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Oleic Acid Induces Adherens Junction Disassembly in Rat Neonatal Cardiomyocytes Through Mechanisms Involving Fyn Kinase and Casein Kinase 1

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Backgrounds: Accumulation of oleic acid (OA) and other nonesterified fatty acids in heart tissue during ischemia and reperfusion contribute to myocardial damage. OA has been shown to induce accumulation of intracellular lipid droplets and to disrupt adherens junctions (AJs). Cardiac N-cadherin is the major cadherin of AJs. This study investigated the role of N-cadherin in OA-induced junctional disassembly in neonatal cardiomyocytes. **Methods:** cardiomyocyte cultures from 2-day-old Wistar rats were used. Immunocytochemistry was performed to detect junctional N-cadherin. The binding of Fyn, phosphotyrosine, or phosphoserine with N-cadherin was assayed by immunopricipitation. **Results:** OA caused the disruption of AJs. In addition, OA promoted the association of N-cadherin with Fyn kinase, a member of Src family kinase, and increased tyrosine phosphorylation of N-cadherin without affecting the protein expression of Fyn and N-cedherin. The Src family kinase inhibitor, PP2, prevented the OA-induced tyrosine phosphorylation of N-cadherin and the loss of N-cadherin from AJs. Moreover, OA increased the expression of casein kinase (CK) 1α and 1δ in a time-dependent manner and the serine phosphorylation of N-cadherin. The CK1 inhibitor, IC261, prevented OA-induced serine phosphorylation of N-cadherin, and this modification is known to accelerate the turnover of N-cadherin. Double-labeling experiment showed partial colocalization of these N-cadherin punctate structures with early endosomes. Either MG132 (a proteasome inhibitor) or NH_4Cl (a lysosomal inhibitor) inhibited OA-induced junctional N-cadherin disruption indicating that OA decreases junctional N-cadherin by the proteasomal and lysosomal degradation. **Conclusions:** OA activates CK1 and Fyn kinases, which modify N-cadherin by serine phosphorylation and tyrosine phosphorylation, respectively, and results in AJ disruption. This study provides an insight into the mechanism of OA-induced junctional abnormality.

Key words: oleic acid, cardiomyocyte, adherens junction, N-cadherin, phosphorylation

INTRODUCTION

Oleic acid (OA) is a monounsaturated 18-carbon fatty acid found in most animal fats and vegetable oils. During ischemia, OA, palmitic acid, linoleic acid, and arachidonic acid were accumulated in heart tissues.[1] Arachidonic acid, linoleic acid, and palmitic acid caused apoptosis of neonatal cardiomyocytes by intracellular overload of Ca^{2+} and Na^{+} and induction of caspase 3.[2] OA is known to suppress gap junction permeability and conductance in neonatal rat cardiomyocytes[3] and vascular smooth muscle A7r5 cell line.[4]

Cadherins are transmembrane adhesion receptors that mediate cell-cell adhesion through calcium-dependent, homophilic interactions in many types of cells.[5] The cytosolic domain of cadherin binds to cytosolic proteins called catenins (ctns), including α-catenin (α-ctn), β-catenin (β-ctn), γ-catenin (γ-ctn) and p120-catenin (p120ctn).[6] Among the members of the cadherin family, N-cadherin is specially expressed in muscles and neurons.[7,8] In rat hearts, cadherins of AJ consists of N-cadherin and T-cadherin, whereas R-cadherin and E-cadherin are virtually absent.[9] N-cadherin is essential for the formation of intercalated disc-like structures in cardiomyocytes and during heart development.[10,11] Deletion of N-cadherin gene in the mouse heart leads to dilated

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cardiomyopathy and impaired cardiac function; muscle tension is lost due to the failure of myofibrils to anchor to the plasma membrane.[12] Based on the importance of N-cadherin in cardiomyocytes, we emphasized on the effect of OA on N-cadherin in this study.

The stability of intercellular junctions and the function of cadherins in establishing and maintaining these junctions are affected by the phosphorylation state of the cadherins and ctns.[13-16] Only a few papers have discussed the modification of N-cadherin at tyrosine residues.[15,16] OA induces the disassembly of AJ via activation of Fyn kinase, which increases tyrosine phosphorylation of p120ctn and β-ctn, resulting in dissociation of p120ctn from N-cadherin and of β-ctn from α-ctn.[17] However, little is known about which kinases are involved in the tyrosine phosphorylation of N-cadherin in primary culture cardiomyocytes.

