ARTICLE
Journal of Cellular Biochemistry 107:408–417 (2009)

Journal of Cellular Biochemistry

Arecoline-Induced Phosphorylated p53 and p21^{WAF1} Protein Expression is Dependent on ATM/ATR and Phosphatidylinositol-3-Kinase in Clone-9 Cells

Wen-Wen Chou,[1] Jinn-Yuh Guh,[2,3,4*] Jung-Fa Tsai,[2,3] Chi-Ching Hwang,[5] Shean-Jaw Chiou,[5] and Lea-Yea Chuang[4,5**]

[1]*Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[2]*Department of Internal Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[3]*Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan*
[4]*Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[5]*Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*

ABSTRACT

Betel-quid use is associated with liver cancer whereas its constituent arecoline is cytotoxic, genotoxic, and induces p53-dependent p21^{WAF1} protein expression in Clone-9 cells (rat hepatocytes). The ataxia telangiectasia mutated (ATM)/rad3-related (ATR)-p53-p21^{WAF1} and the phosphatidylinositol-3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathways are involved in the DNA damage response and the pathogenesis of cancers. Thus, we studied the role of ATM/ATR and PI3K in arecoline-induced p53 and p21^{WAF1} protein expression in Clone-9 cells. We found that arecoline (0.5 mM) activated the ATM/ATR kinase at 30 min. The arecoline-activated ATM/ATR substrate contained p-p53Ser15. Moreover, arecoline only increased the levels of the p-p53Ser6, p-p53Ser15, and p-p53Ser392 phosphorylated p53 isoforms among the known isoforms. ATM shRNA attenuated arecoline-induced p-p53Ser15 and p21^{WAF1} at 24 h. Arecoline (0.5 mM) increased phosphorylation levels of p-AktSer473 and p-mTORSer2448 at 30–60 min. Dominant-negative PI3K plasmids attenuated arecoline-induced p21^{WAF1}, but not p-p53Ser15, at 24 h. Rapamycin attenuated arecoline-induced phosphrylated p-p53Ser15, but not p21^{WAF1}, at 24 h. ATM shRNA, but not dominant-negative PI3K plasmids, attenuated arecoline-induced p21^{WAF1} gene transcription. We conclude that arecoline activates the ATM/ATR-p53-p21^{WAF1} and the PI3K/Akt-mTOR-p53 pathways in Clone-9 cells. Arecoline-induced phosphorylated p-p53Ser15 expression is dependent on ATM whereas arecoline-induced p21^{WAF1} protein expression is dependent on ATM and PI3K. Moreover, p21^{WAF1} gene is transcriptionally induced by arecoline-activated ATM. J. Cell. Biochem.107: 408–417, 2009.

KEY WORDS: ARECOLINE; mTOR; PI3K; ATM/ATR; p21^{WAF1}

Betel-quid (*Areca catechu*) is used by approximately 10% of the world population [Boucher and Mannan, 2002; Guh et al., 2006]. Betel-quid use is associated with oral cancer, obesity, diabetes mellitus, hypertension, hyperlipidemia, and metabolic syndrome [Anonymous, 2004; Guh et al., 2006]. Moreover, we were the first to show that betel-quid is associated with liver cirrhosis and hepatocellular carcinoma [Tsai et al., 2001, 2003, 2004].

Arecoline, the major alkaloid of betel-quid [Guh et al., 2006], induces profound metabolic effects in the liver [Shivapurkar et al., 1978] and is hepatotoxic in mice [Dasgupta et al., 2006]. Arecoline also has cytotoxic and genotoxic effects in various non-liver cells [Trivedi et al., 1993; Jeng et al., 1999; Kumpawat et al., 2003] and hepatocytes [Kevekordes et al., 2001; Chou et al., 2008].

Genotoxic hepatocarcinogens induce deoxyribonucleic acid (DNA) damage and apoptosis while activating p53 and the downstream p21^{WAF1} in the liver [Ellinger-Ziegelbauer et al., 2004]. We have also shown that arecoline induces DNA damage and arrests hepatocytes in the G_0/G_1 phase of the cell cycle [Chou et al.,

Grant sponsor: National Science Council; Grant numbers: NSC95-2314-B-037-096-MY3, 96-2628-B-037-001-MY3; Grant sponsor: Center of Excellence for Environmental Medicine; Grant number: KMU-EM-97-1.3a.

*Correspondence to: Jinn-Yuh Guh, Department of Internal Medicine, Kaohsiung Medical University, 100 Zihyou 1st Rd., Kaohsiung 807, Taiwan. E-mail: guhjy@kmu.edu.tw

**Correspondence to: Prof. Lea-Yea Chuang, PhD, Department of Biochemistry, Kaohsiung Medical University, 100 Zihyou 1st Rd., Kaohsiung 807, Taiwan. E-mail: guhjy1@gmail.com

Received 25 July 2008; Accepted 13 February 2009 • DOI 10.1002/jcb.22137 • 2009 Wiley-Liss, Inc.
Published online 2 April 2009 in Wiley InterScience (www.interscience.wiley.com).

2008]. Moreover, arecoline-induced $p21^{WAF1}$ is dependent on p53 while arecoline-inhibited growth is dependent on both TGF-β and p53.

DNA damage activates the genes ataxia telangiectasia mutated (ATM)/rad3-related (ATR) to phosphorylate (activate) p53, which induces $p21^{WAF1}$ to arrest cells in the G_0/G_1 phase of the cell cycle [Lavin and Kozlov, 2007]. ATM phosphorylates (activates) Akt [Dann et al., 2007; Marone et al., 2008], a downstream effector of phosphoinositide-3-kinase (PI3K) [Marone et al., 2008]. Akt in turn phosphorylates (activates) mammalian target of rapamycin (mTOR) [Nave et al., 1999; Bhaskar and Hay, 2007], which also interacts with p53 [Feng et al., 2005; Lee et al., 2007a]. Interestingly, both Akt and mTOR are involved in DNA damage response [Reiling and Sabatini, 2006; Boehme et al., 2008].

Therefore, we studied the effects of arecoline on ATM, PI3K/Akt, mTOR, p53 and $p21^{WAF1}$ protein expression in Clone-9 (rat hepatocyte) cells, which has been extensively used for the study of hepatotoxicity in vitro [Sahu et al., 2008]. Moreover, the role of ATM, PI3K, and mTOR in arecoline-induced p53 and $p21^{WAF1}$ protein expression was also studied.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Clone-9 (CRL-1439, rat liver epithelial cell, American Type Culture Collection, Manassas, VA) cells were cultured in Ham's F12K medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (Gibco-BRL, Rockville, MD) at 37°C in a humidified atmosphere of 95% air/5%CO_2. Culture materials were obtained from Gibco-BRL. Reagents for LipofectAMINE were purchased from Life Technology (Gaithersburg, MD) while Luciferase substrate was purchased from Promega Corp. (Madison, WI). Arecoline hydrobromide (referred to as arecoline thereafter), caffeine, LY294002, SB203580, PD98059, and all other reagents were obtained from Sigma Chemical Co. unless stated otherwise. Note that rapamycin was thought to inhibit only mTORC1, but not mTORC2, of the mTOR complex [Jacinto et al., 2006; Soulard and Hall, 2007; Lee et al., 2007b]. However, recent studies found that prolonged rapamycin treatment inhibit mTORC2 activity and Akt phosphorylation at serine 473 [Sarbassov et al., 2006].

PLASMIDS

The $p21^{WAF1}$ promoter reporter construct WWP-luc was kindly provided by Dr. Wang [Datto et al., 1995]. pSRα–Δp85 (dominant-negative p85) was a gift from Dr. Wataru Ogawa [Hara et al., 1994]. ATM shRNA, luciferase and GFP shRNA in pLKO.1 plasmids were purchased from the RNAi Consortium at the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA and GFP shRNA plasmids were used as negative controls.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

This was performed as described in our previous study [Guh et al., 2003]. Briefly, cells (10^5 cells/well) were plated onto 6-well plates and grown overnight. Cells were transfected with 0.2 μg of plasmids with the LipofectAMINE method. After treatment with arecoline 0.5 mM for 24 h, luciferase activities were assayed as described in our previous study [Lee et al., 2000]. Luciferase activities were normalized for the amount of protein in cell lysates and were calculated as an average of three independent experiments of duplicate samples.

