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Induction of Apoptosis by L-NMMA, *via* FKHRL1/ROCK Pathway in Human Gastric Cancer Cells

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Objective To investigate the apoptosis-inducing effect of endogenous nitric oxide (NO) suppression in gastric cancer cells and its mechanisms. **Methods** Apoptosis of gastric cancer cells was detected by flow cytometry. Expression of phosphorylated FKHRL1 (thr-32, ser-253) and FKHRL1 in gastric cancer cells was analyzed using Western blotting. Immunofluorescence assay was performed to localize the intracellular phosphorylated FKHRL1 (thr-32, ser-253) and FKHRL1. Transfection of FKHRL1-HA wild type and mutant FKHRL1-HA T32A constructs was performed by lipofectamine plus reagent. NO generation was determined by Griess reaction. **Results** Gastric cancer cells were significantly apoptotic after treatment with N^G-monomethyl-L-arginine (L-NMMA, a nitric oxide synthase inhibitor), compared with the control (P<0.01). The apoptosis of gastric cancer cells induced by L-NMMA was dose-dependent and time-independent. However, the Z-DEVD-fmk, a caspase-3, 6, 7, 8, 10 inhibitor, did not prevent the apoptosis. The immunofluorescence assays showed that FKHRL1 protein was strongly expressed in the nucleu and p-FKHRL1 thr-32 protein was strongly expressed in the cytoplasm of SGC-7901 cells when endogenous nitric oxide generation was blocked by L-NMMA, but no change in FKHRL1 ser-253 phosphorylation. Nevertheless, ROCK protein was strongly expressed in p-FKHRL1 thr-32-positive SGC-7901 cells. The wortmannin, an inhibitor of phosphoinositol-3-OH kinase (PI3K), did not block the phosphorylated FKHRL1 thr-32 protein induced by L-NMMA. However, Y-27632, a specific inhibitor of the protein kinase ROCK, significantly blocked apoptosis induced by phosphorylated FKHRL1 thr-32 (P<0.01), which was mediated by L-NMMA. A significant decrease in NO generation (P<0.01) and a significant increase in apoptosis (P<0.01) were observed when FKHRL1-HA wild-type cells were transfected, which caused increased FKHRL1 thr-32 phosphorylation. **Conclusions** L-NMMA triggers gastric carcinoma cell apoptosis, possibly by promoting FKHRL1 thr-32 phosphorylation and initiating signal of FKHRL1 to ROCK kinase. This apoptotic signaling process is PI3K/Akt as well as caspase-3 independent.

Key words: Gastric carcinoma; Nitric oxide; FKHRL1; Apoptosis; Signaling

INTRODUCTION

Apoptosis is characterized by its specific morphological features in the dying cells, such as chromatin condensation, membrane blebbing, and nuclear fragmentation, distinct from the features observed in cells undergoing necrosis[1]. It has been shown that most of the morphological changes in apoptotic cells are caused by activation of specific caspases[2-3]. Caspases selectively cleave their target proteins and modulate their effects[4]. There are at least two major apoptosis pathways that are involved in the activation of caspases[3,5-6]. One pathway is activated by engagement of pro-apoptotic cytokines to a specific cell surface death receptor. The second pathway is activated in response to pro-apoptotic activity of p53 tumor suppressor protein that leads to changes in the homeostasis in mitochondria. In apoptosis pathways, effector caspases are usually activated proteolytically by an upstream caspase whose activation is regulated by protein-protein interactions[1,3]. However, the actual molecular mechanisms in various apoptosis pathways are extremely complicated and still not fully understood.

