Aldosterone Inhibits Insulin-Induced Glucose Uptake by Degradation of Insulin Receptor Substrate (IRS) 1 and IRS2 via a Reactive Oxygen Species-Mediated Pathway in 3T3-L1 Adipocytes

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Serum aldosterone level is clinically known to correlate with body weight and insulin resistance. Because the underlying molecular mechanism is largely unknown, we examined the effect of aldosterone on insulin-induced metabolic signaling leading to glucose uptake in 3T3-L1 adipocytes. Aldosterone reduced the amounts of insulin receptor substrate (IRS) 1 and IRS2 in a time- and dose-dependent manner. As a result, insulin-induced phosphorylation of Akt-1 and -2, and subsequent uptake of 2-deoxyglucose were decreased. Degradation of IRSs was effectively prevented by a glucocorticoid receptor antagonist and antioxidant N-acetylcysteine, but not by a mineralocorticoid receptor antagonist. Because aldosterone induced phosphorylation of IRS1 at Ser307, responsible kinases were investigated, and we revealed that rapamycin and BMS345541, but neither SP600125 nor calphostin C, conferred for degradation of IRSs. Although lactacystin prevented the degradation of IRSs, glucose uptake was not preserved. Importantly, sucrose-gradient-sediment intracellular fraction analysis revealed that lactacystin did not effectively restore the reduction of IRS1 in the low-density microsome fraction, important for the transduction of insulin’s metabolic signaling. These results indicate that aldosterone deteriorates metabolic action of insulin by facilitating the degradation of IRS1 and IRS2 via glucocorticoid receptor-mediated production of reactive oxygen species, and activation of IκB Kinase β and target of rapamycin complex 1. Thus, aldosterone appears to be a novel key factor in the development of insulin resistance in visceral obesity. (Endocrinology 150: 1662–1669, 2009)

Metabolic syndrome is widely recognized as a causal risk factor for progression of atherosclerosis, leading to stroke and cardiovascular disease (1, 2). The key characteristic on the pathogenesis of hypertension associated with metabolic syndrome is based on the insulin resistance originated from visceral obesity. Accumulation of visceral fats facilitates excess secretion of chemokines and adipocytokines, including TNFα and free fatty acid with concomitant suppression of adiponectin secretion, leading to the development of a state of insulin resistance (3). Recently, angiotensinogen (4) and mineralocorticoid-releasing factor (5) are also shown to be secreted from visceral adipose tissues. The concomitant increase of serum aldosterone levels with growing body of fat mass is related to the elevation of blood pressure in patients with metabolic syndrome or obesity (6, 7). In addition, the obesity related tissue activation of the renin-angiotensin (Ang)-aldosterone system or local production of aldosterone has been shown in peripheral tissues, including adipose tissues (8–10). Because aldosterone is a highly hydrophobic compound, one can speculate that locally produced aldosterone is accumulated in visceral adipose tissue in obese subjects.

Recent evidence indicates that reactive oxygen species (ROS) are involved as a common underlying mechanism in the patho-
genesis of insulin resistance (11–14). Direct exposure of oxidative stress to 3T3-L1 adipocytes causes phosphorylation of both insulin receptor substrate (IRS) 1 and IRS2 at serine residues and subsequent degradation of IRS1 (but not IRS2), resulting in insulin resistance (11). Both TNFα and dexamethasone have induced insulin resistance by increasing ROS production in 3T3-L1 adipocytes (12). In addition, Ang II-induced insulin resistance is caused by ROS production via activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in many tissues, including vascular smooth muscle cells and skeletal muscle cells (13, 14). Furthermore, recent studies have shown that aldosterone causes myocardial fibrosis and glomerular mesangial injury via the activation of NADPH oxidase, independent of Ang II (15, 16). Importantly, the correlation between plasma aldosterone levels and decrease in insulin sensitivity has been notable (17). However, the underlying molecular mechanism by which aldosterone induces insulin resistance in white adipose tissue is largely unknown.

In the present study, we examined the impact of aldosterone on insulin’s metabolic signaling leading to glucose uptake in 3T3-L1 adipocytes. In addition, we aimed to clarify the molecular mechanism by which aldosterone causes insulin resistance. Here, we show that aldosterone causes insulin resistance by promoting phosphorylation of IRS on Ser307, and subsequent degradation of IRS1 and IRS2 via glucocorticoid receptor (GR)-mediated ROS production and activation of IκB kinase β (IKKβ) and target of rapamycin complex (TORC) 1.

