

Metformin suppresses hepatic gluconeogenesis and lowers fasting blood glucose levels through reactive nitrogen species in mice

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Abstract

Aims/hypothesis Metformin, the major target of which is liver, is commonly used to treat type 2 diabetes. Although metformin activates AMP-activated protein kinase (AMPK) in hepatocytes, the mechanism of activation is still not well known. To investigate AMPK activation by metformin in liver, we examined the role of reactive nitrogen species (RNS) in suppression of hepatic gluconeogenesis.

Methods To determine RNS, we performed fluorescence examination and immunocytochemical staining in mouse hepatocytes. Since metformin is a mild mitochondrial complex I inhibitor, we compared its effects on suppression of gluconeogenesis, AMPK activation and generation of the RNS peroxynitrite (ONOO⁻) with those of rotenone, a representative complex I inhibitor. To determine whether

endogenous nitric oxide production is required for ONOO⁻ generation and metformin action, we used mice lacking endothelial nitric oxide synthase (eNOS).

Results Metformin and rotenone significantly decreased gluconeogenesis and increased phosphorylation of AMPK in wild-type mouse hepatocytes. However, unlike rotenone, metformin did not increase the AMP/ATP ratio. It did, however, increase ONOO⁻ generation, whereas rotenone did not. Exposure of eNOS-deficient hepatocytes to metformin did not suppress gluconeogenesis, activate AMPK or increase ONOO⁻ generation. Furthermore, metformin lowered fasting blood glucose levels in wild-type diabetic mice, but not in eNOS-deficient diabetic mice.

Conclusions/interpretation Activation of AMPK by metformin is dependent on ONOO⁻. For metformin action in liver, intra-hepatocellular eNOS is required.

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Metformin · Nitric oxide · Peroxynitrite ·
Reactive nitrogen species

Abbreviations

AMPK	AMP-activated protein kinase
BAEC	Bovine aortic endothelial cells
DCDHF	2,7-Dihydrodichlorofluorescein
eNOS	Endothelial nitric oxide synthase
L-NAME	N ^ω -Nitro-L-arginine methyl ester
NOS	Nitric oxide synthase
OCT1	Organic cation transporter 1
ONOO ⁻	Peroxynitrite
RNS	Reactive nitrogen species

Introduction

Metformin is one of the most commonly used oral glucose-lowering drugs for type 2 diabetes and is recommended as a first-line drug in recent treatment guidelines of the American Diabetes Association and European Association for the Study of Diabetes [1, 2]. The main target tissue of metformin is liver and its major effect is to decrease hepatic glucose output, which occurs largely due to the suppression of gluconeogenesis, leading to lower fasting blood glucose levels without insulin stimulation and weight gain [3–5]. In addition, metformin has beneficial effects on cardiovascular function and reduces cardiovascular risk in type 2 diabetes [6].

Although metformin has been used clinically for several decades, the mechanisms by which it exerts its glucose-lowering effects are still unclear [7]. Recent studies have demonstrated that therapeutic effects of metformin are mediated by activation of AMP-activated protein kinase (AMPK), leading to a decrease in gluconeogenesis and an increase of fatty acid oxidation in liver and of glucose uptake in skeletal muscle [8–10]. AMPK is a serine/threonine kinase that acts as an energy sensor and is activated in response to reductions of cellular energy levels and to environmental stress, including hypoxia, ischaemia, exercise, ATP depletion and oxidative stress [11, 12]. Although it has been known that AMPK is activated by an increase in the AMP/ATP ratio, the AMPK-activating mechanism also involves other pathways that are dependent on upstream AMPK kinases, including LKB1 kinase and calmodulin-dependent protein kinase kinase in liver and skeletal muscle, respectively [13]. Previous studies reported that metformin had an inhibitory effect on mitochondrial complex I; and, indeed, an inhibition of mitochondrial complex I has been found to increase the AMP/ATP ratio [7, 14, 15]. AMPK activation by metformin was therefore thought to be also mediated by an increase in the AMP/ATP ratio. However, recent studies have reported that metformin action may be mediated without a notable inhibition of mitochondrial metabolism [10, 16].

Recently, a possible role of peroxynitrite (ONOO^-), a reactive nitrogen species (RNS), in the mechanism of AMPK activation has been investigated. RNS comprises nitric oxide and its secondary substrates; ONOO^- is generated from superoxide anions (O_2^-) and nitric oxide [17]. Zou et al. reported that metformin activates AMPK through ONOO^- in bovine aortic endothelial cells (BAEC) [18]. However, it is unclear whether RNS generation by metformin is involved in its suppression of hepatic gluconeogenesis or whether RNS generation affects metformin's pharmacological action in lowering of fasting blood glucose levels.

To clarify the mechanism of AMPK activation in liver, we used mouse hepatocytes to investigate the involvement

of the AMP/ATP ratio and RNS in AMPK activation by metformin compared with rotenone, a representative complex I inhibitor. To determine whether endogenous nitric oxide production is required for metformin action in hepatocytes, we also performed experiments using mice lacking endothelial nitric oxide synthase (eNOS) [18–21]. We demonstrated that ONOO^- plays a critical role in AMPK activation by metformin in liver and that eNOS is required for metformin action in vitro and in vivo.