Casein kinase 1 (CK1) are serine/threonine kinases, consisting of seven isoforms (α, β, γ1, γ2, γ3, δ, and ε.[18] CK1 has been implicated in diverse biological functions, such as regulation of DNA repair, cellular morphology, modulation of the Wnt/β-ctn pathway during development, and regulation of circadian rhythms in adulthood.[19,20] CK1 destabilizes β-ctn degradation complex by phosphorylating several components of this complex, thus facilitating Wnt signal.[21] Dupre-Crochet *et al*. reported that CK1δ negatively regulates the function of E-cadherin by phosphorylation of Serine846 residue of E-cadherin and the weakening of its association with β-ctn.[22] In contrast, serine phosphorylation of E-cadherin by CK2 increases the binding of E-cadherin to β-ctn.[15] N-cadherin and E-cadherin share common sequences, especially the Ser846, which is a highly conserved residue between classical cadherins and is targeted by CK1.[23] Whether N-cadherin is affected by CK1 in cardiomyocytes remains unclear.

OA, palmitic and linoleic acid are accumulated in the heart tissues during ischemia and reperfusion,[24] and this caused myocardial damage.[2,4,25] To mimic lipid-laden condition of cardiomyocyes, we incubated the cells with oleate-bovine serum albumin complex according to the method described by Jepson *et al*. (1996).[26] Previously, we have provided evidence for the disruption of gap junction and AJ by OA with special emphasis on connexin and β-ctn of cardiomyocytes.[3,17,27] Whether cardiac N-cadherin was affected by OA remains to be determined. The aims of this study were to evaluate the role of Fyn kinase and CK1 in the OA-induced disassembly of AJs.

METHODS

Cell culture

Cardiomyocyte cultures were prepared from 1- to 2-day-old Wistar rats of both sexes as described previously.[28] Five rat ventricles were minced in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY), then incubated for 15 min at 37 ^{o}C with 5 ml of a mixture of 0.5 mg/ml of collagenase type II (Sigma, St. Louis, MO) and 0.6 mg/ml of pancreatin (Sigma) in HBSS. The digestion was repeated a further two times with fresh enzyme mixture, then the combined cell suspensions from the digestions were mixed with an equal volume of ice-cold plating medium consisting of minimal essential medium (MEM; Gibco) containing 10% fetal bovine serum (Gibco), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (Gibco), and centrifuged at 1,000$\times$g for 10 min at room temperature, then the cells were resuspended in plating medium and preplated for 1 h at 37 ^{o}C in a 100 mm culture dish in a CO_2 incubator. After fibroblast attachment, the cardiomyocytes in the supernatant were collected and plated at a density of 5×10^4 cells/ml on coverslips or 10^6 cells/ml on 35 mm collagen-coated dishes for one day, then the medium was changed to growth medium (MEM containing 10% calf serum (Gibco), 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine). All experiments were performed on day 2 cultures. Day 2 cultures on coverslips were 80% confluent, while those in dishes were 100% confluent. Day 2 cultures were incubated for 1 or 24 h at 37^{o}C with a mixture of 100 μg/ml of OA sodium (Sigma) and 400 μg/ml of bovine serum albumin (BSA) (Sigma) (OA binds to albumin in serum) or with 400 μg/ml of BSA alone (control). To evaluate the role of Src family kinase and CK1 in N-cadherin-mediated AJ disruption following OA treatment, the cells were pretreated for 30 min with for 30 min with a 10 μM concentration of the CK inhibitor IC261 or a 10 μM concentration of the Src family kinase inhibitor PP2 (both from BIOMOL International LP, Plymouth Meeting, PA) before incubation with OA/BSA or BSA for 1 or 24 h.

Immunofluorescence staining

Cells attached to coverslips were washed briefly with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4), then fixed in ice-cold acetone for 10 min at -20^{o}C. For single labeling, the cells were incubated overnight at 4^{o}C with a 1:100 dilution of mouse anti-N-cadherin antibody (BD Biosciences Pharmingen, San Jose, CA) in PBS containing 0.01% Tween 20 and 5% non-fat milk, washed with PBS, and incubated for 1 h at 37^{o}C with FITC-conjugat-

