FULL PAPER *Bacteriology*

Nitric Oxide Causes Anoikis through Attenuation of E-Cadherin and Activation of Caspase-3 in Human Gastric Carcinoma AZ-521 Cells Infected with *Mycoplasma hyorhinis*

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ABSTRACT. *Mycoplasma hyorhinis* (*M. hyorhinis*) infection leads cultured cells to various biological alterations in cell metabolism including apoptosis. Apoptosis induced by *M. hyorhinis* has mainly been considered to be due to mycoplasmal endonucleases. We previously reported that apoptosis in a human carcinoma cell line AZ-521 infected with *M. hyorhinis* was enhanced by addition of L-ascorbic acid to cell cultures. Since both L-ascorbic acid addition and *M. hyorhinis* infection activated cellular iNOS, we examined the hypothesis that nitric oxide (NO) exerts an apoptotic effect on *M. hyorhinis*-infected cells and down-regulates E-cadherin. In this study, we showed that *M. hyorhinis* infection activates iNOS mRNA synthesis, NO production, and caspase-3 activity and attenuates E-cadherin mRNA synthesis by quantitative real-time PCR, Griess assay and fluorescence caspase-3 detection. L-NAME decreased the numbers of apoptotic cells through inhibition caspase-3 activity. Our results indicate that NO causes anoikis throughout attenuation of E-cadherin and activation of caspase-3 in human gastric carcinoma cell line AZ-521 cells infected with *M. hyorhinis*.
KEY WORDS: anoikis, apoptosis, E-cadherin, *Mycopalsma hyorhinis*, nitric oxide.

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Mycoplasmas are the smallest self-replicating prokaryotes, lack rigid cell walls and belong to the class *Mollicutes* [25]. Most mycoplasma species inhabit plants, insects, animals and humans as normal flora in their hosts. Although some are pathogenic, many possess an opportunistic character [22].

Mycoplsma hyorhinis (*M. hyorhinis*) causes polyserositis [7], otitis media [19], arthritis [12] and pneumonia [1] in piglets. These mycoplasmal infections are responsible for economical losses on swine farms. *M. hyorhinis* is also a major and serious contaminant in cell cultures [2]. In addition, it has been demonstrated in human gastric carcinoma tissues [26], and it has been suggested that lipoprotein P37 of *M. hyorhinis* contributes to invasiveness and metastasis of the tumor cells [5, 14].

M. hyorhinis infection has a significant impact on the physiology of cell cultures, including apoptosis, which has previously been explained by mycoplasmal endonucleases [23], production of nitric oxide (NO) by activating cellular inducible NO synthase (iNOS) and production of reactive oxygen species (ROS) in various cell lines [13]. NO is a multifunctional molecule involved in a variety of physiological and pathological processes [17]. While low concentrations of NO can protect cells from apoptosis, excess NO promotes cell death in various cell types [3].

Apoptosis is characterized morphologically by cell shrinkage and chromatin condensation and biochemically by DNA laddering [30]. Detection of caspase activity is a useful assay for apoptosis. The caspase family participates in a series of reactions that are trigged in proapototic signals and result in the cleavage of substrates, and caspases are synthesized as inactive precursors that undergo proteolytic maturation upon apoptotic stimulation [27].

Adhesion of cells to the extracellular matrix (ECM) is important as detachment from the matrix triggers apoptosis referred to as anoikis [7]. It has recently been reported that anoikis is caspase-3 dependent [6]. Epithelial cadherin (E-cadherin) is the prime mediator of intracellular adhesion [28]. Our preliminary examination suggested marked down-regulation of E-cadherin expression in AZ521 cells infected with *M. hyorhinis* based on a microarray analysis (unpublished data). Therefore, we hypothesized that excessive NO produced by *M. hyorhinis* infection leads to anoikis in AZ-521 cells.

We previously reported that apoptosis in human carcinoma cell line AZ-521 infected with *M. hyorhinis* was enhanced by addition of L-ascorbic acid (AsA) to the cell cultures [21]. Since *M. hyorhinis* infection and/or AsA addition enhance iNOS activity [20], we proposed a hypothesis that *M. hyorhinis* infection results in the presence of another external apoptotic pathway including the NO pathway. The aim of the present study was to obtain insights into the role of NO in cell adhesion-dependent apoptosis in cells infected with *M. hyorhinis*.

