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Curcumin-induced histone hypoacetylation: The role of reactive oxygen species

Jiuhong Kang [a,c,*], Jie Chen [b], Yufeng Shi [c], Jie Jia [a], Yuntao Zhang [a]

[a] School of Life Sciences, Institute of Physics, Lanzou University, Lanzou 730000, China
[b] Department of Hematology, General Hospital of Lanzhou, Gansu 730000, China
[c] Shanghai Institute of Cell Biology, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China

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Abstract

Curcumin (Cur), a well-known dietary pigment derived from Curcuma longa, is a promising anticancer drug, but its in vivo target molecules remain to be clarified. Here we report that exposure of human hepatoma cells to Cur led to a significant decrease of histone acetylation. Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are the enzymes controlling the state of histone acetylation in vivo. Cur treatment resulted in a comparable inhibition of histone acetylation in the absence or presence of trichostatin A (the specific HDAC inhibitor), and showed no effect on the in vitro activity of HDAC. In contrast, the domain negative of p300 (a most potent HAT protein) could block the inhibition of Cur on histone acetylation; and the Cur treatment significantly inhibited the HAT activity both in vivo and in vitro. Thus, it is HAT, but not HDAC that is involved in Cur-induced histone hypoacetylation. At the same time, exposure of cells to low or high concentrations of Cur diminished or enhanced the ROS generation, respectively. And the promotion of ROS was obviously involved in Cur-induced histone hypoacetylation, since Cur-caused histone acetylation and HAT activity decrease could be markedly diminished by the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) or their combination, but not by their heat-inactivated forms. The data presented here prove that HAT is one of the in vivo target molecules of Cur; through inhibiting its activity, Cur induces histone hypoacetylation in vivo, where the ROS generation plays an important role. Considering the critical roles of histone acetylation in eukaryotic gene transcription and the involvement of histone hypoacetylation in the lose of cell viability caused by high concentrations of Cur, these results open a new door for us to further understand the molecular mechanism involved in the in vivo function of Cur.

Keywords: Curcumin; Histone acetylation; Histone acetyltransferase (HAT); Histone deacetylase (HDAC); Reactive oxygen species (ROS); p300

Recent studies find that histone acetylation plays an important role in eukaryotic gene transcription, carcinogenesis and the therapy of cancer. Generally, histone acetylation contributes to the formation of a transcriptionally competent environment by 'opening' chromatin and permits access of transcription factors to DNA [1,2], whereas, histone deacetylation contributes to a 'closed' chromatin state and transcriptional repression. The histone acetylation–deacetylation balance favors hypoacetylation in tumor cells [3], while it is accurately maintained through a balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities in normal cells [3,4]. Interestingly, both increasing [4,5] and decreasing [6–10] the histone acetylation of tumor cells results in their cell cycle arrest, redifferentiation or apoptosis.

Curcumin (diferuloylmethane, Cur), a polyphenol derived from the plant Curcuma longa, has been recognized as a promising anticancer drug due to its efficient induction of proliferation arrest and cell death (including apoptosis and necrosis) in a variety of tumor cells [11–16], but its in vivo target molecules remain unclear. Previous studies found that reactive oxygen species (ROS) played a critical role in the anticancer activity. Like most polyphenols, although Cur is a naturally occurring antioxidant, it exhibits prooxidant properties under certain conditions [14,16–20]. For example, high concentrations of Cur (such as at 50 μM) were found to promote ROS generation in different studies [14,16–18], while low Cur (such as at

Abbreviations: Cur, curcumin; HDAC, histone deacetylase; HAT, histone acetyltransferase; ROS, reactive oxygen species; TSA, tricostatin A; SOD, superoxide dismutase; CAT, catalase; p300 DN, p300 domain negative; CHX, cycloheximide

* Corresponding author. Tel.: +86 931 8912563; fax: +86 931 8912561.
E-mail address: kangjiuhong@lzu.edu.cn (J. Kang).

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10 μM) usually diminishes ROS generation [21,22]. Interestingly, both antioxidant and prooxidant activity are believed to be involved in the anticancer activity of Cur [11,15,16,23]. More interestingly, our previous study found that Cur exerts anticancer activity in human leukemia cells via diminishing ROS generation at low concentrations, but via enhancing that at high levels [24].

Reactive oxygen species play important roles in the control of a variety of cell functions, including proliferation, differentiation and apoptosis, and regulation of gene transcription is critically involved in these processes [25,26]. Previous studies indicate that ROS may regulate gene transcription through affecting the activation of transcription factors, such as nuclear factor κ-B (NF-κB), and the DNA binding activity of certain transcription factors, such as activator protein 1 (AP-1) and p53 [27]. Recent studies showed that ROS could also regulate gene transcription through differentially affecting histone acetylation [6,28–33]. Specifically, histone hypoacetylation is involved in the carcinogenicity and cytotoxicity of Ni^{2+} and its-generated ROS [6,28–30], whereas histone hyperacetylation is related with the promotion of ROS on the transcription of proinflammatory gene IL-8 [31–33].

