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Original Contribution

ERK1/2 pathway is involved in renal gluconeogenesis inhibition under conditions of lowered NADPH oxidase activity

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ABSTRACT

The aim of this study was to elucidate the mechanisms involved in the inhibition of renal gluconeogenesis occurring under conditions of lowered activity of NADPH oxidase (Nox), the enzyme considered to be one of the main sources of reactive oxygen species in kidneys. The *in vitro* experiments were performed on primary cultures of rat renal proximal tubules, with the use of apocynin, a selective Nox inhibitor, and TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a potent superoxide radical scavenger. In the *in vivo* experiments, Zucker diabetic fatty (ZDF) rats, a well established model of diabetes type 2, were treated with apocynin solution in drinking water. The main *in vitro* findings are the following: (1) both apocynin and TEMPOL attenuate the rate of gluconeogenesis, inhibiting the step catalyzed by phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of the process; (2) in the presence of the above-noted compounds the expression of PEPCK and the phosphorylation of transcription factor CREB and ERK1/2 kinases are lowered; (3) both U0126 (MEK inhibitor) and 3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione (ERK inhibitor) diminish the rate of glucose synthesis via mechanisms similar to those of apocynin and TEMPOL. The observed apocynin *in vivo* effects include: (1) slight attenuation of hyperglycemia; (2) inhibition of renal gluconeogenesis; (3) a decrease in renal PEPCK activity and content. In view of the results summarized above, it can be concluded that: (1) the lowered activity of the ERK1/2 pathway is of importance for the inhibition of renal gluconeogenesis found under conditions of lowered superoxide radical production by Nox; (2) the mechanism of this phenomenon includes decreased PEPCK expression, resulting from diminished activity of transcription factor CREB; (3) apocynin-evoked inhibition of renal gluconeogenesis contributes to the hypoglycemic action of this compound observed in diabetic animals. Thus, the study has delivered some new insights into the recently discussed issue of the usefulness of Nox inhibition as a potential antidiabetic strategy.

Introduction

Although liver is traditionally thought to be the main glucose buffer in a mammal organism, up to 25% of glucose released into the circulation in the postabsorptive state come from kidneys, the second of the organs capable of gluconeogenesis. Moreover, it is estimated that under diabetic conditions the relative increase in renal gluconeogenesis is substantially greater than that under hepatic conditions (300 versus 30%); i.e., kidneys and liver seem to be an equally important sources of glucose synthesized *de novo* [1–3]. All these findings suggest that renal gluconeogenesis might be a promising target for antidiabetic therapy. However, compared to hepatic gluconeogenesis [4], the mechanisms regulating this process in kidneys are still rather poorly recognized.

NADPH oxidase (EC 1.6.3.1, Nox) catalyzes one electron reduction of molecular oxygen, leading to superoxide radical ($O_2^{\cdot-}$) formation, which initiates the cascade of free radical reactions. Nox was originally discovered in phagocytes, where its activity is responsible for pathogen elimination. However, it has soon found that enzymes exhibiting high homology to phagocyte NADPH oxidase (Nox2) are present in many other tissues and should be considered to be one of the most important intracellular sources of reactive oxygen species (ROS). Currently the family of Nox enzymes consists of seven oxidases: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 [5,6]. Excessive NADPH oxidase activity has been reported to accompany numerous pathological states,

Abbreviations: Nox, NADPH oxidase; PCA, perchloric acid; PEPCK, phosphoenolpyruvate carboxykinase; ROS, reactive oxygen species; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; ZDF rat, Zucker diabetic fatty rat

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especially cardiovascular diseases and nephropathies, including diabetic nephropathies [5–9]. Taking this observation into account, inhibition of NADPH oxidase is widely discussed as a promising novel therapeutic strategy [5,8,9].

Some reports have revealed that, under diabetic conditions, apocynin (acetovanillone, 4′-hydroxy-3′-methoxyacetophenone), a Nox inhibitor, might act as a hypoglycemic agent [10–12]. In our previous paper we pointed out that this phenomenon results from diminished activity of renal gluconeogenesis [12]. The aim of the present study is to elucidate the detailed mechanisms responsible for renal gluconeogenesis inhibition observed under conditions of lowered NADPH oxidase activity. Moreover, we have decided to investigate apocynin's *in vivo* effect on glucose synthesis *de novo* in kidneys of Zucker diabetic fatty (ZDF) rats, a well established model of diabetes type 2 [13].

Materials and methods

Primary cell cultures

Renal proximal tubules were isolated from 12-week-old male WAG (Wistar Albino Glaxo) rats originating from Animal Facility of Faculty of Biology (University of Warsaw), as described by Jarzyna et al. [14], including some modifications of surgical procedures. The animals used for tubules isolation were preeuthanized by intraperitoneal injection of pentobarbital (30 mg/kg body weight), in agreement with the approval of the First Warsaw Local Commission for the Ethics of Experimentation on Animals (decisions No. 944/2009 and No. 316/2012).

