Exogenous nitric oxide induces apoptosis in *Toxoplasma gondii* tachyzoites via a calcium signal transduction pathway

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SUMMARY

The mechanism by which nitric oxide (NO)-dependent cytotoxicity acts against *Toxoplasma gondii* tachyzoites is poorly understood. An NO donor, sodium nitroprusside (SNP), was used to induce death in *T. gondii* tachyzoites *in vitro* as a model for investigating (i) whether NO is capable of inducing apoptosis-like death in tachyzoites and (ii) whether a calcium signal transduction pathway is involved. Exposure to 2 mM SNP resulted in a pattern of tachyzoite death that shares many features with metazoan apoptosis and it may involve a calcium signal transduction pathway. Motility and cell survival in these parasites showed a gradual decline with increasing levels of SNP. Features common to metazoan apoptosis are observed after exposure to 2 mM SNP. Ethylene glycol *bis*-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), Verapamil and *bis*-(o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid/acetoxymethyl ester (BAPTA/AM) partially increased the cell survival concomitant with decreased $[Ca^{2+}]_i$ in cells exposed to SNP. An NO scavenger (*N*-acetylcysteine), the analogue of SNP (devoid of NO), inhibited the rate of apoptosis after SNP treatment compared with SNP treatment without scavenger, but alone did not induce apoptosis. Taken together, the results indicate that SNP is capable of inducing apoptosis in *T. gondii* tachyzoites via a calcium signal transduction pathway.

Key words: *Toxoplasma gondii*, tachyzoites, nitric oxide, apoptosis, calcium signal.

INTRODUCTION

Toxoplasma gondii is an ubiquitous obligate intracellular parasite and a member of the phylum Apicomplexa. The infection is typically asymptomatic, although acute toxoplasmosis can be fatal in immunocompromised individuals and can result in severe birth defects or abortion during the first trimester of pregnancy (Frenkel, 1996). Despite the progress in understanding this infection, many pathophysiological phenomena in the host remain largely unknown.

Apoptosis is a well-established, genetically controlled, cellular 'suicide' mechanism in eukaryotes which leads to elimination of unnecessary or damaged cells. Originally described in mammals (Muzylak & Maslinska, 1992) and later in the nematode *Caenorhabditis elegans* (Driscoll, 1992), apoptosis is a physiological cell suicide programme that is critical for the development and maintenance of healthy tissues (Deveraux & Reed, 1999). Recently, apoptosis has been described in several members of the parasitic protozoa: *Trypanosoma cruzi* (Ameisen *et al*. 1995; Piacenza, Peluffo & Radi, 2001); *T. brucei rhodesiense* (Welburn, Lillico & Murphy, 1999); *Leishmania amazonensis* (Moreira *et al*. 1996); *L. infantum amastigotes* (Sereno *et al*. 2001); *L. donovani* (Das, Mukherjee & Shaha, 2001) and *Plasmodium falciparum* (Picot *et al*. 1997). Apoptosis in *T. gondii*, however, has not previously been described.

Nitric oxide (NO) was originally described as the principal endothelium-derived relaxing factor, but it is now known to serve a variety of functions throughout the body, both physiological and pathophysiological (Culotta & Koshland, 1992). Recently, we and others have shown that endogenous or exogenous NO contributes to the toxoplasmastatic or toxoplasmacidal activity of both intracellular and extracellular *T. gondii* tachyzoites (Adams *et al*. 1990; Langermans *et al*. 1992; Zheng & Lin, 1998). However, the mechanism by which NO-dependent cytotoxicity acts against the tachyzoites (Albina, Cui & Mateo, 1993; Sarih, Souvannavong & Adam, 1993; Ankarcrona, Dypbukt & Brune, 1994; Kitajima, Kawahara & Nakajima, 1994; Shimaoka, Iida & Ohara, 1995) is poorly understood. It has also been shown that NO is capable of inducing apoptosis of a number of cell types, including macrophages, tumour cells, cardiomyocytes and chondrocytes (Kaneto, Fuji & Seo, 1995; Mabbott, Sutherland & Sterberg, 1995; Maciejewski, Selleri & Sato, 1995; Blanco,

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Ochs & Schwarz, 1995). The free cytosolic Ca^{2+} concentration is the key variable governing the intracellular actions of Ca^{2+}. In most eukaryotic cells, Ca^{2+} homoeostasis is achieved by the concerted operation of several Ca^{2+}-transporting systems located in the plasma membrane, endoplasmic reticulum and mitochondria (Carafoli, 1987; Zheng & Lin, 1998). The regulation of Ca^{2+} homeostasis in *T. gondii* is not properly understood.

In this study, we used a NO donor, sodium nitroprusside (SNP), to examine NO-induced death of *T. gondii* tachyzoites *in vitro* to investigate whether NO is capable of inducing apoptosis-like death in *T. gondii* tachyzoites and whether it is dependent on the calcium signal transduction pathway. The results demonstrate that SNP is capable of inducing apoptosis in *T. gondii* tachyzoites through elevating cytoplasmic free calcium concentration, which is mainly the result of the entry of extracellular calcium.

MATERIALS AND METHODS

Parasite

The RH strain of *T. gondii* was obtained from Fujian Sanitary and Epidemic Disease Prevention Station and was maintained by serial intraperitoneal (IP) passages in male BALB/c mice aged 6–8 weeks. Harvested parasites were washed twice in phosphate-buffered saline (PBS, pH 7·2) by centrifugation (460 ***g***, 10 min) and resuspended in PBS without Ca^{2+} and Mg^{2+} at the desired cell density. The vitality of the parasites was monitored by the trypan blue exclusion test (Zheng & Lin, 1998). Tachyzoites were grown under 5% CO_2 air atmosphere at 37 °C.

Reagents

RPMI 1640 was purchased from Gibco/BRL Life Technologies (Burlington, Ontario, Canada) and was supplemented with 100 U of penicillin/ml and 10% heat-inactivated foetal bovine serum. RNase, proteinase K and *in situ* Apoptosis Detection Kit were from Boehringer Mannheim. All other reagents, sodium nitroprusside, *N*-acetylcysteine and potassium ferricyanide, EGTA, fura 2 acetoxymethyl ester (Fura-2/AM), Verapamil, BAPTA/AM were from Sigma.

Electron microscopy

Tachyzoites (5×10^8) were washed in phosphate buffer, fixed initially in 2·5% glutaraldehyde with 0·1% paraformaldehyde, and then in 1% osmic acid. After dehydration, the reagents were embedded in Epoxy resin 618. The resin was polymerized at 50 °C for 24 h, and sections cut at a thickness of 60–90 nm. Sections were stained with uranyl acetate (1·5 h) and lead citrate (0·5 h), washed in 20 mM NaOH followed by double-distilled water, and were examined using a Hu-12A transmission electron microscope.