In the present study, effects of Cur on histone acetylation alteration and ROS generation were studied in human hepatoma Hep3B cells. Cur treatment significantly inhibited histone acetylation through affecting the activity of HAT in cells, and the generation of ROS was diminished or enhanced by low or high concentrations of Cur, respectively. Moreover, the generation of ROS played important roles in the induction of histone hypoacetylation by Cur. These results suggested a new mechanism for the in vivo function of Cur, where HAT is one of its target molecules.

1. Materials and methods

1.1. Materials

Curcumin (Cur), RPMI-1640 medium, trichostatin A (TSA), superoxide dismutase (SOD) and catalase (CAT) were purchased from Sigma. The antibodies recognized histone H3 or H4 acetylated at their *N*-terminal lysine residues were purchased from Upstate Biotechnology. [^{3}H]-acetate and [^{3}H]-acetyl-CoA were purchased from Amersham. The BCA protein assay kit was purchased from Pierce.

1.2. Cell culture and treatment

Human hepatoma Hep3B cells were grown in RPMI-1640 medium with 10% FCS, antibiotics and 5% CO_2 at 37 °C. After culturing the cells (2×10^6 cells/ml) for 24 h, the culture medium was aspirated and replaced with new medium containing Cur and/or appropriate reagents where indicated.

1.3. Histone purification

The preparation of histones from Hep3B cells was performed as described previously [28,34]. The prepared histones were suspended in 4 M urea and stored at −20 °C until used.

1.4. Histone acetylation assay

Cells were plated at a density of 2×10^6 cells/ml, exposed to Cur and other agents indicated in the presence of 10 μCi/ml [^{3}H]acetate (5.0 Ci/mmol) for the indicated times. Before collecting the cells, TSA was or not used to stimulate the cells for 6 h. Histones were isolated and ^{3}H-labelled histones were determined by liquid scintillation counting.

1.5. Western blotting analysis of histone acetylation

Equal amounts of histones (6 μg/lane) were subjected to SDS–PAGE on 15% polyacrylamide gels, and were electrophoretically transferred to a nitrocellulose membrane. Nitrocellulose blots were blocked with 5% milk in TTBS (Tris-buffered saline plus 0.05% Tween 20, pH 7.5) and incubated overnight at 4 °C with an antibody against acetyl-histone H3 or H4 in TTBS containing 5% milk. After incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactivity was visualized by means of enhanced chemiluminescence.

1.6. HAT assay

Crude HAT preparations were extracted from total cellular homogenates of Hep3B cells and the in vitro acetylation assay was performed as described with a minor modification [35,36]. Simply, a typical HAT assay was performed using a 50-μl reaction mixture containing: histone protein (20 μg of core histones extracted from Hep3B cells), 20 μl of crude HAT extract (from 1×10^7 cells), 0–100 μM Cur, 100 ng/ml TSA, 50 mM NaCl, 10 mM HEPES (pH 7.8), 4% glycerol and 0.1 μCi [^{3}H]-acetyl-CoA (5.6 Ci/mmol, Amersham). Reactions were initiated by the addition of [^{3}H]-acetyl-CoA to the mixture, followed by incubation for 1 h at 30 °C. After incubation, the reaction mixture was spotted onto Whatman p81 phosphocellulose paper (Whatman), washed extensively with 0.2 M sodium carbonate buffer (pH 9.2), and then briefly washed with acetone. The dried filters were counted by liquid scintillation.

1.7. HDAC assay

An HDAC assay was performed as previously reported [36]. Radiolabeled histones were prepared from Hep3B cells following incubation with TSA (0.3 μM, 6 h) in the presence of 0.1 μCi/ml [^{3}H]acetate (5.0 Ci/mmol). The

histones were dried and resuspended in distilled water. Crude HDAC preparations were extracted from total cellular homogenates, and then incubated with the ^{3}H-labelled histones in 50 mM Tris–HCl buffer (pH 8.0) for 30 min at 37 °C before the reaction was terminated by the addition of 1N HCl–0.4N acetic acid. The released ^{3}H-labelled acetic acid was extracted and determined by liquid scintillation counting.

1.8. Plasmids and transfections

Wild type p300 and its domain negative (p300 DN) cDNA in pCMVβ vector under the control of the CMV promoter were purchased from Upstate Biotechnology. The p300 and p300 DN cDNA was transfected into Hep3B cells using LF2000 reagent (Invitrogen).