ed goat anti-mouse IgG antibodies (1:25 dilution in PBS; Jackson Laboratory Inc., Bar Habor, MI). For double-labeling for EEA-1 and N-cadherin, cardiomyocytes were incubated overnight at 4°C with a mixture of mouse monoclonal anti-early endosomal entigen-1 (EEA1) antibodies (1:100 dilution, Abcam, Cambridgeshire, UK) and rabbit polyclonal anti-N-cadherin antibodies (1:100 dilution; Takara Bio Inc., Japan) in PBS-Tween. After several PBS washes, the cells were incubated for 1 h at 37°C with a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG antibodies (Sigma) in PBS and Texas red-conjugated goat anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA). For counter staining with DAPI, immunostained cells were stained for 15 min with 1 μg/ml of DAPI in 0.9% NaCl. After brief washes with PBS, the coverslips were mounted in fluorescence mounting medium (Sigma) and sealed with nail polish. Immunofluorescence images were acquired using a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Nikon D1X digital camera (Nikon, Tokyo, Japan).

Immunoprecipitation

After various treatments, cardiomyocytes were washed with cold PBS, scraped off the dishes, ultrasonicated for 20 sec, and extracted for 30 min on a shaker at 4°C with sodium dodecyl sulfate-radioimmunoprecipitation assay (SDS-RIPA) buffer (150 mM NaCl, 1% Nonidet-40, 50% deoxycholate, 1% SDS, 1 mM phenylmethysulfonyl fluoride, 50 mM Tris, pH 7.5). The cell lysates were centrifuged at 12,000×g, 4°C for 10 min and the supernatants collected. A sample of the supernatant (400 μg of protein; about 400 μl) was taken, 1 μg of primary antibodies (4 μl) added, and the mixture incubated overnight at 4°C. These primary antibodies were rabbit anti-N-cadherin, mouse anti-phosphotyrosine (clones PY7E1 and PY20, Zymed Laboratories Inc., San Francisco, CA), and rabbit anti-phosphoserine (Zymed). Protein G Sepharose slurry (50 μl) (Pharmacia, Uppsala, Sweden) was then added and the mixture incubated for 1 h at 4oC on a shaker. After centrifugation at 12,000×g for 30 sec, the precipitates (Sepharose-bound immune complex) were washed and re-suspended in RIPA buffer and a sample taken for protein determination, then the bound proteins were dissolved in reducing SDS sample buffer and analyzed by Western blotting.

Western blotting

After various treatments, the cardiomyocytes were lysed in lysis buffer (0.15% Triton X-100, 60 mM PIPES, 2 mM $MgCl_2$, 10 mM EGTA, 25 mM HEPES, pH 6.9) and the protein concentration determined using the Bradford protein assay (Bio-Rad, Hercules, CA), then 50 μg of the protein samples was resolved on SDS/PAGE (10% gels) and transferred onto a nitrocellulose membrane (Millipore Corporation, Billerica, MA). The membrane strips were blocked in Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris, pH 8.2) containing 5% non-fat milk and 0.1% Tween for 1 h at room temperature, then incubated overnight at 4°C with mouse anti-N-cadherin antibodies (BD Biosciences), mouse anti-rat Fyn antibodies (R&D Systems Inc., Minneapolis, MN), rabbit anti-rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Biovision Inc., Mountain view, CA), rabbit anti-phosphoserine antibodies (Millipore), or mouse anti-phosphotyrosine antibodies (Zymed) in the same buffer. After several washes with TBS containing 0.1% Tween 20, the strips were incubated with alkaline phosphatase-conjugated secondary antibodies and bound antibody visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogen. For chemiluminescence detection, peroxidase-conjugated secondary antibodies were used. After several washes, blots were reacted in ECL substrate developing solution (Millipore). Stained bands were scanned and quantified using Gel-pro Analyzer 3.1 (Media Cybernetics, Silver Spring, MD). Densitometry was performed using Gel Pro 3.1 and the values expressed relative to that of the same band in the control group (zero time or BSA control, as appropriate), taken as 100%. All experiments were performed at least three times and the values are expressed as the mean±SD.

Statistical analysis

Statistical differences between means were evaluated using Student's t test. Differences were considered significant when $p < 0.05$.