DNA isolation and electrophoresis

DNA isolation and electrophoresis were performed as previously described with minor modifications (Herrmann, Lorenz & Voll, 1994). Briefly, 5×10^8 tachyzoites were lysed for 10 sec in DNA lysis buffer containing 1% NP-40, 20 mM EDTA and 50 mM Tris–HCl (pH 7·5). After centrifugation (1600 ***g***, 5 min), the supernatant was first digested with RNase A (1 μg/μl) at 37 °C for 2 h, then with proteinase K (2·5 μg/μl) at 56 °C for 2 h. DNA was precipitated overnight at −20 °C with 2 volumes of absolute ethanol, resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8·0) prior to loading onto a 1·0% agarose gel containing 0·5 μg/ml ethidium bromide. Finally, DNA was visualized and photographed under an ultraviolet lamp.

TUNEL assay

The TUNEL assay was performed following the manufacturer's (Boehringer Mannheim) instructions. Tachyzoites were washed twice in PBS containing 1% bovine serum albumin, and fixed using 4% paraformaldehyde in PBS for 1 h at room temperature. After washing in PBS, they were permeated for 2 min on ice with 0·1% Triton X-100 in 0·1% sodium citrate, washed twice and resuspended in TUNEL reaction mixture at 37 °C for 60 min. Tachyzoites were then washed, incubated with alkaline phosphatase at 37 °C for 30 min, washed again, and stained with nuclei fast red. Apoptosis of tachyzoites was evaluated using light microscopy. Negative controls were produced by labelling in the absence of terminal deoxynucleotidyl transferase.

T. gondii *tachyzoite labelling and flow cytometric analysis*

For evaluation of the degree of apoptosis by DNA staining with propidium iodide (PI), 1×10^6 tachyzoites were washed twice with PBS after different incubation times at 37 °C, and fixed in 5 ml of 70% ethanol for 2 h, washed and resuspended in PBS again containing 0·1% Triton X-100. After 1 h the tachyzoites were washed and incubated for 2 h at room temperature with 10 μg/ml DNase-free RNase before PI was added to a final concentration of 50 μg/ml. The tachyzoites were analysed for DNA content within 2 h using a flow cytometer (EPICS® XL, Coulter, FL, USA) equipped with a 200 mV air-cooled argon ion laser tuned to 488 nm. Fluorescence (FL1) was measured on a linear scale. A total of 10000 events were detected in the DNA analysis with a cell gate defined by side scatter/FL1 characteristics. DNA fragmentation was calculated

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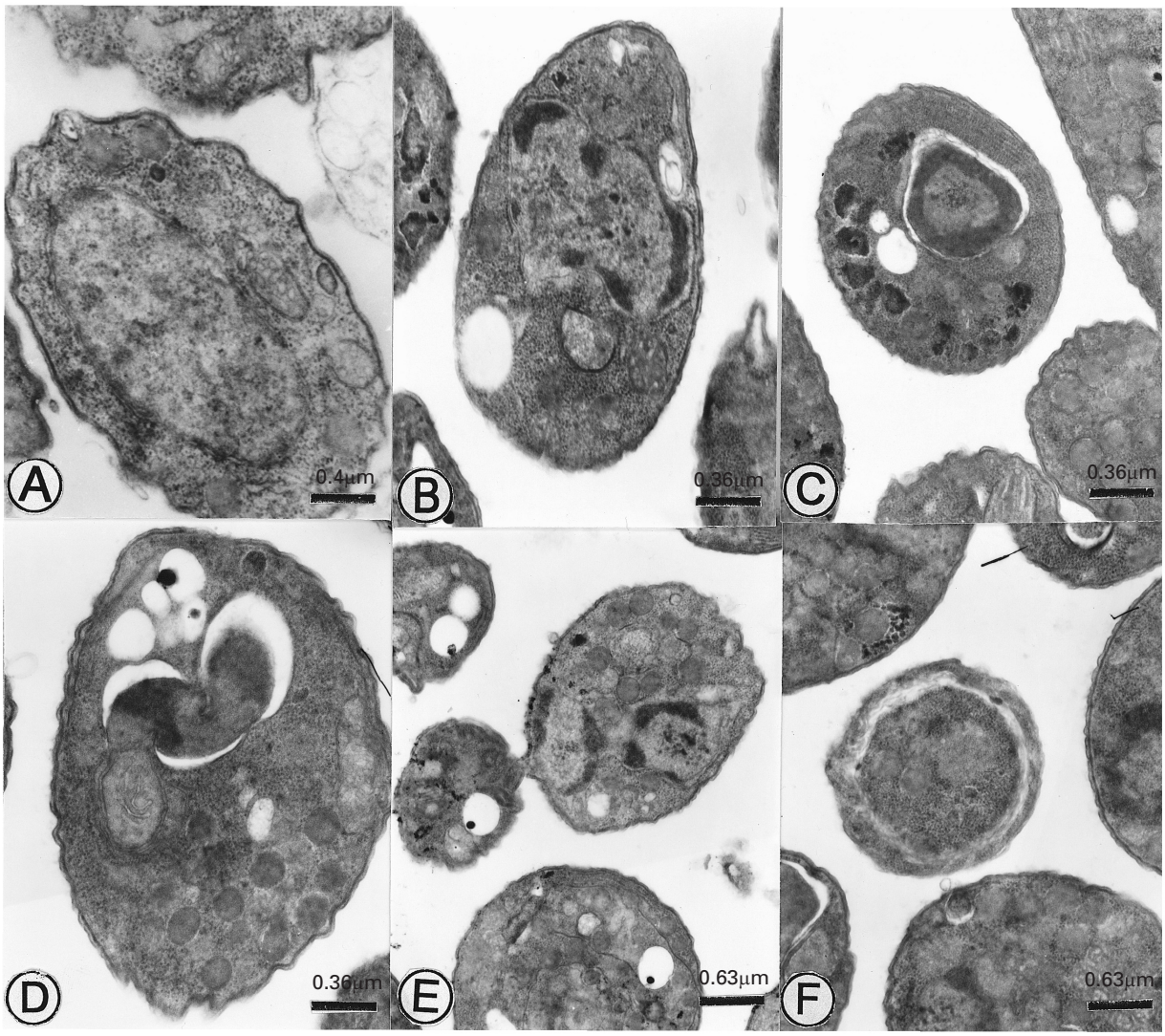

Fig. 1. Ultrastructural features of SNP-induced apoptosis in *Toxoplasma gondii* tachyzoites. (A) *T. gondii* tachyzoite in the control group with normal nuclei and organelles. (B–D) *T. gondii* tachyzoite after incubation with 2 mM SNP for 15 h, showing chromatin condensation below nuclear membrane. (E, F) *T. gondii* tachyzoite after incubation with 2 mM SNP for 20 h, showing chromatin condensation, nuclear pyknosis, and fragmentation.

using lysis II software and plotted as histograms for DNA content (Chen, Chen & Zheng, 1995).

