Original Article

Reactive oxygen species induced by diamide inhibit insulin-induced ATP-sensitive potassium channel activation in cultured vascular smooth muscle cells

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Both insulin resistance and reactive oxygen species (ROS) have been reported to play essential pathophysiological roles in cardiovascular diseases. However, the mechanistic link between ROS and insulin resistance in the vasculature remains unclear. Recently we have shown that insulin causes K_{ATP} channel activation mediated by PI3K in cultured vascular smooth muscle cells (VSMCs). K_{ATP} channel in VSMCs is critical in the regulation of vascular tonus. Here we examined the effects of ROS induced by a thol-oxidizing agent, diamide, on the insulin signalling pathway and K_{ATP} channel activities in cultured VSMCs (A10 cells). Diamide (100 μM) increased intercellular ROS and extracellular signal-regulated kinases (ERK) activitiy. Treatment with 100 μM diamide suppressed significantly insulin-induced IRS and Akt phosphorylation. In addition to IRS and Akt, diamide inhibited insulin receptor auto-phosphorylation. Patch-clamp study showed that diamide suppressed insulin-induced but did not pinacidil-induced K_{ATP} channel activities in A10 cells. From these data, we conclude that ROS inhibit critical insulin signal transduction components including IRS and Akt, and these effects cause down-regulation of insulin's action in the vasculature including K_{ATP} channel activation. This study may contribute to our understanding of mechanisms of insulin resistance-associated cardiovascular disease.

Key Words: Insulin, potassium channel, reactive oxygen species, oxidative stress, smooth muscle cells

INTRODUCTION

Reduction of oxygen by normal cellular metabolism leads to the production of reactive oxygen species (ROS) that include superoxide anion ($^{.}O_2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical. These species are now believed to participate in a variety of cellular signalling mechanisms that transmit transcriptional/translational regulation, cell growth, differentiation, and apoptosis. Accumulating evidence indicates that ROS play important roles in Type 2 diabetes and cardiovascular diseases such as hypertension and atherosclerosis.[1] This seems to be risen from inflammation, glucotoxicity, lipotoxicity and some endocrine mediators.

Insulin exerts important effects on cardiovascular tissue as well as conventional insulin-responsive tissues such as skeletal muscle and adipose tissue. Insulin has important roles in vascular relaxation by activation of PI3K/Akt signaling pathways.[2] We previously have reported that insulin activates K_{ATP} channels mediated by PI3K in cultured VSMCs.[3] Activation of K_{ATP} channels induces membrane hyperpolarization, lowered intracellular calcium concentrations, and vaso-relaxation. In addition to vaso-dilative effect, the opening of K_{ATP} channels has a protective function not only on the cardiovascular systems such as ischemic preconditioning,[4] but also in neuro-adrenal cells in response to vigorous stimuli.[5]

In this study, we examined the effect of ROS for PI3K/ Akt pathway and K_{ATP} channel activities in cultured vascular smooth muscle cell (A10 cells).

MATERIALS AND METHODS

The study was approved by ethics committee of the University of Tokushima for animal studies.

Immunoprecipitation

Embryonic rat thoracic aortic smooth muscle cells from DB1X rat (A10 cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were serum-starved for 1 hour before all experiments. 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). Fluorescence intensities were obtained by microscopy (IX71, Olympus,

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Tokyo, Japan) and analysed with Meta Fluor software (Molecular Devices, Downingtown, PA).

Cell preparation and Western blotting analysis

We examined Western blotting as previous study.[3] After pretreatment with diamide, cells were treated with 100 nM insulin for 5 minutes and solubilized in cold cell lysis buffer. The cell lysates were normalized for protein concentration by using Bicinchoninate (BCA) method (PIERCE, Rockford, IL) and subjected to immunoblotting.

Patch-clamp experiments

Single channel activities were measured using cell-attached patch-clamp recordings as described by our previous study.[3] The resistance of pipettes filled with buffered solution was 7-10 MΩ. The inside of the pipette was voltage-clamped at +50 mV. 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). BIO-PATCH Ver.3.42 software (BIO-LOGIC, Claix, France) was used to analyze recorded data. The channel activities are expressed as NPo, where Po means the open probability and N means the number of the channels active in the patch. The NPo values were determined from recordings lasting longer than 120 seconds.

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 $p < 0.01$ was considered statistically significant.