RESULTS

OA induces tyrosine phosphorylation of N-cadherin by Fyn kinase

In normal serum, OA binds to albumin, so BSA was added together with OA, and BSA alone was used as the control. In BSA-treated control cardiomyocytes, N-cadherin was seen as a continuous linear staining pattern at cell-cell contacts (arrowheads in Fig. 1A). After treatment with OA/BSA for 24 h, cell contact areas were significantly decreased, and N-cadherin staining became discontinuous (arrows in Fig. 1B), accompanied with

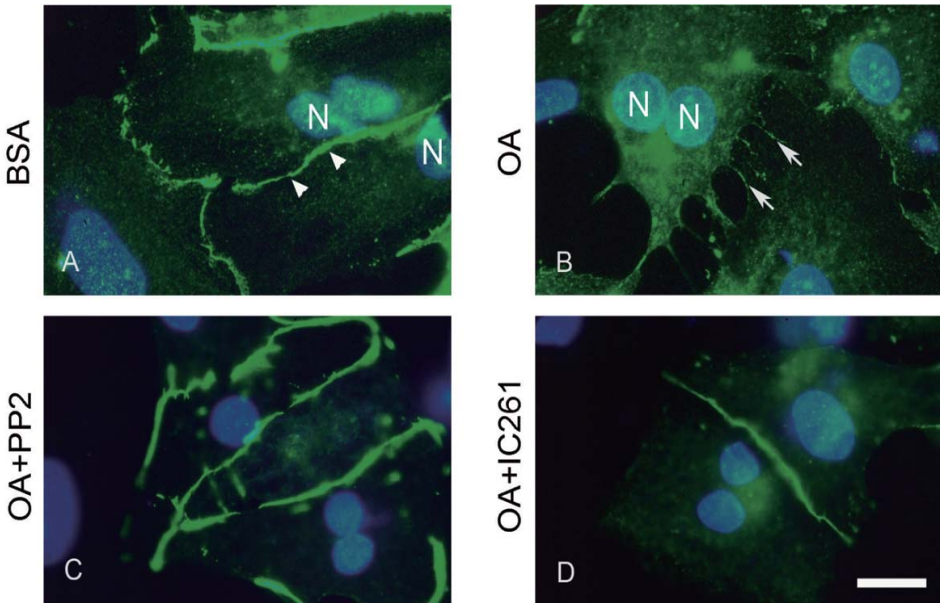

Fig. 1 Effect of OA on distribution of N-cadherin in cardiomyocytes. Cardiomyocytes were treated with BSA alone (A) or OA/BSA for 24 h (B), then immunostained for N-cadherin. Arrowheads indicate junctonal N-cadherin continuously. Arrows indicate junctonal N-cadherin discontinuously. Other groups of cardiomyocytes were incubated for 30 min with 10 μM PP2 (C) or 10 μM IC261 (D), then with OA/BSA for 24 h in the continued presence of the inhibitors before immunolabeling for N-cadherin. N indicates nucleus. Scale bar = 10 μm.

decreased staining intensity (Fig. 1B). The Src family kinase inhibitor PP2 inhibited OA-induced AJ disruption (Fig. 1C).

We next examined whether Fyn kinase was responsible for the OA-induced AJ disruption via tyrosine phosphorylation of N-cadherin. The amount of Fyn kinase associated with N-cadherin in anti-N-cadherin antibody immunoprecipitates was significantly increased by OA/BSA treatment for 1 h (Fig. 2A), while the protein expressions of Fyn and N-cadherin were unaffected by OA treatment for 1 h to 6 h (Fig. 2B). Moreover, OA/BSA treatment also increased tyrosine phosphorylation of N-cadherin and this effect was blocked by pretreatment of the cells with PP2 (Fig. 2C). These data suggest that OA increases the binding of Fyn kinase and N-cadherin and induces Fyn kinase-dependent tyrosine phosphorylation of N-cadherin.

Role of CK1 activation in the serine phosphorylation of N-cadherin

To assess the possible involvement of CK1 in OA-induced N-cadherin detachment from AJ, we evaluated the effect of a CK1 inhibitor IC261. Pretreatment with the CK1 inhibitor, IC261 (10 μM), blocked OA-induced AJ disruption, as shown by N-cadherin staining, (Fig. 1D). CK1ε was hardly detected by blotting. We thus examined CK1α and δ expressions under stimulation with OA/BSA for 0 to 6 h. The expression levels of CK1α and CK1δ were upregulated after OA treatment for 1 h and peaked at 3 h (Fig. 3A and 3B). Next, the involvement of CK1 in OA-induced AJ disassembly and the modification of N-cadherin were examined. The immunoprecipitates obtained using anti-N-cadherin antibodies were analyzed for serine phosphorylation of N-cadherin. OA/BSA treatment for 1 h induced serine phosphorylation of N-cadherin and this effect were blocked by pretreatment with IC261 (Fig. 3C).