IMMUNOPRECIPITATION

The cell lysates were centrifuged and protein concentrations of the lysates were determined by using a BCA protein assay (Bio-Red Laboratories, Hercules, CA). The supernatant (which contained 200 μg total protein/200 μl) was incubated with a specific phospho-(Ser/Thr) ATM/ATR substrate antibody (p-[S/T]Q, Cell Signaling Technology, Danvers, MA) with gentle shocking overnight at 4°C. Followed by adding 20 μl protein A/G agarose (Calbiochem Inc., San Diego, CA) at 4°C for 2 h, the lysates were centrifuged at 12,000 rpm for 5 min at 4°C. The beads were washed two times with 500 μl of lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA,

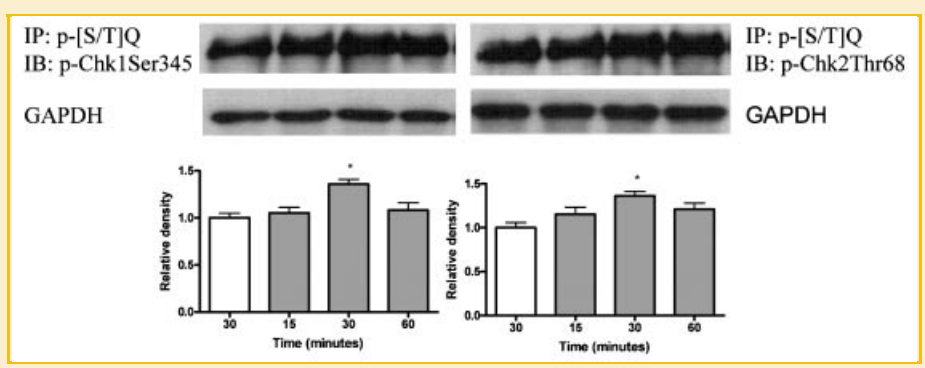

Fig. 1. Time-dependent effects of arecoline on ATM/ATR kinase activity in Clone-9 cells. Serum-starved cells were treated with distilled water (empty bar) for 30 min or 0.5 mM arecoline (gray bars) for 15–60 min. ATM/ATR kinase activity was measured by immunoprecipitating (IP) the cell lysate with p-[S/T]Q (ATM/ATR substrate) antibody followed by immunoblotting (IB) with p-Chk1Ser345 and p-Chk2Thr68 antibodies and was normalized to the expression of GAPDH. The results are shown as the mean of three independent experiments. $^*P < 0.05$ versus lane 1.

1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin). Prior to electrophoresis, an appropriate volume of 60 μl SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 5%-mercaptoethanol, 10% glycerol, 3% SDS, 0.2% bromphenol blue) were boiled for 5 min, then it was subjected to SDS–PAGE on 10% polyacrylamide gels and electro-transferred onto PVDF membranes.

IMMUNOBLOTTING

This was performed as described in our previous study [Guh et al., 2003]. Briefly, total cell lysates were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to membranes and blocked, and then probed with antibodies: $p21^{WAF1}$, β-actin, GAPDH (Santa Cruz Biotechnologies, Santa Cruz, CA), p-p53Ser15, p-AktSer473, Akt, p-mTORSer2448, mTOR, p-Chk1Ser345, and p-Chk2Thr68 (Cell Signaling Technology) and α-tublin (Neomarker, Fremont, CA). The membranes were washed and reprobed with β-actin or α-tubulin antibody to control for protein loading. The bands were detected using the enhanced chemiluminescence system (Perkin-Elmer, Inc., Boston, MA) and quantified by using a scanning densitometer. Relative densities were shown as the ratio between the specific proteins and either β-actin or α-tubulin.

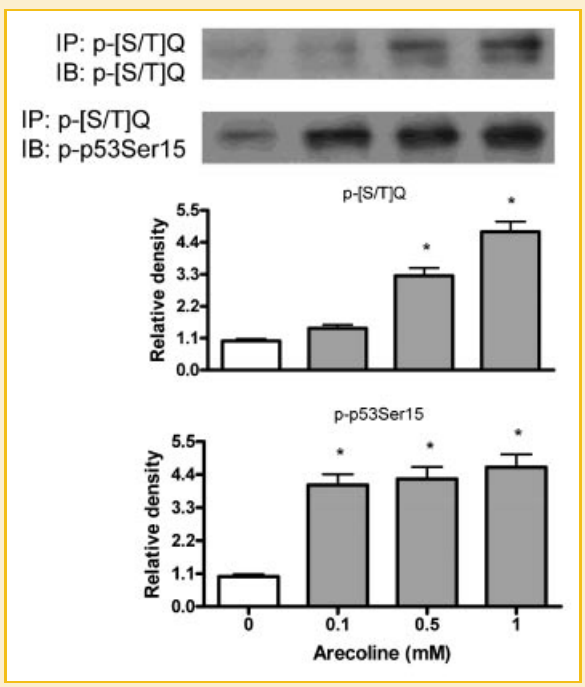

Fig. 2. Dose-dependent effects of arecoline on phosphorylation levels of p-p53Ser15 in Clone-9 cells. Serum-starved cells were treated with distilled water (empty bar) or 0.1–1 mM arecoline (gray bars) for 24 h. The cell lysate was immunoprecipitated (IP) with p-[S/T]Q antibody followed by immunoblotting (IB) with p-[S/T]Q antibody (upper panel). Afterwards, the identity of this band was determined by IP with p-[S/T]Q antibody followed by IB with p-p53Ser15 antibody (lower panel). The results are shown as the mean of three independent experiments. $^*P < 0.05$ versus lane 1.

STATISTICAL ANALYSIS

The results were expressed as the mean ± standard errors of the mean. Unpaired *t*-tests were used to compare between two groups. A *P*-value of less than 0.05 was considered to be statistically significant.

