RESEARCH ARTICLE

The apoptotic inducible effects of salicylic acid on hepatoma cell line: relationship with nitric oxide signaling

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Abstract Clinical and experimental data suggest that salicylic acid (SA) is tumor preventive and NO has a multitude of effects on tumor biology. Therefore, firstly, the aim of our study is to explore the important role of SA in apoptotic induction of liver cancer cells. Secondly, we investigate whether SA mediates the anti-tumor effects by NO signaling pathway. The liver cancer cell line was treated with different concentrations of SA. Cell proliferation was tested using MTS assay and cell apoptosis was assessed by flow cytometry. NO content and NOS activities were measured by biochemical assay. The anti- or pro-apoptotic regulator gene expressions were analyzed by real-time PCR. Our data illustrated that high concentration of SA significantly inhibited liver cancer cell proliferation accompanied by apoptosis induction. In addition, SA led to the release of NO and the increase of NOS activities in above process. Importantly, SA up-regulated a series of apoptosis-related gene expression and reduced the mRNA level of *HMGB1*. Meanwhile, we also found that NOS inhibitor L-NAME and NO scavenger cPTIO attenuated the above SA-induced effects. Thus, we provided the evidence that SA exerted anti-tumor effects in liver cancer cell in part mediated by the NO pathway.

Keywords Salicylic acid · Apoptotic inducible effect · Hepatoma cell line · Nitric oxide

Abbreviations

SA	Salicylic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
L-NAME	N ^G -nitro-L-arginine methyl ester hydrochloride
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt

Introduction

Recently, several decades of research have provided considerable evidence for the prevention role of aspirin for patients with cancer (Annemijn 2012; Menamin et al. 2015; Giampieri et al. 2016; Elwood et al. 2016). Interestingly, salicylic acid, as one of the aspirin metabolite in vivo (Paterson et al. 2001; Klessig 2016), could induce apoptosis selectively in malignant cells in vitro (Chung et al. 2003; Elder 1996; Klampfer 1999; Zitta et al. 2012) and showed anti-tumor effects in vivo (Elwood 2009). However, the mechanistic terms have not been sufficiently explained. Therefore, there is a pressing need to understand the protective mechanisms of salicylic acid against cancer.

Nitric oxide (NO), which is synthesized by three NOS isoforms in vivo, neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2) (Nathan 1997; Klotz et al. 1998), has been invoked in nearly every normal (Palmer et al. 1987) and pathological condition associated with human physiology (Moncada et al. 1991; Xu and Liu 1998). In tumor biology, NO has been characterized and implicated to play

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regulatory roles in various processes of carcinogenesis, including tumor initiation (Puglisi et al. 2015), growth (Jenkins et al. 1995; Reveneau et al. 1999; Ambs et al. 1998), invasion and metastasis (Vakkala et al. 2000), inflammation (Kanwar et al. 2009), and modulation of therapeutic responses (Wink et al. 1997; Hagos et al. 2007). So far, no link between NO generation and salicylic acid anti-tumor effects has been established.

High-mobility group box chromosomal protein 1 (HMGB1), an evolutionarily conserved protein, has been demonstrated to play a potential role in anti-cancer therapy (Sims et al. 2010). HMGB1 could stimulate a number of proteins involved in proliferation of cancer cells and inhibits signals that control cell growth. In addition, HMGB1 were identified as SA targets, which modulate inflammatory responses (Choi et al. 2015). However, the role of HMGB1 in NO signal pathway during the process of SA-induced liver cell apoptosis has not been established.

Hepatocellular carcinoma (HCC) is the most common primary malignancy found in liver, with few effective therapeutic options for this severe disease (Llovet et al. 2003; Ferenci et al. 2010). Therefore, in this article, we investigated the ability of SA to prevent tumorigenic in hepatoma cell line and evaluated the role of NO pathway in salicylic acid-dependent apoptosis induction. As the result, we will provide further information on establishing an impressive synergistic effect of SA.

Materials and methods

Materials

RPMI 1640 medium, FBS, penicillin-streptomycin, and trypsin-EDTA were purchased from HyClone (Thermo Scientific). TRIzol reagent was purchased from Invitrogen (Life Technology, USA). PrimeScript RT Reagent Kit and SYBR Premix EX Tag were purchased from TAKARA (TAKARA Biotechnology, Japan).