OA enhances N-cadherin turnover

The decrease in N-cadherin staining at cell-cell contacts was accompanied by an increase in cytoplasmic punctate structures positive for N-cadherin (oval in Fig. 1B). To identify these structures, we performed double-labeling for N-cadherin and EEA-1, an early endosomal marker. In BSA-treated cardiomyocytes, N-cadherin was located at the AJ (Fig. 4A), and EEA1 appeared in vesicular staining distributed in the perinuclear region (Fig. 4C) and the merged images showed no overlap of these two staining patterns (Fig. 4E). In OA/BSA-treated cardiomyocytes, the number of N-cadherin punctates was significantly increased in the cytoplasm (arrowheads in Fig. 4B) and some of these colocalized with EEA-1 staining (arrowheads in Fig. 4D, F). These results suggest that OA-induced N-cadherin internalization is partially mediated by the endosomal pathway.

MG132 can reversibly block the activity of the 26S proteasome.[29] To examine if the degradation of junctional N-cadherin is mediated by the proteasome pathway, we treated cardiomyocytes with the proteasomal inhibitor MG132 (0.5 μM) and OA/BSA for 24 h, and found that MG132 blocked the OA-induced junctional N-cadherin

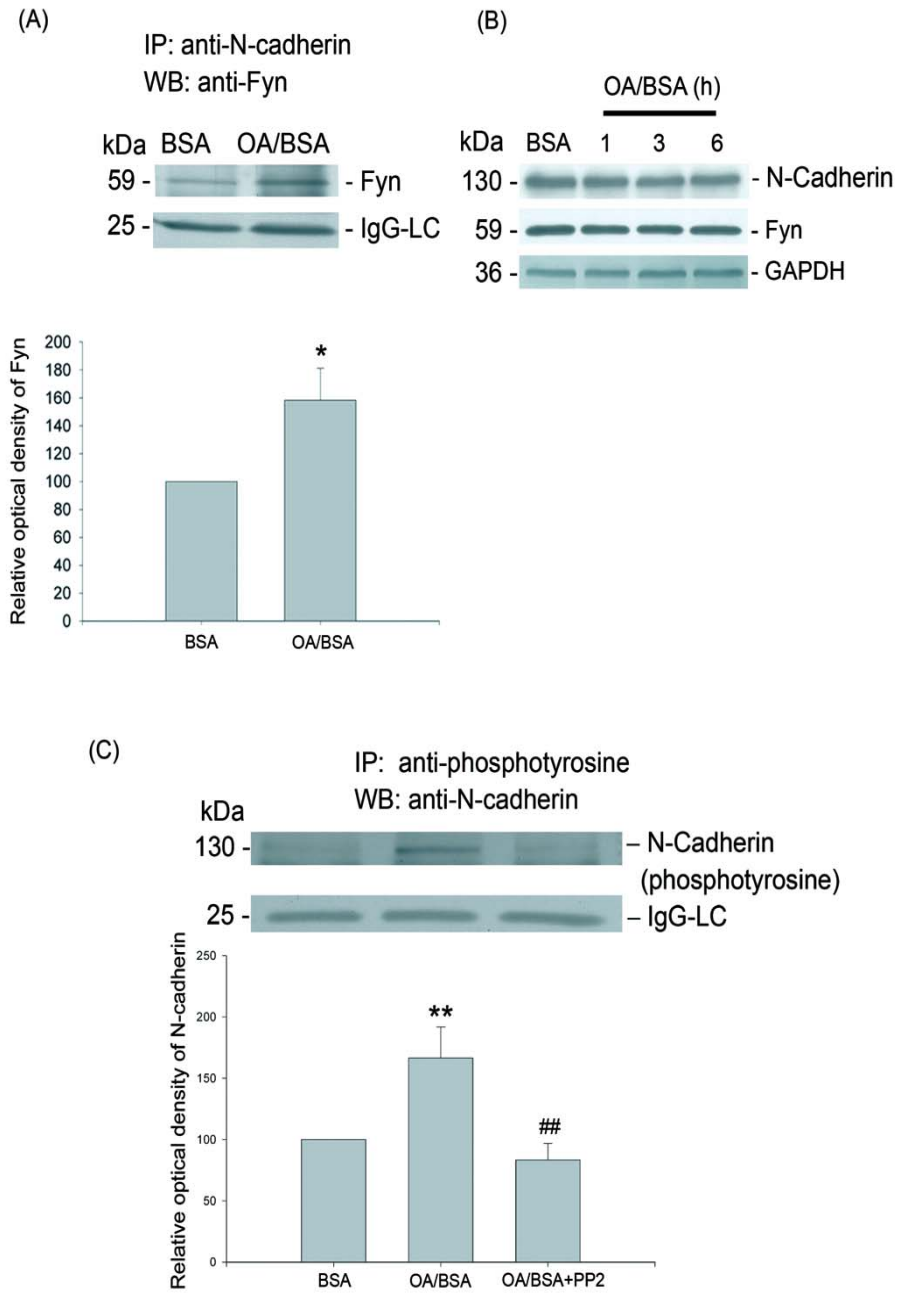

Fig. 2 OA promotes Fyn kinase and N-cadherin association. Cardiomyocytes were treated with BSA or OA/BSA for 1 h or with 10 μM PP2 for 30 min, then for another 1 h with OA/BSA in the continued presence of PP2, then cell lysates were immunoprecipitated with anti-N-cadherin (A) or anti-phosphotyrosine (C) antibodies and the immunoprecipitates analyzed for Fyn kinase (A) or tyrosine phosphorylated N-cadherin (C) and immunoglobulin G light chain (IgG-LC; detected