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Original Article

Inducible Nitric Oxide Synthase Mediates the Triglyceride-induced Death of THP-1 Monocytes

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Triglyceride (TG) accumulation can cause monocytic death and suppress innate immunity. However, the signaling pathways involved in this phenomenon are not fully understood. This study aimed to examine whether inducible nitric oxide synthase (iNOS) is involved in the TG-induced death of THP-1 monocytes. Results showed that iNOS was upregulated in TG-treated THP-1 monocytes, and iNOS inhibition blocked TG-induced monocytic death. In addition, TG-induced poly (ADP-ribose) polymerase (PARP) cleavage and caspase-3 and -7 activation were suppressed by iNOS inhibition. Furthermore, the expression of X-linked inhibitor of apoptosis protein (XIAP) and survivin, which inhibit caspase-3 and -7, was reduced in TG-treated THP-1 monocytes, but iNOS inhibition recovered the TG-induced downregulation of XIAP and survivin expression. Considering that TG-induced monocytic death is triggered by caspase-2 and -8, we investigated whether caspase-2 and -8 are linked to the TG-induced expression of iNOS in THP-1 monocytes. When the activities of caspase-2 and -8 were inhibited by specific inhibitors, the TG-induced upregulation of iNOS and downregulation of XIAP and survivin were restored in THP-1 monocytes. These results suggest that TG-induced monocytic death is mediated by the caspase-2/caspase-8/iNOS/XIAP and survivin/executioner caspase/PARP pathways.

Key Words: Inducible nitric oxide synthase, Triglyceride, Monocytic death, X-linked inhibitor of apoptosis protein, Survivin

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INTRODUCTION

Monocytes are circulating leukocytes that are key components of the innate immune system (Chiu and Bharat, 2016). They are the first line of defense against infections by recognizing foreign molecules (Serbina et al., 2008). Upon recognizing pathogens, monocytes engulf them, become activated, and produce chemokines and proinflammatory cytokines that recruit immune cells to the infection site (Narni-Mancinelli et al., 2011). Hyperlipidemia induces immunosuppression possibly by increasing the amount of cholesterol in the cytoplasmic membrane of immune cells and inhibiting innate immunity (Emruzi et al., 2018). This condition causes immune cell death, which contributes to the impairment of innate immune function. Treatment with postprandial triglyceride (TG)-rich lipoproteins results in apoptosis and S-phase cell cycle arrest in human THP-1 monocytes *in vitro* (Lopez et al., 2007). Similarly, circulating monocytes can engulf excess lipids, which induces not only their activation but also monocytic death in case of hyperlipidemia (Saja et al., 2015). However, the signaling mole cules contributing to monocytic death induced by TG have yet to be identified.

Nitric oxide (NO) contributes to immune reactions by activating immune cells at low concentrations and directly killing target pathogens at high concentrations (Schairer et al., 2012). The expression of inducible nitric oxide synthase (iNOS), a key enzyme in NO generation, is increased in circulating monocytes from patients with immune-related diseases, such as active inflammatory bowel disease (Dijkstra et al., 2002), multiple sclerosis (Lopez-Moratalla et al., 1997), and Graves' disease (Lopez-Moratalla et al., 1996). Moreover, exogenous or endogenous iNOS-derived NO elicits apoptosis in both *in vitro* and *ex vivo* monocyte lines (Taylor et al., 2003). NO mediates monocytic apoptosis by downregulating the expression of cellular inhibitor of apoptosis 1 (cIAP1) and X-chromosome-linked inhibitor of apoptosis (XIAP), which inhibit caspases-3, -7, and -9 (Manderscheid et al., 2001). High concentrations of NO suppress survivin, a member of the inhibitor of apoptosis protein family, in rat primary hepatocytes (Wang et al., 2011), and LPS-mediated iNOS induction promotes BAX/BAK-driven mitochondrial apoptosis in IFNγ-treated macrophages (Simpson et al., 2022).

