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Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway

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The antidiabetic drug metformin can diminish apoptosis induced by oxidative stress in endothelial cells and prevent vascular dysfunction even in nondiabetic patients. Here we tested whether it has a beneficial effect in a rat model of gentamicin toxicity. Mitochondrial analysis, respiration intensity, levels of reactive oxygen species, permeability transition, and cytochrome *c* release were assessed 3 and 6 days after gentamicin administration. Metformin treatment fully blocked gentamicin-mediated acute renal failure. This was accompanied by a lower activity of *N*-acetyl-β-D-glucosaminidase, together with a decrease of lipid peroxidation and increase of antioxidant systems. Metformin also protected the kidney from histological damage 6 days after gentamicin administration. These *in vivo* markers of kidney dysfunction and their correction by metformin were complemented by *in vitro* studies of mitochondrial function. We found that gentamicin treatment depleted respiratory components (cytochrome *c*, NADH), probably due to the opening of mitochondrial transition pores. These injuries, partly mediated by a rise in reactive oxygen species from the electron transfer chain, were significantly decreased by metformin. Thus, our study suggests that pleiotropic effects of metformin can lessen gentamicin nephrotoxicity and improve mitochondrial homeostasis.

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KEYWORDS: acute kidney injury; gentamicin; mitochondria; oxidative stress; renal protection

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The major clinical problem in the use of gentamicin, an aminoglycoside antibiotic extensively used in the treatment of Gram-negative bacterial infection,[1] is its nephrotoxicity even at the lowest therapeutic doses.[2] Indeed, in addition to tubular toxicity, a part of gentamicin-induced renal damage is based on its glomerular effects, especially those altering the function of mesangial cells.[3–5]

Conversely, mitochondria are increasingly believed to have a crucial role in a broad spectrum of renal diseases.[6,7] As the kidney is the main excretory route, through the process of filtration and secretion, for various drugs, some of which may be mitochondrial toxins, mitochondrial dysfunction could have a major role in nephrotoxicity. Previous experiments in a rat model reported that gentamicin inhibited oxidative phosphorylation and reduced ATP levels in renal tubular cells.[8] Evidence also pointed to mitochondrial pathway-dependent oxidative stress in renovascular defects.[9,10] In fact, numerous *in vivo* and *in vitro* studies showed that reactive oxygen species (ROS) were often involved in the onset and progression of these injuries.[11,12] Similarly, ROS-induced cell death processes were proposed to have a relevant role in gentamicin-mediated acute renal failure, characterized by necrosis of proximal tubular cells[13,14] and simultaneous occurrence of glomerular cell proliferation and apoptosis.[15,16] Interestingly enough, gentamicin enhanced ROS formation in isolated cortical mitochondria.[17]

We previously reported that treatment with resveratrol, a natural antioxidant, modulated the toxic effects of gentamicin by preventing increase in oxidative stress.[18] Similar protective effects on renal function in response to gentamicin injection were found with other ROS scavengers.[19–21] However, the exact biochemical mechanisms involved in nephroprotection, as well the specific contribution of mitochondria in this event, were barely examined in most of these studies. On the contrary, data revealing the preventive actions of metformin, a reference medication for prime treatment of type-2 diabetes and its long-term complications,[22] have accumulated over the past few years.

Recent investigations strongly showed that this antidiabetic agent prevented oxidative stress-induced death in several cell types,[23,24] including human endothelial cells,[25] through a mechanism dependent on the mitochondrial permeability transition pore (PTP) opening. Importantly, a randomized comparative trial showed that, for an equivalent effect on glycemic control after years of treatment, metformin was largely superior to other therapeutic measures for reducing vessel diseases and all-cause-related mortality.[26] Therefore, the aim of this study was to examine the potential properties of metformin protecting the kidney from a nephrotoxicant insult, as well to investigate the main underlying mechanisms.

RESULTS

Gentamicin-induced renal dysfunction and associated oxidative stress are both reversed by metformin

Gentamicin exposure led to acute renal failure, as evidenced by decreased creatinine clearance and increased excretion of *N*-acetyl-β-D-glucosaminidase (Table 1). Treated animals also had a lower glomerular filtration rate (Figure 1a), reduced renal plasma flow (RPF) and blood flow (RBF) (Figure 1b and c), as well as higher renal vascular resistance (Figure 1d) than control rats. Metformin fully mitigated this nephrotoxic profile as rats receiving both metformin and gentamicin showed higher glomerular filtration rate, RPF, and RBF than gentamicin-treated rats, the end values even reaching or rising above those in the control group (Figure 1). Expectedly, lipid peroxidation substantially increased with gentamicin as compared with that in the control group (Figure 2a), whereas plasma total antioxidant status (TAS) simultaneously diminished (Figure 2b). Importantly, metformin, when administered before and together with gentamicin, not only normalized lipid peroxidation but also increased TAS (Figure 2).

