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Hydrogen peroxide induces activation of insulin signaling pathway via AMP-dependent kinase in podocytes

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ABSTRACT

Podocytes are cells that form the glomerular filtration barrier in the kidney. Insulin signaling in podocytes is critical for normal kidney function. Insulin signaling is regulated by oxidative stress and intracellular energy levels. We cultured rat podocytes to investigate the effects of hydrogen peroxide (H_2O_2) on the phosphorylation of proximal and distal elements of insulin signaling. We also investigated H_2O_2-induced intracellular changes in the distribution of protein kinase B (Akt). Western blots showed that H_2O_2 (100 μM) induced rapid, transient phosphorylation of the insulin receptor (IR), the IR substrate-1 (IRS1), and Akt with peak activities at 5 min (Δ 183%, $P < 0.05$), 3 min (Δ 414%, $P < 0.05$), and 10 min (Δ 35%, $P < 0.05$), respectively. Immunostaining cells with an Akt-specific antibody showed increased intensity at the plasma membrane after treatment with H_2O_2>. Furthermore, H_2O_2 inhibited phosphorylation of the phosphatase and tensin homologue (PTEN; peak activity at 10 min; Δ −32%, $P < 0.05$) and stimulated phosphorylation of the AMP-dependent kinase alpha subunit (AMPKα; 78% at 3 min and 244% at 10 min). The stimulation of AMPK was abolished with an AMPK inhibitor, Compound C (100 μM, 2 h). Moreover, Compound C significantly reduced the effect of H_2O_2 on IR phosphorylation by about 40% (from 2.07 ± 0.28 to 1.28 ± 0.12, $P < 0.05$). In addition, H_2O_2 increased glucose uptake in podocytes (from 0.88 ± 0.04 to 1.29 ± 0.12 nmol/min/mg protein, $P < 0.05$), and this effect was attenuated by Compound C.

Our results suggested that H_2O_2 activated the insulin signaling pathway and glucose uptake via AMPK in cultured rat podocytes. This signaling may play a potential role in the prevention of insulin resistance under conditions associated with oxidative stress.

1. Introduction

Podocytes are insulin-dependent cells localized in renal glomeruli. They play a key role in the regulation of glomerular filtration. Podocytes have demonstrated similarities to skeletal muscle and fat cells with respect to insulin-stimulated glucose uptake kinetics and the expression of glucose transporters [1–4]. Insulin signaling in the podocyte is critical for normal kidney function [5].

Insulin signaling is initiated by binding to the insulin receptor (IR), with concomitant activation of tyrosine kinase activity in the IR-β subunits. Much of insulin's downstream signaling to affect metabolic events involves the activation of phosphatidylinositol (PI) 3-kinase. Insulin activation causes the p85 subunit of PI3-kinase to dock onto the tyrosine-phosphorylated IR substrates, IRS-1 and IRS-2. This triggers a cascade of distal signaling responses [6]. PI3-kinase is also linked to activation of the protein kinase, Akt (protein kinase B), and further downstream, vesicular translocation of glucose transporter 4 (GLUT4) and activation of glucose transport [7]. The insulin signaling pathway undergoes various types of coordinated regulation. Major regulatory influence is exerted by specific, cellular protein-tyrosine phosphatases (PTP-ases) [8]. PTP-ases are regulated by oxidation/reduction reactions; they require a reduction of the thiol side chain on the catalytic cysteine residue for phosphotyrosine hydrolysis [9]. The phosphatase and tensin homologue deleted on chromosome 10 (PTEN), also expressed in podocytes, opposes PI3K signaling [10]. Activation of PTEN results in attenuation of the Akt pathway, and consequently, impairs insulin signaling [11]. It was previously demonstrated that overexpression of PTEN significantly inhibited insulin-mediated

