Original Research

Apoptosis caused by an inhibitor of NO production in the decidua of rat from mid-gestation

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Abstract

We previously reported that nitric oxide (NO) is first detected in the uterus of a pregnant rat on gestational day 13.5 (GD13.5) and that NO levels peak on GD17.5. In addition, NO production in the uterus is mainly derived from the decidua and not the myometrium. The aim of the present study was to reveal the role of NO that peaked on GD17.5 of gestation in the decidua. To inhibit NO production, pregnant rats were continuously administered by an nitric oxide synthase inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME) for 48 h. In the control group, saline was infused instead of L-NAME. After treatment, the decidua were obtained from GD13.5, GD17.5 and GD21.5 rats. Apoptosis and activated caspase-3-positive cells were observed by transferase-mediated dUTP nick-end labeling (TUNEL) assay and immunohistochemistry, respectively. The caspase-3 enzyme activity was also measured in the cell lysate from the decidua. The numbers of TUNEL-positive cells and activated caspase-3-positive cells each increased and the amount of caspase-3 activity also increased significantly in rats on GD17.5 than in rats in the control group, but no changes were observed in rats on GD13.5 and GD21.5. Furthermore, enzyme activity regarding the initiator caspases, caspase-8 and -9, upstream factors for caspase-3 in the caspase cascade, was measured simultaneously on GD17.5 under the same treatment. Caspase-8 and -9 enzyme activities increased significantly in the control group; an increment of caspase-8 activity was especially prominent. The present results indicate that an inhibitor of NO production caused apoptosis through typical apoptotic signals in the decidua on GD17.5, suggesting that an NO peak in the decidua is essential to cell survival and the maintenance of uterine formation.

Keywords: nitric oxide, decidua, apoptosis

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Introduction

In mammals, nitric oxide (NO) is produced from L-arginine and oxide through the interactions of nitric oxide synthase (NOS) with various cell types such as endothelial cells, nerve cells and macrophages.[1–3] NO has come to be accepted as an important mediator of multiple cellular functions such as smooth muscle relaxation,[4,5] cell growth promotion[6,7] and neurotransmission of the central nerve system.[8,9] The NO binds soluble guanylate cyclase in the cytsol and increases cyclic guanosine monophosphate (cGMP) levels, resulting in the second messenger in the cell.

We previously reported that a mid-gestational increase in NO production was observed in the placenta on gestational day 15.5 (GD15.5) and in the decidua on GD17.5 in rat.[10,11] It has been reported that NO plays a role in adjusting the timing of blastocyst implantation and supporting fetal growth.[12] Relaxation of the womb smooth muscle affects uterine quiescence; furthermore, an inhibition of relaxation causes premature birth in pregnant rats. Thus, it is thought that increased NO production participates in the maintenance of pregnancy by inducing a uterine smooth muscle relaxant effect during gestation.[4,13] However, it is difficult to believe that NO produced in the decidua may contribute to the relaxation of uterine smooth muscle, because NO itself is a very short-lived gaseous molecule that easily oxidizes to NO derivatives such as nitrites or nitrates.[3] Thus, the roles of NO produced in the decidua are not fully understood.

Furthermore, promotive or inhibitory actions of NO *vis-à-vis* apoptosis have been reported in pancreatic B cells and vascular endothelial cells, among others.[14–18] An inhibition of apoptosis induced by NO has also been reported through the inhibitory action of the caspase cascade in endothelial cells and hepatocytes.[17,19,20] These reports,

taken together with the results of our previous study in which NO was produced at mid-gestation, mainly in the decidua,[10] lead us to the hypotheses that NO controls apoptosis and promotes decidual proliferation.

In the present study, we investigated the possible effects of NO on apoptosis in the decidua to clarify the biological roles of NO during mid-gestation. DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL),[21,22] and activated caspase-3-positive cells were observed histologically on the sections from the NO inhibitory model through a constant infusion of the specific NOS inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME); this observation was undertaken on GD17.5 of gestation, when NO is predominantly generated in the decidua. In addition, the enzyme activities of caspase-3, which executes apoptosis and typically initiates caspase-8 and -9, were also examined in the cell lysate from the decidua.

