

Metformin reduces NAD(P)H oxidase activity in mouse cultured podocytes through purinergic dependent mechanism by increasing extracellular ATP concentration

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Hyperglycemia affects the functioning numbers of podocytes and leads to a gradual decline of renal function. The normalization of glucose level is a principle therapeutic goal in diabetic patients and metformin is a popular hypoglycemic drug used in type 2 diabetes mellitus. Metformin activates AMP-activated kinase (AMPK) and decreases NAD(P)H oxidase activity in podocytes leading to reduction of free radical generation. Similar effects are observed after activation of P2 receptors. Therefore, we investigated whether metformin increases extracellular ATP concentration and affects the activities of NAD(P)H oxidase and AMPK through P2 receptors. Experiments were performed on cultured mouse podocytes. NAD(P)H oxidase activity was measured by chemiluminescence and changes in AMPK activity were estimated by immunoblotting against AMPK α -Thr¹⁷²-P. Metformin increased extracellular ATP concentration by reduction of ecto-ATPase activity, decreased NAD(P)H oxidase activity and increased AMPK phosphorylation. A P2 receptor antagonist, suramin (300 μ M), prevented metformin action on NAD(P)H oxidase and AMPK phosphorylation. The data suggests a novel mechanism of metformin action, at least in podocytes. Metformin, which increases extracellular ATP concentration leads to activation of P2 receptors and consequent modulation of the podocytes' metabolism through AMPK and NAD(P)H oxidase which, in turn, may affect podocyte functioning.

Key words: AMP-activated kinase, free radicals, metformin, NAD(P)H oxidase, podocytes, purinoceptors

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INTRODUCTION

Podocytes are highly differentiated, insulin-dependent glomerular epithelial cells contributing to glomerular filtration barrier (Pavenstädt *et al.*, 2003; Diez-Sampedro *et al.*, 2011). Their dysfunction and injury in the course of hyperglycemia lead to changes in glomerular permeability and impairment of renal functions in patients with diabetes mellitus (Li *et al.*, 2007; Reddy *et al.*, 2008). Therefore, a range of glucose-lowering agents with different properties are used to prevent the progression to end-stage renal disease in diabetes mellitus. Metformin (1,1-dimethylbiguanide) is the most widely prescribed drug to treat hyperglycemia in individuals with type 2 diabetes (Viollet *et al.*, 2012). In the blood, metformin is

not bound to plasma proteins but is eliminated into the urine unchanged through glomerular filtration and tubular secretion (Masuda *et al.*, 2006; Graham *et al.*, 2011). It is transported primarily by organic cation transporters (OCTs), particularly 1 and 2, and multi-drug and toxin extrusion proteins (MATEs), namely MATE1, highly expressed in the kidney, and MATE2. Metformin inhibits gluconeogenesis in the liver and stimulates glucose uptake by muscle and fat tissue. The therapeutic action of metformin is mediated by its action on AMP-activated kinase (AMPK) which switches cells from anabolic to catabolic state and restoring energy balance (Zhou *et al.*, 2001; Stephenne *et al.*, 2011). Metformin significantly decreases the urine albumin excretion rate in patients with type 2 diabetes (Amador-Licona *et al.*, 2000) and has beneficial effects in patients with renal function impairment (Ekström *et al.*, 2012). Recent studies in a rat model of type 2 diabetes have provided evidence that metformin suppresses the diabetes-induced loss of podocytes through repression of oxidative injury (Kim *et al.*, 2012). We have shown that metformin decreases NAD(P)H oxidase activity leading to reduction of reactive oxide species generation and activates AMPK in cultured podocytes (Piwkowska *et al.*, 2010). Similar effects regarding the activities of NAD(P)H and AMPK have been observed after short-term activation of P2 receptors in podocytes (Piwkowska *et al.*, 2011). These receptors activated by extracellular nucleotides are part of the purinergic system and are classified into two groups: ATP-gated non-selective cation channels, P2X₁₋₇, and G protein-coupled metabotropic receptors, P2Y_{1,2,4,6,11-14} (Abbracchio *et al.*, 2006; Jarvis & Khakh, 2009). The extracellular ATP concentration is determined by a balance between the capacity of the cells to ATP release and its hydrolysis. Molecular and pharmacological studies have shown that P2X_{4,5,6,7} and P2Y_{1,2,4,6,11} receptors are expressed in podocytes and their activation modulates cell functioning, including glucose uptake (Fischer *et al.*, 2001; Jankowski, 2008).