Recent studies indicate that the forkhead transcriptional factor, FKHRL1, plays an important role in the apoptotic signaling process[7-8]. Phosphorylation at all three regulatory sites of FKHRL1 contributes to the exclusion of FKHRL1 from the nuclei. This leads to a complete repression of FKHRL1-dependent transcription, thereby preventing FKHRL1 from inducing cell cycle arrest and apoptosis[8]. As an effector protein during apoptosis, serine/threonine kinase ROCK-I results

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from caspase-3–mediated activation[9]. Caspase-3-mediated cleavage of ROCK-I induces phosphorylation of the myosin light chain, as well as apoptotic membrane blebbing[10]. However, it was reported that carcinoma cells lacking the caspase-3 gene are sensitive to apoptosis induced by an unknown mechanism[11]. Since iNOS expression and its activities in gastric carcinoma cells are elevated[12-14], we investigated the signaling mechanism of FKHRL1-induced apoptosis, without involving caspase-3, by NO suppression in the human gastric carcinoma cell line SGC-7901.

MATERIALS AND METHODS

Materials

SGC-7901 cell line was obtained from the Shanghai Cell and Biochemistry Institute of the Chinese Academy of Sciences. SNP, L-arginine, L-NMMA (an inhibitor of NO synthase), wortmannin (an inhibitor of phosphoinositol-3-OH kinase (PI3K), Y-27632 (a ROCK kinase inhibitor), and Z-DEVD-fmk (a caspase-3, 6, 7, 8, 10 inhibitor) were purchased from CalBiochem, La Jolla, CA, USA. Monoclonal antibodies, such as anti-FKHRL1, anti-FKHR, anti-p-FKHRL1 thr-32, and ser-256, were purchased from Biotechnology, Lake Placid, NY, USA. Antiphosphorylated-Akt ser-473 and anti-Akt were provided by Cell Signaling, Beverly, MA, USA.

Cell Culture

SGC-7901 cells were cultured in DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in humidified 5% CO_2 atmosphere at 37˚C.

Immunofluorescence Analysis

FKHRL1, p-FKHRL1 thr-32, and p-FKHRL1 ser-253 immunofluorescence analysis was performed according to the following protocol. In brief, SGC-7901 cells were starved for 24 h with 0.1% FBS, and treated with 50 μmol/L L-NMMA in the presence of 0.1% FBS/DMEM on glass coverships in 12-well plates. The SGC-7901 cells were incubated for 24 h, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Coverships were blocked with 5% BSA for 30 min and incubated with primary antibodies (1:500 for anti-FKHRL1, anti-p-FKHRL1 thr-32 and anti-p-FKHRL1 ser-253, 1:600 for anti-p-Akt ser-473 and anti-Akt). After three washe with PBS, samples were incubated with fluorescent secondary antibodies for 1 h at room temperature. Coverships were examined by fluorescent microscopy.

Flow Cytometry Analysis

Apoptotic index of SGC-7901 cells was determined by flow cytometry according to the following protocol. In brief, samples of 5×10^5cells were collected, washed once with PBS, fixed in 70% ice-cold ethanol overnight, treated with 10 μg/mL RNase for 30 min at 37˚C, and then centrifuged. The pellets were resuspended in PBS containing 50 μg/mL of propiduim iodide. The staining was performed at 4˚C for at least 30 min, and samples were analyzed by flow cytometry (Becton Dickinson, NJ, USA).

Western Blotting Analysis

SGC-7901 cells were starved for 24 h with only 0.1% FBS, and treated with 50 μmol/L L-NMMA, 100 μmol/L L-arginine, 100 nmol/L wortmannin, 10 μmol/L Y-27632, and 20 μmol/L Z-DEVD-fmk in the presence of 0.1% FBS/DMEM in a 60-mm tissue culture dish (Corning, NY, USA). At various time points the cells were harvested and lysed in cell lysis buffer (1×PBS，1% NP-40 (v/v)，0.5% deoxycholic acid (w/v), 0.1% SDS (w/v)). The protein concentration of the whole cell extract was determined by the Bratford assay (BioRad, Hercules, CA, USA). The cell lyse (20 μg total protein) was mixed in 20 μL of solution containing 1×SDS gel loading buffer (60 mmol/L Tris，pH 6.8，1%SDS, 1% 2-mercaptoethanol (v/v)，10% glycerol (v/v)，and 0.1% bromophenol blue (w/v)) and boiled for 10 min. The cell lyses were analyzed by SDS-PAGE. The proteins were transferred to a PVDF membrane (Boehringer, Mannheim, Germany) and hybridized with the desired primary antibodies (1:500 for anti-FKHRL1, anti-p-FKHRL1 (thr-32), and anti-p-FKHRL1 (ser-253), 1:600 for anti-p-Akt (ser-473 and anti-Akt), followed by suitable secondary antibodies (1:5000). The ECL reagent (Amersham，Arlington Heights，IL, USA) was used to detect the desired proteins in the membrane.