Methods

Animals Male C57/BL6 (wild-type) mice were obtained from Shimizu (Kyoto, Japan). Male eNOS-deficient (*eNos* [also known as *Nos3*]^{-/-}) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in a temperature-controlled ($25\pm 2^\circ\text{C}$) environment with a 12 h light/dark cycle. The mice had free access to standard laboratory chow and water. All experiments were carried out with mice aged 8 to 10 weeks. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University. All the experiments involving animals were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Hepatocyte preparation and culture Mice hepatocytes were isolated by collagenase digestion as described previously [22]. Primary hepatocytes were prepared by seeding in six well type 1 collagen-coated plates at a density of 1.5×10^6 cells in DMEM (low glucose, 5.6 mmol/l) containing 10% (vol./vol.) FBS, 100 nmol/l regular insulin, 50 U/ml penicillin and 50 $\mu\text{g/ml}$ streptomycin. Hepatocytes were then cultured overnight in a humidified atmosphere (5% CO_2) at 37°C .

Glucose production via gluconeogenesis in hepatocytes Gluconeogenesis was measured as described previously with slight modifications [22, 23]. In brief, freshly isolated hepatocytes from mice fasted for 16 h were treated in 24 well plates (7.5×10^5 cells/well) in 0.5 ml KRB buffer (119.4 mmol/l NaCl, 3.7 mmol/l KCl, 2.7 mmol/l CaCl_2 , 1.3 mmol/l KH_2PO_4 , 1.3 mmol/l MgSO_4 , 24.8 mmol/l NaHCO_3) containing 2% (wt/vol.) BSA, 2 mmol/l oleate, 0.24 mmol/l 3-isobutyl-1-methylxanthine and gluconeogenic substrates (1 mmol/l pyruvate plus 10 mmol/l lactate) treated with metformin (Sigma, St Louis, MO, USA) and rotenone (Nacalai Tesque, Kyoto, Japan). Metformin was dissolved in water. Rotenone was dissolved in dimethyl sulfoxide to a concentration that did not interfere with cell viability (maximally 0.1% vol./vol.).

The glucose content of the supernatant fraction was measured by the glucose oxidation method using an assay kit (Gopod; Megazyme, Wicklow, Ireland). The data were normalised by protein content measured by cell lysates.

Immunoblotting analysis Freshly isolated hepatocytes were treated with metformin, rotenone and ONOO⁻ (Dojindo, Kumamoto, Japan) in KRB buffer containing 2% (wt/vol.) BSA, 2 mmol/l oleate, 0.24 mmol/l 3-isobutyl-1-methylxanthine and gluconeogenic substrates (1 mmol/l pyruvate plus 10 mmol/l lactate). Primary hepatocytes cultured overnight were incubated in FBS-free DMEM (no glucose) treated with metformin and rotenone. The hepatocytes were homogenised in lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 50 mmol/l NaF, 1 mmol/l sodium pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.1 mmol/l benzamidine, 0.1 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l sodium vanadate, 250 mmol/l mannitol, 1% (vol./vol.) Triton X-100 and 5 µg/ml soybean trypsin inhibitor). Cell lysates (50 to 150 µg protein per lane) were subjected to electrophoresis on 8% (vol./vol.) SDS-polyacrylamide gels and transferred on to nitrocellulose membranes (Protran; Schleicher and Schuell, Keene, NH, USA). Blotted membranes were incubated with each primary antibody (1:1,000 dilution). Antibodies against AMPK α and phospho-AMPK α (Thr¹⁷²) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against organic cation transporter 1 (OCT1) and glyceraldehyde-3-phosphate dehydrogenase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were incubated with horseradish peroxidase-linked second antibodies (1:2,000 dilution) (GE Healthcare, Tokyo, Japan) and fluorescent bands were visualised using a western blotting detection system (Amersham ECL Plus; GE Healthcare) and then quantified by densitometry using Image J software from National Institutes of Health (Bethesda, MD, USA).

Determination of reactive nitrogen species ONOO⁻ generation was measured using 2,7-dihydrodichlorofluorescein (DCDHF) diacetate (Cayman Chemical, Ann Arbor, MI, USA) [24–26], which is readily oxidised by ONOO⁻ to the highly fluorescent product, dichlorofluorescein. Alone, nitric oxide, superoxide anions or hydrogen peroxide did not oxidise DCDHF. Freshly isolated hepatocytes were washed in ice-cold PBS and preloaded for 20 min at 37°C with 10 µmol/l DCDHF diacetate (Cayman Chemical) in KRB buffer containing 2% (wt/vol.) BSA, 2 mmol/l oleate, 0.24 mmol/l 3-isobutyl-1-methylxanthine and gluconeogenic substrates (1 mmol/l pyruvate plus 10 mmol/l lactate). Fluorescence was determined using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) with excitation wavelength at 502 nm and emission wavelength at 523 nm. After 1 h incubation in the presence or

absence of metformin, rotenone, ONOO⁻ or hydrogen peroxide with or without RNS scavenger (5 mmol/l α -tocopherol plus 2.3 mmol/l ascorbate) [27], fluorescence was measured and presented as a ratio with respect to the value at time zero.