using alkaline phosphatase-coupled secondary antibody). n=3. *, $p < 0.05$, ** $p < 0.01$ compared to the BSA-treated group. ##, $p < 0.01$ compared to the OA/BSA-treated group. (B) Cardiomyocytes were treated with BSA or OA/BSA for different time intervals, then cell lysates were analyzed for N-cadherin or Fyn kinase. GAPDH is loading control.

disassembly (Fig. 5C). NH4-Cl has been used to inhibit endosome-lysosome system acidification by neutralizing the pH of these lysosomal organelles.[30] Pretreatment with NH_4Cl (5 mM) inhibited OA-induced AJ disruption (Fig. 5D). These results indicate that the degradation of N-cadherin at adherens junction is both proteasomal and endosome-lysosomal dependent.

DISCUSSION

The stability and function of cadherins are affected by the phosphorylation state of cadherins and ctns.[17,23,31] c-Src kinase and Fyn kinase are two Src family kinases that control the stability of AJs.[14,23,32] c-Src is not detected in neonatal cardiomyocytes, and only Fyn kinase is activated by OA treatment.[17] Thus, we paid attention on the Fyn kinase in this study. We showed that the OA-induced tyrosine phosphorylation of N-cadherin was mediated through a Fyn kinase-dependent mechanism. This modification of N-cadherin might accelerate the degradation of N-cadherin by the proteasomal and lysosomal pathways, resulting in disruption of the AJ. Consistent with our results, in *v-src*-transformed cell lines, enhanced tyrosine phosphorylation of cadherin and ctns correlates with decreased cadherin-mediated cell adhesion.[13] Tyrosine phosphorylation of VE-cadherin at two critical tyrosines, Tyr658 and Tyr731, is sufficient to prevent the binding of p120ctn

