7. SERCA activation in involved in A769662-induced relaxations of human renal arteries

Original recordings showing that both vasodilation (top) in human renal arteries (a) and decreases in $[Ca^{2+}]_i$ (bottom) in rat renal arteries evoked by AMPK activation with A769662 (c) were blunted by the inhibitor of sarcoplasmic reticulum calcium ATPase (SERCA) CPA (10 μM). Experiments were performed in endothelium-denuded arteries. Summarized data showing the average changes in relaxation (b) and $[Ca^{2+}]_i$ (d) induced by A769662 in presence of CPA. Data are shown as means ± SEM of n = 6 rats (1 artery per rat) and as the mean ± SEM from 3 patients (2 arteries per patient). Statistical significant differences between means were analyzed by paired Student's t-test *P < 0.05, **P < 0.01, ***P < 0.001.



(caption on next page)

Fig. 8. Activation of AMPK downregulates Nox4-and Nox2-derived ROS generation.

(a,b) Powerful inhibitory effect of AMPK activation on NADPH-stimulated levels $O_2^{\cdot-}$ in renal arteries and cortex samples from human (a) and rat (b) kidney measured by lucigenin-enhanced chemiluminescence. (c) Basal and NADPH-stimulated levels of H_2O_2 in renal arteries and renal cortex from rat measured by Amplex Red fluorescence were reduced by A769662 (30 μ M). Results are expressed in counts per minute (cpm) per mg of tissue for chemiluminescence and in relative fluorescence units (RFU) per mg of tissue for fluorescence. Bars represent mean \pm SEM of 7 patients (2 samples per patient) and 6–10 animals (1–2 samples per animal). Significant differences were analyzed using one-way ANOVA followed by Bonferroni as a *posteriori* test $^{**}P < 0.01$, $^{***}P < 0.001$ versus control before treatment, $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$ versus NADPH-treated. (d) Western blots analysis for Nox4 protein levels in renal arteries and downregulation upon acute *in vitro* treatment with the AMPK activator A769662 (30 μ M). Results were quantified by densitometry and presented as a ratio of density of the NOX4 band vs that of β -actin from the sample. Data are shown as the mean \pm SEM of 8–10 rats. Statistical significance was calculated by unpaired Student's t-test $^*P < 0.05$, $^{**}P < 0.01$. (e) NADPH-stimulated levels $O_2^{\cdot-}$ in samples of renal arteries and cortex from rat kidney were reduced by the selective Nox2 inhibitor Nox2ds-tat (1 μ M) and by A769662 (30 μ M) alone, but there was no further inhibition by combined treatment with Nox2ds-tat plus A769662. (f) Protein kinase C activator phorbol 12,13-dibutyrate PDBu (10 μ M) was used to indirectly stimulate Nox2 and $O_2^{\cdot-}$ production. Basal and PDBu-stimulated levels $O_2^{\cdot-}$ in renal tissues were reduced by the Nox2 inhibitor Nox2ds-tat and by A769662 (f) Results are expressed in counts per minute (cpm) per mg of tissue for chemiluminescence. Bars represent mean \pm SEM of 6–10 rats. Significant differences were analyzed using one-way ANOVA followed by Bonferroni as a *posteriori* test $^{**}P < 0.01$, $^{***}P < 0.001$ versus control before treatment, $\dagger\dagger P < 0.01$; $\dagger\dagger\dagger P < 0.001$ versus NADPH or PDBu-treated.

intrarenal arteries. A7669662 also relaxed mesenteric resistance but not coronary arteries from the same rats, thus suggesting that A-7669662 vasodilator action is vascular tissue-specific. Renal vasodilatation induced by A7669662 was 3 orders of magnitude more potent than that elicited by the pharmaceutical AMPK activator AICAR [10,36,37]. The higher vasodilator potency in the micromolar range found for A-7669662 versus AICAR in intrarenal arteries would confirm its high specificity towards AMPK and is in agreement with that recently reported for other small arteries such as muscular and mesenteric resistance arteries [33].

A7669662 relaxation was found to be associated with phosphorylation of AMPK and of the AMPK downstream target ACC in renal interlobar arteries. A7669662 selectively binds the AMPK β_1 catalytic subunit and allosterically increases kinase activity of the α_1 catalytic subunit, but this compound can also activate AMPK by inhibiting dephosphorylation of AMPK on Thr-172, like the effects of AMP [38]. Accordingly, acute exposure of renal arteries to A769662 enhanced AMPK Thr-172 phosphorylation and also phosphorylation (and thus inhibition) of the AMPK downstream target ACC, rate-limiting enzyme for fatty acid synthesis, the latter indicating that AMPK vasodilator action accompanies kinase regulatory activity of lipid metabolism and energy storage/mobilization in kidney vascular tissues.