Microspectrofluorometric measurements

Fluorescent emission spectra within living tachyzoites were recorded using a microspectrofluometer (RF-5301 PC, Shimadzu, Japan) as previously described (Toborek, Blanc & Kaiser, 1997). Briefly, tachyzoites were washed with serum-free medium and loaded with 2·5 μmol/l Fura-2/AM for 80 min at 37 °C. For each assay, Fura-2/AM-loaded tachyzoites were suspended to 1×10^8/ml with medium containing 0·2% BSA, vibrated at 37 °C and analysed using a fluorospectrophotometer. All unstimulated tachyzoites were removed with cold Balanced Salt Solution (BSS) before analysis. $[Ca^{2+}]_i$ determination was by Dual Wavelength Ratios. $[Ca^{2+}]_i$ was calculated according to the following equation: $[Ca^{2+}]_i = Kd(R\min - R)/(R - R\max).Ff_2/Fb_2$, where R is the ratio of fluorescence emission intensity of the sample in 340 nm and 380 nm, Rmax and Rmin represent the R values in saturated and Ca^{2+}-free tachyzoites, respectively. Ff_2/Fb_2 is the emission intensity ratio of Fura-2/AM in free and saturated Ca^{2+}, and Kd is the apparent dissociation constant of Fura-2/AM for Ca^{2+}. The *in situ* $[Ca^{2+}]_i$ calibration curve from the permeabilized tachyzoites had already been performed. Super Ion Probe Software was used to determine the fluorescence emission spectra in the 300–450 nm range and the ratio of both emission intensities (I_{340} nm/I_{380} nm).

Statistical analysis

Data were expressed as mean ± S.E.M. The results were analysed by Student's t-test. A value of $P < 0{\cdot}05$ is regarded as statistically significant.

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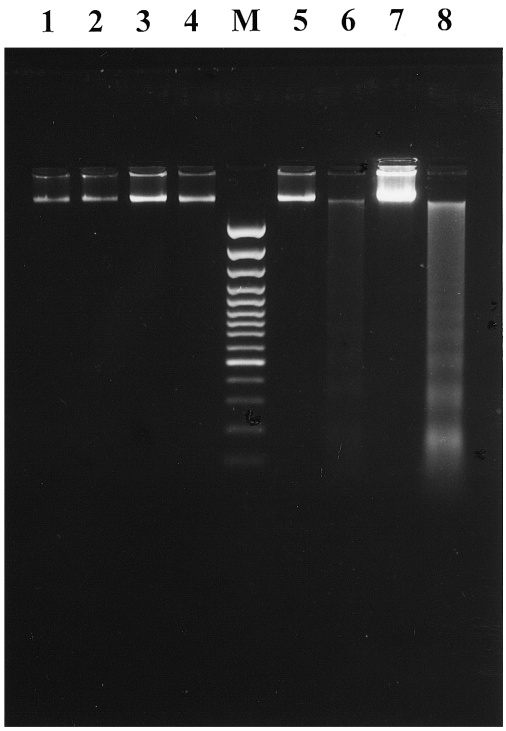

Fig. 2. Agarose gel electrophoresis pattern of SNP-induced apoptosis-like death in *Toxoplasma gondii* tachyzoites. Lanes 1, 3, 5, 7 demonstrate DNA from tachyzoites incubated without SNP for 5, 10, 15 and 20 h, respectively. Lanes 2, 4, 6, 8 show DNA from tachyzoites incubated with SNP for 5, 10, 15 and 20 h, respectively. Marker (M) is GeneRuler 100 bp ladder plus (MBI Fermentas).

RESULTS

SNP induces 'apoptosis' in T. gondii *tachyzoites*

To investigate whether the damaging effects of an NO donor, SNP, might be mediated through inducing tachyzoite apoptosis, tachyzoites were treated with 0 and 2 mM SNP for 1, 5, 10, 15 and 20 h, respectively, before examination by transmission electron microscopy (TEM) to assess features of apoptosis. As shown in Fig. 1, in control tachyzoites after 20 h (Fig. 1A), the nuclei displayed a prominent central or slight ex-central nucleolus, while chromatin was distributed peripherally beneath the nuclear membrane. In tachyzoites exposed to SNP, the morphological changes in the early stages, which were both aggregation of chromatin at the nuclear membrane and condensation of nucleus (Fig. 1B–D) occurred only in a few of tachyzoites treated with 2 mM SNP for 15 h. When treated with 2 mM SNP for 20 h, a number of tachyzoites, undergoing apoptosis, showed characteristic morphological features, such as the formation of apoptotic bodies (Fig. 1E, F). However, regardless of the duration of SNP-treatment, cytoplasmic organelles remain structurally preserved, although an increase in vacuoles and lipid was frequently seen. These findings may represent a sequence of morphological changes induced by SNP.

To analyse kinetic changes in biochemical features of the tachyzoites treated with SNP, both apoptotic DNA nucleosomal fragmentation and the level of total DNA were determined in parasites treated with or without 2 mM SNP for 1, 5, 10, 15 and 20 h prior to agarose gel electrophoresis and flow cytometry. As Fig. 2 shows, a DNA ladder was not visible when tachyzoites were exposed to SNP for 5 and 10 h. The ladder became clear only when the treatment was prolonged. When the tachyzoites were treated with SNP for 20 h, an oligonucleosomal laddering pattern in the genomic DNA was detectable (lane 8). By contrast, a ladder pattern was weak after incubation for 20 h without SNP (lane 7).

As shown in Fig. 3 (A–C) there was a hypoploid apoptotic peak before the G1 phase of the cell cycle in tachyzoites in the presence of SNP. The peak value increased with the elevation of SNP concentration. The peak value of the group treated with 2 mM SNP for 20 h was 5–10 times higher than that of the 0·5 mM SNP-treated group.

Relationship between changes in cytoplasmic free calcium concentration and SNP-induced apoptosis in T. gondii *tachyzoites*

Calcium ions are known to play a key role in many cell events. It is an important second messenger in eukaryotic cells, modulating a wide range of cell processes. Experiments were carried out to determine whether changes in $[Ca^{2+}]_i$ levels correlated with tachyzoite apoptosis induced by SNP. As shown in Fig. 4, both the rate of apoptosis and $[Ca^{2+}]_i$ level increased in a time- and dose-dependent manner after tachyzoites were treated with SNP. However, the $[Ca^{2+}]_i$ induction began at 1 h after SNP treatment and peaked at 10 h, whereas apoptosis in the tachyzoites significantly increased at 10 h and peaked at 20 h after SNP treatment. These data suggest that tachyzoite apoptosis induced by SNP is positively related to the elevation of $[Ca^{2+}]_i$ and calcium may play a critical role in this initiation.