Histological changes due to gentamicin toxicity are prevented by metformin

Light-microscopic examination of kidneys from control and metformin-treated rats showed no structural alterations in renal tissues (Figure 3a and c). Massive and diffuse cell necrosis was observed in the proximal tubules of kidneys from rats injected with gentamicin. In addition, the tubular lumen was frequently filled with hyaline casts or heterogeneous cellular debris (Figure 3b). In contrast, in rats treated with gentamicin and metformin, most of the proximal tubules showed completely viable cells, and manifest necrosis was observed in less than 10% of cells, although most

Table 1 | Urinary flow, NAG activity, proteinuria, and creatinine clearance in control rats, rats that received a daily intraperitoneal injection of gentamicin, rats that received metformin-supplemented drinking water, and rats that received metformin plus gentamicin (*n*=4, performed in duplicate)

	Control	Gentamicin	Metformin	Metformin+gentamicin
Urinary flow (ml day^{-1})	12.5 ± 4.4	7.6 ± 1.5	18.7 ± 10.9	59.8 ± 19.4†,§
NAG activity (AU day^{-1})	0.94 ± 0.17	18.2 ± 4.5†	0.96 ± 0.1	1.6 ± 0.3§
Proteinuria (mg day^{-1})	20.7 ± 5.8	39.9 ± 7.6†	26.3 ± 6.9	15.6 ± 7.9§
Creatinine clearance (ml min^{-1})	1.25 ± 0.015	0.096 ± 0.04†	1.4 ± 0.03	1.3 ± 0.2§

Abbreviation: NAG, *N*-acetyl-β-D-glucosaminidase.
$^{†}P<0.05$ versus control; $^{§}P<0.05$ versus gentamicin.

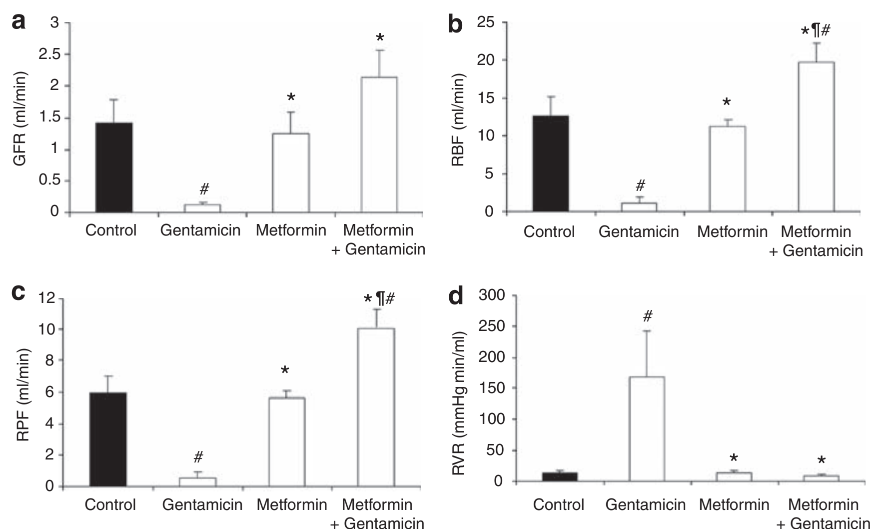

Figure 1 | Effect of metformin on gentamicin-induced renal dysfunction in rats. (**a**) Glomerular filtration rate (GFR), (**b**) renal blood flow (RBF), (**c**) renal plasma flow (RPF), and (**d**) renal vascular resistance (RVR) were determined in all experimental groups mentioned in Table 1 ($n=4$). $^{\#}P<0.05$ versus control; $^{*}P<0.05$ versus gentamicin; $^{¶}P<0.05$ versus metformin.

proximal tubular cells showed signs of initial cellular degeneration (Figure 3d).

Paradoxically, such a protection afforded by metformin against anatomical and functional signs of gentamicin-induced nephrotoxicity was not accompanied by a reduction, but rather by an apparent increase in the renal accumulation of aminoglycoside. Indeed, metformin-treated rats incorporated two times more gentamicin in their renal cortices than did rats treated with only gentamicin (1235 ± 192 versus 666 ± 88 μg/g tissue, $P < 0.05$), whereas low amounts of gentamicin present in the medulla did not significantly vary between both animal groups (103 ± 13 versus 136 ± 34 μg/g tissue).

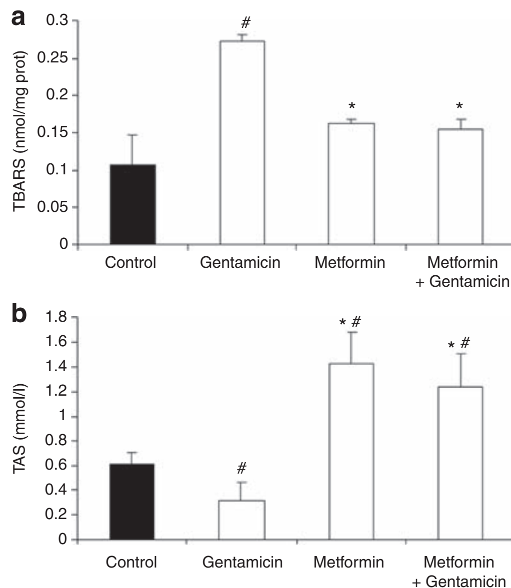

Figure 2 | Effect of metformin on the oxidative stress *in vivo* related to gentamicin-induced nephrotoxicity in rats. (**a**) Lipid peroxidation, as estimated by production of thiobarbituric acid-reactive substances (TBARS) in kidney homogenates and (**b**) total antioxidant systems (TAS) of the plasma were accordingly assessed in the four groups of rats studied. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus gentamicin.