Thus, we checked the hypothesis that metformin induces the changes in AMPK and NAD(P)H oxidase activities through a mechanism involving P2 receptors. We provided evidence that metformin increases extracellular ATP concentration by inhibiting ecto-ATPase activity, thereby leading to activation of the P2 receptors.

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Abbreviations: AMPK, AMP-activated kinase; MET, metformin

MATERIAL AND METHODS

Materials. Cell culture reagents, metformin, suramin and ARL 67156 were from Sigma-Aldrich (St. Louis, MO) and fetal bovine serum (FBS) was from Gibco, Invitrogen (Carlsbad, CA). Reagents for SDS-PAGE were purchased from MP Biochemicals, with the exception of the protein standard (Bio-Rad, Hertfordshire, UK) and protease inhibitor cocktail (Sigma-Aldrich). Primary rabbit polyclonal antibody to AMPK α and rabbit monoclonal antibody to p-AMPK α (Thr172) were from Cell Signaling Technology (Danvers, MA). Alkaline phosphatase-conjugated secondary goat anti-rabbit antibodies were from Santa Cruz Biotechnology. ATP Bioluminescence Assay Kit CLS II was from Roche Diagnostics (Mannheim, Germany). All other agents were purchased from POCh (Gliwice, Poland).

Cell culture. Mouse podocytes from a conditionally immortalized cell line were cultured as described previously (Piwkowska *et al.*, 2011). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/l), and streptomycin (100 μ g/l) in a controlled (5% CO₂) humidified atmosphere. To propagate podocytes, the culture medium was supplemented with 10 U/ml mouse recombinant γ -interferon (γ -INF) and the cells cultivated at 33°C to enhance the expression of the temperature-sensitive large T antigen (permissive conditions). To induce differentiation, podocytes were maintained at 37°C without γ -INF (nonpermissive conditions) for 1 week before starting the experiments.

Western blot analysis. Podocytes were treated with lysis buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol) in the presence of a protease inhibitor cocktail and homogenized at 4°C by scraping. The cell homogenates were centrifuged at 9500 \times g for 20 min at 4°C. Proteins (20 μ g) in the supernatants were separated on an SDS-polyacrylamide gel (10%) and electrotransferred to a nitrocellulose mem-

brane. The membrane was blocked for 1.5 h with Tris-buffered saline (TBS) (20 mM Tris/HCl, 140 mM NaCl, 0.01% NaN₃) containing 3% non-fat dry milk, washed with TBS containing 0.1% Tween-20 and 0.1% bovine serum albumin (BSA), and incubated overnight at 4°C with primary antibody. Anti-AMPK α and anti-AMPK α phosphothreonine 172 antibodies were diluted 1:750 in TBS containing 0.05% Tween-20 and 1% BSA. To detect primary antibodies bound to the immunoblot, the membrane was incubated for 2 h with alkaline phosphatase-labeled secondary antibodies. Protein bands were detected using the colorimetric 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium system. The density of the bands was quantitated using the Quantity One program (Bio-Rad).

