Inhibition of insulin-stimulated hydrogen peroxide production prevents stimulation of sodium transport in A6 cell monolayers

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Markadieu N, Crutzen R, Boom A, Erneux C, Beauwens R. Inhibition of insulin-stimulated hydrogen peroxide production prevents stimulation of sodium transport in A6 cell monolayers. Am J Physiol Renal Physiol 296: F1428–F1438, 2009. First published March 18, 2009; doi:10.1152/ajprenal.90397.2008.—Insulin-stimulated sodium transport across A6 cell (derived from amphibian distal nephron) monolayers involves the activation of a phosphatidylinositol (PI) 3-kinase. We previously demonstrated that exogenous addition of H2O2 to the incubation medium of A6 cell monolayers provokes an increase in PI 3-kinase activity and a subsequent rise in sodium transport (Markadieu N, Crutzen R, Blero D, Erneux C, Beauwens R. Am J Physiol Renal Physiol 288: F1201–F1212, 2005). We therefore questioned whether insulin would produce an intracellular burst of H2O2 leading to PI 3-kinase activation and subsequent increase in sodium transport. An acute production of reactive oxygen species (ROS) in A6 cells incubated with the oxidation-sensitive fluorescent probe 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate was already detected after 2 min of insulin stimulation. This fluorescent signal and the increase in sodium transport were completely inhibited in monolayers incubated with pegylated catalase, indicating that H2O2 is the main intracellular ROS produced upon insulin stimulation. Similarly, preincubation of monolayers with different chelators of either superoxide (O2•−; nitro blue tetrazolium, 100 μM) or H2O2 (50 μM ebselen), or blockers of NADPH oxidase (Nox) enzymes (diphenyleneiodonium, 5 μM; phenylarsine oxide, 1 μM and plumbagin, 30 μM) prevented both insulin-stimulated H2O2 production and insulin-stimulated sodium transport. Furthermore, diphenyleneiodonium pretreatment inhibited the recruitment of the p85 PI 3-kinase regulatory subunit in an anti-phosphotyrosine immunoprecipitate in insulin-stimulated cells. In contrast, PI-103, an inhibitor of class IA PI 3-kinase, inhibited insulin-stimulated sodium transport but did not significantly reduce insulin-stimulated H2O2 production. Taken together, our data suggest that insulin induces an acute burst of H2O2 production which participates in an increase in phosphatidylinositol 3,4,5-trisphosphate production and subsequently stimulation of sodium transport.

ENaC; H2O2; ROS; dichlorodihydrofluorescein; PI 3-kinase

INSULIN AND ALDOSTERONE ARE the major hormones increasing sodium reabsorption in the distal nephron, thereby contributing to the control of extracellular fluid volume and blood pressure. The sodium ion enters into the cell through the epithelial sodium channels (ENaCs) and is then extruded at the basolateral membrane by the Na⁺-K⁺-ATPase (49). The rate-limiting step of this vectorial Na⁺ transport is mainly attributed to the activity of ENaCs (10, 43). In A6 cell (derived from Xenopus laevis kidney) monolayers, insulin has been well documented to increase sodium transport (6, 39) by increasing the activity of a PI 3-kinase (5), which probably belongs to class IA isoform as recently demonstrated in murine cell monolayers (61). The latter enzyme generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) within the basolateral membrane (7, 37), which then diffuses within the inner leaflet of plasma membrane across the tight junctions to the apical membrane of A6 cell monolayers (9). Stimulation of sodium transport can be then explained by direct interactions between ENaCs and PIP3 (32, 33, 44, 45), giving rise to an increase in open probability of these channels (58), a decrease in ENaC retrieval (56), or an increase in ENaC insertion (9, 21). PI 3-kinase is therefore a key mediator of hormone-stimulated sodium transport (5, 8) as any increase in PI 3-kinase activity whatever the stimulatory agents, leads to a proportional increase in sodium transport across A6 cell monolayers (38). In addition to various PI 3-kinase agonists, exogenous addition of H2O2 (46, 55) also increases sodium transport and mimics the early response of insulin-stimulated sodium transport (38). PI 3-kinase activity is increased within 2 min of H2O2 stimulation, and pretreatment of A6 cell monolayers with LY-294002 (a PI 3-kinase inhibitor) (59) prevents both PIP3 generation and any increase in sodium transport. The striking similarity between insulin and H2O2 stimulation of sodium transport suggests that insulin induces the generation of H2O2 within A6 cell monolayers, as observed in various different cell types stimulated not only with insulin (16, 17, 40) but with other growth factors as well (2, 3). For instance in NIH 3T3 adipocytes, insulin activates the NADPH oxidase (Nox) Nox4 by an unknown mechanism, leading to a production of superoxide anion which is converted into H2O2 either spontaneously or by superoxide dismutase (SOD) (35). The level of H2O2 generated as an intracellular signal post-insulin receptor occupancy is low and transient compared with the toxic effects of reactive oxygen species (ROS), encountered with high and sustained productions (12).