The compound 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) was used as a specific NO scavenger. N^G -nitro-L-arginine methylester hydrochloride (L-NAME) was used as the inhibitor of NOS. The other chemicals used, for example, salicylic acid, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Cell line and cell culture

Hepatoma cell line Bel-7402 (moderately differentiated cell line), HepG2 (well-differentiated cell line), and normal liver cell line LO2 were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China) and were maintained in RPMI 1640 medium (HyClone) containing 10% (v/v) FBS (HyClone), 4 mM

glutamine, 100 U penicillin/ml, and 100 mg streptomycin/ml at 37 °C and 5% CO_2 .

Treatments

Control stand for hepatoma cell line (Bel-7402 and HepG2) was grown under normal conditions. In all figures, “S” (100 μM , 500 μM , 1 mM, 5 mM, and 10 mM) stands for hepatoma cell line cells that were incubated at the suitable densities in the presence of SA (100 μM , 500 μM , 1 mM, 5 mM, and 10 mM) for indicated hours. Among them, 5 mM SA was chosen as the suitable treatment because of its inhibition rate $\text{IC}_{50} < 50\%$. The further experimental design thus consisted of the following treatment groups: NOS inhibitor L-NAME (0.1 mM) and NO scavenger cPTIO (0.1 mM) were used, respectively. (S5mM + L-NAME) and (S5mM + cPTIO) stand for 5 mM concentrations of SA treatment in the presence of L-NAME and cPTIO, respectively. In all experiments, assays were performed in duplicate and the above solutions were replaced each day. All tests were carried out in at least three independent sets of experiments with similar results.

Evaluation of cell viability by MTS assay

Cell viability was assessed by the MTS assay. Hepatoma cells were seeded into 96-well plates at a density of 2×10^3 cells/well in 0.1 ml medium with different treatments for 24 and 48 h. After that, all media were aspirated and gently washed with $1 \times \text{PBS}$ twice. Then 100 μl of RPMI 1640 medium was mixed with 20 μl of MTS solution per well. The mixture was placed in an incubator at 37 °C and 5% CO_2 for 1 h. Absorbance was measured at 490 nm (the absorbance on CTL was used to subtract the background absorbance). All experiments were performed in triplicate.

Apoptosis analyses

Hepatoma cells were plated (1×10^5 per well) in a six-well plate and exposed to different treatments. After 24 h, apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit. Briefly, hepatoma cells were washed twice with ice-cold PBS and re-suspended in binding buffer. Cell suspensions were stained with 5 μl of Annexin V-FITC conjugate and 10 μl of PI. Samples were analyzed by FACS Calibur (BD FACS Calibur TM, San Jose, CA) and FlowJo (Tree Star, San Carlos, CA) analysis software.

The measurement of NO

The transient and volatile nature of NO makes it unsuitable for most convenient detection methods; however, two stable breakdown products, nitrate (NO_3^-) and nitrite (NO_2^-), can

Fig. 1 Inhibiting effects of SA on hepatoma cell line Bel-7402 and HepG2 growth after exposure of 24 and 48 h. SA, L-NAME (NOS inhibitor), and cPTIO (NO scavenger) were added to each well of a 96-well plate to yield the final concentrations. The cell viability inhibition rate of LO2 cell line with 5 mM SA treatment was also investigated. Cell viability was determined by the MTS assay and the absorbance was measured at 490 and 630 nm using a microplate reader. Results were expressed as percentages of proliferation compared to the control (mean $\pm$ SE, $n = 6$). Bars denoted by the different letters significantly differed at $P < 0.05$ according to Duncan's multiple range tests

be easily detected by photometric means. In this experiment, the Griess method based on the chemical diazotization reaction was adopted to detect NO, which uses sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.

After cells were incubated according to the aforementioned grouping, 50 μ l culture solutions of each well were collected and put into the counterpart well of another plate. Then NO production in cells was measured according to the indication on the NO assay kit (Beyotime, China). The data were analyzed with Statistical Program for Social Sciences.