In the present study, we examined the role of NO in apoptosis induced by *M. hyorhinis* infection. We used a general competitive inhibitor of NOS, N^{w}-nitro-L-arginine methyl ester (L-NAME), to measure NO based on the Griess assay

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and analyzed iNOS and E-cadherin expression by real-time PCR (RT-PCR) and caspase-3 detection. This study may also promote understanding of the mechanism of piglet diseases caused by *M. hyorhinis*.

MATERIALS AND METHODS

Cell cultures: Human gastric carcinoma cell line AZ-521 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and was maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, U.S.A.), 1% penicillin and streptomycin (100 U/m*l*) at 37°C in a humidified 5% CO_2 atmosphere. To minimize the pH change by L-NAME (Nacalai Tesque Inc., Kyoto, Japan) addition to the cell culture, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5) was added to the DMEM at a final concentration of 50 mM. The cell culture was tested for the absence of mycoplasma contamination by using a PCR Mycoplasma Detection Set (TaKaRa-Bio, Shiga, Japan) according to the manufacturer's instructions. The cells were seeded into chamber flasks at 8.5×10^5 cells per chamber (Asahi Techno Glass, Tokyo, Japan).

Five hundred μM of L-NAME were added to the final concentration as indicated. The vehicle for L-NAME was distilled water, and distilled water was used as a control. Addition of distilled water or 500 μM of L-NAME to AZ-521 cells did not produce any differences in this study (data not shown) using trypan blue staining after 30 hr of incubation in order to monitor the cytotoxic effect of distilled water [16]. The reagents were freshly prepared and dissolved in distilled water before pretreatment.

M. hyorhinis strain and its growth conditions: The BTS-7 strain of *M. hyorhinis* was used in present study. *M. hyorhinis* was grown at 37°C in PPLO broth (Difco, Detroit, MI, U.S.A.) supplemented with 20% horse serum (Gibco BRL, Grand Island, NY, U.S.A.), 5% fresh yeast extract (ICN Biomedicals, Inc., OH, U.S.A.) and 0.5% glucose instead of bacteriological mucin [15]. The propagation of *M. hyorhinis* was expressed as Colony-forming units (CFU). *M. hyorhinis* was inoculated into the AZ-521 cell culture at a multiplicity of infection (MOI) of 10^3 after seeding the cells into Chamber Slide II wells (Asahi Techno Glass, Tokyo, Japan). *M. hyorhinis* grown in PPLO broth containing 500 μM of L-NAME and HEPES (pH 7.5) was calculated after 30 hr of incubation at 37°C. Viability of the AZ-521 cells and *M. hyorhinis* strain were not affected by incubation with distilled water or 500 μM of L-NAME after 30 hr post-inoculation (data not shown).

Quantitative RT-PCR for iNOS and E-cadherin mRNA transcription: Total RNA was extracted from AZ-521 cells inoculated with *M. hyorhinis*, after 20, 24 and 28 hr using RNA-Bee™ (Tel-Test, Inc., Pearland, TX, U.S.A.), precipitated by isopropanol and dissolved in diethyl pyrocarbonate-treated water.

Similarly, total RNA was extracted from AZ-521 cells inoculated with *M. hyorhinis* and treated or not treated with 100, 250 and 500 μM of L-NAME after 24 and 28 hr. The RNA was transcribed with *Moloney murine leukemia* virus reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. First-strand complementary DNA was quantitatively analyzed for the expression of iNOS and E-cadherin gene in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with TaKaRa Ex *Taq* R-PCR (TakaRa Bio, Shiga, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 2.5 μl of 10 × reaction buffer, 20 nmol of each deoxynucleotide, 0.25 μl of 250 mM $MgCl_2$, 0.2 μl (5 U/μl) TaKaRa Ex *Taq* HS DNA polymerase (TaKaRa Bio, Shiga, Japan), 2.5 μl of 1:3,000 SYBR Green I (TaKaRa Bio, Shiga, Japan) and water to a volume of 23 μl. Finally, 2 μl of RNA as a template was added to this mixture. The conditions of amplification were as follows: after initial melting at 94°C for 2 min, amplification was performed with 40 cycled of 94°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec. The primer sequences used were as follows: 5'- CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3', forward, and 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3', reverse, for iNOS (394 bp) [13]; 5'-AGA ATG ACA ACA AGC CCG AAT-3', forward, and 5'-CGG CAT TGT AGG TGT TCA CA-3', reverse for E-cadherin (132 bp) [4]; and 5'- GTC TTC ACC ACC ATG GAG AAG GCT-3', forward, and 5'-CAT GCC AGT GAG CTT CCC GTT CA-3', reverse, for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 420 bp) [13]. The amount of target gene transcription was calculated from the standard curves and was normalized using the transcription of the housekeeping GAPDH gene as an internal control. The value for the mock-infected control was scored as one.