NAD(P)H oxidase assay. NAD(P)H oxidase activity in podocytes was measured by the lucigenin-enhanced chemiluminescence method with modifications (Piwkowska *et al.*, 2010). To measure superoxide anion generation, cell homogenates (50 μ g protein) were added to PBS buffer containing 1 mM EDTA and 5 μ M lucigenin. The assay was initiated by adding 100 μ M NADPH. Photon emission, in terms of relative light units, was measured every 30 s for 12 min in an FB12 luminometer (Berthold). There was no measurable activity in the absence of NADPH. The amounts of superoxide were calculated by integrating the area under the signal curve. These values were compared with a standard curve generated using xanthine/xanthine oxidase. Protein content was measured with the Lowry method.

Measurement of extracellular concentration and intracellular content of ATP. Podocytes were incubated for a short-time (15 min) with various metformin concentrations in the range of 0.01–2 mM for determination of extracellular ATP concentration or for a long-time (120 min) with 2 mM metformin for determination of the intracellular pool of ATP. Some experiments were performed in the presence of ARL 67156, an inhibitor of ecto-ATPase (100 μ M, 15-min pre-incubation). Incu-

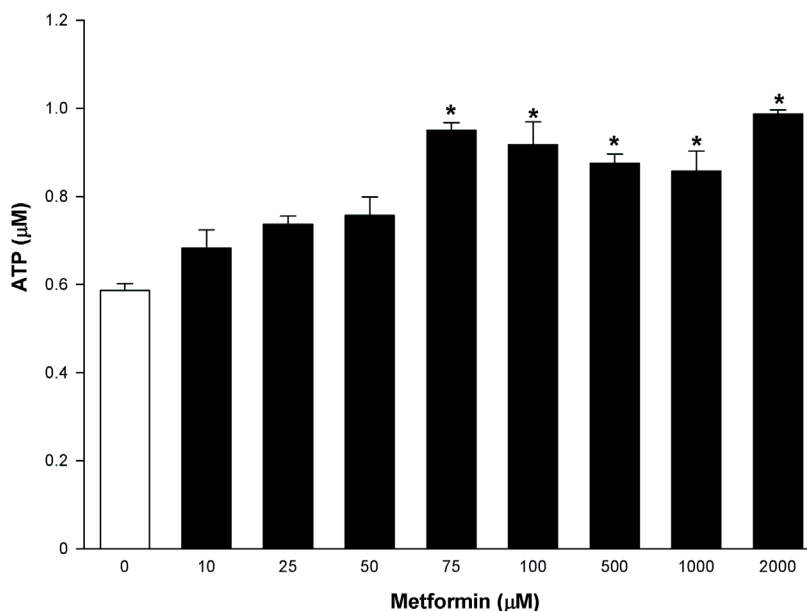


Figure 1. Time course of the effect of metformin on hydrolysis of extracellular ATP by cultured mouse podocytes.

Following 15-min preincubation without or with metformin in a range of concentrations 0.01–2 mM cells were incubated for 3 min with 1 μ M ATP and supernatant was taken to measure ATP concentration using luciferin-luciferase method. Values are the mean \pm S.E.M. of 3–4 independent experiments. * P < 0.05 with respect to absence of metformin.

bation medium and homogenized cells were heated separately at 99°C for 2 minutes, centrifuged (4°C, 800×g, 10 min) and immediately used for ATP determination. Concentration of ATP was measured using the luciferin-luciferase method with an FB12 luminometer (Kimmich *et al.*, 1975).

Determination of ecto-ATPase activity. Podocytes were incubated without or with metformin (2 mM, 15 min) and then the rate of hydrolysis of exogenously added ATP (1 μM) was determined by luminometric analysis of ATP concentration in supernatants collected at suitable time intervals.

Statistical analysis. Statistical analyses were performed by one-way ANOVA followed by the Student-Newman-Keuls test to determine significance. Values are reported as means ± S.E.M. Significance was set at $P < 0.05$.