NO synthase activity determination

Total and inducible NO synthase activity was measured by monitoring the NO concentration using a nitric oxide synthase assay kit (Beyotime Corporation). Cells were cultured with different treatments for 24 h, and the cultured cells were collected and transferred to a 96-well plate. Assay buffer, the L-arginine substrate, and the tracer DAF-FMDA (3-amino-4-aminomethyl-20, 70-difluorescein diacetate) were added, followed by addition of 0.1 mM NADPH to the reaction mixtures to initiate the reactions. The plate was incubated at 37 $^{\circ}$ C for 30 min, centrifuged, and washed three times in PBS. After keeping at room temperature for 20 min, the fluorescence of the plate was measured by a Bio-Rad iMark instrument (ex = 495 nm, em = 515 nm). Instructions from the manufacturer were followed strictly, and each compound was tested in triplicate; the value given is an average of three determinations.

RNA extraction and real-time PCR analysis

Briefly, total RNA was isolated from cells with TRIzol reagent. Oligo(dT)-primed RNA (1 μ g) was reverse-transcribed with PrimeScript RT Reagent Kit according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of inducible nitric oxide synthase (NOS2), endothelial nitric oxide synthase (NOS3), BCL2-Like11 (BCL2L11), BCL2-Like13 (BCL2L13), BCL2-Like14 (BCL2L14), Caspase 9 (CASP9), and HMGB1 by real-time PCR with Taq DNA polymerase (TAKARA). GAPDH was used as an internal control. The Ct value of each cell was recorded, and the data were analyzed by the comparative Delta-delta Ct method. The primers used for amplification are listed as follows:

Gene	Primer
1.NOS2	Forward: 5'-TTCAGTATCACAACCTCAGCAAG-3' Reverse: 5'-TGGACCTGCAAGTAAAAATCCC-3'
2.NOS3	Forward: 5'-TGATGGCGAAGCGAGTGAAG-3' Reverse: 5'-ACTCATCCATACACAGGACCC-3'
3.BCL2L11	Forward: 5'-TAAGTTCTGAGTGTGACCGAGA-3' Reverse: 5'-GCTCTGTCTGTAGGGAGGTAGG-3'
4.BCL2L13	Forward: 5'-AGGACTATTCGGCAGAGTACAT-3' Reverse: 5'-TGATTCCAGGGTATTCCTCCTC-3'
5.BCL2L14	Forward: 5'-CAAAATCCTCGCTACTACACC-3' Reverse: 5'-GACTCATTGTCTGAACAATTCCC-3'
6.CASP9	Forward: 5'-CTCAGACCAGAGATTCGAAAC-3' Reverse: 5'-GCATTTCCCTCAAACCTCTCAA-3'
7.HMGB1	Forward: 5'-TCGCGAATTATCATGGCAAAGGAG-3' Reverse: 5'-GCTTTTATTCATCATCATC-3'
8.GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

Fig. 2 Cell apoptosis of Bel-7402 with different treatments for 24 h were determined by flow cytometry and dyed by Annexin V/PI double staining

Statistical analysis

All data were analyzed by SPSS 16.0 software (SPSS Inc., Chicago, IL) and presented as mean $\pm$ SE from at least three independent experiments performed in triplicate. Different measurements were subjected to analysis of variance (ANOVA) using SPSS with significances of $P < 0.05$, respectively.

Results

Salicylic acid inhibited hepatoma cell line proliferation

The MTS assay showed that the cell proliferation of Bel-7402 and HepG2 cancer cells was inhibited by SA treatment in a time- and dose-dependent manner (Fig. 1). Compared with the control group, SA at lower concentrations (100 and 500 μ M) showed little inhibition effects on cell viability. However, the proliferation levels of 1, 5, and 10 mM SA treatment groups were obviously decreased at 24 and 48 h. In both cell lines, SA at the concentration of 5 mM caused less than half of cell growth inhibition at 24 h, but had little impact on the normal liver cell line LO2 ($P > 0.05$). Therefore, this concentration was used in all further experiments.

Salicylic acid induced hepatoma cell line apoptosis

To investigate the anti-tumor effects of salicylic acid, the induction of apoptotic cell death was measured by Annexin V-FITC/PI assay after 24 h of treatment with different concentrations of SA (Fig. 2). The analysis of the Bel-7402 cells demonstrated that there was an increase in the number of cells in the early stages of apoptosis (control, 2.5%; 100 μ M, 3.7%; 500 μ M, 6.8%, 1 mM, 8.5%; 5 mM, 12.7%; 10 mM, 14.5%). Moreover, treatments with higher concentrations of SA caused an obvious increase in the late stages of cell apoptosis (control, 3.8%; 5 mM, 25.8%; 10 mM, 32.6%). The apoptosis of HepG2 cells were also induced by SA at the 24 h time point (figure not shown).