Measurement of NO oxidation products, nitrite and nitrate: NO is chemically unstable and undergoes rapid oxidation to nitrite, and cellular components catalyze its further oxidation to nitrate. Therefore, production of NO was determined by measuring the formation of the stable oxidation products of NO, nitrite and nitrate as described previously [10]. After the cells had been infected with *M. hyorhinis* for 20, 24 and 28 hr, total nitrite and nitrate concentrations in the cell culture were determined based on the Griess reaction by using a Total Nitric Oxide Assay Kit (Assay Designs Inc., Ann Arbor, MI, U.S.A.) according to manufacturer's instructions. The cell culture was collected after centrifugation at 1,000 × g for 5 min. The collected supernatant fluid was stored at −80°C until measurement of nitrate. The absorbance was measured at a wavelength of 570 nm in an NJ-2000 multiwell plate reader (InterMed, Tokyo, Japan).

Fluorescent microscopic analysis for apoptotic cell count and caspase-3 activity detection: The AZ-521 cells were examined for apoptotic changes by using a fluorescent dye, Hoechst 33258 (Dojin Chemicals, Kumamoto, Japan). We observed fluorescent nuclei showing apoptotic changes, mainly due to chromatin condensation, at 400 × magnification by using 352 and 461 nm band pass filters for excitation and emission, respectively, on an Eclipse E400 fluorescent

microscope (Nikon, Tokyo, Japan). At 24 and 28 hr post-inoculation, we counted the number fluorescent cells infected with BTS-7 showing apoptotic changes at 400 × magnification in 200 AZ-521 cells each treated with or without 100, 250 or 500 μM of L-NAME. Apoptosis was expressed as the percentage of cells with an apoptotic nuclear morphology in relation to the total cell number.

Furthermore, to detecte caspase-3 activity in AZ-521 cells, we used an APO LOGIX^{TM} FAM-DEVD-FMK Carboxyfluorescein Caspase Detection Kit (Cell Technology Inc., Mountain View, CA, U.S.A.). AZ-521 cells grown in the DMEM containing 500 μM of L-NAME were examined more than 24 and 28 hr after inoculation with *M. hyorhinis*, according to manufacturer's instructions. Caspase-3 activity of AZ-521 cells was examined by counting the number fluorescent cells that were positive for caspase-3 in 200 AZ-521 cells at 400 × magnification.

Statistical analysis: The data was analyzed using analysis of variance (ANOVA) for comparison between groups using StatView ver. 5 (Hulinks Inc., Tokyo, Japan). Differences were accepted as significant values at $P<0.05$. Each test was repeated three times. Data were expressed as mean ± SEM values.

RESULTS

RT-PCR analysis of iNOS and E-cadherin transcription: The transcription levels of iNOS and E-cadherin were examined by using RT-PCR (Fig. 1). At 20, 24 and 28 hr post-inoculation, a significant amount of iNOS mRNA was detected in the *M. hyorhinis*-infected AZ-521 cells. On the other hand, no significant difference in iNOS mRNA transcription was observed in the mock-infected controls at any hour post-inoculation. These results suggested that *M. hyorhinis* induced iNOS mRNA expression in the AZ-521 cells.