Materials and Methods

Materials

Human recombinant insulin was provided from Novo Nordisk Pharmaceutical Co. (Copenhagen, Denmark). A polyclonal anti-insulin receptor (IR) β-subunit antibody, monoclonal anti-Akt1 antibody, and monoclonal anti-IR substrates 1 (PY99) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A polyclonal anti-IRS1 antibody, polyclonal anti-IRS2 antibody, and polyclonal anti-Akt2 antibody were from Upstate Biotechnology Inc. (Lake Placid, NY). A polyclonal anti-Ser307 phospho-specific IRS1 antibody, polyclonal anti-Ser473 phospho-specific Akt antibody, polyclonal anti-p44/p42 MAPK antibody, and polyclonal anti-Thr202/Tyr210 phospho-specific p44/p42 MAPK antibody were obtained from Cell Signaling Technologies (Beverly, MA). Aldosterone was from Biogenesis Ltd. (Poole, UK). Spironolactone, RU486, cycloheximide, lactic acid, rapamycin, BMS45541, calphostin C, and SP600125 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Eplerenone and olmesartan were provided from Pfizer Inc. (New York, NY) and Sankyo Co. Ltd. (Tokyo, Japan), respectively. Enhanced chemiluminescence reagents were purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). DMEM, MEM vitamin mixtures, and MEM amino acid solutions were from Life Technologies, Inc. (Tokyo, Japan). All other reagents were of analytical grade and purchased from Sigma-Aldrich or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture and differentiation of 3T3-L1 adipocytes

3T3-L1 fibroblasts were grown and passaged in DMEM supplemented with 10% newborn calf serum. The cells of 2–3 d after confluence were used for differentiation. The differentiation medium contained 10% fetal calf serum (FCS), 250 μM dexamethasone, 0.5 μM isobutyl methylxanthine, and 500 nM insulin. After 3 d the differentiation me-

remium was replaced with post-differentiation medium containing 10% FCS and 500 nM insulin. After a further 3 d, post-differentiation medium was replaced with DMEM supplemented with 10% FCS. 3T3-L1 adipocytes of 10–12 d after initial differentiation were used for experiments.

Immunoprecipitation and Western blotting

Western blotting was performed as described previously (18, 19). In brief, 3T3-L1 adipocytes grown in six-well multiplates were serum starved for 16 h with or without various inhibitors, followed by incubation with aldosterone for indicated times. The cells were then treated with 17 nM insulin at 37°C for the specified times. The cell lysates or immunoprecipitates were separated by SDS-PAGE, transferred onto membranes, and immunoblotted. Densitometric analysis was conducted directly from the blotted membrane by using LAS-4000 lumino-image analyzer system (Fujifilm, Tokyo, Japan).

Adenoviral expression of myrP110

Adenovirus vectors encoding a constitutively active form of phosphatidylinositol 3 (PI3) kinase (bovine p110α with 30% myristylation signal sequence at the N terminus; myr-p110) or control LacZ virus encoding β-galactosidase were transiently expressed in differentiated 3T3-L1 adipocytes by adenovirus-mediated gene transfer as described before (19). In brief, a multiplicity of infection of 20 plaque-forming units per cell was used to infect 3T3-L1 adipocytes with the virus being left on the cells for 16 h before removal. Subsequent measurement of glucose uptake was conducted 24–48 h after initial addition of the virus. The efficiency of adenovirus-mediated gene transfer was approximately 95%.

Measurement of insulin-induced [3H]2-deoxy-glucose (2DG) uptakes

3T3-L1 adipocytes grown in six-multiwell plates were serum starved overnight and further incubated in Krebs-Ringer phosphate (KRP)-HEPES buffer containing 1% BSA for 3 h at 37°C, without or with various concentrations of aldosterone. Indicated inhibitors were pre-treated 30 min before addition of aldosterone. The cells were then stimulated with various concentrations of insulin for 15 min. Subsequently, 0.1 μCi of 2DG was applied for 4 min. The reaction was stopped by the addition of 10 μM cytochalasin B. The cells were washed three times with PBS, and solubilized with 0.2 mM sodium dodecyl sulfate/0.2 N NaOH. The radioactivity incorporated into the cells was measured by liquid scintillation counting.