1.9. Measurement of intracellular ROS generation

The level of intracellular ROS was measured by the alteration of fluorescence resulting from oxidation of 29,79-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) [37]. DCFH-DA was dissolved in DMSO to a final concentration of 20 mM before use. For the measurement of ROS, cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min, then the excess DCFH-DA was washed with RPMI-1640 media prior to the treatment with Cur and/or other reagents for a time period indicated in the figure legends. The intensity of fluorescence was recorded using a flow cytometry (Becton Dickenson), with an excitation filter of 485 nm and an emission filter 535 nm. The ROS level was calculated as a ratio: ROS = - mean intensity of exposed cells: mean intensity of unexposed cells.

1.10. RT-PCR

Cells were plated at a density of 2×10^{6} cells/ml, exposed to different concentrations of Cur for 20 h, total RNA was extracted. The IL-8 mRNA level was detected by RT-PCR.

1.11. MTT assay

The viability of cells after various treatments was estimated in terms of their ability to reduce the dye (3,4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) to blue purple formazan crystal. The products were dissolved in DMSO for quantification by measuring the absorption at 570 nm using a microplate spectrophotometer reader (Multiskan Ascent, Labsystems).

1.12. Miscellaneous

Protein concentrations were determined with the BCA protein assay (Pierce) using bovine serum albumin as a standard. Statistical analysis was performed by analysis of variance (ANOVA post hoc Bonferroni), and p values less than 0.05, 0.01 or 0.001 were denoted as *, ** or *** respectively.

2. Results

2.1. Inhibition of Cur on histone acetylation in Hep3B cells

Firstly, effect of Cur on histone acetylation was detected by assaying the incorporation of [^{3}H] acetate in histones in Hep3B cells. At low concentrations (on more than 20 μM), Cur treatment led to no obvious alteration of histone acetylation, while at high concentrations (no less than 25 μM), Cur resulted in a concentration- and time-dependent decrease in histone acetylation (Fig. 1A and B). The inhibition of histone acetylation was sustained for all tested times (24 h). This inhibitory effect of Cur was further confirmed by its significant inhibition on both histone H3 and H4 acetylation in the same conditions (Fig. 2A and B). In Western blotting and histone acetylation assay, the reduction of H3 and H4 acetylation showed a similar trend with those of global histone acetylation, thus in the following study, the alteration of histone acetylation was

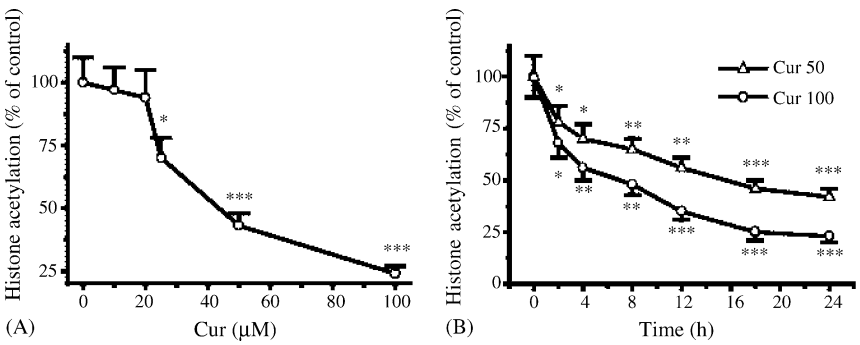

Fig. 1. Effect of Cur on the global acetylation of histones. In the presence of 10 μCi/ml [^{3}H]acetate, Hep3B cells were incubated with different concentrations of Cur for 20 h in (A), or with 50 and 100 μM Cur for the indicated times in (B). The radioactivities of the [^{3}H]acetylated histones isolated from the cells were determined by liquid scintillation counting. Means ± S.D. of three parallel experiments were indicated. $n = 3 \times 3$ cultures per conditions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the corresponding control group.

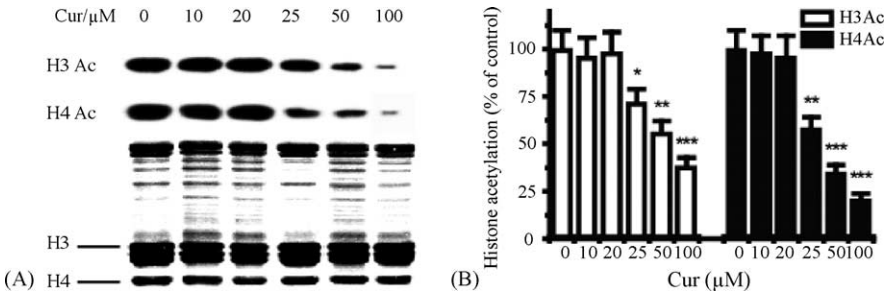

Fig. 2. Effect of Cur on the acetylation of histone H3 and H4. Hep3B cells were incubated with different concentrations of Cur for 20 h in (A). Histones were isolated and separated on 15% SDS–PAGE gels (6 μg per lane) and then subjected to Western blotting with an antibody specific for acetylated Histone H3 or H4 (the upper panels in A). 15 μg of histones used in the experiments were stained with Coomassie Blue (the lower panels in A) to confirm the equal amount of histones were added in each lane. The triplicate experiments were performed and the levels of histone H3 or H4 acetylation were quantified and normalized on the basis of control cells (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group.

evaluated by quantitating the incorporation of [^{3}H] acetate in histones.

