

ORIGINAL**Hydrogen peroxide inhibits insulin-induced ATP-sensitive potassium channel activation independent of insulin signaling pathway in cultured vascular smooth muscle cells**

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Abstract : Both reactive oxygen species (ROS) and insulin resistance have been reported to play essential pathophysiological roles in cardiovascular diseases, such as hypertension and atherosclerosis. However, the mechanistic link between ROS and insulin resistance in the vasculature remains unclear. Recently we have shown that insulin causes membrane hyperpolarization *via* ATP-sensitive potassium (K_{ATP}) channel activation, which is mediated by phosphatidylinositol 3-kinase (PI3-K) in cultured vascular smooth muscle cells (VSMCs). K_{ATP} channel in the vasculature is critical in the regulation of vascular tonus. Here we examined the effects of ROS induced by hydrogen peroxide (H_2O_2) on insulin-induced K_{ATP} channel activities in cultured VSMCs, A10 cells. H_2O_2 (10 μM) increased significantly intercellular ROS in A10 cells. By using a cell-attached patch clamp experiment, 10 μM H_2O_2 suppressed significantly insulin-induced K_{ATP} channel activation without inhibition of insulin receptor signal transduction component including IRS and Akt in A10 cells. Furthermore 10 μM H_2O_2 suppressed significantly pinacidil-induced K_{ATP} channel activation in A10 cells. These data suggest that H_2O_2 might inhibit directly K_{ATP} channel independent of insulin signaling pathway. This study may contribute to our understanding of mechanisms of insulin resistance-associated cardiovascular disease. *J. Med. Invest.* 59 : 36-44, February, 2012

Keywords : insulin, K_{ATP} channel, ROS, oxidative stress, VSMCs

INTRODUCTION

Reduction of oxygen by normal cellular metabolism leads to the production of reactive oxygen

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species (ROS) that include superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\bullet$) (1). These species are now believed to participate in a variety of cellular signaling mechanisms that transmit transcriptional/translational regulation, cell growth, differentiation, and apoptosis (2, 3). In this regard, extracellular signal-regulated kinases (ERK) and other members of the mitogen-activated protein kinase (MAPK) family are up-regulated as a result of ROS stimulation (4, 5). In addition, several

protein tyrosine kinases (PTK) are reported to be activated by ROS in various cell lines (6, 7). Accumulating evidence indicates that ROS play important roles in cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after angioplasty (8, 9). Besides cardiovascular disease, oxidative stress, exerted by the intracellular accumulation of ROS, has been implicated in Type 2 diabetes as well as in beta cell failure and insulin resistance (10, 11). There is now much emerging evidences suggesting that several factors that cause cardiovascular diseases and insulin resistance have a common pathway in the excessive formation of ROS (12, 13). This seems to be risen from inflammation, glucotoxicity, lipotoxicity and some endocrine mediators.

Insulin exerts important biological roles on vasculature as well as conventional insulin-responsible tissue such as skeletal muscle and adipose tissue (14-17). Insulin enhances glucose uptake in skeletal muscle cells and adipocytes, and induces vasodilation through nitric oxide production from vascular endothelial cells (14-17). These effects of insulin mediate insulin receptor signaling pathway-catalyzed tyrosine phosphorylation of insulin receptor substrate (IRS) and the subsequent activation of phosphatidylinositol 3 kinase (PI3-K) and downstream molecules such as protein kinase B (Akt) (14-17). Recently we had reported that insulin activates ATP-sensitive potassium (K_{ATP}) channel and causes membrane hyperpolarization through insulin receptor signaling pathway including PI3-K in cultured vascular smooth muscle cells (VSMCs) (18). K_{ATP} channel in vascular smooth muscle likely plays an important role in vaso-dilation and control of blood pressure (19, 20). Activation of K_{ATP} channels induces membrane hyperpolarization, lowered intracellular calcium concentrations, and vaso-relaxation (19, 20). In addition to vaso-dilative effect, the opening of K_{ATP} channels has a protective function on the cardiovascular systems such as ischemic preconditioning (21). Thus insulin has important roles in vascular relaxation by activation of PI3-K/Akt signaling pathways. However, the relationship between oxidative stress and the effects of insulin on the vasculature remains unclear.