Materials and methods

Animals and treatments

Female Crj: Wistar rats (Charles River Japan, Yokohama, Japan), which were 10–15 weeks old at the time of mating, were used in the study. They were maintained on a commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and tap water *ad libitum* and kept at a temperature of $22 \pm 3^{\circ}C$, with a relative humidity of $55 \pm 10\%$ and a constant 12L:12D schedule. Three female rats were placed with a single male rat overnight; the next morning, the presence of sperm was detected from a vaginal smear. Noon of the day on which sperm was found was designated as GD0.5, and the females were individually maintained thereafter. The NOS-specific inhibitor, L-NAME (Sigma-Aldrich, St Louis, MO, USA), was administered to pregnant rats from GD11.5, GD15.5 and GD19.5 for 48 h each, at a constant infusion of 65 μg/min (subcutaneous), using an osmotic mini-pump (2ML1; Alzet, Los Angeles, CA, USA) that had been implanted under the dorsal neck skin (L-NAME group; $n = 4$). In the control group ($n = 4$), saline was administered instead of L-NAME under the same treatment conditions. After 48 h of treatment, uteri were removed from rats on GD13.5, GD17.5 and GD21.5 under ether anesthesia. To examine them histologically, whole uteri were fixed in 10% phosphate buffer formalin, dehydrated in a graded series of ethanol, embedded in paraffin and sectioned serially at 4 μm. To evaluate the caspase enzyme activity, uterine cavities were opened and fetuses, embryonic membranes and placentas were carefully removed; the deciduae were then obtained from the uteri. These decidual samples were snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until analysis.

Electron paramagnetic resonance spectroscopy measurement for NO levels in the decidua

To confirm the inhibitory effect of NO production by L-NAME, L-NAME was administered to pregnant rats from GD15.5 for 48 h, at a constant infusion of 30 or 65 μg/min (subcutaneous). In the control group, saline was administered instead of L-NAME under the same conditions described above. NO levels were monitored in the decidua by spin trapping, followed by electron paramagnetic resonance (EPR) analysis as reported previously.[23] Briefly, for spin trapping of NO, Fe-*N*-(dithiocarboxy) sarcosine (Fe-DTCS, Dojin, Kumamoto, Japan) complex was injected subcutaneously at a dose of 500 mg/kg-body wt for 0.5 h before sampling. Rats were decapitated under ether anesthesia. Blood was taken rapidly from the abdominal vein, after which, only the decidua was removed and minced with surgical scissors. Each sample was transferred to a quartz EPR tube and frozen immediately in liquid nitrogen.

EPR spectra were recorded with an EPR spectrometer (JES-TE3X; JEOL, Tokyo, Japan) under the following conditions: microwave power, 10 mW; center field and width, 320 mT; temperature, $-196^{\circ}C$; measurement time, 4 min; and time constant, 0.3 s. In some experiments, MnO powder was used as a standard and the heights of the NO-Fe-DTCS and MnO signals were measured simultaneously. The ratio of these signal heights was used to quantify NO production levels, as it has been demonstrated that the signal height of the NO-Fe-dithiocarbamate complex can be used as an index for NO formation.[24] All the procedures were performed under the guidance of the Committee for Animal Experimentation at Azabu University.

TUNEL assay

Fragmented DNA was observed on the tissue sections using the TUNEL technique.[21,22] After deparaffinization, the sections were stained with a Vaso TACS *in situ* apoptosis detection kit (R&D Systems Inc, Minneapolis, MN, USA), according to the manufacturer's instructions.

Immunohistochemical detection of activated caspase-3

Immunohistochemical analysis was performed on deparaffinized sections using monoclonal antibody against activated caspase-3 (1:200; Cell Signaling Technology, Danvers, MA, USA) with peroxidase-conjugated goat anti-rabbit IgG antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and a peroxidase stain DAB kit (Nakarai Tesque, Inc, Kyoto, Japan).[25] Sections were then counterstained with Mayer's hematoxylin.