RESULTS

Effects of metformin on extra/intracellular pool of ATP

Metformin significantly inhibited hydrolysis of exogenous 1 μM ATP (Fig. 1); the effect was concentration dependent until a maximum was reached at 75 μM metformin ($0.95 \pm 0.02 \mu\text{M}$ vs $0.52 \pm 0.01 \mu\text{M}$) followed by a plateau up to 2 mM metformin (Fig. 1). The magnitude of the inhibitory effect of metformin was similar to that of 100 μM ARL 67156, a known inhibitor of ecto-ATPase. No additional effect of metformin was observed during co-incubation with ARL 67156 (Fig. 2A). Furthermore, as it is presented in Fig. 2B, the inhibitory effect of 2 mM metformin was significant also at shorter time intervals. In contrast to its effect on extracellular ATP stability, metformin did not significantly affect the intracellular content of ATP (51.2 ± 6.8 vs $49.3 \pm 6.7 \mu\text{mol/mg protein}$).

Effect of metformin on AMPK phosphorylation

Next, we estimated the effect of metformin on AMPK activity (Fig. 3A and B). Exposing the podocytes to 2 mM metformin for 2 hours resulted in AMPK phosphorylation assessed as the densitometric ratio of its phosphorylated form to the total AMPK protein (1.41 ± 0.12 vs. 1.06 ± 0.06). Suramin, a non-selective P2 receptor antagonist, was used to explore the involvement of P2 receptors in the metformin-induced AMPK phosphorylation. Pre-incubating podocytes with suramin (300 μM, 5 min) abolished the effect of metformin on AMPK phosphorylation. These results suggest that the metformin-induced AMPK phosphorylation is mediated *via* P2 receptors.

Effect of metformin on NAD(P)H oxidase activity

As it is shown in Fig. 3C, metformin (2 mM, 2 h) significantly decreased by 32% the NAD(P)H oxidase activity (1.76 ± 0.03 vs. $2.78 \pm 0.06 \text{ nmol/min/mg protein}$, $P < 0.05$). Preincubation of the podocytes with suramin (300 μM, 5 min) abolished the effect of metformin on the NAD(P)H oxidase activity. These results suggest an involvement of P2 receptors in the metformin-mediated changes in rate of $\text{O}_2^{\cdot-}$ generation in cultured mouse podocytes.

DISCUSSION

In the present study we provide evidence for a novel mechanism of metformin action. Exposure of cultured podocytes to relatively low concentrations of metformin (10–75 μM) affects purinergic signaling through inhibition of ecto-ATPase causing an increase of the extracellular ATP concentration and activation of P2 receptors with subsequent activation of AMPK and reduction of NAD(P)H oxidase activity. Thus, the biological effects of metformin may be modified by purinergic signaling,