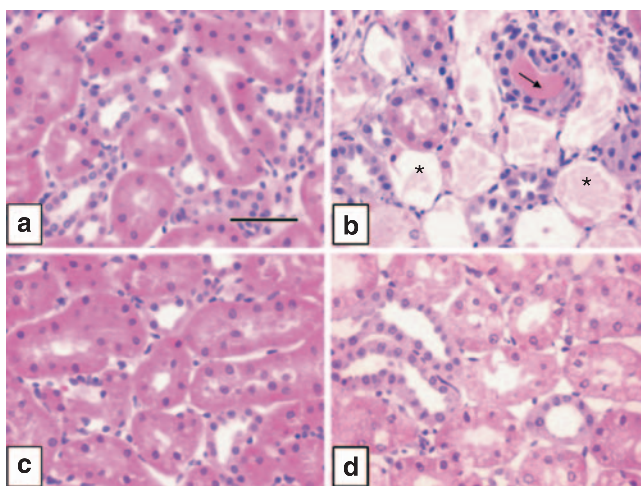

Figure 3 | Light micrographs of sections from kidneys stained with hematoxylin–eosin. (**a**, **c**) Normal histology of kidney tissue in control and metformin-treated rats. (**b**) Massive necrosis in proximal tubules (asterisk) with hyaline cast (arrow) is observed in animals treated with gentamicin (150 mg/kg body weight per day) for 1 week. (**d**) Sections from gentamicin—metformin-treated rats reveal almost complete prevention of histopathological alterations by the biguanide drug. Bar = 50 μm.

Role of mitochondria in the mechanism of gentamicin-induced kidney damage, as well as in the protective response initiated by metformin: key implication of PTP

We propose that a profound mitochondrial dysfunction is at the origin of gentamicin-caused pathogenesis, leading ultimately to necrotic degradation of kidney cells. To test this theory, we addressed the questions of whether PTP opening and cytochrome *c* release were involved in gentamicin toxicity, and how metformin modulated these events *in vivo*. This experimental set was mainly conducted using rats receiving only three doses of gentamicin to assess the early occurrence of mitochondrial changes. Similar to that observed in the liver,[27] kidney mitochondria that were energized with glutamate–malate or succinate absorbed and retained Ca^{2+} until the final increase in fluorescence (Figure 4a), indicative of mitochondrial permeability transition induced by calcium overload, and were also sensitive to cyclosporine-A (CsA), the reference PTP inhibitor. Metformin increased the Ca^{2+} amount required for PTP opening, irrespective of the nature of respiratory substrates. Importantly, addition of Ca^{2+} to mitochondria from rats treated with either three or six doses of gentamicin, stimulated a release of accumulated Ca^{2+} under all conditions, a phenomenon that was hindered by metformin and CsA (Figure 4b). We next studied cytochrome *c* compartmentalization during gentamicin treatment. For this purpose, the cytosolic and mitochondrial fractions were isolated from the renal cortex (Figure 5). After three doses of gentamicin, cytochrome *c* release into the cytosol was obvious. Nevertheless, the released part was <25% of the total cytochrome *c* and, hence, mitochondrial cytochrome *c* did not show a clear decrease. Metformin ameliorated this cytochrome *c* delocalization, indicating that PTP inhibition by metformin provides a potential means of reducing the nephrotoxicity of gentamicin.

Gentamicin-mediated defects in mitochondrial bioenergetics are reversed by metformin

Table 2 summarizes all mitochondrial respiration data from groups receiving gentamicin for 3 or 6 days, either alone or with metformin, before administration to rats for 1 week. Compared with control values, gentamicin treatment resulted in alterations in respiratory chain function independent of the respiratory fuels used, after as few as three doses of this nephrotoxicant. Gentamicin mildly reduced the oxygen consumption rates (Jo_2) under the phosphorylating condition, that is, in the presence of ADP (state-3), and in the