Transfection and NO Assay

The FKHRL1 human cDNA was tagged at the C-terminus with the HA epitope and subcloned into the expression vector, pcDNA 3.1, as FKHRL1-HA wild type. In addition, the mutant of FKHRL1 at position thr-32 (FKHRL1-HA T-32A) was generated by polymerase chain reaction.

Cells were cotransfected with the pcDNA 3.1

wild-type FKHRL1-HA vector and mutant FKHRL1-HA T-32 with green fluorescent protein was cotransfected with the pcDNA3 vector, using the Lipofectamine-PLUS method provided by the supplier (Gibco, OK, USA). After 24 h, the cells positively stained with the green fluorescent protein indicated the transfection efficiency. Cells were then incubated for 30 min in PBS with 0.1% FBS. Supernatants were collected, and NO_2^- and NO_3^- were detected with the colorimetric assay kit using the Griess reaction[15] and spectrophotometric analysis based on manufacturer's instructions (Becton Dickinson, NJ, USA).

Statistical Analysis

Results are expressed as $\bar{x}\pm s$. Experimental data were analyzed using SSPS 8.0 software. *P* value less than 0.05 was considered statistically significant.

RESULTS

NO Suppression Promoted Apoptosis of SGC-7901 Cells

To confirm the induction of apoptosis by NO suppression, we initially examined the apoptotic index of SGC-7901 cells by flow cytometry after 12 h, 24 h, and 48 h of L-NMMA (10-50 μmol/L) treatment. We determined the NO production of SGC-7901 cells at different concentrations of L-NMMA (10-50 μmol/L) treatment by measuring the concentrations of NO_2^- and NO_3^- content with the Griess reagent. The results showed that NO production of SGC-7901 cells was significantly degraded after treatment with L-NMMA, compared with the control (P<0.01) (Fig. 1). SGC-7901 cells were significantly apoptotic after treatment with L-NMMA, compared with the control (P<0.01). The apoptotic index of SGC-7901 cells by NO suppression was time-independent. However, after the treatment of SGC-7901 cells with increasing concentrations of L-NMMA (10-50 μmol/L), the apoptotic index of SGC-7901 cells increased (P<0.01) (Table1), indicating that NO suppression induced the apoptosis of SGC-7901 cells in a dose-dependent manner. The Z-DEVD-fmk (20 μmol/L), a caspase-3, 6, 7, 8,10 inhibitor, did not prevent apoptosis.

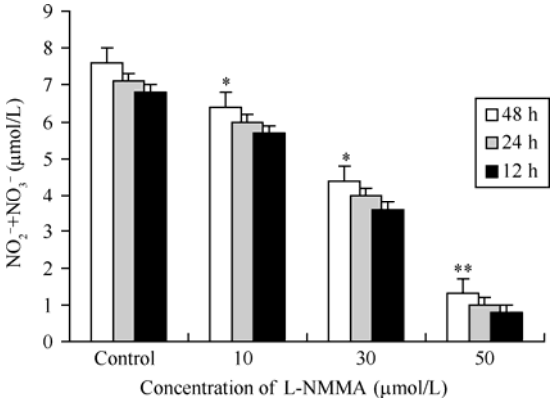

FIG. 1. Levels of $NO_3^- + NO_3^-$ measured in culture medium of SGC-7901 cells incubated (12 h, 24 h, 48 h) in the absence (control) or presence of L-NMMA (10-50 μmol/L). After the treatment of SGC-7901 cells with increasing concentrations of L-NMMA (10-50 μmol/L), the NO production of SGC-7901 cells was degrading. However, NO suppression was time-independent. $^*P<0.05$, $^{**}P<0.01$ *vs* control.

