REPORT

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# The induction of thioredoxin-1 by epinephrine withdraws stress via interaction with β-arrestin-1

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**Keywords:** CHOP, chronic epinephrine stress, DNA damage, p53, Thioredoxin-1, β-arrestin-1

**Abbreviations:** ASK1, Apoptosis signal-regulating kinase 1; CHOP, C/EBP homologous protein; GPCR, G protein-coupled receptors; γ-H2AX, Phosphorylation of histone H2AX; MAPK, Mitogen-activated protein kinase; MDA, Malondialdehyde; MDM2, Murine double minute 2; PKA, Protein Kinase A; TBP-2, Thioredoxin binding protein-2; Trx-1, Thioredoxin-1; Txnip, thioredoxin interacting protein.

Stress regulates a panel of important physiological functions and disease states. Epinephrine is produced under stresses threaten to homeostasis. Thioredoxin-1(Trx-1) is a redox regulating protein which is induced to resist stresses and related with various diseases. Thus, it is important to examine whether Trx-1 is induced by epinephrine and to understand the underlying molecular mechanisms that Trx-1 modulates epinephrine stress. Here, we show that the expression of Trx-1 was induced by epinephrine via β-adrenergic receptor/Cyclic AMP/protein kinase A (PKA) signaling pathway in PC12 cells. The down-regulation of Trx-1 by siRNA aggravated accumulation of γ-H2AX and further decreased expression of p53 by epinephrine. Accordingly, Trx-1 overexpression alleviated accumulation of γ-H2AX and restored the expressions of p53 and C/EBP homologous protein (CHOP) in the cortex, hippocampus and thymus of mice. Moreover, Trx-1 overexpression reduced the malondialdehyde concentration by epinephrine. We further explored the mechanism on p53 and γ-H2AX regulated by Trx-1. We found that overexpression of Trx-1 suppressed β-arrestin-1 expression through interaction with β-arrestin-1. Consequently, the downregulation of β-arrestin-1 suppressed the cell viability and the expressions of γ-H2AX and cyclin D1, and increased p53 expression. Taken together, our data suggest that Trx-1/β-arrestin-1 interaction may represent a novel endogenous mechanism on protecting against stress.

## Introduction

The human mind and body respond to stress,[1] a state of perceived threat to homeostasis, by activating the sympathetic nervous system and secreting the catecholamines epinephrine and norepinephrine in the `fight-or-flight' response. These hormones can bind directly to G protein-coupled adrenergic receptors on the surface throughout the body cells.[2,3] And the effects of these hormones are similar to those associated with neural activation during stress but may have more long-lasting consequences.[4] When chronic, the stress response can be associated with disease symptoms such as peptic ulcers or cardiovascular disorders.[5] The epidemiological studies strongly indicate that chronic stress leads to DNA damage.[6,7] This stress-induced DNA damage may promote aging,[8] tumorigenesis,[6,9] neuropsychiatric conditions,[10,11] and miscarriages.[12] Epinephrine and norepinephrine promote the intracellular expression of oxidative stress gene.[13] Oxidative stress results in proto-oncogene activation, tumor suppressor gene inactivation, DNA damage, and genomic instability.[14] It has been reported that catecholamines can induce DNA damage via production of hydroxyl radicals.[15] However, the mechanisms underlying the deleterious effects of stress remain largely unknown.

Thioredoxin-1(Trx-1) is a small ubiquitous protein with 2 redox-active cysteine residues in its active site sequence:-Cys-Gly-Pro-Cys-,[16] which is essential to maintain the redox homeostatic state of the cells. It contains a redox-active disulfide bridge in its oxidized form, which is reduced by the NADPH-dependent thioredoxin reductase.[17] Trx-1 has been shown to scavenge singlet oxygen and hydroxyl radicals.[18] Furthermore, Trx-1 can protect cells from hydrogen peroxide, UV irradiation and focal ischemic brain damage.[19,20] Accumulating evidence has shown that Trx-1 plays a cytoprotective role against cellular damage and stressful perturbations.[21] Trx-1 transgenic mice display elongated life span and resistance against diabetes and toxicity caused by environmental stressors.[22] Previous study showed that Trx-1 had a protective role in the DNA damage under ultraviolet A radiation.[23-25] It has been reported that Trx-1 is closely related to various cancers.[26-28] However, the involvement of Trx-1 in a chronic stress by epinephrine exposure has not been reported.

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The aim of the present study is to explore the relationship between Trx-1 and chronic epinephrine exposure. We found that Trx-1 was induced by epinephrine. The chronic exposure to stress hormone epinephrine resulted in DNA damage, oxidative stress, and the decreases of suppressors of cancer, p53 and C/EBP homologous protein (CHOP). Moreover, overexpression of Trx-1 could reverse effects by chronic epinephrine stimulation. Our results suggest Trx-1 may be a key marker of chronic stress and regulate the molecules related to chronic epinephrine stress.

## Results

### Chronic epinephrine stimulation induced Trx-1 expression in PC 12 cells, cortex, hippocampus and thymus in mice

Trx-1 is usually induced under various stresses, including X-ray and UV irradiation, hydrogen peroxide, viral infection and ischemic reperfusion. However, it is unknown whether the Trx-1 is involved in a chronic stress by epinephrine exposure. So we detected Trx-1 expression upon epinephrine exposure. We found that epinephrine induced Trx-1 expression in a dose- and time-dependent manner (**Figs. 1A** and **B**). Furthermore, the signaling pathway on Trx-1 expression by epinephrine in PC12 cells was also investigated. PC12 cells were pretreated with propranolol, SQ22536 or H-89 for 30 min and then stimulated with 10 μM epinephrine. Epinephrine-induced Trx-1 expression was inhibited by propranolol, SQ22536 or H-89 (**Fig. 1C–E**). These data indicate that epinephrine induces Trx-1 expression via β-adrenergic receptor/Cyclic AMP/PKA signaling pathway. Consistent with effects of epinephrine *in vitro*, chronic stimulation of epinephrine induced Trx-1 expression in the cortex, hippocampus and thymus of mice, which responded prominently to acute or chronic stress (**Fig. 1F–H**).

### Trx-1 siRNA further aggravated $\gamma$-H2AX expression and decreased p53 expression by chronic epinephrine stimulation in PC 12 cells

To evaluate the role of Trx-1 in epinephrine-induced accumulation of DNA damage, we investigated the effect of downregulation of Trx-1 with siRNA on phosphorylation of histone H2AX($\gamma$-H2AX), one of the earliest indicators of DNA damage. 50 nM of Trx-1 siRNA reduced the expression of Trx-1 in PC12 cells by Western blot analysis (**Fig. 2A**). Downregulation of Trx-1 by siRNA resulted in the enhanced increase of $\gamma$-H2AX (**Fig. 2B**). It has been reported that p53 and CHOP are induced after DNA damage and try to induce the DNA repair or to induce the cell arrest or apoptosis, so we also detected the effect of Trx-1 downregulation on p53 and CHOP expressions. Downregulation of Trx-1 by siRNA further aggravated the decrease of p53 by epinephrine (**Fig. 2C**). We could not examine the CHOP change regulated by Trx-1 siRNA due to CHOP could not be detected in PC12 cells.