Caspase activity assay

Enzyme activities of caspase-3, -8 and -9 were determined using appropriate caspase colorimetric activity assay kits (Chemicon Co. Ltd, Temecula, CA, USA) according to the manufacturer's instructions. Individual deciduae were resuspended in 1 mL of cell lysis buffer and homogenized on ice. After incubation on ice for 10 min, cell debris was removed by centrifugation at 10,000*g* for 5 min, and protein concentrations in the supernatant were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (10 μg per reaction mixture) were added to the assay buffer and applied to colorimetric

assays of caspase-3, -8 and -9 activities. Caspase activity was detected using a spectrophotometer (405 nm) on a microtiter plate reader.[26] Each enzyme activity is shown as a quantity of substance per minute that had been degraded using 1 μg of protein extracted from the tissue. Caspase-3 activity in the decidua was measured on GD13.5, GD17.5 and GD21.5, while those of caspase-8 and -9 were measured on GD17.5.

Statistical analysis

Data are expressed as means $\pm$ SEM. Differences among groups of rats were assessed by analysis of variance or Student's *t*-test. If significant differences among the groups were demonstrated, Scheffe's test was used to determine which groups were different. P values <0.05 were considered to be statistically significant.

Results

Inhibitory effect of L-NAME infusion on the NO levels in the decidua

The EPR spectra of the standard samples of the NO-Fe-DTCS complex in fresh blood showed a typical triplet signal ($g = 2.038$) that was previously identified as NO-Fe-DTCS by Yoshimura *et al.*[24] (Figure 1A). The EPR spectrum with Fe-DTCS trapping in the decidua of rats pretreated with L-NAME (for 24 h at a constant infusion of 65 μg/min) was identified as the $g\perp$ signal of the Cu-dithiocarbamate complex[27] (Figure 1C); the EPR spectrum showing NO-Fe-DTCS was almost diminished. The EPR spectrum in the decidua of rats from GD15.5 was identified as the signal of the NO-Fe-DTCS superimposed on the $g\perp$ signal of the Cu-dithiocarbamate complex[27] (Figure 1B). The NO-Fe-DTCS and MnO signal heights were measured simultaneously, and the ratio of these heights was used to quantify NO levels. NO levels in the decidua of rats pretreated with L-NAME (30, 65 μg/min) from GD15.5 were significantly lower than in the saline group, and the NO levels decreased in a dose-dependent manner of L-NAME (Figure 1D). In addition, the difference did not occur in NO production of the whole uterus, which included the decidua after infusion of L-NAME for 24 or 48 h (data not shown). Therefore, a constant infusion rate of 65 μg/min for 48 h is sufficient to inhibit NO production by L-NAME in the decidua. The following experiment assumed an appropriate dosing regimen.

Effect of NO on apoptosis in decidua, revealed by TUNEL assay

NO production was inhibited by a constant infusion of a specific NOS inhibitor, L-NAME (65 μg/min for 48 h) to pregnant rats from GD11.5, GD15.5 or GD19.5 for 48 h. Generally, when apoptosis is induced in a cell, the genomic DNA of the cell is fragmented into nucleosome unit segments (about 180 bp each) by caspase-activated DNase (CAD) or DNase γ.[28,29] Therefore, when apoptosis is induced in the decidua, a TUNEL assay makes it possible

