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Superoxide is responsible for apoptosis in rat vascular smooth muscle cells induced by α-tocopheryl hemisuccinate

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Abstract

We investigated the mechanism of cell toxicity of α-tocopheryl hemisuccinate (TS). TS concentration- and time-dependently induced the lactate dehydrogenase release and DNA fragmentation of rat vascular smooth muscle cells (VSMC). Exogenous addition of superoxide dismutase, but not catalase, significantly inhibited the cell toxicity of TS. The NADPH-dependent oxidase activity of VSMC was stimulated by TS treatment. The cell toxicity of TS was inhibited by NADPH oxidase inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride. Consequently, TS-induced apoptosis of VSMC was suggested to be caused by exogenous O_2^- generated via the oxidase system activated with TS.

Keywords: α-Tocopheryl hemisuccinate; Apoptosis; Superoxide; Vascular smooth muscle cell

1. Introduction

α-Tocopherol hemisuccinate (TS), an amphiphilic succinyl ester of α-tocopherol (T), has been reported to have various biological activities such as inhibition of acetylcholine esterase [1], prevention of function of the transcriptional factor nuclear factor kappa B [2–5], and suppression of the growth of various cancer cells [6–10]. Previously, as it has been found that TS induced apoptosis in various cell lines such as human breast cancer cells, neuroblastoma cells and lymphoblastoid cells [11–21], apoptosis has been considered to be responsible for the cell toxicity of TS. In the process of TS-induced apoptosis, TS activated the expressions of genes of several proteins such as c-*jun*, transforming growth factor-β [11–17,19]. Recently, it was reported that TS-mediated apoptosis involves Fas signaling [14,16,19]. Yu et al. suggested that TS converts Fas-resistant human cancer cells to a Fas-sensitive phenotype by translocation of cytosolic M_r 43 000 Fas to the membrane [16,19]. Furthermore, it was also reported that the dysfunction of mitochondria and activation of caspase cascades were caused in the process of TS-induced apoptosis [17,20]. However, the trigger event of TS-induced apoptosis is still unclear. In this study, to obtain further information about the cell toxicity of TS, we examined the effect of TS on rat vascular smooth muscle cells (VSMC).

2. Materials and methods

2.1. Materials

α-Tocopheryl hemisuccinate (TS) and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol (T) was kindly provided by Eisai Co. (Tokyo, Japan). Other reagents were of the highest grade commercially available.

2.2. Treatment of VSMC with TS

Vascular smooth muscle cells (VSMC) were isolated from rat thoracic aorta using the proteases elastase and collagenase as described previously [22]. The VSMC (1.0×10^6 cells) were seeded into 35-mm dishes, and were

Abbreviations: TS, α-tocopheryl hemisuccinate; VSMC, vascular smooth muscle cells; LDH, lactate dehydrogenase; T, α-tocopherol; SOD, superoxide dismutase; PBS, phosphate-buffered saline; Cat, catalase; AsA, sodium ascorbate; DPI, diphenyleneiodonium; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; HPLC, high-performance liquid chromatography

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cultured for 24 h in Dulbecco's modified Eagle's medium with 10% fetal calf serum in a CO_2-incubator at 37°C with CO_2 in humidified air. Then, the medium containing serum was removed, and the cells were washed with phosphate-buffered saline (PBS). Next, 1 ml of the medium containing various concentrations of TS without serum was added into the dishes. After certain periods, the cells were subjected to various assays.

2.3. Assay of lactate dehydrogenase released into the culture medium

Release of lactate dehydrogenase (LDH) from cells into the culture medium was measured as LDH activity. As LDH catalyzes the reduction of pyruvic acid to lactic acid using NADH, LDH activity can be measured as change of absorbance at 340 nm of NADH. LDH activity in the culture medium was measured as follows. The culture medium was collected in a 1.5-ml sample tube, and centrifuged at $5000\times g$ for 1 min at 4°C and the supernatant was used as the sample. Measurement of LDH activity was started by the addition of 0.15 ml of the sample solution to 1.35 ml of phosphate buffer (pH 7.2) containing 0.1 mM NADH and 1 mM pyruvic acid, and the absorbance change at 340 nm was monitored with time in a Shimazu UV-1600 spectrophotometer. The percentage release of LDH into the culture medium was estimated by comparison of the initial velocity of absorbance change of the sample solution with that of a whole cell lysate.

