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Research Article

Mitochondrial dysfunction-induced amphiregulin upregulation mediates chemo-resistance and cell migration in HepG2 cells

C.-J. Chang[a, b, +], P.-H. Yin.[c], D.-M. Yang[c, d], C.-H. Wang[a], W.-Y. Hung[a], C.-W. Chi[a, c], Y.-H. Wei[b, †, *] and H.-C. Lee[a, †, *]

[a] Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, 112, Taiwan (Republic of China), Fax: +886-2-28264372, e-mail: hclee2@ym.edu.tw
[b] Department of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, 112, Taiwan (Republic of China), Fax: +886-2-28264843, e-mail: joeman@ym.edu.tw
[c] Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, 112, Taiwan (Republic of China)
[d] Institute of Biophotonics, National Yang-Ming University, Taipei, Taiwan (Republic of China)

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Abstract. The aim of this study was to investigate the contribution of mitochondrial dysfunction to chemo-resistance and migration of hepatoma cells. We found that inhibition of mitochondrial respiration and mitochondrial DNA (mtDNA) depletion resulted in induction of amphiregulin (AR) expression in HepG2 cells. Upon oligomycin treatment of HepG2 cells, the cytosolic Ca^{2+} was significantly raised after 30 min, and the intracellular level of reactive oxygen species (ROS) was elevated 2.2-fold after 4 h. Moreover, the condition medium of oligomycin-treated HepG2 cells was found to stimulate the migration of SK-Hep-1 cells. On the other hand, oligomycin-induced cisplatin-resistance and cell migration of HepG2 cells were attenuated by AR-specific RNA interference (#L-017435, Dharmacon) and a neutralizing antibody (MAB262, R&D Systems), respectively. Together, these findings suggest that mitochondrial dysfunction induced Ca^{2+} mobilization, and ROS overproduction, which modulated the chemo-resistance and migration of hepatoma cells through the induction and activation of AR.

Keywords. ROS, Ca^{2+}, ADAM17, EGFR, oligomycin, mtDNA depletion.

Introduction

Mitochondria play important roles in energy production, Ca^{2+} homeostasis, as well as in the integration and execution of the apoptotic pathways in mammalian cells [1]. Mitochondrial genome instability and reduction in the copy number of mitochondrial DNA (mtDNA) might lead to serious consequences, such as defective energy metabolism, increased mitochondrial oxidative stress, and apoptosis. About eight decades ago, Otto Warburg noticed that tumor cells mainly utilize glycolysis instead of oxidative phosphorylation for energy supply even under aerobic conditions [2]. In 1950 s, Warburg proposed that mitochondrial respiratory function impairment may contribute to the initiation and/or progression of cancers [3].

+ Present address: Department of Food, Health and Nutrition Science, School of Agriculture, Chinese Culture University, Taipei, 111, Taiwan, Republic of China. e-mail: zjr6@faculty.pccu.edu.tw
† These authors contribute equally to this work.
* Corresponding authors.

In the past few decades, mitochondrial defects have been identified in various human cancers, including hepatoma [4–9]. Mitochondrial defects including mtDNA mutations, altered expression of mitochondrial genes, and impairment of respiratory enzymes have been well documented in cancer tissues. In hepatoma, not only accumulation of somatic mtDNA mutations in the D-loop region but also reduction of the copy number of mtDNA were observed in cancer tissues [10–15]. For pathological aspects, it was shown that mtDNA mutations might aggravate cancer progression by preventing apoptosis and increase tumorigenicity [16, 17]. These observations suggest that mtDNA mutation and mitochondrial dysfunction may play important roles in the development and progression of human hepatoma. Detailed mechanisms, however, remain unclear.

Cisplatin, doxorubicin and other chemotherapeutic drugs were used in combination to treat patients with metastatic hepatoma in previous clinical trials [18–20]. These combination chemotherapies for patients with metastatic hepatoma showed modest efficacy. Besides, doxorubicin and cisplatin have been used as postresectional adjuvant intraportal chemotherapy for patients with hepatoma. This therapy was effective for patients with stages I and II tumor-node-metastasis but not for those with stages III and IV hepatoma [21]. Thus, we have been interested in exploring whether mitochondrial dysfunction affects the sensitivity to chemotherapeutic agents and cell migration in hepatoma cells. Mitochondrial dysfunction-induced production of reactive oxygen species (ROS) were reported to affect the phenotype and invasive behavior of cancer cells [22–24]. A recent study revealed that mitochondrion may use ROS as signaling molecules to modulate cell cycle progression [25]. In one of our previous studies, we observed that mtDNA depletion and mitochondrial dysfunction are correlated with the tumor stages of hepatoma [11]. Therefore, one of the aims of this study was to investigate whether mitochondrial dysfunction induces ROS activation and modulates malignant phenotype of hepatoma cells.

One of our previous studies revealed that the mitochondrial respiratory dysfunction may affect biological processes, such as protein metabolism and modification, in HepG2 cells [26]. In that study, mitochondrial respiratory dysfunction was induced by a mtDNA transcriptional inhibitor (ethidium bromide; EtBr) and a mitochondrial translational inhibitor (chloramphenicol; CAP), respectively, in HepG2 cells [27, 28]. We observed that these treatments could significantly deplete mtDNA and inhibit mitochondrial respiration in HepG2 cells (Supplemental Fig. 1). Furthermore, we performed whole-genome cDNA microarray analysis and found that the expression of amphiregulin (AR) was upregulated by mitochondrial dysfunction in HepG2 cells (Supplemental Table).

AR was identified as a protective protein in liver tissue because AR expression was not detected in healthy liver, but was induced in liver with chronic injury [29]. However, a previous study showed that overexpression of AR mRNA correlated with liver cirrhosis and hepatoma [30]. Moreover, the overexpression of AR was upregulated through the activation of epidermal growth factor receptor (EGFR) autocrine and/or paracrine loop, which was shown to be involved in the transformation of hepatoma [31]. In addition, recent studies suggested that AR was critical to resistance to doxorubicin, gefitinib, and cisplatin, respectively, in breast and lung cancers [32, 33]. Thus, it is very likely that AR overexpression and autocrine/paracrine loop may play an important role in hepatoma progression. In this study, we intended to investigate the role of AR in the modulation of the effect of mitochondrial dysfunction on chemo-resistance and cell migration of hepatoma HepG2 cells.