Subcellular fractionation

3T3-L1 adipocytes were washed twice with PBS and once with HES buffer (255 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 2 μg/ml aprotinin, and 50 μg/ml okadaic acid [pH 7.4]), and immediately homogenized by 20 strokes with a motor-driven homogenizer in HEPES EDTA sucrose ([HES) buffer in 4°C. The homogenates (two 10 cm diameter dishes per condition) were subjected to subcellular fractionation as described previously to isolate plasma membrane (PM), high-density microsome, low-density microsome (LDM), and cytosol (19, 20). In brief, the homogenates were centrifuged at 10,000 g for 20 min to remove PM fraction, organelles, and nuclei. The resulting supernatant was centrifuged at 400,000 g for 90 min, yielding LDM as pellet. Remaining supernatant was concentrated by centrifugation of 250,000 x g for 90 min, yielding LDM as pellet. Remaining supernatant was concentrated by Centrificon-30 (Amicon Inc., Beverly, MA) and used as cytosol fraction. All fractions were adjusted to a final protein concentration of 1–3 mg/ml, which was measured by the Bradford method, and stored at −80°C until use.

Statistical analysis

The data are represented as means ± SE. P values were determined by one-way ANOVA with the Bonferroni test. P < 0.05 was considered statistically significant.
Results

Aldosterone decreased expressions of IR, IRS1, and IRS2

Recent evidence indicates that the suppression of excessive renin-angiotensin-aldosterone system activity has beneficial effects on whole body glucose metabolism in addition to regulate salt and water homeostasis and blood pressure (21–23). Although these effects are generally recognized as Ang II-mediated actions through its type 1 receptor (24), little is known about the direct effect of aldosterone on glucose homeostasis in the metabolic target tissue of insulin. Therefore, we first examined the dose-dependent effect of aldosterone on the protein levels of IR, IRS1, and IRS2 in 3T3-L1 adipocytes (Fig. 1, A–C). Treatment with 10^{-5} M aldosterone for 16 h slightly reduced the amount of IR to 75.8 ± 3.1%. On the other hand, treatment with aldosterone markedly decreased the protein levels of IRS1 and IRS2 in a dose-dependent manner. In accordance with the decreased protein levels, insulin-induced tyrosine phosphorylation of IRS1 and IRS2 was also significantly suppressed by aldosterone (data not shown). Interestingly, the time courses of the effects of aldosterone were different among these proteins (Fig. 1, D–F). The decrease in the protein levels of IRS1 was started after 3 h treatment with aldosterone, whereas that of IRS2 was started after 12 h treatment with aldosterone, indicating that the amount of IRS1 is more susceptible to aldosterone exposure, compared with that of IRS2. The total amount of β-actin was not affected by a dose- and time-dependent treatment with aldosterone (Fig. 1, G and H).

Aldosterone suppressed insulin-induced phosphorylations of Akt and p44/42 MAPK

Because treatment with aldosterone affected early signaling events of insulin, we next examined the effect of aldosterone on phosphorylations of insulin-induced Akt and p44/42 MAPK, two major downstream factors involved in insulin signaling. Consistent with the decreased amounts of IRS1 and IRS2, insulin-induced phosphorylations of Akt1 (Fig. 2A), Akt2 (Fig. 2B), and p44/42 MAPK (Fig. 2C) were significantly reduced by treatment with 10^{-5} M aldosterone. The degree of phosphorylations of Akt1, Akt2, and p44/42 MAPK at 5 min insulin stimulation was decreased to 60.0 ± 4.8, 52.6 ± 6.2, and 57.6 ± 5.6%, respectively. The total amount of Akt1, Akt2, and p44/42 MAPK was not altered by treatment with aldosterone (Fig. 2, A–C).