2.4. DNA fragmentation assay

DNA fragmentation was determined by extraction of DNA followed by electrophoresis. Cells in three dishes (10^6 cells per 35-mm dish) were collected in a 1.5-ml sample tube, and centrifuged at $2000\times g$ for 5 min. The supernatant was removed and 40 μl of extraction solution containing 192 mM Na_2HPO_4 and 4 mM citric acid was added for extraction of DNA. The mixture was incubated for 1 h at room temperature, then the extracted DNA solution was collected by centrifugation at $2000\times g$ for 5 min. Then 3 μl of 10 mg/ml RNase A and 3 μl of 0.25% Triton X-100 were added to the extract, and the mixture was incubated at 37°C for 1 h. After addition of 3 μl of 10 mg/ml proteinase K, the extract was reincubated at 50°C for 30 min. DNA was precipitated by the addition of 20 μl of 5 M NaCl and 100 μl of isopropanol. The DNA solution was electrophoresed in 2% agarose gel containing ethidium bromide and photographed under UV light.

2.5. Measurement of O_2^- generation

Superoxide generation by NADPH-dependent oxidase of cultured VSMC was measured as the reduction of cytochrome *c* [23]. VSMC pretreated with TS were removed from dishes after addition of trypsin, and the cell suspension (1.0×10^6 cells/ml) was added to PBS containing 50 μM cytochrome *c*. Then, superoxide generation was started by addition of NADPH (final concentration 100 μM), and change of absorbance at 550 nm was monitored at 37°C with stirring.

2.6. Analysis of the amount of TS transferred to the cells

The amounts of TS transferred to the cells and remaining in the culture medium were quantified by HPLC as follows. After the addition of TS, the culture medium and the cells were collected at various incubation periods. Then lipids were extracted from them by the method of Bligh and Dyer [24]. Lipid extracts were dried under N_2, and redissolved in methanol. The extracted samples were subjected to HPLC using a column of Mightysil Si60 (150×4.5 mm, Kanto Chemical, Tokyo) monitored at 286 nm corresponding to λ_{max} of TS. The solvent system used was methanol/water (99:1, v/v).

3. Results

We examined the cytotoxic effect of α-tocopheryl hemisuccinate (TS) on cultured rat vascular smooth muscle cells (VSMC). Fig. 1 shows the time courses of lactate dehydrogenase (LDH) release from VSMC treated with different concentrations of TS. TS induced slowly the LDH release in a concentration-dependent manner; on 96 h treatment, 50 μM TS caused about 60% release of total LDH. TS also caused DNA fragmentation, which is known as a typical apoptotic event, concentration- and time-dependently (Fig. 2). These results suggest that apoptosis participates in the cytotoxic effect of TS on VSMC. In addition, α-tocopherol (T) and/or succinic acid did not

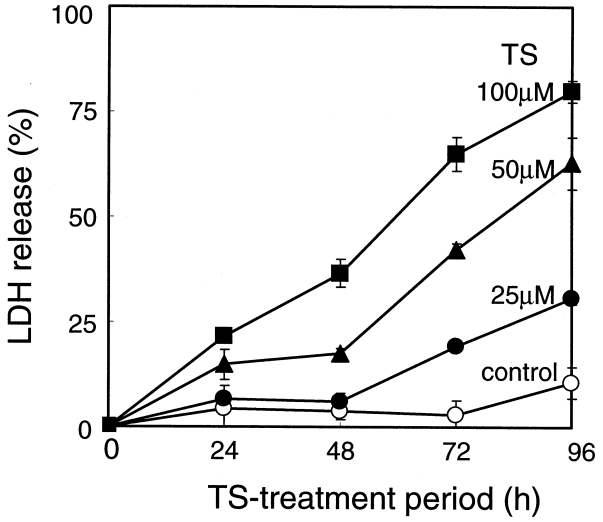

Fig. 1. TS-induced LDH release of VSMC into culture medium. Values are mean ± S.D. ($n=3$).