To determine the effect of NO donor, SGC-7901 cells were pretreated with L-NMMA (50 μmol/L) for 60 min, and then with SNP (1.0 mmol/L) for 30 min. The apoptosis induced by L-NMMA could be prevented by an exogenous NO donor (data not shown).

Inhibition of NO Production in SGC-7901 Cells Triggered FKHRL1 Thr-32 Phosphorylation

To investigate whether NO suppression triggers FKHRL1 phosphorylation, immunofluorescence assay was performed to examine p-FKHRL1 thr-32

TABLE 1

Apoptotic Index of SGC-7901 Cells Induced by L-NMMA ($\bar{x}\pm s$)

Different Concentrations of L-NMMA (μmol/L)	Treatment Hours (Apoptotic Index)		
	12	24	48
0	0.0311±0.018	0.0367±0.020	0.0324±0.022
10	0.0831±0.021	0.0923±0.042	0.1138±0.039
20	0.1221±0.023*	0.1673±0.040*	0.1923±0.019**
30	0.1314±0.041*	0.1756±0.038**	0.2116±0.043**
40	0.1587±0.022*	0.1934±0.043**	0.2317±0.038**
50	0.1853±0.018**	0.2123±0.041**	0.2542±0.044**

Note. $^*P$<0.05 when compared with the control at the same time point. $^{**}P$<0.01 when compared with the control at the same time point.

protein and p-FKHRL1 ser-253 protein expressed in SGC-7901 cells when iNOS activity was inhibited by L-NMMA. We found that phosphorylation of FKHRL1 at position thr-32 occurred when NO generation was blocked. The results of immunofluorescence assay showed that FKHRL1 protein was strongly expressed in nuclei of SGC-7901 cells (Fig. 2A). However, p-FKHRL1 thr-32 protein was strongly expressed in but cytoplasm, not in nuclei of SGC-7901 cells (Fig. 2B). Maximal expression of p-FKHRL1 thr-32 protein was observed between 40 and 60 min. Phosphorylation of FKHRL1 thr-32 protein occured quickly, starting 15 min after treatment with L-NMMA and ending 90 min later. The NOS inhibitor, L-NMMA, did not cause any change in FKHRL1 ser-253 phosphorylation. (Fig. 2C)