The K_{ATP} channel consists pore-forming heterooctamer containing four inwardly rectifying K^+ channel (Kir) subunit and four sulfonylurea receptor (SUR) subunits (22). The K_{ATP} channels have different isotypes among expressed tissue, such as VSMCs (Kir6.1/SUR2B), pancreatic β -cells (Kir6.2/SUR1), and skeletal muscle cells (Kir6.2/SUR2A)

(23). Pinacidil is a well known K_{ATP} channel opener which binds SUR subunits and induces relaxation of endothelium-denuded aorta through activating K_{ATP} channel in smooth muscle cells (24). Erdös *et al.* reported that insulin-resistant rats with oxidative stresses were observed weaker pinacidil-induced vaso-dilation than control subjects (25). Furthermore Yang *et al.* recently reported that H_2O_2 suppressed pinacidil-induced activity of smooth muscle isotype K_{ATP} channel, Kir6.1/SUR2B, which was expressed in human embryonic kidney (HEK) cells by genetically modification technique, because H_2O_2 induced *S*-glutathionyl modulation of K_{ATP} channel (26). However the effect of H_2O_2 on the activity of native K_{ATP} channel expressed in VSMCs remains unknown. In this study, we demonstrated the effect of extracellular administration of H_2O_2 on pinacidil-induced K_{ATP} channel activities in cultured VSMCs by using patch-clamp experiments. Furthermore to investigate the effect of H_2O_2 on insulin's action in vasculature, we also observed insulin-induced K_{ATP} channel activities and signaling pathway in cultured VSMCs.

MATERIALS AND METHODS

Cell culture

A10 cells, which are embryonic rat thoracic aortic smooth muscle cells from DB1X rat, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). A10 cells is an useful model of VSMCs for the observation about K_{ATP} channel activity, because A10 cells showed close similar activities about pinacidil- and insulin-induced K_{ATP} channel with primary isolated VMSCs from Wistar rat thoracic aorta (18). Cells were cultured at 37°C in a humidified atmosphere with 95% air and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO, USA) containing 0.5 mg/mL gentamicin (Sigma) and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were serum-starved for 1 hour before experiments.

Determination of intercellular reactive oxygen species

Changes in intercellular ROS were indicated using the fluorescence dye, carboxy- H_2DCFDA [6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester)] (Molecular Probes, Eugene, OR, USA). When the intercellular ROS was increased, the dye was deacetylated and oxidated

into the fluorescent product (DCF) and fluorescence intensities increased (27). A10 cells were subcultured on glass cover-slips. After loading with carboxy-H₂DCFDA at 37°C for 30 minutes, cells were placed in a chamber filled with HEPES buffered solution (HBS) containing (mM) NaCl 145, KCl 5, MgCl₂ 1, CaCl₂ 1, D-glucose 5.5, HEPES 10, pH 7.2. Fluorescence intensities at an excitation wavelength of 490 nm were obtained by microscopy (IX71, Olympus, Tokyo, Japan) and analyzed with Meta Fluor software (Molecular Devices, Downingtown, PA, USA). All experiments were performed at 37°C. Fluorescence intensities were continuously recorded every 30 seconds. Fluorescence intensities of an identical area adjacent to cells were subtracted as background. To evaluate changes in fluorescence intensity, we calculated their ratio before and after H₂O₂ treatment.

Cell preparation and Western blotting analysis

After incubation in HBS for 1 hour, cells were pretreated with or without H₂O₂ for 5 minutes and then treated with 100 nM insulin for 5 minutes. After treatment with insulin, the cells were rinsed with cold phosphate buffered saline (PBS) and solubilized in cold cell lysis buffer containing (mM) Tris-HCl 20, pH 8.0, NaCl 140, MgCl₂ 1, CaCl₂ 1, dithiothreitol 1, sodium vanadate 0.5, sodium pyrophosphate 20, phenylmethanesulfonyl fluoride 0.02, 10% glycerol (v/v), 1% Nonidet P40 (v/v).

The lysate samples were normalized for protein concentration by using Bicinchoninate (BCA) method (PIERCE, Rockford, IL, USA) and subjected to immunoblotting. The normalized lysates were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schnell Bioscience, Dassel, Germany), which were incubated with specific primary antibodies for 12 hours at 4°C. Following incubation with horseradish-peroxydase conjugated secondary antibodies, immunoreactive bands were visualized using an enhanced chemi-luminescence substrate. The membranes were stripped by incubating with stripping buffer containing (mM) Tris-HCl 62.5, and 2-mercaptoethanol 100, 2% SDS, pH 6.8, at 50°C for 30 minutes for a second round immunoblotting.