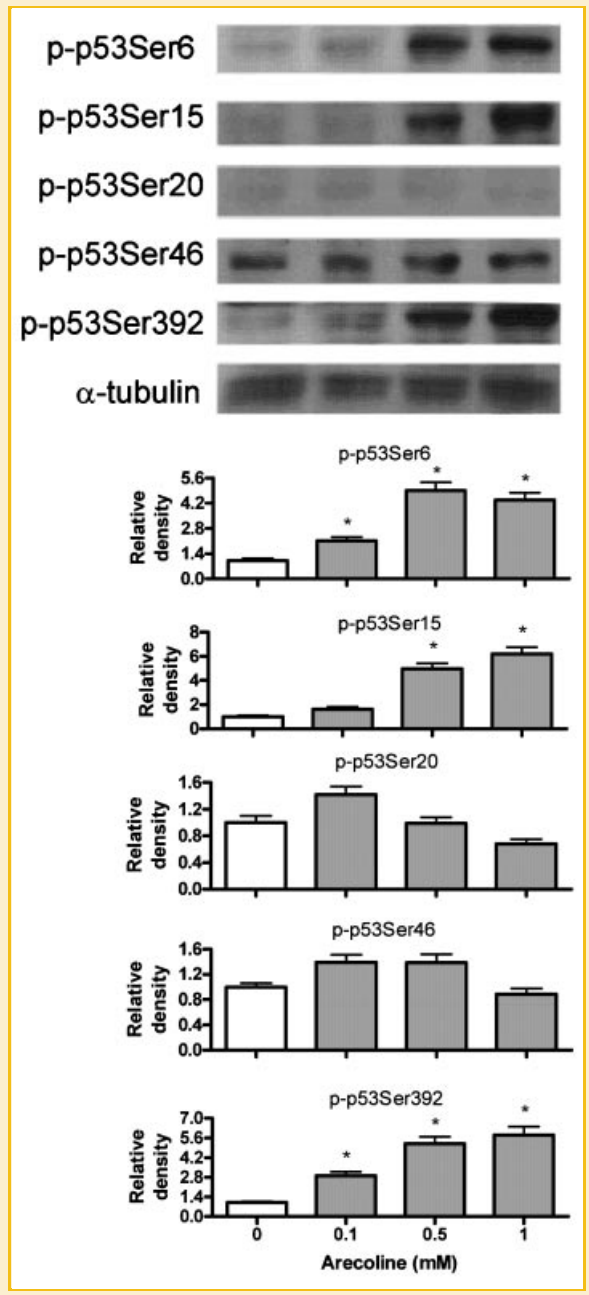

Fig. 3. Dose-dependent effects of arecoline on phosphorylation levels of various forms of p-p53 in Clone-9 cells. Serum-starved cells were treated with distilled water (empty bar) or 0.1–1 mM arecoline (gray bars) for 24 h. Phosphorylation levels of p-p53Ser6, p-p53Ser15, p-p53Ser20, p-p53Ser46, and p-p53Ser392 were performed by immunoblotting and normalized to those of α-tubulin. The results are shown as the mean of three independent experiments. $^*P < 0.05$ versus lane 1.

RESULTS

TIME-DEPENDENT EFFECTS OF ARECOLINE ON ATM/ATR KINASE ACTIVITY IN CLONE-9 CELLS

We have found that arecoline induces DNA damage while activating p53 in Clone-9 cells in a previous study [Chou et al., 2008]. Because DNA damage activates ATM/ATR [Lavin and Kozlov, 2007], we measured ATM/ATR kinase activity by immunoprecipitation of the cell lysate with p-[S/T]Q (ATM/ATR substrate) antibody followed by immunoblotting with p-Chk1Ser345 and p-Chk2Thr68 antibody. As shown in Figure 1, arecoline (0.5 mM) activated ATM/ATR (i.e., increased p-Chk1Ser345 and p-Chk2Thr68 expression) at 30 min.

DOSE-DEPENDENT EFFECTS OF ARECOLINE ON PHOSPHORYLATION LEVELS OF p-p53SER15 IN CLONE-9 CELLS

Figure 1 showed that arecoline activated two of the ATM/ATR substrates: Chk1 and Chk2. Among the other ATM/ATR substrates, p53 is known to be phosphorylated by ATM/ATR at serine 15 [Lavin and Kozlov, 2007]. Thus, phosphorylation levels of p53 protein were determined by immunoprecipitation of the cell lysate with p-[S/T]Q antibody followed by immunoblotting with p-p53Ser15 antibody. As shown in Figure 2, arecoline dose-dependently (0.5–1 mM) increased the ATM/ATR substrate at the authentic p-p53 site at 24 h. Additionally, arecoline (0.1–1 mM) evenly increased phosphorylation levels of p-p53Ser15 at 24 h.

DOSE-DEPENDENT EFFECTS OF ARECOLINE ON PHOSPHORYLATION LEVELS OF VARIOUS FORMS OF p-p53 IN CLONE-9 CELLS

The protein p53 can be serine phosphorylated at multiple locations by serine–threonine kinases, such as: serine 15 (and serine 6) by ATM/ATR [Lavin and Gueven, 2006; Toledo and Wahl, 2006], serine 20 by checkpoint kinase 2 (Chk2) [Lavin and Gueven, 2006; Toledo and Wahl, 2006], serine 46 by homeodomain-interacting protein kinase-2 [Lavin and Gueven, 2006; Toledo and Wahl, 2006], serine 392 by casein kinase-2 and double-stranded RNA-dependent protein kinase [Lavin and Gueven, 2006; Toledo and Wahl, 2006]. Thus, phosphorylation levels of p-p53Ser6, p-p53Ser15, p-p53Ser20, p-p53Ser46, and p-p53Ser392 were measured by immunoblotting. Among these phosphorylated p53, only phosphorylation levels of p-p53Ser6, p-p53Ser15, and p-p53Ser392 were dose-dependently increased by arecoline (0.5–1 mM) at 24 h (Fig. 3).

ROLE OF ATM/ATR IN ARECOLINE-INDUCED PHOSPHORYLATION LEVELS OF p-p53SER15 AND PROTEIN LEVELS OF $p21^{WAF1}$ IN CLONE-9 CELLS

DNA damage-induced ATM/ATR activates p53 and induces $p21^{WAF1}$ [Lavin and Kozlov, 2007]. Thus, phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$ were measured by immunoblotting. As shown in Figure 4, caffeine, an ATM/ATR inhibitor [Bohm et al., 2003] used at a dose (5–10 mM) similar to a previous study [Chang et al., 2004], attenuated arecoline (0.5 mM)-induced phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$ at 24 h. Similarly, ATM (but not luciferase) shRNA also

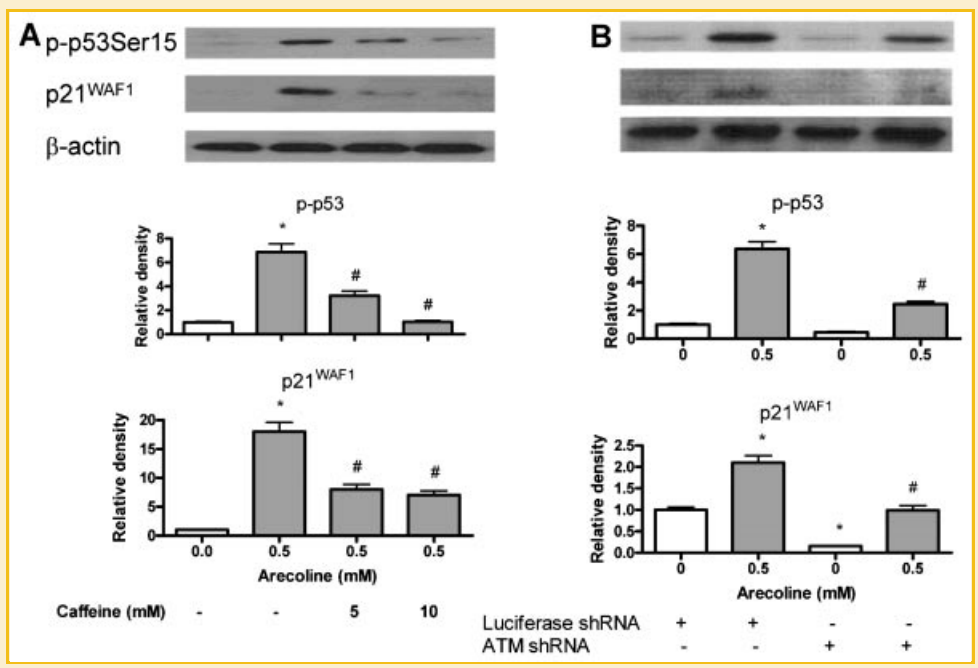

Fig. 4. Role of ATM in arecoline-induced phosphorylation levels of p-p53Ser15 and levels of $p21^{WAF1}$ in Clone-9 cells. Serum-starved cells were treated with distilled water (empty bar) or 0.5 mM arecoline (gray bars) for 24 h. Phosphorylation levels of p-p53Ser15 and levels of $p21^{WAF1}$ were measured by immunoblotting and normalized to those of β-actin. A: Caffeine (5–10 mM) was added in lanes 3 and 4. B: Transient transfection of ATM shRNA and the negative control (luciferase shRNA) was performed as described in Materials and Methods Section. The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1. $^{\#}P < 0.05$ versus lane 2.