Materials and methods

Cell culture. HepG2, and SK-Hep-1 hepatoma cell lines were cultured according to the instructions of American Type Culture Collection (ATCC), and supplemented with 50 units/mL penicillin G and 50 μg/mL streptomycin sulfate. Human osteosarcoma 143B cells and mtDNA-less (rho^0) cells were grown in DMEM supplemented with 100 μg/mL pyruvate, 50 μg/mL uridine, 50 units/mL penicillin G, and 50 μg/mL streptomycin sulfate. All of the reagents for cell culture were obtained from Gibco/BRL Co. (Bethesda, MD). Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Small interfering RNAs transfection. The small interfering RNAs (siRNA) of mitochondrial transcription factor A (siTFAM; #M-019734), AR (siAR; #L-017435), a disintegrin and metalloproteinase domain 17 (ADAM17) (siADAM17; #L-003453) and non-targeting control siRNA (siCon; #D-001206) were obtained from siGENOME SMARTpool of Dharmacon Research (Lafayette, CO). Each siRNA set was designed to target four different regions of the specific gene. Cells were transfected with the siRNA at a concentration of 100 nM using the DharmaFECT siRNA transfection reagent in a serum-free medium. After 24 h of transfection, the medium was replaced with the complete culture medium containing vehicle or the indicated reagent.

DNA extraction and measurement of mtDNA copy number. The mtDNA copy number was determined according to a previously reported method [8]. For quantification of mtDNA, the threshold cycle (Ct) values of the 18S gene and the mitochondrial NADH dehydrogenase 1 (ND1) gene were analyzed in each individual run of the quantitative PCR. The mtDNA content in a cell is represented by $-\Delta\Delta Ct$ (ND1 gene to 18S gene). (Supplemental Fig. 2)

RNA extraction and quantitative real-time reverse transcription (RT)-PCR analysis. The gene expression level was assessed according to a previously reported method [15]. The alteration in the expression level of AR and ADAM genes was determined relative to the 18S rRNA gene in the sample (AR gene: forward, 5'- GGA GAA GCT GAG GAA CGA AA -3'; reverse, 5'- TGG CTA TGA CTT GGC AGT GA -3'. ADAM17 gene: forward, 5'- CTG TGG TGC AAA AGC AGA AA -3'; reverse, 5'- TGC CAA ATG CCT CAT ATT CA-3'; 18S gene: forward, 5'- ATC AAC TTT CGA TGG TAG TCG-3'; reverse 5'- TCC TTG GAT GTG GTA GCC G-3').

Enzyme-linked immunosorbent assay. An aliquot of 10^5 cells was seeded in a six-well plate and incubated with drugs to induce mitochondrial dysfunction. Condition medium was obtained after culture of cells at indicated periods of time. The concentration of AR in the condition medium was measured by the AR DuoSet ELISA kit (DY262; R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer.

Measurement of ATP content. Cells (1×10^5) were seeded in a six-well plate and incubated with 0.5 μg/mL oligomycin for indicated periods of time. The ATP content in the cells was then measured using an ATP bioluminescence assay kit (Roche Applied Science, Mannheim, Germany) according to the instruction of the manufacturer.

Measurement of oxygen consumption rate. An aliquot of 5×10^5 cells was seeded in a 10-cm petri dish and incubated with 0.5 μg/mL oligomycin for indicated periods of time. The rate of oxygen consumption of the cells was measured according to a previously reported method [34].

Cell cycle analysis by flow cytometry. After 36 h of treatment with cisplatin (10 μM; Platinex® Bristol-Myers Squibb, New York, NY), the HepG2 cells were trypsinized and resuspended in 70% ethanol solution. Cell suspension was stored at 4 °C until further analysis. Cell cycle analysis was performed according to a previously reported method [35]. In each measurement, a minimum of 15 000 cells were analyzed. Data were acquired and analyzed using the CellQuest™ software (BD Bioscience, San Diego, CA).

Preparation of condition medium. HepG2 cells (1×10^7) were seeded in a 15-cm petri dish, and mitochondrial dysfunction was then induced by treatment with oligomycin (0.5 μg/mL) for 1 h. To exclude the potential non-specific effect of oligomycin, the cultured medium was discarded and cells were washed twice with serum-free medium. HepG2 cells were then incubated in 25 mL of serum-free medium for 12 h. The condition medium was collected for following assays. Moreover, the AR neutralizing antibody (MAB262, R&D Systems) and mouse IgG_1 isotype control antibody (MAB002, R&D Systems) were used to examine the effects of AR paracrine on cell migration of hepatoma SK-Hep-1 cells.

Transwell migration assay. Transwell migration assay was carried out with a 24-well chamber (Costar 3422, Corning Inc., Corning, NY). The lower and upper chambers were separated by a polycarbonate membrane (8 μm pore size). Approximately 8×10^3 SK-Hep-1 cells were resuspended in 0.1 mL condition medium of HepG2 cells with and without pretreatment of 0.5 μg/ml oligomycin, respectively. The DMEM containing 20% FBS was added to the lower chamber. Cells were allowed to migrate for 16 h at 37 °C in a humidified atmosphere containing 5% CO_2. The membrane was fixed in methanol for 10 min at 4 °C, and then stained with modified Giemsa stain solution for 30 min. Cells on the upper side of the membrane were removed by PBS-rinsed cotton swabs. Cells on the lower side of the membrane were counted under a light microscope with the 10× objective lens. Two individuals blinded to the treatment of the transwell filter counted cells from four random fields in each of two wells per treatment; and the results were pooled. Each experiment was performed in triplicate.