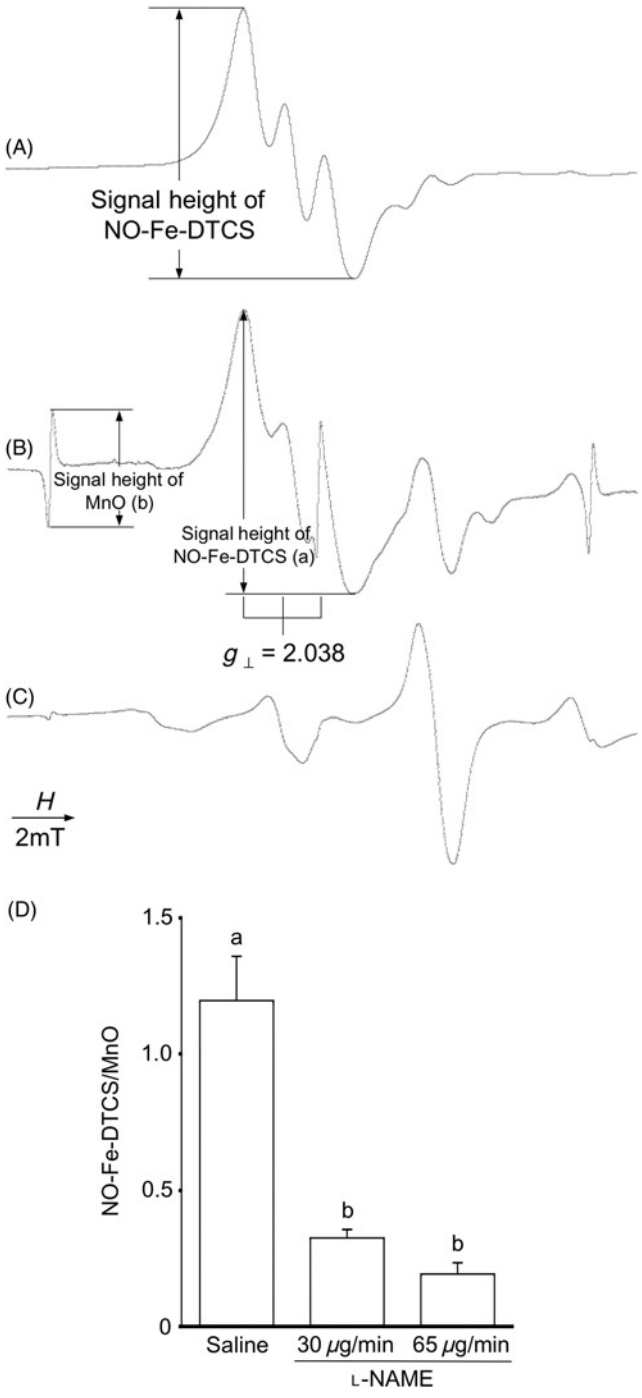

Figure 1 Typical EPR spectra of the NO-Fe-DTCS complex and NO levels after L-NAME treatment. (A) EPR spectrum of a standard sample of the NO-Fe-DTCS complex in fresh blood. (B) EPR spectrum of the NO-Fe-DTCS complex detected in the decidua on GD15.5. The heights of the NO-Fe-DTCS (a) and MnO (b) signals were measured simultaneously. The ratio of the signal heights (a/b) was used to quantify the levels of NO production. (C) EPR spectrum with Fe-DTCS trapping of the decidua after administration of L-NAME (for 24 h at a constant infusion of 65 μg/min) on GD15.5. (D) NO levels in the decidua after constant infusion of L-NAME. NO levels were quantified by the spin trapping followed by EPR analysis in the decidua after administration of L-NAME for 24 h at a constant infusion of 30, 65 μg/min from GD15.5. Data are expressed as the means $\pm$ SEM of five individual experiments. Bars with different letters at the top differ significantly ($P < 0.05$). EPR, electron paramagnetic resonance; NO, nitric oxide; L-NAME, N^G-nitro-L-arginine-methyl ester; GD, gestational day; Fe-DTCS, Fe-*N*-(dithiocarboxy)sarcosine

for the terminal deoxynucleotidyl transferase to label 3′-OH terminals of fragmented DNA with biotin-labeled dUTP on a section of the decidua. On GD13.5, TUNEL-positive cells were conspicuously detected in the L-NAME-treated and control (saline) groups; there was no significant difference between the groups (Figures 2A and B). In contrast, on GD17.5, few TUNEL-positive cells were detected in the control group (Figure 2C); meanwhile, positive cells in the L-NAME-treated group on GD17.5 were detected as similar to on GD13.5 (Figure 2D) from the NO inhibitory group. However, positive cells in the decidua were not detected at all on GD21.5 in either group (Figures 2E and F).

Immunohistochemical observation of activated caspase-3 in the decidua

Recently, apoptosis lacking DNA fragmentation has been reported,[30] but it is rash to conclude that apoptosis can be decided solely by the TUNEL method. The apoptosis induction signal is transmitted inside the cell via a cascade reaction consisting of a group of cysteine proteases classified in the caspase family. Effector caspases such as activated caspase-3 are involved in activating CAD, by inactivating inhibitors of CAD and DNA fragmentation factor 45; effector caspases also function as apoptosis execution factors in the most downstream region of the cascade.[31] Activated caspase-3 expression was detected by immunohistochemical analysis in the decidua of rats on GD13.5, GD17.5 and GD21.5. There were few differences between the distributions of the TUNEL-positive cells and activated caspase-3-positive cells in the decidua. On GD13.5, regardless of the presence or absence of L-NAME administration, activated caspase-3-positive cells were easily detected in the decidua (Figures 3A and B). On GD17.5, activated caspase-3-positive cells were conspicuous only in the L-NAME-treated group (Figures 3C and D). Activated caspase-3-positive cells were observed in the perivascular areas of both groups on GD17.5; in addition, the activated caspase-3-positive cells were also observed in the stroma of the L-NAME-treated group. The distribution patterns of these activated caspase-3-positive cells were in good agreement with those of the TUNEL-positive cells. On GD21.5, few activated caspase-3-positive cells were detected in either group, as per the results of TUNEL assays (Figures 3E and F).