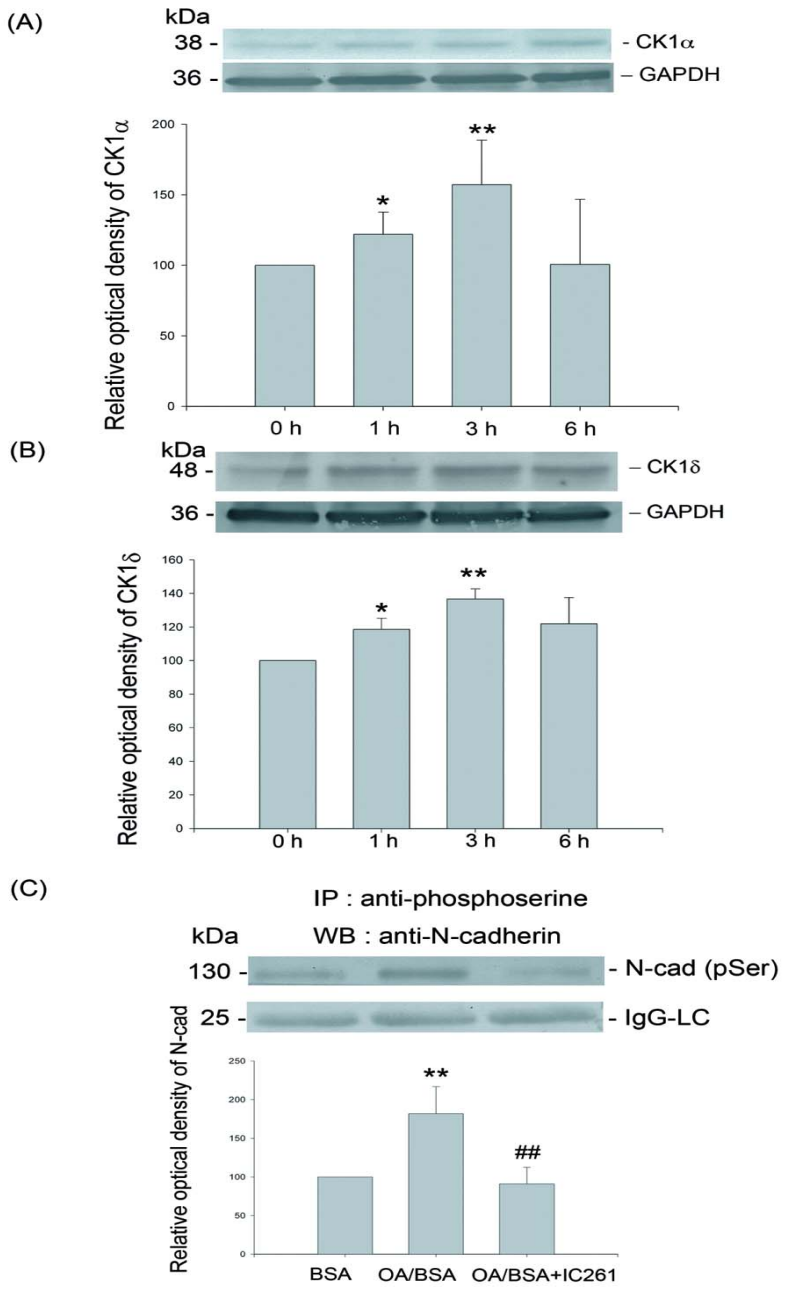

Fig. 3 OA stimulates CK1 expression and induces CK1-dependent serine phosphorylation of N-cadherin. (A) CK1 expression. Cardiomyocytes were treated with OA/BSA for 0, 1, 3, or 6 h, then total cell lysates were analyzed for CK1α (A) and CK1δ (B) by immunoblotting. n = 3. *, $p < 0.05$, **, $p < 0.01$, compared to the 0 h group. (C) CK1-dependent serine phosphorylation of N-cadherin. Cardiomyocytes were treated for 1 h with BSA alone or OA/BSA, or pretreated for 30 min with IC261 (10 μM), then treated for 1 h with OA/BSA in the continued presence with IC261. Cell lysates were immunoprecipitated with anti-phosphoserine antibodies and the immunoprecipitates analyzed for N-cadherin and the immunoglobulin G light chain (IgG-LC). ** $p < 0.01$ compared to the BSA group. ##, $p < 0.01$ compared to the OA/BSA-treated group. n =3.

and β-ctn, respectively, to the cytoplasmic tail of VE-cadherin.[33]

CK1 proteins have been assumed to be constitutively active serine/threonine kinases.[34] Overexpression of CK1δ increased CK1δ enzyme activity and downregulation of CK1δ by siRNA decreased CK1δ activity.[35] Thus, the time-dependent increase in CK1δ expression induced by OA treatment might represent an increase in CK1δ activity. It has been reported that dephosphorylation of autophosphorylation sites or C terminus truncated of CK1 elevated its activity.[36-38] However, it is still unclearly known what physiologically regulates CK1 activity.[22] IC261 inhibited serine phosphorylation of OA induced by OA, and prevented OA-induced splitting of AJ. These data support that serine phosphorylation of N-cadherin contributes to AJ disruption. The concentration of 10 μM IC261 used in this study inhibits three CK1 isoforms, α, δ, and ε.[39,40] We exclude the possible involvement of CK1ε, since CK1ε is absent in rat cardiomyocytes by immunoblotting in this study (data not shown). The possible link between Fyn kinase and CK1 kinase remains to be studied.

We also observed that the number of cytoplasmic N-cadherin punctates increased with time of OA treatment. It is possible that these N-cadherin punctates represent protein aggregates of tyrosine- or serine-phosphorylated N-cadherin associated with proteasomes or lysosomes/endosomes. However, double-labeling for EEA-1 and N-cadherin showed that only some N-cadherin punctates were co-distributed with early endosomes. Whether the majority of the cytosolic punctates are localized in another structure(s) remains to be determined. Saitoh *et al.* reported that proteasome inhibition stabilizes cell