Immunoprecipitation and Western-blot analysis. Approximately 2×10^6 cells were seeded in a 10-cm petri dish and incubated with desired chemicals. At the indicated period of time, the treated cells were harvested and analyzed according to the reported method [36]. The activation of EGFR was assessed by immunoprecipitation with an anti-EGFR antibody (BD Biosciences, San Jose, CA), and probed with an anti-phospho-tyrosine antibody (clone 4G10, Upstate Biotechnology Inc., New York, NY) at 4 °C overnight and followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Health-

care Bio-Sciences Co., Piscataway, NJ). Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents (Immobilon, Millipore Co., Billerica, MA).

Determination of intracellular ROS. ROS production in cells was measured using the probe DCFH-dA (Molecular Probes-Invitrogen, Carlsbad, CA) according to a previously reported method [37]. Cells were incubated with 5 μM DCFH-dA in culture medium for 1 h at 37 °C, washed, and resuspended in 0.3 mL PBS, and subjected to flow cytometric analysis.

Live cell imaging for measurement of $[Ca^{2+}]_i$. The Ca^{2+} imaging by using Fura-2 was carried out by a method described previously [38]. The basal values of the Fura-2 ratio (F_{340}/F_{380}) recorded for 30 s were the average of results from three random fields of views in each experiment.

Cell viability assay. The effects of AR on anticancer drug-resistance of HepG2 cells were estimated by the release of lactate dehydrogenase (LDH). HepG2 cells were transfected with siAR or siCon one day before treatment with oligomycin to induce mitochondrial dysfunction. Approximately 5×10^3 cells were seeded in a 96-well culture plate with 100 μL of complete medium, and then incubated overnight. Oligomycin was added to the medium at a final concentration of 0 or 0.5 μg/mL for 1 h, and the medium was then replaced by the complete medium containing cisplatin 0, 4, and 8 μM, respectively. Cells were incubated for 48 h before LDH assay according to the procedure recommended by the manufacturer (Promega, Madison, WI).

Statistics. Data are shown as the mean ± SEM except where indicated. Statistical comparison of data between groups was performed using one-way analysis of variance (ANOVA), followed by Student's t-test. A P value < 0.05 is considered statistically significant.

Results

Mitochondrial dysfunction upregulates the expression and secretion of AR. Firstly, quantitative real-time RT-PCR was performed to verify the expression level of AR mRNA in HepG2 cells with mitochondrial dysfunction. As shown in Figure 1A, AR mRNA expression was significantly induced in EtBr and CAP-treated HepG2 cells. Additionally, we found that lack of mtDNA in the rho^0 cell is associated with a higher expression of AR as compared with parental 143B cells. To exclude the potential non-specific effect of mtDNA depletion induced by chemicals, we depleted mtDNA by transfection of siRNA of mitochondrial transcription factor A (TFAM; controls the transcription, replication, damage sensing, and repair of mtDNA). The results showed that knockdown of TFAM decreased the copy number of mtDNA and increased AR expression of HepG2 cells to 0.33- and 2.4-fold of the control, respectively (Fig. 1B). Thus, the AR induction was correlated with mitochondrial dysfunction in HepG2 and osteosarcoma 143B cells. To test whether increased expression of AR is a common response of human cells to mtDNA depletion and mitochondrial respiratory function defect, we treated HepG2 cells with non-lethal concentrations of oligomycin, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), rotenone, and antimycin A, respectively. The results showed that all these treatments caused an increase in the AR expression of HepG2 cells (Fig. 1C) but did not significantly affect cell viability under these treatments.

To examine whether the excessively expressed AR was secreted to the medium, we measured AR release in the condition medium of HepG2 cells that had been subjected to mitochondrial stress. As shown in Figure 1D, under both genetic and metabolic stress conditions, AR release was significantly increased in HepG2 cells. Additionally, the treatment of oligomycin induced AR transcription in a time-dependent manner (Fig. 1E).

Treatment of oligomycin induces mitochondrial dysfunction, chemo-resistance and cell migration. In this study, we treated HepG2 cells with oligomycin, a mitochondrial ATP synthase inhibitor, up to 24 h to rapidly induce mitochondrial dysfunction. Figure 2A shows that after 6 h of treatment with 0.5 μg/mL oligomycin, the ATP level was significantly decreased as compared with that of the control. Additionally, the oxygen consumption rate was significantly decreased after treatment of cells with oligomycin for 1 min (Fig. 2B). This result indicated that the treatments of oligomycin effectively induced mitochondrial dysfunction in HepG2 cells.

To evaluate the effects of mitochondrial dysfunction on chemo-sensitivity, HepG2 cells were treated with oligomycin for 1 h, and then transferred to a fresh medium containing 10 μM of cisplatin to allow the cells to grow for 36 h. The results showed that the cisplatin-induced accumulation of cells at S/G2M phase was attenuated by oligomycin-induced mitochondrial dysfunction (Fig. 2C). The effects of mitochondrial dysfunction on the migration of hepatoma cells were also analyzed. To exclude the potential non-specific effect of oligomycin, we washed and transferred cells to fresh serum-free medium after 1 h of