Effect of NO on the enzyme activities of caspase-3, -8 and -9

The enzyme activity of caspase-3, one of the final exacting caspase family cysteine proteases, is located in the most

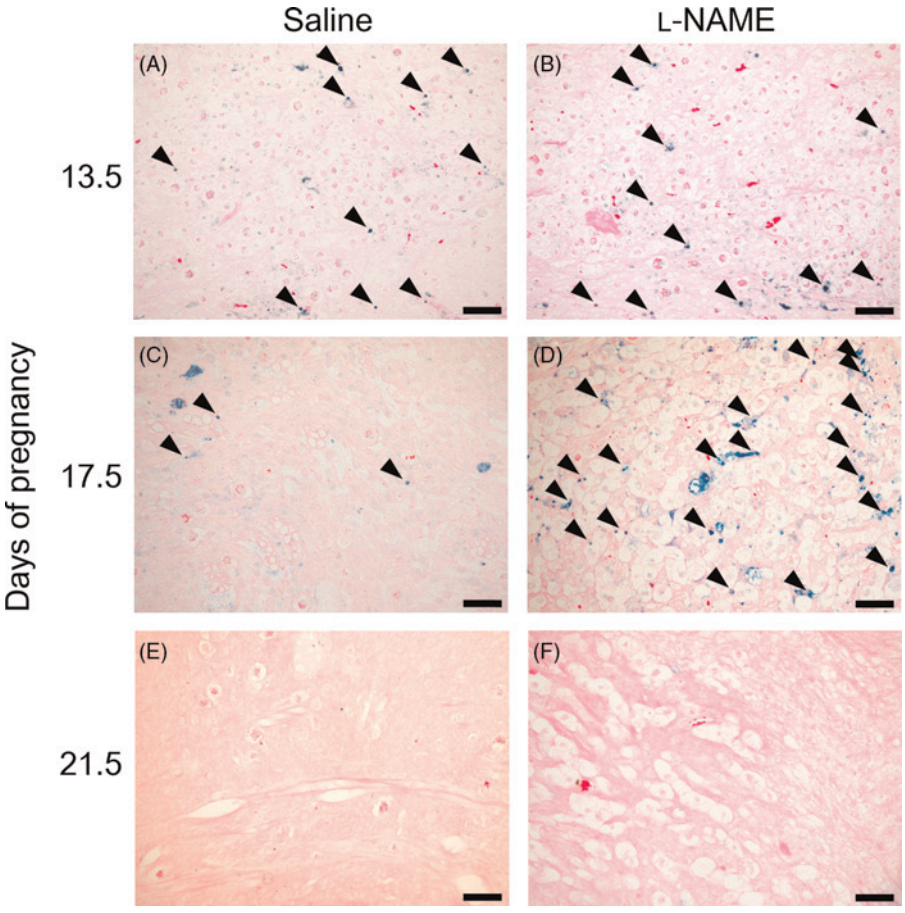

Figure 2 Detection of apoptosis by TUNEL assay in the decidua. Apoptosis cells in the decidua on GD13.5 (A, B), GD17.5 (C, D) and GD21.5 (E, F) were detected by TUNEL assay in the presence (B, D, F) or absence (A, C, E) of the NOS inhibitor L-NAME. Arrowheads indicate TUNEL-positive cells that included fragmented genomic DNA and were dyed dark blue. Scale bar, 100 μm. L-NAME, N^G-nitro-L-arginine-methyl ester; GD, gestational day; TUNEL, transferase-mediated dUTP nick-end labeling; NOS, nitric oxide synthase