RESULTS

Diamide increases ERK phosphorylation and ROS generation in A10 cells

It is well known that stimulation of ROS enhanced the activities of ERK. Diamide treatment (1−1000 μM) for 10 minutes increased Thr202/Tyr204 phosphorylation of ERK in A10 cells (Figs. 1A−B). We also checked effect of diamide on ROS generation by using a ROS-reactive fluorescent dye, carboxy-H_2DCFDA (Fig. 1C). Treatment with 100 μM diamide increased significantly ($p < 0.01$) intercellular ROS after 2.5 minutes and kept increasing that for 10 minutes. The effect of 100 μM diamide for intercellular ROS generation was about one third that of 10 μM H_2O_2 (Fig. 1C). These data suggest that 100 μM diamide has effective intensity as oxidative stress in this study.

Diamide inhibits insulin signalling pathway in A10 cells

To determine the effect of diamide treatment on insulin signalling pathway in A10 cells, we firstly examined the for 10 minutes and then stimulated with 100 nM insulin for 5 minutes. Treatment with 100μM diamide suppressed insulin-stimulated IRS phosphorylation (Figs. 2A−B). Similarly with IRS, insulin-stimulated Akt phosphorylation was suppressed by treatment with 100 μM diamide (Figs. 2A and 2C).
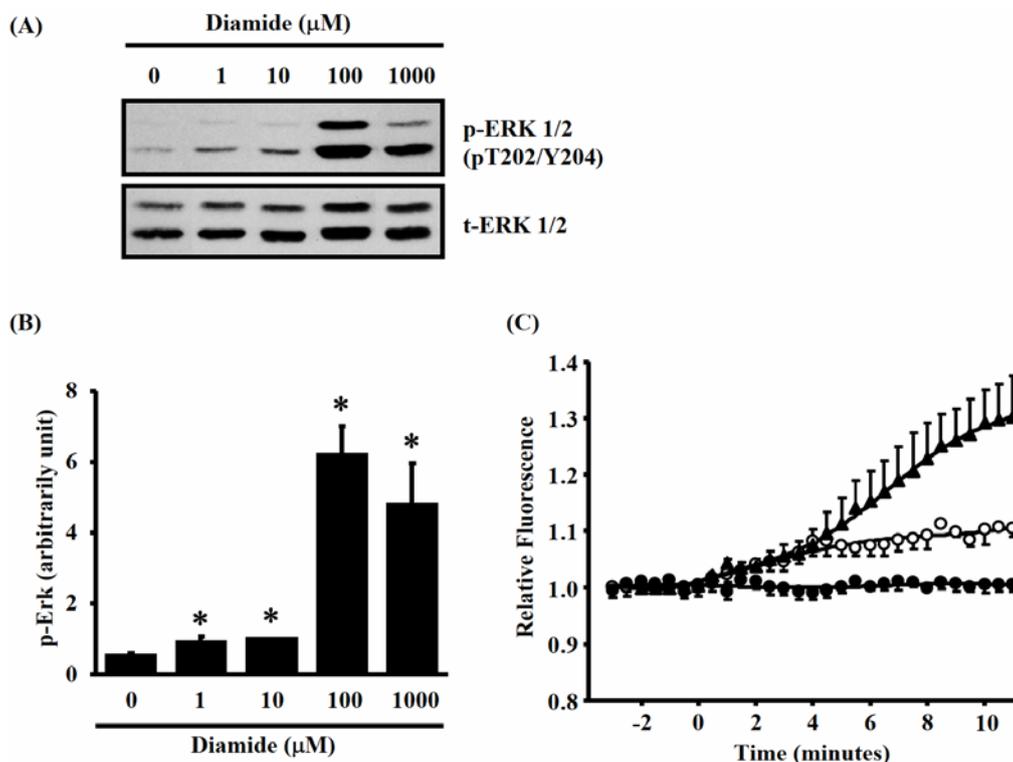