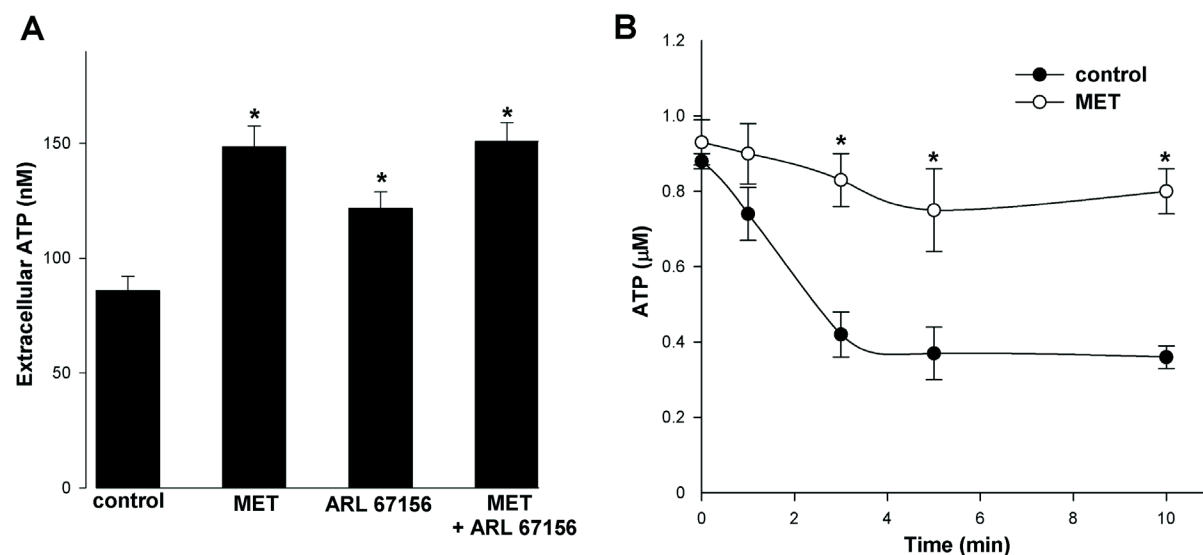


Figure 2. Effect of metformin on extracellular ATP hydrolysis by cultured mouse podocytes.

Following 15-min preincubation without or with 100 μM ARL 67156 cells were incubated for 15 min with 2 mM metformin and then ATP concentration in incubation medium was measured (A) or cells were incubated for 15 min with 2 M metformin, then 1 μM ATP was added to cells for indicated time and ATP concentration in incubation medium was measured using luciferin-luciferase method. Values are the mean ± S.E. of 3–4 independent experiments. * $P < 0.05$ with respect to appropriate control.

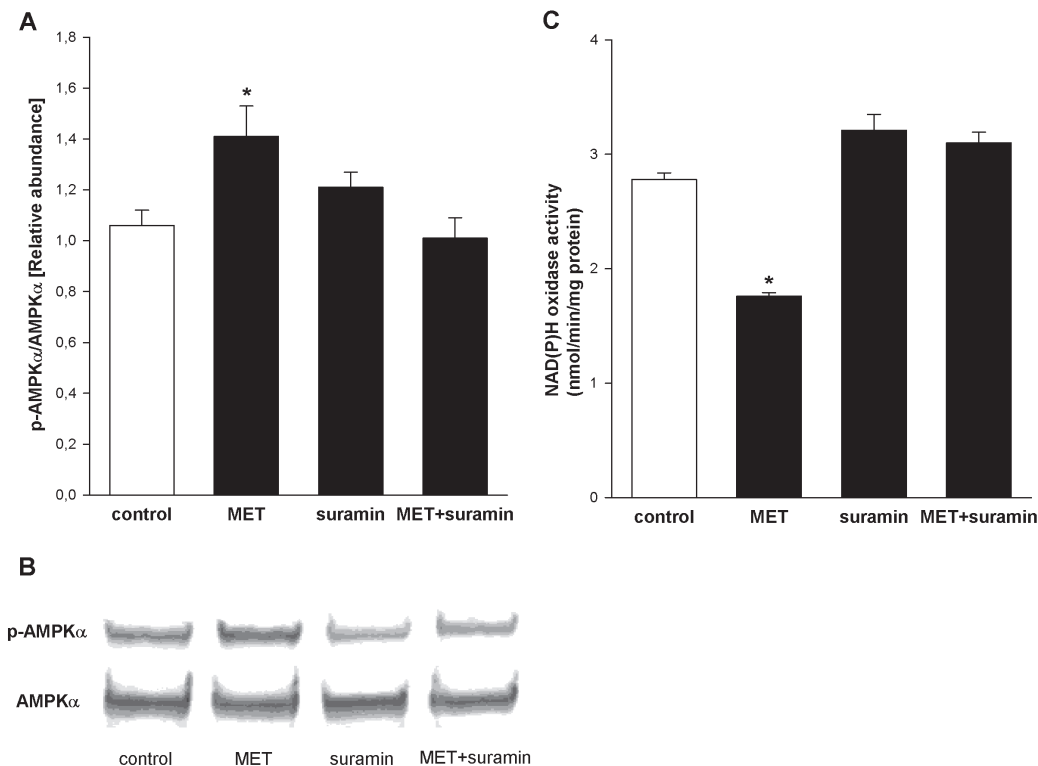


Figure 3. Effect of suramin on metformin-induced changes in AMPK phosphorylation in cultured mouse podocytes.