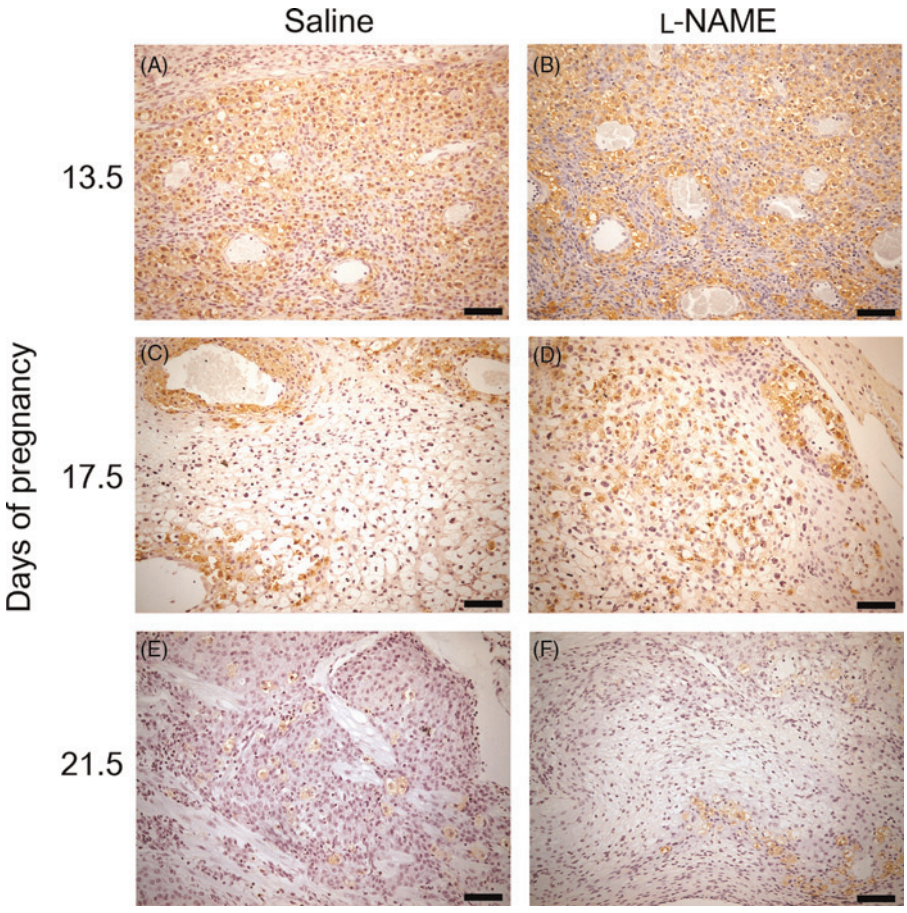

Figure 3 Immunohistochemical detection of caspase-3 in the decidua. Existence of activated caspase-3 in the decidual cells on GD13.5 (A, B), GD17.5 (C, D) and GD21.5 (E, F) were detected by immunohistochemical analysis, using monoclonal antibody against activated caspase-3 in the presence (B, D, F) or absence (A, C, E) of the NOS inhibitor L-NAME. Activated caspase-3-positive cells were dyed by bistre. Scale bar, 100 μm. L-NAME, N^G-nitro-L-arginine-methyl ester; GD, gestational day; NOS, nitric oxide synthase

downstream region of the apoptosis signal transduction pathway. This activity was quantitatively determined in the cell lysate of the decidua from rats on GD13.5, GD17.5 and GD21.5 (Figure 4A). At three analyzing points, the enzyme activities of caspase-3 in the L-NAME-treated and control groups showed a declining trend until the end of the gestation period. In addition, on GD17.5, the caspase-3 enzyme activity increased more significantly in the L-NAME-treated group (85.49 ± 5.3 pmol/min/μg protein) than in the control group (68.06 ± 8.78 pmol/min/μg protein, $n = 4$; $P < 0.05$). Meanwhile, there were no significant differences between the other two groups, on GD13.5 or GD21.5 (Figure 4A).