Figure 1. (A) Representative data of reactive oxygen species (ROS)-induced phosphorylated extracellular regulated kinase (ERK) with diamide treatment in A10 cells by using Western blotting. (B) Densitometric analysis for phosphorylated forms of ERK. (n = 4−8). (∗) $p < 0.01$, versus vehicle treatment. (C) Intercellular ROS measurements in A10 cells by using a ROS reactive fluorescence dye. The data indicate relative fluorescence intensity for the following conditions. (●) Vehicle treatment. (○) 100 μM diamide. (▲) 10 μM hydrogen peroxide (H_2O_2). 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.
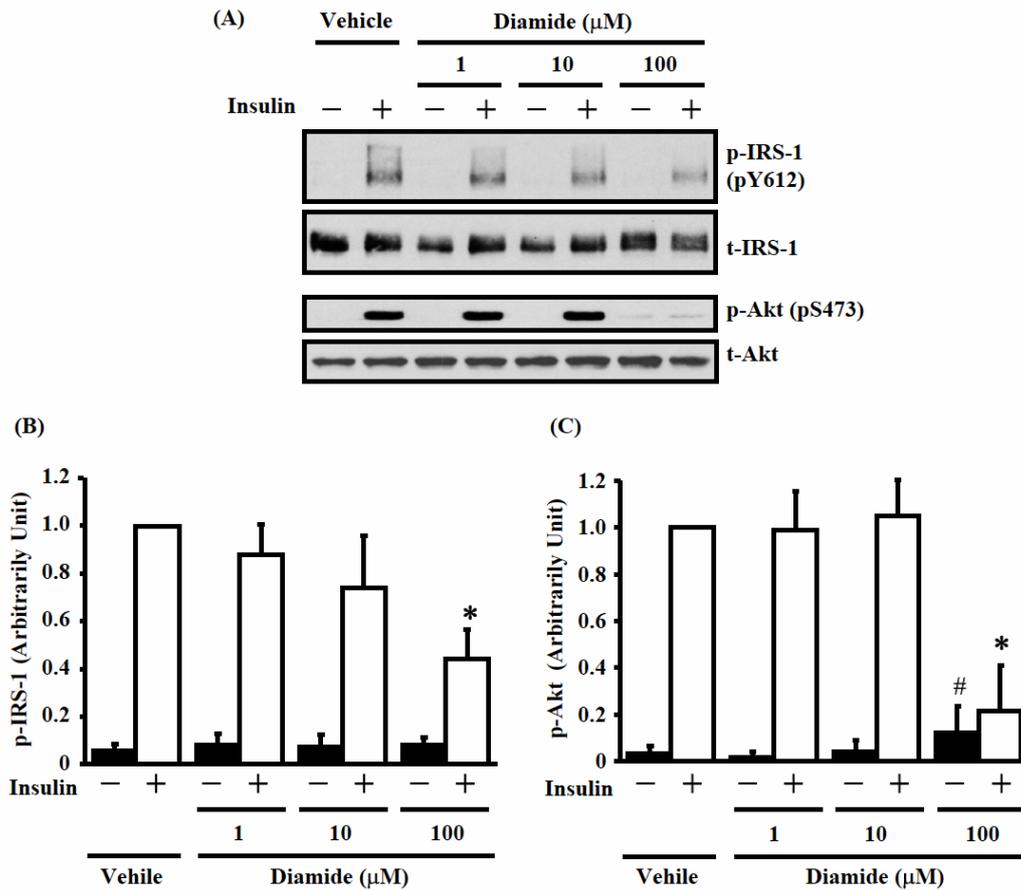
Figure 2. Effect of diamide treatment on insulin signaling pathway in A10 cells. Whole cell lysates were subjected to immunoblot analysis with specific antibodies to phosphorylated forms (upper panels) and total (lower panels) IRS and Akt protein. (B−C) Densitometric analysis for phosphorylated forms of IRS and Akt respectively ($n = 5$). Values are shown as means ± S.D. Significant difference ($p < 0.01$), versus vehicle treatment in (#) absence or (∗) presence of insulin.
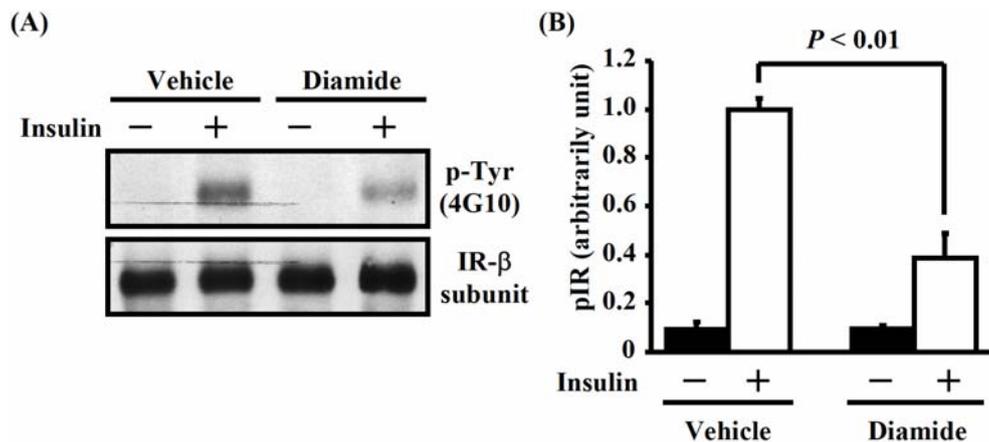

Figure 3. Effect of diamide treatment for insulin receptor (IR) auto-phosphorylation in A10 cells. The cell lysates were immunoprecipitated with anti-IR-β subunit antibody and then subjected to immunoblot analysis with anti-phosphorylated tyrosine (4G10) antibody. (B) Densitometric analysis for tyrosine phosphorylated forms of IR ($n = 3$).