Effect of aldosterone on insulin- and myrP110-induced glucose uptake

Because aldosterone effectively suppressed phosphorylation of Akt2, which mainly mediates metabolic signals of insulin (25), we next examined the effect of aldosterone on insulin-induced 2DG uptakes. Treatment with aldosterone for 16 h inhibited insulin-induced 2DG uptake in a dose-dependent manner (Fig. 2D). Aldosterone at 10^{-7}, 10^{-6}, and 10^{-5} M significantly reduced 1.7 nm insulin-stimulated glucose uptake to 85.2 ± 6.2, 82.1 ± 2.6, and 55.3 ± 2.5%, respectively, and treatment with 10^{-6} and 10^{-5} M aldosterone suppressed 17 nm insulin-stimulated glucose uptake to 77.1 ± 1.0 and 64.9 ± 1.7%, respectively, compared with those in aldosterone-untreated control cells. Instead, 2DG uptake observed in the cells expressing a constitutive active form of PI3 kinase (myrP110) was not affected by the treatment with 10^{-5} M aldosterone (Fig. 2E). The amount of glucose transporter (Glut) 1 and Glut4, major Gluts in 3T3-L1 adipocytes, was not affected by treatment with aldosterone for 16 h at any concentrations (supplemental Fig. 1, which is published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). These results indicate that aldosterone induces insulin resistance mainly by degrading IRS1 and IRS2 in 3T3-L1 adipocytes.

Aldosterone induced degradation of IRS1 and IRS2 through GR but not mineralocorticoid receptor (MR)

Adipose tissue is known to express both MR and GR (26). In fact, we confirmed mRNA expression of both MR1 and GR1 in 3T3-L1 adipocytes by RT-PCR (data not shown). We examined which receptor is involved in the aldosterone-induced IRS degradation leading to insulin resistance in 3T3-L1 adipocytes by using specific receptor antagonists (Fig. 3). Pretreatment with 10 μM eplerenone (a specific MR antagonist) had no significant effect on the aldosterone-induced degradation of IRS1 (Fig. 3A) and IRS2 (Fig. 3B). Treatment with further increment of eplerenone again did not alter the degradation (data not shown). In contrast, pretreatment with RU486 (a selective GR antagonist) effectively prevented aldosterone-induced degradation of IRS1 (Fig. 3A) and IRS2 (Fig. 3C).
Ablation of ROS prevented aldosterone-induced degradation of IRS1 and IRS2

Increasing evidence suggests the contribution of ROS to the pathogenesis of insulin resistance (11, 12, 24, 27). Aldosterone is known to induce activation of NADPH oxidase in cardiomyocytes and mesangial cells (15, 16). To clarify the involvement of ROS in aldosterone-induced insulin resistance, we started treatment of 3T3-L1 adipocytes with N-acetyl cysteine (NAC) or coenzyme Q10, two potent ROS scavengers, 30 min before an addition of aldosterone, and examined the protein levels of IRS1 and IRS2. Either pretreatment with NAC (Fig. 4, A and B) or coenzyme Q10 (data not shown) effectively prevented the aldosterone-induced degradation of IRS1 and IRS2. In addition, NAC effectively restored aldosterone-induced reduction of 2DG uptake (Fig. 4C). These results indicate that aldosterone induces insulin resistance possibly via ROS-mediated degradation of IRS proteins in 3T3-L1 adipocytes.

Aldosterone phosphorylated Ser307 residues of IRS1 via TORC1 pathway

During the development of insulin resistance, phosphorylation of serine residues on IRS proteins precedes their degradation (28). We examined the effect of aldosterone on the Ser307 phosphorylation of IRS1, one of the main phosphorylation sites. Interestingly, 10^{-6} M aldosterone induced significant phosphorylation of IRS1 at Ser307 (2.1 ± 0.3-fold) (supplemental Fig. 2), despite the significant reduction of IRS1 protein.

We further investigated the crucial protein kinases involved in the aldosterone-induced serine phosphorylation and degradation of IRSs by using inhibitors of serine kinases. Aldosterone degraded both IRS1 and IRS2 partly at 10^{-6} M and almost completely at 10^{-5} M. Preincubation with BMS345541 (an IKKβ inhibitor) effectively attenuated both 10^{-6} and 10^{-5} M aldosterone-induced degradation of IRS1 and IRS2. Although rapamycin (an inhibitor of a TORC1 pathway) effectively inhibited the degradation caused by 10^{-6} M aldosterone, the impact was limited at 10^{-5} M aldosterone-treated cells. In contrast, neither calphostin C [a protein kinase C (PKC) inhibitor] nor SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor] affected the aldosterone-induced degradation of IRSs (Fig. 5, A and B). In accordance with the degradation of IRS1, aldosterone-induced phosphorylation of IRS1 at Ser307 residue was also suppressed by pretreatment with either BMS345541 or rapamycin (supplemental Fig. 2B). It is noteworthy that aldosterone-induced phosphorylation of nuclear factor-κB and S6 kinase (downstream targets of IKKβ and TORC1, respectively), and these phosphorylations were effectively inhibited by pretreatment with BMS345541 or rapamycin, respectively. In contrast, aldosterone did not induce phosphorylation of either JNK or PKC (supplemental Fig. 3).