In the present study, we aimed to elucidate the molecular mechanisms underlying TG-induced monocytic death. We observed that TG-mediated cell death is mediated by iNOS in THP-1 cells. We also revealed that TG-induced iNOS expression is mediated by caspase-2 and -8. We suggest a signaling pathway through which TG mediates monocytic death. This study may serve as a reference to using iNOS inhibitors as a potential therapeutic strategy to prevent TG-induced monocytopenia.

MATERIALS AND METHODS

Materials

Lipofundin® MCT/LCT 20% obtained from B. Braun Melsungen AG (Melsungen, Germany) was used to deliver TG into the cells following a previously described method (Aronis et al., 2005). iNOS inhibitor 1,400 W was obtained from Sigma-Aldrich (St. Louis, MO, USA). Caspase-3 and -7 substrate Ac-DEVD-pNA was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies against cleaved caspase-3, cleaved caspase-7, poly (ADP-ribose) polymerase (PARP), XIAP, and survivin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin, iNOS, and z-VDVAD-fmk (caspase-2-specific inhibitor) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase-8-specific inhibitor z-IETD-fmk was obtained from R&D Systems (Minneapolis, MN, USA).

Cell culture

The human THP-1 acute monocytic leukemia cell line (No. TIB-202™) was purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum, streptomycin (50 μg/mL), and penicillin (50 U/mL) at 37℃ in a humidified atmosphere with 5% CO_2.

Trypan blue dye exclusion assay

Trypan blue stain solution (10 μL) was mixed with the

cell suspension (10 μL) to enumerate viable cells, and unstained viable cells were counted with a hemocytometer (Marienfeld, Lauda-Königshofen, Germany). Each experiment was performed at least three times, and the results are expressed as the mean ± SEM for each group.

Measurement of caspase activity

Caspase-3 and -7 activities were determined as previously described (Imre et al., 2012). Briefly, THP-1 cells were lysed with phosphate buffered saline (PBS) containing 1% Triton X-100 and then centrifuged at 19,000 × *g* for 10 min at 4℃. The supernatant was collected, and the total protein concentration was quantified. To detect caspase-3 and -7 activities, we mixed 90 μg of protein with Ac-DEVD-pNA in PBS. The reactions were incubated for 3 h at 37℃, and the optical density at 405 nm was measured using a Nano-Quant Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Western blotting

THP-1 cells were washed with PBS and lysed at 4℃ in lysis buffer containing 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Roche, Mannheim, Germany), and PBS. Lysates were clarified, and the supernatants were subjected to western blotting, as described previously (Jo et al., 2016).

RNA extraction and semi-quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from THP-1 macrophages using TRIzol® reagent (Invitrogen, CA, USA) in accordance with the manufacturer's instructions. cDNA was generated by reverse transcription from 2 μg of total RNA, 0.25 μg of random hexamer (Invitrogen), and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 10 min at 25℃, 50 min at 37℃, and 15 min at 70℃. cDNA amplification was performed with Prime Taq premix PCR kit (Genet Bio, Chungnam, Korea) of 0.2 U in a thermocycler using specific primers. Primer sequences were as follows: iNOS, 5'-ACA AGC TGG CCT CGC TCT GGA AAG A-3' (forward), 5'-TCC ATG CAG ACA ACC TTG GGG TTG AAG-3' (reverse); GAPDH, 5'-CGG GAA GCT TGT CAT CAA TGG-3' (forward), and 5'-GGC AGT GAT GGC ATG GAC TG-3' (reverse). GAPDH was used as an internal control. PCR products were electrophoresed on 2% (w/v) agarose gels containing 0.5 μg/mL ethidium bromide, and the product size was determined by comparison to a 100 bp DNA ladder marker (Intron, Gyeonggi, Korea). Gel images were obtained using the Gel Doc™ XR+ system (Bio-Rad, Hercules, CA, USA). The PCR product band intensity was measured and normalized against GAPDH using Image Lab™ software (version 4.1, Bio-Rad).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The *P*-values were calculated using Student's *t*-test. Data are presented as mean ± standard error of the mean (SEM). Each experiment was conducted three times, and data were pooled for analysis. The differences were considered statistically significant at $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