The aim of this study was to detect, first, an intracellular production of H2O2 following insulin stimulation of A6 cell monolayers, second, to investigate the effect of different chelators or inhibitors of ROS production on insulin-stimulated sodium transport, third, to investigate the effect of PI-103 (24), a class IA PI 3-kinase inhibitor on sodium transport, and, finally, to possibly establish a link among H2O2 cell production, PI 3-kinase activity, and sodium transport.

EXPERIMENTAL PROCEDURES

Materials. Porcine insulin, nitro blue tetrazolium (NBT), ebselen, diphenyleneiodonium (DPI), phenylarsine oxide (PAO), plumbagin, polyethylene glycol-conjugated catalase (catalase-PEG), and protein A-Sepharose were purchased from Sigma (St. Louis, MO). Mowiol was purchased from Merck Biosciences (Nottingham, UK). The PI

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3-kinase inhibitor PI-103 was purchased from Alexis (San Diego, CA). The oxidation-sensitive probe 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCF-DA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA). Protease inhibitor tablets were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Millicell inserts were purchased from Millipore (Bedford, MA), and Transwell inserts were purchased from Corning Costar (Cambridge, MA). Tissue culture flasks were purchased from Sarstedt (Nümbrecht, Germany). Electrophysiological measurements were realized by use of an epithelial voltohmometer (EVom) from World Precision Instruments, (Sarasota, FL). The current evaluated by this technique is called an epithelial open-circuit Na$^+$ current. Antibodies to the PI 3-kinase p85 subunit and to phosphotyrosine were purchased from Upstate (Lake Placid, NY). Thr 308 and Ser 473 Phospho-PKB polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Blotted reaction with catalase-PEG prevented any detection of fluorescence present in each “maximum picture” file (512 × 512 “max pixels”). The integration with the ImageJ software quantitates the amount of fluorescence present in each “maximum picture” file. These amounts are reported to the amount present in the control paired monolayers to express the relative content of fluorescence.

Fluorescence of dichlorofluorescein (DCF), i.e., oxidized H$_2$DCF, was observed mainly attributed to a H$_2$O$_2$ production, as preincubation with catalase-PEG prevented any detection of fluorescence induced by insulin (36).

Western blot analysis. Immunodetection of phospho-PKB on Thr 308 and Ser 473 residues was performed on extracts of cells grown for a minimum of 10 days on Transwell inserts preincubated in the presence or absence of different inhibitors of ROS (100 μM NBT, 30 min; 50 μM ebense, 30 min; 5 μM DPI, 60 min; 1 μM PAO, 30 min; 30 μM plumbagin, 60 min) and then stimulated or not with insulin (100 nM, basolateral, 5 min). Cells were scraped into ice-cold lysis buffer (150 mM KCl, 10 mM Tris-HCl, 2 mM EDTA, 1 mM sodium orthovanadate, 100 μM NaF, 10 nM okadaic acid, 0.5% NP-40, 0.1% β-mercaptoethanol, 300 μg/ml pefabloc, and 10 μg/ml leupeptin, pH: 7.4) at 4°C. Cell extracts were maintained at 4°C for 1 h and centrifuged (13,000 rpm; 20 min); the pellet was discarded. Proteins were separated onto 9% polyacrylamide gels. Nitrocellulose membranes were incubated for 1 h in a solution of Odyssey blocking buffer/PBS (1:1), incubated for 2 h at room temperature with anti-phospho-PKB antibodies prepared in a solution of Odyssey blocking buffer/0.1% Tween 20 PBS (1:1), and then incubated for 40 min in the dark with infrared-labeled secondary antibodies (rabbit 680 nm) prepared in a solution of Odyssey blocking buffer/0.1% Tween 20 PBS (1:1). After washing in 0.1% Tween 20 PBS, membranes were analyzed using the Odyssey Infrared Imaging system.