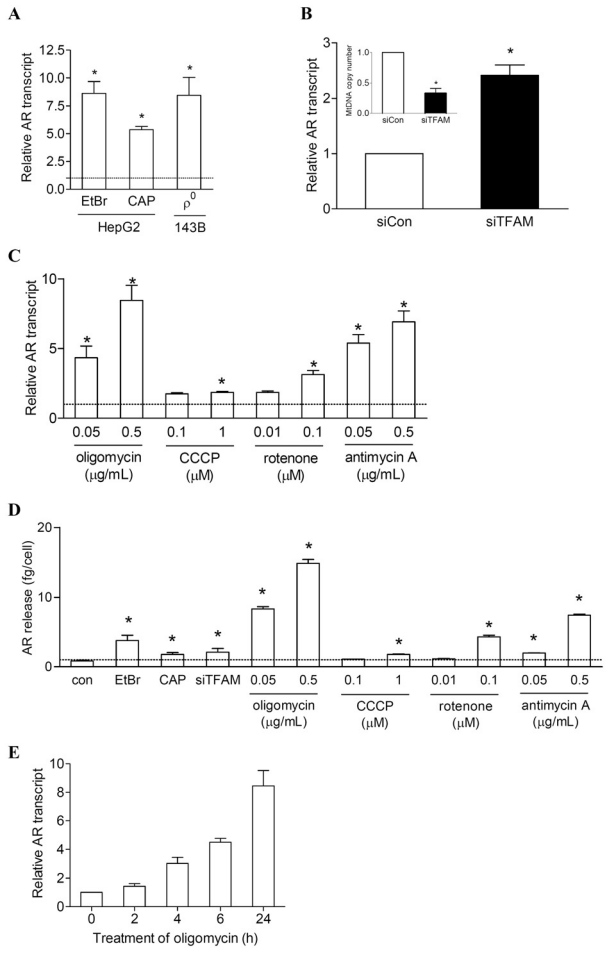

Figure 1. Mitochondrial dysfunction induced amphiregulin (AR) overexpression and release in HepG2 cells. (*A*) The mitochondrial genetic stress was induced by treatment of cells with EtBr for seven days, and CAP for two days, as well as by (*B*) small interfering RNA knocked down the mitochondrial transcription factor A (siTFAM). (*C*) Mitochondrial metabolic stress was induced by 24 h treatment of HepG2 cells with an ATP synthase inhibitor (oligomycin), mitochondrial uncoupling agent (carbonyl cyanide m-chlorophenylhydrazone; CCCP), Complex I inhibitor (rotenone), and Complex III inhibitor (antimycin A), respectively. Gene expression level was measured by quantitative real-time PCR. The AR and mitochondrial ND1 (mtND1) gene expression levels were normalized by 18S rRNA (18S) in cDNA and 18S nDNA (18S) in genomic DNA, respectively. The AR expression levels in osteosarcoma 143B parental and rho^o cells were also measured. (*D*) Genetic and metabolic stress in mitochondria induced AR release in HepG2 cells as measured by ELISA. (*E*) Treatment of HepG2 cells with 0.5 μg/mL oligomycin induced AR expression in a time-dependent manner. Data are the mean ± SEM of the results from three independent experiments. * $P<0.05$ as compared with vehicle.

treatment with oligomycin to induce mitochondrial dysfunction in HepG2 cells. Due to the low mobility of the HepG2 cell line, a highly invasive SK-Hep-1 cell line was selected to evaluate the potential of HepG2 paracrine to induce the migration of hepatoma cells. SK-Hep-1 cells were resuspended in the 12-h condition medium of the control cells and the cells with mitochondrial dysfunction, respectively. We found that the transwell migration behavior of SK-Hep-1 was significantly increased by the condition medium of oligomycin-treated HepG2 cells (Fig. 2D). These results suggest that mitochondrial dysfunction may modulate chemo- resistance and cell migration of hepatoma cells.

Mitochondrial dysfunction-induced alterations of cytosolic Ca^{2+}, and intracellular ROS regulate overexpression and release of AR. We further examined whether oligomycin could increase cytosolic Ca^{2+}, and intracellular ROS. Figure 3A shows that the cytosolic Ca^{2+} was significantly raised after 30 min of treatment with oligomycin (Fig. 3A). In addition, the level of ROS was elevated by 2.2-fold as compared with that of the control (Fig. 3B). These results indicated that oligomycin could increase cytosolic Ca^{2+} as well as ROS in HepG2 cells.

Next, we examined whether mitochondrial dysfunction-elicited oxidative stress, and whether a rise of cytosolic Ca^{2+} was involved in the overexpression and release of AR. HepG2 cells were pretreated with a Ca^{2+} chelator BAPTA-AM, and antioxidants N-acetyl cysteine (NAC), and vitamin E, respectively, in the presence or absence of oligomycin for 24 h. The results revealed that these compounds significantly alleviated AR overexpression and secretion in the cells with mitochondrial dysfunction (Fig. 4A and 4B). These results suggest that mitochondrial dysfunction induced cytosolic Ca^{2+} rise, and oxidative stress, which were involved in the induction and secretion of AR.

Mitochondrial dysfunction activates AR autocrine loop via EGFR. We then investigated whether mitochondrial dysfunction activates AR autocrine loop in HepG2 cells. The signal transduction blocker of EGFR (AG1478), and a transcriptional inhibitor actinomycin D, as well as an AR neutralizing antibody and its isotype control were used to pre-treat HepG2 cells 2 h before oligomycin treatment. Figure 5A shows that these inhibitors and the neutralizing antibody attenuated AR overexpression. Moreover, AG1478 inhibited oligomycin-induced AR release (Fig. 5B). These results suggest that mitochondrial dysfunction induced a self-perpetuating AR autocrine loop. In addition, activation of EGFR was found to be involved in the regulation of this AR loop (Fig. 5C). Furthermore, we determined whether AR release was regulated by *de novo* protein synthesis or occurred through the ADAM17 (a disintegrin and metalloproteinase domain 17)-mediated pro-AR cleavage. The HepG2 cells were treated with cycloheximide, a general inhibitor of protein synthesis, or inhibitor of ADAM17 (GM6001) 2 h before induction of mitochondrial dysfunction by oligomycin. The