Salicylic acid induced apoptosis in association with the release of NO

Nitric oxide (NO), a pleiotropic regulator, may have both genotoxic and angiogenic properties in tumor cells. In order to explore the role of NO in SA-induced apoptosis of hepatoma cell line, we assessed the effect of SA stimulation on NO production and discovered that SA had a dose-dependent inducing function on NO release (Fig. 3). For example, in the 100 μ M, 500 μ M, and 1 mM SA treatments for 24 h, the total NO population of each group were 109.8, 337.2, and 730.4 nmol/l, respectively in Bel-7402 cell line. With the increase in incubation concentration, 5 mM SA could lead to the maximum release of NO, approximately 6.7 times compared

with control treatment. As predicted, increased NO production was also observed in HepG2 cell line in a concentration-dependent manner.

Salicylic acid induces the boost of NO in hepatoma cell line apoptosis related to the augment of NOS activities

In previous studies, we identified that SA increases NO production in hepatoma cells, but the underlying mechanism was not clear. Various studies have shown that nitric oxide synthases (NOS) stimulates angiogenesis by generating nitric oxide. In our study, we investigated the possibility that SA stimulates NOS expression and discovered that the extent of total NOS and inducible NOS activities were increment in both Bel-7402 and HepG2 cells, which were in accordance with NO content after different concentrations of SA treatments. For instance, in Bel-7402 cell line,

Fig. 3 NO release in SA-induced apoptosis of Bel-7402 and HepG2 cell line after different treatments for 24 h. Bars denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range tests

500 μ M and 1 mM SA treatments for 12 h could bring about the slight increase of total NOS and inducible NOS activities (Fig. 4a, b). Moreover, the inducing effect of 5 mM SA treatment was the most significant, approximately up to 7.8 times compared with control. Besides, the level of *NOS2* transcripts was also obviously higher in 5 mM SA treatment group (Fig. 4c).

Salicylic acid up-regulated the expression of apoptotic regulator genes

BCL-2 family members act as anti- or pro-apoptotic regulators which are involved in a wide variety of cellular activities. BCL-2L11, BCL-2L13, and BCL-2L14 have been shown to act as an apoptotic activator (Zamzami et al. 1998; Cory and Adams 2002; Misaka et al. 2015). To further explore the molecular mechanism involved in the significant anti-tumor effects of salicylic acid, Bel-7402 and HepG2 cell lines were collected and the change of apoptotic target genes with different concentrations of SA treatments was investigated. Real-time PCR analysis reveals that after being treated with 5 mM salicylic acid for 6 h, *BCL-2L11*, *BCL-2L13*, *BCL-2L14*, and *CASP9* were obviously up-regulated (Fig. 5). However, lower concentrations of SA (100 μ M, 500 μ M) have slight inducing effects on these genes' expression described above.

L-NAME, the NOS inhibitor, and cPTIO, NO scavenger, relieved SA-induced changes in the apoptosis of hepatoma cell line

Our previous study validated that NO level and NOS expression were elevated in SA-induced hepatoma cell line apoptosis. Further, L-NAME and cPTIO were used to evaluate whether NO modulated SA-induced hepatoma cell apoptosis. Our experiment data suggested that L-NAME and cPTIO administration significantly diminished not only the proliferation inhibition (Figs. 1 and 2) effects but also relative gene mRNA levels induced by SA (Figs. 5 and 6). L-NAME and cPTIO treatment alone showed little function.

Salicylic acid depresses the transcript levels of *HMGB1*

In order to investigate the mechanisms as to how SA induces apoptotic regulator gene expression in hepatoma cell line apoptosis, the transcript level of *HMGB1* was determined. As shown in Fig. 6, the expression of *HMGB1* was decreased at 6 h exposed to different doses of SA treatment. Additionally, L-NAME and cPTIO combination reverses the depression effect of *HMGB1* exerted by SA.