### Trx-1 overexpression suppressed expressions of $\gamma$-H2AX and cyclin D1, restored the expressions of p53 and CHOP by chronic epinephrine stress

Trx-1 plays a cytoprotective role against cellular damage and stressful perturbations. To further study the roles of Trx-1 in regulating the molecules involved in chronic epinephrine stress, we next detected whether overexpression of Trx-1 could suppress the expression of $\gamma$-H2AX by chronic epinephrine stimulation. The PC12 cells were transfected with GFP-tagged human Trx-1. The Trx-1 overexpression in the PC12 cells was observed by fluorescence microscope (**Fig. 3A**). As shown in **Figs. 3B** and **C**, Trx-1 overexpression suppressed the increase of $\gamma$-H2AX and restored the expression of p53 by epinephrine. Consistent with the effects of epinephrine *in vitro*, chronic infusion of epinephrine-induced increase of $\gamma$-H2AX was suppressed in the cortex, hippocampus and thymus in hTrx-1 Tg mice (**Figs. 3D**, **H**, and **L**). These results suggest that overexpression of Trx-1 attenuates accumulation of $\gamma$-H2AX by chronic epinephrine stimulation. Trx-1 overexpression also restored the expressions of p53 (**Figs. 3E**, **I** and **M**) and CHOP (**Figs. 3F**, **J**, and **N**). In addiction, Trx-1 overexpression suppressed the expression of cyclin D1 by epinephrine (**Figs. 3G**, **K**, and **O**).

### Trx-1 overexpression suppressed MDA by epinephrine in cortex, hippocampus and thymus in mice

Meanwhile, we detected the concentration of malondialdehyde(MDA), biomaker for oxidative stress, and found significant increases of MDA in the cortex, hippocampus and thymus in WT mice treated with epinephrine which were suppressed in hTrx-1 Tg mice (**Fig. 4**). The data suggest that the decreasing levels of MDA in hTrx-1 Tg mice may be associated with the protective role of Trx-1 against oxidative stress.

### The relationship between Trx-1 and β-arrestin-1 by chronic epinephrine stimulation

Arrestin isoforms 1 and 2 are widely expressed cytosolic proteins that are recruited to mediate desensitization of G-protein-coupled receptors (GPCRs) upon agonist binding, more recent work has shown that arrestin recruitment to agonist-occupied receptors also leads to activation of a variety of signaling pathways.[29,30] It has been reported that β-arrestin-1 is induced in stress model. Either Trx-1 or β-arrestin-1 was induced by stress, thus, it is interesting to explore the relationship between Trx-1 and β-arrestin-1. We examined the effect of Trx-1 siRNA on the expression of β-arrestin-1. As shown in **Fig. 5A**, Trx-1 siRNA further increased the expression of β-arrestin-1 induced by epinephrine stimulation. Trx-1 overexpression in PC12 cells suppressed the increase of β-arrestin-1 by epinephrine (**Fig. 5B**). Accordingly, the epinephrine-induced increase of β-arrestin-1 expression was inhibited in the cortex, hippocampus and thymus in hTrx-1 Tg mice (**Fig. 5C–E**). We further studied the possible mechanism on inhibition of β-arrestin-1 by Trx-1 after epinephrine stimulation. Trx-1 is translocated from cytoplasm to nucleus in response to various stresses. So we studied the relative distribution of Trx-1 in cytosolic and nuclear fractions after