RESULTS

iNOS is involved in TG-induced THP-1 monocytic death

High TG concentrations in the serum are correlated with high iNOS expression and TG levels are related to NO production, which can mediate cell death (Jaiswal et al., 2000; Oleson et al., 2014; Baydoun et al., 2015). Based on these reports, we first investigated whether iNOS is involved in TG-induced cell death in THP-1 monocytes. THP-1 monocytes were treated with the indicated concentrations of TG (0, 0.1, 0.2, 0.5, or 1.0 mg/mL) for 24 h or with TG (1.0 mg /mL) for the indicated times (0, 0.5, 3, 6, 12, or 24 h), and iNOS levels were examined using RT-PCR and western blotting. In TG-treated monocytes, iNOS mRNA and protein levels were upregulated in a time- and dose-dependent manner (Fig. 1A, B). To determine whether the increased expression of iNOS is responsible for monocytic death, we treated THP-1 monocytes with TG in the presence or absence of iNOS-specific inhibitor 1,400 W for 24 h and then assessed cell viability. TG-induced monocytic death was restored by iNOS inhibition in a dose-dependent manner (Fig. 1C). These results demonstrate that iNOS is associated

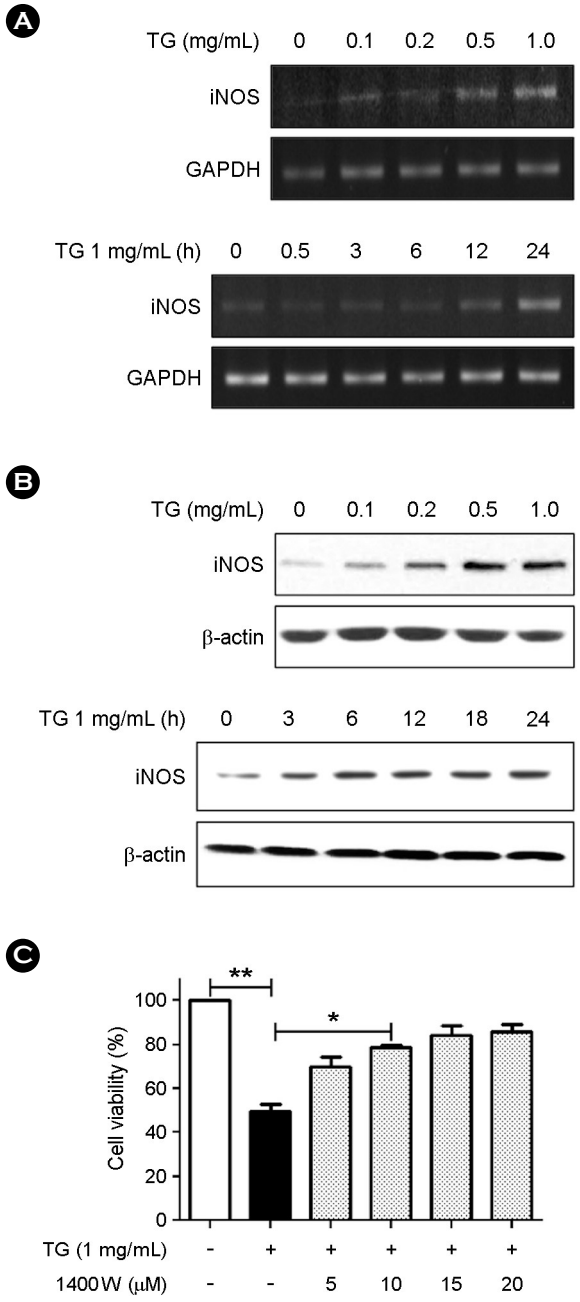

Fig. 1. Involvement of iNOS in TG-induced monocytic death. THP-1 monocytes were treated with the indicated concentrations of TG (0, 0.1, 0.2, 0.5, or 1.0 mg/mL) for 24 h or with 1.0 mg/mL TG for the indicated times (0, 0.5, 3, 6, 12, or 24 h). **(A)** The mRNA level of iNOS was analyzed using RT-PCR. GAPDH was used as an internal control. **(B)** Western blotting was performed with an anti-iNOS antibody for assaying the protein level of iNOS. β-actin was used as an internal control. **(C)** THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the iNOS inhibitor 1,400 W (0, 5, 10, 15, or 20 μM) for 24 h, and the trypan blue exclusion assay was performed for enumerating viable cells. The number of viable cells in THP-1 monocytes without TG treatment was set as 100%. All data are expressed as the mean ± SEM of three independent experiments. *P*-values were determined with Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