Immunoprecipitation. Immunoprecipitation was carried as previously described (37). Briefly, cell lysates were incubated overnight with antibodies to phosphotyrosine and subsequently with protein A-Sepharose for 2 h. The beads were washed three times in PBS and collected by centrifugation. After boiling, samples were loaded onto 9% gel polyacrylamide, and membranes were probed with anti-p85 antibodies. Immunodetection was then carried out as described in the previous section, using the Odyssey technique.

Statistics. Data are presented as means ± SE. Paired t-tests were used to compare the experimental vs. control group.

RESULTS

Insulin stimulation elicits an acute concentration-dependent production of H$_2$O$_2$ in A6 cell monolayers. The dye CM-H$_2$DCF-DA is a redox indicator that is trapped intracellularly after cleavage into 2',7'-dichlorodihydrofluorescein (H$_2$DCF) by cellular esterases. Oxidation of H$_2$DCF into DCF by ROS generates a fluorescent signal which can be detected by confocal microscopy (2, 36).

In response to insulin stimulation, A6 cell monolayers generate ROS intracellularly (Fig. 1, A and B). Cells loaded for 30 min with the probe CM-H$_2$DCF-DA produce a fluorescent signal when monolayers are stimulated by insulin (100 nM). While unstimulated cells (control) show a very weak fluores-
cence, 2 min of insulin stimulation are sufficient to induce a significant increase in intracellular ROS production. The intensity of the fluorescence signal is maximal after 5 min of insulin stimulation (Fig. 1B) and then decreases but remains higher than at basal condition up to 30 min of insulin stimulation (Fig. 1B). The intensity of fluorescence detected is dependent on insulin concentration. Monolayers loaded with CM-H2DCF-DA for 30 min and stimulated with insulin for the last 5 min of incubation show a higher signal when stimulated with 100 nM compared with 20 or 10 nM insulin (Fig. 2, A and B). Monolayers stimulated with H2O2 and those pretreated with a permeant form of catalase (catalase-PEG) were used, respectively, as positive and negative controls. While addition of H2O2 (1 mM, 1 min) to the apical bathing medium elicits an increase in fluorescence, overnight pretreatment of monolayers with catalase-PEG (15,000 IU/ml, basolateral) prevents any signal de-

Fig. 1. Effect of insulin incubation period on the detection of oxidized 2′,7′-dichlorodihydrofluorescein (H2DCF) fluorescence in A6 cell monolayers. A: maximum picture files constituted of the z-projection of each maximum pixel of each set of 21 optical sections (x-y axis, 0.5-μm intervals from apical to basal side). Monolayers were incubated for 30 min with 5 μM 5,6-chloromethyl (CM)-H2DCF-diacetate (DA) and 100 nM insulin for various time periods before the end of CM-H2DCF-DA incubation. a: Untreated monolayer incubated with only CM-H2DCF-DA (control). b: 2-min insulin stimulation. c: 5 min. d: 10 min. e: 15 min. f: 30 min. B: quantitation of the amount of oxidized H2DCF fluorescence. The amount of fluorescence was expressed relative to that of the paired control monolayer. Values are means ± SE; n = 3. *P < 0.05 vs. control.
tection in insulin- or H₂O₂-stimulated monolayers (Fig. 2, A and B). Moreover, the fluorescence of oxidized H₂DCF is dependent on H₂O₂ concentration (Fig. 3) validating the method used for its detection. These experiments indicate that fluorescence of oxidized H₂DCF detected is mainly attributed to an intracellular H₂O₂ production.

Pretreatment of monolayers with catalase inhibits insulin- and H₂O₂-stimulated sodium transport. The enzyme catalase, mainly found in peroxisomes, is well known to convert H₂O₂ into H₂O and O₂ (49). Monolayers were treated with a derivative of catalase, catalase-PEG, which can diffuse across the cell membrane. Overnight pretreatment with 15,000 IU/ml catalase-PEG prevented insulin and H₂O₂ stimulation of sodium transport (Fig. 4). Catalase-PEG was added to the basolateral medium, and at t = 0 min monolayers were or not stimulated by insulin (100 nM, basolateral) or by H₂O₂ (1 mM, apical). These experiments stress an important early role of an intracellular H₂O₂ production in the stimulation of sodium transport.