AMPK cardiovascular actions have mainly been attributable to AMPK-activating eNOS in endothelial cells and cardiomyocytes, although AMPK is expressed in both endothelium and VSM and its activation can induce endothelium-independent vasodilation [33,36,39]. In endothelial cells AMPK can be regulated by stimuli affecting cellular ATP levels such as hypoxia, as well as by fluid shear stress, Ca^{2+} -elevating agonists and hormones such as insulin, leptin, adiponectin and thyroid hormones, through upstream kinases including LKB1 or CAMKK β [2,8]. Activation of AMPK by AICAR in endothelial cells of both large and small resistance arteries has been demonstrated to increase phosphorylation of eNOS at Ser1177 and Ser635, resulting in increased NO production and vasodilatation [5–7,10,40]. In agreement with these data, A769662-induced relaxations of both human and rat intrarenal arteries were found to be partially inhibited by mechanical endothelial cell removal and by pharmacological blockade of eNOS, suggesting the involvement of the eNOS/NO pathway in renal vasodilatation induced by AMPK. However, a significant amount of the A769662-induced relaxant response persisted in arteries denuded from endothelium indicating that AMPK activation can directly relax VSM of renal arteries.

VSM expresses AMPK α_1 -subunit and both β_1 and β_2 subunits, predominantly β_1 -AMPK [36,39]. AMPK is activated by metabolic stress in arterial smooth muscle and has been involved in endothelium-independent vasodilation of both large and small arteries [33,36,39] and in the regulation of VSM proliferation and inflammation [9,41]. The present findings demonstrate that AMPK activation causes relaxation of renal arteries mainly by reducing VSM $[Ca^{2+}]_i$ and to a lesser extent through Ca^{2+} -independent mechanisms decreasing calcium sensitivity.

While recent reports demonstrate that AMPK activators reduce VSM $[Ca^{2+}]_i$ of resistance arteries through hyperpolarization mediated by smooth muscle BK_{Ca} channels [33], the present findings suggest that stimulation of IK_{Ca} rather than BK_{Ca} channels is involved in the decrease of smooth muscle $[Ca^{2+}]_i$ of renal arteries. Thus, while the inhibitor of BK_{Ca} channels iberiotoxin had no effect on A769662 vascular responses, selective pharmacological blockade of IK_{Ca} channels with TRAM reduced both relaxation of human renal arteries and the decrease in VSM intracellular Ca^{2+} elicited by A769662. The differences between our findings and those reported by Schneider et al. (2015) [33] in small muscle and mesenteric arteries support again vascular tissue-specificity of the A769662 action and are likely to be related to the features of the endothelium-dependent hyperpolarization (EDH)-type dilatation of intrarenal arteries. Thus, EDH-elicited response is initiated by stimulation of intermediate- and small conductance K_{Ca} channels and hyperpolarization of renal endothelial cells which further spreads through myoendothelial gap junctions to the underlying VSM thus inducing hyperpolarization and vasorelaxation, greatly reduced by blockade of IK_{Ca} channels with TRAM both *in vitro* and *in vivo* [31,42,43]. The inhibitory effect of TRAM-34 on both decreases in VSM $[Ca^{2+}]_i$ and relaxation elicited by A-769662 would be consistent with the AMPK activator stimulating endothelial IK_{Ca} channels and further producing VSM relaxation in intrarenal arteries. These findings would be supported by recent studies demonstrating that (EDH)-type dilatation of small arteries is lost after endothelium-specific knock out of the AMPK α_1 -subunit, thus suggesting that AMPK mediates EDH-type responses of microvessels therefore being involved in blood pressure regulation [44].

Interestingly, our findings first provide evidence that AMPK can also activate VSM K_{ATP} channels and thus contribute to renal arterial relaxation, since the sulfonylurea glibenclamide markedly inhibited both vasodilation of human renal artery and the decrease in VSM $[Ca^{2+}]_i$ elicited by A-769662. While AMPK is activated in response to metabolic stress usually associated with ATP depletion and increase in the AMP/ATP ratio [1], sarcolemmal K_{ATP} channels are likewise stimulated by decrease in intracellular ATP, these channels functioning as metabolic sensors which couple intracellular metabolic state to membrane excitability [45]. In the kidney, K_{ATP} channels are functionally expressed in renal arteries and arterioles and K_{ATP} activators dilate afferent arterioles *in vitro* and increase renal blood flow *in vivo* and in the isolated perfused kidney [46–48]. K_{ATP} channels were early involved in metabolic vasodilation of preglomerular arterioles wherein glucose deprivation elicited dilation that was reversed by glibenclamide [46]. Therefore, stimulation of renal arterioles K_{ATP} channels by AMPK activation, as shown in the present study, might represent a mechanism to couple renal blood flow and metabolism. Relationship between K_{ATP} channels and AMPK activation has been reported in the heart wherein AMPK stimulates sarcolemmal K_{ATP} channel activity and recruitment in cardiac myocytes thus contributing to cardioprotection evoked by preconditioning [49]. In the kidney, both AMPK activators and K_{ATP}

channels openers have independently been reported to provide protection against renal ischemia/reperfusion injury through antioxidant, antiapoptotic and prosurvival actions [17,19,50], while the present findings first show that K_{ATP} channels are downstream targets for the AMPK vasodilator action probably implicated in the metabolic regulation of renal blood flow.

