SirT1 activator represses the transcription of TNF-α in THP-1 cells of a sepsis model via deacetylation of H4K16

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Abstract. Sepsis is a systemic inflammatory response resulting from the excessive production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α. Sirtuin 1 (SirT1) actively deacetylates histone proteins, and facilitates chromatin compaction and gene silencing. In the present study, a cell model of sepsis, comprising lipopolysaccharide (LPS)-tolerant THP-1 cells, was used to investigate whether the SirT1 activator, resveratrol, repressed the transcription of TNF-α. Chromatin immunoprecipitation and real-time PCR were used to determine the transcription of the TNF-α promoter. The result revealed that the binding of SirT1 to the TNF-α promoter was decreased by LPS stimulation in normal cells. However, in LPS-tolerant cells, nuclear protein levels of SirT1 remained elevated, and LPS stimulation had no significant effect on the binding of SirT1 to the TNF-α promoter. However, the activity of SirT1 was increased and binding of ace-H4K16 to the TNF-α promoter was decreased with resveratrol treatment in the tolerant cells. It was concluded that resveratrol stimulated sirtuin activity in LPS-tolerant THP-1 cells, and repressed TNF-α transcription through the deacetylation of H4K16, without affecting the methylation of H3K9. Resveratrol offers potential as an infective candidate to alleviate inflammation in patients with sepsis.

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Introduction

Sepsis is a systemic inflammatory response mediated by various innate immune cells, including neutrophiles, monocytes and macrophages, upon severe infection (1). Normally, the moderate production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8 assist in confining infection and tissue damage; with the eradication of infectious agents, the inflammatory response can recover to homeostasis. However, excessive and prolonged production of inflammatory cytokines can lead to an overwhelming inflammatory response, which is referred to as sepsis (2). The mortality rates of severe sepsis can reach as high as 70% and the number of cases of sepsis continues to increase due to the continued increase in the number of immunocompromised patients (3,4). However, the molecular mechanism of sepsis remains to be fully elucidated. Studies have revealed that several mechanisms may contribute to the occurrence of sepsis, including the continued activation of neutrophils and macrophages/monocytes, upregulation of lymphocyte costimulatory molecules (5,6), rapid lymphocyte apoptosis and delayed neutrophil apoptosis, and excessive necrosis of cells and tissues (7,8). Of these, the overwhelming production of TNF-α is considered to be important in the occurrence and development of sepsis (9).

Sirtuin 1 (sirT1), an NAD^{+}-dependent deacetylase, has been well-established as a major component in the regulation of cellular stress responses, including apoptosis, autophagic DNA damage repair and metabolic disorders through histone and non-histone deacetylation. It is not surprising that SirT1 has a number of roles in multiple tissues through its effects on diverse physiological processes (10,11). Previous studies have further revealed the role of SirT1 in sepsis. For example, nuclear SirT1 has been found to guide RelB to promote mitochondrial biogenesis, which alters bioenergetics during sepsis adaptation (12). Resveratrol, an activator of SirT1, has been revealed to protect against sepsis-induced liver injury through promoting the SirT1-mediated nucleocytoplasmic translocation of high mobility group box 1 (HMGB1) (13). Other previous studies have examined the association between SirT1 and inflammatory cytokines in sepsis. For example,

acute hyperglycemia in sepsis is considered to repress the transcription and translation of SirT1, and then promote the transcription and translation of TNF-α and IL-1β (14). Studies have shown that SirT1 inhibits acute lung inflammation during sepsis by repressing the inflammasome activation pathway, including the activation of nuclear factor (NF)-κB, signal transducer and activator of transcription 3 and extracellular signal-regulated kinase (ERK)1/2 (15). In particular, the NF-κB transcription factor, RelA/p65, is considered to be the primary target of SirT1 in regulating the transcription of TNF-α through deacetylating RelA/p65 in innate cells (16,17).

It is well-known that SirT1 is also involved in chromatin compaction and gene silencing through deacetylating H4K16, H3K9 and H1K26 (18,19). Studies have also shown that SirT1 deacetylates H4K16 and promotes termination of the NF-κB-dependent transcription of TNF-α during initial lipopolysaccharide (LPS) stimulation in normal THP-1 cells (20). However, the exact role of SirT1 in epigenetic modifications of inflammatory gene promoters in sepsis remains to be fully elucidated. In the present study, epigenetic modifications by SirT1 and resveratrol were assayed in a cell model of sepsis, namely LPS-mediated tolerance in THP-1 promonocytes (21), to reveal whether SirT1 activation can repress the transcription of TNF-α in the sepsis model, and offer potential as a promising candidate for sepsis therapy.