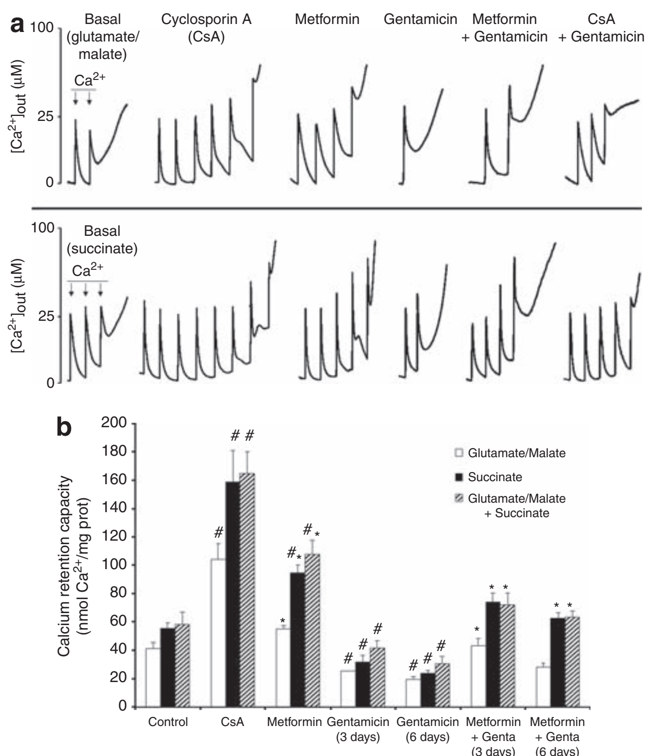

Figure 4 | Calcium retention capacity of kidney mitochondria isolated from control or gentamicin-treated rats (3 and 6 days), and subjected or not to metformin treatment. (**a**) Typical traces obtained after suspension of renal mitochondria in an incubation medium with respiratory substrates and calcium green. Where indicated, pulses of 25 μM Ca^{2+} were added every minute. CsA, the common inhibitor of the PTP, was directly added to mitochondria isolated from control rats, treated or not with gentamicin. (**b**) Quantitative analysis of the calcium retention capacity for each experimental set with glutamate–malate (GM,□), succinate (S,■), glutamate–malate + succinate (GMS,▨) as energizing substrates. $^{\#}P<0.05$ versus control; $^{*}P<0.05$ versus gentamicin. No significant difference was found between the two groups of rats treated for different durations with gentamicin alone. PTP, permeability transition pore.

uncoupling state, after DNP addition. Jo_2 with TMPD-ascorbate, used to assess the maximal activity of cytochrome oxidase, was not altered by gentamicin. After six doses of this antibiotic, all respiratory values were significantly different from that of the control; respiration rate in state 4, that is, in the presence of ATP synthase inhibitor oligomycin, was also below normal, resulting in no significant change in the respiratory control index or RCR (state-3/state-4). Metformin, when administrated before gentamicin, prevented this progressive mitochondrial dysfunction almost completely (Table 2).

The fact that metformin weakly reduced glutamate/malate-dependent respiration may be consistent with a physiological inhibitory effect on the functionally isolated complex-I, which we previously reported in a quite different setting.[28] Here, complex-I activity from the control mitochondria was significantly reduced by metformin, and this inhibition was even preserved in gentamicin-treated rats. On the contrary, the fact that gentamicin itself drastically lowered complex-I activity

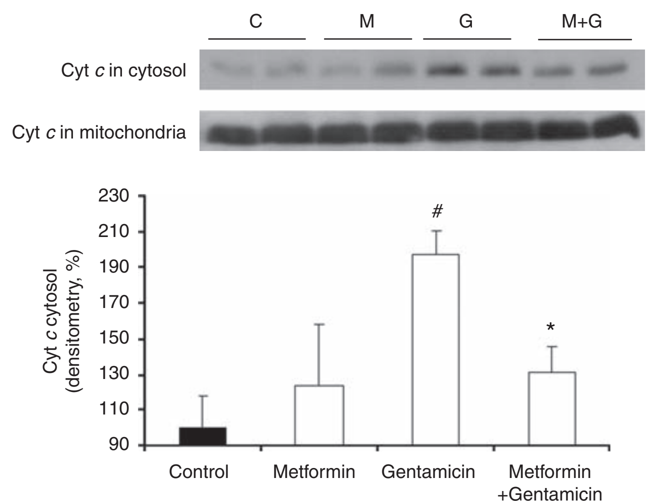

Figure 5 | Cytochrome *c* release during gentamicin nephrotoxicity and prevention by metformin. After 3 days of gentamicin treatment in the absence or presence of metformin, cortical tissues were freshly collected to isolate the cytosolic and mitochondrial fractions for cytochrome *c* immunoblotting. Total protein concentration is 50 μg per lane in all samples. Cytochrome *c* release depicts changes in mitochondrial membrane permeability. $^{\#}P<0.05$ versus control; $^{*}P<0.05$ versus gentamicin.

after 6 days of treatment, would be linked more to molecular alterations (proteolysis, conformational change) in this huge complex, rather than to a generalized damage of mitochondria, as citrate synthase activity, an usual indicator of the respiratory chain content in tissues, was only poorly attenuated (Table 3).

Decline of gentamicin-induced mitochondrial oxidative stress by metformin

There is a trend toward considering that alterations in the respiratory chain are responsible for the formation of excessive amounts of ROS, thereby contributing to cell damage.[29,30] Although hydrogen peroxide (H_2O_2) levels were barely affected by gentamicin, when glutamate–malate or succinate was individually used without inhibitors (Figure 6c and d), a decrease in NADH fluorescence was found in both situations (Figure 6a and b). As the main NADH-oxidizing pathway was blocked by gentamicin (Table 3), an escape of NADH from mitochondria would therefore be envisaged, possibly through the open PTP. A gentamicin-related increase in ROS was especially sizeable in mitochondria respiring on both glutamate–malate and succinate (Figure 6e). Remarkably, metformin prevented the mitochondrion-driven oxidative stress by lowering or normalizing ROS production along the respiratory chain (Figure 6c–e). Moreover, gentamicin-induced depletion of mitochondrial NADH was highly recovered with metformin treatment under either glutamate–malate or succinate conditions (Figure 6a and b).