Activation of SERCA has been involved in lowering smooth muscle $[Ca^{2+}]_i$ and relaxation, and both redox regulation and more recently phosphorylation of SERCA2b, the major SERCA isoenzyme in VSM, increase pump activity to introduce Ca^{2+} in the SR and therefore to reduce VSM $[Ca^{2+}]_i$ and relax arterial smooth muscle [33,51–53]. In our study, pharmacological blockade of SERCA and subsequent SR store depletion by treatment with CPA greatly reduced both relaxation of human renal arteries and the VSM $[Ca^{2+}]_i$ decreasing effects induced by AMPK activation, which suggests that SERCA pump is also a target for AMPK phosphorylation in renal VSM accounting for the AMPK-induced vasorelaxation of kidney arteries. This is in agreement with that recently reported for other small arteries wherein AMPK activation augmented VSM calcium transients elicited by SR store depletion with caffeine and inhibition of SERCA reduced the relaxations induced by A769662 [33]. Moreover, in the latter study relaxation induced by A769662 was associated with increased phosphorylation of the SERCA modulator phospholamban at the regulatory T17 site which stimulates SERCA activity [33]. Therefore, the present findings in intrarenal arteries confirm that stimulation of SERCA and reduction of VSM $[Ca^{2+}]_i$ is a powerful universal mechanism for the relaxant effects of AMPK activators in small arteries and arterioles.

In the cardiovascular system, AMPK activity can be regulated by oxidative stress and AMPK in turn is able to modulate both mitochondrial ROS generation and gene expression of antioxidant defenses in endothelial cells [21,54]. Hence, pharmacological activation of AMPK in the vascular endothelium has been proposed to be beneficial in metabolic disease not only because of its bioenergetic effects but also due to its ability to counteract oxidative stress [54]. In the present study, we first provide evidence for Nox subunits being targets of AMPK in kidney vascular tissues and demonstrate that AMPK regulates both Nox expression and ROS generating activity. Up-regulation of Nox4 induced by high glucose in podocytes and high fat diet in renal tubular cells has been reported to be inhibited by activation of AMPK both *in vitro* and *in vivo* [13,20,55]. Nox4, is highly expressed in the kidney and is a source of endothelial vasodilator H_2O_2 and protective of vascular function under physiological conditions [30]. In line with this scenario, we have found that acute treatment of renal interlobar arteries with the AMPK activator A-769662 induced a powerful inhibition of NADPH-derived ROS associated to a marked down-regulation of Nox4 expression and reduced H_2O_2 generation. Nox4 produces H_2O_2 rather than O_2 and is a functional source of ROS in the mitochondria of kidney cortex and renal endothelium [30,56]. Despite AMPK negatively regulates Nox4-dependent oxidative stress and apoptosis in diabetes and obesity [17,55], the present findings suggest that AMPK can also modulate Nox4-dependent H_2O_2 production and vasoactive actions in the kidney under normal physiological conditions.

On the other hand, AMPK has also been shown to reduce activity and expression of other Nox subunits such as Nox2; thus, AMPK α 1 knockout mice displayed increased vascular oxidative stress, Nox2 up-regulation and endothelial dysfunction mediated by enhanced NF- κ B activation in endothelial cells, these effects being reversed by AICAR [21,57,58]. The present findings also support a role for AMPK as a physiological regulator of Nox2 activity in intrarenal arteries, since acute exposure to A-769662 did not further inhibited Nox-derived ROS production under conditions of Nox2 blockade, whereas it suppressed O_2^- generation stimulated by phorbol esters, which reduces translocation and phosphorylation of p47phox thus inhibiting Nox2 activation, as reported for cardiac myocytes and human neutrophils [59,60].

In summary, we provide evidence here for new physiological targets of AMPK activation in the kidney leading to renal vasodilation and

downregulation of vascular ROS production. These vasorelaxant and antioxidant effects would be consistent with the cardiovascular protective actions of antidiabetic drugs like metformin whose actions have been shown to be mediated by AMPK activation [26,61]. Amelioration of renal fibrosis and inflammation reported for pharmacological AMPK activators [17,25] along with the benefits of vascular AMPK activation suggest that these drugs may be useful therapeutic tools in the treatment of metabolic disease-associated nephropathy and chronic kidney disease.

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Declaration of competing interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101575>.

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