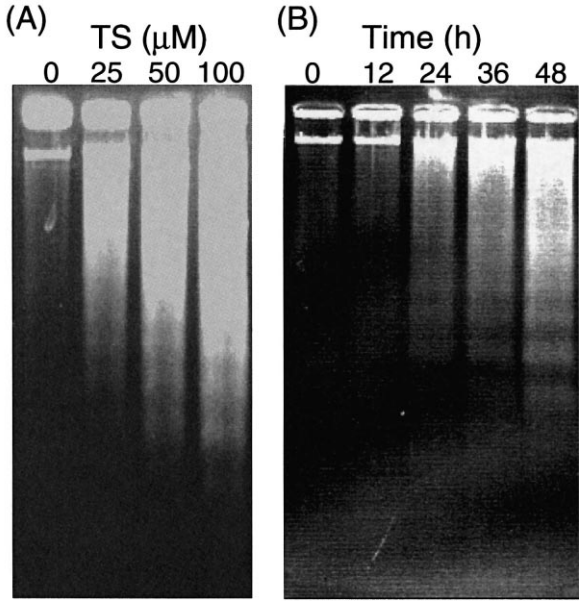

Fig. 2. TS-induced DNA fragmentation of VSMC. Extracted DNA of VSMC treated with (A) various concentrations of TS for 48 h and (B) with 50 μM TS for various periods was analyzed by agarose-gel electrophoresis.

cause LDH release or DNA fragmentation of VSMC (data not shown), indicating that TS itself caused apoptosis.

Next, we examined the effect of various reagents (100 μM T, 500 units/ml superoxide dismutase (SOD), 500 units/ml catalase (Cat) and 100 μM sodium ascorbate (AsA)) on the cell toxicity induced with 50 μM TS. As shown in Fig. 3, the TS-induced LDH release and DNA fragmentation of VSMC were inhibited by exogenous addition of SOD, but not by Cat and AsA. While these inhibitions were partially, we confirmed that a higher concentration of SOD (2000 units/ml) completely inhibited DNA fragmentation (data not shown). These results indicate that O_2^-, but not H_2O_2, would participate in the TS toxicity. Furthermore, we found that the coexistence of T inhibited the toxic effect of TS.

NADPH oxidase is well known to be an O_2^- generating system in various cells [25]. Fig. 4A shows the effect of TS treatment on NADPH-dependent oxidase activity of VSMC. In the control VSMC just after removal of serum, addition of NADPH (final concentration 100 μM) caused a small, but significant increase in the absorbance at 550 nm due to reduction of cytochrome *c*. This may be explained by increased permeability of exogenously added NADPH through the cell membrane, which would be caused with the trypsin treatment in the cell preparation. The rate of the cytochrome *c* reduction remained constant for 48 h in control cells. However, in cells treated with 50 μM TS, the rates of NADPH-dependent reduction of cytochrome *c* were significantly higher than those of control cells even at 12 h after the treatment (Fig. 4). It should be mentioned that this increase in NADPH-dependent cytochrome *c* reduction preceded the DNA fragmentation induced by TS shown in Fig. 2. The absorbance change of cytochrome *c* in TS-treated VSMC was inhibited by addition of diphenyleneiodonium (DPI) or 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), which are specific inhibitors of flavin-containing oxidases such as NADPH oxidase [26,27], although the inhibitions were not complete. This result indicates that the absorbance change should be due to O_2^- exogenously generated by NADPH-dependent flavin-containing oxidase system (Fig. 5), although we could not exclude completely the possibility that O_2^- generated by intracellular NAPDH-dependent oxidase under our experimental conditions. The absorbance change was

