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Tumor necrosis factor-α induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes

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Abstract

Objective: It has been demonstrated that tumor necrosis factor-α (TNFα) induces apoptosis in cardiac myocytes. However, its mechanism of action is still not well understood. In the present study, we hypothesized that TNFα induces myocardial apoptosis by induction of inducible nitric oxide synthase (iNOS). **Methods:** Neonatal cardiac myocytes were isolated from iNOS (−/−) mutant and C57BL6 wild type mice. Cells were cultured for 3 days before treatment with an NO donor or TNFα. Following treatment with *S*-nitroso-N-acetyl-penicillamine (SNAP) or TNF-α, cells were tested for apoptosis by terminal deoxynucleotidyl transfer-mediated end labeling (TUNEL) staining and cell death detection ELISA. NO production was measured by nitrite concentration in the culture medium. Cardiomyocyte expression of iNOS and TNF type 1 receptor (TNFR1) mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). **Results:** SNAP (0.01–100 μM) induced apoptosis of cardiac myocytes in a concentration-dependent manner in the wild type mice ($n=5$, $P<0.01$). TNFR1 mRNA was expressed in neonatal cardiomyocytes from both wild type and iNOS (−/−) mutant mice. TNFα induced a concentration-dependent increase in iNOS mRNA expression and nitrite production as well as significant apoptosis of cardiomyocytes in the wild type mice ($n=4$, $P<0.01$). However, without iNOS expression, the apoptotic effects of TNF-α were significantly attenuated in cardiomyocytes from iNOS (−/−) mutant mice ($n=4$, $P<0.05$). **Conclusion:** TNFα induces apoptosis via iNOS expression and NO production in neonatal mouse cardiomyocytes.

Keywords: Apoptosis; Myocytes; Nitric oxide

1. Introduction

Apoptosis, the programmed cell death is a process by which cells undergo inducible non-necrotic cellular suicide [1,2]. In contrast to necrotic cell death, apoptosis depends on de novo synthesis of proteins that initiate a cellular suicide program in response to specific stimuli. Characteristic features of apoptosis include chromatin condensation, membrane blebbing, and internucleosomal DNA fragmentation [1–3]. Nitric oxide (NO), a free-radical molecule produced by NO synthase (NOS), is known to play an important role in the regulation of a variety of physiological functions including cardiovascular and immune systems [4,5]. Excess amounts of NO produced by inducible NOS are involved in myocardial damage including myocardial apoptosis in rats with myosin-induced autoimmune myocarditis [6] and myocardial infarction [7]. NO has also been shown to induce apoptosis in neonatal rat cardiomyocytes [8], activated macrophages of mice [9], rat vascular smooth muscle cells [10,11], and rat neurons [12].

TNFα is a pro-inflammatory cytokine that has been implicated in the pathogenesis of cardiovascular disease [13]. In fact, cardiomyocytes have recently been shown to be an important source of TNFα production in disease conditions such as myocardial infarction and heart failure [14,15]. TNFα inhibits myocardial contractility and induces apoptosis of cardiomyocytes via stimulation of TNF type 1 receptor (TNFR1) [3,13]. Studies from adult rat cardiomyocytes have demonstrated that the pro-apoptotic

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effect of TNFα is mediated by the formation of sphingosine [16]. However, it is not known whether iNOS is involved in TNFα-induced apoptosis. Expression of iNOS and high levels of NO induced by interleukin-1β (IL-1β) and interferon-γ have been demonstrated to induce apoptosis in cardiomyocytes [17]. While TNFα in combination with IL-1β and interferon-γ has been shown to induce cardiomyocyte iNOS expression, TNFα alone at the concentrations tested did not induce iNOS expression or apoptosis in neonatal rat cardiomyocytes [17–19]. Adult rat cardiomyocytes express TNFR1 [16]. The fact that Kinugawa et al. [19] did not demonstrate any TNFα-induced iNOS expression while Muller-Werdan et al. [20] showed a weak effect of TNFα on iNOS expression and NO production in neonatal rat cardiomyocytes, suggests that the concentration of TNFα used in these studies may be too low to produce a pathological effect rather than the lack of TNFR1 in these cardiomyocytes. Although the pathophysiological levels of TNFα are reported to be 2 ng/ml in whole blood of septic patients [21] and about 1 ng/g tissue in myocardium after ischemia–reperfusion [22], the actual levels of TNFα at the vicinity of the cell or inside the cell may be much higher. In the present study, we hypothesized that TNFR1 is expressed in neonatal mouse cardiomyocytes. We further hypothesized that TNFα induces apoptosis by iNOS expression and NO production in the cultured neonatal mouse cardiomyocytes. To test this hypothesis, different concentrations of TNFα were used. The specific role of iNOS in TNFα-induced apoptosis was investigated by employing iNOS (−/−) mutant mice.