Similarly, E-cadherin mRNA transcription was examined in AZ-521 cells treated with or without 100, 250 or 500 μl of L-NAME at 24 and 28 hr post-inoculation (Fig. 2). *M. hyorhinis* infection caused decreases of E-cadherin mRNA transcription at 24 and 28 hr post-inoculation as compared with the mock-infected controls. Also, the amount of E-cadherin mRNA was significantly decreased in the *M. hyorhinis*-infected AZ-521 cells treated with 500 μl of L-NAME at 24 hr and in those treated with 250 and 500 μl of L-NAME at 24 hr and 28 hr post-inoculation. *M. hyorhinis* infection did not alter GAPDH mRNA transcription. These results suggested that *M. hyorhinis* infection attenuates E-cadherin mRNA transcription by NO-dependency in AZ-521 cells.

Measurement of NO oxidation products, nitrite and nitrate: To determine whether or not the expressed iNOS in AZ-521 cells produces NO, NO synthesis was assayed by measuring the accumulation of the NO end products, nitrite and nitrate, in the medium (Fig. 3). At 20 hr post-inocula-

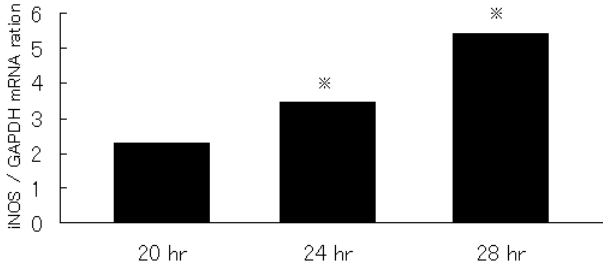

Fig. 1. Expression of iNOS in AZ-521 cells was analyzed by quantitative real-time PCR. The cells were treated with *M. hyorhinis* for 24 and 28 hr. The intensity of iNOS mRNA expression was normalized by that of GAPDH, and the value for mock was estimated as 1. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

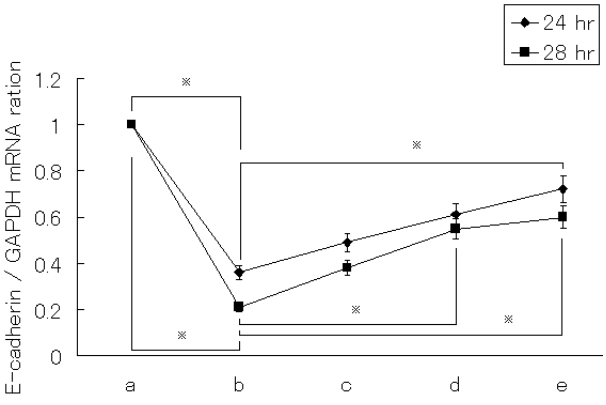

Fig. 2. E-cadherin expression in AZ-521 cells was analyzed by quantitative real-time PCR. The cells were infected with *M. hyorhinis* and treated with (b) no L-NAME, (c) 100, (d) 250 or (e) 500 μM of L-MAME or (a) mock for 24 and 28 hr. The intensity of E-cadherin mRNA expression was normalized by that of GAPDH. The value for mock was set at 1. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

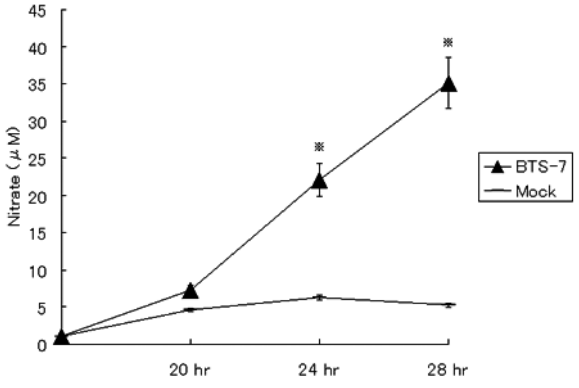

Fig. 3. Nitrate production in AZ-521 cells infected with *M. hyorhinis* at 20, 24 and 28 hr post inoculation. Means ± SEM for two separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