Abbreviations: AMPK, AMP-dependent kinase; CC, Compound C; GLUT4, glucose transporter 4; H_2O_2, hydrogen peroxide; IR, insulin receptor; IRS1, insulin receptor substrat 1; PI3-kinase, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue; PTP-ase, protein-tyrosine phosphatase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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glucose uptake in adipocytes [12]. Oxidative stress can modulate these insulin signaling effectors to enhance glucose transport in variety of cultured cells [13]. Insulin elicits the generation of hydrogen peroxide (H_2O_2) [14], which is associated with enhanced insulin signaling [15] and the reversible, oxidative inhibition of cellular PTP-ase activity [16]. Moreover, exposure of cells to H_2O_2 resulted in the oxidation of PTEN in a time- and concentration-dependent manner, and this caused PTEN inhibition [17]. Furthermore, exposure to H_2O_2 enhanced the glucose transport system by activating insulin signaling pathway elements (IR, PI3-kinase, and Akt) [18]. Glucose transport is also stimulated by activation of 5′-AMP-dependent kinase (AMPK) and Ca^{2+}-dependent factors [19]. AMPK is postulated to be a cellular energy sensor that plays a role in restoring energy homeostasis by activating ATP generation and inhibiting ATP consumption pathways. Several studies have demonstrated that increased intracellular concentrations of H_2O_2 resulted in the activation of AMPK and the enhancement of AMPK-mediated cellular adaptation [20–22]. Additionally, AMPK activation appeared to exert positive regulation on insulin signaling [19,23]. The activity of AMPK is suppressed in disorders associated with insulin resistance [24]; however, its pathophysiological role in podocytes, particularly in the context of oxidative stress, is not well recognized.

No study to date has investigated whether oxidative stress can modulate the glucose transport system in rat cultured podocytes. Therefore, we investigated the short-term effects of H_2O_2 on the insulin signaling cascade (including IR, IRS1, and Akt), PTEN phosphorylation, AMPK activity, and glucose uptake. We also determined the role of AMPK activation in the oxidative stress-induced enhancement of insulin signaling and glucose transport.

2. Material and methods

2.1. Preparation and culture of rat podocytes

All experiments were approved by the local ethics committee (No. 11/2007). Female Wistar rats weighing 100–120 g were anesthetized with thiopental (70 mg/kg body weight, i.p.). The kidneys were excised and minced with a scalpel and then pressed through a system of sieves with decreasing pore diameters (160, 106, and 53 mm) to obtain a suspension of glomeruli in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The final suspension of glomeruli was plated in 75 cm^2 type I collagen-coated culture flasks (Becton Dickinson Labware, Beckton, UK) and maintained at 37 °C in an atmosphere of 95% air/5% CO_2 for 5–7 days. The outgrowing podocytes were trypsinized and passed through sieves with 33-mm pores to remove the remaining glomerular cores. The suspension of podocytes was seeded in culture flasks and cultivated at 37 °C in an atmosphere of 95% air/5% CO_2. Experiments were performed using podocytes cultivated for 12–20 days. The phenotype of the podocytes and cell viability were determined as described previously [4].

2.2. 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 (Sigma–Aldrich) and homogenized at 4 °C by scraping. The cell homogenates were centrifuged at 9500×*g* for 20 min at 4 °C. Supernatant proteins (20 μg) were separated on an SDS–polyacrylamide gel (10%) and electrotransferred to a nitrocellulose membrane. The membrane was blocked for 1.5 h with Tris-buffered saline (TBS) (20 mM Tris–HCl, 140 mM NaCl, 0.01% NaN_3) 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. The following primary antibodies were diluted in TBS containing 0.05% Tween-20 and 1% BSA: anti-AMPKα (1:750, Cell Signaling Technology), anti-p-AMPK (Thr^{172}) (1:750, Cell Signaling Technology), anti-Akt 1/2/3 (1:600, Santa Cruz Biotechnology), anti-p-Akt 1/2/3 (Ser^{473}) (1:600, Santa Cruz Biotechnology), anti-insulin Rβ (1:200, Santa Cruz Biotechnology), anti-p-insulin Rβ ($Tyr^{1150/1151}$) (1:200, Santa Cruz Biotechnology), anti-IRS1 (1:200, Santa Cruz Biotechnology), anti-p-IRS1 (Ser^{374}) (1:200, Santa Cruz Biotechnology), anti-PTEN (1:300, Santa Cruz Biotechnology), anti-p-PTEN (Ser^{380}/$Thr^{382/383}$) (1:300, Santa Cruz Biotechnology) and anti-actin (1:3000, Sigma–Aldrich). To detect primary antibodies bound to the immunoblot, the membrane was incubated for 2 h with the appropriate alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit IgG-AP, goat anti-mouse IgG-AP, or goat anti-rabbit IgG-AP, Santa Cruz Biotechnology). The protein bands were detected using the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium (BCIP/NBT) system. The density of the bands was measured quantitatively using the Quantity One program (Bio-Rad). Protein content was measured using the Lowry method. The ratio of phosphorylated P-AMPKα to total AMPKα expression was used as an index of AMPK activity [25].