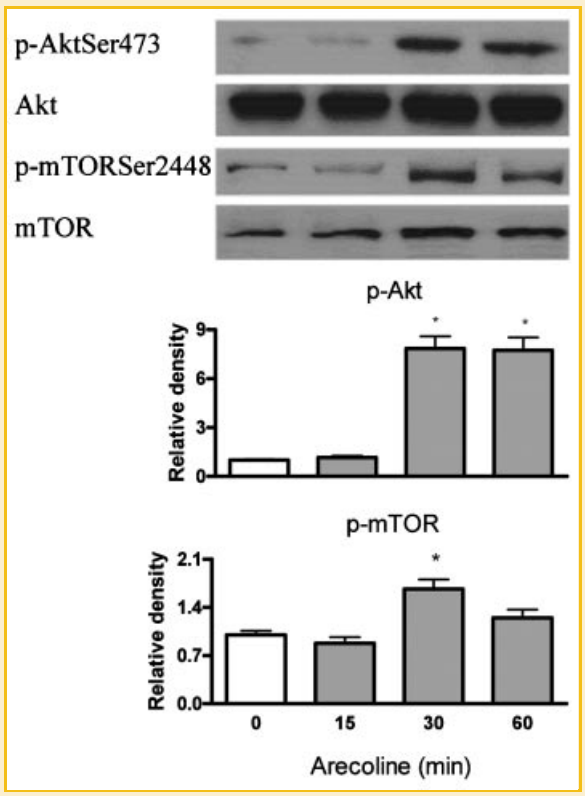

Fig. 5. Time-dependent effects of arecoline on phosphorylation levels of p-AktSer473 and p-mTORSer2448 in Clone-9 cells. Serum-starved cells were treated with distilled water (empty bar) or 0.5 mM arecoline (gray bars) for 15–60 min. Phosphorylation levels of p-AktSer473 (first panel) and p-mTORSer2448 (third panel) were measured by immunoblotting and normalized to those of Akt or mTOR. The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1.

attenuated arecoline (0.5 mM)-induced phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$ at 24 h.

TIME-DEPENDENT EFFECTS OF ARECOLINE ON PHOSPHORYLATION LEVELS OF p-AktSer473 AND p-mTORSER2448 IN CLONE-9 CELLS

Phosphorylation levels of p-AktSer473 and p-mTORSer2448 were measured by immunoblotting. As shown in Figure 5, arecoline (0.5 mM)-induced phosphorylation levels of p-AktSer473 at 30–60 min. In contrast, arecoline (0.5 mM)-induced phosphorylation levels of p-mTORSer2448 only at 30 min.

ROLE OF PI3K IN ARECOLINE-INDUCED PHOSPHORYLATION LEVELS OF p-p53SER15 AND $p21^{WAF1}$ PROTEIN EXPRESSION IN CLONE-9 CELLS

The role of PI3K was studied by LY294002. Phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$ were measured by immunoblotting. As shown in Figure 6, LY294002, used at a dose (20 μM) similar to a previous study [Banerjee et al., 2008], attenuated arecoline (0.5 mM)-induced phosphorylation levels of p-pAkt473, p-p53Ser15, and protein levels of $p21^{WAF1}$ at 24 h. Because LY294002 is a non-specific PI3K inhibitor, which also inhibits the

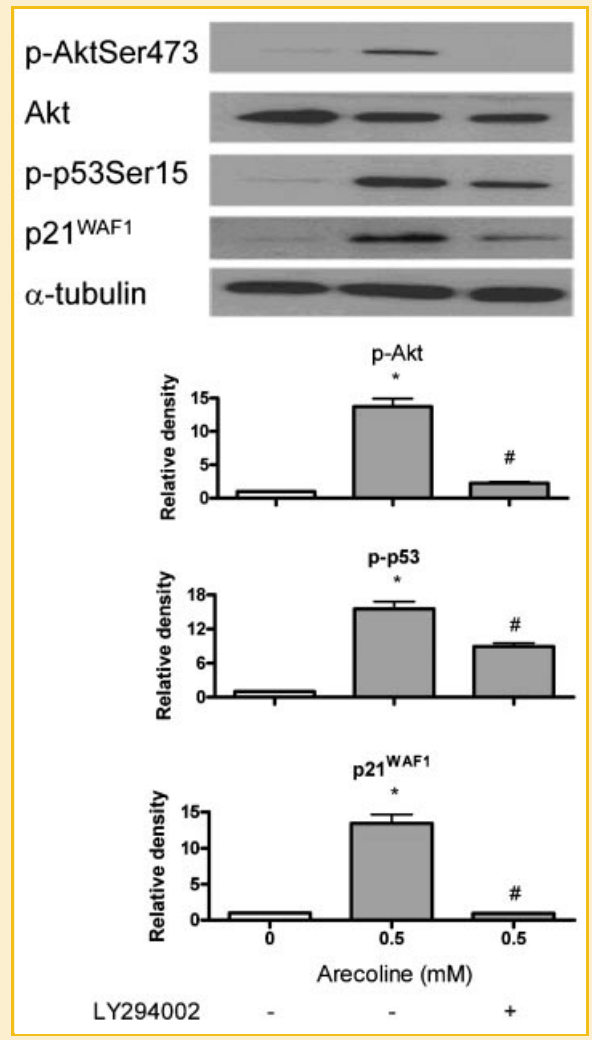

Fig. 6. Role of PI3K in arecoline-induced phosphorylation levels of p-p53Ser15 and levels of $p21^{WAF1}$ in Clone-9 cells. Serum-starved cells were treated with 0.2% dimethylsulfoxide (DMSO, empty bar) or arecoline (0.5 mM in 0.2% DMSO, gray bars) for 24 h. LY294002 (20 μM in 0.2% DMSO) was added in lane 3. Phosphorylation levels of p-AktSer473 (First panel), p-p53Ser15 (third panel) and levels of $p21^{WAF1}$ (fourth panel) were measured by immunoblotting and normalized to those of α-tubulin. The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1. $^{\#}P < 0.05$ versus lane 2.

PI3K-related kinases such as ATM [Marone et al., 2008], we also used the specific PI3K inhibitor dominant-negative p85 (Δp85) plasmid. Thus, we found that the Δp85 plasmid attenuated arecoline (0.5 mM)-induced phosphorylation levels of p-pAkt473 and protein levels of $p21^{WAF1}$ at 24 h (Fig. 7). Conversely, Δp85 plasmid did not attenuate arecoline (0.5 mM)-induced phosphorylation levels of p-p53Ser15 at 24 h (Fig. 7).

ROLE OF mTOR IN ARECOLINE-INDUCED PHOSPHORYLATION LEVELS OF p-p53Ser15 AND $p21^{WAF1}$ PROTEIN EXPRESSION IN CLONE-9 CELLS

Figures 6 and 7 showed that PI3K was required for arecoline-induced phosphorylation levels of p-p53Ser15 and protein expres-