Materials and methods

Cell culture model of sepsis and resveratrol treatment. THP-1 cells, obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Gibco RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10 U/ml penicillin G, 10 μg/ml streptomycin, 2 mM L-glutamine and 10% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C and 5% CO_2 in a humidified incubator. The sepsis phenotype (LPS-tolerant) was mimicked by stimulation of the THP-1 cells with LPS (0111:B4; 1 μg/ml) overnight (21). The normal and LPS-tolerant group THP-1 cells (1x10^6 cells/sample) were washed once with RPMI-1640, re-suspended in FBS supplemented RPMI-1640 medium at 1x10^6 cells/ml, and stimulated by 1 μg/ml LPS (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 3 h at 37°C and 5% CO_2. The normal+resveratrol group and tolerant+resveratrol group were treated with resveratrol (10 μmol/l; Sigma-Aldrich; Merck Millipore) (22) for 30 min at 37°C and 5% CO_2 prior to the LPS stimulation described above.

Chromatin immunoprecipitation (ChIP) assay. To assess the binding of SirT1, H3K9me2 and H4K16ace around the NF-κB binding site of the TNF-α proximal promoter in the SirT1-treated tolerant THP-1 cells, ChIP assays (Upstate Biotechnology, Inc., Lake Placid, NY, USA) were performed with the following modifications. Proteins from 5x10^6 cells in each sample were cross-linked to DNA using 1% formaldehyde for 10 min at room temperature. Each sample with sheared chromatin was divided into two sample groups, providing an 'input' sample, which was not incubated with any antibodies. The other sample was incubated with antibodies specific for

SirT1 (cat. no. sc-135792; 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), H3K9me2 (cat. no. 6814-25; 1:600; BioVision, Inc., Milpitas, CA, USA), H4K16ace (cat. no. sc-8662; 1:500; Santa Cruz Biotechnology, Inc.), and IgG (1:800; cat. no. sc-2027; Santa Cruz Biotechnology, Inc.) for the negative control at 4°C overnight.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The ChIP DNA for each treatment group were analyzed quantitatively through the amplification of a sequence in the human TNF-α proximal promoter region containing the κB3 site at -98 bp, relative to the transcription start site (23). The primers were as follows: Forward, 5'TAC CGCTTCCTCCAGATGAG-3' and reverse, 5'-TGCTGGCTG GGTGTGCCAA-3'. The probe was: 5'-6-FAM TTGGTGGAG AAACC-TAMRA-3' (Sangon Biotech Co., Ltd., Shanghai, China). The PCR reaction (total 20 μl) contained 2 μl DNA, 10 μl 2x TaqMan Universal Master Mix, 300 nM each primer and 100 nM dNTPs. The PCR procedure was as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles with 15 sec at 95°C and 15 sec at 60°C, using an ABI 7500 fast detection system (Thermo Fisher Scientific, Inc.). Data were normalized to the input DNA and are presented as the fold change, relative to DNA from the untreated cells.

To measure the mRNA expression of TNF-α, total RNA was isolated from cells using a Qiagen RNA mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The RNA (1 μg) was reverse transcribed into cDNA in a 20 μl volume containing 5 mM $MgCl_2$, 1 mM dNTP, 2.5 μM Oligo d (T), 2.5 U/μl MuLV Reverse Transcriptase (Thermo Fisher Scientific, Inc.). The qPCR was performed using 3 μl cDNA TNF-α and GAPDH predesigned TaqMan primer/probe sets (Thermo Fisher Scientific, Inc.) under the conditions described above. Data were normalized to GAPDH mRNA, which was analyzed using GAPDH pre-designed TaqMan primer/probe kits (Thermo Fisher Scientific, Inc.) and are presented as the fold change, relative to mRNA from the untreated cells. Sample data were normalized to GAPDH mRNA values and are presented as the fold changes, relative to mRNA from the untreated cells (23).