Patch-clamp experiments

Single channel activities were measured using cell-attached patch-clamp recordings as described by Hamill *et al* (28). Cells on glass cover-slips

were placed in a chamber in a solution containing (mM) KCl 140, MgCl₂ 1, CaCl₂ 0.1, D-glucose 5.5, HEPES 10, pH 7.2. The buffered solution in patch pipettes contained (mM) KCl 140, D-glucose 5.5, HEPES 10, pH 7.2. Patch pipettes were made from soft-glass capillaries (DRUMMOND SCIENTIFIC, Broomall, PA, USA) using an electrode puller (PP-830, Narishige, Tokyo, Japan). The resistance of pipettes filled with buffered solution was 7-10 MΩ. All drugs were added into the dishes. The inside of the pipette was voltage-clamped at +50 mV. Experiments were performed at 37°C.

Currents were recorded with a patch clamp amplifier (L/M-EPC7, List-Medical, Darmstadt, Germany) and converted into digital files using DigiData 1200 (Axon Instruments, Foster, CA, USA). The noises of current signals were removed using a low-pass filter of 1 kHz. pClamp version 6 software (Axon Instruments) was used for recording data. BIO-PATCH Ver. 3.42 software (BIO-LOGIC, Claix, France) was used to analyze recorded data. The channel activities were expressed as NPo. NPo was determined from current amplitude histograms and calculated as follows,

$$NPo = \left(\sum_{j=1}^N t_{jj} \right) / T$$

where Po is the open probability ; T, the duration of the measurement ; t_j, the time spent at the current level corresponding to j = 1, 2 ... N channels in the open state ; N, the number of the channels active in the patch. The NPo values were determined from recording data for longer than 120 seconds.

Reagents

Pinacidil and glibenclamide were purchased from SIGMA. Insulin and H₂O₂ was from WAKO chemicals (Tokyo, Japan). Anti-phosphorylated (Tyr612) and anti-total insulin receptor substrate (IRS) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of anti-phosphorylated Akt (Ser478), anti-total Akt, anti-phosphorylated ERK (Thr202/Tyr204), and anti-total ERK were from Cell Signaling (Beverly, MA, USA).

Statistical analysis

Statistical analysis of differences was estimated using ANOVA plus Bonferroni multiple comparison tests. Student's *t*-tests for paired data were used when appropriate. A value of *P* < 0.05 was considered statistically significant.

RESULTS

H₂O₂ increases ERK phosphorylation and ROS generation in A10 cells

It is well known that stimulation of ROS enhanced the phosphorylation of ERK (4, 5). To demonstrate the effects of extracellular administration with H₂O₂ on ERK phosphorylation, we treated A10 cells with various concentration (1-100 μM) of H₂O₂ for 5 minutes by using Western blot analysis (Fig. 1A). H₂O₂ treatment increased Thr202/Tyr204 phosphorylation of ERK from the concentration in 10 μM. By using densitometric analysis for Western blotting, the effect of H₂O₂ on ERK phosphorylation was significant increased in both 10 and 100 μM (Fig. 1B).

Next, to check effect of extracellular administration with H₂O₂ on ROS generation in A10 cells, we observed the change of intercellular ROS by using a ROS-reactive fluorescent dye, carboxy-H₂DCFDA (Fig. 1C). Treatment with 10 μM H₂O₂ increased intercellular ROS after 2.5 minutes and intercellular ROS was kept increasing for 10 minutes (Fig. 1C). In addition, we observed obvious morphological changes of A10 cells with 100 μM H₂O₂ treatment under a microscopy, but did not in the cells with under 10 μM (data not shown). These data suggested that 10 μM H₂O₂ had sufficient effect on elevation

of both intercellular ROS and ERK phosphorylation without morphological changes in cultured vascular smooth muscle A10 cells.

H₂O₂ suppresses pinacidil-induced K_{ATP} channel activity in A10 cells.