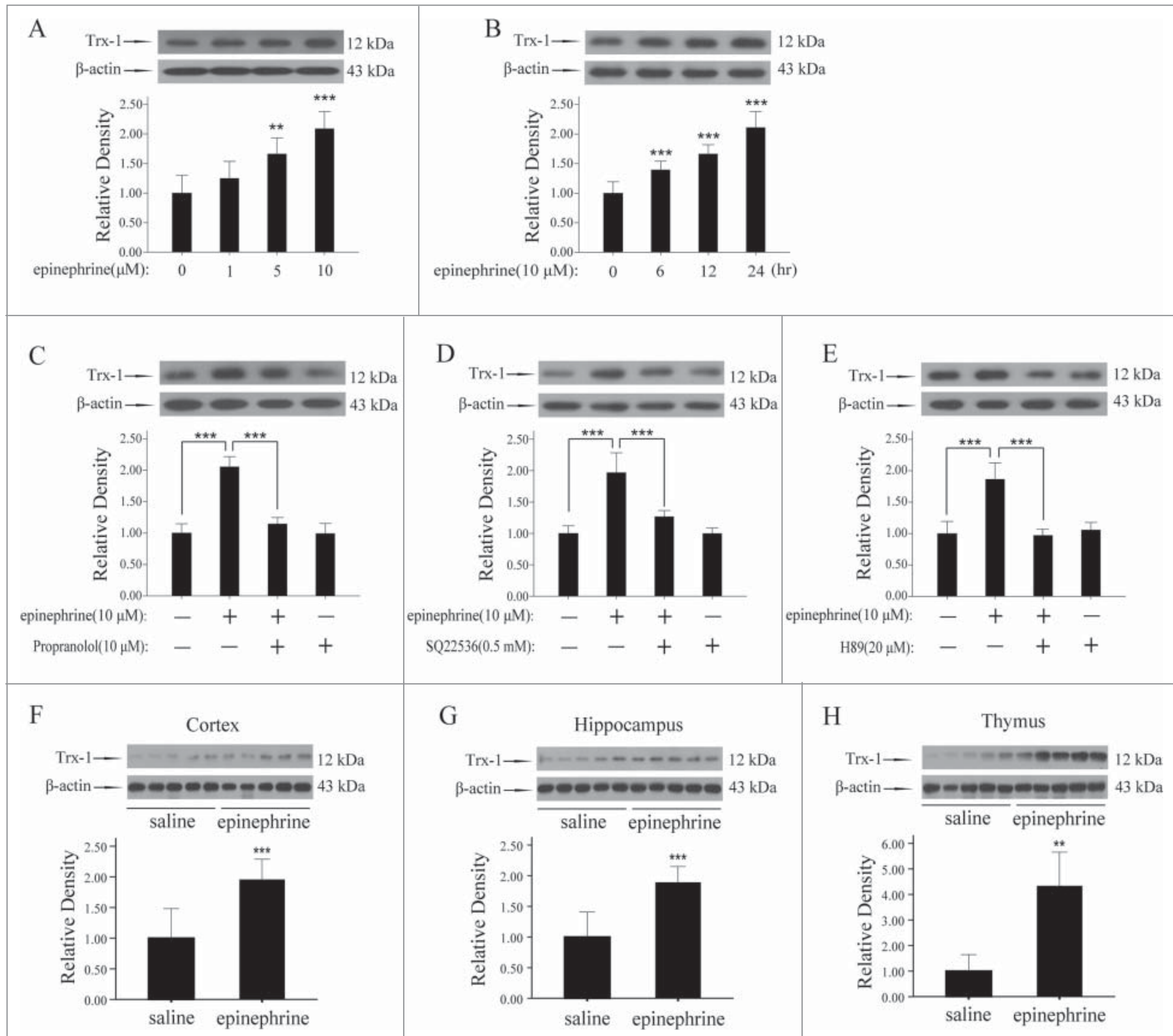


**Figure 1.** Chronic epinephrine stimulation induced Trx-1 expression. (**A**) Western blot analysis on Trx-1 expression in PC12 cells incubated with epinephrine (0, 1, 5 and 10 μM) for 24 hr. (**B**) Western blot analysis on Trx-1 expression in PC12 cells incubated with epinephrine (10 μM) for 0, 6, 12 and 24 hr. (**C**) Western blot analysis on Trx-1 expression in PC12 cells pretreated with propranolol (10 μM) for 30 min followed by incubated with epinephrine (10 μM) for 24 hr. (**D**) Western blot analysis on Trx-1 expression in PC12 cells pretreated with SQ22536 (0.5 mM) for 30 min followed by incubated with epinephrine(10 μM) for 24 hr. (**E**) Western blot analysis on Trx-1 expression in PC12 cells pretreated with H89 (20 μM) for 30 min followed by incubated with epinephrine (10 μM) for 24 hr. (**F**) Western blot analysis on Trx-1 expression in the cortex of WT mice infused with epinephrine (0.2 mg/kg/d) for 2 weeks (n = 5). (**G**) Western blot analysis on Trx-1 expression in the hippocampus of WT mice infused with epinephrine(0.2 mg/kg/d) for 2 weeks (n = 5). (**H**) Western blot analysis on Trx-1 expression in the thymus of WT mice infused with epinephrine (0.2 mg/kg/d) for 2 weeks (n = 5). All experiments *in vitro* were repeated for 3 times. Asterisks indicate statistical significance (**$P < 0.01$, ***$P < 0.001$).

epinephrine stimulation. After 12 hr and 24 hr of epinephrine stimulation, Trx-1 protein levels were increased in both cytosolic and nuclear fractions (**Figs. 5F** and **G**), indicating that Trx-1 is imported to nucleus and exerts its function after chronic stimulation of epinephrine. Previous study showed that nuclear β-arrestin-1 played an important role in catecholamine-induced accumulation of DNA damage, so we also studied the relative distribution of β-arrestin-1 in cytosolic and nuclear fractions. After 12 h and 24 h of epinephrine stimulation, β-arrestin-1 protein level was increased in nuclear fraction (**Fig. 5I**), but β-arrestin-1 protein level in cytosolic fraction was not changed (**Fig. 5H**), suggesting that chronic epinephrine stimulation leads to nuclear import of β-arrestin-1. Considering that epinephrine induced nuclear imports of Trx-1 and β-arrestin-1, we examined the interaction of Trx-1 and β-arrestin-1 and observed the endogenous binding between these 2 molecules in PC12 cells (**Fig. 5J**). Our results suggest that Trx-1 may play roles in suppressing expression of β-arrestin-1 by epinephrine stimulation through binding β-arrestin-1.

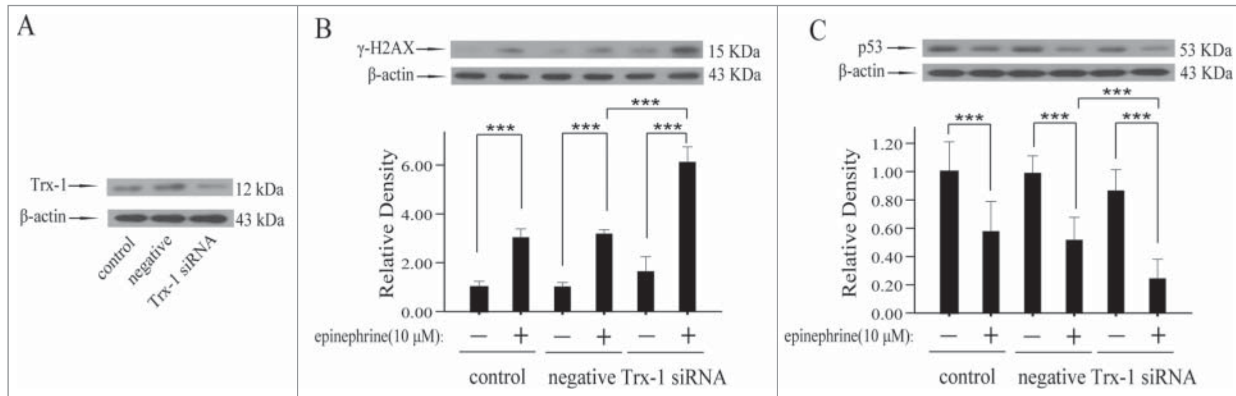


**Figure 2.** Effect of Trx-1 downregulation on $\gamma$-H2AX and p53 expressions. (**A**) Western blot analysis on Trx-1 expression by siRNA in PC12 cells. (**B**) Effect of Trx-1 downregulation on $\gamma$-H2AX expression by treatment with epinephrine (10 μM) for 24 hr in PC12 cells (**C**) Effect of Trx-1 downregulation on p53 expression. All experiments were repeated for 3 times. Asterisks indicate statistical significance (***$P < 0.001$).

**β-arrestin-1 siRNA restored the p53 and suppressed the expressions of $\gamma$-H2AX and cyclin D1 and the proliferation by epinephrine in PC12 cells**

It has been reported that β-arrestin-1 is related to cancer proliferation.[31] We further examined the effects of β-arrestin-1 siRNA on $\gamma$-H2AX and p53 by epinephrine. The expression of β-arrestin-1 was reduced by rat β-arrestin-1 siRNA in PC12 cells after incubated with β-arrestin-1 siRNA for 24 hr. (**Fig. 6A**). Furthermore, $\gamma$-H2AX expression was reduced after β-arrestin-1 downregulation by siRNA (**Fig. 6B**). The expression of p53 was also significantly restored by β-arrestin-1 siRNA (**Fig. 6C**). Cyclin D1 is overexpressed in various cancers and promotes the cell proliferation. So we also examined the effect of β-arrestin-1 siRNA on the expression of cyclin D1. As shown in **Fig. 6D**, β-arrestin-1 siRNA suppressed the increase of cyclin D1 by epinephrine. Consistent with this result, β-arrestin-1 siRNA also suppressed the proliferation by epinephrine in PC12 cells (**Fig. 6E**).