2.2. Role of HAT and HDAC in Cur-induced histone hypoacetylation

To evaluate the role of HDAC activity in Cur-induced histone hypoacetylation, effect of Cur on the global histone acetylation was detected in the presence of 100 nM TSA, the specific inhibitor of HDAC [38]. Although TSA significantly elevated the histone acetylation in both control and Cur-treated cells (the values of histone acetylation in the control and TSA alone-treated cells were 191 ± 16 or 426 ± 38 dpm/μg protein, respectively), Cur induced a comparable inhibition of histone acetylation in the absence or presence of TSA (Fig. 3A), indicating that HDAC may be not involved in the inhibition of Cur on histone acetylation. The in vitro HDAC assay further confirmed this conclusion since Cur did not affect HDAC activity at all (Fig. 3B). In contrast, Cur significantly inhibited the in vitro acetylation of core histones catalyzed by HAT extracted from Hep3B cells (Fig. 3C). Coincidently, the HAT extracts from cells treated with Cur also showed a lower HAT activity than those from the control cells (Fig. 3D). Collectively, these results indicate that Cur inhibited histone acetylation through directly inhibiting HAT activity. In addition, p300 overexpression significantly increased histone acetylation in Hep3B cells, Cur also inhibited this increase, while p300 DN transfection completely blocked the inhibition of Cur on histone acetylation (Fig. 3E), further confirming that the inhibition of Cur on histone acetylation resulted from its inhibition on HAT activity.

2.3. ROS generation in Hep3B cells exposed to Cur

It has been previously shown that in biological systems, Cur diminished ROS generation at low concentrations [21,22,24], but promoted that at high concentrations [16–19]. DCFH-DA is commonly used to detect the generation of reactive oxygen intermediates in cells [37]. To evaluate the induction of ROS in Cur-treated cells, cells preloaded with DCFH-DA were exposed to Cur for the indicated times. Consistent with previous reports, at low concentrations (no more than 20 μM), Cur obviously decreased the ROS generation in hepatoma cells, while at high concentrations (no less than 25 μM), Cur treatment resulted in a concentration- and time-dependent increase in ROS generation (Fig. 4). In addition, the inhibitor of protein synthesis, cycloheximide (CHX), did not affect the increase of ROS by Cur (Fig. 4B).

2.4. Role of ROS generation in Cur-induced histone hypoacetylation

ROS decrease is obviously not involved in Cur-induced histone hypoacetylation, since Cur did not affect histone acetylation at the concentrations of no more than 20 μM in Hep3B cells (Figs. 1A and 2A and B). To evaluate whether the promotion of ROS generation participates in Cur-induced histone hypoacetylation, we used superoxide dismutase (SOD, an antioxidant enzyme specifically scavenges O_2^-), catalase (CAT, an antioxidant enzyme specifically scavenges H_2O_2), or their combination to diminish the high Cur-induced ROS generation. Neither the addition of SOD at 400 U/ml or CAT at 5000 U/ml alone, nor the addition of their combination, induced a significant histone acetylation alteration in Hep3B cells (data not shown). On the contrary, SOD, CAT or their combination was efficient on diminishing the promotion of 100 μM of Cur on ROS generation (Fig. 5A), the inhibition of Cur on the HAT activity in vitro (Fig. 5B) and in vivo (Fig. 5C), as well as the inhibition of Cur on histone acetylation (Fig. 5D). Combined with that the heat-inactivated SOD and CAT affected neither the promotion of Cur on ROS generation nor its inhibition on HAT activity and histone acetylation (Fig. 5), these data herein indicate an important role of ROS in the induction of histone hypoacetylation by Cur. The inhibition of 200 μM H_2O_2 on HAT activity (Fig. 5B and C) and histone acetylation (Fig. 5D) further supporting this view.