PCR analysis. A standard PCR reaction (total 25 μl) was composed of 2 μl ChIPed DNA or 2 μl Input DNA, 1 μM of each primer (as above), 2 mM $MgCl_2$, 0.2 M dNTPs and 0.03 U/μl AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Inc.) was performed to confirm the results of the RT-qPCR analysis. The PCR conditions were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C, 58°C and 72°C for 30 sec each, and a final cycle at 72°C for 5 min. Equal volumes of PCR product was visualized using 1.5% agarose gels and images were captured using Quantity One Imager version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

SirT1 histone deacetylase (HDAC) activity assay. SirT1 activity was assayed according to a previous study (24). Briefly, the THP-1 cells were homogenized using a Diagenode Bioruptor sonicator (Tosho Denki Co., Ltd., Tokyo, Japan). Total protein was extracted from 1.5x10^6 cells/sample (21). Briefly, cells were collected and washed twice with PBS, resuspended in 100 μl RIPA buffer (cell lysis buffer containing 0.45% NaCl,

0.5% deoxycholate, 0.5% Triton X-100, 0.05% sodium dodecyl sulfate, 0.005 M Tris) and incubated on ice for 20 min. Next, the cells were vortexed for 10 sec and centrifuged at 12,000 x g for 5 min, supernatant was retained. Protein quantity was assayed with Bradford method protein assay kit (Amresco, LLC, Solon, OH, USA). Each sample (containing 30 μg/10 μl total protein) was used for the measurement of SirT1 activity. The activity of HDAC was determined using an SirT1 Fluorimetric Drug Discovery kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's protocol. The THP-1 cell protein extracts were incubated in assay buffer with β-nicotinamide adenine dinucleotide (NAD$^+$) substrate at 37°C for 45 min. The fluorescence density was determined using a multimode detector (DTx880; Beckman Coulter, Brea, CA). The SirT1 activity was determined, relative to that in the untreated control cells.

Western blot analysis. Total nuclear protein was assayed using western blot analysis. The methods for protein extraction are the same as described previously (21). Whole-cell protein (20 μg) or nuclear protein (30 μg) was separated by SDS-PAGE and transferred to PVDF membranes. SirT1 antibodies (cat no. sc-135791; 1:800; Santa Cruz Biotechnology, Inc.) were used to visualize and quantify protein levels following incubation at 37°C for 30 min using Image Quant software 4.6.2 (GE Healthcare Life Sciences). IgG (cat. no. sc-69917; 1:1,000) was used as negative control.

Results

Resveratrol treatment suppresses the mRNA transcription of TNF-α in LPS-tolerant THP-1 cells. To assess whether resveratrol treatment repressed the mRNA transcription of TNF-α in a cell model of sepsis, RT-qPCR analysis was used to assess the mRNA levels of TNF-α. As indicated in Fig. 1, the mRNA levels of TNF-α rapidly and markedly increased in the normal cells stimulated with LPS, and then reduced over the 4 h period. By contrast, in the tolerant cells, the peak increase in the mRNA transcription of TNF-α was relatively lower following LPS re-stimulation, compared with the normal cells, which indicated that the sepsis model using THP-1 cells had been successfully established. Following resveratrol treatment for 30 mins, the mRNA levels of TNF-α decreased significantly in the tolerant+resveratrol group, compared with the tolerant group, particularly at the 1 h time point. In the normal cells, resveratrol treatment also suppressed the mRNA transcription of TNF-α, as indicated in the normal+resveratrol group, compared with the normal group.

Protein levels of SirT1 are not altered significantly with resveratrol treatment in tolerant cells. To analyze whether resveratrol treatment affected the protein transcription and translation of SirT1, western blot analysis was used to measure the protein levels of SirT1 in the THP-1 cells of each group 0, 0.5, 1 and 3 h following stimulation by LPS. As shown in Fig. 2, nuclear SirT1 protein decreased and then recovered partially in the normal and normal+resveratrol groups following LPS stimulation. However, the nuclear protein levels of SirT1 remained substantially higher over the 4 h assessment
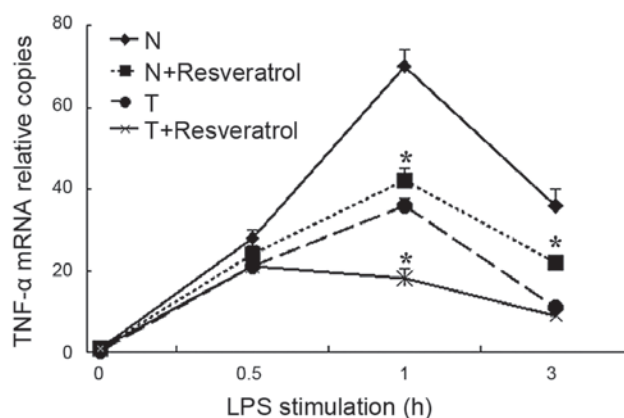