To test whether NO released by SNP was responsible for the elevation of cytoplasmic free calcium and apoptosis-like death, tachyzoites were incubated with 5 mM *N*-acetylcysteine (NO scavenger) or 2 mM potassium ferricyanide (NO analogue) in the presence or absence of 2 mM SNP for 20 h. As shown in Table 1, the NO scavenger inhibited the increase in $[Ca^{2+}]_i$ and apoptosis induced by SNP ($P<0{\cdot}01$), but *N*-acetylcysteine or potassium ferricyanide alone did not induce changes in $[Ca^{2+}]_i$ and apoptosis in tachyzoite, compared with the controls ($P>0{\cdot}05$).

Origin of increase in $[Ca^{2+}]_i$

As demonstrated above, the SNP-induced apoptosis in tachyzoites correlated with an increase in $[Ca^{2+}]_i$

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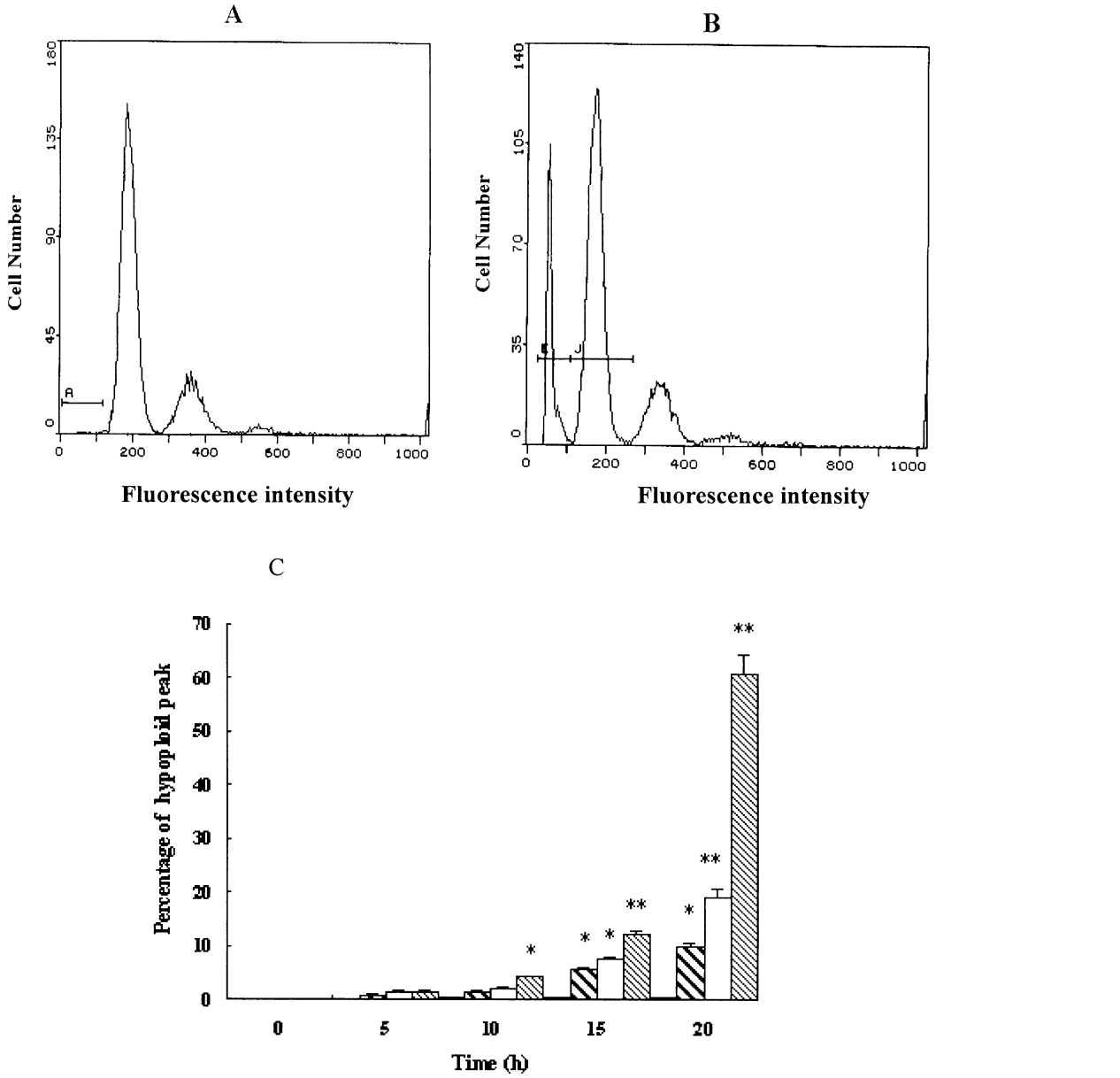

Fig. 3. Flow cytometric analysis of *Toxoplasma gondii* tachyzoite DNA content showing the frequency of apoptotic tachyzoites in the presence of serial doses of SNP. Tachyzoites treated with 2 mM SNP for 10 h (A) and 20 h (B). They were then stained with PI and analysed by flow cytometry. The frequency of apoptotic tachyzoites was expressed as mean ± S.E.M of triplicates. (C) Time/dose response of SNP on tachyzoite DNA content. Concentration of SNP is 0 mM (■), 0·5 mM (▨), 1·0 mM (□) and 2·0 mM (▨). The rate of hypoploid-peak enhanced with prolonged time; at each time-point, the higher the concentration, the higher the percentage of hypoploid-peak. Data are means ± S.E.M. of 4 experiments. $*P<0·05$, $**P<0·001$, significantly different compared with 2·0 mM SNP treated for 5 h.

level. In order to identify the origin of the calcium signal in such a system, tachyzoites were incubated with EGTA (2 mM), Verapamil (50 μM) and BAPTA/AM (25 μM) in the presence or absence of 2 mM SNP for 20 h before assessing $[Ca^{2+}]_i$ by Fura-2/AM, DNA content by cytometry, and apoptosis in tachyzoites by TUNEL assay, respectively. As shown in Fig. 5, chelation of extracellular calcium with EGTA, inhibited an increase in $[Ca^{2+}]_i$ ($P<0·01$) and the voltage-dependent calcium channel blocker (Verapamil) only partially inhibited the increase in $[Ca^{2+}]_i$ ($P<0·01$) concomitant with a corresponding improved survival of tachyzoites following treatment with SNP.