Immunocytochemistry Primary hepatocytes were plated on cover glass coated with 0.01% (vol./vol.) poly-L-lysine (Sigma) in six-well plates (5.0×10^5 cells per well). Hepatocytes were then incubated with FBS-free DMEM (no glucose) in the presence or absence of rotenone, metformin, metformin with RNS scavenger (5 mmol/l α -tocopherol plus 2.3 mmol/l ascorbate) and metformin with 1 mmol/l of the nitric oxide synthase (NOS) inhibitor *N*^w-nitro-L-arginine methyl ester (L-NAME) for 2 h, or in the presence or absence of ONOO⁻ for 5 min. The hepatocytes were fixed in 3.7% (wt/vol.) paraformaldehyde and incubated with rabbit polyclonal anti-nitrotyrosine antibody (1:100 dilution; Millipore; Billerica, MA, USA). Next, cells were incubated with goat anti-rabbit IgG fluorescein-conjugated secondary antibody (1:100 dilution; Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA). Fluorescence in cells was monitored using a laser scanning microscope (LSM 510; Carl Zeiss, Tokyo, Japan) for confocal microscopy and a software package (LSM 510 Meta; Carl Zeiss) for image acquisition.

Measurement of adenine nucleotide content After freshly isolated hepatocytes were incubated in KRB buffer containing 2% (wt/vol.) BSA, 2 mmol/l oleate, 0.24 mmol/l 3-isobutyl-1-methylxanthine and gluconeogenic substrates (1 mmol/l pyruvate plus 10 mmol/l lactate) in the presence or absence of metformin or rotenone for 2 h, or of ONOO⁻ for 5 min, treatment was stopped by rapid addition of 0.1 ml of 2 mol/l HClO₄, followed by mixing by vortex and sonication in ice-cold water for 3 min. Cell lysates were then centrifuged for 3 min at 3,000×g and 4°C, and a fraction (0.4 ml) of the supernatant fraction was mixed with 0.1 ml of 2 mol/l HEPES and 0.1 ml of 1 mol/l Na₂CO₃. Adenine nucleotide contents were measured by a lumino-metric method as previously described [28, 29].

Effect of metformin on plasma glucose levels and AMPK phosphorylation in liver tissue of wild-type and *eNos*^{-/-} diabetic mice Mice were made diabetic by intraperitoneal injection of streptozotocin (120 mg/kg) into male C57/BL6 and *eNos*^{-/-} mice at 8 weeks of age. At 1 week after injection of streptozotocin, the animals were confirmed to be diabetic by high fed blood glucose levels (≥ 13.8 mmol/l) and other diabetic features, including polyuria, polydipsia and hyperphagia. After fasting for 16 h, the blood glucose levels were measured and mice were immediately injected intraperitoneally with metformin (250 mg/kg) in 0.9%

sterile saline or 0.9% (wt/vol.) sterile saline only, a similar treatment to that described previously [8, 18]. Blood glucose levels were measured again after 1 h. Diabetic mice received injections of metformin or vehicle as described above for three consecutive days and blood glucose levels were measured again after fasting for 16 h. Immediately after the final measurement of blood glucose levels, the abdomen was cut open and liver tissue of each group was collected and homogenised in lysis buffer. Tissue lysates (50 µg protein/lane) were used for immunoblotting assay of AMPK phosphorylation using antibodies against AMPK α and phospho-AMPK α (Thr¹⁷²).

Statistical analysis Results are expressed as mean \pm SE per number (*n*) of animals. Statistical significance was evaluated by ANOVA, unpaired *t* test (not noted) and paired *t* test (noted). A value of *p* < 0.05 was considered statistically significant.

Results

Effects of metformin and rotenone on gluconeogenesis and AMPK α phosphorylation in C57/BL6 mice hepatocytes Hepatic gluconeogenesis and AMPK α phosphorylation

were measured using freshly isolated hepatocytes. After 2 h exposure to metformin, hepatic gluconeogenesis was significantly and dose-dependently suppressed at doses between 0.5 and 50 mmol/l metformin; it was also suppressed by exposure to 100 nmol/l rotenone (control 115.4 \pm 2.5 nmol/mg protein, 2 mmol/l metformin 92.1 \pm 3.3 nmol/mg protein, *p* < 0.05 vs control; 100 nmol/l rotenone 91.5 \pm 8.7 nmol/mg protein, *p* < 0.05 vs control; Fig. 1a). Gluconeogenesis at 2 mmol/l metformin and 100 nmol/l rotenone were similar (*p* = NS metformin vs rotenone). After 2 h exposure, metformin (0.5–50 mmol/l) and 100 nmol/l rotenone each stimulated phosphorylation of Thr¹⁷² of AMPK α (Fig. 1b, c). Increments of phosphorylation relative to control in hepatocytes exposed to 2 mmol/l metformin and 100 nmol/l rotenone were almost equivalent (fold increase relative to control 1.79 \pm 0.11 [metformin] and 1.85 \pm 0.12 [rotenone], *p* = NS, metformin vs rotenone). Similar results were observed using primary cultured hepatocytes (Fig. 1d, e). In the time course study of exposure to 2 mmol/l metformin, the suppressing effects on gluconeogenesis appeared after 120 min (*p* < 0.05 vs corresponding control; Fig. 1f). In addition, after 60 min exposure to 2 mmol/l metformin stimulated phosphorylation of Thr¹⁷² of AMPK α (*p* < 0.05 vs pre-exposure; Fig. 1g, h).