2.3. Immunofluorescence

Podocytes were seeded on type-I collagen-coated coverslips (Becton Dickinson Labware, Beckton, UK) and cultured in RPMI 1640 supplemented with 10% FBS. Cells were fixed in PBS containing 2% formaldehyde for 10 min at room temperature. The coverslips were placed on ice, and the cells were permeabilized with 0.3% Triton-X 100 for 3–4 min and then blocked with PBSB solution (PBS containing 2% FBS, 2% BSA, and 0.2% fish gelatin) for 60 min. After blocking, cells were incubated with anti-Akt1/2/3 antibody in PBSB (1:100) at 4 °C for 1 h. For non-specific staining, the primary antibodies were substituted with PBSB. Next, cells were washed three times with cold PBS and incubated with Cy3-conjugated anti-goat (1:100) secondary antibodies for 45 min. After three 5-min washes, the coverslips were attached to slides with Mowiol 4-88 diluted in glycerol-PBS (1:3, v:v), and the cells were viewed under a confocal laser scanning microscope (Olympus FluoView FV10i).

2.4. Measurement of glucose uptake

Podocytes were seeded at a density of 3×10^4/well on type I collagen-coated 24-well plates. Cells were preincubated for 2 h with serum-free RPMI 1640 medium and then were exposed to 100 μM CC or buffer for 2 h. The measurement was started by the addition of [1,2-^{3}H]-DOG diluted in non-radioactive glucose (50 μM final concentration) with 100 μM H_2O_2 or insulin (300 nM, 3 min) for 3 min. Glucose transport was initiated by the addition of 1 μCi 2-deoxy-[1,2-^{3}H]glucose (2-DG). The 2-DG uptake was determined over a 3-min period at 37 °C. Next, the plates were placed on ice and experimental medium was rapidly removed from above cell layer for the determination of extracellular radioactivity. The layer of podocytes was then washed 3 times with ice-cold PBS and lysed by shaking for 90 min in 0.05 mM NaOH at room temperature. Intracellular and extracellular radioactivities were determined by liquid scintillation counting (Wallac 1409). Protein content was measured using a modified Bradford method. In each experiment glucose transport was calculated from the mean of at least triplicate determinations.

2.5. Measurement of ATP concentration

Podocytes were incubated 15 min at 37 °C in the presence/absence of 100 μM ARL 67156 (ecto-ATPase inhibitor). Incubation

medium and homogenized cells solution were placed at 99 °C for 2 min, centrifuged (4 °C, 800 g, 10 min) and immediately used for ATP determination. Concentration of ATP was measured using luciferin-luciferase methods as described previously [26]. Luminescence was measured in room temperature with a photon-counting luminometer (TD-20/20).

2.6. 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 ± SEM. Significance was set at $P < 0.05$.