with the death of TG-treated THP-1 monocytes.

iNOS mediates cell death via activation of caspase-3 and -7 and cleavage of PARP in TG-treated THP-1 monocytes

TG induces apoptotic cell death by activating executioner caspases, including caspase-3 and -7 (Jung et al., 2023). Activated executioner caspases participate in cell death through the direct cleavage of PARP, which is a specific marker for apoptotic cell death (Puig et al., 2001). Therefore, we examined whether iNOS affects the activities of executioner caspases and the cleavage of PARP in TG-treated THP-1 monocytes. When THP-1 monocytes were treated with TG in the absence or presence of 1,400 W (0, 2.5, 5, or 10 μM) for 24 h, the TG-induced cleavage of PARP was reduced in THP-1 monocytes (Fig. 2A). We also found that iNOS inhibition suppressed TG-induced caspase-3 and -7 activation in THP-1 monocytes (Fig. 2B). These results indicate that iNOS leads to cell death in TG-treated THP-1 monocytes by activating executioner caspases, which consequently cleave PAPR.

iNOS downregulates XIAP and survivin in TG-treated THP-1 monocytes

XIAP and survivin block the activities of executioner caspases, such as caspase-3 and -7 (Obexer and Ausserlechner, 2014; Jaiswal et al., 2015; Qin et al., 2015). XIAP and survivin are involved in cell survival, and the expression of these proteins is regulated by iNOS (Razavi et al., 2005; Engels et al., 2008). Therefore, we investigated whether XIAP and survivin are associated with TG-induced, iNOS-mediated monocytic death. As shown in Fig. 3A, XIAP and survivin levels decreased in the TG-treated THP-1 monocytes in a time- and dose-dependent manner. Moreover, the TG-induced downregulation of XIAP and survivin was significantly recovered by the presence of 1,400 W in a dose-dependent manner (Fig. 3B). These results suggest that iNOS downregulates XIAP and survivin, which consequently lead to the activation of caspase-3 and -7, resulting in caspase-dependent cell death in TG-treated THP-1 monocytes.