In addition, the enzyme activity of caspase-8 or -9, either of which initiates caspase-3, was determined on GD17.5, when caspase-3 enzyme activity significantly increased by administering L-NAME (Figure 4B). On GD17.5, the caspase-8 enzyme activity of the L-NAME-treated group (55.00 ± 8.94 pmol/min/μg protein) significantly increased (1.7-fold) as compared with that of the control group (33.35 ± 7.85 pmol/min/μg protein; $P < 0.01$). Similarly, the caspase-9 enzyme activity of the L-NAME-treated group (71.10 ± 6.86 pmol/min/μg protein) significantly increased (1.2-fold) as compared with that of the control group (58.03 ± 5.75 pmol/min/μg protein; $P < 0.05$).

Discussion

The present study revealed that the inhibition of NO causes the following effects: (i) increases the number of apoptotic cells and activated caspase-3-positive cells; (ii) increases the enzyme activities of caspase-3, -8 and -9; and (iii) induces more prominent increments in caspase-8 enzyme activity in the decidua. These effects were prominent on GD17.5, when NO production peaked, as previously reported.[10] On the basis of the findings of the present study, together with the facts that the cellular density in the decidua decreases in NOS 2 knockout mice[12] and that NOS 2 was the main producer of NO production in the mid-gestational decidua in rat,[10] it can be asserted that NO may be indispensable for cell proliferation or decidua maintenance, in line with fetal growth.[12] In the present study, DNA fragmentation and an increment of activated caspase-3-positive cells appeared after a constant infusion of L-NAME in the decidua on GD17.5. In addition, the activities of initiator caspase-8 and -9, each of which

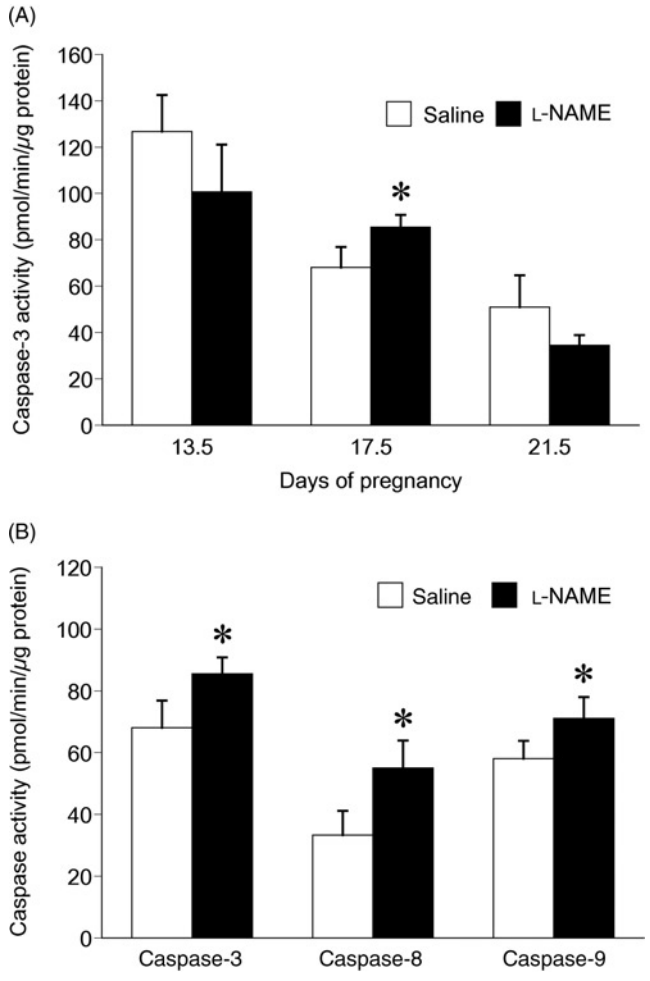

Figure 4 Changes to caspase activity in the mid-gestational decidual cell lysate. (A) Caspase-3 activity in the decidua, from GD13.5 to GD21.5. (B) Caspase-3, -8 and -9 activities in the decidua on GD17.5. Each enzyme activity is shown as quantity of substance per minute that was degraded by using 1 μg of protein extracted from the tissue. Data are expressed as means $\pm$ SEM of four individual experiments. Data that differ significantly ($P < 0.05$) from the saline group are indicated by asterisks at the tops of the bars. L-NAME, N^G-nitro-L-arginine-methyl ester; GD, gestational day

upregulate an activated caspase-3, increased significantly in the decidua. However, on comparing both increments of enzyme activity, caspase-8 was found to be 1.7-fold higher than the control group, whereas caspase-9 was 1.2-fold higher than the control.