Following 5-min preincubation without or with 300 μ M suramin cells were incubated for 15 min with 2 mM metformin. Cells were lysed and immunoblotted with anti-AMPK α (Thr¹⁷²-P) and anti-AMPK α antibodies (A, B) or were harvested to measure NAD(P)H oxidase activity using the lucigenin enhanced method (C). Values are the mean \pm SE of 3–4 independent experiments (A, C). * P < 0.05 with respect to control. A. The relative abundance of optical density of the bands. B. Representative Western blot of podocytes stimulated with metformin in the presence or absence of suramin.

at least in cultured podocytes. This novel mechanism could hopefully constitute a new pharmacological target in patients with type 2 diabetes mellitus, especially those with diabetic nephropathy/podocytopathy.

The podocytes are insulin-dependent renal glomerular cells forming an outer layer of the glomerular filtration barrier. Their dysfunction, called podocytopathy, is observed in diabetes mellitus and leads to renal function impairment (Pavenstädt *et al.*, 2003; Diez-Sampedro *et al.*, 2011). The factors involved in the pathogenesis of diabetic podocytopathy are multifaceted. Among them reactive oxygen species are important. They can induce actin filament polymerization leading to cytoskeletal dysfunction and consequent structural changes of podocytes affecting glomerular permeability. The main source of reactive oxygen species in podocytes is the multi-subunit NAD(P)H oxidase generating the superoxide anion. All components of the NAD(P)H oxidase complex, including p22^{phox}, p47^{phox}, and the NOX isoforms are expressed in podocytes (Eid *et al.*, 2009). Additionally, diabetes mellitus is associated with disturbances in diverse extra/ intracellular metabolic signalling pathways, including the purinergic ones. The latter comprise extracellular hydrolases e.g., ecto-nucleotide pyrophosphatase/phosphodiesterases (E-EPPs) and enzyme synthesizing the nucleotides, as well as P2 receptors of two types: P2X and P2Y. Their activation results in a rise of the concentration of calcium ions in the cytoplasm, which triggers a variety of intracellular events depending on cell type. Moreover, activation of P2Y receptors may also result in

changes of intracellular AMP concentration (Abbracchio *et al.*, 2006; Jarvis & Khakh, 2009). Extracellular nucleotides modulate a number of intracellular signal transduction pathways (da Silva *et al.*, 2006; Burnstock, 2007). It has been shown that activation of P2 receptors influences the activity of AMPK (da Silva *et al.*, 2006). This enzyme, a major cellular energy sensor and a key regulator of metabolic homeostasis, is activated by Ca²⁺ and AMP-dependent pathways. It is activated (phosphorylation of Thr¹⁷² on α subunit of the heterotrimeric complex) under conditions leading to a rise of the intracel-

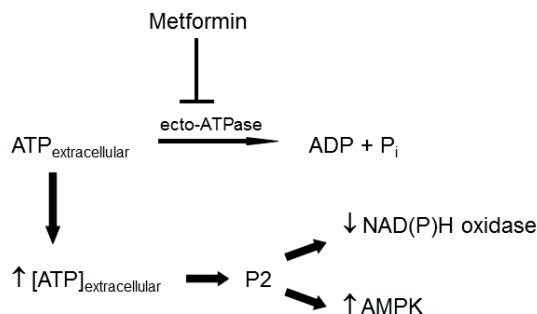


Figure 4. Proposed novel mechanism of metformin action. Metformin inhibits ecto-ATPase activity leading to an increased extracellular ATP concentration, subsequent activation of P2 receptors and eventual reduction of NAD(P)H oxidase activity and enhancement of AMPK activity.