Fig. 2. Effect of different insulin concentrations, H₂O₂ and catalase pretreatment on the detection of oxidized H₂DCF fluorescence in A6 cell monolayers. A: maximum picture files constituted of the z-projection of each maximum pixel of each set of 21 optical sections (x-y axis, 0.5-μm intervals from apical to basal side). Monolayers were pretreated overnight with 15,000 IU/ml catalase-polyethylene glycol (PEG), incubated for 30 min with 5 μM 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA), and for the last minutes of incubation stimulated with different concentrations of insulin (5 min) or 1 mM H₂O₂ (1 min). a: Untreated monolayer incubated with only CM-H₂DCF-DA (control). b: Monolayer stimulated with 10 nM insulin. c: 20 nM insulin. d: 100 nM insulin. e: Monolayer stimulated with H₂O₂ (positive control). f: Incubation with catalase-polyethylene glycol (PEG) followed by H₂O₂ stimulation (negative control). g: Incubation with catalase-PEG followed by 100 nM insulin stimulation (negative control). B: quantitation of the amount of oxidized H₂DCF fluorescence. The amount of fluorescence was expressed relative to that of the paired control monolayer. Values are means ± SE; n = 3. *P < 0.05 vs. control. §P < 0.05 vs. 1 mM H₂O₂. #P < 0.05 vs. 100 nM insulin.
Pretreatment of monolayers with different inhibitors of ROS production decreases oxidized H$_2$DCF fluorescence and thereby inhibits insulin-stimulated production of H$_2$O$_2$. The treatment of H$_2$DCF-loaded monolayers with different inhibitors or chelators of ROS prevented the insulin-stimulated emission of fluorescence from oxidized H$_2$DCF (Fig. 5). While monolayers stimulated with insulin for 5 min generate an intense fluorescent signal, cells treated bilaterally with either inhibitors of NADPH oxidase (5 μM DPI, 60 min; 30 μM plumbagin, 60 min; or 1 μM PAO, 30 min) or with either a chelator of H$_2$O$_2$ (50 μM ebselen, 30 min) or a chelator of O$_2^•$ (100 μM NBT, 30 min) show a decreased fluorescence. Although all the inhibitors decrease significantly the oxidized H$_2$DCF fluorescence, NBT and PAO are slightly less potent in decreasing the fluorescence intensity produced by insulin (paired t-test, *P* < 0.05). Monolayers preincubated with DPI (5 μM, bilateral) for 1 h do not show any fluorescence when cells are then stimulated by insulin.

The effect of these NADPH oxidase inhibitors highly suggests that the source of ROS in A6 cells exposed to insulin is O$_2^•$ (subsequently transformed into H$_2$O$_2$), most probably generated by some NADPH oxidase activity. Pretreatment of monolayers with different inhibitors of ROS production inhibits insulin-stimulated sodium transport. The addition of inhibitors of ROS to A6 cell monolayers significantly diminishes the increase in sodium transport induced by insulin stimulation (Fig. 6). While pretreatment of cells for 1 h with DPI (5 μM), plumbagin (30 μM) or for 30 min with ebselen (50 μM) prevents the increase in sodium transport (Fig. 6, A–C), preincubation for 30 min with NBT (1 μM) or with PAO (1 μM) slightly reduces the insulin-stimulated sodium transport (Figs. 6, D and E). This is in keeping with the fact that the fluorescence intensity was not totally abolished when cells are pretreated with NBT or PAO. These experiments using various unrelated inhibitors of ROS oxidation establish a correlation with the magnitude of ROS generated (fluorescence intensity due to oxidized H$_2$DCF) and the increase in sodium transport induced by insulin (Table 1).

Pretreatment of monolayers with inhibitor of PI 3-kinase PI-103 prevents insulin-stimulated sodium transport. To get more insight into the PI 3-kinase dependence of insulin- and H$_2$O$_2$-stimulated sodium transport, A6 cells were treated with 1 μM PI-103, which is an inhibitor of the p110-α isoform of class IA PI 3-kinase (24). Pretreatment of monolayers with PI-103 prevents the stimulation of sodium transport. The inhibitor was added bilaterally and at 0 min monolayers were or were not stimulated by either insulin (100 nM, basalateral) or H$_2$O$_2$ (1 mM, apical) (Fig. 7). Activation of a class IA PI 3-kinase is therefore required for insulin- and H$_2$O$_2$-stimulated sodium transport in A6 cells monolayers. These results confirm recent data obtained by Wang et al. (61) for insulin- and

![Graph](http://ajprenal.physiology.org/DownloadedFrom)
aldosterone-stimulated sodium transport in mpkCCD monolayers.