Next, to determine effect of diamide treatment on the upstream of IRS in insulin signaling pathway, we obseved tyrosine phosphorylation of insulin receptor (IR)-β subunit (Fig. 3). The cells were pretreated with vehicle or 100 μM diamide for 10 minutes and then stimulated with 100 nM insulin for 5 minutes. Insulin-stimulated tyrosine phosphorylation of IR was suppressed by treatment with 100 μM diamide (Figure 3). Inhibitory effect of 100 μM diamide for IR phosphorylation (61.4 ± 14.1% inhibition) had about same intensity for IRS in the presence of insulin (Figs. 2B and 3B). From these results, we suggested that diamide suppressed IR and then its downstream IRS and Akt in insulin signalling pathways in A10 cells.
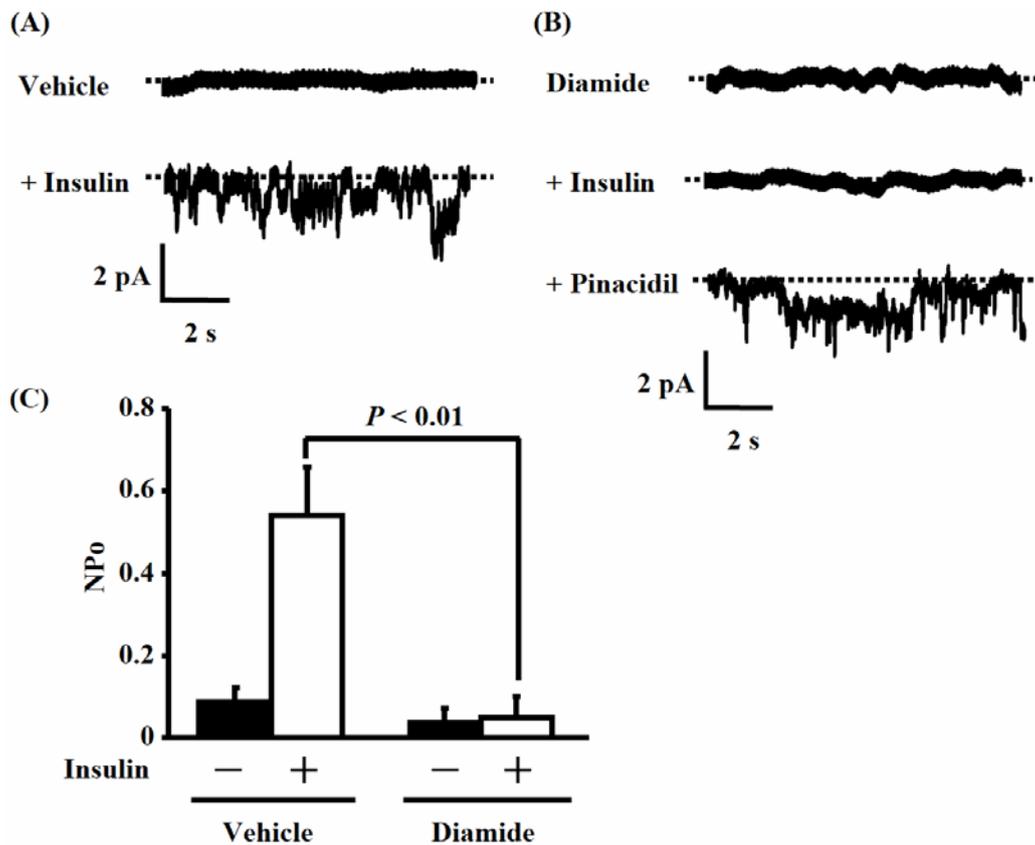
Figure 4. Effect of diamide for insulin-induced K_{ATP} channel activities in A10 cells. Single channel currents were measured by cell-attached patch-clamp assay. The pipette solution was voltage-clamped at +50 mV. The cells were pretreated with (A) vehicle or (B) 100 μM diamide and then stimulated with 100 nM insulin. Pinacidil, a KATP channel opener, increased single channel currents in diamide-treated cells. (C) K_{ATP} channel activities are shown as NPo (where N is the number of channels present in the patch and Po is the single channel open probability). Values are shown as means ± S.E. ($n = 6-19$).