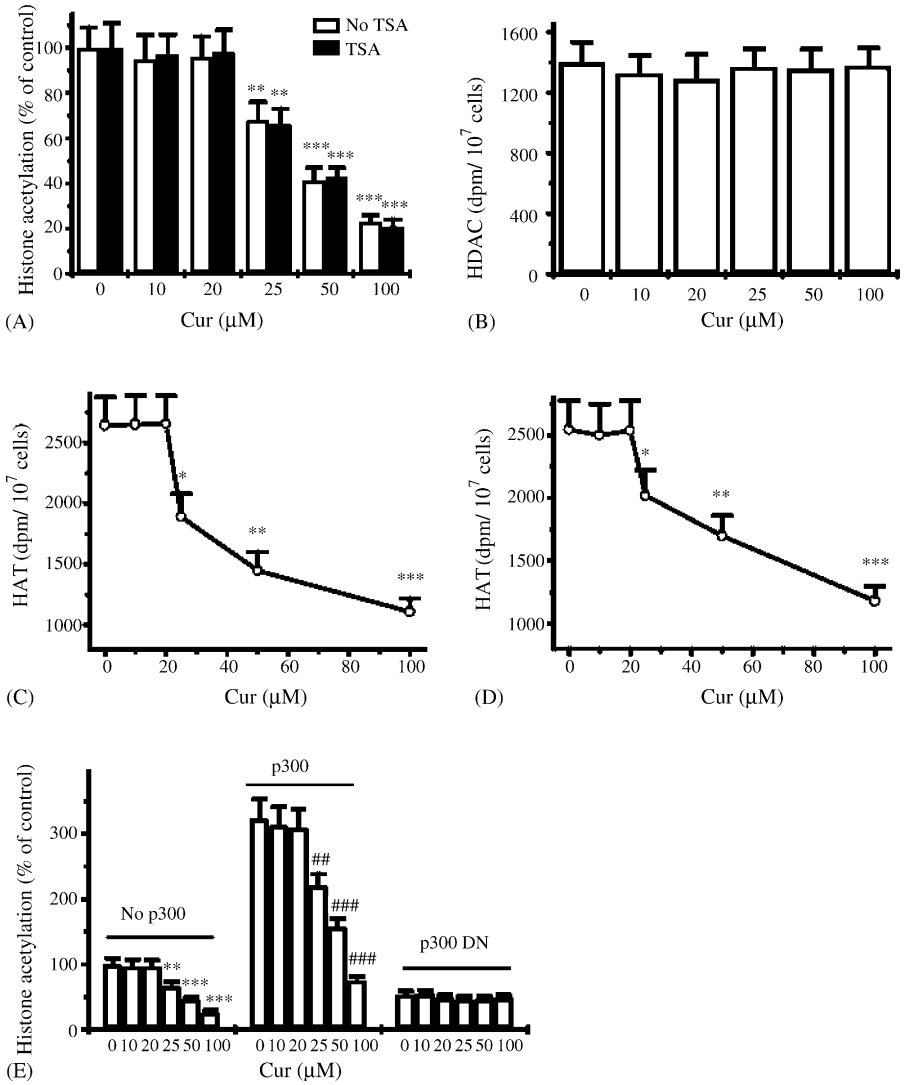

Fig. 3. Role of HDAC and HAT in Cur-induced histone hypoacetylation. (A) Inhibition of Cur on histone acetylation in the absence or presence of 0.1 μM TSA. Hep3B cells (2×10^6) were exposed to different concentrations of Cur alone or combined with 100 nM TSA together for 20 h in the presence of 10 μCi/ml [^{3}H]acetate. The radioactivities of the [^{3}H]acetylated histones isolated from the cells were determined by liquid scintillation counting. (B) Effect of Cur on the in vitro activity of HDAC. Acetylated core histones were isolated from the nuclei of Hep3B cells, crude HDAC preparations were extracted from total cellular homogenates of Hep3B. Activities of HDAC in the presence of various concentrations of Cur were determined as described in Section 2. The means of three parallel experiments are plotted. (C) Effect of Cur on the in vitro activity of HAT. In the presence of various concentrations of Cur, HAT assay was performed using the free core histones and the crude HAT preparations extracted from total cellular homogenates of Hep3B. (D), Effect of Cur on the in vivo activity of HAT. HAT preparations were extracted from total cellular homogenates of Hep3B cells treated with different concentrations of Cur for 20 h, and then HAT assay was performed using the free core histones and the crude HAT preparations. Activities of HAT in both C and D were determined as described in Material and methods, and the means of three parallel experiments are plotted. (E), Effect of p300 and p300 domain negative (p300 DN) on Cur-caused histone hypoacetylation. Hep3B cells were transfected with p300 or p300 DN cDNA using LF2000 reagent. After 24 h incubation, cells were treated with different concentrations of Cur in the presence of 10 μCi/ml [^{3}H]acetate for another 20 h. Their effect on histone acetylation was detected as indicated in Section 2. The means of three parallel experiments are plotted. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group transfected with no p300, while $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the control group transfected with p300 alone.