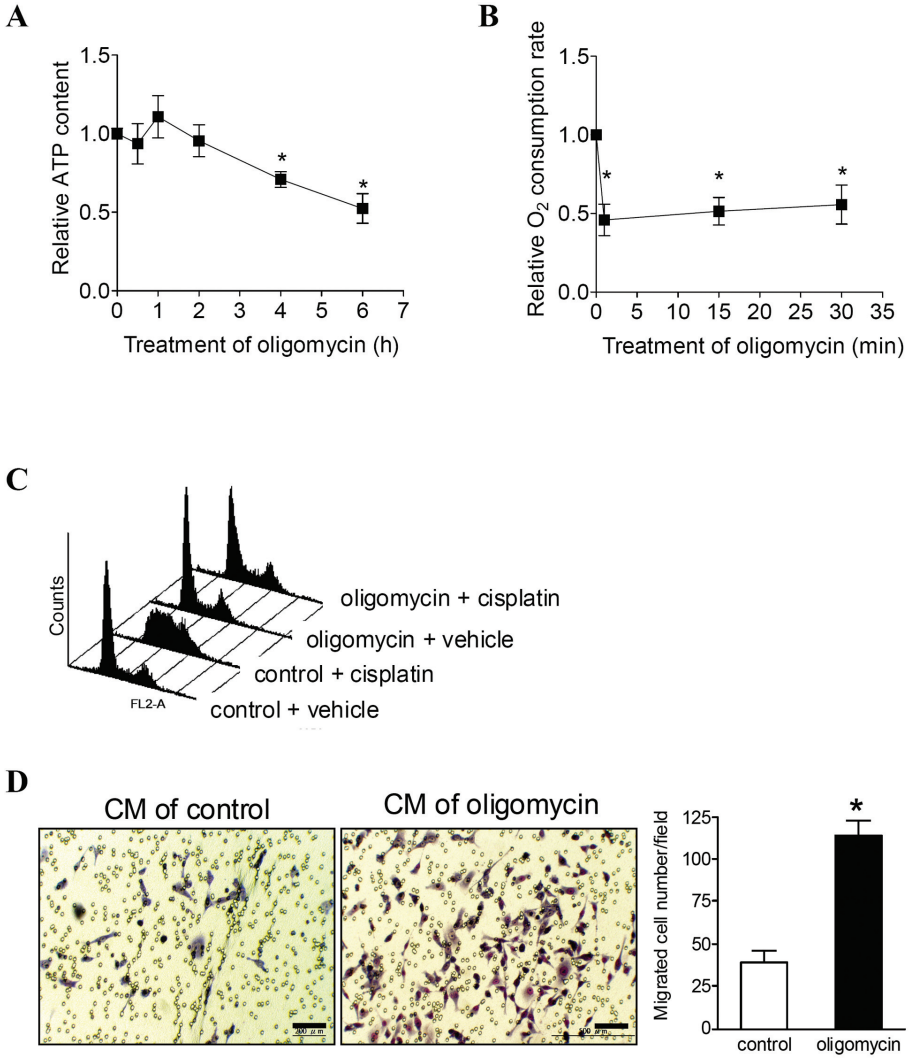

Figure 2. Mitochondrial dysfunction induced cisplatin-resistance of HepG2 cells and cell migration of SK-Hep-1 cells. Treatment of cells with 0.5 μg/mL oligomycin induced mitochondrial dysfunction, such as (*A*) depletion of ATP, (*B*) decrease of oxygen consumption in HepG2 cells. The intracellular content of ATP, and oxygen consumption rate of HepG2 cells were measured at indicated periods of time. The data were normalized by that of the control group at the same time. *$P < 0.05$ as compared with the 0 h group. (*C*) Mitochondrial dysfunction was induced by 1 h treatment with 0.5 μg/mL oligomycin in HepG2 cells. Cells were then maintained in the fresh complete medium containing cisplatin (0 or 10 μM). After 36 h of incubation, the DNA content was measured by flow cytometry. (*D*) After induction of mitochondrial dysfunction by 1 h treatment of HepG2 cells with 0.5 μg/mL oligomycin, the medium was replaced by a fresh serum-free medium. Condition medium (CM) of HepG2 was collected after 12 h of incubation. Hepatoma SK-Hep-1 cells were resuspended in CM of HepG2 cells with and without oligomycin treatment, respectively. Transwell migration assay was performed as described in Materials and methods. Data are the mean ± SEM of the results from three independent experiments. *$P < 0.05$ as compared with the control.

results showed that pretreatment with cycloheximide or GM6001 blocked the AR release triggered by oligomycin-induced mitochondrial dysfunction (Fig. 5B). These findings indicate that mitochondrial dysfunction- induced AR release is dependent on the *de novo* protein synthesis and ADAM17 activity. To dissect the role of ADAM17 in the regulation of mitochondrial dysfunction-induced AR induction, the ADAM17 was suppressed by transfection with specific siRNA (siADAM17). As shown in Figure 5D, knock-down of ADAM17 transcript (reduced to 40% of the non-target control) significantly suppressed mitochondrial dysfunction-induced AR induction. The results indicate that the ADAM17 is crucial for AR induction in HepG2 cells with dysfunctional mitochondria. Additionally, the results shown in Figures 4 and 5 suggest that mitochondrial dysfunction in HepG2 cells uses cytosolic Ca^{2+} and ROS as signaling molecules to regulate the overexpression and autocrine loop of AR.

AR involvement in mitochondrial dysfunction-induced chemo-resistance. To further investigate the role that AR may play in mitochondrial dysfunction-induced

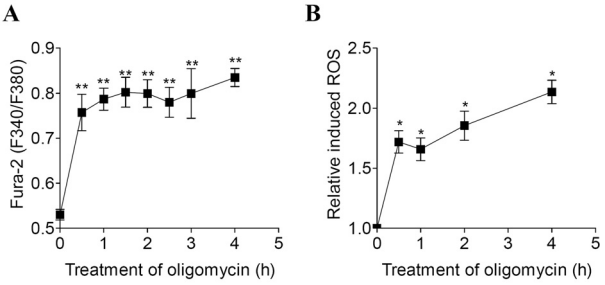

Figure 3. Treatment of oligomycin induced increase of cytosolic Ca^{2+} and production of ROS in HepG2 cells. (*A*) After treatment of the HepG2 cells with 0.5 μg/mL oligomycin for indicated periods of time, cells were stained with 5 μM AM form of fura-2 in the loading buffer for 30 min at 37 °C. The images of the ratiometric Fura-2 (F340/F380) were illuminated by a xenon lamp within a monochrometer, collected by a high-speed cooled CCD camera and recorded using SimplePCI 6.0. The peak values of the Fura-2 ratio were calculated and analyzed by normalizing the image data against the basal level (the first 10 s before treatment). (*B*) Cells were treated with DMSO or 0.5 μg/mL oligomycin for indicated periods of time. Before cells were subjected to determination of the production of ROS by flow cytometry, cells had been washed and incubated with 5 μM DCFH-dA in the culture medium for 1 h at 37 °C. Data are the mean ± SEM of the results from three independent experiments. * $P < 0.05$, or ** $P < 0.001$ as compared with control.