Insulin activates KATP channels in A10 cells

To observe the effect of insulin for the changes in potassium ion transport on the membrane of VSMCs, we directly measured single channel activities by using cell-attached patch-clamp experiments (Fig. 4A). Ion channels were activated 5 minutes after insulin stimulation (Fig. 4A). Insulin-activated currents showed inward rectification and were inhibited significantly by treatment with 3 μmol/L glibenclamide, a K_{ATP} channel blocker (data not shown). These data indicate that insulin activates K_{ATP} channel in A10 cells.

Diamide treatment inhibits insulin-induced K_{ATP} channel activation in A10 cells

We previously have reported that insulin activates K_{ATP} channels via IR–IRS–PI3K pathway in cultured VSMCs.[6] Therefore we observed the effect of ROS on insulin-induced K_{ATP} channel activities in A10 cells (Figs. 4B−C). The cells were pretreated with 100 μM diamide (Fig. 4B) for 10 minutes and then stimulated with 100 nM insulin for 5 minutes. Treatment of 100 μM diamide significantly suppressed insulin-induced channel activities. However pinacidil, a K_{ATP} channel opener, increased channel activities in spite of treatment with diamide (Fig. 4B). These data suggested that diamide-induced ROS had an inhibitory effect for insulin-induced K_{ATP} channel activities but not direct effect for K_{ATP} channels.

DISCUSSION

Here we find that diamide increases intercellular ROS and inhibits insulin-induced K_{ATP} activities in cultured VSMCs. These effects of diamide are caused by inhibition of insulin signaling pathway including IR and its downstream molecules, IRS and Akt. To our knowledge, this is the first report that oxidative stress inhibits insulin's effect, especially K_{ATP} channel activation in VSMCs. Our data may provide further insight into the relationship with PI3K/Akt pathway and oxidative stress.

Emerging evidence also support an important role of ROS in various forms of insulin resistance.[6] One mechanism of insulin desensitization was believed that various so-called stress kinases including mitogen-activated protein kinase (MAPK) family such as ERK are involved. A common denominator for these kinases seems to be their ability to attenuate directly or indirectly the kinase activities of IR and IRS. Here we demonstrated that 100 μM diamide suppressed IR, IRS, and Akt phosphorylation in the presence of insulin (Figs. 2 and 3). Our data are supported by the studies for the effect of oxidative stress on insulin signalling by using angiotensin II (Ang II) and H_2O_2 as oxidative stress in VSMCs.[7,8] Ang II increases production of ROS and then activates ERK in VSMCs.[9] Izawa et al. have reported that insulin-induced IRS and Akt activation are inhibited by Ang II in VSMCs.[7] Similarly with Ang II, H_2O_2 treatment suppresses insulin-induced IRS and Akt activation in VSMCs.[8]

Furthermore H$_2$O$_2$ treatment suppresses insulin-induced IR-β subunit auto-phosphprylation in VSMCs.[8] These are thought to be particularly involved inhibition of receptor binding.[8] We demonstrated that diamide suppressed IR-β subunit auto-phosphorylation in A10 cells (Fig. 3). These data suggested that the inhibitory effects of diamide for insulin signalling pathway might be involved inhibition of receptor binding.

K$_{ATP}$ channel was considered to play an important role in adaptation of vigorous stress by counteracting inward current. There are several reports that insulin activates K$_{ATP}$ channels in neuronal cells,[10,11] pancreatic β-cells,[12] and skeletal muscle cells.[13] 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. In previous study in VSMCs, we report that insulin-activated K$_{ATP}$ channels are not mediated the changes of intracellular ATP levels.[6] We and others have found that insulin-induced K$_{ATP}$ channel activities are mediated by PI3K in VSMCs and other cells.[3,11-13] PI3K is main downstream molecule in insulin signalling pathway.[2] Recently, epigalocathechin-3-gallate and ginsenosides Re also act on this pathway.[14,15] From these data, we suggested that the inhibitory effect of diamide for K$_{ATP}$ channel activities was also involved inhibition the PI3K/Akt pathway.

In conclusion, we have demonstrated that diamide suppress insulin-induced K$_{ATP}$ channel activities in cultured VSMCs. Our findings suggest that insulin has important roles of the regulation of vascular tonus, which mediates K$_{ATP}$ channel activation, but ROS induces impairment of these effects. Our data should provide further insight into the effect of ROS on PI3K/Akt pathway in the vasculature.

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