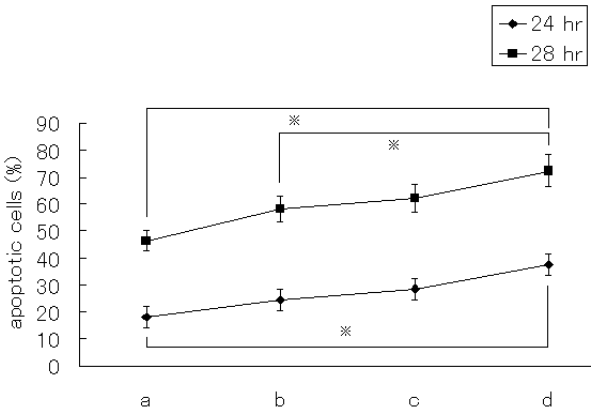

Fig. 4. The effect of L-NAME on *M. hyorhinis*-induced apoptosis in AZ-521 cells. The cells were infected with *M. hyorhinis* treated with (a) 500, (b) 250 or (c) 100 μM of L-MAME or with (d) no L-NAME for 24 and 28 hr. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

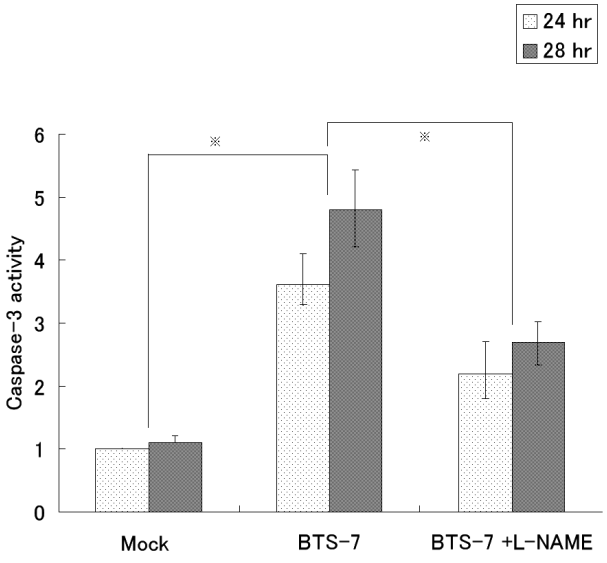

Fig. 5. Caspase-3 activity of AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post inoculation. AZ-521 cells were treated with 500 μM of L-NAME. AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post inoculation; in the non-treated AZ-521cells, the number of caspase-3 positive cells was scored as 1, and the numbers of caspase-3 positive AZ-521 cells were counted in *M. hyorhinis*-infected cells treated with 500 μM L-NAME or without. Means ± SEM for 2 separate replications are shown. Statistical significance was determined using ANOVA. * $p<0.05$.

tion, no significant NO production was evident among the different *M. hyorhinis*-infected AZ-521 cells or in the mock-infected controls. Together with the augmentation of iNOS mRNA expression, nitrite and nitrate increased in the *M. hyorhinis*-infected AZ-521 cells at 24 and 28 hr post-inoculation compared with the mock-infected controls AZ-521 cells. These results corresponded to the results of the RT-PCR analyses for iNOS expression.

Fluorescent microscopic analysis for apoptotic cell counts and caspase-3 activity detection: The AZ-521 cells were examined for apoptotic changes by using a fluorescent microscope. Addition of 100, 250 and 500 μM L-NAME to AZ-521 cells infected with *M. hyorhinis* at 24 and 28 hr post-inoculation showed that L-NAME significantly inhibited apoptosis depending on the concentrations, except for 100 μM of L-NAME at 28 hr (Fig. 4).

To examine whether caspase-3 was involved in apoptosis induced by BTS-7 infection, we examined caspase-3 activity in AZ-521 cells by using an FAM-DEVD-FMK kit (Fig. 5). *M. hyorhinis*-infected AZ-521 cells showed significant caspase-3 activity at 24 and 28 hr post-inoculation compared with the mock-infected controls. This indicated that *M. hyorhinis* has another apoptotic pathway other than its endonucleases as previously reported [23]. Furthermore, at both 24 and 28 hr after inoculation with 500 μM of L-NAME, *M. hyorhinis*-infected AZ-521 cells were significantly less caspase-3 positive than non-treated *M. hyorhinis*-infected AZ-521 cells. These results suggested that NO produced by AZ-521 cells infected with *M. hyorhinis* plays an important role in activating caspase-3 and finally promoting apoptosis.