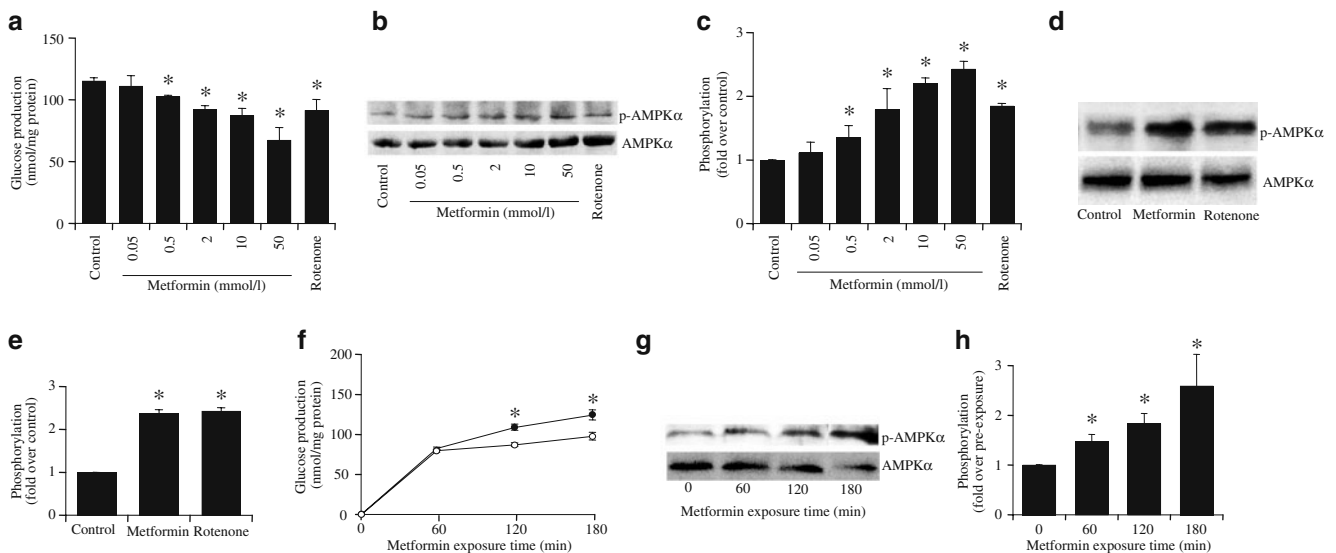


Fig. 1 Metformin and rotenone suppress gluconeogenesis and stimulate AMPK α phosphorylation in hepatocytes isolated from C57/BL6 mice. **a** Gluconeogenesis after 2 h exposure to metformin and rotenone. Metformin (dose-dependently between 0.5 and 50 mmol/l) and rotenone (100 nmol/l) significantly suppressed gluconeogenesis. **b, c** Effects of metformin and rotenone on activation of AMPK. After 2 h exposure, AMPK α phosphorylation in freshly isolated hepatocytes was significantly stimulated by metformin (dose-dependently as above [a]) and rotenone (100 nmol/l). Data are expressed as fold stimulation over control. **d, e** Effects of metformin and rotenone on activation of AMPK in primary cultured hepatocytes.

After 2 h exposure, AMPK α phosphorylation was significantly stimulated by metformin (2 mmol/l) and rotenone (100 nmol/l). Data are expressed as fold stimulation over control. **f** Time course of gluconeogenesis with exposure to metformin. Suppressing effects on gluconeogenesis by 2 mmol/l metformin (white circles) compared with control (black circles) were detected after 120 min. **g, h** Time course of AMPK activation upon exposure to metformin (2 mmol/l), which after 60 min stimulated phosphorylation of AMPK α in freshly isolated hepatocytes. Data are expressed as fold stimulation over pre-exposure. Values (all bar graphs) are means \pm SE (*n* = 6), **p* < 0.05 vs control (**a–f**) and pre-exposure (**h**)

ATP content and AMP/ATP ratio in C57/BL6 mice hepatocytes In wild-type mice, exposure of freshly isolated hepatocytes to 100 nmol/l rotenone for 2 h decreased ATP content and increased the AMP/ATP ratio compared with control (Table 1). However, 2 h exposure to 2 mmol/l metformin did not alter ATP content or AMP/ATP ratio compared with control. ATP content and the AMP/ATP ratio at 2 mmol/l metformin and 100 nmol/l rotenone were significantly different ($p < 0.01$ metformin vs rotenone).

RNS production by metformin In freshly isolated hepatocytes, exposure to 2 mmol/l metformin for 1 h increased DCDHF fluorescence, revealing an increase of ONOO⁻ generation, whereas 300 μmol/l hydrogen peroxide or 100 nmol/l rotenone had no effect on DCDHF fluorescence (Table 2). Co-administration of RNS scavengers (vitamin E plus vitamin C) completely suppressed RNS production by metformin.

Immunocytochemical staining of primary cultured hepatocytes with anti-nitrotyrosine antibody was performed to detect ONOO⁻ (Fig. 2). ONOO⁻ (10 μmol/l) incubated for 5 min in primary hepatocytes increased nitrotyrosine staining. Exposure to 2 mmol/l metformin, but not to 100 nmol/l rotenone for 2 h increased nitrotyrosine staining (Fig. 2a). Similarly to the DCDHF fluorescence study, co-administration of RNS scavengers (vitamin E plus vitamin C) suppressed nitrotyrosine staining by metformin. Co-administration of L-NAME, a NOS inhibitor, suppressed ONOO⁻ generation by metformin (Fig. 2b).