2. Methods

2.1. Animals

Breeding pairs of iNOS (−/−) mutant (Stock no. 2609) and C57BL6 wild type mice were purchased from Jackson Laboratory. Genotyping of adult iNOS (−/−) mutant mice was performed by a PCR method using genomic DNA extracted from the tail. A breeding program was carried out to produce neonates. The study protocol was approved by the Animal Care Committee at the University of Western Ontario, Canada.

2.2. Isolation and culture of cardiac myocytes

Cell culture of neonatal mouse cardiomyocytes was prepared by methods previously described for neonatal rat ventricular myocytes [23,24] with minor modifications. Briefly, ventricles from C57BL6 wild type or iNOS (−/−) mutant mice born within 24 h were minced in a nominally Ca^{2+} and Mg^{2+} free Hanks balance solution. Cardiac myocytes were dispersed by the addition of 0.625 mg/ml collagenase (type II; Worthington Biochemicals, Freehold, NJ, USA) and incubated at 37°C for 40 min. The cell suspension was filtered through a polypropylene macroporous filter (mesh opening 105 μm, Spectra/Mesh, Spectrum Medical Industries) and centrifuged at 1200 rpm for 5 min to obtain a cell pellet. Cells were then suspended in M199 medium supplemented with 5% fetal calf serum (FCS) and 5 mM D-glucose, and preplated for 40 min to remove non-cardiomyocytes. The cardiac myocytes were plated at a density of 2×10^6 cells/ml in M199 supplemented with 10% FCS on 18-mm square coverslips or in 35-mm petri dishes precoated with 1% gelatin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. A confluent monolayer of spontaneously beating cells was formed within 2 days. Using this method, we routinely obtained cultures with <5% non-cardiomyocytes. Cells were switched to serum-free M199 for 24 h before they were treated with NO donor, *S*-nitroso-N-acetylpenicillamine (SNAP, Sigma) or mouse TNFα (recombinant, Sigma) for 6–18 h. Concentrations of SNAP and TNFα in the cell culture were 0.01–100 μM and 100–300 ng/ml, respectively.

2.3. Electron microscopy

The ultrastructural features of apoptosis were confirmed using electron microscopy. Cardiomyocytes grown in petri dishes treated with and without SNAP were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. Cells were then postfixed in 1% osmium tetroxide in phosphate buffer for 1 h and dehydrated in graded series of alcohol. Subsequently, cells were gently scraped away from the petri plates. The resultant sheet of cells was transferred to an Eppendorf tube and spun. All subsequent embedding steps were carried out in Eppendorf tubes with centrifugations between the solution exchange steps. Cells were embedded in a Polybed–Araldite mixture and polymerized at 60–65°C overnight. Ultrathin silver sections, 90–100 nm thick, were prepared and stained in 2% uranyl acetate in 70% ethanol and Reynolds lead citrate and viewed with a transmission microscope (Philip 410, The Netherlands).

2.4. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay

After treatment with SNAP or TNFα in serum-free medium for 18 h, cells seeded on coverslips were fixed in 4% paraformaldehyde in PBS for 30 min. The in situ TUNEL assay was then performed in accordance with the manufacturer's protocol for cultured cells (Boehringer Mannheim, Indianapolis, IN, USA). Cells were counterstained with hematoxylin. Apoptosis in cardiomycytes was quantified by the number of apoptotic nuclei in the total nuclei in 50 continuous microscopic fields under 500× magnification by using the following formula: percent apoptosis=(apoptotic nuclei/total nuclei)×100. The inter-

and intra-observer variances of apoptosis quantification were 3.7 and 2.5%, respectively.