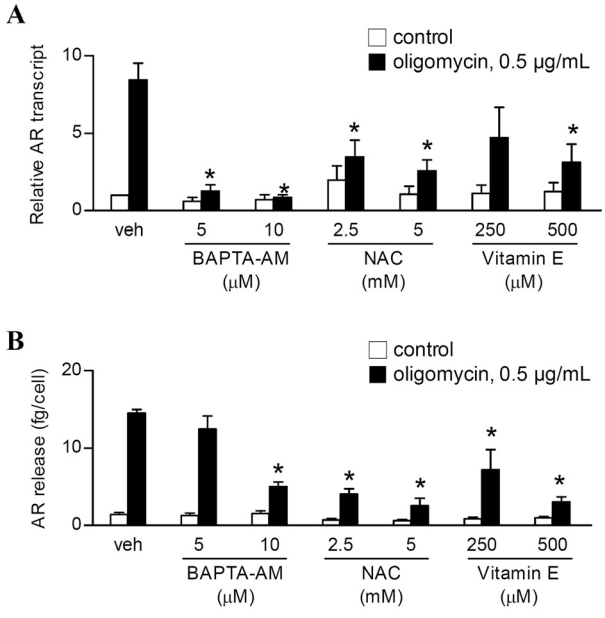

Figure 4. Involvement of mitochondrial dysfunction-induced cytosolic Ca^{2+} and ROS in AR induction and release. Ca^{2+} chelator (BAPTA-AM), and antioxidants (N-acetyl cysteine, NAC; and vitamin E) were added to the medium 2 h before induction of mitochondrial dysfunction by 0.5 μg/mL oligomycin in HepG2 cells. After treatment of cells with oligomycin for 24 h, the (*A*) AR expression and (*B*) AR release were measured by quantitative real-time PCR and ELISA, respectively. * $P < 0.05$ as compared with vehicle.

chemo-resistance, we examined the cell cycle and cytotoxicity of cisplatin and/or doxorubicin in HepG2 cells after AR knockdown by siRNA. We found that AR release of HepG2 cells was reduced to 52 % of the control after siAR transfection (Fig. 6A). The suppression of AR expression resulted in an increase of the sensitivity of HepG2 cells to cisplatin (Fig. 6A and 6B) and doxorubicin (Fig. 6C). This suggests that mitochondrial dysfunction-induced chemo-resistance was related to AR induction. Nevertheless, the addition of recombinant AR could not induce resistance of HepG2 cells to cisplatin (Supplemental Fig. 3).

Involvement of AR paracrine in mitochondrial dysfunction-induced cell migration. To further test whether mitochondrial dysfunction-induced AR paracrine could mediate invasion of SK-Hep-1 cells, we added the AR neutralizing antibody to the condition medium of HepG2 cells. HepG2 cells were treated with oligomycin for 1 h, and then transferred to a serum-free fresh medium for another 12 h. The concentration of AR in the condition medium was then measured by ELISA. The results showed that treatment of cells with oligomycin induced AR release in a time-dependent manner. Moreover, a significant increase in the AR release began at 4 h after oligomycin treatment. The condition medium after 12-h growth of HepG2 cells contained about 40 and 500 pg/mL of AR in the control cells and cells with mitochondrial dysfunction, respectively (Supplemental Fig. 4).

As shown in Figure 6D, the cell migration induced by the condition medium of HepG2 cells was abolished when the AR neutralizing antibody had been added. Accordingly, AR is a key factor to confer the ability of migration of SK-Hep-1 cells. Since SK-Hep-1 is an AR-null cell line [30], this AR-regulated cell migration was solely contributed by the AR in the condition medium of HepG2 cells.

Discussion

Mitochondrial genome instability and mtDNA alteration were frequently observed in human hepatoma. In this study, we demonstrated that mitochondrial dysfunction induced the expression of AR in HepG2 cells. Mitochondrial dysfunction was found to induce a cytosolic Ca^{2+} rise and ROS overproduction, which elicited the overexpression and activation of AR. Meanwhile, mitochondrial dysfunction modulated chemo-resistance and cell migration of hepatoma cells via the AR autocrine and paracrine. These results imply that AR plays a key role in mitochondrial dysfunction-mediated malignancy of hepatoma.