To establish more precisely the role of $[Ca^{2+}]_i$ during apoptosis induced by SNP, experiments were carried out to investigate the effects of an intracellular calcium chelator BAPTA/AM, an extracellular calcium chelator EGTA and Verapamil, the calcium channel antagonist, on changes of DNA content of tachyzoites induced by SNP. As shown in Table 2, when tachyzoites were treated with BAPTA/AM (25 μM), EGTA (2 mM) or Verapamil (50 μM) alone for 20 h before analysis by flow cytometry, the ratio

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Table 1. Inhibitory effect of NO scavenger on elevation of cytoplasmic free calcium and apoptosis in tachyzoites induced by SNP

(Tachyzoites were treated with different SNP-regulators (SNP, 2 mM; potassium ferricyanide, 2 mM; *N*-acetylcysteine, 5 mM) for 20 h and were then stained with TUNEL for direct detection of apoptosis. Apoptotic tachyzoites show pronounced violet-blue nuclei with condensed chromatin, whereas nuclei were unstained in viable tachyzoites. Free cytoplasmic calcium was detected using Fura-2/AM. Data are means ± S.E.M.)

Treatment	Rate of apoptosis (%)	$[Ca^{2+}]_i$ (nM)
Control	1·25 ± 0·71	113·00 ± 5·33
SNP	46·37 ± 2·07**	372·25 ± 17·09**
Potassium ferricyanide	1·75 ± 0·87*	115·58 ± 5·16*
N-acetylcysteine	1·88 ± 0·64*	116·23 ± 5·75*
SNP + *N*-acetylcysteine	8·00 ± 1·85***	230·27 ± 16·56***

* $P > 0{\cdot}05$ *vs* control group; ** $P < 0{\cdot}01$ *vs* control group; *** $P < 0{\cdot}01$ *vs* SNP treatment group.

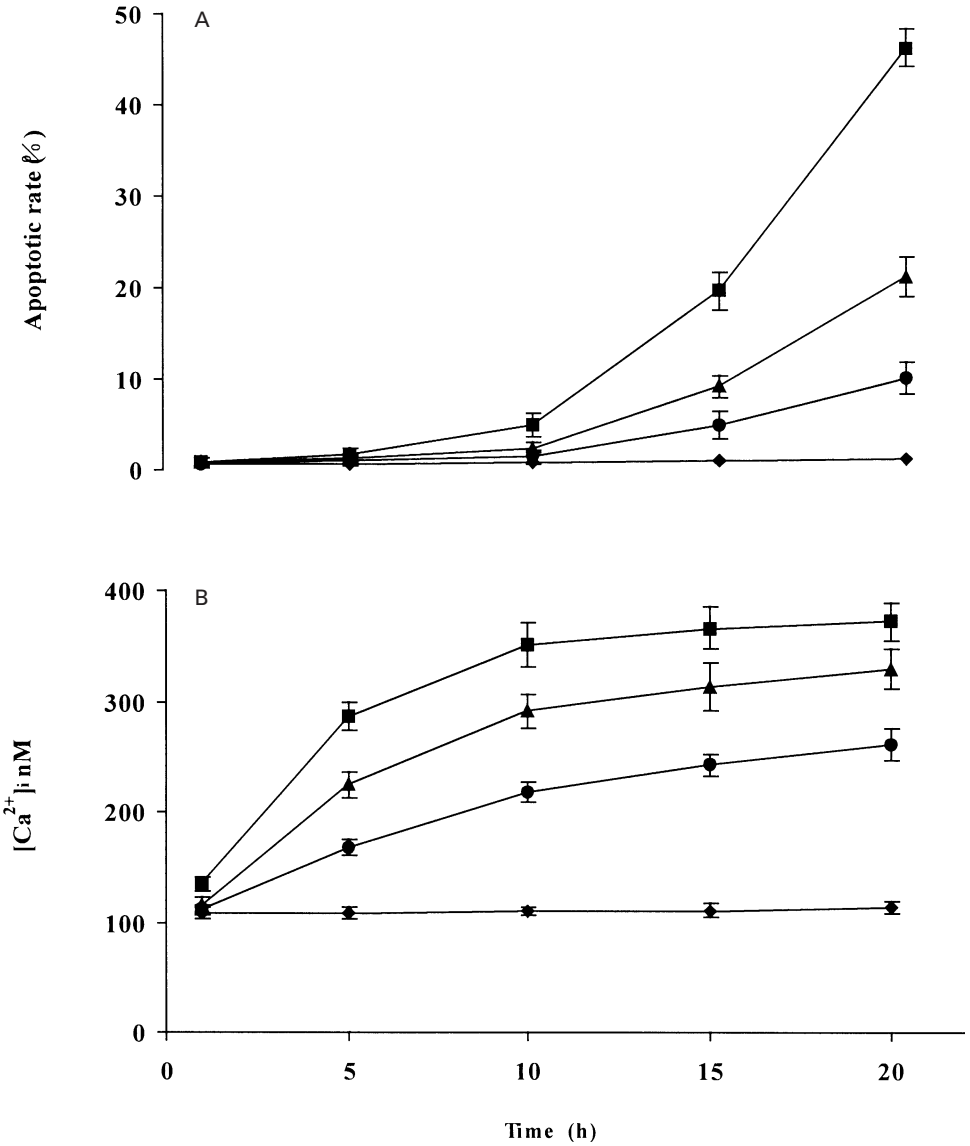

Fig. 4. Correlation of apoptosis with $[Ca^{2+}]_i$ level in *Toxoplasma gondii* tachyzoites treated with SNP. SNP concentrations: 0 mM (◆), 0·5 mM (●), 1 mM (▲), 2 mM (■), respectively. (A) Apoptotic rate versus SNP concentration and time ($P < 0{\cdot}01$). (B) Intracellular calcium concentration $[Ca^{2+}]_i$ versus time in tachyzoites treated with 2 mM SNP. No significant difference was noted at (10, 15 and 20 h, $P > 0{\cdot}05$).