2.5. Enzyme immunoassay for cytoplasmic histone-associated DNA fragments

For quantitative determination of apoptosis, we measured cytoplasmic histone-associated DNA fragments (mono- and oligonucleotides) using a photometric enzyme immunoassay (cell death detection ELISA, Boehringer Mannheim) according the manufacturer's instructions. Briefly, floating cells were first collected from the culture medium by centrifugation. The attached cells were harvested with 0.25% trypsin and the cell suspension was pelleted by centrifugation. Both floating and attached cells were lysed and the supernatant (cytoplasmic fraction) was obtained by centrifugation at 20 000 *g* for 10 min at 4°C. After 1:10 dilution, the supernatant (100 μl) in duplicate was added to the microtiter plate coated with anti-histone antibody and incubated for 90 min. The samples were washed and anti-DNA peroxidase was added to each well and incubated for 90 min. The plate was washed again and 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) was added for colour development. Absorbance was measured at 405 nm and the reading represented the amount of cytoplasmic DNA fragments in 10^3 cells.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of TNFR1 and iNOS was determined by RT-PCR. Total RNA was isolated from cells according to the method of Chomozynski and Sacchi [25] with Trizol reagent (Gibco-BRL). The RNA was extracted with the use of phenol–chloroform, precipitated by isopropanol and quantified by spectrophotometry. Subsequently, RNA was reverse transcribed into first-strand cDNA by using a Moloney murine leukemia virus (M-MLV) reverse transcriptase system. The cDNA was amplified by PCR using a programmable thermal cycler (Progene, Techne, Duxford, Cambridge, UK). The forward and reverse primers for mouse TNFR1 gene (Genbank accession, no. M60468) were 5′-TTG GTG ACC GGG AGA AGA GG-3′ and 5′-TGA CAT TTG CAA GCG GAG GA-3′, respectively. Primers for mouse iNOS gene (Genbank accession, no. M84373) were 5′-ACC CCT GTG TTC CAC CAG GAG ATG TTG AA -3′ (forward) and 5′-TGA AGC CAT GAC CTT TCG CAT TAG CAT GG-3′ (reverse) to amplify the 1583–1771 bp region of the iNOS gene which is disrupted in iNOS (−/−) mutant mice [26]. To ensure that equal amounts of reverse-transcribed cDNA were added to the PCR mixture, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Genbank accession, no. M17701) was also amplified by using the following primers: forward primer 5′-AAA GGG CAT CCT GGG CTA CA-3′; reverse primer, 5′-CAG TGT TGG GGG CTG AGT TG-3′. The logarithmic ranges of amplification were established for TNFR1, iNOS and GAPDH (40, 35 and 25 cycles, respectively) as previously described to ensure that the amplified PCR product reflected the original mRNA level [27]. The PCR product was separated on a 2% agarose gel and visualized under UV light. The predicted length of the amplification product for TNFR1, iNOS and GAPDH was 510, 189 and 297 bp, respectively. Both TNFR1 and iNOS PCR products were confirmed by DNA sequencing.

2.7. Measurement of nitrite concentration

The formation of nitrite in culture medium was used as an indicator of NO release by neonatal cardiac myocytes. Medium in the culture petri dishes was collected and centrifuged at 3000 rpm for 5 min at 4°C to remove cellular debris. The nitrite content in the supernatant was measured by mixing 100 μl of medium with 1 ml of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% H_3PO_4), and the concentration of the resultant chromophore was determined spectrophotometrically at 543 nm using known concentrations of sodium nitrite as a standard [28]. The protein concentration of the attached cells was measured by the method of Lowry [29] and the nitrite concentration was expressed as nmol/mg cell protein.

2.8. Statistical analysis

All data are presented as mean±S.E.M. Statistical analysis was performed by using SIGMASTAT for Windows version 1.0 (Jandel, CA, USA). ANOVA followed by Student–Newman–Keuls test was used to compare differences between groups. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Exogenous NO induces apoptosis in cardiomyocytes

Cultured neonatal mouse cardiomyocytes from both iNOS (−/−) knockout and wild type mice were exposed to a NO donor, SNAP for 18 h. The amount of apoptosis was quantified by TUNEL staining. SNAP induced concentration-dependent increases in apoptosis in cardiomyocytes from both iNOS (−/−) knockout and wild type mice (Fig. 1). Furthermore, SNAP-induced apoptosis was similar between iNOS (−/−) knockout and wild type mice (P=n.s.).

To confirm that TUNEL-positive cells were indeed apoptotic, electron microscopy was used to examine the ultrastructural changes in SNAP-induced cardiomyocytes. Under control conditions, normal cellular features were observed in both iNOS (−/−) knockout and wild type

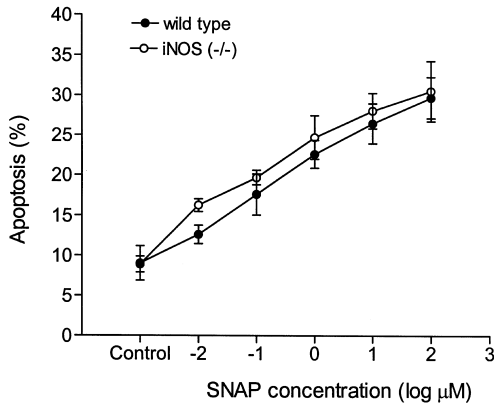

Fig. 1. Concentration-dependent apoptosis induced by *S*-nitroso-N-acetyl-penicillamine (SNAP) in cultured neonatal mouse cardiomyocytes from wild type ($n=5$) and iNOS ($-/-$) mutant mice ($n=3$). *N* represents the number of experiments. Each experiment was performed on a litter of five to eight mice. $P=$n.s. wild type vs. iNOS ($-/-$).