Figure 1. Resveratrol treatment alleviates the mRNA transcription of TNF-α in THP-1 cells induced by LPS stimulation. The mRNA level of TNF-α in the T+resveratrol group was significantly lower, compared with that in the T group, particularly at 1 h. The mRNA level of TNF-α in the N+resveratrol group was significantly lower, compared with levels in the N group at 1 and 3 h. Data are presented as the mean ± standard error of the mean from three independent experiments and are presented as the increase in copy number relative to that at 0 h (set as an arbitrary unit of one). *P<0.05 vs. N group. N, normal; T, tolerant; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide.

period in the tolerant group and tolerant+resveratrol group. In addition, the nuclear protein levels of SirT1 in the tolerant group and tolerant+resveratrol groups were significantly higher, compared with those in the normal and normal+resveratrol groups. Of note, the whole cell protein levels of SirT1 in the normal+resveratrol group, tolerant group and tolerant+resveratrol group were higher, compared with that in the normal group. However, it was clear that resveratrol treatment had no effect on the protein levels of SirT1 in the tolerant cells.

Resveratrol treatment promotes SirT1 activity and the binding of SirT1 to the TNF-α promoter in tolerant cells. To investigate the effect of resveratrol on SirT1 activity, THP-1 cells in each group were collected at 0 and 2 h-post LPS stimulation, and activities of SirT1 HDAC were analyzed (Fig. 3). Although the activities of SirT1 in the normal cells (N group) were reduced 2 h-post LPS stimulation, the decreases were not statistically significant. No significant alterations were found in the activities of SirT1 in the normal cells or tolerant cells at 2 h-post LPS stimulation. However, the activity of SirT1 in the tolerant cells was higher, compared with that in the normal cells. Following resveratrol treatment, the activities of SirT1 were increased significantly in the normal cells and tolerant cells. To further assess the binding of SirT1 to the TNF-α promoter κB3 site at the core promoter, the functionally important NF-κB site for the activation of TNF-α transcription (23), ChIP assays were used. It was shown that, in the normal group, binding of SirT1 to the TNF-α promoter was decreased 2 h post-LPS stimulation (Fig. 4), whereas binding of SirT1 was increased significantly in the normal+resveratrol group. In the tolerant group, the binding of SirT1 remained consistently higher, compared with that in the normal cells under LPS stimulation, and resveratrol promoted the binding of SirT1 to the TNF-α promoter, as indicated in the tolerant+resveratrol group.
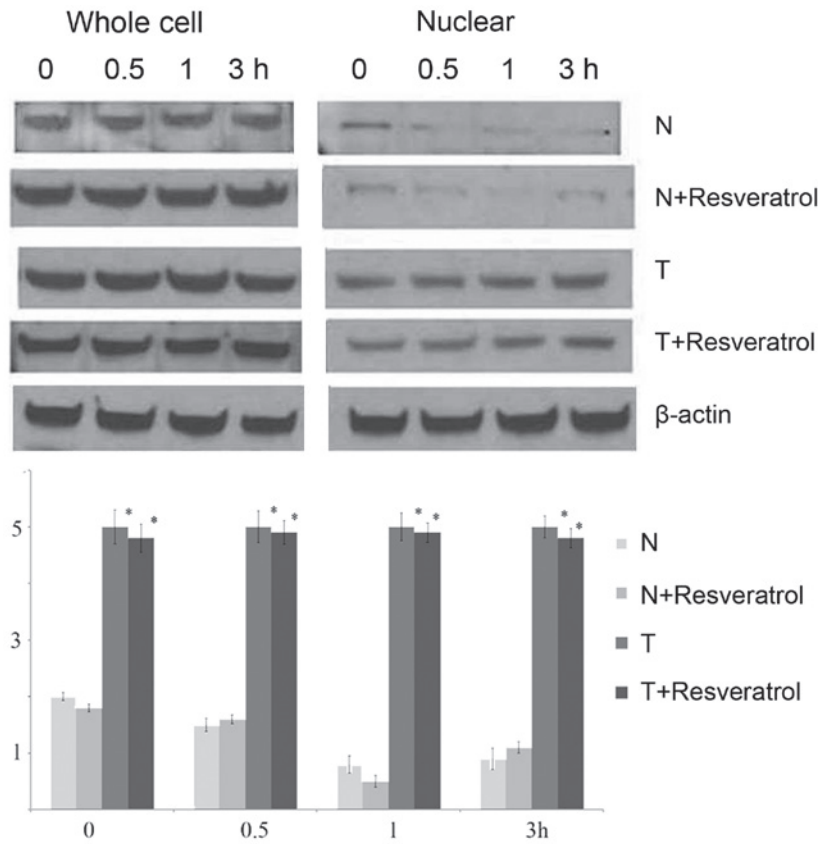
Figure 2. Resveratrol treatment has no significant effect on protein levels of SirT1 in tolerant cells. In the T group of THP-1 cells, resveratrol did not affect protein levels of SirT1 in the cytosol (left) or nucleus (right). In the N group of cells, whole cell protein levels of SirT1 increased following resveratrol treatment. Nuclear protein levels of SirT1 decreased initially and then partially recovered in the N and N+resveratrol groups. In the T and T+resveratrol groups, nuclear protein levels of SirT1 were sustained at a high level. *P<0.05. N, normal; T, tolerant; SirT1, sirtuin 1.
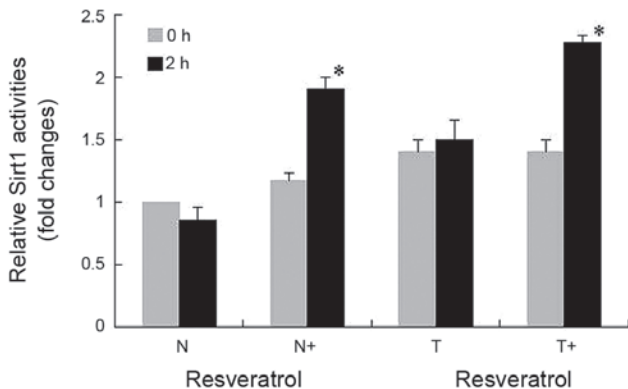