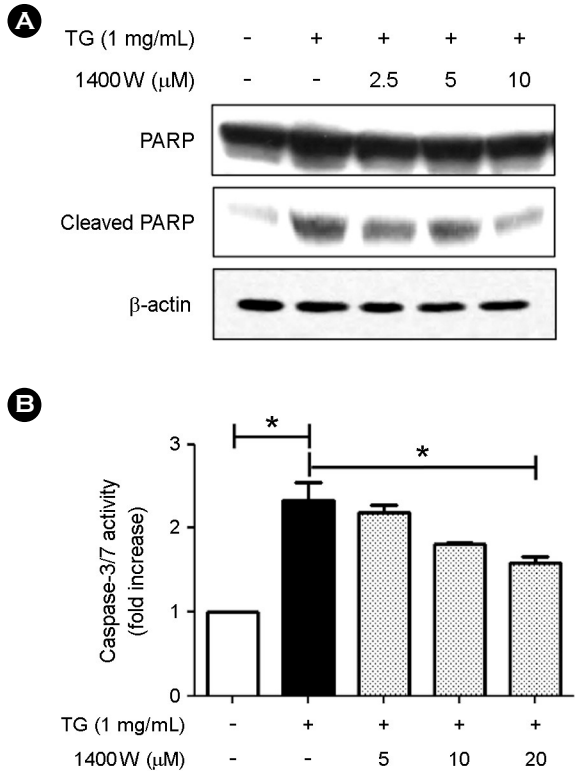

Fig. 2. iNOS-mediated activation of caspase-3 and -7 and cleavage of PARP in TG-induced monocytic death. (A) THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the iNOS inhibitor 1,400 W (0, 2.5, 5, or 10 μM) for 24 h. The cleaved form of PARP was detected using western blotting. **(B)** THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the iNOS inhibitor 1,400 W (0, 5, 10, or 20 μM) for 24 h, and caspase-3 and -7 activities were measured. All data are expressed as the mean ± SEM of three independent experiments. *P*-values were determined with Student's *t*-test. **P* < 0.05.

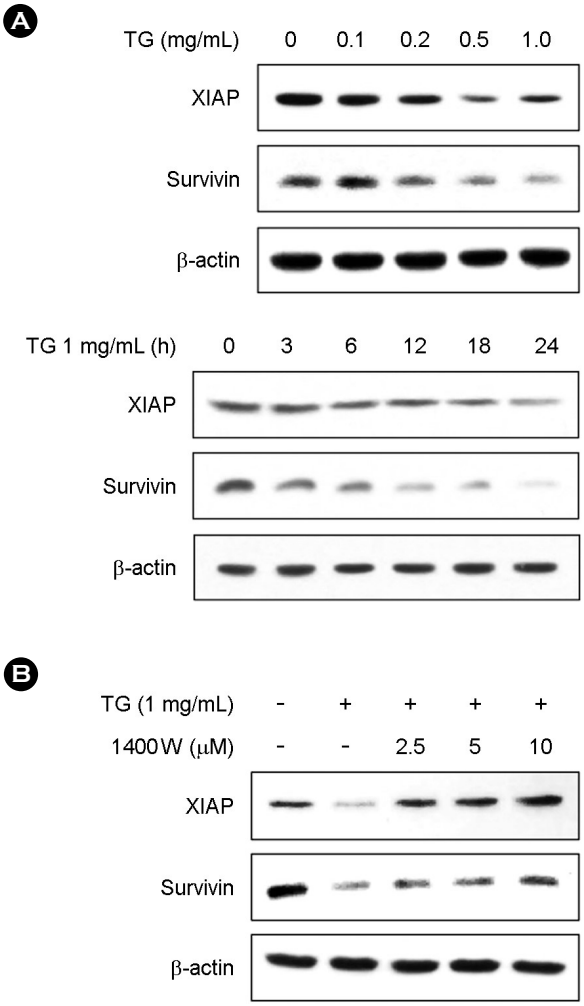

Fig. 3. iNOS-mediated downregulation of XIAP and survivin in TG-induced monocytic death. (A) THP-1 monocytes were treated with the indicated concentrations of TG (0, 0.1, 0.2, 0.5, or 1.0 mg/mL) for 24 h or with TG (1.0 mg/mL) for the indicated times (0, 0.5, 3, 6, 12, or 24 h). XIAP and survivin were detected using western blotting. **(B)** THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the iNOS inhibitor 1,400 W (0, 2.5, 5, or 10 μM) for 24 h. Western blotting was performed with anti-XIAP and anti-survivin antibodies for assaying the protein levels of XIAP and survivin.