DISCUSSION

Although gentamicin continues to be an irreplaceable treatment against life-threatening infections, its use remains

Table 2 | Oxygen consumption rates (nmol O_2/min/mg protein) in kidney mitochondria isolated from control rats or rats treated with gentamicin for 3 or 6 days, and subject or not to treatment with metformin (*n*=4–6)

		Control	Gentamicin (3 days)	Gentamicin (6 days)	Metformin	Metformin+ Genta (3 days)	Metformin+ Genta (6 days)
Glutamate/malate (GM)	State-4	17.6 ± 0.4	20.1 ± 1.3	10.2 ± 1.1†	15.8 ± 1.3†	24.9 ± 2.6	15.1 ± 0.9†,§
	State-3	148.9 ± 7.2	128.9 ± 9.2†	80.6 ± 2.1†	119.8 ± 6.9†	155.5 ± 3.3§	126.3 ± 8.1§
	DNP	150.3 ± 11	103.4 ± 7.1†	76.1 ± 2.2†	129.5 ± 14.4	148.9 ± 15.7§	111.8 ± 7.2†,§
	RCR	8.2 ± 0.2	6.4 ± 0.09	7.8 ± 0.4	8.1 ± 0.1	7.1 ± 0.4	8.2 ± 0.3
Succinate+ rotenone	State-4	48.4 ± 2.7	52.2 ± 6.1	28.1 ± 1.5†	44.6 ± 3.3	52.6 ± 14.1	40.9 ± 2.3§
	State-3	285.9 ± 8.7	235.7 ± 9.2†	125.6 ± 18.2†	286.6 ± 6.7	294.8 ± 20.5§	206.1 ± 12.7†,§
	DNP	290.9 ± 16.8	255.8 ± 16.7†	150.9 ± 18.5†	300 ± 18.2	297.3 ± 17.5§	252.8 ± 21.4§
	RCR	6.4 ± 0.1	4.7 ± 0.4	5.1 ± 0.2†	6.6 ± 0.3	6.1 ± 0.35	5.8 ± 0.1
	TMPD-ascorbate	514.8 ± 42.7	492.5 ± 25.3	330.6 ± 48.6†	503.9 ± 27.1	502.8 ± 19.3	484.5 ± 61.9§
GM+succinate	State-4	40.8 ± 2.2	43.7 ± 3.6	24.6 ± 2†	40.1 ± 4	58.7 ± 3.2	39.2 ± 1.4§
	State-3	302.7 ± 9.8	231.3 ± 27.3†	145.1 ± 5.3†	314.4 ± 7.7	327.1 ± 17.8§	258.4 ± 24.3§
	DNP	296.4 ± 10	210 ± 23	132.7 ± 11.4†	312.1 ± 13.9	318.2 ± 25§	243.5 ± 16.3§
	RCR	7.1 ± 0.1	5.05 ± 0.34	6.75 ± 0.3	7.3 ± 0.4	5.8 ± 0.2	6.85 ± 0.2

Abbreviations: DNP, dinitrophenol-uncoupled respiration; RCR, respiratory control ratio; State-3, ADP-stimulated respiration; State-4, basal respiration.
$^†P<0.05$ versus control; $^§P<0.05$ versus gentamicin (3 or 6 days accordingly).

Table 3 | Measurement of functionally isolated respiratory chain complex-I and matrix CS activities in kidney mitochondria isolated from control or gentamicin-treated rats, and subject or not to treatment with metformin (*n*=6)

	Control	Gentamicin	Metformin	Metformin+gentamicin
CS activity (nmol CoA/min/mg protein)	303.1 ± 6.6	275.2 ± 6.8	302.9 ± 6.7	294.1 ± 6.2
Rotenone-sensitive activity of complex-I (nmol NADH/min/mg protein)	91.7 ± 2.8	38 ± 4.1†	77.6 ± 2.4†	71.7 ± 8.8§
Complex-I/CS (nmol NADH/unit of CS)	0.31 ± 0.01	0.13 ± 0.02†	0.27 ± 0.01†	0.25 ± 0.02§

Abbreviation: CS, citrate synthase.
$^†P<0.05$ versus control; $^§P<0.05$ versus gentamicin.

seriously limited by its nephrotoxicity. Although various studies reported on the benefits of several agents in gentamicin-induced renal poisoning,[31] the basis of nephroprotection remains elusive. To the best of our knowledge, this report is the first to show that metformin, a drug widely used in the treatment of diabetes, prevents functional, histological, and biochemical kidney injuries in the setting of gentamicin insult. Of utmost importance, all these impressive effects, seen at a metformin dosage corresponding to the clinically evidenced therapeutic range,[32] partly proceed from a normalization of *in vivo* oxidative stress and restoration of mitochondrial functional integrity.

Our findings corroborate those of earlier studies demonstrating that an enhanced endogenous oxidative stress has a major role in the severity of gentamicin-induced acute renal failure.[3,17] Although having no effect alone, metformin blunted alterations in the hemodynamics induced by the daily injection of a nephrotoxic dose of gentamicin. In addition, RPF, RBF, and urinary flow even increased significantly in metformin plus gentamicin-treated rats as compared with that under baseline conditions. Although these remarkable outcomes cannot be readily explained, one may conceive that such a protection afforded by metformin could be mediated, at least in part, by the *in vivo* antioxidant features of this antidiabetic agent recently found,[33,34] as well by its ability to prevent gentamicin-induced lipid peroxidation and to enhance antioxidant defenses in the control and treated animals (this study). One of the early sensitive markers of tubular injury after exposure to aminoglycosides is increased excretion of lysosomal enzymes. Our data showing that metformin considerably prevented the increase of gentamicin-induced urinary *N*-acetyl-β-D-glucosaminidase excretion, suggest evident protection against structural and functional tubular alterations. Strikingly, metformin-reduced gentamicin toxicity occurs in spite of larger intrarenal amounts of toxicant. This paradoxical result of high cortical concentrations of gentamicin with preservation of renal function was similarly reported by others using rats treated with a combination of gentamicin and polyaspartic acid.[35] As a cationic drug, metformin is known to be transported across the kidney through organic cation transporter-2.[36] In the case of gentamicin, the endocytotic receptor megalin has been reported to be responsible for its tubular accumulation, which is directly related to its toxicity.[37] The fact that metformin did not diminish gentamicin accumulation reflects a lack of interaction between both ways of transport, and suggests that protection conferred by biguanide occurs from within renal cells.