2.5. Role of histone hypoacetylation in Cur-induced cell viability loss

Cur treatment for 20 h led to a marked decrease in the number of viable cells in a dose-dependent manner (Fig. 6A). As we expected, 100 nM of TSA significantly attenuated the loss of cell viability caused by high concentrations of Cur (Fig. 6B), suggesting the involvement of histone hypoacetylation in the in vivo function of Cur, such as in its-induced cell viability decrease.

3. Discussion

Curcumin is a well-known dietary pigment derived from the plant Curcuma longa. Previous studies indicated that it

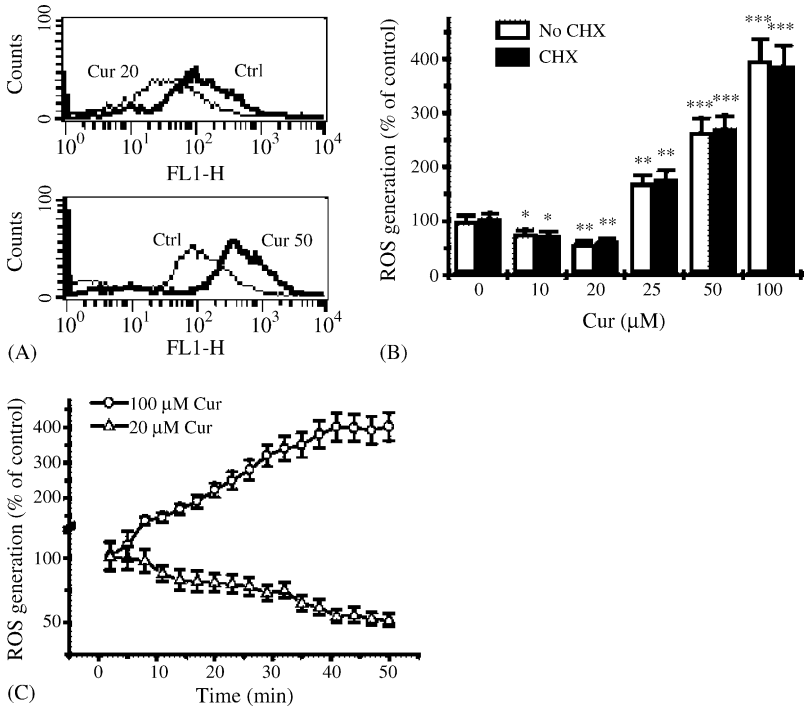

Fig. 4. Effects of Cur on ROS generation. In the absence or presence of 5 μg/mL cycloheximide (CHX), relative ROS generation in Hep3B cells exposed to different concentrations of Cur for 8 h (A, B), or exposed to 20 and 100 μM of Cur for the indicated times (C). A representative flow cytometry histogram of cells incubated without Cur or with 20 and 100 μM Cur is shown in A. All data represent mean values of six independent measurements ±S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group in (A).

efficiently induces the proliferation arrest and cell death (including apoptosis and necrosis) in a variety of tumor cells [11–16], but its in vivo target molecules and anticancer mechanisms remain to be clarified. The present study suggests that inhibition of histone acetylation was one new mechanism for the anticancer activity of Cur, where HAT serves as its molecular target, and the generation of ROS plays important roles.

Since HAT and HDAC are the enzymes controlling the state of histone acetylation in vivo [3,4], two possible mechanisms can be proposed to explain Cur-induced histone hypoacetylation. One model postulates the direct or indirect inhibition of Cur on the overall HAT activity. The other one involves the upregulation of HDAC activity by Cur, where histone deacetylation occurs at an increased rate, thereby reducing the extent of histone acetylation. Interestingly, although resveratrol, a compound structurally related to Cur, has been found to activate the human deacetylase of SIRT1 [39], the present data show that: (1) Cur did not affect the activity of HDAC in vitro; (2) TSA, an inhibitor of HDAC [38], could not rescue the inhibition of histone acetylation by Cur; (3) Cur at no less than 25 μM significantly inhibited the HAT activity in vitro; (4) Cur-treated cells possessed a lower HAT activity than their controls; and (5) most importantly, p300 DN completely blocked the inhibition of Cur on histone acetylation. Considering that p300 is an important HAT protein in Hep3B cells [40], these results indicated that it is not HDAC, but HAT that is mainly involved in the inhibition of Cur on histone acetylation.