Caspase-2 and -8 act as upstream molecules of iNOS in TG-induced THP-1 monocytic death

We previously reported that TG activates caspase-2 and -8 to induce THP-1 monocytic death (Jung et al., 2023). Other studies have shown that caspase-2 and -8 are associated with iNOS expression and NO production in several cell types (Burguillos et al., 2011; Fauconnier et al., 2011; Ivey et al., 2014). Therefore, we examined the association between iNOS and caspase-2 and -8 during TG-induced THP-1 monocytic death. To examine whether caspase-2 contributes to iNOS expression in TG-treated THP-1 monocytes, we incubated THP-1 monocytes with TG in the absence or presence of the caspase-2 inhibitor z-VDVAD-fmk for 24 h and then assayed iNOS through western blot. As shown in Fig. 4A, the TG-induced upregulation of iNOS was inhibited by treatment with caspase-2 specific inhibitors in a dose-dependent manner. In addition, caspase-2 inhibition recovered the TG-induced downregulation of XIAP and survivin (Fig. 4B). Likewise, caspase-8 inhibition by z-IETD-fmk treatment restored the TG-induced upregulation of iNOS and TG-induced downregulation of XIAP and

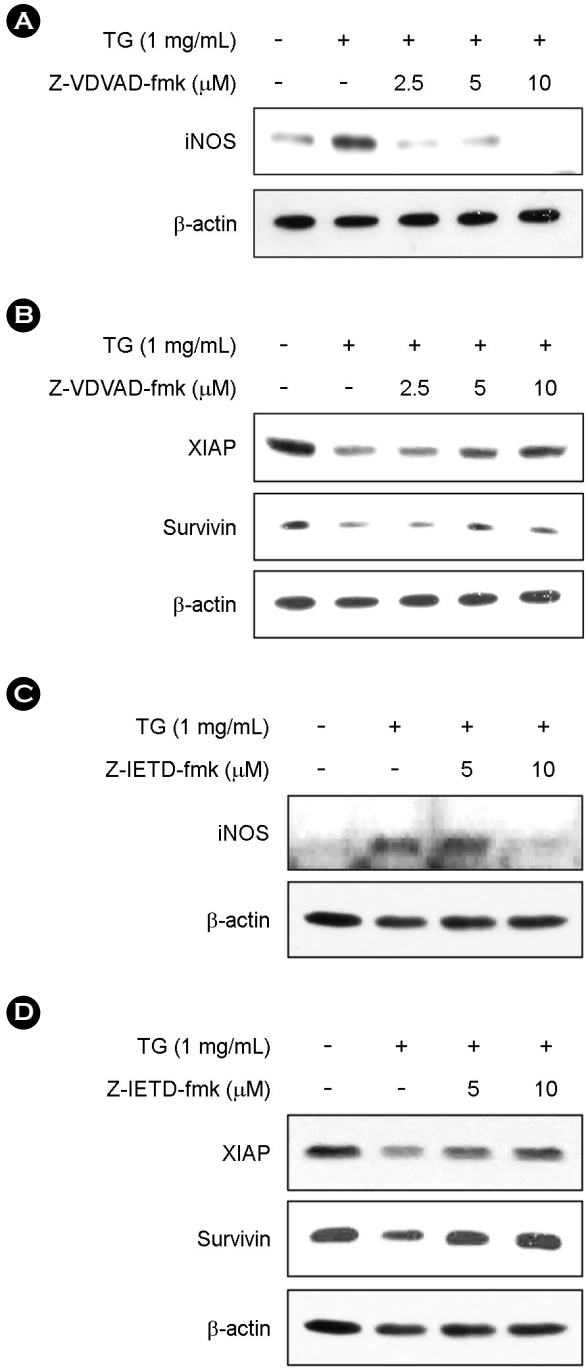

Fig. 4. Regulation of iNOS by caspase-2 and -8 in TG-induced monocytic cell death. THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the caspase-2 inhibitor z-VDVAD-fmk (0, 2.5, 5, or 10 μM) for 24 h, and iNOS **(A)**, XIAP, and survivin **(B)** levels were detected using western blotting. THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the caspase-8 inhibitor z-IETD-fmk (0, 5, or 10 μM) for 24 h, and iNOS **(C)**, XIAP, and survivin **(D)** levels were detected using western blotting.

survivin in TG-stimulated THP-1 monocytes (Fig. 4C, D). These results suggest that caspase-2 and -8 regulate iNOS expression, which subsequently downregulates XIAP and survivin, thereby contributing to TG-induced THP-1 monocytic death.