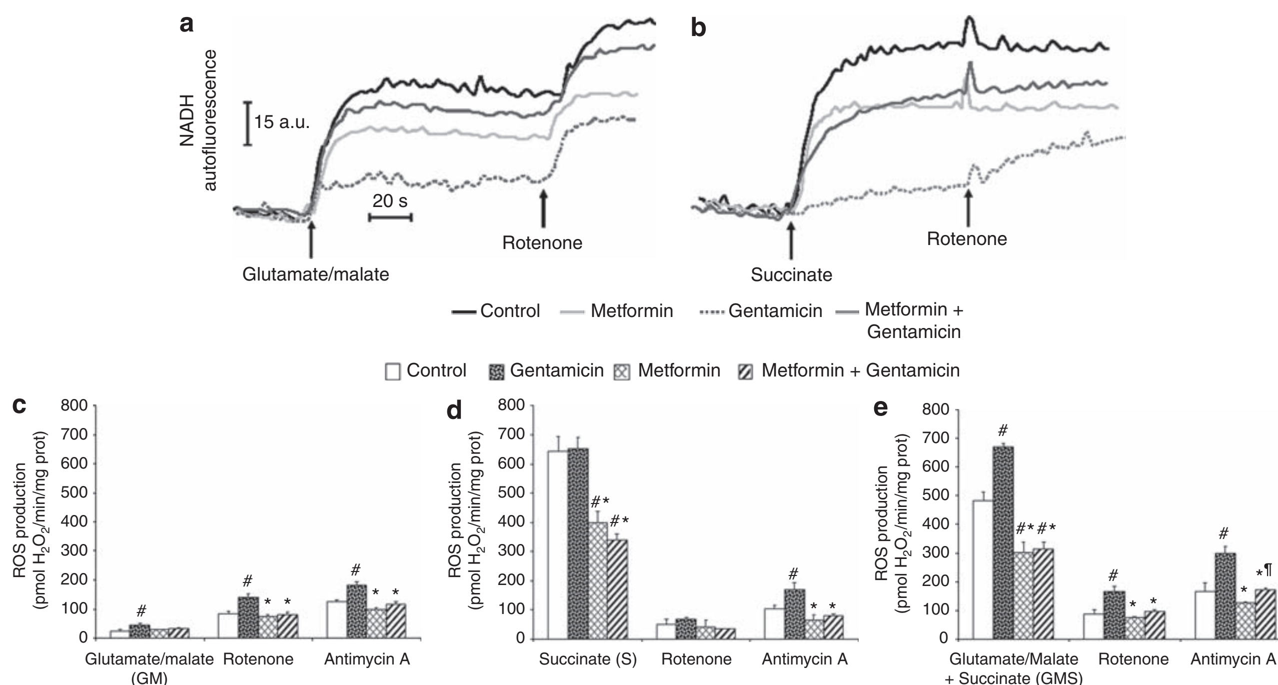

Figure 6 | NADH fluorescence and ROS formation in kidney mitochondria incubated with glutamate–malate and/or succinate. Once mitochondria were isolated from control, gentamicin-, metformin-, and metformin + gentamicin-treated rats, NADH was followed up by determining its spontaneous autofluorescence; one typical experiment is presented (**a**, **b**). Conversely, H_2O_2 production was assessed by amplex red/horseradish peroxidase for all the experimental groups ($n = 6$) (**c**–**e**). Where indicated, substrates and/or inhibitors were sequentially added; each reaction was thus started with either glutamate–malate (**a**, **c**), succinate (**b**, **d**), or both substrates (**e**). Thereafter, 1 μM rotenone (for both mitochondrial parameters) and 0.125 μM antimycin-A (for ROS measurement only) were added. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus gentamicin; $^{\P}P < 0.05$ versus metformin. ROS, reactive oxygen species.

Another potentially important finding of this study is that gentamicin induces drastic mitochondrial changes that largely precede overt cell necrosis and acute renal failure. After three doses of gentamicin, mitochondrial damage included PTP opening and leakage of cytochrome *c* into the cytosol, whereas no evident differences in the histology of treated rats as compared with that in the controls were found, except for cytoplasmic vacuolization in most of the proximal tubules (data not shown). It is noteworthy that these initial mitochondrial abnormalities were improved by metformin. A progressive deterioration in mitochondrial function and cellular integrity was observed with increasing gentamicin dosage. After six doses, PTP opening was higher than that with three doses and, expectedly, light microscopic examination revealed serious lesions and necrosis in a large number of proximal tubular cells. Thus, necroses and shedding of dead cells into the urine would account for the sharp decline in cortical gentamicin concentrations. As the harmful effect of ROS on tissues is widely appreciated, we provide enough evidence that these cytotoxic effects for gentamicin are partly associated with mitochondrial oxidative stress. Despite the presence of non-physiological substrate concentrations, specific conditions of dual-electron entry at both respiratory chain site-1 (glutamate/malate) and site-2 (succinate), as it is most likely the case in living cells, led to higher H_2O_2 contents after gentamicin treatment, indicating major derangements along all electron transfer chain segments and excess generation of ROS. This result correlates with the reported depletion in plasma antioxidant capacity. Among the various effects of ROS on cell metabolism, they are recognized to induce pore opening in either *in vitro* or *in vivo* settings.[38] The fact that gentamicin successively triggered PTP opening, cytochrome *c* release, and cell necrosis, whereas metformin prevented almost all these events, strongly argues that cytotoxicity of gentamicin-mediated stress is likely related to lowered mitochondrial permeability.