Effect of direct exposure to ONOO⁻ on AMPKα phosphorylation and AMP/ATP ratio The direct effect of exogenous ONOO⁻ on AMPK phosphorylation in the absence of metformin was examined. Exposure to ONOO⁻ for 5 min stimulated phosphorylation of AMPKα by 1 to 100 μmol/l ($p < 0.05$ vs control) (Fig. 3a, b). Exposure to

10 μmol/l ONOO⁻ for 5 min did not affect ATP content (pre-exposure 0.49 ± 0.05 nmol/mg protein; 5 min ONOO⁻ 0.50 ± 0.05 nmol/mg protein, $p = \text{NS}$ vs pre-exposure, $n = 5$) or the AMP/ATP ratio (pre-exposure 0.99 ± 0.06 , 5 min ONOO⁻ 0.98 ± 0.05 , $p = \text{NS}$ vs pre-exposure, $n = 5$).

No effect of metformin on gluconeogenesis, AMPKα phosphorylation or ONOO⁻ generation in hepatocytes lacking eNOS In freshly isolated hepatocytes from *eNos*^{-/-} mice, 2 h exposure to 2 mmol/l metformin did not suppress gluconeogenesis, whereas exposure to 100 nmol/l rotenone suppressed gluconeogenesis to a similar degree to that observed in wild-type hepatocytes (control 110.1 ± 4.4 nmol/mg protein, metformin 107.0 ± 3.9 nmol/mg protein, $p = \text{NS}$ vs control; rotenone 81.6 ± 8.8 nmol/mg protein, $p < 0.05$ vs control; Fig. 4a). Metformin did not stimulate AMPKα phosphorylation in freshly isolated hepatocytes from *eNos*^{-/-} mice, whereas rotenone significantly stimulated AMPKα phosphorylation (fold increase relative to control at 2 h: metformin 0.96 ± 0.12 , $p = \text{NS}$ vs control; rotenone 1.94 ± 0.13 , $p < 0.05$ vs control; Fig. 4b, c). Similarly, in primary cultured hepatocytes, metformin also did not stimulate, whereas rotenone significantly stimulated AMPKα phosphorylation (Fig. 4d, e). Metformin also did not increase nitrotyrosine staining in primary cultured hepatocytes from *eNos*^{-/-} mice, indicating no generation of ONOO⁻ (Fig. 4f). In addition, nitrotyrosine staining was not induced by 2 h exposure to rotenone. Exposure of *eNos*^{-/-} freshly isolated hepatocytes to 100 nmol/l rotenone also decreased ATP content and increased the AMP/ATP ratio, whereas exposure to metformin had no effect (Table 1). Recently, it was reported that metformin is first transported across the plasma membrane before exerting its cellular action, a step mediated by OCT1 [30]. To exclude involvement of OCT1 in *eNos*^{-/-} mice, we confirmed that levels of OCT1 protein in freshly isolated hepatocytes from *eNos*^{-/-} mice were similar to those in wild-type mice hepatocytes (Fig. 4g).

Table 1 Effect of metformin or rotenone on ATP content and AMP/ATP ratio in hepatocytes

Treatments per mouse type	ATP (nmol/mg protein)	AMP/ATP ratio
Wild-type mice		
Control	0.45 ± 0.08	0.98 ± 0.07
Metformin	0.47 ± 0.05	0.96 ± 0.12
Rotenone	$0.13 \pm 0.02^{**}$	$1.94 \pm 0.13^{**}$
<i>eNos</i> ^{-/-} mice		
Control	0.42 ± 0.07	1.22 ± 0.11
Metformin	0.41 ± 0.06	1.27 ± 0.16
Rotenone	$0.11 \pm 0.03^{**}$	$2.34 \pm 0.23^{**}$

Values are means \pm SE ($n = 5$)

** $p < 0.01$ vs control

Essential role of eNOS in lowering of glucose levels by metformin in diabetic mice in vivo To determine whether metformin lowers fasting blood glucose levels in the absence of eNOS, metformin (250 mg/kg) was injected intraperitoneally into streptozotocin-induced diabetic wild-type or *eNos*^{-/-} mice. Characteristics of wild-type and *eNos*^{-/-} mice used in the experiments showed no significant differences in body weight, fasting blood glucose levels or fed blood glucose levels before streptozotocin injection at 8 weeks of age among the four groups (Table 3).

Fasting blood glucose levels were lowered by about 3.9 mmol/l at 1 h after single administration of metformin in overnight-fasted wild-type diabetic mice, whereas those in overnight-fasted *eNos*^{-/-} diabetic mice were not altered

Table 2 Effect of metformin on RNS production

Treatments	No addition	Addition of vitamins E and C
Control	1.03±0.01	0.94±0.01**
Metformin (2 mmol/l)	1.15±0.04*	0.95±0.01**
Rotenone (100 nmol/l)	1.03±0.02	0.99±0.01**
Hydrogen peroxide (300 μmol/l)	1.02±0.02	0.98±0.01**
ONOO ⁻ (10 μmol/l)	1.21±0.05*	1.00±0.01**

Data are expressed as the value at 60 min divided by the value at time zero (fold increase); values are means ± SE (n=8)

p*<0.05 vs control; *p*<0.01 vs corresponding values without RNS scavengers

(Table 3). Administration of vehicle (saline) alone in overnight-fasted wild-type diabetic mice did not alter fasting blood glucose levels after single administration, as was also found in overnight-fasted *eNos*^{-/-} diabetic mice (Table 3). Following the first injection, daily administration of metformin was continued for two more days. Administration of metformin for three consecutive days lowered fasting blood glucose levels by about 7.1 mmol/l in wild-type diabetic mice, whereas it had no lowering effect on fasting blood glucose in diabetic *eNos*^{-/-} mice (Table 3). Administration of vehicle (saline) alone in overnight-fasted wild-type mice did not alter fasting blood glucose levels