## Discussion

Epinephrine, produced during psychological stress, may have more long-lasting consequences such as genomic instability leading to DNA damage and tumorigenesis.[4,6] β-Adrenergic receptors are prototypical G protein-coupled receptors(GPCR)[32,33] that are identified throughout the body, including in the brain, lung, skeletal muscle and bone marrow.[34,35] Stimulation of the β-adrenergic receptors leads to Gs-dependent adenylyl cyclase activation and Cyclic AMP production, followed by the activation of PKA.[36,37] Mammalian Trx-1 has been shown to be a stress-inducible protein.[38] However, whether Trx-1 is induced in a chronic stress model of epinephrine exposure is unknown. In the present study, we found that Trx-1 was induced by epinephrine via β-adrenergic receptor/Cyclic AMP/PKA signaling pathway in PC12 cells (**Fig. 1A–E**). Consistent with the effects of epinephrine *in vitro*, Trx-1 expression was increased in cortex, hippocampus and thymus after mice were infused with epinephrine for 2 weeks (**Fig. 1F–H**). These results suggest that cellular defense mechanisms are activated to resist the effects of chronic epinephrine stimulation.

It has been studied that expressions of $\gamma$-H2AX and p53 are regulated in stress model. Then we examined whether Trx-1 induction was related with these effects by epinephrine. We firstly investigated the effect of downregulation of Trx-1 expression with siRNA on epinephrine-induced $\gamma$-H2AX expression. We found that Trx-1 siRNA resulted in the enhanced increase of $\gamma$-H2AX expression (**Fig. 2B**) and further aggravated the decrease of p53 by epinephrine in PC 12 cells (**Fig. 2C**). We next detected whether overexpression of Trx-1 could attenuate chronic epinephrine stimulation-induced effects. Contrary to downregulation of Trx-1, Trx-1 overexpression suppressed the expression of $\gamma$-H2AX and restored the expression of p53 by chronic epinephrine stimulation (**Figs. 3B–E, H, I, L**, and **M**). These results suggest that Trx-1 plays roles in suppressing DNA damage by chronic epinephrine stimulation and restoring DNA damage response. This result is explained by previous study, in which Trx-1 augmented the DNA binding activity of p53 and also further potentiated Ref-1-enhanced p53 activity. Trx-1 dependent redox regulation of p53 activity indicates coupling of the oxidative stress response and p53-dependent repairing mechanism.[39]

Besides p53, CHOP is a transcriptional regulator induced downstream of DNA damage and endoplasmic reticulum stress, which can promote or inhibit apoptosis according to context. Also our previous study reported that overexpression of Trx-1 suppressed CHOP in Parkinson disease model.[40] Interestingly, in the present study the CHOP was decreased by epinephrine and overexpression of Trx-1 restored the CHOP expression (**Figs. 3F, J**, and **N**). CHOP recovery may be related with the p53 increase by Trx-1 overexpression. CHOP usually plays a role in regulating DNA damage through same pathway with p53.[41] The mechanism needs to be studied further. It is interesting that overexpression of Trx-1 suppressed the cyclin D1 by epinephrine (**Figs. 3G, K**, and **O**). We think this is from p53 increase by Trx-1 overespression, then cyclin D1 was suppressed by p53.[42] Moreover, previous study showed the paradoxical suppression of