Figure 3. Resveratrol treatment promotes SirT1 activity. Analysis of the activity of SirT1 demonstrated that resveratrol treatment increased the activity of SirT1 substantially in the N and T cells. *P<0.05 vs. N group. N, normal; T, tolerant; SirT1, sirtuin 1.

Binding of H4K16ace to the TNF-α promoter decreases with resveratrol treatment in tolerant cells. The epigenetic regulation in the TNF-α promoter was assessed using ChIP assays to analyze the binding of H4K16ace and H3K9me2 to the κB3 site (Fig. 5). In the normal group, H4K16ace was increased and H3K9me2 was decreased at 2 h post-LPS stimulation. In the normal+resveratrol group, H4K16ace binding decreased with an accompanied increase in H3K9me2 binding at 2 h post-LPS stimulation. In the tolerant cells, prominent H4K16ace and H3K9me2 binding to the TNF-α promoter was sustained constantly regardless of the time course of LPS stimulation, whereas H4K16ace binding decreased significantly with resveratrol treatment (tolerant+resveratrol group) without effects on H3K9me2 binding.

Discussion

In the present study, the SirT1 activator, resveratrol, successfully repressed the transcription of TNF-α through deacetylating H4K16 at the TNF-α promoter, which indicated that resveratrol may be a promising therapeutic candidate for sepsis. Human SirT1 consists of 747 amino acids, divided into four major regions: N-terminal domain (residues 1-182), allosteric site (residues 183-243), catalytic core (residues 244-498) and C-terminal domain (residues 499-747) (25). SirT1 