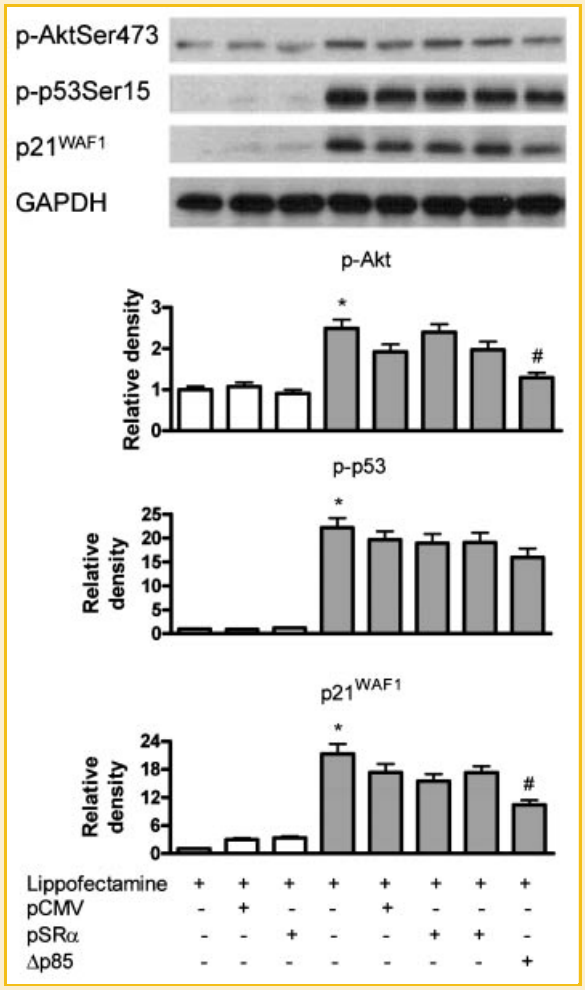

Fig. 7. Role of PI3K in arecoline-induced phosphorylation levels of p-p53Ser15 and levels of $p21^{WAF1}$ in Clone-9 cells. Serum-starved cells were treated with 0.2% dimethylsulfoxide (DMSO, empty bar) or arecoline (0.5 mM in 0.2% DMSO, gray bars) for 24 h. Transient transfection of the dominant-negative PI3K (Δp85) plasmid, the negative control (pSRα empty vector), and the irrelevant plasmid (pCMV) was performed as described in Materials and Methods Section. Phosphorylation levels of p-AktSer473 (first panel), p-p53Ser15 (second panel) and protein levels of $p21^{WAF1}$ (third panel) were measured by immunoblotting and normalized to those of GAPDH. The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1. $^{\#}P < 0.05$ versus lane 4.

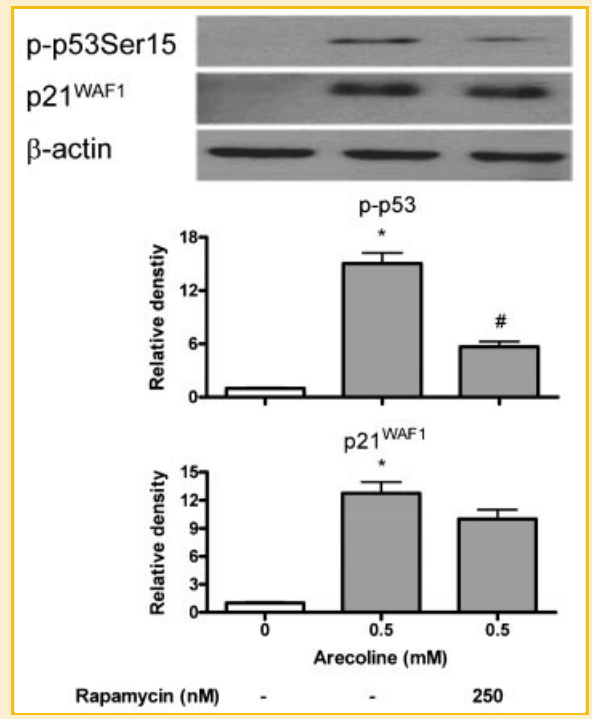

Fig. 8. Role of mTOR in arecoline-induced phosphorylation levels of p-p53Ser15 and levels of $p21^{WAF1}$ in Clone-9 cells. Serum-starved cells were treated with 0.2% DMSO (empty bar) or arecoline (0.5 mM in 0.2% DMSO, gray bars) for 24 h. Rapamycin (250 nM in 0.2% DMSO) was added in lane 3. Phosphorylation levels of p-p53Ser15 (upper panel) and levels of $p21^{WAF1}$ (lower panel) were measured by immunoblotting and normalized to those of β-actin. The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1. $^{\#}P < 0.05$ versus lane 2.

sion of $p21^{WAF1}$ protein expression. Because mTOR is a downstream effector of PI3K/Akt, the role of mTOR was studied by rapamycin, an mTOR inhibitor. Phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$ were measured by immunoblotting. As shown in Figure 8, rapamycin, used at a dose (250 nM) similar to a previous study [Moller et al., 2004], attenuated arecoline (0.5 mM)-induced phosphorylation levels of p-p53Ser15, but not protein levels of $p21^{WAF1}$ at 24 h.

ROLE OF PI3K, ERK1/2, p38 KINASE, AND p53 IN ARECOLINE-INDUCED $p21^{WAF1}$ PROTEIN EXPRESSION IN CLONE-9 CELLS

Mitogen-activated protein kinase induces $p21^{WAF1}$ in some circumstances [Pruitt and Der, 2001]. Thus, the role of extracellular signal regulated kinase 1/2 and p38 kinase was studied by PD98059 (a MEK inhibitor) and SB203580 (a p38 kinase inhibitor). As shown in Figure 9, neither PD98059 (20 μM) nor SB203580 (20 μM) attenuated arecoline (0.5 mM)-induced $p21^{WAF1}$ protein expression at 24 h. In contrast, LY294002 (20 μM) and pifithrin-α (a p53 inhibitor, 20 μM) attenuated arecoline (0.5 mM)-induced $p21^{WAF1}$ protein expression at 24 h.

ROLE OF ATM/ATR AND PI3K IN ARECOLINE-INDUCED $p21^{WAF1}$ GENE TRANSCRIPTION IN CLONE-9 CELLS

The role of ATM/ATR was studied by using caffeine, an ATM/ATR inhibitor [Bohm et al., 2003]. Gene transcriptional activity of $p21^{WAF1}$ was measured by the promoter activity of the transiently transfected full-length $p21^{WAF1}$ gene promoter construct p21P. As shown in Figure 10A,C, caffeine (5–10 mM) and ATM (but not GFP) shRNA attenuated arecoline (0.5 mM)-induced $p21^{WAF1}$ gene transcription at 24 h. LY294002 (20–40 μM) also attenuated arecoline (0.5 mM)-induced $p21^{WAF1}$ gene transcription at 24 h (Fig. 10B). However, dominant-negative p85 (Δp85) plasmid increased arecoline (0.5 mM)-induced $p21^{WAF1}$ gene transcription at 24 h (Fig. 10D).

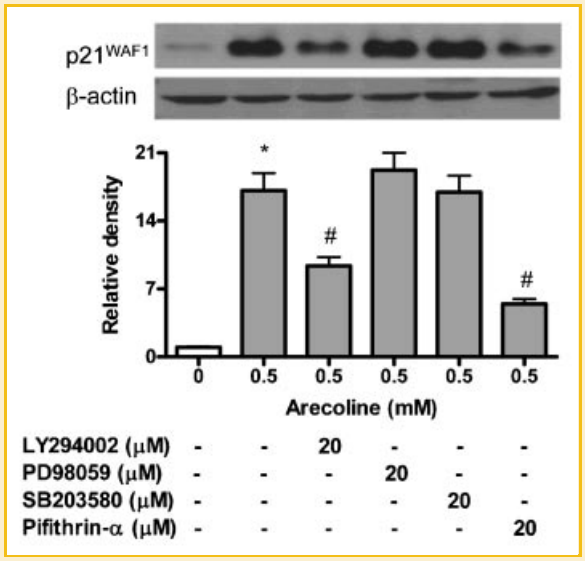

Fig. 9. Role of PI3K, ERK1/2, p38 kinase, and p53 in arecoline-induced protein expression of $p21^{WAF1}$ in Clone-9 cells. Serum-starved cells were treated with 0.2% DMSO (empty bar) or arecoline (0.5 mM in 0.2% DMSO, gray bars) for 24 h. LY294002 (20 μM in 0.2% DMSO, lane 3), PD98059 (20 μM in 0.2% DMSO, lane 4), SB203580 (20 μM in 0.2% DMSO, lane 5) or pifithrin-α (20 μM in 0.2% DMSO, lane 6) was added. Protein expression of $p21^{WAF1}$ was measured by imunooblotting and was normalized to that of β-actin. The results are shown as the mean of three independent experiments. Note that lane 2 was only compared to lane 1 whereas lanes 3–6 were only compared to lane 2. $^{*}P < 0.05$ versus lane 1, $^{\#}P < 0.05$ versus lane 2.