To confirm the results with inhibitors, we used small interfering RNA-based gene silencing against IKKβ and S6 kinase (downstream targets of IKKβ and TORC1, respectively), and these phosphorylations were effectively inhibited by pretreatment with BMS345541 or rapamycin, respectively. In contrast, aldosterone did not induce phosphorylation of either JNK or PKC (supplemental Fig. 3).
Phosphorylation and degradation of IRSs were prevented by either knockdown or inhibition with TORC1 and IKKβ pathways. Interestingly, these two kinases are activated by ROS, which relates to the development of insulin resistance via degradation of IRS1 (11–14, 27, 28). Indeed, oxidative stress-induced impairment of insulin signaling was reversed by expression of dominant-negative IkB and rapamycin (11, 27, 30). In this regard, aldosterone impaired vascular reactivity by decreasing expression of glucose-6-phosphate dehydrogenase and increasing oxidative stress in endothelial cells (31). Furthermore, aldosterone promoted vascular remodeling (31–33), myocardial fibrosis (15), and renal injury (16) via ROS production. Consistently, we demonstrated that the ROS production is involved in the mechanism of aldosterone-induced degradation of IRS in 3T3-L1 adipocytes. Although Ang II is also known to induce Ser307 phosphorylation and degradation of IRS1 via ROS-mediated signals (24), the observed effect of aldosterone on the levels of IRS proteins and glucose uptake is not mediated by Ang II because the Ang II type 1 receptor antagonist had no effect. We further consider that although pretreatment with NAC partly prevented aldosterone-induced degradation of IRSs, this level of recovery appears to be sufficient for the efficient restoration of the impaired glucose uptake. Together, these results suggest that induction of ROS by aldosterone triggers the activation of both TORC1 and IKKβ pathways, and that these kinases coordinately promote the degradation of IRS protein and inhibit subsequent insulin actions.

In our studies the relatively higher concentrations of aldosterone are needed to induce insulin resistance (34). Aldosterone at

Discussion

In the present study, we demonstrated that aldosterone inhibited insulin-induced glucose uptake via degradation of IRS proteins. Phosphorylation of IRS1 on serine residues plays an important role in subsequent degradation of IRS1 (14, 20, 28). Ser307 is one of the key serine residues on IRS1, and can be phosphorylated by TNFα via JNK and IKKβ, by free fatty acid via PKCθ, and by prolonged insulin treatment or osmotic shock via TORC1 pathway (28). Among these kinases, we demonstrated the involvement of IKKβ and TORC1 because aldosterone-induced serine

FIG. 4. Effect of NAC on aldosterone-induced degradations of IRS1 and IRS2. 3T3-L1 adipocytes were preincubated with 1 mM NAC. After 30 min incubation, cells were treated with 10−6 M aldosterone (Aldo) for 16 h. A and B, Cell lysates were subjected to SDS-PAGE, and immunoblotted with anti-IRS1 (A) or anti-IRS2 (B) antibody. The amounts of IRS1 and IRS2 were quantitated by densitometry. Results are means ± SE of five (A and