DISCUSSION

The present study is the first to provide data showing that the BTS-7 strain of produces excessive amounts of NO and attenuates E-cadherin expression, leading to anoikis.

Two regular apoptotic pathways have previously been identified. The extrinsic pathway is triggered by receptor/ligand interaction such as TNF receptor-1/TNF-alpha [31] and is mediated by "initiator" caspase-8 to the death receptor complex [24]. This leads to activation of "executioner" caspase-3, which can further activate downstream substrates involved in apoptotic changes [27]. In the present study, we showed that caspase-3 activity increased in AZ-521 cells infected by *M. hyorhinis*, and L-NAME-treated AZ-521 cells infected by *M. hyorhinis* had decreased caspase-3 activity as compared with the mock-infected AZ-521 cells (Fig. 5). Activation of caspase-3 is a downstream event in apoptosis and may occur before apoptosis. Thus, the "true" caspase-3 activity may be higher than in this study (at 24 and 28 hr). The more NO increased, the more cellular apoptosis and caspase-3 activity increased.

Different stress situations may alter matrix proteins, and such alteration may affect cell-matrix adhesion [28]. During cell-matrix adhesion, adhesion molecules on cell membrane surfaces recognize their matrix protein receptors as an initial step of adhesion. The present study showed that *M. hyorhinis* infection decreased E-cadherin mRNA expression in AZ-521 cells (Fig. 2). Since E-cadherin is the prime mediator of intracellular adhesion [28], E-cadherin down-regulation means the collapse of cell-matrix adhesion,

occurs anoikis in dependent on caspase-3.

NO can promote or inhibit cell death [3] in various cells depending on the concentration of intracellular NO in combination with the intracellular environment and its interactions with other biological molecules such as oxygen or superoxide. Excess NO induces apoptosis in various cells [3]. Also, NO rapidly reacts with superoxide anion radicals to form peroxynitrite, which is an oxidant substance producing cytotoxic effects in many cells [29]. It is known that peroxynitrite induces apoptosis [29]. Overproduction of NO resulting from *M. hyorhinis* infection may induce a caspase cascade. The present paper presents evidence showing that the unselective NOS inhibitor L-NAME prevented apoptosis induced by *M. hyorhinis* infection (Fig. 4). This suggests that excess amounts of NO or the NO reactant peroxynitrite resulting from *M. hyorhinis* decrease E-cadherin expression (Fig. 2) and lead to anoikis in AZ-521 cells.

M. hyorhinis infection in cell cultures causes diverse biological effects. For example, it has been reported that *M. hyorhinis* infection activates nNOS expression in human neuroblastoma cell line YT-nu [9]. Therefore, it is necessary to ascertain the absence of mycoplasma in cell cultures prior to study [11].

In conclusion, we first showed that *M. hyorhinis* induces anoikis in AZ-521 cells through NO production and E-cadherin down-regulation. Although we have obtained similar results in MDBK cells infected with *M. hyorhinis* (unpublished data), our results await application to other host cell types. Since *M. hyorhinis* is very common in nasal and tracheobronchial secretions of young swine [22], a few of strains of *M. hyorhinis* may induce apoptosis *in vitro*. In fact, we have shown that there is a difference in the amount of induced iNOS mRNA expression, NO production and apoptosis in AZ-521 cells between *M. hyorhinis* strains (data not shown). The difference of virulence to piglets among *M. hyorhinis* strains may depend on the ability to produce NO and induce apoptosis *in vivo*. However, we did not elucidate the relation between pathogenecity to piglets and the difference in ability to produce NO by cells infected with *M. hyorhinis* strains. It might be necessary to confirm whether serious lesion of piglets infected with *M. hyorhinis* presents mainly apoptotic cells. The present study will lend us further understanding of the mechanism of the piglet diseases caused by *M. hyorhinis*. Our results also underline the potential application of NOS inhibitor in the treatment of *M. hyorhinis*-induced swine diseases. However, the molecular mechanism of effect of NO on swine diseases needs further exploration.

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