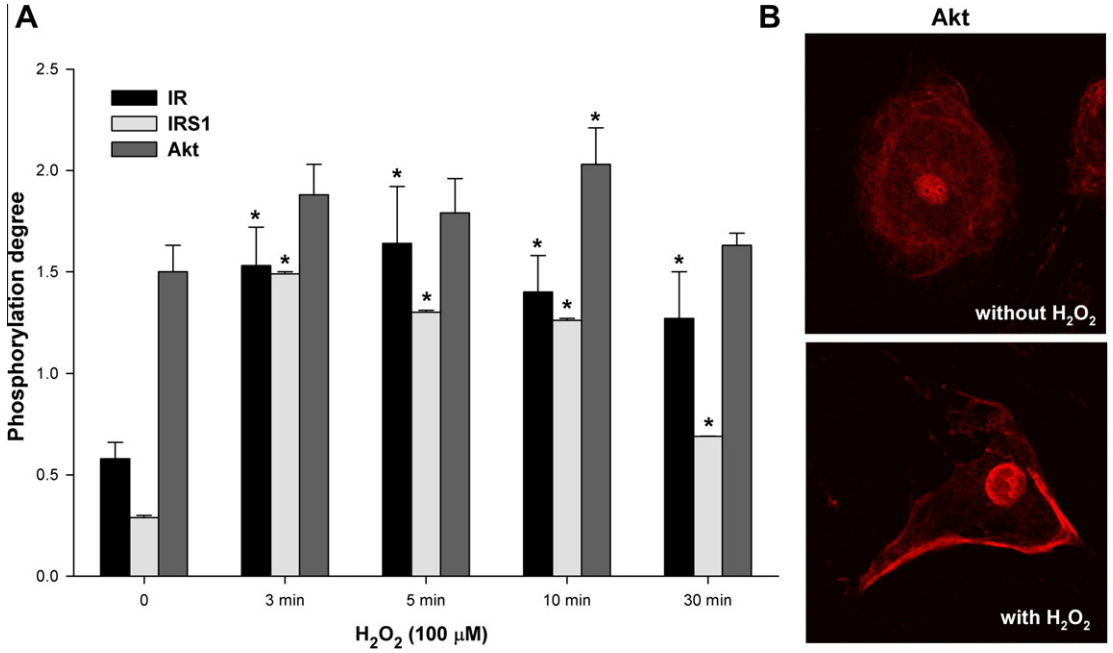

Fig. 1. Hydrogen peroxide enhances insulin signal transduction in cultured rat podocytes. Cells were incubated with 100 μM H_2O_2 for 0, 3, 5, 10 and 30 min. (A) Cell lysates were immunobloted with anti-IR, anti-p-IR ($Tyr^{1150/1151}$), anti-IRS1, anti-p-IRS1 (Ser^{374}), anti-Akt1/2/3 and anti-p-Akt1/2/3 (Ser^{473}). Values are the mean ± SEM of four independent experiments. *$P < 0.05$ compared to time 0. (B) The effect of hydrogen peroxide on Akt distribution. Rat podocytes, seeded onto coverslips, were incubated for 3 min in absence (upper part) or presence (lower part) of 100 μM H_2O_2. Cells were then immunostained with anti-Akt1/2/3 antibody.