catalyzes the protein deacetylation reaction in its catalytic core, which consists of two subdomains for NAD⁺ and substrate binding (26). Adjacent to the N-terminal in the catalytic core is the compacted allosteric domain, to which SirT1 activators, including resveratrol, bind and positively regulate sirtuin activity (27). Resveratrol has been shown to activate SirT1 and suppress the overexpression of pro-inflammatory molecules in a dose-dependent manner in a mouse model of sepsis induced by LPS (28). The results of the present further supported that resveratrol decreased the transcription of TNF-α under LPS stimulation in tolerant cells and in normal cells.
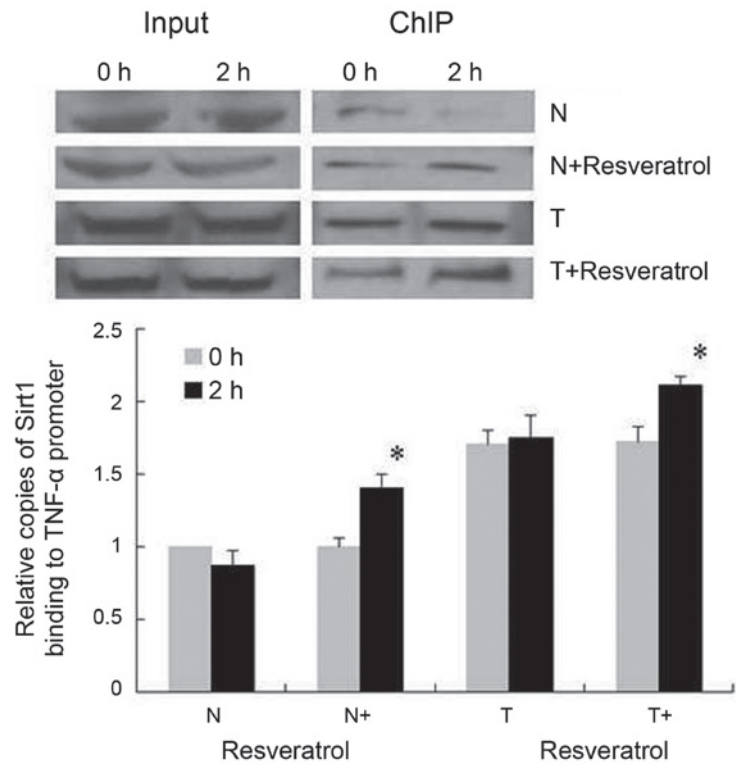
Figure 4. Resveratrol treatment increases the binding of SirT1 to the TNF-α promoter in N and T cells. Results of the PCR analysis (above) and RT-qPCR analysis (below) are shown. ChIP analyses indicated that the binding of SirT1 to the TNF-α promoter increased at 2 h in the N and T cells. Data for RT-qPCR are presented as the mean ± standard error of the mean of three independent experiments and are presented as the increase in binding relative to that of N cells at 0 h (arbitrary unit of one). *P<0.05 2 h group vs 0 h group. The standard PCR results of the ChIP samples with the same primer sets as in the RT-qPCR are representative of three independent experiments. PCR, polymerase chain reaction; N, normal; T, tolerant; SirT1, sirtuin 1; TNF-α, tumor necrosis factor-α.
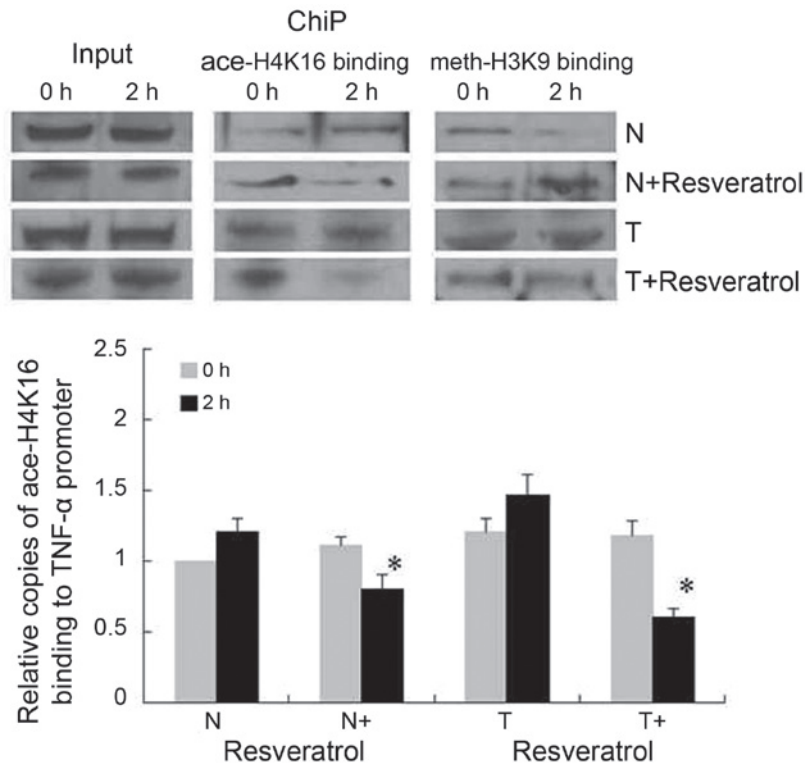