Previous studies found that high concentrations of Cur (such as at 50 μM) promoted ROS generation in different systems [14,16–18], while low Cur (such as at 10 μM) usually diminished that [21,22]. Our previous study also found that in human leukemia cells, Cur diminished the ROS generation at low concentrations, but enhanced that at high levels [24]. Consistent with these reports, Cur diminished the ROS generation of Hep3B cells at no more than 20 μM, but promoted that at no less than 25 μM (Fig. 4). The relationship between the histone hypoacetylation and the ROS generation caused by Cur may be complicated, because on the one hand, the state of histone acetylation regulates many different gene expressions, the ROS generation by high Cur may result from the regulation of histone hypoacetylation on some members of the ROS generating complex; on the other hand, ROS generation has been reported to differently regulate histone acetylation [6,28–33]. In our study, the translation inhibitor, CHX showed no effect on Cur-caused ROS generation, indicating that the histone hypoacetylation and its caused gene suppression are not involved in the ROS generation. Combined with that some polyphenols could diminish or enhance ROS generation through affecting the Fenton reaction [20], and that Cur could induce metal ion-mediated oxidative DNA damage [17], the alteration of ROS generation in our system maybe also results from the effect of Cur on the in vivo Fenton reaction. This needs to

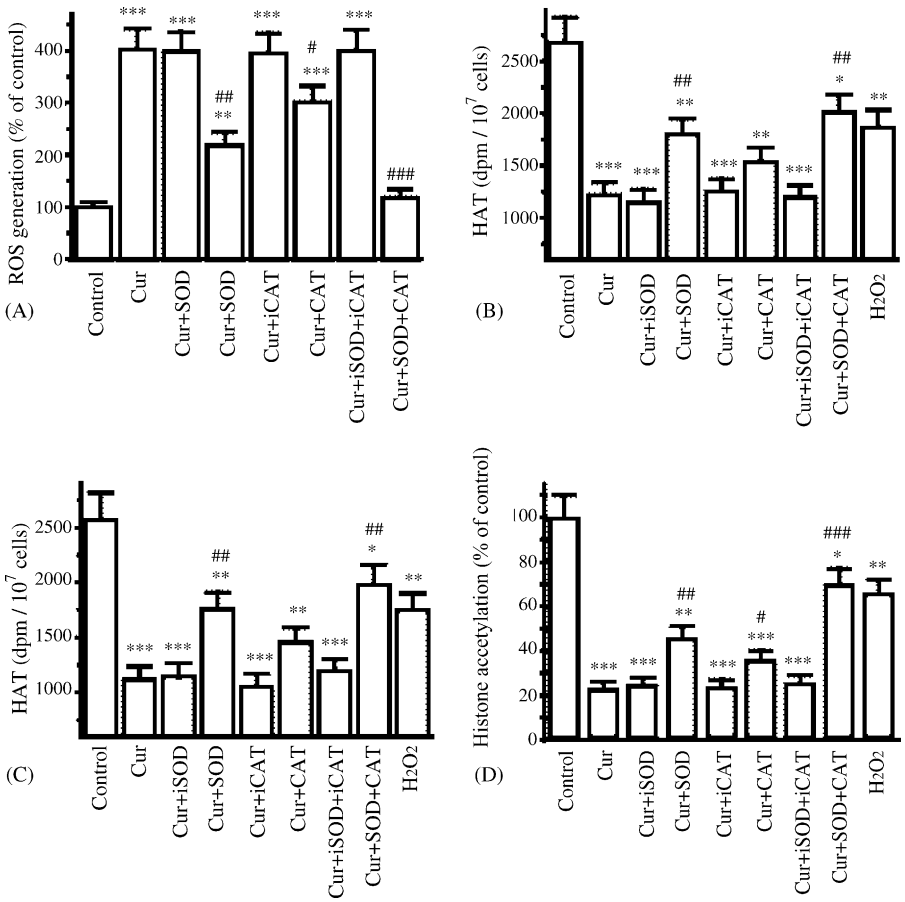

Fig. 5. Effects of antioxidant enzymes on Cur-induced ROS generation, HAT activity decrease and histone hypoacetylation. Cells were treated as indicated for 8 h in A, 24 h in B-D. iSOD and iCAT indicate heat-inactivated superoxide dismutase and catalase, respectively. 400 U/ml SOD, 5000 U/ml CAT and 200 μM H_2O_2 were used in these experiments. Means of three parallel experiments are displayed (means ± S.D.), *, **, *** indicate $p < 0.05, 0.01, 0.001$ as compared with the control; while #, ##, ### indicate $p < 0.05, 0.01, 0.001$ as compared with the 100 μM Cur-treated group, respectively. Effect of the antioxidant enzymes on relative ROS generation (A), the in vitro HAT activity (B), the in vivo HAT activity (C), and the status of histone acetylation were analyzed as described in Section 2.

be further studied. In contrast with that the ROS generation is not the result of Cur-induced histone hypoacetylation, the promotion of Cur on ROS generation may be involved in its induced histone hypoacetylation, since SOD, CAT or their combination could simultaneously diminish the ROS generation and the inhibition of both histone acetylation and HAT activity (in vitro or in vivo) by Cur, while their heat-inactivated forms showed neither such effects. The inhibition of exogenously introduced 200 μM H_2O_2 on both HAT activity and histone acetylation further supported this conclusion. However, we have noticed that although very important, ROS generation may be not the sole mechanism for Cur to inhibit the HAT activity and histone acetylation, since the combination of SOD and