It should be stressed that, in the experimental group receiving six doses of gentamicin, the resting, ADP-dependent, and uncoupled respirations all substantially reduced below control values, yet RCR was marginally altered. Importantly, gentamicin lowered the respiratory flux to cytochrome *c* oxidase, whereas citrate synthase activity did not vary. Thus, no indication of oxidative phosphorylation uncoupling by gentamicin was found in this study, contrary to what others have shown in this field.[8] Blockade of respiration should therefore be explained by a loss of key mitochondrial components, including cytochrome *c*, which we already evidenced above, and also probably NADH. Our data showing the destructive effect of gentamicin on complex-I activity, together with the collapse of NADH autofluorescence irrespective of the substrates used, indirectly suggest that part of NADH has left the mitochondrial matrix. Similarly, this process could be attributed to PTP opening. Indeed, we recently showed that permanent mitochondrial

pore opening in intact cells inhibited NADH-consuming processes and allowed the export of NADH outside the mitochondria.[39] It is noteworthy that the unique feature of metformin, namely a regulatory effect on reverse electron flux-related ROS formation through mild inhibition of complex-I that has been depicted using mitochondria isolated from livers perfused *ex vivo* with the biguanide,[40] was kept in the kidney under gentamicin insult. Indeed, metformin suppressed this specific ROS production in both control and gentamicin-treated rats when mitochondria respired on succinate. This response was also accompanied by complete recovery of NADH autofluorescence levels. Finally, even though a weak inhibitory effect of metformin on complex-I was recorded in either case, we can assume that these repeated inhibitions and their advantages in terms of cell survival throughout the treatment have likely had a preconditioning role in gentamicin toxicity.

Apart from complex-I, it is widely thought that AMP-activated protein kinase (AMPK), the energy sensor in cell metabolism, is another crucial target for metformin.[41] However, the true underlying mechanisms remain elusive, even contradictory, and there is actually no simple explanation for the differences between data from a lot of published studies. Furthermore, there are few relevant results in the recent literature about the specific action of metformin on AMPK in the kidney. No significant effect on AMPK phosphorylation was found in this study with metformin alone (data not shown). This result is possibly related to the fact that our conditions are far from protocols using renal cells incubated with AMPK activators for a short time.[42] Further *in vivo* studies intended to clarify the exact role of AMPK in mitochondrial metabolism at the kidney level are thus mandatory.

As both PTP dysregulation and mitochondrial oxidative stress underlie, at least partly, the toxicity of gentamicin, diverse strategies to maintain the integrity of these organelles and cellular viability could be of therapeutic relevance. To a certain extent, the immunosuppressor CsA that delayed gentamicin-induced pore opening would be a putative candidate, but, due to wide-ranging cellular actions that entail side effects and nephrotoxicity,[43] its clinical use is still limited. For its part, metformin might be envisaged as a prospective alternative, even better than other antioxidants,[44] to hamper gentamicin nephrotoxicity by virtue of net prevention, which seems to depend on the interrelationship between complementary processes. In this regard, it is worth keeping in mind that metformin is eliminated unchanged through the kidney by glomerular filtration and tubular secretion.[45] This can promote its potential accumulation in the condition of renal impairment and lead to the rare but most undesirable metformin complication, namely, lactic acidosis.[46] However, a recent study underlined that mild acute renal injury enhanced metformin amounts in the diabetic rat cortex, without further worsening renal function or increasing plasma lactate levels.[47] Although metformin is undoubtedly contraindicated in situations of renal insufficiency, our data along with this latter report now conduce to reconsider more carefully the limitations previously defined about the therapeutic applications of this longstanding medication.[48]

In conclusion, our results suggest that metformin treatment protects the kidney from gentamicin-induced toxicity, and that this protection is based, at least in part, on the correction of gentamicin-induced mitochondrial alterations.

MATERIALS AND METHODS

Animals

Male rats were placed individually into metabolic cages in a temperature-, humidity-, and light-controlled animal house. They were divided into six experimental groups: a control group, a metformin group that received the antidiabetic drug dissolved in drinking water (100 mg/kg/day) for 13 days, two gentamicin groups that received a daily intraperitoneal injection of gentamicin (150 mg/kg), for 3 and 6 days each, and two metformin plus gentamicin groups that received metformin before and together with gentamicin for 10–13 days. Rats were treated in accordance with the protocols of the following institutions: Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18th December 1986) and Spanish Govern-ment (published in Boletín Oficial del Estado N. 67, pp. 8509–8512, 18th March 1988, and Boletín Oficial del Estado N. 256, pp. 31349–31362, 28th October 1990).