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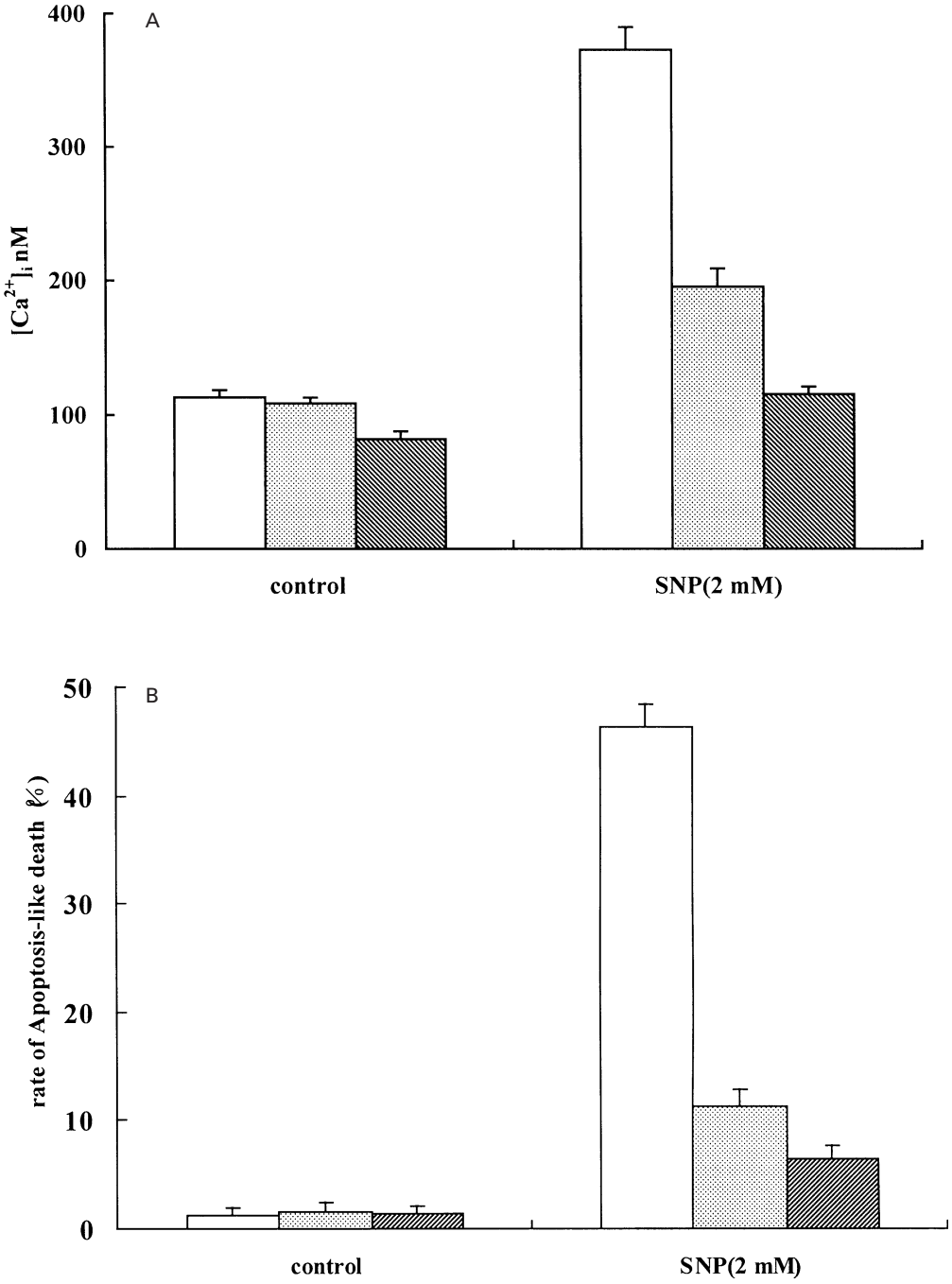

Fig. 5. Inhibitory effect of EGTA and Verapamil on the increase in cytoplasmic $[Ca^{2+}]_i$ and apoptosis in *Toxoplasma gondii* tachyzoites induced by SNP. Tachyzoites were incubated in the presence of SNP with Verapamil/EGTA. (□), No additional treatment; (▨) added Verapamil; (▨) added EGTA for 20 h, and then stained with TUNEL to determine apoptosis and with Fura-2/AM for cytoplasmic $[Ca^{2+}]_i$. (A) Verapamil + SNP and EGTA + SNP group, apoptosis rates are lower than in SNP group ($P<0{\cdot}01$), but still higher than in control ($P<0{\cdot}01$). (B) EGTA group, $[Ca^{2+}]_i$ is lower than control ($P<0{\cdot}01$), but no significant difference was noted between the Verapamil group and control group ($P<0{\cdot}05$). In the Verapamil + SNP group, $[Ca^{2+}]_i$ level decreased compared with the SNP group but was not markedly different from the control ($P>0{\cdot}05$).

of hypoploid peak (named A0) was 1·8%, 1·0% and 1·0%, respectively. The A0 peak was 56·7% in the SNP (2 mM)-treated group. However, when SNP was combined with BAPTA/AM, EGTA or Verapamil respectively, the ratio of the hypoploid peak was decreased to 7·2%, 10·9% and 20·6% ($P<0{\cdot}01$), concomitant with an increase in G0/G1 and a decrease in G2/M, compared to the homologous control.

DISCUSSION

NO can induce apoptosis in a wide range of mammalian cells (Shimaoka *et al*. 1995; Sarih, Souvannavong & Adam, 1993; Albina *et al*. 1993; Kitajima *et al*. 1994; Ankarcrona *et al*. 1994; Kaneto *et al*. 1995; Mabbott *et al*. 1995; Maciejewski *et al*. 1995; Blanco *et al*. 1995), and NO has a more important

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Table 2. Effect of EGTA, BAPTA/AM and Verapamil on changes of tachyzoite caused by SNP

(Flow cytometry detected the DNA content of tachyzoites stained with PI. SNP-treated for 20 h, the peak value appeared before the G1 peak, which was expressed as the apoptotic peak (A0). The value of the A0 peak changed with treatment with calcium-regulators.)

	A0			G0/G1		G2/M	
Treatment	Channel	No. of tachyzoites	%	Channel	%	Channel	%
Control	74·4	50	0·5	188·7	73·5	422	26·0
BAPTA/AM	90·2	176	1·8	305·5	72·0	405	26·2
EGTA	85·8	100	1·0*	288·9	72·7	375	26·3
Verapamil	67·7	100	1·0*	177·8	72·8	368	26·2
BAPTA/AM+SNP	78·4	721	7·2***	286·3	66·8	415	26·0
EGTA+SNP	86·6	1087	10·9***	270·5	59·4	348	29·7
Verapamil+SNP	99·0	2058	20·6***	173·2	48·6	413	30·8
SNP	60·3	5668	56·7**	236·5	30·4	129	12·9

* $P>0{\cdot}05$ *vs* control group; *** $P<0{\cdot}001$ *vs* control group; *** $P<0{\cdot}05$ *vs* single SNP treatment group.

role as an immune effector *in vivo* (Esposito *et al*. 2002). The present study investigated whether NO can induce apoptosis-like death in *T. gondii* tachyzoites, and the role of calcium in this process.