Recent report showed that H₂O₂ suppressed pinacidil-induced activity of smooth muscle K_{ATP} channels, Kir6.1/SUR2B isotype, which were expressed in HEK cells by genetically modification technique (26). To consider direct effect of H₂O₂ on the activities of native K_{ATP} channel expressed in VSMCs, we measured the effect of H₂O₂ for pinacidil-induced K_{ATP} activities in cultured A10 cells using cell-attached patch clamp experiments (Fig. 2). The cells were pretreated with vehicle or 10 μM H₂O₂ for 5 minutes and then stimulated with 100 μM pinacidil for 5 minutes. Pinacidil increases channel activation without H₂O₂ treatment (Fig. 2A). Treatment with 3 μM glibenclamide, a K_{ATP} channel blocker, significantly inhibited pinacidil-induced channel activation (data not shown). Treatment with H₂O₂ suppressed significantly pinacidil-induced channel activity in A10 cells (Figs. 2B and 2C). These results suggested that H₂O₂ suppress K_{ATP} channel activity in cultured VSMCs.

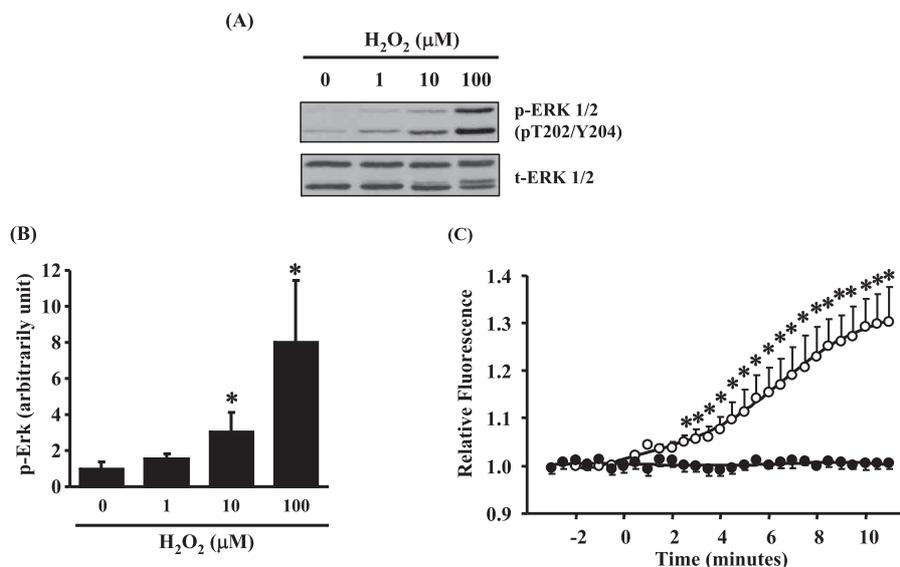


Fig. 1 Measurements of reactive oxygen species (ROS). (A) Representative data of ROS-induced phosphorylated extracellular regulated kinase (ERK) by extracellular administration with H₂O₂ in A10 cells. The cells were treated with the indicated concentrations of H₂O₂ for 5 minutes. Whole cell lysates were subjected to immunoblot analysis with specific antibodies to phosphorylated form (upper panels) and total (lower panels) ERK protein. (B) Densitometric analysis for phosphorylated forms of ERK. (n=5). (*)*P* < 0.05 versus vehicle treatment. (C) Intercellular ROS measurements in A10 cells. Intercellular ROS measurements were measured using the ROS reactive fluorescence dye, carboxy-H₂DCFDA [6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester)]. The data indicate relative fluorescence intensity for the following conditions. (●) Vehicle treatment. (○) 10 μM H₂O₂. Values are shown as means ± S.E.. All curves are representative of triplicate independent experiments and each point is the mean of triplicate values. Independent experiment typically included 6 cells. (*)*P* < 0.01 versus vehicle treatment.