Caspase-8 amplifies apoptosis signals to activate caspase-9 via the BH3 interacting domain (Bid), which accelerates the mitochondrial release of cytochrome *c*.[32,33] It is suggested that high levels of caspase-8 activity induce apoptosis via a death receptor; in the present study, caspase-9 activation amplified the apoptosis signal via Bid. Although Bid activity was not examined in the present study, it is presumed that Bid can be activated and accelerated by a mitochondrial release of cytochrome *c*, because caspase cascades that induce apoptosis are well-conserved among many organism species.[34–36] NO has been shown to promote apoptosis in some cells and inhibit apoptosis in others. In pancreatic B cells, for example, it has been reported that NO triggers the endoplasmic reticulum stress pathway and accelerates apoptosis.[15,16]

Meanwhile, in a cerebral embolism rat model, NO produced by NOS 3 activation in the capillary endothelial cells of the brain inhibits apoptosis in the nerve cells through the tyrosine nitration of caspase-3.[18] Upregulation of some proteins that have cytoprotective functions, such as heat shock protein 70,[37] hemoxygenase[38] and Bcl-XL,[39] is involved in the antiapoptotic effect of NO. These complex relationships between NO and apoptosis are influenced by the amount of NO production involved, or the interaction between NO and the metal ions, thiol and tyrosine of proteins or reactive oxygen species.[40]

Tumor necrosis factor (TNF)-α, produced by natural killer (NK) cells, plays an important role in mid-gestational placental formation in the decidua.[41] Additionally, NO production in NK cells was accelerated to induce the expression of NOS 2 in the uteri of normal mice.[41] In mice in which NK cells had been eradicated by the antibody to Ly-49G2 (a surface maker of the NK cell), miscarriages on GD12–GD14 have occurred, following placental growth insufficiency, thickening of a vascular wall and clot formation in the decidua.[42] In rat, the expression of TNF-α in uterine and placental cells was reported.[43] TNF-α induces NO production in bovine endometrial stromal cells, and NO stimulates production of prostaglandin E2 and F2α.[44] When pregnant rats were continuously administered L-NAME (50 mg/day) from GD8 to GD14, the blood TNF-α level increased six-fold over the control group on GD21.[45] These several lines of evidence, together with the findings of the present study, suggest that NO contributes to the maintenance of uterine function just enough to inhibit excessive apoptosis, and that it contributes to cell proliferation in the decidua. These findings also suggest that NO produced mid-gestationally in the decidua was locally regulated by the autocrine or paracrine actions of proinflammatory cytokines, such as TNF-α.

Activated caspase-3-positive cells in the decidua are located around blood vessels, as shown in Figure 3. TNF-α, which increased in the blood to inhibit NOS in an earlier report,[45] affects the cells that regulate blood-vessel circumference. Meanwhile, whether the effect is induced directly or indirectly via the change of blood flow, it is known that inducible nitric oxide synthetase (NOS 2) is induced by TNF-α. Additionally, NO that is produced in the decidua is derived from NOS 2.[10] Thus, it was suggested that NO production in the decidua from mid-gestation of pregnancy is locally regulated by the autocrine or paracrine effect of proinflammatory cytokines such as TNF-α. When NO is closely regulated to appropriate production levels at each stage of pregnancy by various adjustment factors such as TNF-α, NO inhibits excessive apoptosis in the decidua. This reaction would, in turn, contribute just enough to maintain placental function as well as cell proliferation.[12]

In summary, we examined the effect of NO on apoptosis in mid-gestational decidua in rat. On the basis of our previous data, NO production peaked on GD17.5. An inhibition of NO production increased the numbers of TUNEL-positive cells and activated caspase-3-positive cells, increased the enzyme activities of caspase-3, -8 and -9, and induced more prominent increments in caspase-8

enzyme activity in the decidua on GD17.5. The present results indicate that an inhibitor of NO production caused apoptosis through typical apoptotic signals in the decidua on GD17.5, suggesting that the NO peak in the decidua is essential to cell survival and the maintenance of uterine formation.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; CN, TK, RM and TS conducted the experiments, and TS, KT, HM, MS and TT wrote the manuscript.

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