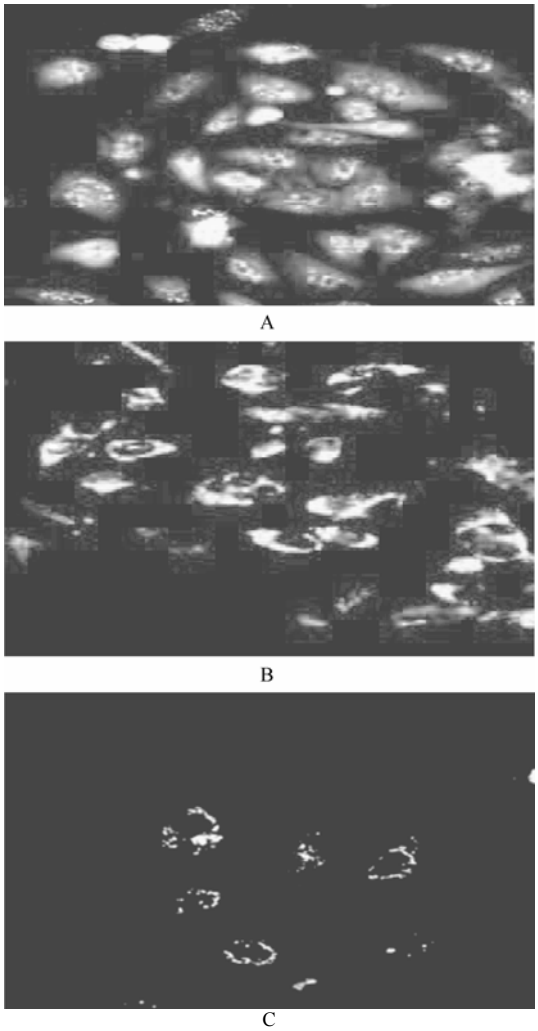

FIG. 2. p-FKHRL1 thr-32 immunofluorescence staining of SGC-7901 cells, ×400. Cells were treated (60 min) with L-NMMA (A, B, C), and then immunofluorescence staining was performed with anti-FKHRL1 (1:500), anti-p-FKHRL1 thr-32 (1:500) and anti-p-FKHRL1 ser-253 antibody. A: Strong expression of FKHRL1 in the nuclei of SGC-7901 cells. B: Phosphorylation of FKHRL1 thr-32 protein strongly expressed in the cytoplasm of SGC-7901 cells. C: p-FKHRL1 ser-253 protein weakly expressed in the cytoplasm of SGC-7901 cells.

Induction of FKHRL1 Thr-32 Phosphorylation by NO Suppression Was Independent of PI3K/Akt Pathway

To determine whether the PI3K/Akt pathway regulates FKHRL1 thr-32 phosphorylation induced by L-NMMA, Western blotting analysis was performed using anti-p-FKHRL1 thr-32 antibody and anti-p-Akt ser-473 antibody when PI3K activity was inhibited by wortmannin (a specific inhibitor of the PI3K activity). SGC-7901 cells were pretreated with wortmannin (100 nmol/L) for 45 min, and then with L-NMMA (50 μmol/L). From 15 min to 90 min after treatmeant, the cells were harvested for Western blotting analysis. As shown in Fig. 3, phosphorylation at FKHRL1 thr-32 induced by L-NMMA was not blocked by wortmannin. L-arginine was used at a concentration of 100 μmol/L, which had no effect on FKHRL1 thr-32 phosphorylation. In addition, there was no change in Akt ser-473 phosphorylation by L-NMMA or L-arginine in SGC-7901 cells (Fig. 4).

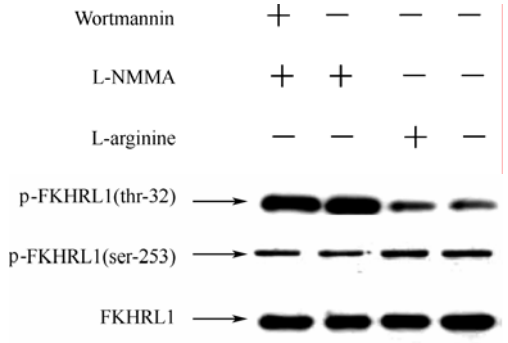

FIG. 3. Western blot analysis of phosphorylated FKHRL1 thr-32 protein induced by 50 μmol/L L-NMMA was not blocked by 100 nmol/L wortmannin. Treatment lasted 45 minutes. L-Arginine was used at a concentration of 100 μmol/L, which had no effect on FKHRL1 thr-32 phosphorylation.

Induction of Apoptosis of Gastric Carcinoma Cells by NO Suppression Through the FKHRL1/ROCK Pathway

To investigate whether L-NMMA-mediated apoptosis of SGC-7901 cells requires the biological role of the protein kinase ROCK, we determined the expression of ROCK protein in SGC-7901 cells by immunofluorescence analysis, and examined the apoptotic index of SGC-7901 cells by flow cytometry.