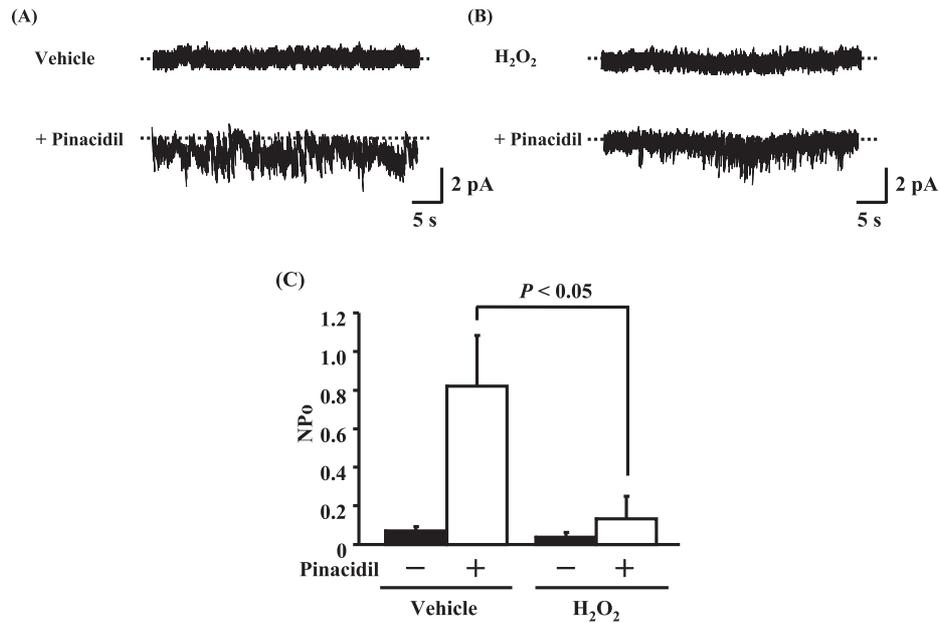


Fig. 2 Effect of H₂O₂ for pinacidil-induced K_{ATP} channel activities in A10 cells. Single channel-currents were measured by the cell-attached patch-clamp experiments. The pipette solution was voltage-clamped at +50 mV. The cells were pretreated with (A) vehicle, or (B) 10 μ M H₂O₂ for 5 min and then stimulated with 100 μ M pinacidil for 5 min. (C) K_{ATP} channel activities are shown as NPo. Values are shown as means \pm S.E. ($n=5$).

Insulin activates K_{ATP} channels in A10 cells.

We previously have reported that insulin causes membrane hyperpolarization *via* K_{ATP} channels activation in cultured VSMCs (18). To observe the effect of H₂O₂ for insulin-induced potassium ion transport on the membrane of A10 cells, we directly measured single channel activities using cell-attached

patch-clamp experiments (Fig. 3). Ion channels were activated 5 minutes after insulin stimulation without H₂O₂ treatment (Fig. 3A). Insulin-activated currents showed inward rectification; the conductance of the positive pipette voltage was 28.6 pS, whereas that of the negative pipette voltage was 21.8 pS. The conductance of insulin-induced currents in A10 cells was similar to that of vascular

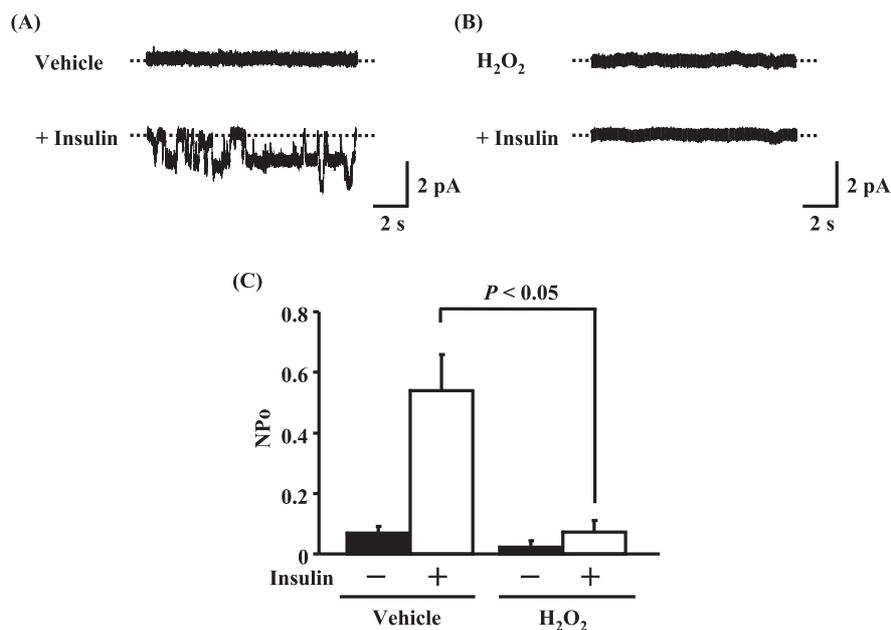


Fig. 3 Effect of H₂O₂ for insulin-induced K_{ATP} channel activities in A10 cells. Single channel-currents were measured by the cell-attached patch-clamp analysis as Fig. 2. The cells were pretreated with (A) vehicle, or (B) 10 μ M H₂O₂ for 5 min and then stimulated with 100 nM insulin for 5 min. (C) K_{ATP} channel activities are shown as NPo. Values are shown as means \pm S.E. ($n=12-19$).

smooth muscle type K_{ATP} channel (18). These data indicate that insulin activates K_{ATP} channel in A10 cells. Treatment of 10 μM H_2O_2 significantly suppressed insulin-induced channel activities (Figs. 3B and 3C).