Isolated renal proximal tubules were sown into 6-well polystyrene CellBind plates (Corning GmbH, Kaiserslautern, Germany) and cultured at 37 °C under an atmosphere of 95% O_2 + 5% CO_2. The culture medium, composed of Dulbecco's modified Eagle's medium enriched with streptomycin (0.1 mg/ml), penicillin (0.1 mg/ml), 15 mM Hepes, 1.5 μM thymidine, 1.5 μM biotin, 0.5 μM vitamin B_{12}, 50 mM hydrocortisone, transferrin (5 μg/ml), sodium selenite (5 ng/ml), gluconeogenic substrates (2 mM glutamine, 2 mM sodium lactate, and 5 mM glycerol), and proper effectors, was changed every 24 h.

Following the final replacement of the culture media, i.e., after 48 h of incubation, renal tubules were cultured for the next 5 h. Then, media samples for glucose concentration measurements were withdrawn (according to the procedure described in section 2.3), and cell extracts for determination of gluconeogenic intermediates (cf. section 2.6.2) and cell lysates for Western blot analyses (cf. section 2.5) were prepared.

Experimental design of in vivo studies

The *in vivo* experiments were performed on male ZDF rats purchased from Charles River Laboratories (ZDF–Lepr^{fa}/Crl). The animals were fed with Purina 5008 diet, with free access to water and food. At the beginning of the experiment the rats were 12 weeks old. Obese diabetic ZDF rats (homozygous fa/fa) were randomly divided into two groups of five animals each: (1) untreated (fa/fa); (2) treated with apocynin (fa/fa + Apo). A group of five untreated control (nondiabetic) lean ZDF rats (fa/+ or +/+ genotype, i.e., ?/+) was also included. Apocynin was applied as a solution in drinking water, at a commonly used dose of 2 g/L [15]. Untreated rats (both fa/fa and ?/+) were given tap water.

Glycemia was measured weekly in blood withdrawn from tail veins of 24 h starved animals. Samples (ca. 0.2 ml) were collected into heparinized tubes placed on ice and then centrifuged in order to separate blood cells. The supernatants dedicated for glucose determination (cf. section 2.6.1) were deproteinized with 35% perchloric acid (PCA; 1:10, v/v) and then neutralized with 3 M K_2CO_3 (1:10, v/v).

Following 8 weeks of the experiment the animals were starved for 24 h and subsequently euthanized by intraperitoneal injection of pentobarbital (30 mg/kg body weight). Right kidneys were immediately collected for the determinations of NADPH activity and PEPCK activity and expression. From the left kidneys proximal tubules were isolated according to the method of Jarzyna et al. [14], including some modifications of surgical procedures. All animal treatment procedures were approved by the First Warsaw Local Commission for the Ethics of Experimentation on Animals (decisions Nos. 944/2009 and 316/2012).

Incubation of freshly isolated proximal tubules

Freshly isolated (cf. section 2.2) proximal tubules (ca. 3 mg protein) were incubated at 37 °C, under an atmosphere of 95% O_2 + 5% CO_2, in 25 ml plastic Erlenmeyer flasks containing 1.5 ml of Krebs-Ringer bicarbonate buffer with 2 mM sodium lactate or 2 mM glutamine. After 60 min of incubation, 1 ml samples were withdrawn, acidified with 0.1 ml of 35% PCA, and centrifuged. Then, supernatants were neutralized with 3 M K_2CO_3 (1:10, v/v) and used for glucose determination (cf. section 2.6.1).

Measurement of enzymatic activities

NADPH oxidase

Samples for NADPH oxidase activity determinations were prepared as described by Chen et al. [16]. Superoxide anion production by renal NADPH oxidase was determined using the lucigenin-enhanced chemiluminescence method [16,17]. Chemiluminometric measurements were performed using a Thriatler 425-014 luminometer (Hidex Ltd., Turku, Finland).

PEPCK

Cytosolic fractions for PEPCK activity determinations were prepared as described by MacDonald et al. [18]. PEPCK activity was determined spectrophotometrically according to Petrescu et al. [19]. Spectrophotometric measurements were performed using a Cary 50Bio spectrophotometer (Varian Ltd., Melbourne, Australia).

Western blot analysis

Lysates were prepared in ice-cold buffer, pH 7.0, containing 20 mM Tris, 0.5% Igepal C630, 3 mM benzamidine, 1 μM leupeptin, pepstatin A (1.6 μg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 2 mM EGTA, 30 mM NaF, 1 mM sodium orthovanadate, 60 mM β-glycerophosphate, and 20 mM sodium pyrophosphate. Samples were incubated for 30 min at 4 °C and then centrifuged. Just before electrophoresis the samples were denaturalized in Laemmli buffer (5 min, 100 °C). Aliquots (15 μg protein/lane) were applied to 10% polyacrylamide gels (Lonza, Basel, Switzerland) and electrophoresed, followed by electroblotting to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA).

After blocking in 5% nonfat dried milk, the membranes were incubated overnight at 4 °C with primary antibodies diluted according to the manufacturers' instructions. Then unbound primary antibodies were removed and the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:1,000). HRP-linked mouse anti-β-actin antibodies (1:50,000) were used to verify the equability of sample application to the gel. The previously stripped membranes were incubated with them for 1 h at room temperature.