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Effects of Ser/Thr kinase inhibitors on aldosterone-induced degradation of IRSs and suppression of glucose uptake. 3T3-L1 adipocytes were preincubated with 20 μM SP600125 (JNK inhibitor), 100 nM calphostin C (CalC) (PKC inhibitor), 10 μM BMS345541 (BMS) (IKKβ inhibitor), or 20 nM rapamycin (Rapa) (mTORC1 inhibitor) for 30 min. A and B, Cells were treated with \(10^{-7}\) to \(10^{-5}\) M aldosterone (Aldo) for 16 h. Cell lysates were subjected to SDS-PAGE, and immunoblotted with anti-IRS1 antibody (A) or anti-IRS2 antibody (B). C and D, Cells pretreated with BMS345541 or rapamycin for 30 min were treated with \(10^{-8}\) or \(10^{-5}\) M aldosterone (Aldo) for 16 h. Cells were stimulated with various concentrations of insulin (Ins) for 15 min. 2DG uptake for 4 min was then quantitated by a liquid scintillation counter. Each measurement was performed in duplicate. Results are means ± SEM of five (A) or six (B–D) separate experiments. *, \(P < 0.05\) and **, \(P < 0.01\) vs. objected cells as indicated in each panel by one-way ANOVA with the Bonferroni test.

FIG. 5. Effects of Ser/Thr kinase inhibitors on aldosterone-induced degradation of IRSs and suppression of glucose uptake. 3T3-L1 adipocytes were preincubated with 20 μM SP600125 (JNK inhibitor), 100 nM calphostin C (CalC) (PKC inhibitor), 10 μM BMS345541 (BMS) (IKKβ inhibitor), or 20 nM rapamycin (Rapa) (mTORC1 inhibitor) for 30 min. A and B, Cells were treated with \(10^{-7}\) to \(10^{-5}\) M aldosterone (Aldo) for 16 h. Cell lysates were subjected to SDS-PAGE, and immunoblotted with anti-IRS1 antibody (A) or anti-IRS2 antibody (B). C and D, Cells pretreated with BMS345541 or rapamycin for 30 min were treated with \(10^{-8}\) or \(10^{-5}\) M aldosterone (Aldo) for 16 h. Cells were stimulated with various concentrations of insulin (Ins) for 15 min. 2DG uptake for 4 min was then quantitated by a liquid scintillation counter. Each measurement was performed in duplicate. Results are means ± SEM of five (A) or six (B–D) separate experiments. *, \(P < 0.05\) and **, \(P < 0.01\) vs. objected cells as indicated in each panel by one-way ANOVA with the Bonferroni test.

10^{-7} M only partly suppressed insulin-induced glucose uptake, and more than 10^{-6} M aldosterone is needed to suppress insulin actions significantly. Concerning this issue the secretion of angiotensinogen and mineralocorticoid releasing factor from adipose tissues appears to contribute to the elevation of serum aldosterone levels by the hypersecretion from adrenal gland in obese individuals (4–6). In addition, local production of aldosterone with elevation of tissue angiotensin-converting enzyme has been documented in heart, vessels, or kidney (8, 9). Ang II is also known to stimulate aldosterone production in human monocytes (35). Because macrophage accumulation in enlarged visceral fat closely correlates with insulin resistance (3), adipose tissue might be one of the major extra-adrenal sources of aldosterone. It is possible that accumulation of aldosterone in adipose tissues may occur due to the highly lipophilic nature of aldosterone.

In our study, aldosterone-induced degradation of IRS appears to be mediated through GR because a MR antagonist but not a MR antagonist restored the aldosterone-induced inhibition of insulin actions. In this regard, high doses of aldosterone can bind both MR and GR by a mechanism of sharing the considerable structural homology (36). The distinct mechanism of aldosterone-induced insulin resistance has been reported (37). Aldosterone stimulated production of proinflammatory cytokines, including TNFα, IL6, and monocyte chemoattractant protein-1, and simultaneous suppression of insulin-sensitizing adiponectin production in 3T3-L1 preadipocytes. In addition, administration of a MR antagonist effectively ameliorated insulin resistance seen in db/db mice (37). Furthermore, aldosterone-induced reduction of IRS1 in vascular smooth muscle cell was abolished by pretreatment with either a ROS scavenger or an MR antagonist (38). On the other hand, our results are relatively consistent with a recent report that aldosterone stimulated gene expression of hepatic gluconeogenic enzymes including glucose-6-phosphatase and phosphoenolpyruvate carboxykinase through GR in primary cultured mouse hepatocytes (39). Interestingly, regardless of the similar experimental condition used, aldosterone did not affect signaling molecules of insulin, including IRS1 and Akt, in hepatocytes. Although the precise mechanisms of the difference are uncertain, it is possible that the response to aldosterone is different in cell types. In any case, we would like to stress that aldosterone appears to play important roles in the pathogenesis of insulin resistance, not only through MR-mediated production of adipokines, but also the cross activation of GR in adipocytes.