Pretreatment of monolayers with PI-103 does not prevent insulin-stimulated production of H$_2$O$_2$. To begin to dissect the intracellular cascade of insulin action from H$_2$O$_2$ production to the stimulation of sodium transport, i.e., to get insight into whether the H$_2$O$_2$ target is before or after class IA PI 3-kinase activation, A6 cell monolayers were pretreated with PI-103 before H$_2$DCF loading and insulin or H$_2$O$_2$ addition (Fig. 8). While H$_2$DCF-loaded monolayers stimulated with 100 nM insulin produce a fluorescent signal of high intensity, cells pretreated for 30 min with PI-103 (bilateral, 1 µM), loaded with H$_2$DCF and stimulated with insulin do not show a significant decrease in fluorescence (paired $t$-test, $P > 0.05$). However, H$_2$O$_2$-stimulated monolayers show a significant decrease in oxidized H$_2$DCF fluorescence (paired $t$-test, $P < 0.05$) when previously treated with 1 µM PI-103.

Blocking intracellular H$_2$O$_2$ production inhibits insulin-stimulated recruitment of PI 3-kinase regulatory subunit p85 in an anti-phosphotyrosine complex. As H$_2$O$_2$ produced by NADPH oxidase has been shown to be required to enhance PI 3-kinase activity in insulin-stimulated 3T3-L1 adipocytes by inhibition...
Three effects consequent to insulin stimulation in A6 monolayers are summarized: H₂O₂ production at 5 min (amount of oxidized H₂DCF fluorescence)

The relative value obtained after insulin stimulation (100 nM) alone or following preincubation with of each inhibitor is presented. The effect of each inhibitor was compared with that of insulin alone. *P < 0.05 (paired t-test; n = 3 or 4).

Fig. 7. Effect of phosphoinositol (PI)-103 treatment on insulin- and H₂O₂-stimulated sodium transport. Monolayers were pretreated for 30 min with PI-103 (1 μM, bilateral) and at t = 0 min, stimulated with 100 nM insulin or 1 mM H₂O₂. Values are means ± SE; n = 3.

Table 1. Summary of the relative effects of the various inhibitors tested

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Oxidized H₂DCF Fluorescence</th>
<th>Sodium Transport</th>
<th>Phosphorylation of PKB (Thr 308)</th>
<th>Phosphorylation of PKB (Ser 473)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Insulin</td>
<td>325 ± 49</td>
<td>421 ± 24</td>
<td>321 ± 6</td>
<td>233 ± 2</td>
</tr>
<tr>
<td>DPI + insulin</td>
<td>82 ± 11*</td>
<td>89 ± 11*</td>
<td>158 ± 18*</td>
<td>100 ± 9*</td>
</tr>
<tr>
<td>Plumbagin + insulin</td>
<td>135 ± 13*</td>
<td>139 ± 32*</td>
<td>126 ± 25*</td>
<td>110 ± 5*</td>
</tr>
<tr>
<td>Ebselen + insulin</td>
<td>106 ± 14*</td>
<td>105 ± 18*</td>
<td>301 ± 20</td>
<td>189 ± 19</td>
</tr>
<tr>
<td>NBT + insulin</td>
<td>143 ± 24*</td>
<td>215 ± 29*</td>
<td>191 ± 13*</td>
<td>127 ± 9*</td>
</tr>
<tr>
<td>PAO + insulin</td>
<td>146 ± 27*</td>
<td>242 ± 30*</td>
<td>343 ± 12</td>
<td>228 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE. H₂DCF, 2',7'-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium; NBT, nitro blue tetrazolium; PAO, phenylarsine oxide.

Pretreatment of monolayers with different inhibitors of ROS production decreases PKB phosphorylation in response to insulin. As the PI 3-kinase pathway is associated with an increase in PKB activity, the phosphorylation of PKB was used as a readout of an increase in the PIP₃ level in A6 cell monolayers stimulated or not with insulin (100 nM, basolateral, 5 min) (37). While insulin-stimulated monolayers display a high level of phospho-PKB on both Thr 308 and Ser 473, phosphorylation is quite weak for unstimulated cells. The intensity of phosphorylated PKB was compared between insulin-stimulated monolayers and cells preincubated with different ROS inhibitors before insulin stimulation (Fig. 10, A and B). Pretreatment with DPI (5 μM, 60 min), plumbagin (30 μM, 60 min), or NBT (100 μM, 30 min) decreases (paired t-test, #P < 0.05) the phosphorylation of PKB. This was not observed with ebselen (50 μM, 30 min) or PAO (1 μM, 30 min) in response to insulin (on both phospho-PKB residues). These results are in agreement with our previous fluorescence data and the effect on sodium transport observed after inhibitors treatment and insulin stimulation (Table 1).