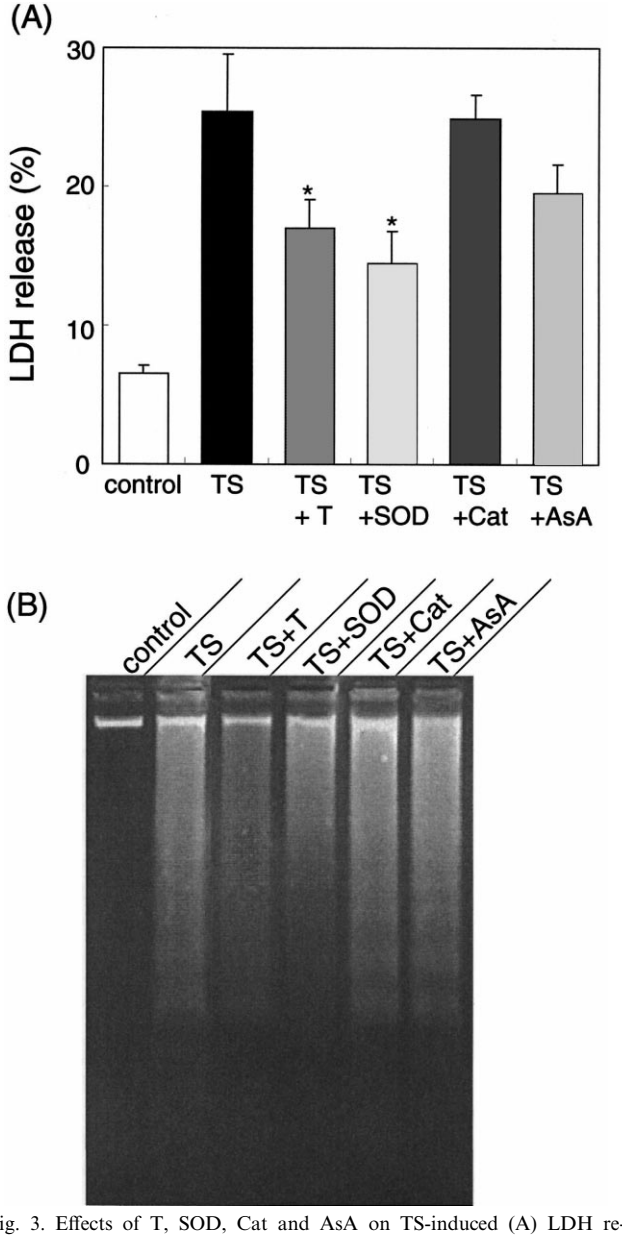

Fig. 3. Effects of T, SOD, Cat and AsA on TS-induced (A) LDH release and (B) DNA fragmentation of VSMC. The concentrations of T, SOD, Cat and AsA were 100 μM, 500 units/ml, 500 units/ml and 100 μM, respectively. DNA was extracted from VSMC 48 h after 50 μM TS-treatment. $^*P < 0.01$. Values are mean ± S.D. ($n = 3$).