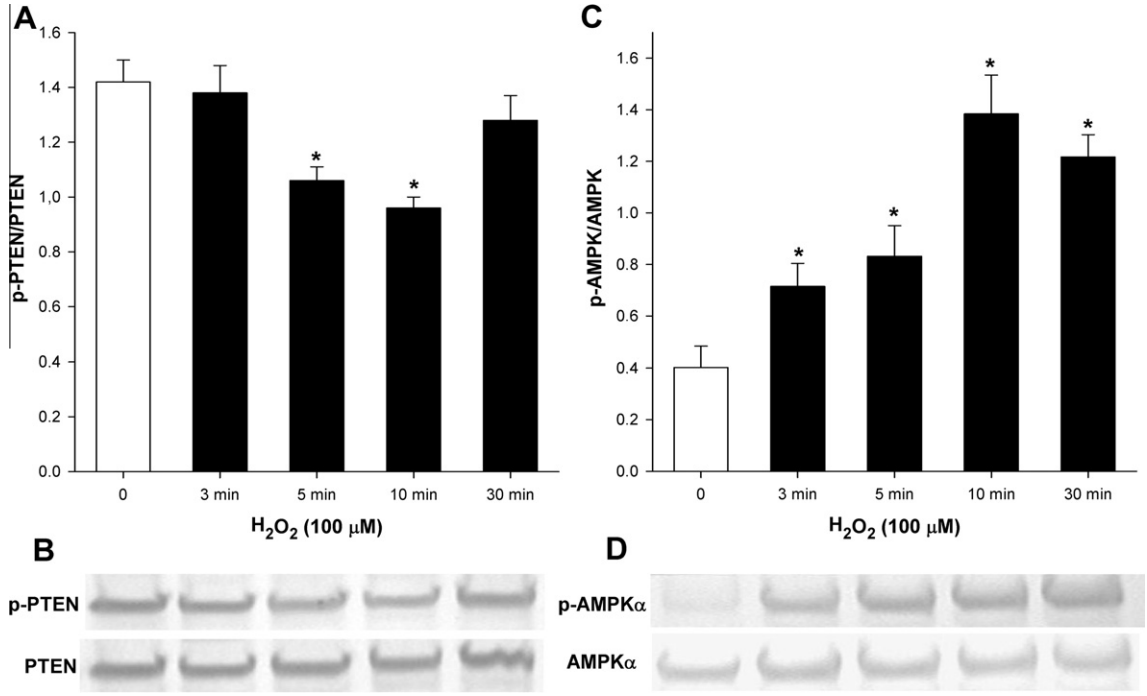

Fig. 2. Time course of the effects of hydrogen peroxide on PTEN and AMPK phosphorylation in cultured rat podocytes. Cells were incubated with 100 μM H_2O_2 for 0, 3, 5, 10 or 30 min. Cell lysates were immunobloted with anti-p-PTEN (Ser^{380}/$Thr^{382/383}$) and anti-PTEN (A) or with anti-p-AMPKα (Thr^{172}) and anti-AMPKα (C). Values are the mean ± SEM of four independent experiments. *$P < 0.05$ compared to time 0. Representative Western blot of podocytes stimulated by H_2O_2 for PTEN (B) and AMPK (D).

3. Results

3.1. Hydrogen peroxide enhances insulin signal transduction in cultured rat podocytes

We first investigated the time-dependent effects of H_2O_2 on phosphorylation of proteins involved in insulin signal transduction. Exposing the podocytes to 100 μM H_2O_2 resulted in rapid, transient phosphorylation of IR, IRS1, and Akt (Fig. 1A). The maximum effects were reached at 5 min (183%, $P < 0.05$) for IR, 3 min (414%, $P < 0.05$) for IRS1, and 10 min (35%, $P < 0.05$) for Akt. Moreover, immunofluorescence experiments showed that H_2O_2 caused substantial changes in the subcellular localization of the Akt protein in cultured rat podocytes (Fig. 1B). The intensity of Akt immunostaining increased at the plasma membrane after treatment with H_2O_2 (100 μM, 3 min).

Next, we examined the time-dependent effects of H_2O_2 on phosphorylation of PTEN. Significant decreases in PTEN phosphorylation were observed after 5 min (Δ −25%, $P < 0.05$) and 10 (Δ −32%, $P < 0.05$) incubations with H_2O_2; phosphorylation was restored to control levels after 30 min (Fig. 2A).

3.2. Hydrogen peroxide induces time-dependent changes in AMPK phosphorylation in cultured rat podocytes

To examine the role of H_2O_2 in AMPK activation, podocytes were incubated for 0, 3, 5, 10, and 30 min with 100 μM H_2O_2. As shown in Fig. 2B, the percentage of phosphorylation on the alpha subunit (AMPKα) increased continuously after incubation with H_2O_2. Phosphorylation increased by 78% at 3 min, 107% at 5 min, and 244% at 10 min ($n = 4$). A longer incubation (30 min) led to an insignificant decrease in phosphorylated AMPKα.

H_2O_2 had no effect on the intracellular ATP concentration. Podocytes exposed to H_2O_2 for 10 min showed an intracellular ATP concentration of 4.38 ± 0.03 μM, vs. 4.62 ± 0.32 μM ($n = 3$) in control cells; after 30 min, the ATP concentration was 4.56 ± 0.05 μM in treated cells vs. 4.92 ± 0.09 μM in control cells ($n = 3$).

3.3. Hydrogen peroxide enhances insulin signaling pathways via AMPK activation

Both H_2O_2 and insulin (300 nM, 3 min) increased the levels of phosphorylated AMPKα by 66% (Fig. 3A, $P < 0.05$), and they increased the levels of phosphorylated IR by about 276% and 113%, respectively (Fig. 3B, $P < 0.05$). We hypothesized that H_2O_2 induced changes in insulin receptor phosphorylation through activation of AMPK. To test this hypothesis, podocytes were incubated with H_2O_2 (100 μM, 3 min) in the presence of the AMPK inhibitor, Compound C (100 μM, preincubation 2 h). Compound C alone had no significant effect on AMPK or IR phosphorylation. The effect of H_2O_2 on AMPK phosphorylation was abolished in the presence of Compound C (Fig. 3A, $P < 0.05$). Moreover, the effect of H_2O_2 on IR phosphorylation was significantly reduced by about 40% (from 2.07 ± 0.28 to 1.28 ± 0.12, $P < 0.05$) in the presence of Compound C. Compound C also abolished the effects of insulin on AMPK and IR phosphorylation (Fig. 3). These results suggested that H_2O_2 could regulate the insulin signaling pathway via AMPK activation in cultured rat podocytes.