**DISCUSSION**

Oxidative cell stress induces an increase in ROS, and this has been well documented in several pathological conditions including cancer, diabetes mellitus, atherosclerosis, aging, and neurodegenerative diseases (4, 25). In these cases, a sustained high level of ROS is produced. More recently, a signaling role for increased intracellular ROS has been demonstrated in various cell types following growth factor stimulation and insulin (2, 3, 16, 35, 42). These increases are more transient and never reach such comparably high levels (12). Although the insulinomimetic action of H₂O₂ has been already demonstrated in the 1907s (40, 41), only the recent elegant studies of the Goldstein group (16, 17, 34) have highlighted the role of H₂O₂ in the transduction of insulin signaling, at least in the model of adipocytes. Insulin action is initiated by its binding to the α-subunit of the plasma membrane insulin receptor (IR), of tyrosine phosphatases (34, 35), we studied the effect of ROS production on the recruitment of the PI 3-kinase-regulatory subunit, p85, in an anti-phosphotyrosine complex. Two minutes of insulin stimulation are required to increase the recruitment of p85. A pretreatment of monolayers with the NADPH inhibitor DPI prevents the increase in recruitment of p85 in anti-phosphotyrosine immunoprecipitates (Fig. 9). ROS and H₂O₂ generated by NADPH oxidase are therefore required to recruit p85 in an anti-phosphotyrosine complex, a prerequisite in the activation of PI 3-kinase and stimulation of sodium transport.

![Figure 7](http://ajprenal.physiology.org/)

**Fig. 8.** Effect of PI-103 treatment on oxidized H₂DCF fluorescence in insulin- and H₂O₂-stimulated A6 cell monolayers. Monolayers were incubated for 30 min with 5 μM CM-H₂DCF-DA and treated with PI-103 (1 μM, bilateral) and, for the last 5 min of this incubation period, stimulated with 100 nM insulin or 1 mM H₂O₂. The amount of oxidized H₂DCF fluorescence was quantitated and reported to the amount present in the paired control monolayer. Values are means ± SE; n = 3. *P < 0.05 vs. control. §P < 0.05 vs. 1 mM H₂O₂.
whose β-subunit then becomes phosphorylated on intracellular tyrosine residues; this stimulates its tyrosine kinase activity and allows the recruitment of adaptor proteins like the insulin receptor substrate (IRS) and Shc. These adaptors then become phosphorylated on tyrosine residues, allowing the docking of the p85-regulatory subunit of class IA PI 3-kinase. Thus the catalytic p110 subunit generates PIP3 within the inner leaflet of the plasma membrane. It has been demonstrated that activation of PI 3-kinase is required for GLUT4 translocation in the adipocyte (33) as well as for activation of the ENaC in distal nephron models (45, 61), including A6 cell monolayers (5, 37, 58). As we previously observed that exogenous addition of H2O2 reproduces the early natriferic action of insulin in A6 cell monolayers, we decided to investigate whether insulin also induces a burst of ROS in A6 cell monolayers. We show that A6 cell monolayers stimulated by insulin elicit an acute production of ROS, which is maximal after 5 min and then decreases, although remaining sustained over the basal condition up to at least 30 min after insulin addition. The method was validated with a positive and a negative control either by the addition of H2O2 to the incubation media for 1 min or by preincubation of the monolayer overnight with catalase-PEG, which completely prevented ROS generation 5 min after insulin addition (i.e., at its maximum) or 1 min after exogenous H2O2. In both instances, catalase-PEG preincubation inhibited the increase in sodium transport. This indicates that H2O2 accounts for the major part of the intracellular ROS produced in these conditions.

In separate sets of experiments, the dependence on the concentration of insulin and H2O2 was studied for its effect on intracellular H2O2 production. A concentration-dependent increase in intracellular H2O2 was observed in both instances, reminiscent of the concentration dependence of the natriferic effect. Therefore, intracellular ROS production might be an intermediary step of the intracellular events leading to the increase in sodium transport. Whether intracellular H2O2 production is required in the intracellular cascade triggered by insulin in A6 cells was actually the main focus of this study. In addition, as all agents increasing the PIP3 level increase sodium transport across A6 monolayers (37), immunodetection of phospho-PKB was used as a readout for detection of PIP3 in response to insulin.