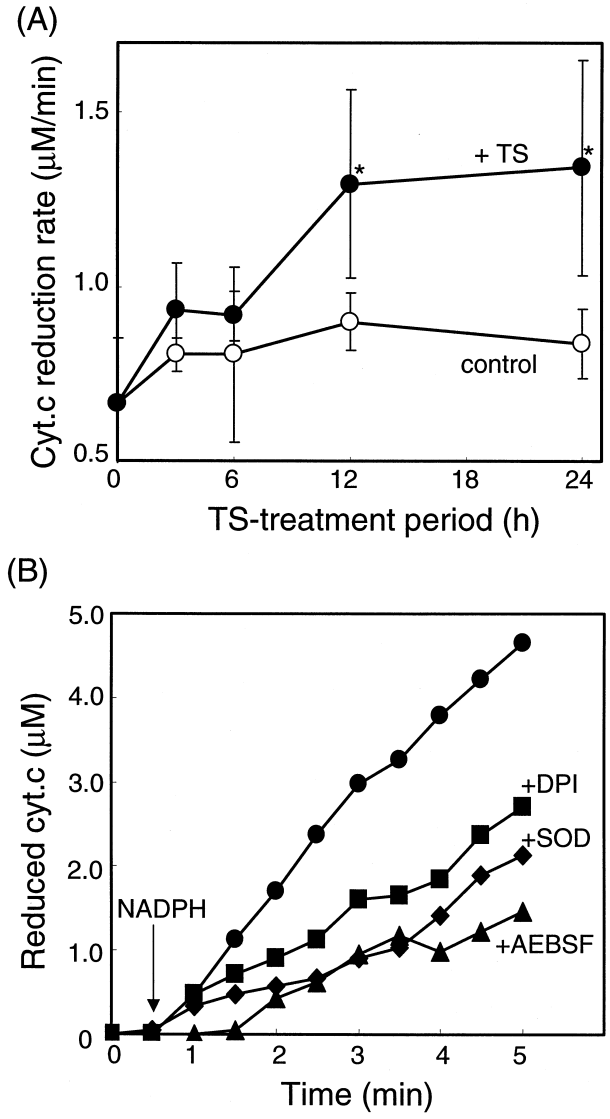

Fig. 4. TS-induced NADPH-dependent reduction of cytochrome *c* (A), and its inhibition by DPI, AEBSF and SOD. (A) ●, 50 μM TS-treated cells; ○, untreated control cells; Cyt.c, cytochrome *c*. Reduction of cytochrome *c* was started by the addition of 100 μM NADPH to VSMC suspension. $*P < 0.05$. The values are mean ± S.D. ($n = 3$). (B) Effect of 500 units/ml SOD (♦), 40 μM DPI (■) and 400 μM AEBSF (▲) were examined on the cytochrome *c* reduction enhanced by the treatment with 50 μM TS for 24 h.

also inhibited by addition of SOD (Fig. 4), suggesting exogenous O_2^- generation by the NADPH-dependent oxidase in VSMC.

We further examined the effect of the NADPH oxidase inhibitor AEBSF on the TS cytotoxicity. The coexistence of 200 μM AEBSF inhibited the LDH release and DNA fragmentation induced with 50 μM TS (Fig. 5), although the inhibition was not complete. AEBSF itself showed an apoptotic effect on VSMC at the concentration more than 200 μM (data not shown), and therefore we could not confirm the complete inhibition of TS-induced apoptosis.

We analyzed the transfer of TS in the culture medium to the cells by using HPLC. As shown in Fig. 6, TS contents in the cells increased biphasically with increase in the incubation period. The cellular contents of TS increased at first immediately up to about 60% just after addition of TS to culture medium, and then gradually. Finally, TS transferred completely to the cells 12h after the addition. In addition, no T was detected in the cells and culture medium throughout the incubation, indicating TS was not hydrolyzed in our experimental conditions. Furthermore, exogenous additions of 50 μM T or 500 units/ml SOD did not affect the transfer of TS to the cells (data not shown). These results rule out the possibility that the cytoprotec-