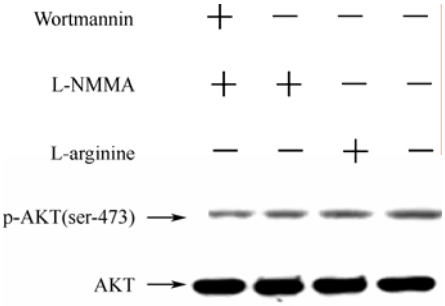

FIG. 4. Western blotting analysis of Akt ser-473 phosphorylation. No change in phosphorylation of Akt ser-473 was seen 45 min after treatment with L-NMMA (50 μmol/L) or L-arginine (100 μmol/L) in SGC-7901 cells.

The expression of ROCK protein in SGC-7901 cells after treatment with 50 μmol/L L-NMMA was significantly stronger (Fig. 5B) than that in the control (Fig. 5A). Especially, ROCK protein was strongly expressed in p-FKHRL1 thr-32-positive SGC-7901 cells. However, Y-27632, a specific inhibitor of the protein kinase ROCK, significantly FKHRL1 thr-32 (P<0.01), which was mediated by L-NMMA (Fig. 6). Flow cytometry also revealed that apoptotic index of SGC-7901 cells after treatment

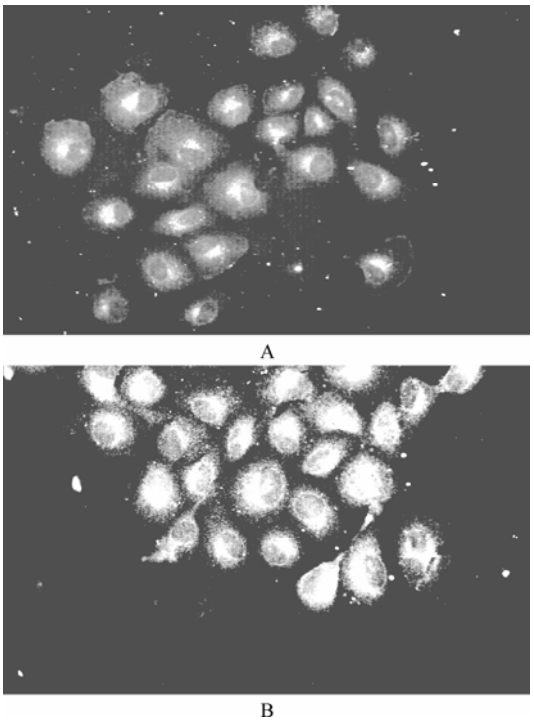

FIG. 5. ROCK immunofluorescence staining of SGC-7901 cells. Cells were treated (60 min) with (B) or without (A) L-NMMA, and then immunofluorescence staining was performed with anti-ROCK protein antibody. A: Weak expression of ROCK protein in the cytoplasm of SGC-7901 cells. B: Strong expression of ROCK protein in the cytoplasm of FKHRL1 thr-32-positive SGC-7901 cells.

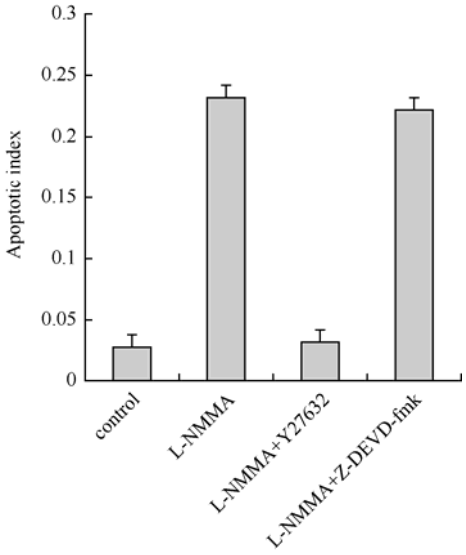

FIG 6. Signaling of FKHRL1 to ROCK causes apoptosis in gastric carcinoma cells. SGC-7901 cells were harvested after 24 hours of L-NMMA treatment and analyzed for apoptosis by flow cytometry. Gastric carcinoma cells were significantly (P<0.01) apoptotic after treatment with 50 μmol/L L-NMMA, compared with the control. Z-DEVD-fmk (20 μmol/L) did not prevent apoptosis. However, the specific ROCK inhibitor, Y-27632 (10 μmol/L), significantly decreased apoptosis (P<0.01), compared with L-NMMA and L-NMMA plus Z-DEVD-fmk treatment.