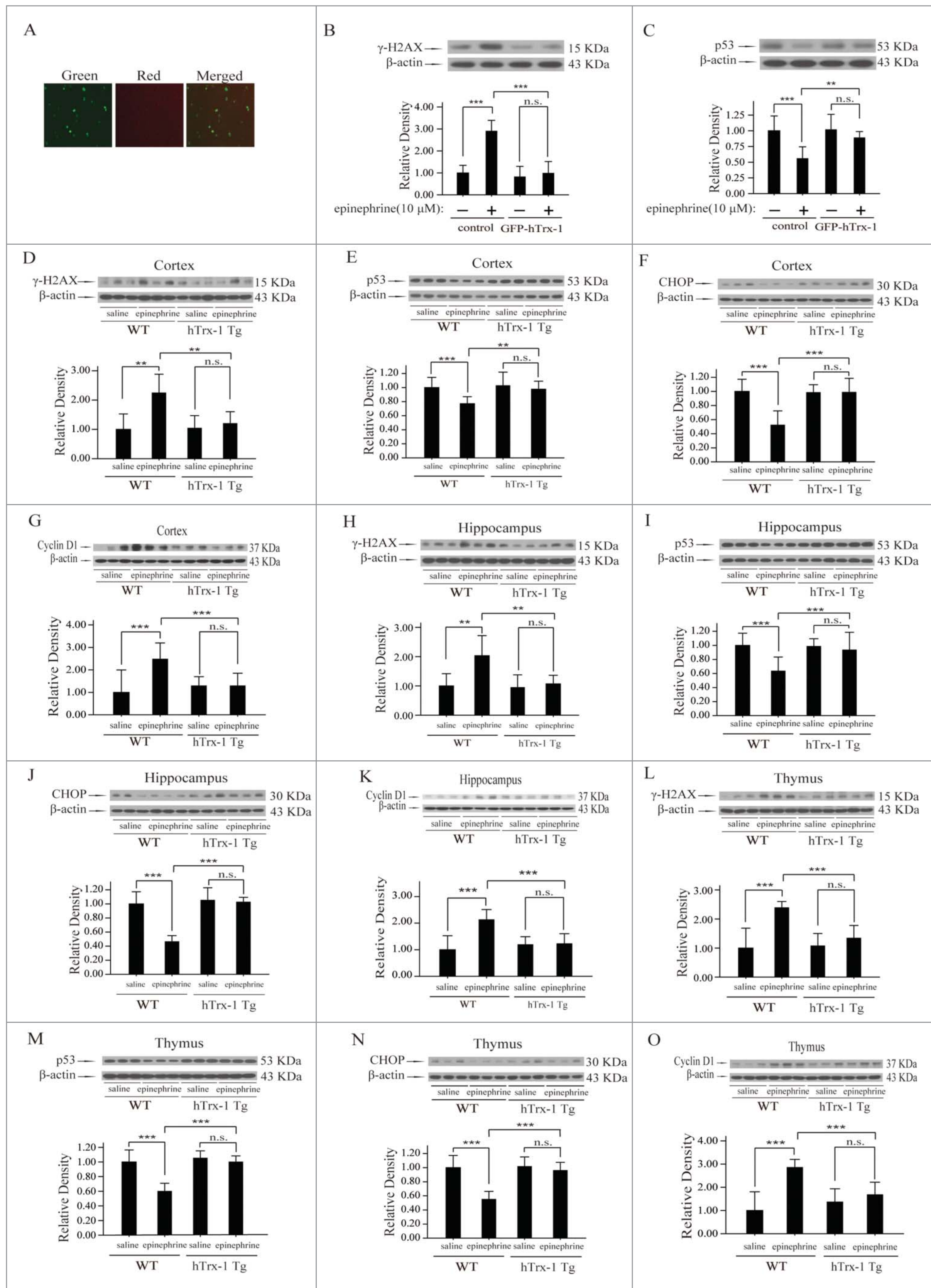


**Figure 3.** For figure legend, see page 3126.

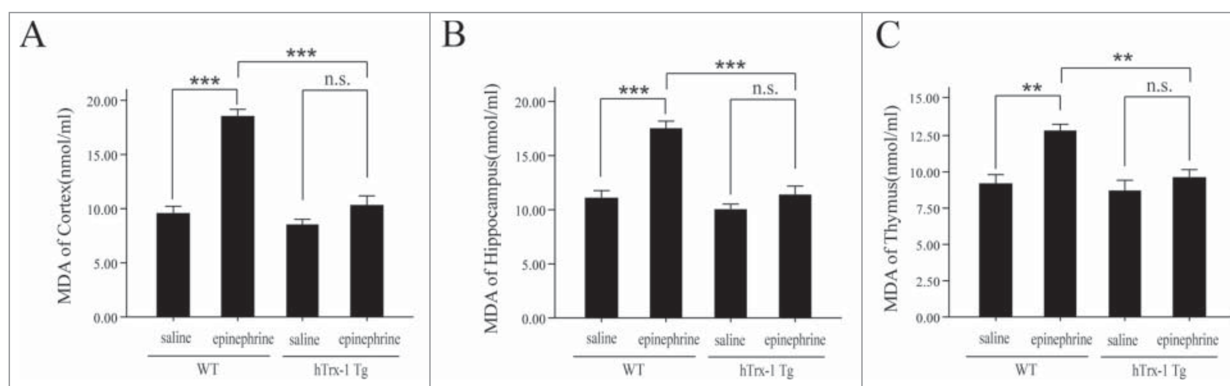


**Figure 4.** Trx-1 overexpression suppressed MDA concentration by chronic epinephrine stimulation in mice. (**A**) Trx-1 overexpression suppressed MDA concentration in the cortex (n = 5). (**B**) Trx-1 overexpression suppressed MDA concentration in the hippocampus (n = 5). (**C**) Trx-1 overexpression suppressed MDA concentration in the thymus (n = 5). Asterisks indicate statistical significance (**$P < 0.01$, ***$P < 0.001$).

senescence by p53, however, cyclin D1 was elevated in senescent cells.[43] Our result is different from previous study, in which Trx-1 up-regulated cyclin D1 expression.[44] The different results may due to the different stimulations.

Besides the DNA damage, upon chronic secretion of epinephrine and stimulation of β-adrenergic receptors, G-protein leads to increased oxidative stress.[45] Oxidative stress generated through Gs-PKA signaling[46] and appears when catecholamine increase, which contributes to DNA damage.[47] As a common by-product of lipid peroxidation, MDA is a well-accepted biomarker of the oxidative stress. In the present study, increased MDA concentration by chronic epinephrine stimulation in the cortex, hippocampus and thymus was inhibited by Trx-1 overexpression in hTrx-1 Tg mice(**Fig. 4A–C**). These data suggest that oxidative stress is induced by chronic epinephrine exposure, which is suppressed in hTrx-1 Tg mice.

In addition oxidative stress contributes to accumulation of DNA damage, β-arrestin-1 is one key component that coordinately regulates DNA damage in response to chronic exposure to stress.[48] DNA damage induced by catecholamine is abrogated in β-arrestin-1-knockout mice. β-arrestin is a scaffold/adaptor protein that interacts with various signaling regulators such as mitogen-activated protein kinase(MAPK) and apoptosis signal-regulating kinase 1(ASK1) and has a variety of functions including the regulation of endocytosis and degradation of surface receptors such as GPCR.[2,49] It has been reported that β-arrestin-1 expression was induced by stress in other study.[48] Thioredoxin binding protein-2 (TBP-2)/thioredoxin interacting protein(Txnip), acting as a negative regulator of Trx-1,[50,51] belongs to the α-arrestin family and has arrestin domains with similarity to those of β-arrestins.[52] So we further examined the relationship between Trx-1 and β-arrestin-1. We detected the effect of Trx-1 downregulation or overexpression on β-arrestin-1 expression, we found that Trx-1 acted as a negative regulator of β-arrestin-1 after chronic epinephrine stimulation (**Fig. 5A–E**). Since cytosolic β-arrestin-1 mediates epinephrine-induced activation of Murine double minute 2(MDM2), whereas nuclear β-arrestin-1 serves as an adaptor for MDM2-dependent ubiquitination of p53, which triggers the accumulation of DNA damage.[48] Trx-1 is translocated from cytoplasm to nucleus in response to various stresses.[53] So we measured the localization of β-arrestin-1 and Trx-1 after chronic epinephrine stimulation. Subcellular fractionation showed that chronic epinephrine stimulation led to increases of Trx-1 and β-arrestin-1 in nuclear fraction (**Figs. 5G** and **I**), indicating that chronic epinephrine stimulation induces β-arrestin-1 and Trx-1 nuclear translocations. The endogenous interaction between these 2 molecules was also observed (**Fig. 5J**). More importantly, the binding between Trx-1 and β-arrestin-1 was increased after epinephrine stimulation (**Fig. 5J**).

Since β-arrestin-1 expression was inhibited by overexpression of Trx-1, we further examined effects of β-arrestin-1 knockdown on accumulation of DNA damage. Thus, we examined whether β-arrestin-1 expression affected the levels

**Figure 3 (See previous page).** Effect of Trx-1 overespression on the molecules involved in chronic epinephrine stress. (**A**) PC12 cells were transfected with GFP-tagged human Trx-1 for 24 hr. The efficiency of transfection was observed by fluorescence microscope. The green channel was GFP and the red was PC12 cells. (**B**) Western blot analysis on $\gamma$-H2AX expression after PC12 cells were transfected with GFP-tagged human Trx-1 for 24 hr. (**C**) Western blot analysis on p53 expression after PC12 cells were transfected with GFP-tagged human Trx-1 for 24 hr. (**D**) Effect of Trx-1 overexpression on $\gamma$-H2AX expression in the cortex in mice (n = 5). (**E**) Effect of Trx-1 overexpression on p53 expression in the cortex in mice (n = 5). (**F**) Effect of Trx-1 overexpression on CHOP expression in the cortex in mice (n = 5). (**G**) Effect of Trx-1 overexpression on cyclin D1 expression in cortex in mice (n = 5). (**H**) Effect of Trx-1 overexpression on $\gamma$-H2AX expression in the hippocampus in mice (n = 5). (**I**) Effect of Trx-1 overexpression on p53 expression in the hippocampus in mice(n = 5). (**J**) Effect of Trx-1 overexpression on CHOP expression in the hippocampus in mice (n = 5). (**K**) Effect of Trx-1 overexpression on cyclin D1 expression in hippocampus in mice. (**L**) Effect of Trx-1 overexpression on $\gamma$-H2AX expression in the thymus in mice (n = 5). (**M**) Effect of Trx-1 overexpression on p53 expression in the thymus in mice (n = 5). (**N**) Effect of Trx-1 overexpression on CHOP expression in the thymus in mice (n = 5). (**O**) Effect of Trx-1 overexpression on cyclin D1 expression in thymus in ice. All experiments *in vitro* were repeated for 3 times. Asterisks indicate statistical significance (**$P < 0.01$, ***$P < 0.001$).