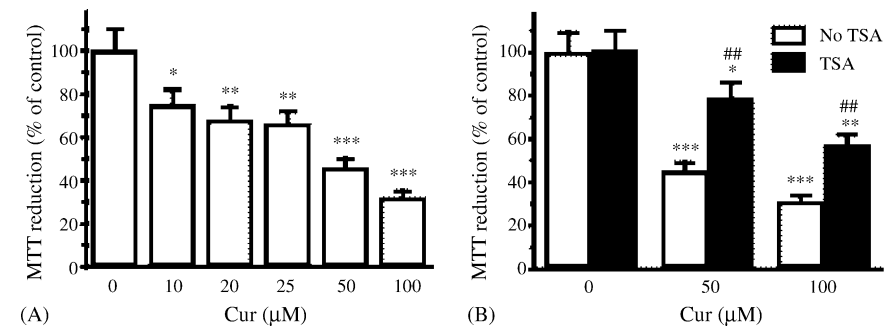

Fig. 6. Effects of Cur on the viability of hepatoma cells. (A) Cells were treated with different concentration of Cur for 20 h. (B) Cells were treated with 50 and 100 μM Cur in the presence or absence of 0.1 μM TSA for 20 h. Means of three parallel experiments are displayed (means ± S.D.), *, **, *** indicate $p < 0.05$, 0.01, and 0.001 as compared with the control cells; while $^{\#\#}p < 0.01$ as compared with the 50 or 100 μM Cur-treated group, respectively.

CAT completely suppressed the ROS generation, but did not completely block the inhibition of Cur on histone acetylation and HAT activity (Fig. 5).

The effect of ROS generation on histone acetylation remains a controversial issue. Some reports claim that ROS generation promotes histone acetylation, NF-κB activation, and the transcription of the proinflammatory gene IL-8 [31–33,41]. On the contrary, other reports have concluded that histone hypoacetylation is involved in the carcinogenicity and cytotoxicity of Ni^{2+} and its-generated ROS [6,28–30]. In our study, Cur treatment caused no significant change in the mRNA level of IL-8 (data not shown), but led to a significant decrease of histone acetylation. The mechanism underlying the inhibition of HAT activity by Cur-generated ROS is unclear. One possible explanation for that is the impairment of ROS on the activity of HAT proteins through oxidative modification of their critical cysteine and histidine-rich domains. This hypothesis is supported by the facts that: (1) p300/CBP, an important HAT protein in Hep3B cells [40], possesses 3 cysteine- and histidine-rich domains (CH1, CH2 and CH3), and any mutation in these cysteine or histidine residues significantly impairs their activation on transcription [42]; and (2) in our study, p300 DN completely blocked the inhibition of Cur on histone acetylation. Coincidently, one very recent study showed that Cur specifically inhibited the activity of p300 both in vitro and in Hela cells [43]. Although additional work is needed to understand how Cur and its generated ROS affect HAT activity, our study indicates that they inhibit overall HAT activity either directly or indirectly.

Histone hypoacetylation plays dual functions in determining cell fate. On the one hand, hypoacetylation results in increased cell viability and decreased cell differentiation and apoptosis, and hence sometimes cell malignancy [3,4]. On the other hand, histone hypoacetylation also leads to the arrest of cell cycle and the loss of cell viability including cell apoptosis [6–10]. Thus, the physiological consequence of histone hypoacetylation is obviously determined by the stimuli that caused hypoacetylation. In our system, histone hypoacetylation is also involved in the in vivo function of Cur, at least in its induced cell viability loss. Although as we have noticed, only high concentrations of Cur cause ROS generation and inhibition of HAT activity (IC_{50} for HAT activity between 25 and 50 μM), our study may be relevant to the mechanisms of Cur underlying its anticancer activity in vivo. This is because Cur is safe in human clinical trials even when administered at doses up to 10 g/day [11], and hence high doses of Cur could be used in patients. To investigate the effect of Cur on HAT activity in animals and humans is interesting.

In summary, we conclude that ROS generation is promoted during exposure of cells to high concentrations of Cur, and this ROS generation is involved in the Cur inhibition of histone acetyaltion, where HAT serves as the target molecule. And inhibition of HAT activity represents one mechanism for Cur to exert its in vivo functions. Considering the critical roles of histone hypoacetylation in the suppression of eukaryotic gene transcription and carcinogenesis [3,4], this study opens a new door for us to further understand the anticancer mechanism of Cur, and informs us that except for the clinical purpose, high doses of Cur should not be taken as a dietary supplement or as an antioxidant.

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