First, chelators of the superoxide anion and H2O2 were studied. Preincubation with ebselen, a H2O2 chelator, nearly completely abolished H2O2 generation and the increase in

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Fig. 9. Effect of DPI, an inhibitor of Nox enzymes, on insulin-stimulated docking of p85 at the plasma membrane. The presence of the PI 3-kinase p85 subunit in anti-phosphotyrosine (pY) immunoprecipitates was evaluated by Western blotting. A6 cell extracts from monolayers incubated or not with DPI and insulin (2 min) were immunoprecipitated with anti-pY and then immunoblotted with anti-p85. From left to right: lane 1, recruitment of p85 by pY in untreated A6 cell monolayer (control); lane 2, in insulin-stimulated monolayer; lane 3, in DPI-pretreated monolayer; lane 4, in monolayer incubated with DPI before insulin stimulation.

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Fig. 10. Effect of ROS inhibitors on the phosphorylation of PKB induced by insulin. Phospho-PKB (Thr 308 (A) and Ser 473 (B)) was immunodetected by Western blotting in A6 cell extracts from monolayers pretreated with different ROS inhibitors (DPI, plumbagin, ebselen, NBT, PAO) and stimulated with insulin (5 min). The amount of phospho-PKB was quantitated and reported to the amount present in the control extract. Values are means ± SE; n = 3. *P < 0.05 vs. control. #P < 0.05 vs. 100 nM insulin.
sodium transport induced by insulin. Yet PKB phosphorylation was not significantly reduced after 5-min exposure to insulin. A possible explanation would be that ebselen did not prevent a short-lived burst of H$_2$O$_2$ leaving, at 5 min, PKB phosphorylated to a level still comparable to that observed with insulin. Preincubation with NBT, an O$_2^-$ chelator, reduced each effect nearly to the same extent (Table 1). This suggests a competition for the superoxide anion between the oxidation-sensitive dye H$_2$DCF and SOD. Thus the superoxide anion could be generated first, transformed into H$_2$O$_2$ by intracellular SOD, and then eventually either into other ROS or into H$_2$O by peroxisomal catalase (31, 50).

Other inhibitors tested were chosen to try to understand the mechanism of H$_2$O$_2$ production after exposure to insulin. In theory, an oxidative burst could be result from enhanced ROS produced by the mitochondrial enzymes of the oxidative phosphorylation cascade, by the xanthine-xanthine oxidase system, and overall by activation of one of the Nox-dual oxidase (Duox) isoenzymes. All Nox-Duox family members are transmembrane proteins that transport electrons across biological membrane, reducing oxygen to the superoxide anion (Nox1-5) (4, 23, 27), which in the Duox enzymes is immediately converted into H$_2$O$_2$ extracellularly. Thus, to test the possible involvement of the Nox isoenzyme, we tested a general and potent inhibitor of all Nox-Duox enzymes, DPI, which at low concentration (5 μM), completely abolished ROS generation, phosphorylation of PKB, and the natriferic action of insulin in A6 cell monolayers. This highly supports the implication of some Nox-Duox isoenzyme in the action of insulin. Although less specific, PAO, an inhibitor of the oxidase component of Nox-Duox (18), also inhibited ROS generation in A6 cell monolayers and greatly slowed down the increase in sodium transport induced by insulin. Phosphorylation of PKB at 5 min was, however, not significantly affected. Again, whether PKB phosphorylation decreased faster than with insulin alone was not investigated here. Interestingly, PAO is known to inhibit insulin-activated glucose transport in 3T3-L1 adipocytes (13).

Nox enzymes are mostly inactive under basal conditions, although this has been questioned for Nox4 (27). However, in the adipocyte model, Mahadev et al. (34) demonstrated unequivocally that insulin activates Nox4, even though the mechanism of activation is still obscure. Activation by intracellular calcium has been reported for Nox5 (53) and the Duox enzymes (19, 20, 47), which contain an EF-hand motif. However, there is no general agreement on whether intracellular Ca$^{2+}$ concentration actually enhances or diminishes Na$^+$ transport activity in A6 cells monolayer, and the possible involvement of Nox5 or the Duox isoenzymes was not considered. First described, Nox2, the phagocyte homolog, produces large amounts of O$_2^-$ as a host defense mechanism for killing microbes (30). Like Nox1 and Nox3, Nox2 requires the assembly of several cytoplasmic subunits to the membrane for its activation, among p47-phox and the small GTPase Rac (22).