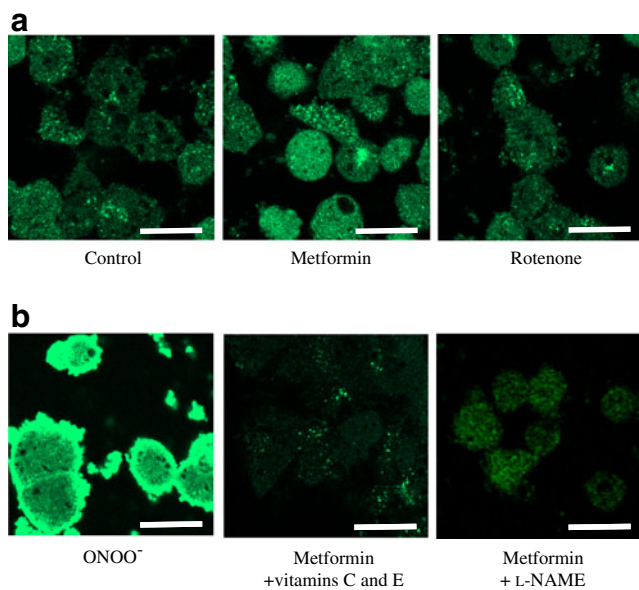


Fig. 2 Immunocytochemical staining with anti-nitrotyrosine antibody for detection of ONOO⁻ generation. ONOO⁻ (10 μmol/l) incubated for 5 min was used as a positive control. **a** Exposure to metformin (2 mmol/l) for 2 h increased staining, but exposure to rotenone (100 nmol/l) for the same time did not. **b** ONOO⁻ generation induced by metformin was decreased by co-administration with RNS scavengers (5 mmol/l α-tocopherol [vitamin E] plus 2.3 mmol/l ascorbate [vitamin C]) and a NOS inhibitor (1 mmol/l L-NAME), respectively. Confocal microscopy, magnifications ×100; scale bars 50 μm

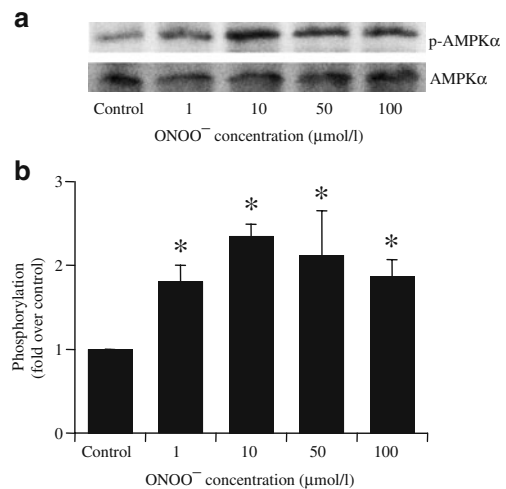


Fig. 3 Exogenous ONOO⁻ stimulates AMPKα phosphorylation in freshly isolated hepatocytes. **a** Blot showing that direct exposure to ONOO⁻ for 5 min at doses ranging from 1 to 100 μmol/l stimulated AMPKα phosphorylation. **b** Quantification with data expressed as fold stimulation over control. Values are means ± SE (n=4), **p*<0.05 vs control

after administration for three consecutive days, as was also the case in *eNos*^{-/-} mice (Table 3).

Lack of effects of metformin in vivo on AMPKα phosphorylation in liver tissues lacking eNOS In liver tissue samples collected after three consecutive days of administration, metformin stimulated phosphorylation of AMPKα in wild-type mice (metformin 2.17±0.30 [fold increase relative to vehicle], *p*<0.05 vs vehicle; Fig. 5a, b). However, stimulation of AMPKα phosphorylation by metformin was not observed in liver tissues of *eNos*^{-/-} mice (metformin 0.97±0.12 [fold increase relative to vehicle], *p*=NS vs saline; Fig. 5a, c).

Discussion

In the present study, we show for the first time that activation of AMPK and the inhibitory effect on hepatic gluconeogenesis by metformin are mediated by generation of the RNS, ONOO⁻. We also showed that eNOS plays an important role in metformin action in liver.

We investigated the metformin–RNS–AMPK pathway for its suppressing effects on hepatic gluconeogenesis. Because recent studies have shown that metformin activates AMPK through the RNS, ONOO⁻, in BAEC [18], we evaluated RNS production in liver, the major target of metformin action. We found that metformin increased ONOO⁻ generation and that ONOO⁻ itself activates AMPK, which is induced in only 5 min. A previous study found that AMPK phosphorylation by metformin does not appear within 10 min but only after 30 min [31]. Consistent