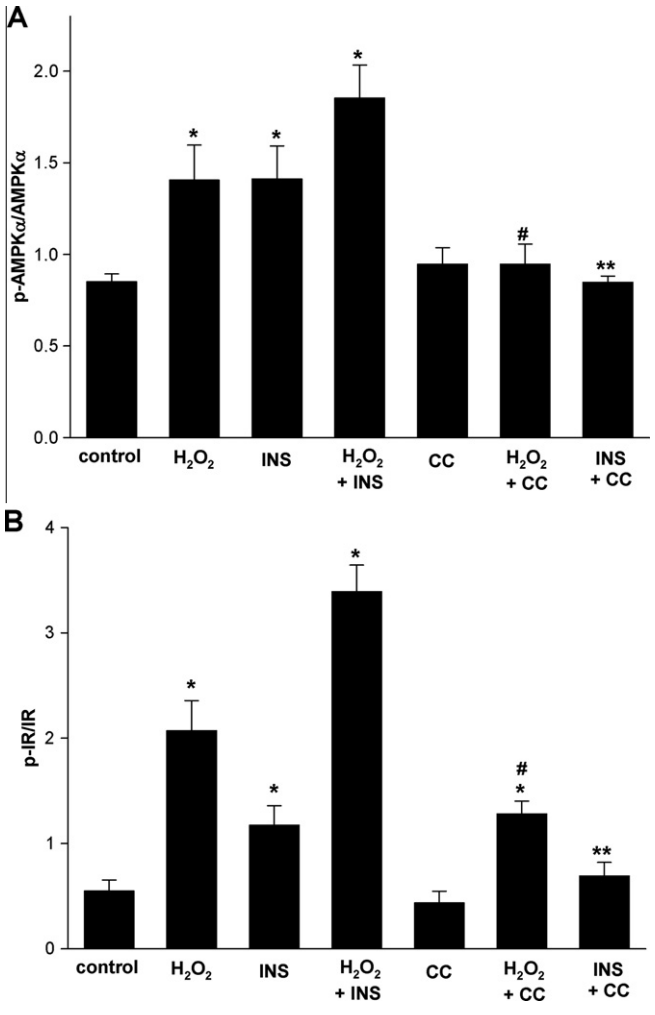

Fig. 3. Hydrogen peroxide induced enhance of insulin signaling pathways via AMPK activation. Before hydrogen peroxide (100 μM, 3 min) or insulin (300 nM, 3 min) stimulation cells were preincubated with AMPK inhibitor, Compound C (CC, 100 μM for 2 h). The cell lysates (20 μg) were analyzed via Western blotting. Quantitative densitometric analysis was used to determine the ratio of (A) anti-p-AMPKα (Thr^{172}) to anti-AMPKα and (B) anti-p-IRβ ($Tyr^{1150/1151}$) to anti-IRβ. Values are the mean ± SEM ($n = 4$), $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. control with hydrogen peroxide, $^{**}P < 0.05$ vs. control with insulin.