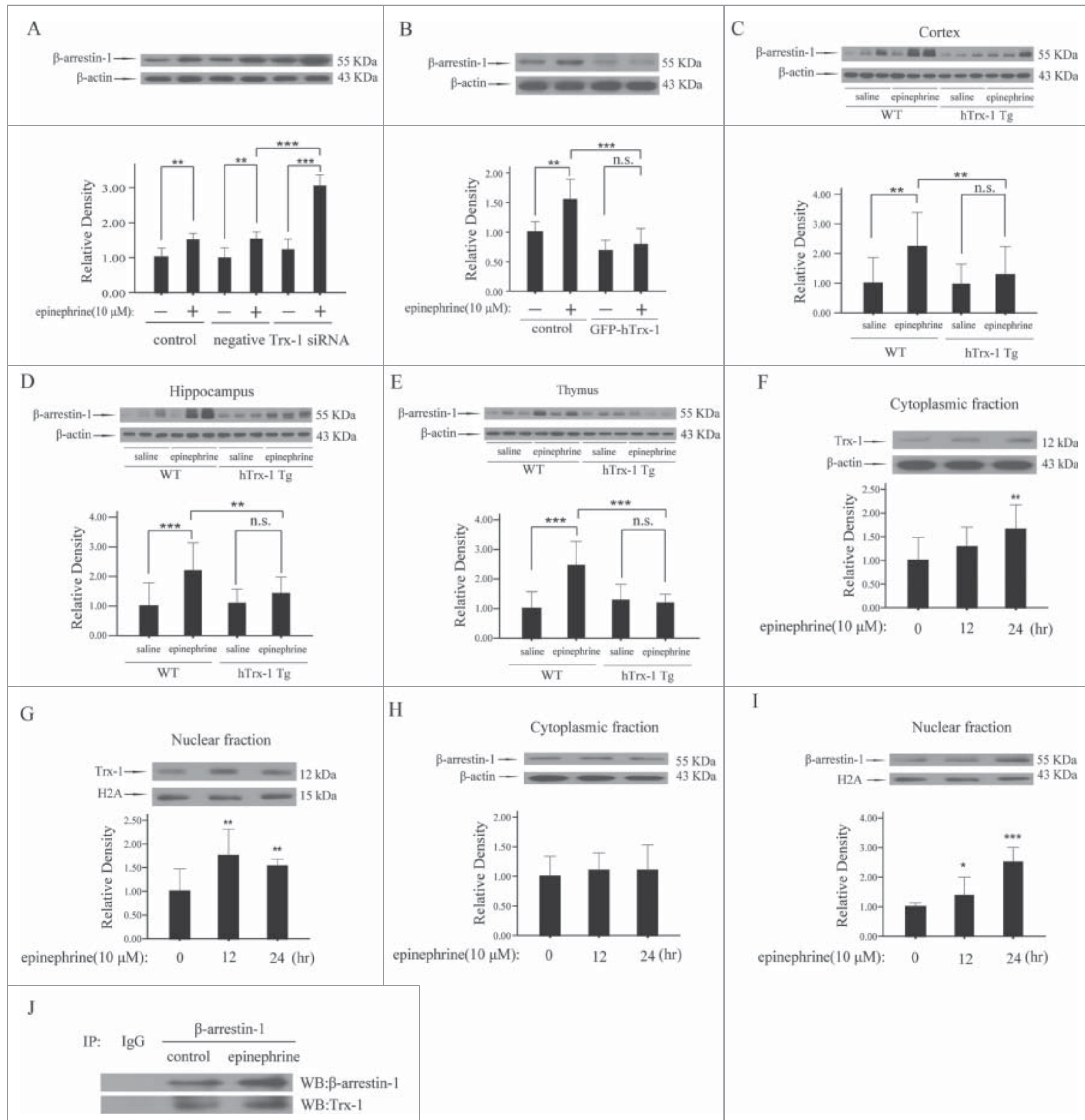


**Figure 5.** The relationship between Trx-1 and β-arrestin-1. (**A**) Effect of Trx-1 downregulation on β-arrestin-1 expression. (**B**) Western blot analysis of β-arrestin-1 expression after PC12 cells were transfected with GFP-tagged human Trx-1 for 24 hr. (**C**) Effect of Trx-1 overexpression on β-arrestin-1 expression in the cortex of hTrx-1 Tg mice (n = 5). (**D**) Effect of Trx-1 overexpression on β-arrestin-1 expression in the hippocampus in mice (n = 5). (**E**) Effect of Trx-1 overexpression on β-arrestin-1 expression in the thymus in mice (n = 5). (**F**) Western blot analysis on Trx-1 expression in cytoplasmic fraction. (**G**) Western blot analysis on Trx-1 expression in nuclear fraction. (**H**) Western blot analysis on β-arrestin-1 expression in cytoplasmic fraction. (**I**) Western blot analysis on β-arrestin-1 expression in nuclear fraction. (**J**) Endogenous binding of β-arrestin-1 and Trx-1. Cell lysates from PC12 cells were used for immunoprecipitation (IP) with anti-β-arrestin-1 antibody or normal IgG, and analyzed by western blot analysis with anti-Trx-1 antibody. All experiments were repeated for 3 times. Asterisks indicate statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

of γ-H2AX and p53. Downregulation of β-arrestin-1 significantly decreased the expression of γ-H2AX (**Fig. 6B**) and restored level of p53 (**Fig. 6C**) by epinephrine in PC12 cells, suggesting that β-arrestin-1 siRNA reduces chronic epinephrine stimulation-induced accumulation of DNA damage, which is consistent with the result that pharmacological blockade of β-arrestin-1 prevents the accumulation of DNA damage in a behavioral stress model.[45] These results suggest β-arrestin-1 is required for accumulation of DNA damage by chronic epinephrine stimulation. β-arrestin-1, a negative regulator of p53,[54] facilitates the activation of MDM2 and also promotes MDM2 binding to, and degradation of p53.[48] Nuclear export of p53 has been shown to be involved in its degradation.[55] Importantly, downregulation of β-arrestin-1 significantly decreased the expression of cyclin D1 (**Fig. 6D**) and the viability of PC 12 cells (**Fig. 6E**) by epinephrine.