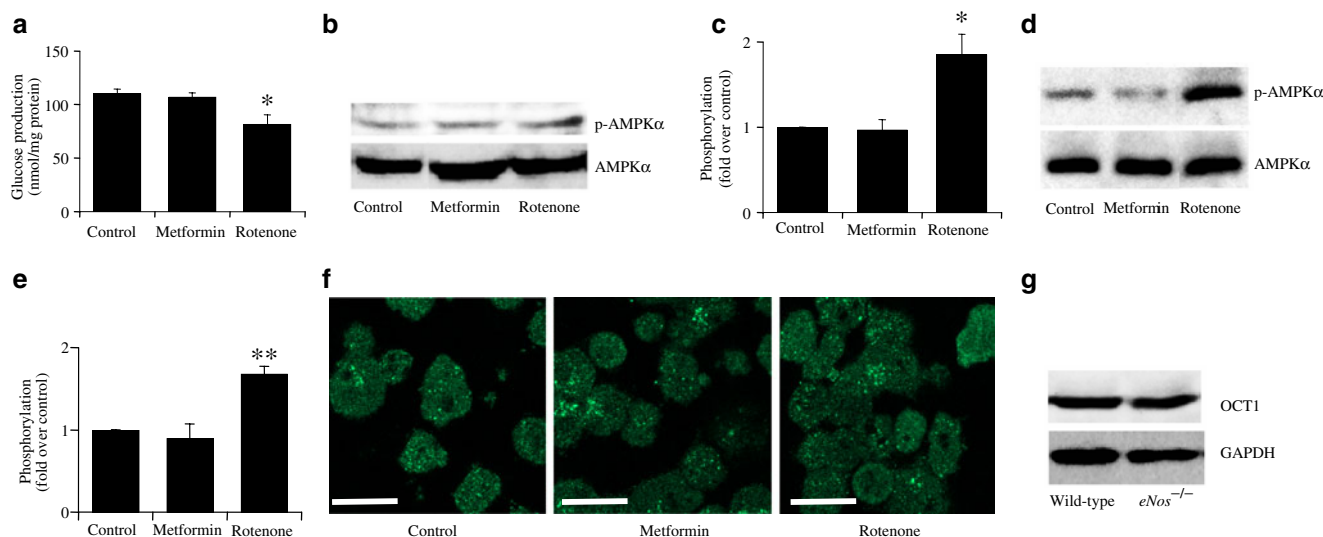


Fig. 4 Lack of effects of metformin on suppression of gluconeogenesis, AMPK α phosphorylation and ONOO $^-$ generation in hepatocytes lacking eNOS. **a** Metformin (2 mmol/l) did not suppress gluconeogenesis after 2 h exposure in hepatocytes lacking eNOS, but rotenone (100 nmol/l) suppressed gluconeogenesis to a similar degree to that observed in wild-type hepatocytes. Values are means \pm SE ($n=6$), $*p < 0.05$ vs control. **b** Blot showing that AMPK α phosphorylation was not stimulated by metformin (2 mmol/l), but was stimulated by rotenone (100 nmol/l) after 2 h exposure in freshly isolated hepatocytes; **(c)** quantification with data expressed as fold stimulation over control. Values are means \pm SE ($n=4$), $*p < 0.05$ vs control. **d** Blot showing that AMPK α phosphorylation was not stimulated by metformin (2 mmol/l),

but was stimulated by rotenone (100 nmol/l) after 2 h exposure in primary cultured hepatocytes, with **(e)** bar graph showing data expressed as fold stimulation over control. Values are means \pm SE ($n=5$), $**p < 0.01$ vs control. **f** Immunocytochemical staining (confocal microscopy) with anti-nitrotyrosine antibody in hepatocytes lacking eNOS. Exposure to metformin (2 mmol/l) and rotenone (100 nmol/l) for 2 h did not increase staining. Magnification $\times 100$, scale bar 50 μ m. **g** Levels of OCT1 protein in wild-type and $eNos^{-/-}$ mice hepatocytes. OCT1 levels in $eNos^{-/-}$ mice hepatocytes were similar to those in wild-type mice hepatocytes. Findings normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

with that study, our data showed that AMPK phosphorylation by metformin did not appear within 15 min, but only after more than 30 min (data not shown). Thus, ONOO $^-$ generation appears to precede AMPK phosphorylation after exposure to metformin. ONOO $^-$ is generated by nitric oxide and superoxide anions; intra-hepatocellular nitric oxide is produced by NOS. In the present study, the NOS inhibitor, L-NAME, suppressed ONOO $^-$ production by metformin.

This suggests that nitric oxide production by hepatocellular NOS is required for ONOO $^-$ production by metformin. Since eNOS is the representative subtype of the NOS family for generation of ONOO $^-$ in liver [17], we sought to determine whether eNOS is required for ONOO $^-$ production by metformin. Using eNOS-deficient mice, we were able to demonstrate that eNOS is essential for metformin action in liver. Thus metformin increases ONOO $^-$ produc-

Table 3 Effect of metformin on blood glucose levels in wild-type and $eNos^{-/-}$ diabetic mice

Treatments per mouse type	Pre streptozotocin			Post streptozotocin		
	Body weight (g)	FBG (mmol/l)	Fed BG (mmol/l)	FBG (mmol/l)	BG (mmol/l) at 1 h PM	FBG (mmol/l) after 3 days met
Wild-type mice						
Saline	20.3 \pm 0.4	3.7 \pm 0.2	8.2 \pm 0.5	16.0 \pm 2.7	17.2 \pm 3.1	16.6 \pm 3.3
Metformin	20.4 \pm 0.3	3.7 \pm 0.2	8.0 \pm 0.3	16.6 \pm 2.8	12.7 \pm 3.0**	9.5 \pm 1.9**
$eNos^{-/-}$ mice						
Saline	20.5 \pm 0.3	3.6 \pm 0.2	7.9 \pm 0.4	15.0 \pm 1.9	17.3 \pm 2.4	16.9 \pm 2.7
Metformin	20.7 \pm 0.3	3.5 \pm 0.2	8.1 \pm 0.5	15.8 \pm 1.6	19.0 \pm 1.9	16.5 \pm 2.1