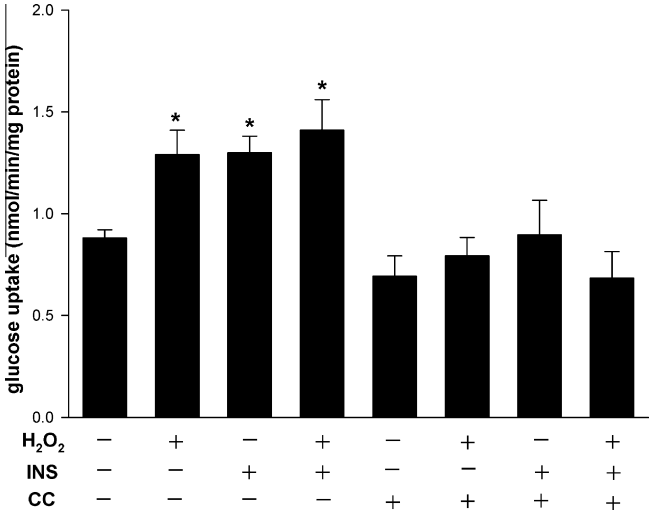

Fig. 4. Effect of AMPK inhibitor on hydrogen peroxide-stimulated glucose uptake in cultured rat podocyte. Before H_2O_2 or insulin stimulation cells were preincubated with Compound C (CC) for 2 h. Uptake measurement was started with the addition of 1 μCi of [1,2-^{3}H]-deoxy-D-glucose diluted in non-radioactive glucose to final concentration of 50 μM and 100 μM H_2O_2 or/and 300 nM insulin for 3 min. Values are the mean ± SEM ($n = 4$), $^{*}P < 0.05$ compared to the control.

3.4. Hydrogen peroxide stimulation of glucose uptake was AMPK-dependent

As shown in Fig. 4, H_2O_2 induced significant increases in glucose uptake in podocytes (from 0.88 ± 0.04 to 1.29 ± 0.12 nmol/min/mg protein, $P < 0.05$). Podocytes were insulin-sensitive, and insulin stimulation increased glucose uptake by about 50% (Fig. 4). To determine the role of AMPK on H_2O_2-induced glucose uptake, we evaluated the effect of Compound C. We found that Compound C attenuated H_2O_2-induced increases in glucose uptake and blocked insulin-dependent glucose uptake. This indicated that AMPK played a critical role in this process (Fig. 4). Compound C alone had no significant effect on transport-mediated glucose uptake.

4. Discussion

In the present study, we showed that H_2O_2 activated the insulin signaling pathway and glucose uptake via AMPK kinase in cultured rat podocytes.

For many years, reactive oxygen species (ROS) have been viewed as the unwanted by-product of aerobic metabolism. However, recently, oxidants were recognized for their crucial roles in homeostatic control of mammalian cells in the healthy state. NAD(P)H oxidase is the major source of superoxide anion ($O_2^{\bullet-}$), the predominant form of ROS in podocytes [27,28]. The $O_2^{\bullet-}$ is metabolized to H_2O_2 intracellularly by superoxide dismutase (SOD). Hydrogen peroxide mimics insulin by triggering many of the same physiological responses [29,30]. We have demonstrated in the present investigation that H_2O_2 activated glucose uptake in insulin-sensitive cultured rat podocytes. These findings were consistent with findings in other studies that utilized a variety of insulin-sensitive cells, including myocytes and adipocytes [13]. We also showed that oxidant stimulation engaged the insulin signaling pathway to affect glucose transport. Time-dependent exposure to H_2O_2 activated both proximal elements (IR and IRS1) and distal elements (Akt) of the pathway. Moreover, we used an inhibitor of AMPK (Compound C) to demonstrate that the positive effects exerted by H_2O_2 on insulin signal transduction and glucose transport depended on AMPK activity.

It is well known that AMPK signaling functions as an integrator of cellular and whole body glucose and energy regulation [31,32]. In the present investigation, we reported that H_2O_2 could increase phosphorylation of the α isoform of AMPK. This may represent an additional mechanism that enables oxidative stress to modulate, directly or indirectly (via modulation of the insulin signaling pathway), glucose uptake in podocytes. Other authors also showed that H_2O_2 induced large increases in AMPK activity in NIH-3T3 cells [33]. Recently, it was demonstrated that physiologically relevant concentrations of H_2O_2 could activate AMPK through oxidative modification of the AMPKα subunit [34]. We also previously demonstrated a close relationship between the activity of NAD(P)H oxidase and AMPK activity in podocytes [35].

Many reports have focused on the significance of PTEN in insulin signaling [36,37]. In a separate study, we also made the observation that H_2O_2 caused a time-dependent change in PTEN phosphorylation. PTEN phosphatase activity could be inactivated by phosphorylation of Ser^{380} and $Thr^{382/383}$ [38]. Similarly, exposure of cells to H_2O_2 resulted in PTEN oxidation and subsequent inhibition [17].

In conclusion, this study provided evidence that, in podocytes, H_2O_2 modulated the insulin signaling pathway and glucose transport. Moreover, we demonstrated that the oxidant stress-induced enhancement of glucose transport depended on the engagement of oxidative stress-activated AMPK.

Acknowledgments

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