Our results demonstrated that an exogenous NO donor, SNP, is capable of inducing apoptosis-like death in *T. gondii* tachyzoites as assessed by the following observations. (1) The tachyzoites treated by SNP showed the characteristic ultrastructural features of apoptosis, such as aggregation of chromatin, condensation of the nucleus, fragmentation of cells and presence of apoptotic bodies. (2) DNA of SNP-treated tachyzoites showed the appearance of the 'DNA ladder' in agarose gel electrophoresis. (3) A hypoploid peak appeared before the G1 phase of the cell cycle in tachyzoites treated with SNP determined by flow cytometric analysis. (4) *N*-acetylcysteine, an NO scavenger, greatly inhibited SNP-induced tachyzoite apoptosis, but potassium ferricyanide, an analogue of SNP devoid of NO, did not have the same effect.

Ultrastructural change is an important hallmark of apoptosis (Cohen, 1993). In the present study, the ultrastructural characteristics of apoptosis in *T. gondii* tachyzoites, such as condensation of chromatin, nuclear pyknosis, nuclear fragmentation and apoptotic bodies, were observed by TEM after exposure to 2 mM SNP as in other protozoa. The morphological changes were related to the duration of SNP treatment. When treated with SNP (2 mM) for 1, 5 and 10 h, no apoptotic features were found. Only a few tachyzoites treated with SNP for 15 h appeared to be undergoing nuclear chromatin aggregation. More tachyzoites appeared to have nuclear and cytoplasmic changes after treatment with SNP for 20 h.

In addition, the other two types of cell death: necrosis and oncosis, which were described by Majno (Majno & Joris, 1995), were observed by chance in tachyzoites treated by SNP (data not shown). Many researchers considered that *in vivo* apoptotic bodies are rapidly recognized and phagocytosed by either macrophages or adjacent epithelial cells (Savil, Oransfield & Hogg, 1990; Wyllie, 1980). *In vitro*, due to lack of this efficient mechanism for the removal of apoptotic cells, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed 'secondary necrosis'.

Another hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event leading to cell death. In many systems DNA fragmentation had been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units generating mono- and oligo-nucleosomal DNA fragments (Vielhaber *et al*. 2002; Yoshimura *et al*. 2002). These DNA fragments, after agarose gel electrophoresis, show a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit (Wyllie, 1980). In this study, it was shown that when tachyzoites were treated with SNP (2 mM) for 15–20 h, a DNA ladder was detected on agarose gel electrophoresis and the DNA content was reduced. These data have demonstrated that SNP is capable of inducing apoptosis-like death in *T. gondii* tachyzoites. But at present, little is known about the mechanism involved.

Ionized calcium (Ca^{2+}) is the most common signal transduction element in cells ranging from bacteria to specialized neurons (Majno & Joris, 1995) and deregulation of calcium homeostasis is an important event in cell damage (Trump & Berezesky, 1995). During every stage of apoptosis induced by SNP, cytoplasmic $[Ca^{2+}]_i$ was raised in tachyzoites in a time- and dose-dependent manner. Cultured with 2 mM SNP for 1 h, $[Ca^{2+}]_i$ began to rise, and the concentration reached a rather high level at 10 h. Despite this, apoptotic tachyzoites were still low in number even when detected by the sensitive TUNEL assay. After 10 h $[Ca^{2+}]_i$ rose continuously to a saturation concentration ($P>0{\cdot}05$), when the

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percentage of tachyzoites undergoing apoptosis started to increase greatly. It is clear that $[Ca^{2+}]_i$ was increased in a very early stage of apoptosis, indicating a key role for $[Ca^{2+}]_i$ in initiating apoptosis of tachyzoites.

Normally, intracellular Ca^{2+} levels at $\sim$100 nM are 20 000-fold lower than 2 mM concentration found extracellularly. The elevation of cytoplasmic free calcium in eukaryotes (Silvia, Moreno & Zhong, 1996) has 2 possible sources: one is Ca^{2+} flow inside through the opening of Ca^{2+} channels, the other is a release from Ca^{2+} stock. Enhancement of cytoplasmic $[Ca^{2+}]_i$ in tachyzoites is through both pathways. In the present study, we found that chelation of extracellular Ca^{2+} with EGTA can inhibit both the elevation of $[Ca^{2+}]_i$ and apoptosis in the tachyzoites induced by SNP, and Verapamil can partly inhibit it. Similarly, BAPTA/AM, EGTA and Verapamil were able to inhibit the effects of SNP on DNA content. The data further demonstrate that the increase in cytoplasmic free $[Ca^{2+}]_i$ participated directly in the apoptosis of tachyzoites induced by SNP. A possible mechanism is that NO promotes Ca^{2+} flowing into the cell and $[Ca^{2+}]_i$ levels rise, leading to apoptosis in tachyzoites.

In summary, the present work shows that an NO donor, SNP, is capable of inducing apoptosis in *T. gondii* tachyzoites by elevating cytoplasmic free calcium concentration, which is mainly the result of the entry of extracellular calcium. These findings (i) suggest that NO-dependent cytotoxicity against *T. gondii* tachyzoites is exerted through a form of cell death resembling metazoan apoptosis and (ii) point out the potential chemotherapeutic importance.

REFERENCES

ADAMS, L. B., HIBBS, J. B. Jr, TAINTOR, R. R. & KRAHENBUHL, J. L. (1990). Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology* **144**, 2725–2729.

ALBINA, J. E., CUI, S. & MATEO, R. B. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *Journal of Immunology* **15**, 5080–5085.

AMEISEN, J. C., IDZIOREK, T., BILLAUT-MULOT, O., LOYENS, M., TISSIER, J. P., POTENTIER, A. & OUAISSI, A. (1995). Apoptosis in a unicellular eukaryote (*Trypanosoma cruzi*): implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival. *Cell Death and Differentiation* **2**, 285–300.

ANKARCRONA, M., DYPBUKT, J. M., BRUNE, B. & NICOTERA, P. (1994). Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Experimental Cell Research* **213**, 172–177.

BLANCO, F. J., OCHS, R. L. & SCHWARZ, H. (1995). Chondrocyte apoptosis induced by nitric oxide. *American Journal of Pathology* **146**, 75–85.

CARAFOLI, E. (1987). Intracellular calcium homeostasis. *Annual Review of Biochemistry* **56**, 395–433.

CHEN, G., CHEN, F. & ZHENG, H. (1995). Biological significance of DNA and protein contents and form features of *Toxoplasma gondii* tachyzoite population. *Acta Parasitology Medical Entomology Sina* **11**, 5–8.