Induction of insulin resistance by administration of glucocorticoid is well known. However, pretreatment of a glucocorticoid dexamethasone degraded only IRS1 protein, and protein levels of IRS2 were not apparently affected (40, 41), whereas both IRS1 and IRS2 were degraded in response to aldosterone in 3T3-L1 adipocytes in the present study. In addition, treatment with aldosterone did not alter the amount of Glut1 (supplemental Fig. 1), whereas dexamethasone is known to down-regulate the expression of Glut1 (40). Although the precise mechanism is not currently known, it is possible that aldosterone may bind to GR with different interactive characteristics compared with dexamethasone because the recruitment of transcriptional coactivator or corepressor to the nuclear receptors is modified by different ligand binding (42). Further investigation will be needed to clarify this issue.

Prolonged exposure to insulin and/or high glucose promotes progestational degradation of IRS1 (20, 28, 43), whereas it reduces the levels of IRS2 mRNA in rat adipose tissue (44). The reduction of IRS2 via progestational degradation is controversial in the state of insulin resistance (44). Interestingly, aldosterone-induced decrease in the amount of both IRS1 and IRS2 was blocked by pretreatment with either lactacystin or MG132. These results clearly indicate that aldosterone induces progestational degradation of both IRS1 and IRS2. It is of note that the reduction of IRS1 and IRS2 was started at 3 and 12 h after treatment with aldosterone, respectively. Thus, the susceptibility to the aldosterone-induced degradation appears to be different between these two types of IRS proteins. Previous studies with various IRS1/IRS2 chimera revealed that the N-terminal region of IRS1 is essential for the progestational degradation of IRS induced by prolonged insulin treatment (43). Structural differences between IRS1 and...
IRS2, including the serine phosphorylation sites, may alter the susceptibility to the proteasomal degradation. We also examined the effects of short-term exposure (for up to 60 min) to aldosterone. However, neither the levels of IRS proteins nor insulin signaling was affected at any concentration (data not shown).

In addition, preincubation with cycloheximide, a potent inhibitor of protein synthesis, effectively protected aldosterone-induced degradation of IRS1 and IRS2 (data not shown). These results indicate that aldosterone-induced degradation of IRSs is mediated through genomic action of aldosterone in 3T3-L1 adipocytes (45).

Although the aldosterone-induced decrease in the amounts of IRS proteins in whole cell lysates was prevented in the presence of lactacystin, the glucose uptake was not preserved. Recent studies indicate that intracellular localization of signaling molecules is a crucial factor for the subsequent signal transduction (19, 20, 29). IRS1 located in LDM fraction is important for activation of PI3-kinase leading to glucose uptake, and phosphorylated IRS1 on serine residues is transferred from LDM to cytosol fraction for the degradation in down-regulation of signaling machinery (19, 20). Importantly, aldosterone effectively reduced the amount of IRS1 located in LDM fraction, and pretreatment with lactacystin did not prevent the reduction of IRS1 in the LDM fraction, whereas it effectively prevented that in cytosol (data not shown). These results indicate that aldosterone-induced phosphorylation at serine residues may facilitate translocation of IRS1 molecule from LDM to cytosol, resulting in degradation by proteasome. Because proteasomal inhibitors did not restore the level of IRS1 in LDM fraction, subsequent insulin’s metabolic signaling does not appear to be adequately transmitted, regardless of the restoration of IRS in other fractions.

In conclusion, we precisely investigated the molecular mechanism underlying aldosterone-induced insulin resistance in 3T3-L1 adipocytes, and found that aldosterone promotes proteasomal degradation of IRS proteins via its serine phosphorylation by TORC1 and IKKβ (supplemental Fig. 6). In particular, the decrease in the amount of IRS1 localized in LDM fraction is a critical step for the impairment of downstream insulin signaling for glucose uptake. Moreover, GR-mediated ROS production appears to be involved in the activation of TORC1 and IKKβ pathway. Thus, the present findings that aldosterone induces insulin resistance independently of Ang II provide new insight into the strategy for amelioration of insulin resistance in patients with hypertension and primary aldosteronism, as well as metabolic syndrome.

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