Values are means \pm SE ($n=8$)

** $p < 0.01$ vs the value of pre-injection intraperitoneally with metformin in saline or saline only, paired t test

BG, blood glucose; FBG, fasting blood glucose; met, metformin; PM, post-metformin

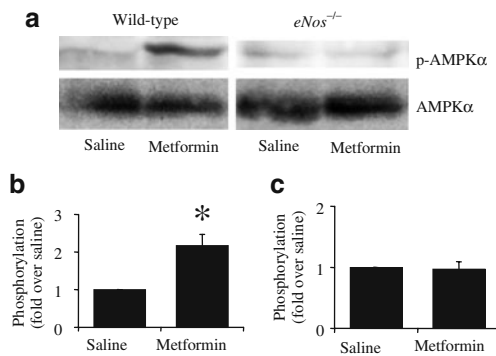


Fig. 5 Lack of effects of metformin in vivo on AMPK α phosphorylation in liver tissues deficient in eNOS. **a** Blot showing that metformin stimulated phosphorylation of AMPK α in liver tissues of wild-type diabetic mice after administration for three consecutive days. **b** Quantification of blot for wild-type and **(c)** *eNos*^{-/-} mice. Metformin did not stimulate **(a, c)** phosphorylation of AMPK α in liver tissues of *eNos*^{-/-} diabetic mice after metformin administration for three consecutive days. Data **(b, c)** are expressed as fold stimulation over saline. Values are means \pm SE ($n=5$), * $p<0.05$ vs vehicle

tion, which is followed by AMPK activation and suppression of gluconeogenesis.

Although metformin has been reported not to affect the ATP content of hepatocytes [32], several studies have found that metformin decreased ATP content and/or increased the AMP/ATP ratio in hepatocytes [23, 33], possibly a result of metformin's suppressive effect on complex I activity in the respiratory chain [34] and one that plays an important role in AMPK activation by metformin. While metformin was found not to affect ATP content and the AMP/ATP ratio in the present study, the AMP/ATP ratio might nevertheless play an important role in AMPK activation by metformin because AMPK is sensitive to changes in the AMP/ATP ratio at levels too slight to be detected by measurement of the total adenine nucleotide content of whole cells [35]. Interestingly, metformin activates AMPK with a smaller increase in the AMP/ATP ratio than that effected by mitochondrial uncoupler and rosiglitazone [16] and without affecting the ADP/ATP ratio [10]. These results suggest that, apart from increases in the AMP/ATP ratio, other important mechanisms may be involved in AMP activation by metformin.

Rotenone inhibits complex I of the mitochondrial respiratory chain and decreases oxidative phosphorylation, leading to ATP depletion and an increase in the AMP/ATP ratio, which results in stimulation of AMPK phosphorylation. In the present study we observed that while 2 mmol/l metformin and 100 nmol/l rotenone had similar effects on gluconeogenesis and AMPK phosphorylation, the AMP/ATP ratio increased prominently only upon exposure to rotenone but not upon exposure to metformin. These results indicate that complex I inhibition alone is unlikely to

explain the action of metformin. Interestingly, metformin significantly increased RNS in contrast to the lack of effect of rotenone on RNS. Furthermore, a decrease in metformin-induced RNS production by eNOS disruption abolished activation of AMPK by metformin. These results demonstrate that RNS is a regulator distinct from the AMP/ATP ratio in AMPK activation by metformin.

Some groups have reported that eNOS acts upstream of AMPK activation in BAEC [18], while other groups have reported that eNOS acts downstream of AMPK activation in capillary endothelial cells and in cardiomyocytes [21]. In the present study, we show that, in wild-type hepatocytes, direct exposure to ONOO⁻ activates AMPK and that rotenone activates AMPK without increase in ONOO⁻ production, supporting the former notion [18] in hepatocytes.

It is well known that high levels of RNS have deleterious effects on cell function and viability [17]. On the other hand, the low levels of RNS seen in physiological conditions are required for maintaining normal cell functions such as signal transduction [36]. For example, it has been reported that RNS production induced by skeletal muscle contraction is correlated with glucose uptake [20]. Thus, RNS has protective and damaging effects on cells. Indeed, the RNS produced by metformin at a dose used in the present study (2 mmol/l) should have beneficial effects on hepatic glucose metabolism through AMPK activation.

We demonstrate in the present study that AMPK activation by metformin in hepatocytes is dependent on RNS. We also demonstrate that eNOS plays an important role in suppressing hepatic gluconeogenesis in vitro as well as in lowering fasting blood glucose levels in vivo. It is generally accepted that fasting blood glucose levels are determined by hepatic gluconeogenesis, which suggests that eNOS is required for metformin's action on fasting blood glucose levels.

In the present study, we have elucidated a novel mechanism for metformin action. However, some limitations of this study must be considered. In our in vivo metformin experiments, the mice were injected intraperitoneally with 250 mg/kg metformin in 0.9% sterile saline, which is a similar dosage to that used previously [8, 18]. This protocol using a high dose of metformin for rodents may cause a very distinct acute response. Therefore, we cannot exclude the possibility that the acute hepatocellular response to AMPK activation by metformin in the present study differs from the clinical effects of metformin when used to treat patients with type 2 diabetes. To elucidate the detailed mechanisms of AMPK activation by metformin in liver, which may provide novel therapeutic targets for type 2 diabetes, further investigations are required.

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