DISCUSSION

We found that arecoline activates ATM/ATR while increasing phosphorylation levels of p-p53Ser15, p-AktSer473, p-mTORSer2448, and protein levels of $p21^{WAF1}$ in Clone-9 cells. Moreover, ATM/ATR, PI3K, and mTOR played differential roles in arecoline-induced phosphorylation levels of p-p53Ser15 and protein levels of $p21^{WAF1}$.

This is the first demonstration that arecoline activates ATM/ATR in hepatocytes. Additionally, we found that the phosphorylated ATM/ATR substrate contains p-p53Ser15. This finding corroborates our previous study showing that arecoline (0.5 mM) induces DNA damage while activating p53 in Clone-9 cells [Chou et al., 2008].

ATM phosphorylates p53 at serine 6 and serine 15 [Lavin and Gueven, 2006; Lavin and Kozlov, 2007]. Thus, arecoline increased p-p53Ser6 and p-p53Ser15 among the multiple phosphorylated p53 in this study. Moreover, both caffeine and ATM shRNA attenuated arecoline-induced phosphorylated levels of p-p53Ser15. This finding is corroborated by a previous study showing that caffeine attenuates diethylnitrosamine-induced p-p53Ser15 in hepatocytes [Silins et al., 2001]. Arecoline, a muscarinic receptor agonist [Langmead et al., 2008], also increased p-p53Ser392 in this study. Interestingly, p-p53Ser392 is known to be increased by casein kinase-2 [Lavin and Gueven, 2006], which in turn is increased by muscarinic receptor agonists [Torrecilla et al., 2007].

We found that arecoline increased phosphorylation levels of p-AktSer473. This finding has been corroborated by previous studies showing that muscarinic receptor agonists activate the PI3K/Akt pathway [Bommakanti et al., 2000; Oldenburg et al., 2002; Ma et al., 2004; Gerthoffer, 2005] and that DNA damage activates the insulin/insulin-like growth factor-1 (IGF-1)-PI3K/Akt pathway [Matsuoka et al., 2007].

In contrast, our finding that arecoline activates mTOR (by increasing p-mTORSer2448 protein expression) in hepatocytes is a novel one. This finding is consistent with the view that Akt phosphorylates (activates) mTOR at serine 2448 [Nave et al., 1999; Bhaskar and Hay, 2007] whereas ATM phosphorylates (activates) Akt at serine 473 [Viniegra et al., 2005; Bhaskar and Hay, 2007]. Interestingly, p-mTORSer2448 is increased in 40% of patients with hepatocellular carcinoma [Sieghart et al., 2007].

A previous study found that PI3K is required for some of arecoline-induced effects [Yang et al., 2008]. In view of the fact that PI3K/Akt stabilizes and increases $p21^{WAF1}$ protein expression [Li et al., 2002], we found that that PI3K is required for arecoline-induced $p21^{WAF1}$ protein expression. This finding is compatible with the notion that cell survival (e.g., PI3K and mTOR) and cell cycle inhibitory (e.g., p53 and $p21^{WAF1}$) pathways are interconnected [Maddika et al., 2007].

Conversely, we found that the dominant-negative PI3K plasmids did not attenuate arecoline-induced phosphorylation levels of p-p53Ser15. This finding is compatible with the notion that Akt destabilizes and decreases levels of p53 [Lavin and Gueven, 2006].

Our finding that rapamycin attenuated arecoline-induced phosphorylation levels of p-p53Ser15 is similar to a previous study showing that mTOR enhances DNA damage-induced p-p53Ser15, which is attenuated by rapamycin [Lee et al., 2007b]. Conversely, we found that rapamycin did not attenuate arecoline-induced $p21^{WAF1}$ protein expression. This finding can be explained by the fact that $p21^{WAF1}$ gene expression is controlled by many factors other than p53 [Gartel and Tyner, 1999].

It is known that ATM activates p53 [Lavin and Kozlov, 2007], which transcriptionally induces the $p21^{WAF1}$ gene [Maddika et al., 2007; Chou et al., 2008]. Thus, we found that both caffeine and ATM shRNA attenuated arecoline-induced $p21^{WAF1}$ gene transcription. Conversely, we found that the dominant-negative PI3K plasmids increased arecoline-induced $p21^{WAF1}$ gene transcription. This finding can be explained by the fact that Akt decreases levels of p53 [Lavin and Gueven, 2006], thereby inhibiting $p21^{WAF1}$ gene transcription (Fig. 11).

A hypothetical scheme for arecoline-induced p53-$p21^{WAF1}$ via the ATM/ATR and PI3K/Akt pathways is shown in Figure 11. Note that the functional relationship between ATM/ATR and PI3K/Akt is more complex than the modulation of p53. For example, all of the downstream targets of Akt [Sale and Sale, 2008] (e.g., mTOR) are potential common targets of both ATM/ATR and PI3K. Moreover, signal transducers and activators of transcription-3 and NF-κB are two of the common targets of both ATM/ATR and PI3K [Zhang et al., 2003; Liu et al., 2006; Neumann and Naumann, 2007]. Interestingly, both STAT3 and NF-κB can induce $p21^{WAF1}$ in cancer cells [Wuerzberger-Davis et al., 2005; Moran et al., 2008].

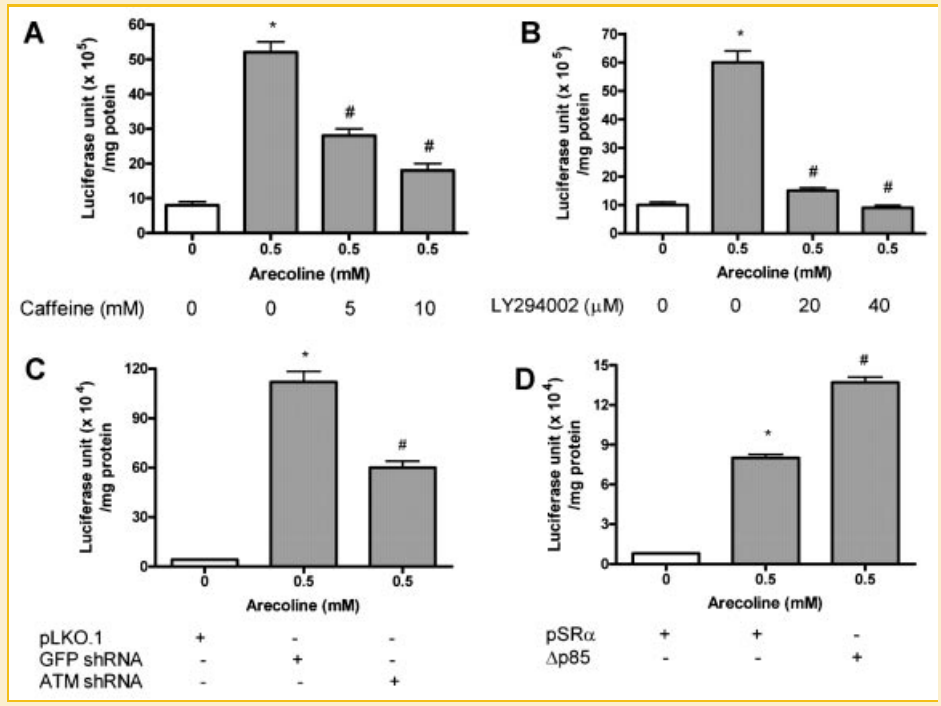

Fig. 10. Role of PI3K and ATM in arecoline-induced p21^{WAF1} gene transcription in Clone-9 cells. Serum-starved cells (10^5 cells/well) were treated with 0.2% DMSO (empty bar) or arecoline (0.5 mM in 0.2% DMSO, gray bars) for 24 h. Caffeine (A) or LY294002 (B) was added in lanes 3 and 4. Gene transcriptional activity of p21^{WAF1} was measured by the promoter activity of the transiently transfected full-length p21^{WAF1} gene promoter construct p21P. A: Caffeine (5–10 mM in 0.2% DMSO) attenuated arecoline-induced p21^{WAF1} gene transcription. B: LY294002 (20–40 μM in 0.2% DMSO) attenuated arecoline-induced p21^{WAF1} gene transcription. C: Transient transfection of pLKO.1 (empty vector), GFP shRNA, and ATM shRNA. D: Transient transfection of the dominant-negative PI3K (Δp85) plasmid and the negative control (pSRα empty vector). The results are shown as the mean of three independent experiments. $^{*}P < 0.05$ versus lane 1, $^{\#}P < 0.05$ versus lane 2.