Apocynin, which is an inhibitor of p47-phox translocation (57), does not alter oxidized H$_2$DCF fluorescence or sodium transport stimulated by insulin (data not shown), rendering Nox1-3 less likely. Furthermore, plumbagin, claimed to be specific for Nox4 (11), strongly inhibits ROS generation, phosphorylation of PKB, and the increase in sodium transport induced by insulin in A6 monolayers. This points toward Nox4 as the possible source of increased superoxide generation under the influence of insulin, as in adipocytes, but this important question remains to be established. Nox4, earlier called Renox, was first isolated from the kidney (14, 15) but was also detected later in many different cells, including endothelial cells, vascular smooth muscle cells (1), fibroblasts (48), adipocytes (35), and osteoclasts (52). Its activation by insulin stimulation, has been firmly demonstrated (16, 17, 34–36), although the mechanism remains unknown. In A6 cell monolayers, activation of some Nox by insulin relies on the effect of three structurally and functionally unrelated Nox inhibitors. They all inhibited ROS generation, the increase in sodium transport, and the phosphorylation of PKB induced by insulin, except for ebselen and PAO for this last effect. However, the time course of PKB phosphorylation was not systematically investigated for each inhibitor, as this was not the main focus of our study. The correlation in inhibition observed for each drug is nevertheless impressive (Table 1).

Regardless of the source of H$_2$O$_2$ generation, its precise target(s) is the next obvious question. Preincubation of A6 monolayers with PI-103, an inhibitor of p110α of class IA PI 3-kinase, before insulin stimulation does not prevent the intracellular generation of H$_2$O$_2$, while it totally inhibited the increase in sodium transport. This suggests that H$_2$O$_2$ production lies before and probably contributes to activation of PI 3-kinase. An amplification loop with PI 3-kinase secondarily increasing Nox4 activity has been suggested in endothelial cells (54). This possibility cannot be excluded here, as PI-103 slightly although significantly reduced the fluorescent signal induced by exogenous H$_2$O$_2$, and the PI 3-kinase inhibitor LY-294002 similarly slightly and significantly reduced intracellular H$_2$O$_2$ production (data not shown). Finally, precipitation of p85 with anti-phosphotyrosine antibodies showed that preincubation with the general and potent Nox inhibitor DPI prevents the insulin-stimulated recruitment of p85 in the anti-phosphotyrosine immunoprecipitated complex. This is in agreement with H$_2$O$_2$ production lying at an early step before PI 3-kinase stimulation and possibly even participating in its stimulation. In adipocytes, H$_2$O$_2$ reversibly oxidizes cysteine residues within the active site of the protein tyrosine phosphatase PTP1B, which normally serves as a negative regulator of insulin action via the dephosphorylation of IR/IRS. Thus a reasonable hypothesis is that the production of H$_2$O$_2$ within A6 cells is also necessary for the reversible inhibition of the IR/IRS protein tyrosine phosphatases (likely overall PTP1B), allowing efficient phosphorylation of the tyrosine residues of IR/IRS, recruitment of the p85 subunit of PI 3-kinase at the plasma membrane, and in turn enhancing PIP$_3$ generation. Importantly, ROS and particularly H$_2$O$_2$ have been shown to reversibly inactivate not only the protein tyrosine phosphatase PTP1B but also the PIP$_3$-phosphatase PTEN (26, 28, 29, 51).

While the effect of inhibition of PTP1B is upstream of p85 docking at the plasma membrane, the oxidative inactivation of PTEN is downstream of PI 3-kinase activation. Both effects result in increased accumulation of PIP$_3$. In this study, we show that pretreatment of monolayers with Nox inhibitors or ROS chelators also prevents the insulin-stimulated phosphorylation of PKB, suggesting that the inhibitors used decreased PIP$_3$ levels, which accounts for the lack of insulin-induced increase in sodium transport upon exposure to these agents.

Recently Yu et al. (60) reported that 24-h exposure to aldosterone substantially increases O$_2^-$ production in A6
cells, using dihydroethidium fluorescence to visualize changes in intracellular O$_2$$^-$$^+$ levels. These authors examined mainly the interaction between O$_2$ and nitric oxide in the regulation of ENaC open probability. We did not investigate such a possible negative interaction following insulin stimulation and cannot exclude a possible contribution to the increase in sodium transport brought by insulin. However, a strong correlation has been demonstrated between the increase in sodium transport and PIP$_2$ level. We therefore hypothesized that, at least in the case of insulin, the main effect of O$_2$$^-$$^+$ will probably lie at an early step in insulin signaling, controlling PIP$_3$ levels.

In conclusion, as previously shown for insulin-induced glut-4 translocation in adipocytes, our data in A6 cell monolayers demonstrate for the first time a direct link among intracellular production of O$_2$$^-$$^+$ and H$_2$O$_2$, increased PIP$_3$ levels, and the insulin-induced increase in sodium transport.

**REFERENCES**

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H₂O₂ PRODUCED BY INSULIN INCREASES SODIUM TRANSPORT