with 50 μmol/L L-NMMA was significantly higher than that in the control (P<0.01). The Z-DEVD-fmk, a caspase-3, 6, 7, 8, 10 inhibitor, could not block the apoptosis induced by L-NMMA (data not shown). These findings indicated that NO suppression induced apoptosis of SGC-7901 cells through the FKHRL1/ROCK pathway.

Transfected FKHRL1 Caused NO Suppression and Apoptosis in Gastric Carcinoma Cells

In order to validate that FKHRL1 plays an important role in apoptosis induced by L-NMMA, we transfected SGC-7901 cells with FKHRL1-HA pcDNA 3.1 wild type and the mutant FKHRL1-HA T32A. Generation of NO was measured by the Griess reaction. We found a significant decrease (P<0.01) in NO generation (Fig. 7A) and a significant increase (P<0.01) in apoptosis (Fig. 7B) in SGC-7901cells when FKHRL1-HA wild type was transfected. Furthermore, Western blotting analysis of the same experimental samples showed the increased expression of FKHRL1 thr-32 phosphorylation after transfection (Fig. 7C). This decrease in NO generation caused an increase in FKHRL1 thr-32 phosphorylation, which was responsible for the activation of ROCK kinase. This finding supports a previous observation[16] that NO is decreased in patients with a high mortality, indicating that NO

decrease could initiate the signaling process for apoptosis.

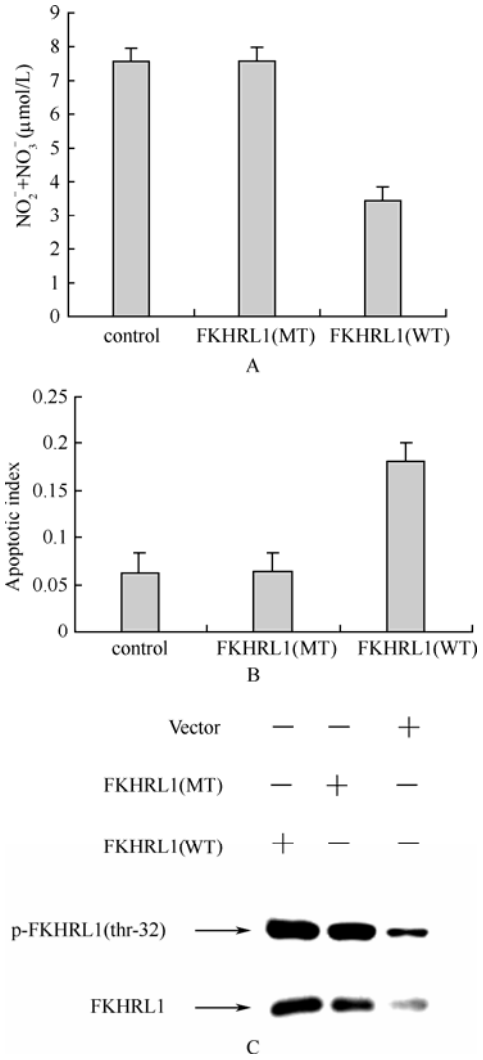

FIG. 7. Transfected FKHRL1 causes nitric oxide (NO) suppression and apoptosis. SGC-7901 cells generated 7.6±0.3 μmol/L of NO at the basal level. Transfected SGC-7901 cells with FKHRL1-HA wild type (wt) caused a significant (P<0.01) decrease in NO production (A) and a significant (P<0.01) increase in apoptosis (B). The FKHRL1-HA T-32A mutant (mt) did not affect production of NO (A) or apoptosis (B). In a Western blot analysis of the same experimental samples, increased expressions of FKHRL1 threonine-32 phosphorylation (caused by L-NMMA) and FKHRL1 protein after transfection were observed (C).