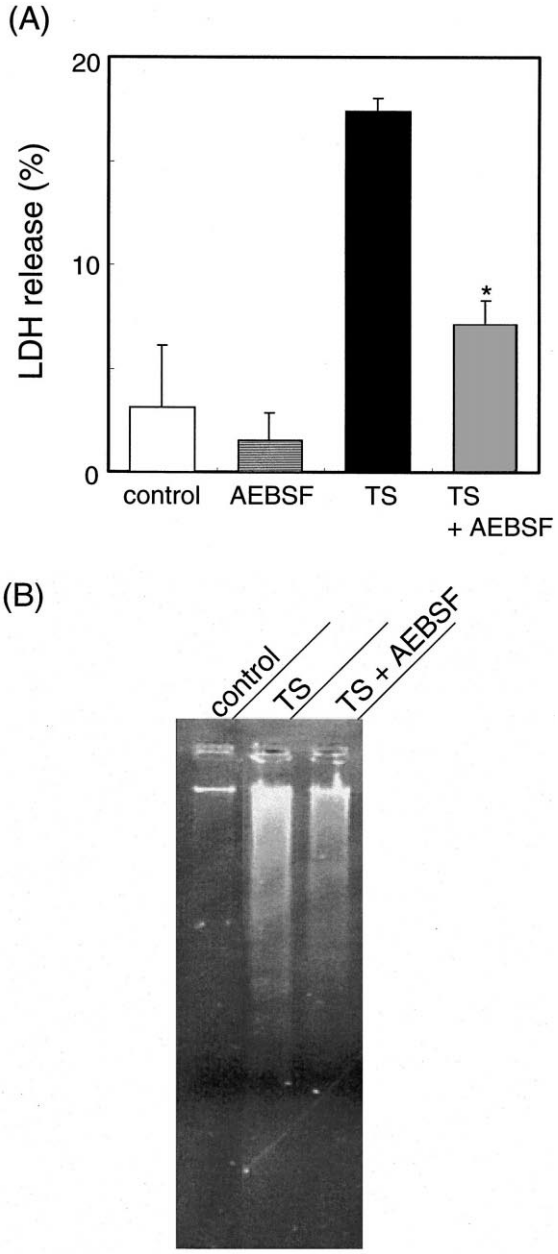

Fig. 5. Effect of AEBSF on TS-induced (A) LDH release and (B) DNA fragmentation of VSMC. The concentrations of TS and AEBSF were 50 and 200 μM, respectively. DNA was extracted from VSMC 48 h after TS treatment. $*P < 0.01$. Values are mean ± S.D. ($n = 3$).

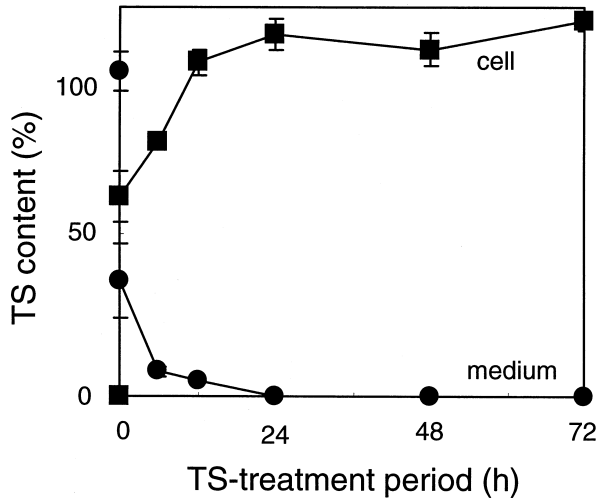

Fig. 6. Time-dependent transfer of TS from culture medium to VSMC. TS contents in the medium (●) and cells (■) were quantified by using HPLC. The concentration of TS in the culture medium at time 0 was 50 μM. Values are mean ± S.D. ($n = 3$).

tion observed with the addition of exogenous T and SOD are related to preventing TS cellular uptake and thus attenuating intracellular TS concentrations and its ability to induce cell death.

4. Discussion

In this study using VSMC, we found that TS showed the apoptotic effects consistent with previous reports on other cells [11–21]. The concentration of TS needed for induction of apoptosis of VSMC was almost the same as those in Jurkat T cells and HL-60 cells, but the time for its induction in VSMC (Fig. 2) was much longer than those in the cell lines [17,20]. The almost of TS added in the culture medium transferred to VSMC without its hydrolysis to T (Fig. 6). Similar findings on human acute monocytic leukemia cells were reported by Nakamura et al. [5]. Our results indicate that the transferred TS itself is responsible for the cell toxicity, although it is unclear whether the TS were taken up into the inside of cell or stayed at the membrane surface.