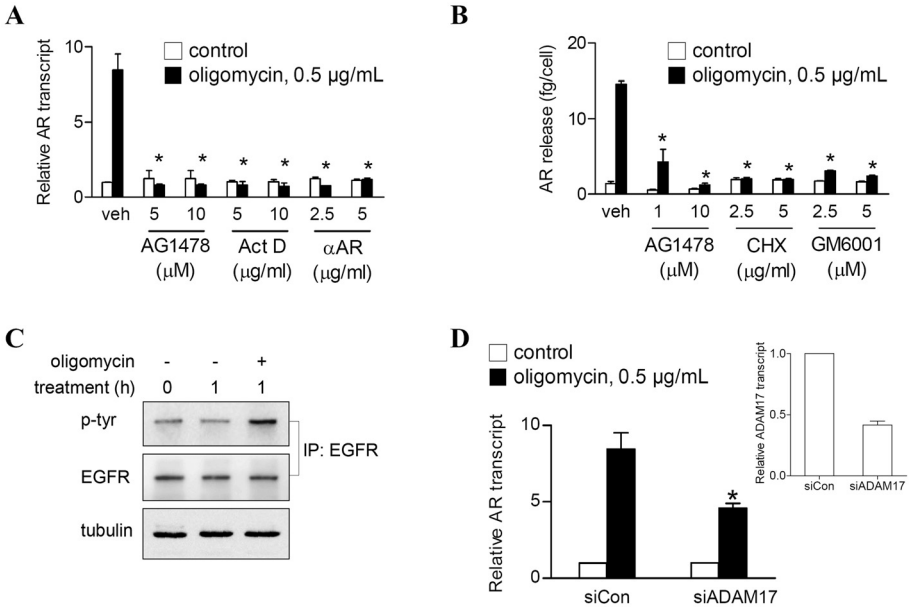

Figure 5. Mitochondrial dysfunction-induced AR autocrine loop, and the ADAM metallopeptidase domain 17 mediated the AR release in HepG 2 cells. (*A*) HepG2 cells were pretreated with EGFR-specific signal transduction blocker (AG1478), transcriptional inhibitor (actinomycin D; Act D), AR neutralizing antibody (αAR), and its isotype control, respectively, for 2 h before treatment with 0.5 μg/mL oligomycin. After 24 h, AR expressions were measured by real-time RT-PCR. (*B*) HepG2 cells were pretreated with AG1478, cycloheximide (CHX) or inhibitor of ADAM17 (GM6001) for 2 h, and mitochondrial dysfunction was then induced by treatment with 0.5 μg/mL oligomycin for 24 h. AR concentration in the condition medium was measured by ELISA. (*C*) Activation of EGFR signaling in HepG2 cells with mitochondrial dysfunction were analyzed by immunoprecipitation and Western-blotting as described in Materials and methods. (*D*) HepG2 cells were transfected with the small interfering RNAs of non-targeting control (siCon) and ADAM17 (siADAM17), respectively, for 24 h before treatment with 0.5 μg/mL oligomycin. After 24 h of incubation, gene expression levels were measured by real-time RT-PCR. Data are the mean ± SEM of the results from three independent experiments. *, $P < 0.05$ as compared with vehicle.

It was reported that the activation of EGFR autocrine and/or paracrine loop and overexpression of AR are important events contributing to the transformation of hepatoma [31]. In this study, we showed that mitochondrial dysfunction triggered AR overexpression, which in turn activated the AR autocrine loop to mediate chemo-resistance and cell migration in HepG2 cells. In addition to HepG2 cells, we also investigated the effects of mitochondrial dysfunction in other hepatoma cell lines including HA22T/VGH, Hep3B, Huh7, and SK-Hep-1. We observed similar upregulation of AR transcription in HA22T/VGH and Hep3B cells, but not in Huh7 and SK-Hep-1 cells under mitochondrial stress (data not shown). Unlike HepG2 cells, no significant AR secretion, cisplatin-resistance or transwell migration were observed in the HA22T/VGH and Hep3B cells. These findings thus suggest that mitochondrial dysfunction-induced AR secretion, but not AR overexpression, is critical for the malignancy of hepatoma cells. Therefore, we further investigated the role of ADAM17 in mitochondrial dysfunction-induced AR release. By knockdown of the ADAM17 transcript, we demonstrated that ADAM17 is an important modulator of mitochondrial dysfunction-induced AR release (Fig. 5). On the other hand, a recent study suggested that ADAM17-mediated release of AR and subsequent activation of EGFR can corrupt the evolution of cancer [39]. Collectively, the detailed mechanism as to how the mitochondrial dysfunction-induced signaling to regulate the activation of ADAM17 to shed pro-AR from cell surface and mediate cancer progression warrants further investigation.

An increase of AR mRNA and elevation of serum level of AR were reported to correlate with gefitinib-resistance and mortality of patients with non-small cell lung carcinoma [33, 40]. A previous study also showed that AR modulates the IGF2/IGF1R survival pathway, which may affect the IGF-1R/EGFR signaling crosstalk in the response to gefitinib of hepatoma cells [41]. In this study, we found that in the absence of AR, the doxorubicin-sensitivity of HepG2 cells with mitochondrial dysfunction was reversed to the level of control (Fig. 6C). This finding suggests that AR contributes to the resistance to doxorubicin induced by mitochondrial dysfunction. Moreover, cisplatin treatment of HepG2 cells showed no significant difference in sensitivity between cells with and without mitochondrial dysfunction (Fig. 6B). However, knockdown of AR significantly increased the sensitivity of HepG2 cells with mitochondrial dysfunction toward cisplatin-induced cell death. Consequently, AR was essential for survival when cells were treated