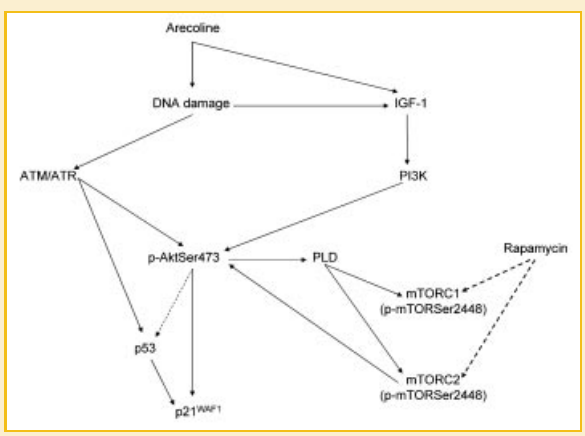

Fig. 11. A hypothetical scheme for arecoline-induced p53-p21^{WAF1} via the ATM/ATR and PI3K/Akt pathways. Arecoline induces both DNA damage [Chou et al., 2008] and insulin-like growth factor-1 (IGF-1) [Tsai et al., 2005]. DNA damage activates ATM/ATR, which increases levels of phosphorylated p-p53Ser15 and the insulin/IGF-1-downstream mediator p-AktSer473 [Matsuoka et al., 2007]. The activated p53 transcriptionally induces p21^{WAF1}. PI3K activates Akt, which activates PLD [Sun et al., 2008; Toschi et al., 2009]. PLD activates both mTOCR1 and mTORC2 [Sun et al., 2008; Toschi et al., 2009], which are inhibited by rapamycin [Sarbassov et al., 2006]. mTORC2 in turn activates Akt [Jacinto et al., 2006]. Akt destabilizes and decreases levels of p53 [Lavin and Gueven, 2006]. Conversely, Akt stabilizes and increases p21^{WAF1} protein expression [Li et al., 2002]. Solid lines are stimulatory whereas dotted lines are inhibitory pathways.

Apart from oral cancer and hepatocellular carcinoma, betel-quid has been associated with cancers of the lung, esophagus, stomach, and uterine cervix [Anonymous, 2004]. The relevance of our study to the betel-quid–associated cancers is in the relevance of DNA damage, ATM/ATR, PI3K/Akt, mTOR, p53 and p21^{WAF1} to the pathogenesis, and treatment of cancers [Maddika et al., 2007]. For example, clinical trials of various inhibitors of PI3K or mTOR for various cancers are in progress [Marone et al., 2008; Yap et al., 2008].

In conclusion, arecoline activates the ATM/ATR-p53-p21^{WAF1} and the PI3K/Akt-mTOR-p53 pathways in Clone-9 cells. Arecoline-induced phosphorylated p-p53Ser15 expression is dependent on ATM whereas arecoline-induced p21^{WAF1} protein expression is dependent on ATM and PI3K. Moreover, p21^{WAF1} gene is transcriptionally induced by arecoline-activated ATM.

ACKNOWLEDGMENTS

This research was supported by the National Science Council, Republic of China (NSC95-2314-B-037-096-MY3 to Lea-Yea Chuang, 96-2628-B-037-001-MY3 to Jinn-Yuh Guh) and the Center of Excellence for Environmental Medicine (KMU-EM-97-1.3a) in Kaohsiung Medical University.

REFERENCES

Anonymous IARC Working group on the evaluation of carcinogenic risks to humans. 2004. Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC Monogr Eval Carcinog Risks Hum 85:1–334.

Banerjee S, Saito K, Ait-Goughoulte M, Meyer K, Ray RB, Ray R. 2008. Hepatitis C virus core protein upregulates serine phosphorylation of insulin receptor substrate-1 and impairs the downstream akt/protein kinase B signaling pathway for insulin resistance. J Virol 82:2606–2612.

Bhaskar PT, Hay N. 2007. The two TORCs and Akt. Dev Cell 12:487–502.

Boehme KA, Kulikov R, Blattner C. 2008. p53 stabilization in response to DNA damage requires Akt/PKB and DNA-PK. Proc Natl Acad Sci USA 105:7785–7790.

Bohm L, Roos WP, Serafin AM. 2003. Inhibition of DNA repair by Pentoxifylline and related methylxanthine derivatives. Toxicology 193:153–160.

Bommakanti RK, Vinayak S, Simonds WF. 2000. Dual regulation of Akt/protein kinase B by heterotrimeric G protein subunits. J Biol Chem 275:38870–38876.

Boucher BJ, Mannan N. 2002. Metabolic effects of the consumption of Areca catechu. Addict Biol 7:103–110.

Chang KL, Kung ML, Chow NH, Su SJ. 2004. Genistein arrests hepatoma cells at G2/M phase: Involvement of ATM activation and upregulation of p21waf1/cip1 and Wee1. Biochem Pharmacol 67:717–726.

Chou WW, Guh JY, Tsai JF, Hwang CC, Chen HC, Huang JS, Yang YL, Hung WC, Chuang LY. 2008. Arecoline-induced growth arrest and p21WAF1 expression are dependent on p53 in rat hepatocytes. Toxicology 243:1–10.

Dann SG, Selvaraj A, Thomas G. 2007. mTOR Complex1-S6K1 signaling: At the crossroads of obesity, diabetes and cancer. Trends Mol Med 13:252–259.

Dasgupta R, Saha I, Pal S, Bhattacharyya A, Sa G, Nag TC, Das T, Maiti BR. 2006. Immunosuppression, hepatotoxicity and depression of antioxidant status by arecoline in albino mice. Toxicology 227:94–104.

Datto MB, Yu Y, Wang XF. 1995. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem 270:28623–28628.

Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ. 2004. Characteristic expression profiles induced by genotoxic carcinogens in rat liver. Toxicol Sci 77:19–34.

Feng Z, Zhang H, Levine AJ, Jin S. 2005. The coordinate regulation of the p53 and mTOR pathways in cells. Proc Natl Acad Sci USA 102:8204–8209.

Gartel AL, Tyner AL. 1999. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res 246:280–289.

Gerthoffer WT. 2005. Signal-transduction pathways that regulate visceral smooth muscle function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles. Am J Physiol Gastrointest Liver Physiol 288:G849–G853.

Guh JY, Chuang TD, Chen HC, Hung WC, Lai YH, Shin SJ, Chuang LY. 2003. Beta-hydroxybutyrate-induced growth inhibition and collagen production in HK-2 cells are dependent on TGF-beta and Smad3. Kidney Int 64:2041–2051.

Guh JY, Chuang LY, Chen HC. 2006. Betel-quid use is associated with the risk of the metabolic syndrome in adults. Am J Clin Nutr 83:1313–1320.

Hara K, Yonezawa K, Sakaue H, Ando A, Kotani K, Kitamura T, Kitamura Y, Ueda H, Stephens L, Jackson TR. 1994. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc Natl Acad Sci USA 91:7415–7419.

Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. 2006. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127:125–137.

Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC. 1999. Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. J Oral Pathol Med 28:64–71.

Kevekordes S, Spielberger J, Burghaus CM, Birkenkamp P, Zietz B, Paufler P, Diez M, Bolten C, Dunkelberg H. 2001. Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: Results with 15 naturally occurring substances. Anticancer Res 21:461–469.