Figure 5. Resveratrol treatment decreases the binding of ace-H4K16 to the TNF-α promoter in N and T cells. Results of PCR (above) and RT-qPCR (below) analyses are shown. ChIP analyses indicated that the binding of ace-H4K16 decreased in the N+resveratrol and T+resveratrol groups at 2 h. However, in the T cells, the binding of meth-H3K9 to the TNF-α promoter remained unaltered at 2 h. Data for the RT-qPCR are presented as the mean ± standard error of the mean of three independent experiments and are presented as the increase in binding relative to that of N cells at 0 h (arbitrary unit of one). *P<0.05 2 h group vs 0 h group. Standard PCR results of ChIP samples with the same primer sets are representative of three independent experiments. PCR, polymerase chain reaction; N, normal; T, tolerant; SirT1, sirtuin 1; TNF-α, tumor necrosis factor-α; ace, acetylated; meth, methylated.

A previous study indicated that the protein levels of SirT1 decrease transiently, followed by a substantial increase between 8 and 24 h of LPS stimulation, in THP-1 cells, which is attributed to increased SirT1 protein synthesis in tolerant cells (20). The present study also found higher protein levels of SirT1 in tolerant cells, compared with normal cells. However, rather than analyzing alterations in whole cell SirT1 protein, the present study assessed the levels of nuclear SirT1 protein in each group. In normal THP-1 cells, the level of nuclear SirT1 protein decreased initially, and then recovered partially under LPS stimulation, which was similar to the observations in whole cell SirT1 protein reported previously (20). It is possible that nuclear SirT1 repressed the transcription of inflammatory genes in basal conditions by repressing TNF-α promoter transcription. Under LPS stimulation, nuclear SirT1 decreased, possibly through transferring to the cytosol, indicated by the fact that no significant alterations were found in total SirT1 protein in the present study. In accordance with this, the transcription of TNF-α was increased.

In addition to activating SirT1, resveratrol has been revealed to increase the mRNA and protein levels of SirT1 in Wistar rats, although the mechanism remains to be elucidated (29). In the present study, elevated protein levels of SirT1 were observed in normal cells treated with resveratrol, compared with untreated cells. However, resveratrol appeared to have no effect on the protein level of SirT1 in tolerant cells. These results suggested that, in the tolerant cells, the transcription and translation of SirT1 may have peaked and responded minimally to resveratrol treatment. Accordingly, the nuclear protein level of SirT1 in the LPS-tolerant cells was sustained at high levels, and binding of SirT1 to the TNF-α promoter was more marked, compared with that in normal cells in the present study. This indicated that SirT1 constantly repressed the transcription of TNF-α in tolerant cells, which showed a high mRNA level of TNF-α, but were hyporesponsive to LPS re-stimulation (30).

Studies have revealed that SirT1 limits inflammation through several non-histone proteins. It has been reported that SirT1 deacetylates H4K16 and promotes silencing of the transcription of TNF-α on initial LPS stimulation in normal THP-1 cells (20). The present study demonstrated that SirT1 was involved in epigenetic modifications in the TNF-α promoter of cells in sepsis. The binding and activity of SirT1 at the TNF-α promoter were increased in tolerant cells, compared with normal cells. Resveratrol treatment further promoted the activity and binding of SirT1 to the TNF-α promoter in the tolerant cells. As expected, H3K9 methylation decreased with LPS stimulation in normal cells due to chromatin relaxation and gene transcription (31). Resveratrol appeared to repress the transcription of TNF-α through promoting H3K9 methylation in normal cells. However, resveratrol had no effect on H3K9 methylation in tolerant cells, which already had higher levels of H3K9 methylation at the TNF-α promoter, compared with normal cells. Of note, the contradictory finding of the coexistence of a high level of H4K16 acetylation and H3K9 methylation in the TNF-α promoter in tolerant cells demonstrated that pro- and anti-inflammatory activities were active at the same time. These results indicated that resveratrol further promoted the activity of SirT1 in LPS-tolerant THP-1 cells and repressed the transcription

TNF-α through the deacetylation of H4K16 without affecting the methylation of H3K9. Taken together, the results of the present study indicated that resveratrol, as an activator of SirT1, offers potential as an infective subsidiary treatment to alleviate inflammation in patients with sepsis.

Acknowledgements

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