DISCUSSION

Experimental models have shown that cytokine-induced NO, or NO produced as the result of iNOS transfection, may exert an antitumor activity *in vivo* by promotion of cellular apoptosis, leading to suppression of tumorigenicity and metastasis formation[17-18]. In contrast with induced NO production, results of the present study indicated that endogenous NO suppression promoted apoptosis in the human gastric carcinoma cell line. Endogenous NO suppression induced the apoptosis of SGC-7901 cells in a dose-dependent manner. The apoptosis induced by L-NMMA could be prevented by an exogenous NO donor, SNP. These data suggest that constructively produced endogenous NO is an antiapoptotic factor in human gastric carcinoma cells.

The Forkhead transcriptional factors FKHRL1, FKHR, and AFX, members of the FOXO group, cause growth suppression and cell cycle arrest when present in nuclei of a variety of cell lines. Efficient phosphorylation of FKHRL1 at all three regulatory sites is crucial for preventing FKHRL1 from inducing apoptosis[19]. When phosphorylated on all three regulatory sites, inactivated FKHRL1 leads to its translocation from nuclei to cytoplasm and inhibits FKHRL1-dependent transcription[8,20]. However, Zivotije *et al.*[21] reported that NO suppression promotes FKHRL1 thr-32–enhanced phosphorylation and triggers apoptosis in human breast carcinoma cells. In this study, the inhibition of NO by L-NMMA significantly enhance FKHRL1 thr-32 phosphorylation and induced apoptosis in SGC-7901 cells. This process was PI3K/Akt independent. These results support the Zivotije's view that NO suppression promotes FKHRL1 thr-32–enhanced phosphorylation and triggers apoptosis[21].

Apoptotic signals are generally believed to be mediated through either of two distinct pathways, namely, caspase initiators (e.g., caspase-8, 9, 10) and effectors (e.g., caspase-3). The ser/thr kinase, ROCK, plays a crucial role in membrane blebbing and apoptosis[10]. ROCK I, but not ROCK II, is cleaved during apoptosis by activated caspase-3. However, some authors have confirmed that the directly activated ROCK I kinase is sufficient to drive cell contraction and membrane blebbing without caspase-3 activation[9,22], which is consistent with the direct effect of ROCK-induced apoptosis. In the current study, the signaling pathway from FKHRL1 thr-32 phosphorylation to ROCK protein kinase was caspase-3 independent. This signaling phenomenon is induced by NO suppression in human gastric carcinoma cells.

In this study, a significant decrease in NO generation and a significant increase in apoptosis were observed when FKHRL1-HA wild type was transfected in SGC-7901 cells. The decrease in NO generation caused an increase in FKHRL1 thr-32 phosphorylation, which is responsible for the activation of ROCK kinase. This finding supports a previous observation that NO is decreased in patients with a high mortality, indicating s that NO decrease

initiates the signaling process for apoptosis[16]. The mechanism and role of single FKHRL1 phosphorylation at position thr-32 during decreased NO production are not known. However, we have demonstrated that this apoptotic signaling pathway initiated by phosphorylated FKHRL1 thr-32 directly activates ROCK kinase, which is PI3K/Akt and caspase-3 independent.

In conclusion, NO suppression triggers gastric carcinoma cell apoptosis, which promotes FKHRL1 thr-32–enhanced phosphorylation and initiates signaling of FKHRL1 to ROCK kinase. This apoptotic signaling process is PI3K/Akt as well as caspase-3 independent.

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