H₂O₂ has no effects for insulin-induced IRS-1 and Akt phosphorylation in A10 cells

We previously have reported that insulin activates K_{ATP} channels *via* insulin receptor signaling pathway, which contains insulin receptor substrate (IRS) and Akt, in cultured VSMCs (18). To determine the effect of H_2O_2 treatment on insulin receptor signaling pathway in A10 cells, we examined the phosphorylation state of IRS-1 and that downstream molecule Akt using Western blotting analysis (Fig. 4). The cells were pretreated with vehicle or 1-10 μM H_2O_2

for 5 minutes and then stimulated with 100 nM insulin for 5 minutes. Treatment with H_2O_2 had no effect for insulin-stimulated IRS phosphorylation (Figs. 4A and 4B). Similarly with IRS, insulin-stimulated Akt phosphorylation was not changed by treatment with H_2O_2 (Figs. 4A and 4C). From these results, we suggested that H_2O_2 inhibited insulin-induced K_{ATP} channel activation independent of insulin receptor signaling pathway including IRS and Akt.

DISCUSSION

Here we found that 10 μM H_2O_2 increases intercellular ROS and inhibits pinacidil-induced K_{ATP} channel activities in cultured VSMCs. This result was similar with the report using smooth muscle

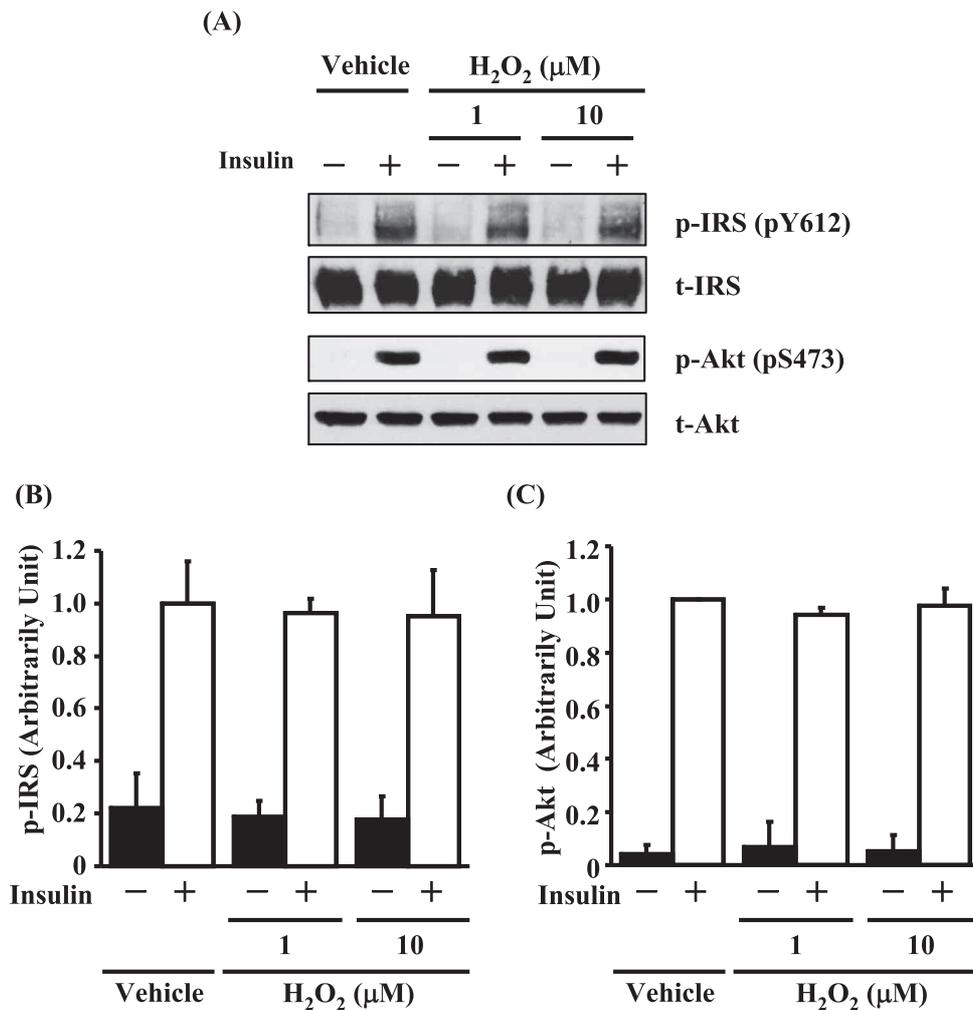


Fig. 4 Effect of H_2O_2 treatment for insulin signaling pathway in A10 cells. The cells were pretreated with the indicated concentration of H_2O_2 for 5 minutes and then stimulated with 100 nM insulin for 5 minutes. Whole cell lysates were subjected to immunoblot analysis with specific antibodies to phosphorylated forms (upper panels) and total (lower panels) insulin receptor substrate (IRS) and protein kinase B (Akt) protein. (B-C) Densitometric analysis for phosphorylated forms of IRS and Akt respectively ($n=5$). Values are shown as means \pm S.E..

K_{ATP} channel expressed HEK cells (26). Furthermore we found that H₂O₂ suppressed insulin-induced K_{ATP} channel activities independent of insulin signaling pathway, including IRS and Akt, in cultured VSMCs. To our knowledge, this is the first report that H₂O₂ inhibits both pinacidil- and insulin-induced K_{ATP} channel activities in VSMCs. The *in vitro* studies have reported that a K_{ATP} channel blocker glibenclamide increases muscle tone and causes depolarization in VSMCs (29, 30). Furthermore, the *in vivo* studies also have showed that glibenclamide significantly increases vascular resistance and decreased arterial diameter (31). In the study for the mouse disrupted the gene encoding Kir6.1, vascular smooth muscle type K_{ATP} channel is critical in the regulation of vascular tonus, especially in the coronary arteries (32). Our data may provide further insight into the relationship among K_{ATP} channel and oxidative stress on cardiovascular diseases.