Fig. 4 SA anti-tumor effects might mediate by NO pathway. Nitric oxide synthesis activities (a) and inducible nitric oxide synthesis activities (b) were determined by DAF-FMDA method after indicated treatments for 12 h. c Relative mRNA expression level of *iNOS* (*NOS2*) and *eNOS*

(*NOS3*) in Bel-7402 and HepG2 cell line received indicated treatments above for 6 h. Bars denoted by the different letters significantly differed at $P < 0.05$ according to Tukey's multiple range tests

Fig. 5 Apoptosis-related gene expression levels were up-regulated after SA treatment for 12 h in Bel-7402 and HepG2 cell line, respectively. The mRNA expression levels of *BCL-2-Like11*, *13*, *14*, and *Caspase 9* in the indicated cells were measured by real-time PCR compared with control treatment. Bars denoted by the different letters significantly differed at $P < 0.05$ according to Tukey's multiple range tests

Discussion

Accumulating lines of evidence showed that salicylic acid, one of the aspirin metabolites, could inhibit the occurrence and development of cancer (Chung et al. 2003; Elder 1996; Klampfer 1999; Zitta et al. 2012). However, the related mechanisms about anti-liver carcinoma effects of salicylic acid were rarely conducted. Our research demonstrated that SA could inhibit the proliferation and induce the apoptosis of hepatoma cell line in a time- and dose-dependent manner, which implies a potential application of SA as an anti-cancer agent.

Nitric oxide (NO) in cancer cells was produced at sub-micromolar levels to activate signaling pathways leading to

Fig. 6 The transcripts levels of *HMGB1* were depressed with different treatments for 6 h in Bel-7402 and HepG2 cell line, respectively. Bars denoted by the different letters significantly differed at $P < 0.05$ according to Tukey's multiple range tests

evasion of apoptosis on the one hand and promotion of cell proliferation, migration, and metastatic invasion on the other (Tan et al. 2013; Li et al. 2015). A few reports (Karmohapatra et al. 2007) have demonstrated that aspirin could activate nitric oxide synthase from human blood platelets. In this study, we also provided the evidence that SA could induce NO release generated by NOS in the apoptosis process of hepatoma cell line. In addition, the ameliorating effects of SA were prevented when NOS inhibitor L-NAME and NO scavenger cPTIO were administrated. These results might propose a link between NO generation and cell viability inhibition due to SA treatment.

Furthermore, we gained insight into the molecular mechanism of the cytotoxic effect exerted by SA on hepatoma cell line. A series of genes as stimulators which will trigger the apoptotic process might participate in this pathway. *BCL-2-Like11*, *13*, and *14* are key members of apoptosis-related genes in mitochondrial apoptosis pathway, which are able to promote apoptosis. Therefore, the expressions of *BCL-2-Like11*, *13*, and *14* were selected as apoptosis markers for anti-tumor medicine examination in many studies (Zamzami et al. 1998; Cory and Adams 2002; Misaka et al. 2015). By using HCC cell lines, we provided the evidence that SA increased the mRNA expressions of *BCL-2-Like11*, *13*, and *14* while activating the *caspase-9* cascade, which in turn leads to toxic effects on inducing liver cancer cell apoptosis.

Inside cells, HMGB1 is found mainly in the cell nucleus, where it participates in replication, recombination, transcription, and DNA repair processes. Following release into the extracellular space, HMGB1 becomes a proinflammatory cytokine which stimulates formation of new blood microvessels, enhances cell migration, activates the inflammatory condition, and affects cell proliferation (Jiang and David 2006). In our

study, we discovered that SA could suppress the expression of *HMGB1* from hepatoma cells and inhibiting NOS will increase *HMGB1* mRNA level, which suggests that *HMGB1* may act as a downstream effector of NO.

Based on these results, we deduced that SA triggered the NO pathway by activation of NOS expression and depression of *HMGB1* mRNA level, which in the end led to induce the apoptosis of hepatoma cell line.

Conclusion

In summary, our findings highlighted a novel mechanism for SA to anti-liver carcinoma and for the first time suggested that the apoptosis process of hepatoma cell line by SA is partly mediated via NO pathway, which up-regulated the apoptosis corresponding gene expression. Therefore, it could be a potential therapeutic agent for HCC treatment in the future.

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