Kumpawat K, Deb S, Ray S, Chatterjee A. 2003. Genotoxic effect of raw betelnut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells. Mutat Res 538:1–12.

Langmead CJ, Watson J, Reavill C. 2008. Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol Ther 117:232–243.

Lavin MF, Gueven N. 2006. The complexity of p53 stabilization and activation. Cell Death Differ 13:941–950.

Lavin MF, Kozlov S. 2007. ATM activation and DNA damage response. Cell Cycle 6:931–942.

Lee TH, Chuang LY, Hung WC. 2000. Induction of p21WAF1 expression via Sp1-binding sites by tamoxifen in estrogen receptor-negative lung cancer cells. Oncogene 19:3766–3773.

Lee C, Kim JS, Waldman T. 2007a. Activated PI3K signaling as an endogenous inducer of p53 in human cancer. Cell Cycle 6:394–396.

Lee CH, Inoki K, Karbowniczek M, Petroulakis E, Sonenberg N, Henske EP, Guan KL. 2007b. Constitutive mTOR activation in TSC mutants sensitizes cells to energy starvation and genomic damage via p53. EMBO J 26:4812–4823.

Li Y, Dowbenko D, Lasky LA. 2002. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. J Biol Chem 277:11352–11361.

Liu AM, Lo RK, Wong CS, Morris C, Wise H, Wong YH. 2006. Activation of STAT3 by G alpha(s) distinctively requires protein kinase A, JNK, and phosphatidylinositol 3-kinase. J Biol Chem 281:35812–35825.

Ma W, Li BS, Zhang L, Pant HC. 2004. Signaling cascades implicated in muscarinic regulation of proliferation of neural stem and progenitor cells. Drug News Perspect 17:258–266.

Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, Eshraghi M, Manda KD, Wiechec E, Los M. 2007. Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. Drug Resist Updat 10:13–29.

Marone R, Cmiljanovic V, Giese B, Wymann MP. 2008. Targeting phosphoinositide 3-kinase: Moving towards therapy. Biochim Biophys Acta 1784:159–185.

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316:1160–1166.

Moller MT, Samari HR, Seglen PO. 2004. Toxin-induced tail phosphorylation of hepatocellular S6 kinase: Evidence for a dual involvement of the AMP-activated protein kinase in S6 kinase regulation. Toxicol Sci 82:628–637.

Moran DM, Mattocks MA, Cahill PA, Koniaris LG, McKillop IH. 2008. Interleukin-6 mediates G(0)/G(1) growth arrest in hepatocellular carcinoma through a STAT 3-dependent pathway. J Surg Res 147:23–33.

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. 1999. Mammalian target of rapamycin is a direct target for protein kinase B: Identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J 344(Pt 2): 427–431.

Neumann M, Naumann M. 2007. Beyond IkappaBs: Alternative regulation of NF-kappaB activity. FASEB J 21:2642–2654.

Oldenburg O, Qin Q, Sharma AR, Cohen MV, Downey JM, Benoit JN. 2002. Acetylcholine leads to free radical production dependent on K(ATP) channels, G(i) proteins, phosphatidylinositol 3-kinase and tyrosine kinase. Cardiovasc Res 55:544–552.

Pruitt K, Der CJ. 2001. Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett 171:1–10.

Reiling JH, Sabatini DM. 2006. Stress and mTORture signaling. Oncogene 25:6373–6383.

Sahu SC, Garthoff LH, Robl MG, Chirtel SJ, Ruggles DI, Flynn TJ, Sobotka TJ. 2008. Rat liver clone-9 cells in culture as a model for screening hepatotoxic potential of food-related products: Hepatotoxicity of deoxynivalenol. J Appl Toxicol 28:765–772.

Sale EM, Sale GJ. 2008. Protein kinase B: Signalling roles and therapeutic targeting. Cell Mol Life Sci 65:113–127.

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 22:159–168.

Shivapurkar NM, Bhide SV, Ranadive KJ. 1978. Biochemical studies of betel nut constituents. Indian J Pharmacol 10:191–200.

Sieghart W, Fuereder T, Schmid K, Cejka D, Werzowa J, Wrba F, Wang X, Gruber D, Rasoul-Rockenschaub S, Peck-Radosavljevic M, Wacheck V. 2007. Mammalian target of rapamycin pathway activity in hepatocellular carcinomas of patients undergoing liver transplantation. Transplantation 83:425–432.

Silins I, Finnberg N, Stahl A, Hogberg J, Stenius U. 2001. Reduced ATM kinase activity and an attenuated p53 response to DNA damage in carcinogen-induced preneoplastic hepatic lesions in the rat. Carcinogenesis 22: 2023–2031.

Soulard A, Hall MN. 2007. SnapShot: mTOR signaling. Cell 129:434.

Sun Y, Fang Y, Yoon MS, Zhang C, Roccio M, Zwartkruis FJ, Armstrong M, Brown HA, Chen J. 2008. Phospholipase D1 is an effector of Rheb in the mTOR pathway. Proc Natl Acad Sci USA 105:8286–8291.

Toledo F, Wahl GM. 2006. Regulating the p53 pathway: In vitro hypotheses, in vivo veritas. Nat Rev Cancer 6:909–923.

Torrecilla I, Spragg EJ, Poulin B, McWilliams PJ, Mistry SC, Blaukat A, Tobin AB. 2007. Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. J Cell Biol 177:127–137.

Toschi A, Lee E, Xu L, Garcia A, Gadir N, Foster DA. 2009. Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid—A Competition with Rapamycin. Mol Cell Biol 29:1411–1420.

Trivedi AH, Dave BJ, Adhvaryu SC. 1993. Genotoxic effects of nicotine in combination with arecoline on CHO cells. Cancer Lett 74:105–110.

Tsai JF, Chuang LY, Jeng JE, Ho MS, Hsieh MY, Lin ZY, Wang LY. 2001. Betel quid chewing as a risk factor for hepatocellular carcinoma: A case-control study. Br J Cancer 84:709–713.

Tsai JF, Jeng JE, Chuang LY, Ho MS, Ko YC, Lin ZY, Hsieh MY, Chen SC, Chuang WL, Wang LY, Yu ML, Dai CY, Ho C. 2003. Habitual betel quid chewing as a risk factor for cirrhosis: A case-control study. Medicine (Baltimore) 82:365–372.

Tsai JF, Jeng JE, Chuang LY, Ho MS, Ko YC, Lin ZY, Hsieh MY, Chen SC, Chuang WL, Wang LY, Yu ML, Dai CY. 2004. Habitual betel quid chewing and risk for hepatocellular carcinoma complicating cirrhosis. Medicine (Baltimore) 83:176–187.

Tsai CH, Yang SF, Chen YJ, Chou MY, Chang YC. 2005. The upregulation of insulin-like growth factor-1 in oral submucous fibrosis. Oral Oncol 41:940–946.

Viniegra JG, Martinez N, Modirassari P, Losa JH, Parada CC, Lobo VJ, Luquero CI, varez-Vallina L, Cajal S, Rojas JM, Sanchez-Prieto R. 2005. Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. J Biol Chem 280:4029–4036.

Wuerzberger-Davis SM, Chang PY, Berchtold C, Miyamoto S. 2005. Enhanced G2-M arrest by nuclear factor-{kappa}B-dependent p21waf1/cip1 induction. Mol Cancer Res 3:345–353.

Yang SF, Tsai CH, Chang YC. 2008. The upregulation of heat shock protein 47 expression in human buccal fibroblasts stimulated with arecoline. J Oral Pathol Med 37:206–210.

Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P. 2008. Targeting the PI3K-AKT-mTOR pathway: Progress, pitfalls, and promises. Curr Opin Pharmacol 8:393–412.

Zhang Y, Cho YY, Petersen BL, Bode AM, Zhu F, Dong Z. 2003. Ataxia telangiectasia mutated proteins, MAPKs, and RSK2 are involved in the phosphorylation of STAT3. J Biol Chem 278:12650–12659.