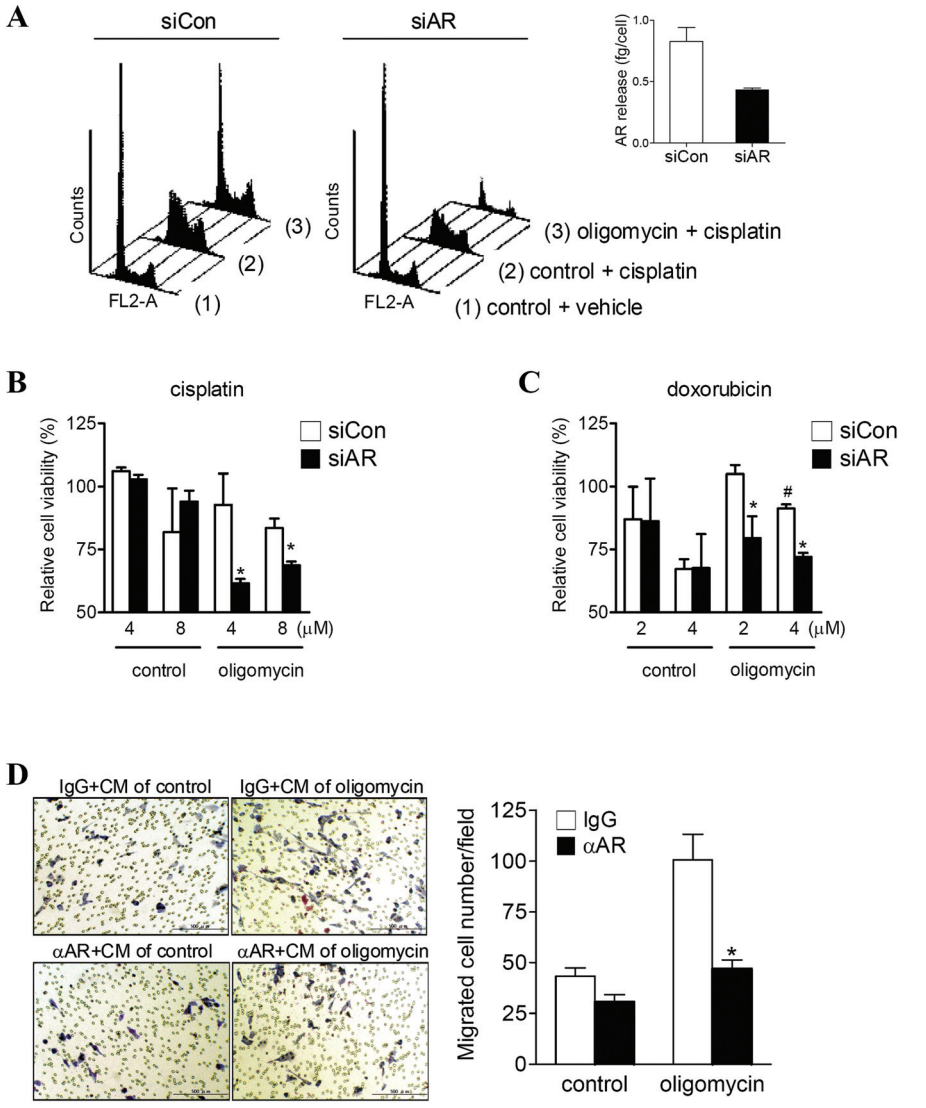

Figure 6. Mitochondrial dysfunction-induced chemo-resistance in HepG2 cells and cell migration in SK-Hep-1 cells was attenuated by AR-specific RNA interference and a neutralizing antibody, respectively. Mitochondrial dysfunction was induced by 1 h treatment of HepG2 cells with 0.5 μg/mL oligomycin one day after transfection with either the small interfering RNAs of non-targeting control (siCon) or AR (siAR). HepG2 cells were then maintained in the fresh complete medium containing indicated concentrations of cisplatin or doxorubicin. (*A*) After 36 h of incubation, the DNA content was measured by flow cytometry. (*B, C*) After 48 h of incubation, the cell viability was measured by the release of lactate dehydrogenase (LDH). * $P < 0.05$ as compared with siCon. # $P < 0.05$ as compared with control. (*D*) SK-Hep-1 migration driven by mitochondrial dysfunction induces AR paracrine was abolished by the AR neutralizing antibody. After induction of mitochondrial dysfunction by 1 h treatment of HepG2 cells with 0.5 μg/mL oligomycin, the medium was replaced by a fresh serum-free medium. Condition medium (CM) of HepG2 was collected after 12 h of incubation, and then mixed with the AR neutralizing antibody (αAR) or mouse IgG_1 isotype control (IgG) to 5 μg/mL of the final concentration. Hepatoma SK-Hep-1 cells were resuspended in CM of HepG2 cells with and without oligomycin treatment, respectively. Transwell migration assay was performed as described in Materials and methods. Data are the mean ± SEM of the results from three independent experiments. *$P < 0.05$ as compared with the IgG control.

with mitochondrial inhibitors plus cisplatin. These observations support the notion that there is a link between mitochondrial dysfunction-induced AR secretion and anticancer drug resistance and poor prognosis of hepatocellular carcinoma.

This study substantiates the importance of AR expression in HepG2 cells with mitochondrial defect, which were subject to the regulation of Ca^{2+}, and ROS. It was reported that AR is under strong regulation by cyclic AMP in various cell types [42]. Moreover, in a 37-bp segment upstream of the TATA box of the pro-AR gene there is a cyclic AMP response element [43], which has been shown to be able to constitutively bind to the CRE binding protein. In addition, a Wilms'

tumor (WT) suppressor responsive element was also found in a 55-bp segment upstream of the TATA box of the pro-AR gene [44]. Consequently, the roles of these two response elements in the mitochondrial retrograde regulation of pro-AR gene expression are worthy of further investigations.

Mitochondrial retrograde signaling is thought to be involved in cancer progression. This mitochondria -to-nucleus stress signaling was initially demonstrated in yeast and further confirmed in murine C2C12 myoblasts and human pulmonary carcinoma A549 cells [22, 45–48]. Under pathological conditions, mitochondria regulate nuclear gene transcription and cellular functions in a retrograde fashion. In mammalian cells, mtDNA depletion caused by drugs or mutagens can elicit disruption of mitochondrial membrane potential, a subsequent increase of cytosolic free Ca^{2+} and activation of calcineurin, which influence regulatory factors such as NFκB, calmodulin- dependent protein kinase IV, and/or their downstream signaling pathways [22, 49]. Here, we show that the mitochondrial dysfunction-induced increase of cytosolic Ca^{2+} as well as production of ROS and their effects on the expression and secretion of AR may account for the increase in chemo-resistance and cell migration of hepatoma cells.

In conclusion, we have demonstrated that mitochondrial dysfunction plays an important role in chemo-resistance and cell migration of hepatoma cells via the modulation of AR secretion. Although the detailed mechanism as to how the induction and release of AR result in poor prognosis of hepatoma patients remains unclear, the observation that AR expression is modulated by mitochondrial dysfunction has provided useful information that may lead to the development of new combination therapies for cancer patients.

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