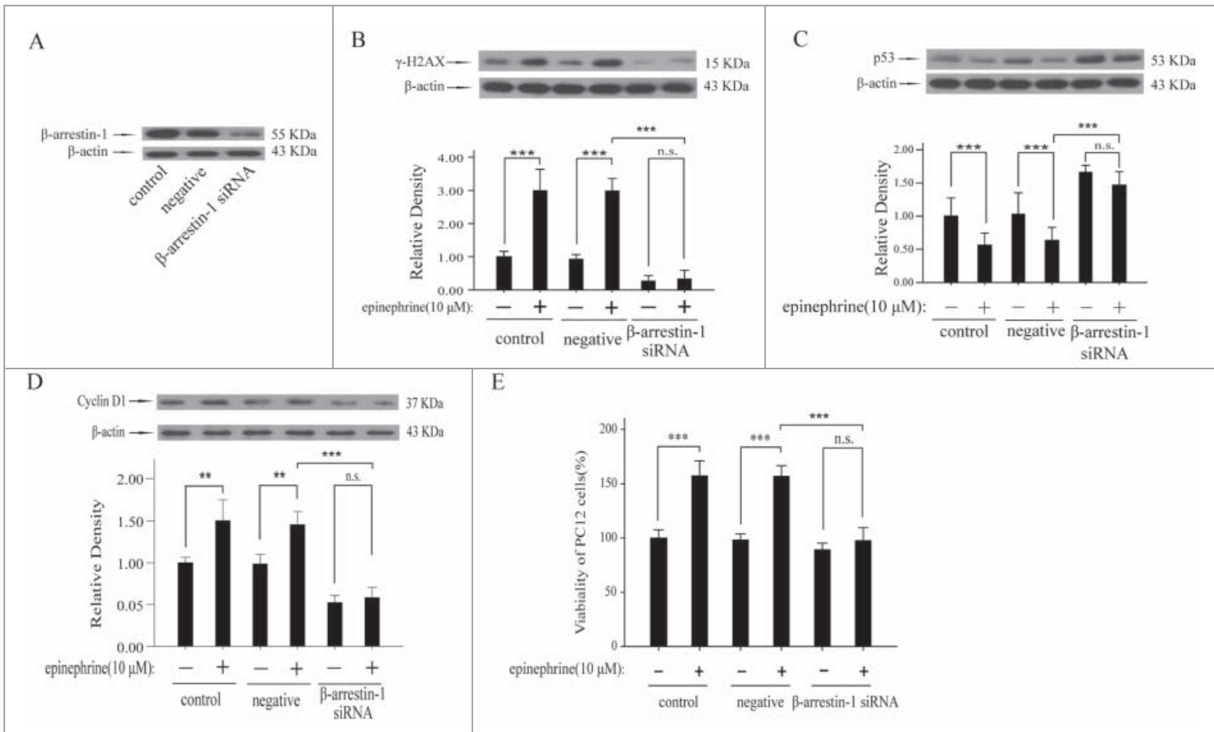


**Figure 6.** Effects of β-arrestin-1 siRNA on accumulation of DNA damage, cyclin D1 and proliferation by epinephrine. (**A**) Western blot analysis on β-arrestin-1 expression by siRNA in PC12 cells. (**B**) Effect of β-arrestin-1downregulation on γ-H2AX expression by treatment with epinephrine (10 μM) for 24 hr in PC12 cells (**C**) Effect of β-arrestin-1 downregulation on p53 expression. (**D**) Effect of β-arrestin-1 downregulation on cyclin D1 expression. (**E**) Effect of β-arrestin-1 downregulation on cell viability of PC12 cells. All experiments were repeated for 3 times. Asterisks indicate statistical significance (n.s. > 0.05, ***$P < 0.001$).

The cyclin D1 expression and cell viability was inhibited by siRNA β-arrestin-1 can be explained by the study, in which β-arrestin-1 increased cyclin D1 expression.[56]

When DNA damage occurs, DNA repair mechanisms are usually activated. If DNA repair mechanisms fail, cells are then programmed to undergo apoptosis. However, if apoptosis is prevented, somatic mutations can accumulate and lead to abnormal cell growth with DNA damage.[57] Although it has been reported that Trx-1 contributes to many of the hallmarks of increased proliferation, resistance to cell death and angiogenesis increase,[26] in present study, chronic epinephrine stimulation induced γ-H2AX, decreased expressions of p53 and CHOP, increased expression of cyclin D1 and proliferation of PC 12 cells, which were reversed by overexpression of Trx-1 through suppressing β-arrestin-1 expression (**Fig.** 7). Trx-1 is induced by epinephrine and can withdraw the effects by epinephrine *in vitro* and *in vivo*. Thus, Trx-1 is a good maker for epinephrine stimulation and plays protective roles against stress.

## Materials and Methods

### Chemicals

Epinephrine hydrochloride was obtained from Shenyang First Pharmaceutical Factory (Shenyang, China). The primary antibodies against β-arrestin-1, p53, CHOP, cyclin D1 and β-actin were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse Trx-1 rabbit polyclonal antibody was obtained from Redox Bioscience, Inc. (Kyoto, Japan). Anti-human Trx-1 rabbit polyclonal antibody was owned by our laboratory and GFP-tagged human Trx-1 plasmid was made by Yongwon Kwon. The primary antibody against γ-H2AX and H2A were purchased from Cell Signaling Technology (Boston, MA, USA). SQ22536, H-89 and propranolol were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Protease inhibitor cocktail tablets and Protein A/G agarose beads were from Merck Millipore(Billerica, MA, USA). LipofectamineTM 2000 and Opti MEM were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

### Cell culture

PC12 cells of the rat pheochromocytoma tumor cell line were purchased from Kunming Institute of Zoology (Kunming, China). PC12 cells were maintained in RPMI1640 medium with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) at 37°C in a humid atmosphere containing 5% $CO_2$.

### siRNA preparation and cell transfection

Trx-1 siRNA was chemically synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). siRNA was diluted to

20 μM with a universal buffer. The sequences were as follows: Rat Trx-1 siRNA, sense: 5′-CAG-GAUGUUGCUGCAGACU-GUtt-3′; and anti-sense:5′-ACAGUCUGCAGCAACAUC-CUGtt-3′. Rat β-arrestin-1 siRNA, sense: 5′-UUCUCC-GAACGUGUCACGUtt-3′; and anti-sense: 5′-ACGUGACAC-GUUCGGAGAAtt-3′. Negative control siRNA, sense: 5′-UUCUCCGAACGUGUCAC-GUtt-3′; and anti-sense: 5′-ACGUGACACGUUCGGA-GAAtt-3′.

PC12 cells were plated in 6-well plates at a density of $2 \times 10^5$ and allowed to adhere for 12 hr. The contents of 0.33 μg siRNA and 5 μl lipofectamine™ 2000 per well were diluted separately in serum-free Opti MEM for final volume of 250 μl, gently mixed, and incubated for 5 min at room temperature. Then, the diluted siRNA solution and the diluted lipofectamine™ 2000 were mixed gently and incubated for 20 min at room temperature. The diluted siRNA/lipofectamine™ 2000 complex was added to the plates. After transfection with siRNA for 24 hr, cells were stimulated with epinephrine for 24 hr and then harvested for assay.