Clearance studies

On day 5 after the start of treatment in all concerned experimental groups, urine samples were collected to assess (i) urinary flow, (ii) proteinuria using the Bradford method, (iii) creatinine clearance by the Jaffé kinetic reaction, and (iv) the activity of *N*-acetyl-β-D-glucosaminidase by a photometric procedure from Roche (Roche Applied Science, Indianapolis, IN, USA). At the end of the treatment, the animals were anesthetized with sodium pentobarbital (40 mg/kg) and placed on a heated board. A tracheotomy was then performed to facilitate breathing throughout the experiment. Rats were surgically prepared for clearance studies, while continuously recording their mean arterial pressure. Urine was collected using a bladder catheter into vials containing 0.5 ml water-stabilized mineral oil. An isotonic infusion containing [^{3}H]inulin and [^{14}C]amino-hippuric acid was started at 3 ml/h using a venous catheter to allow clearance determinations. After equilibration, three urine samples were collected at 30 min intervals, with blood sampling at the beginning and the end of each clearance period. Packed cell volume was determined by the microcapillary method. [^{3}H] and [^{14}C] rates were measured of blood and urine samples using a two-channel liquid scintillation counter (Wallac 1409 DSA, Turku, Finland). Inulin and [^{14}C]aminohippuric acid clearances were calculated to measure glomerular filtration rate and RPF, respectively. RBF was calculated from RPF and packed cell volume. Renal vascular resistance was calculated from RBF and mean arterial pressure.

Measurement of *in vivo* oxidative stress

Lipid peroxidation was estimated from a tissue homogenate by measuring thiobarbituric acid-reactive substances as described previously.[49] Blood samples were collected at day 5 of treatment for analysis of TAS using a commercial kit (Randox Laboratories LTD, UK).

Light microscopy imaging

Rats used for 24 h urine collections were anesthetized and the abdomen was opened by means of a midline incision. A perfusion

catheter was inserted into the abdominal aorta and a renal perfusion with cold isotonic saline was performed to wash out the blood. Kidney pieces were trimmed sagitally, fixed by immersion in 4% buffered formalin for 24 h, embedded in paraffin, sectioned (3 μm thick), then counterstained with hematoxylin–eosin.

Determination of gentamicin concentrations

The gentamicin concentration in kidney samples was analyzed by HPLC on the basis of a derivatization with *O*-phthaldialdehyde and by fluorescence detection.[50] Samples of 20 μl were injected into the chromatograph, and elution was achieved at a flow rate of 1.5 ml/min.

Mitochondrial respiratory chain functioning

Mitochondria, isolated by differential centrifugation from kidneys of fasted rats as earlier described,[51] were used to measure bioenergetic parameters. After renal mitochondria were suspended in incubation medium supplemented with glutamate (5 mM)/malate (2.5 mM) or with succinate (5 mM) in the presence of rotenone as energizing substrates, Jo_2 was recorded at 30°C in a constantly stirred oxygraph vessel after successive additions of 1 mM ADP (state 3), 0.75 $\mu g\,ml^{-1}$ oligomycin (state 4), 75 μM DNP (uncoupling), and TMPD–ascorbate (1:5 mM). In addition, respiratory chain complex I and matrix citrate synthase activities were determined spectrophotometrically using protocols that we previously described.[24]

H_2O_2 and NADH autofluorescence levels

Mitochondrial H_2O_2 generation was measured by monitoring H_2O_2-induced fluorescence of probe amplex red (excitation: 560 nm; emission: 583 nm) under the catalysis of horseradish peroxidase.[40] ROS production was stimulated in the presence of respiratory substrates (the same as that used for Jo_2 measurements), then 1.25 μM rotenone and 2 μM antimycin-A were sequentially added to determine the maximun rate of H_2O_2 production of complexes-I and I + III of the electron transfer chain, respectively. Mitochondrial NADH levels were monitored by recording its relative fluorescence intensity (excitation: 340 nm; emission: 460 nm).

Calcium retention capacity and cytochrome *c* release

The sensitivity of the mitochondrial PTP was evaluated fluorimetrically by measuring the calcium retention capacity of mitochondria incubated in a suited buffer supplemented with 0.25 μM calcium green (excitation: 506 nm; emission: 532 nm). The respiratory substrates were the same as before, and pulses of 25 μM Ca^{2+} were added every minute until pore opening. The specificity of this assay was assessed by adding 1 μM CsA, the standard inhibitor of PTP.

After 3 days of gentamicin treatment, in the absence or presence of metformin, kidneys were perfused with saline solution, then renal cortical tissues were freshly collected, homogenized, and fractionated by differential centrifugation in a cold mitochondrial extraction buffer as described elsewhere.[52] The resulting cytosolic and mitochondrial fractions were collected, protein contents were quantified, and 50 μg was next fractionated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and probed with a mouse-purified antibody to cytochrome *c* (1:1000; BD Pharmingen, San Jose, CA, USA). Protein expression was quantified by densitometry using the Scion Image software.

Statistical analysis

All data are represented as mean ± s.e.m. Comparisons were made either by analysis of variance followed by Scheffe's test for repeated measurements, or by an appropriate Student's *t*-test for a non-matched series. $P < 0.05$ was taken to indicate statistical significance.

DISCLOSURE

All the authors declared no competing interests.

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