COHEN, J. J. (1993). Apoptosis: the physiologic pathway of cell death. *Hospital Practice* **28**, 35–43.

CULOTTA, E. & KOSHLAND, D. E. Jr. (1992). NO news is good news. *Science* **258**, 1862–1865.

DAS, M., MUKHERJEE, S. B. & SHAHA, C. (2001). Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *Journal of Cell Science* **114**, 2461–2469.

DEVERAUX, Q. L. & REED, J. C. (1999). IAP family proteins-suppressors of apoptosis. *Genes and Development* **13**, 239–252.

DRISCOLL, M. (1992). Molecular genetics of cell death in the nematode *Caenorhabditis elegans*. *Journal of Neurobiology* **23**, 1327–1351.

ESPOSITO, G., LIGRESTI, A., IZZO, A. A., BISOGNO, T., RUVO, M., DI ROSA, M., DI MARZO, V. & IUVONE, T. (2002). The endocannabinoid system protects rat glioma cells against HIV-1 Tat-induced cytotoxicity: Mechanism and regulation. *Journal of Biological Chemistry* **277**, 50348–50354.

FRENKEL, J. K. (1996). The stage-conversion time of *Toxoplasma gondii*: Interpretation of chemical-biologic data out of parasitologic or host context. *Parasitology Research* **82**, 656–658.

HERRMANN, M., LORENZ, H.-M. & VOLL, R. (1994). A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Research* **22**, 5506–5507.

KANETO, H., FUJI, J. & SEO, H. G. (1995). Apoptotic cell death trigered by nitric oxide in pancreatic beta-cells. *Diabetes* **44**, 733–738.

KITAJIMA, I., KAWAHARA, K. & NAKAJIMA, T. (1994). Nitric oxide-mediated apoptosis in murine mastocytoma. *Biochemical and Biophysical Research Communications* **204**, 244–251.

LANGERMANS, J. A., VAN DER HULST, M. E., NIBBERING, P. H., HIEMSTRA, P. S., FRANSEN, L. & VAN FURTH, R. (1992). IFN-gamma-induced L-arginine-dependent toxoplasmastatic activity in murine peritoncal macrophages is mediated by endogenous tumor necrosis factor-alpha. *Journal of Immunology* **148**, 568–574.

MABBOTT, N. A., SUTHERLAND, I. A. & STERBERG, J. M. (1995). Suppressor macrophages in *Trypanosoma brucei* infection: nitric oxide is related to both suppressive activity and lifespan *in vivo*. *Parasite Immunology* **17**, 143–150.

MACIEJEWSKI, J. P., SELLERI, C. & SATO, T. (1995). Nitric oxide suppression of human hematopoiesis *in vitro*. Contribution to inhibitory action of interferon-gamma and tumor necrosis factor-alpha. *Journal of Clinical Investigation* **96**, 1085–1092.

MAJNO, G. & JORIS, I. (1995). Apoptosis, oncosis, and necrosis: an overview of cell death. *American Journal of Pathology* **146**, 3–15.

MOREIRA, M. E., DEL PORTILLO, H. A., MILDER, R. V., BALANCO, J. M. & BARCINSKI, M. A. (1996). Heat Shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania* (*Leishmania*) *amazonensis*. *Journal of Cellular Physiology* **167**, 305–313.

MUZYLAK, M. & MASLINSKA, D. (1992). Neurotoxic effect of vincristine on ultrastructure of hypothalamus in rabbits. *Folia Histochemica et Cytobiologica* **30**, 113–117.

PIACENZA, L., PELUFFO, G. & RADI, R. (2001). L-arginine dependent suppression of apoptosis in *Trypansoma cruzi*: contribution of the nitric oxide and polyamine pathways. *Proceedings of the National Academy of Sciences, USA* **98**, 7301–7306.

PICOT, S., BURNOD, J., BRACCHI, V., CHUMPITAZI, B. F. & AMBROISE-THOMAS, P. (1997). Apoptosis related to chloroquine sensitivity of the human malaria parasite *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **91**, 590–591.

SARIH, M., SOUVANNAVONG, V. & ADAM, A. (1993). Nitric oxide synthase induces macrophage death by apoptosis. *Biochemical and Biophysical Research Communications* **191**, 503–508.

SAVIL, J., ORANSFIELD, I. & HOGG, N. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature, Nature* **343**, 170–173.

SERENO, D., HOLZMULLER, P., MANGOT, I., CUNY, G., OUAISSI, A. & LEMESRE, J. L. (2001). Antimonial-mediated DNA fragmentation in *Leishmania infantum* amastigotes. *Antimicrobial Agents and Chemotherapy* **45**, 2064–2069.

SHIMAOKA, M., IIDA, T. & OHARA, A. (1995). NOC, a nitric-oxide-releasing compound, induces dose dependent apoptosis in macrophages. *Biochemical and Biophysical Research Communications* **209**, 519–526.

SILVIA, N. J., MORENO S. N. & ZHONG, L. (1996). Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *The Biochemical Journal* **313**, 655–659.

TOBOREK, M., BLANC, E. M. & KAISER, S. (1997). Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured vascular endothelial cells. *Journal of Lipid Research* **38**, 2155–2167.

TRUMP, B. F. & BEREZESKY, I. K. (1995). Calcium-mediated cell injury and cell death. *Federation of American Societies for Experimental Biology* **9**, 219–228.

VIELHABER, S., JAKUBICZKA, S., SCHRODER, J. M., SAILER, M., FEISTNER, H., HEINZE, H. J., WIEACKER, P. & BETTECKEN, T. (2002). Facioscapulohumeral muscular dystrophy with EcoRI/BlnI fragment size of more than 32 kb. *Muscle Nerve* **25**, 540–548.

WELBURN, S. C., LILLICO, S. & MURPHY, N. B. (1999). Programmed cell death in procyclic form *Trypanosoma brucei rhodesiense*-identification of differentially expressed genes during Con A induced death. *Memorias do Instituto Oswaldo Cruz, Rio de Janeiro* **94**, 229–234.

WYLLIE, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature, London* **284**, 555–557.

YOSHIMURA, A., URA, K., ASAKURA, H., KOMINAMI, R. & MISHIMA, Y. (2002). Mononucleosomes assembled on a DNA fragment containing (GGA/TCC)(n) repeats can form a DNA-DNA complex. *Biochemical and Biophysical Research Communications* **290**, 16–22.

ZHENG, C. & LIN, J. (1998). The inhibition effect of nitroprusside on *Toxoplasma gondii* infection macrophage *in vitro*. *Acta Parasitology Medical Entomology Sina* **5**, 65–70.