Kline and coworkers reported that coexistence of T inhibited TS-induced apoptosis in various cells such as EL4 T lymphoma cells [12,15]. In this study, we also confirmed that the coexistence of 100 μM T inhibited LDH release and DNA fragmentation induced with 50 μM TS (Fig. 3), suggesting that the mechanism of TS-induced apoptosis of VSMC is the same as those of various cell lines, although the mechanism of the inhibitory effect of T is still unclear.

Our results showed that exogenous addition of SOD inhibited TS-induced LDH release and DNA fragmentation (Fig. 3). Thus, O_2^- generated on the outside of the cells seemed to be responsible for cell toxicity of TS, although it has generally been believed that O_2^- production by activated NADPH oxidase in VSMC occurs mainly intracellular [28]. There are some reports that oxygen radicals generated by NADPH oxidase contribute to apoptosis in various cells such as sympathetic neurons and human leukemia cells [29,30]. Recently, Marumo et al. [31] reported that in human VSMC, DPI-inhibitable exogenous O_2^- generation was observed by treatment with platelet-derived growth factor. In addition, O'Donnell and Azzi [23] reported that exogenous O_2^- generation was caused by the addition of NADPH in human fibroblasts. Consistent with these previous reports, NADPH-dependent O_2^- generation was increased in TS-treated VSMC, and we found that the increase was inhibited by DPI and AEBSF (Fig. 4). Furthermore, in this study, AEBSF was found to inhibit significantly TS-induced LDH release and DNA fragmentation (Fig. 5). These results suggested that the exogenous O_2^- generation by NADPH-dependent oxidase is responsible for the TS-induced cell toxicity.

There have been many reports about the mechanism of TS-induced apoptosis [11–21]. However, since almost of all these reports were about the apoptotic cascade inside the cytoplasm, there is no information about the trigger of apoptosis induced by TS. In this study, we found for the first time the trigger event of TS-induced apoptosis in rat VSMC, i.e., exogenous O_2^- generation via oxidase systems including NADPH-dependent oxidase activated by TS, is responsible for the apoptosis in VSMC. Activation of NADPH oxidase in neutrophils has been suggested to be associated with the influx of Ca^{2+} into cytoplasm from extracellular space [32,33]. Recently, Yamamoto et al. [20] reported that TS increased transiently the intracellular concentration of Ca^{2+} of promyelocytic leukemia cells HL-60. From these findings, we supposed that TS might activate the NADPH-dependent oxidase due to an increase in the intracellular Ca^{2+} level. The mechanisms of the TS-induced activation of O_2^- generation and of O_2^--dependent apoptosis in VSMC are unclear. More detailed studies are in progress to clarify the TS-induced apoptosis in our laboratory.

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References

[1] J. Chelliah, J.D. Smith, M.W. Fariss, Biochim. Biophys. Acta 1206 (1994) 17–26.
[2] Y.J. Suzuki, L. Packer, Biochem. Biophys. Res. Comuun. 193 (1993) 277–283.
[3] Y.J. Suzuki, L. Packer, Biochem. Mol. Biol. Int. 31 (1993) 693–700.
[4] E. Wolfgang, C. Weber, C. Wardemann, P.C. Weber, Am. J. Physiol. 273 (1997) H634–H640.