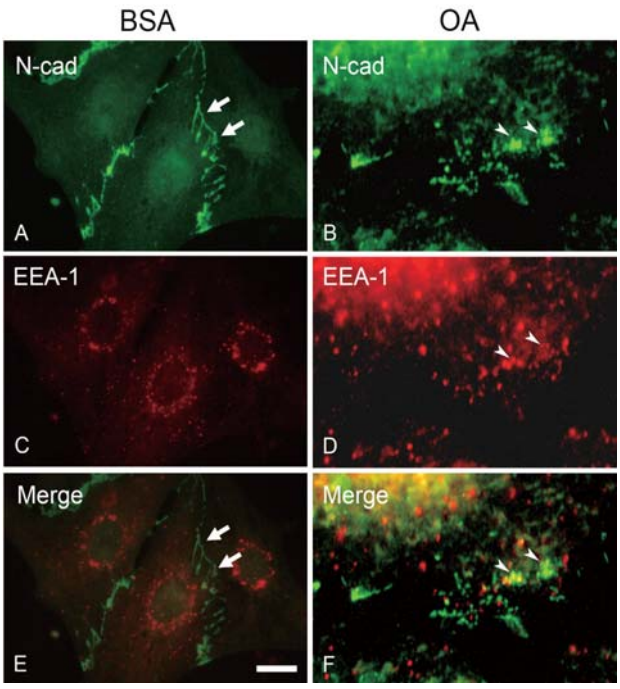

Fig. 4 Distribution of EEA-1 and N-cadherin in cardiomyocytes. Cardiomyocytes were treated with BSA alone (A, C, E) or OA/BSA (B, D, F) for 3 h, then immunostained for the early endosome marker, EEA-1 (C, D) and N-cadherin (A, B). The arrowheads in B indicate N-cadherin punctates in the cytosol. In the BSA-treated group, N-cadherin is found only at cell-cell contacts (arrows in A, E), while EEA-1 is distributed in perinuclear vesicles (endosomes), and the merged image (E) does not show any colocalization. In the OA-treated cardiomyocytes, cytosolic N-cadherin punctates are increased (arrowheads, B). The arrowheads in the merged image (F) indicate endosomes containing N-cadherin. scale bar = 10 μm.

surface E-cadherin during TGF-β-induced epithelial-mesenchymal transition.[41] The movement of some viral particles from the early endosome to the caveosome is blocked by NH_4Cl.[42] NH_4Cl was therefore used as an endosome-lysosome inhibitor. We observed that MG132 or NH_4Cl effectively prevented the OA-induced disruption of junctional N-cadherin. It is possible that phosphorylated N-cadherin is degraded by proteasomal or lysosomal pathway. In summary, the present experiments provide novel information on the signaling pathway involved in the OA-induced tyrosine and serine phosphorylation of N-cadherin and AJ disassembly.

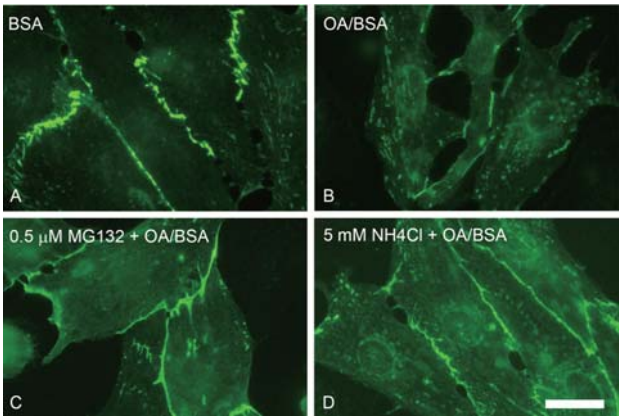

Fig. 5 Proteasomal and lysosomal inhibitors prevent OA-induced AJ disassembly. Cardiomyocytes were treated with BSA alone (A) or OA/BSA for 24 h (B), then cotreated for 24 h with proteasomal inhibitor MG132 (0.5 μM) and OA/BSA (C) or with lysosomal inhibitor NH_4Cl (5 mM) and OA/BSA (D), then stained for N-cadherin. Scale bar: 10 μm.

DISCLOSURE

The authors declare that they have no conflict of interest.

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ABBREVIATIONS USED

AJ, adherens junction; BSA, bovine serum albumin; ctns, catenins; CK, casein kinase; CTN, catenin; HBSS, Hank's balanced salt solution; MEM, minimal essential medium; OA, oleic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; PTP, protein tyrosine phosphatase; SDS-RIPA, sodium dodecyl sulfate-radioimmunoprecipitation assay; TBS, Tris-buffered saline.

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