DISCUSSION

Hyperlipidemia, characterized by increased serum cholesterol and TG, exerts detrimental effects on innate immunity (Lei et al., 2013; Emruzi et al., 2018). We previously reported that TG triggers the activation of caspase-2 and -8, leading to DNA damage and eventual cell death of monocytes, one of the major cell types of the innate immune system (Jung et al., 2023). In the current study, the TG-induced activation of caspase-8 and -2 upregulated iNOS expression, which consequently downregulated XIAP and survivin expression and led to the death of TG-treated monocytes.

Numerous studies have investigated the association between hyperlipidemia and iNOS expression. Excess lipids upregulate iNOS expression in both *in vitro* myocytes and *in vivo* rat models (Puthanveetil et al., 2011). In addition, treatment with cholesterol upregulates iNOS expression in the immortalized rat liver stellate cell line HSC-T6 (Anavi et al., 2015). By contrast, the hyperlipidemic rat model shows lower myocardial NO content than normal rats without altered NO synthase activity (Onody et al., 2003). Similarly, TG accumulation in primary rat hepatocytes reduces nitrite levels and iNOS mRNA expression (Ilan et al., 2005). In the current study, treatment with TG upregulated iNOS expression in THP-1 monocytes, and iNOS inhibition suppressed the death of TG-treated monocytes, implying that iNOS was involved in TG-induced monocytic death. Previously, we found that TG induces DNA damage, leading to monocytic death. Further studies are warranted to examine whether the TG-induced upregulation of iNOS expression and the resultant increase in NO production cause DNA damage in TG-treated monocytes.

NO-induced downregulation of cIAP1 and XIAP is reportedly involved in NO-mediated monocytic death. In the current study, TG treatment downregulated XIAP expression in TG-treated monocytes. To the best of our knowledge,

the current study is the first to demonstrate that survivin expression is downregulated during TG-induced monocytic death. We previously found that TG activates caspase-3 and -7 in TG-induced monocytic death. In the current study, iNOS inhibition suppressed the activities of caspase-3 and -7. These results imply that TG increases iNOS, which subsequently downregulates XIAP and survivin expression and activates caspase-3 and -7 in TG-treated monocytes. Further studies are warranted to elucidate other upstream molecules of caspase-3 and -7 in TG-induced monocytic death.

The iNOS-related signaling pathway in immune cells has been well documented. Lipopolysaccharide (LPS) stimulation of immune cells activates mitogen-activated protein kinases, nuclear factor-κB, and Janus tyrosine kinase (JAK) and subsequently upregulates iNOS expression (Hegazy et al., 2015; Palikhe et al., 2019). In addition, interferon-γ stimulates iNOS expression by inducing interferon regulatory factor-1, resulting in the apoptosis of microglial cells (Lee et al., 2001). Caspase-8 and -2 are upstream molecules of iNOS in TG-induced monocytic death. Further studies must be conducted to examine the upstream molecules of caspase-8 and -2 in TG-induced monocytic death.

NO activates the JAK2/STAT-1 pathway to activate caspase-8, which subsequently activates caspases-3, leading to apoptosis (Li et al., 2007; Dubey et al., 2016). Simpson et al. reported that LPS activates caspase-8, subsequently upregulating iNOS expression in IFNγ-sensitized macrophage (Simpson et al., 2022). The current study shows that caspase-8 is an upstream molecule of iNOS in TG-induced monocytic death. Therefore, the upstream molecules of iNOS and caspase-8 in various cellular signal transduction pathways can be determined by the triggers and cell types used in the study.

In conclusion, the current study demonstrated that iNOS is involved in TG-mediated monocytic death, suggesting that iNOS inhibition is a potential therapeutic strategy for this condition.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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