There are several reports that insulin activates K_{ATP} channels in neuronal cells (33, 34), pancreatic β -cells (35), and skeletal muscle cells (36). K_{ATP} channels are sensitive to intracellular ATP levels: their activity is suppressed by increases in ATP and activated by increases in ADP and other nucleoside diphosphates (37). In previous study in VSMCs, we reported that insulin-activated K_{ATP} channel activation was not mediated the changes of intracellular ATP levels (18). Furthermore we and others have found that insulin-induced K_{ATP} channel activities are mediated by PI3-K in VSMCs and other cells (18, 34-36). PI3-K is main downstream molecule in insulin receptor signaling pathway (14-17). Some reports showed ROS-generating agents, such as angiotensin II, could suppress insulin-receptor signaling pathways (38). We have also previously reported that diamide, a thiol-oxidizing agent, inhibited critical insulin signal transduction component including IRS and Akt (39). In addition, diamide suppressed insulin-induced K_{ATP} channel activities, but did not change pinacidil-induced activities in A10 cells (39). However here we showed 10 μ M H₂O₂ suppressed insulin-induced K_{ATP} channel activities without inhibition of insulin-receptor signaling (Figs. 3 and 4). Yang *et al.* recently reported that H₂O₂ inhibited the activities of VSMC type K_{ATP} channels (Kir6.1/SUR2B) expressed in HEK cells, because H₂O₂ induced conformational changes of the channels as closed state *via* S-glutathionylation of Kir6.1 Cys¹⁷⁶, a cysteine residue in the core domain (40). From these data, some agents of oxidative stress may

induce oxidation to different target molecules for K_{ATP} channel activities in VSMCs.

In conclusion, we have demonstrated that H₂O₂ suppresses insulin-induced K_{ATP} channel activities in vascular smooth muscle A10 cells. Oxidative stress is increased in the diabetic state; hyperglycemia leads to production of H₂O₂ within the cells (41). Our findings suggest that insulin has maybe important roles of the regulation of vascular tonus, which mediates K_{ATP} channel activation, but ROS induces impairment these effects on the vasculature. Our data should provide further insight into the effect of ROS on insulin's action in the vasculature.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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