[5] T. Nakamura, M. Goto, A. Matsumoto, I. Tanaka, BioFactors 7 (1998) 21–30.
[6] M.W. Fariss, M.B. Fortuna, C.K. Everett, J.D. Smith, D.F. Trent, Z. Djuric, Cancer Res. 54 (1994) 3346–3351.
[7] M. Simmons-Menchaca, M. Qian, W. Yu, B.G. Sanders, K. Kline, Nutr. Cancer 24 (1995) 171–185.
[8] Z. Djuric, L.K. Heilbrun, S. Lababidi, C.K. Everett-Bauer, M.W. Fariss, Cancer Lett. 111 (1997) 133–139.
[9] J.M. Turley, F.W. Ruscetti, S.J. Kim, T. Fu, F.V. Gou, M.C. Birchenall-Roberts, Cancer Res. 57 (1997) 2668–2675.
[10] M.P. Malafa, L.T. Neitzel, J. Surg. Res. 93 (2000) 163–170.
[11] J.M. Turley, S. Funakoshi, F.W. Ruscetti, J. Kasper, W.J. Murphy, D.L. Longo, M.C. Birchenall-Roberts, Cell Growth Differ. 6 (1995) 655–663.
[12] M. Qian, J. Kralova, W. Yu, H.R. Bose Jr., M. Dvorak, B.G. Sanders, K. Kline, Oncogene 15 (1997) 223–230.
[13] W. Yu, K. Heim, M. Qian, M. Simmons-Menchaca, B.G. Sanders, K. Kline, Nutr. Cancer 27 (1997) 267–278.
[14] J.M. Turley, T. Fu, F.W. Ruscetti, J.A. Mikovits, D.C. Bertolette III, M.C. Birchenall-Roberts, Cancer Res. 57 (1997) 881–890.
[15] W. Yu, B.G. Sanders, K. Kline, Nutr. Cancer 27 (1997) 92–101.
[16] W. Yu, K. Israel, Q.Y. Liao, C.M. Aldaz, B.G. Sanders, K. Kline, Cancer Res. 59 (1999) 953–961.
[17] J. Neuzil, I. Svensson, T. Weber, C. Weber, U.T. Brunk, FEBS Lett. 445 (1999) 295–300.
[18] P.J. Pussinen, H. Lindner, O. Glatter, H. Reicher, G.M. Kostner, A. Wintersperger, E. Malle, W. Sattler, Biochim. Biophys. Acta 1485 (2000) 129–144.
[19] K. Israel, W. Yu, B.G. Sanders, K. Kline, Nutr. Cancer 36 (2000) 90–100.
[20] S. Yamamoto, H. Tamai, R. Ishisaka, T. Kanno, K. Arita, H. Kobuchi, K. Utsumi, Free Radic. Res. 33 (2000) 407–418.
[21] J. Neuzil, T. Weber, A. Schröder, M. Lu, G. Ostermann, N. Gellert, G.C. Mayne, B. Olejnicka, A. Nègre-Salvayre, M. Stícha, R.J. Coffey, C. Weber, FASEB J. 15 (2001) 403–415.
[22] A. Tokumura, M. Toujima, Y. Yoshioka, K. Fukuzawa, Lipids 31 (1996) 1251–1258.
[23] V.B. O'Donnell, A. Azzi, Biochem. J. 318 (1996) 805–812.
[24] E.G. Bligh, W.J. Dyer, J. Biochem. Physiol. 37 (1959) 911–917.
[25] B.M. Babior, Blood 93 (1999) 1464–1476.
[26] J.A. Ellis, S.J. Mayer, O.T.G. Jones, Biochem. J. 251 (1988) 887–891.
[27] V. Diatchuk, O. Lotan, V. Koshkin, P. Wikstroen, E. Pick, J. Biol. Chem. 272 (1997) 13292–13301.
[28] K.K. Griendling, D. Sorescu, M. Ushio-Fukai, Circ. Res. 86 (2000) 494-501.
[29] W. Hiraoka, N. Vazquez, W. Nieves-Neira, S.J. Chanock, Y. Pommier, J. Clin. Invest. 102 (1998) 1961–1968.
[30] S.P. Tammariello, M.T. Quinn, S. Estsu, J. Neurosci. 20 (2000) RC53.
[31] T. Marumo, V.B. Schini-Kerth, B. Fisslthaler, R. Busse, Circulation 96 (1997) 2361–2367.
[32] J.P. Wang, L.T. Tsao, S.L. Raung, P.L. Lin, C.N. Lin, Free Radic. Biol. Med. 26 (1999) 580–588.
[33] F. Watson, S.W. Edwards, Biochem. Biophys. Res. Commun. 247 (1998) 819–826.