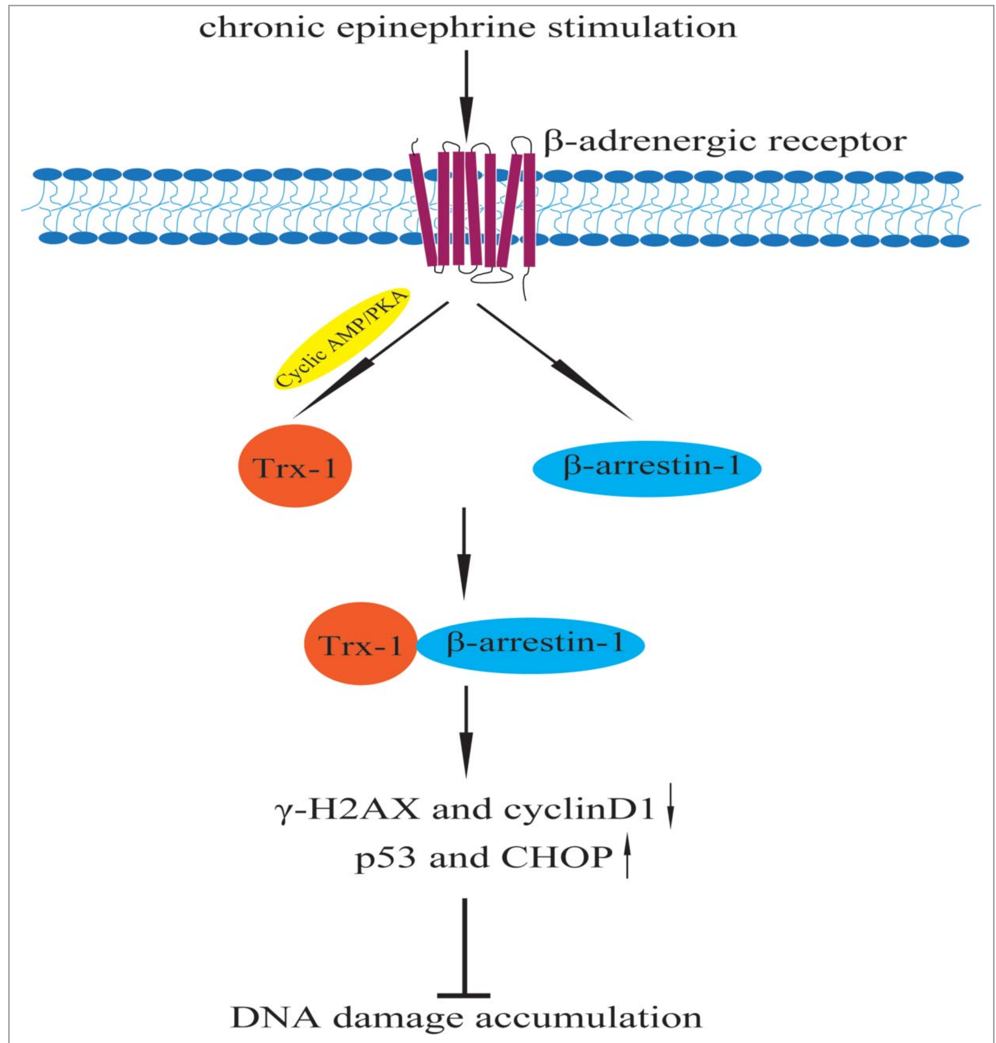


**Figure 7.** Molecular mechanism on Trx-1 regulating chronic epinephrine stress. The expression of Trx-1 was induced by epinephrine via β-adrenergic receptor/Cyclic AMP/protein kinase A signaling pathway and Trx-1 played a protective role in chronic epinephrine stress-induced DNA damage through regulating β-arrestin-1 expression by binding β-arrestin-1.

**Trx-1 overexpression in PC12 cells**

4 μg GFP-human Trx-1 plasmid and 10 μl lipofectamine™ 2000 per well were diluted separately in serum-free Opti MEM for final volume of 250 μl, gently mixed, and incubated for 5 min at room temperature. Then, the diluted plasmid solution and the diluted lipofectamine™ 2000 were mixed gently and incubated for 20 min at room temperature. The diluted plasmid/lipofectamine™ 2000 complex was added to the plates. After transfection with plasmid for 24 hr, cells were stimulated with epinephrine for 24 hr and then harvested for assay.

**Epinephrine infusion in mice**

Wild-type (C57BL/6), human Trx-1 transgenic mice were subcutaneously implanted with AlZET Model 2004 osmotic pumps (DURECT Corporation, Cupertino, CA, USA) to administer saline or epinephrine hydrochloride ($0.2\ mg\bullet kg^{-1}\bullet d^{-1}$) continuously for 2 weeks following the manufacturer's procedure. After administration, animals were killed and the indicated organs were dissected out. All animals used in these studies were adult male mice of 8-12 weeks of age. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Committee on Animal Use and Protection.

**Western blot analysis**

Protein lysates were prepared using the solubilizing solution (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO4$, 1 mM β-glycerolphosphate and 1 mg/ml leupeptin). Protein concentration was determined using Bio-Rad protein assay reagent (Hercules, CA, USA). An equal quantity of proteins was

separated by 10%(for p53) or 15% (for Trx-1, CHOP, $\gamma$-H2AX and β-arrestin-1) SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was soaked in 10% skim milk (in PBS, pH 7.2, containing 0.1% Tween-20) or 3% BSA(in TBS, pH 7.2, containing 0.1% Tween-20)overnight at 4°C, then incubated with primary antibodies against β-arrestin-1(1:1000), p53(1:1000), CHOP (1:1000), β-actin(1:1000), Trx-1(1:10000) and $\gamma$-H2AX (1:1000) followed by peroxidase-conjugated anti-mouse (1:10000) or anti-rabbit(1:10000) IgG (KPL, Inc., Gaithersburg, MD, USA). The epitope was visualized by an ECL Western blot detection kit (Millipore Corporation, Billerica, MA, USA). Densitometry analysis was performed by using ImageJ software.

### ELISA analysis

Malondialdehyde(MDA) was quantified using Quantikine MDA ELISA kit (RD, USA). Cortex or hippocampus tissue was homogenized in PBS. The assay was performed according to the manufacturer's instructions. Plates were read in an ELISA reader (Hercules, CA, USA) at 450 nm. The values thus obtained were plotted into the standard plot prepared by using serial dilutions of the standard provided with the kit and MDA concentration was calculated. MDA was expressed as nmol/ml protein.

### Immunoprecipitation (IP)

Cells were harvested in coimmunoprecipitation buffer (150 mM NaCl, 0.01 mM $Na_3PO_4$, 2 mM EDTA, 0.5% Triton X-100, and 5% glycerol plus protease inhibitor cocktail tablets). β-arrestin-1 complex was immunoprecipitated with anti-β-arrestin-1 antibody and detected with anti-Trx-1 antibody. Protein A/G-Sepharose beads were added to bind the complex from solution. The complex was brought down in the pellet by centrifugation and boiled in the presence of SDS to liberate antigen. The rest procedures of Western blot analysis were described as mentioned above. Naive IgG was used as negative control.

### Cell viability

PC12 cells were seeded into a 96-well plate overnight and then were incubated with β-arrestin-1 siRNA for 24 hr followed by cultured in the absence or presence of epinephrine in final volume of 0.2 ml for 24 hr. Thereafter, cell viability was measured by using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay according to the manufacturer's instructions. The viability = mean OD of one group/mean OD of the control.

### Data analysis

Data were expressed as means ± SD values. Statistical analysis was performed by using SPSS software. The one-way ANOVA followed by a post hoc multiple comparison test was used to compare control and treated groups. P values of less than 0.05 were considered statistically significant. All blots are representative of experiments that were performed at least 3 times.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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