lular AMP/ATP ratio (e.g., glucose deprivation, hypoxia) or upon paracrine/autocrine stimulation (Zhang & Zhou, 2009; Foretz & Viollet, 2011). Recently, we have shown that a diabetic milieu affects purinergic modulation of glucose transport into cultured podocytes (Karczewska *et al.*, 2007). Taken together, an increased glucose concentration leads to many cellular disturbances and therefore, the main goal of treatment of diabetic patients is normalization of glycemia. Metformin is an insulin-sensitizing biguanide with anti-hyperglycemic properties that is widely used to treat patients with type 2 diabetes mellitus (Viollet *et al.*, 2012). The beneficial effects of metformin are well established and have also been demonstrated in patients with renal impairments in a population-based observational study (Ekström *et al.*, 2012). Despite the established place of metformin in pharmacotherapy of hyperglycemia, its mechanism of action is not fully understood. There is evidence that metformin affects the rate of glucose metabolism through reducing the intracellular energy charge or/and by AMPK activation (Zhou *et al.*, 2001). Our recent studies have shown that extracellular ATP through P2 receptors plays a role in restoring energy homeostasis after oxidative stress in podocytes (Piwkowska *et al.*, 2011). ATP stimulates AMP-activated protein kinase *via* Ca²⁺/calmodulin-dependent kinase kinase-β and LKB1-STRAD-MO25 complex, and in turn decreases NAD(P)H oxidase activity, the main source of reactive oxide species in podocytes. Now, we have shown that metformin increases extracellular ATP concentration. Two types of experiments suggest that metformin does it by inhibiting the extracellular hydrolysis of ATP. First, ARL 67156, a specific inhibitor of ecto-ATPase did not affect the metformin action, suggesting that these two agents affect extracellular ATP level by the same mechanism. Second, metformin was shown directly to significantly reduce the hydrolysis rate of extracellular ATP. Importantly, extracellular ATP concentration affects intracellular signaling and this action is mediated through P2 receptors. In podocytes, where ENPP1 is abundantly expressed metformin, even at a low concentration, would prolong the effect of endogenously released ATP on P2 receptors. Using suramin, a specific but non-selective antagonist of P2 receptors, we have shown that effect of metformin is indeed dependent on P2 receptors. However, further molecular studies using siRNA should be performed to identify the purinoceptors involved in metformin action on podocytes.

Our present results do not exclude the participation of a product of ATP hydrolysis (i. e., adenosine) in metformin action since we have previously shown that adenosine-induced AMPK phosphorylation also depends on the adenosine uptake via nucleoside transporters (Karczewska *et al.*, 2007; Piwkowska *et al.*, 2011).

To our knowledge this is the first report describing an involvement of purinoceptors in metformin action. The involvement of purinergic signaling has been investigated for nebivolol and carvedilol, third-generation β-adrenoreceptor antagonists in glomeruli. We have provided evidence that the vasodilator effect of nebivolol and carvedilol in renal glomerular microvasculature is associated with the activation of ATP efflux with consequent stimulation of P2Y-purinoceptor-mediated liberation of NO from glomerular microvascular endothelial cells (Kalinowski *et al.*, 2003). Moreover, these drugs have beneficial effects on urinary albumin excretion both in experimental models and in patients with type 2 diabetes (Bakris *et al.*, 2005; Whaley-Connell *et al.*, 2009).

Based on our present results we propose a novel mechanism of metformin action. It inhibits ecto-ATPase

activity leading to an increased extracellular ATP concentration, subsequent activation of P2 receptors and eventual reduction of NAD(P)H oxidase activity and enhancement of AMPK activity. Both these changes may have beneficial effects for podocytes during metformin treatment in diabetic patients. We suggest that modulation of purinergic signaling may be a novel pharmacological target during metformin treatment in patients with diabetes type 2.

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