neonatal cardiomyocytes (Fig. 2A). However, treatment of cardiomyocytes from both iNOS ($-/-$) mutant and wild type mice with 100 μM SNAP showed characteristic ultrastructural features of apoptosis, i.e., the condensation of chromatin in the periphery of the nucleus and cellular shrinkage. A typical example of SNAP-induced apoptosis in cardiomyocytes from wild type mice is shown in Fig. 2B.

3.2. TNFR1 is present in cardiomyocytes

The mRNA expression of TNFR1 was determined by RT-PCR. TNFR1 mRNA was equally expressed in neonatal cardiomyocytes from both wild type and iNOS ($-/-$) mutant mice. Furthermore, TNFα treatment did not alter TNFR1 mRNA expression in either group (Fig. 3). Since adult myocardium is known to express TNFR1 receptor, the myocardium from an adult mouse was used as a positive control. TNFR1 mRNA was detected by the same RT-PCR method in the adult mouse myocardium and neonatal mouse cardiomyocytes (data not shown).

3.3. TNF-α induces iNOS expression and NO production in cardiomyocytes

The expression of iNOS mRNA was determined by RT-PCR. Under control conditions, iNOS mRNA was not detectable in cardiomyocytes from wild type mice. Treatment with TNFα (100 and 300 ng/ml) for 18 h induced a concentration-dependent expression of iNOS mRNA while there was no change in GAPDH transcript levels in wild type mouse cardiomyocytes (Fig. 3). However, iNOS transcripts were not detectable in either control or TNFα-treated cardiomyocytes from iNOS ($-/-$) mutant mice.

NO production was measured by nitrite concentration in the culture medium. Treatment with TNFα for 18 h resulted in a concentration-dependent increase in nitrite production in neonatal cardiac myocytes from wild type mice ($P<0.05$; Fig. 4). However, TNFα did not cause any significant changes in nitrite production in the culture medium of neonatal cardiomyocytes from iNOS ($-/-$) mutant mice ($P=$n.s., Fig. 4).

3.4. TNF-α induces apoptosis in cardiomyocytes

Similarly to TNFα-induced iNOS mRNA expression and nitrite production, TNFα treatment for 18 h induced concentration-dependent apoptosis determined by TUNEL assay in wild type neonatal mouse cardiomyocytes ($P<0.05$, Fig. 5A). However, the apoptotic effects of TNFα were significantly blunted in cardiomyocytes from iNOS ($-/-$) mutant mice ($P<0.05$, Fig. 5A). TNFα-induced apoptosis in cardiomyocytes from wild type mice was further confirmed by cell death ELISA which measures cytoplasmic histone-associated DNA fragments (Fig. 5B).

3.5. Time-dependent increases in NO production and apoptosis by TNFα

Neonatal cardiomyocytes from wild type mice were treated with TNFα (300 ng/ml) for 6, 12 and 18 h. Time-dependent increases in nitrite production and apoptosis were observed ($P<0.01$, Fig. 6).

4. Discussion

The major finding in the present study is that TNFα induces apoptosis in cultured neonatal mouse cardiomyocytes by iNOS expression and NO production. This was supported by the fact that TNFα-induced apoptosis paralleled NO production in cardiomyocytes of the wild type mice and these effects of TNFα were abrogated in cardiomyocytes from iNOS ($-/-$) mutant mice. Our results suggest that iNOS expression and NO production represent an important pathway of TNFα-induced apoptosis in cultured neonatal mouse cardiomyocytes.

Exogenous NO induces apoptosis. Two recent studies have demonstrated that NO donors such as SNAP and *S*-nitrosoglutathione cause apoptosis in cardiomyocytes by activation of soluble guanylate cyclase, leading to increased levels of intracellular cGMP, and subsequent activation of a cGMP-dependent protein kinase (PKG) [8,17]. Consistent with this notion, we demonstrated in the present study that treatment with SNAP caused concentration-dependent apoptosis in neonatal mouse cardiomyocytes. The fact that the amount of apoptosis induced by SNAP was similar between iNOS ($-/-$) mutant and wild type mice, suggests that there is no defect in the NO-induced apoptotic pathway in cardiomyocytes from iNOS ($-/-$) mutant mice.

Endogenous NO is produced from three NOS isozymes, NOS1 (nNOS), NOS2 (iNOS) and NOS3 (eNOS) [5,30].

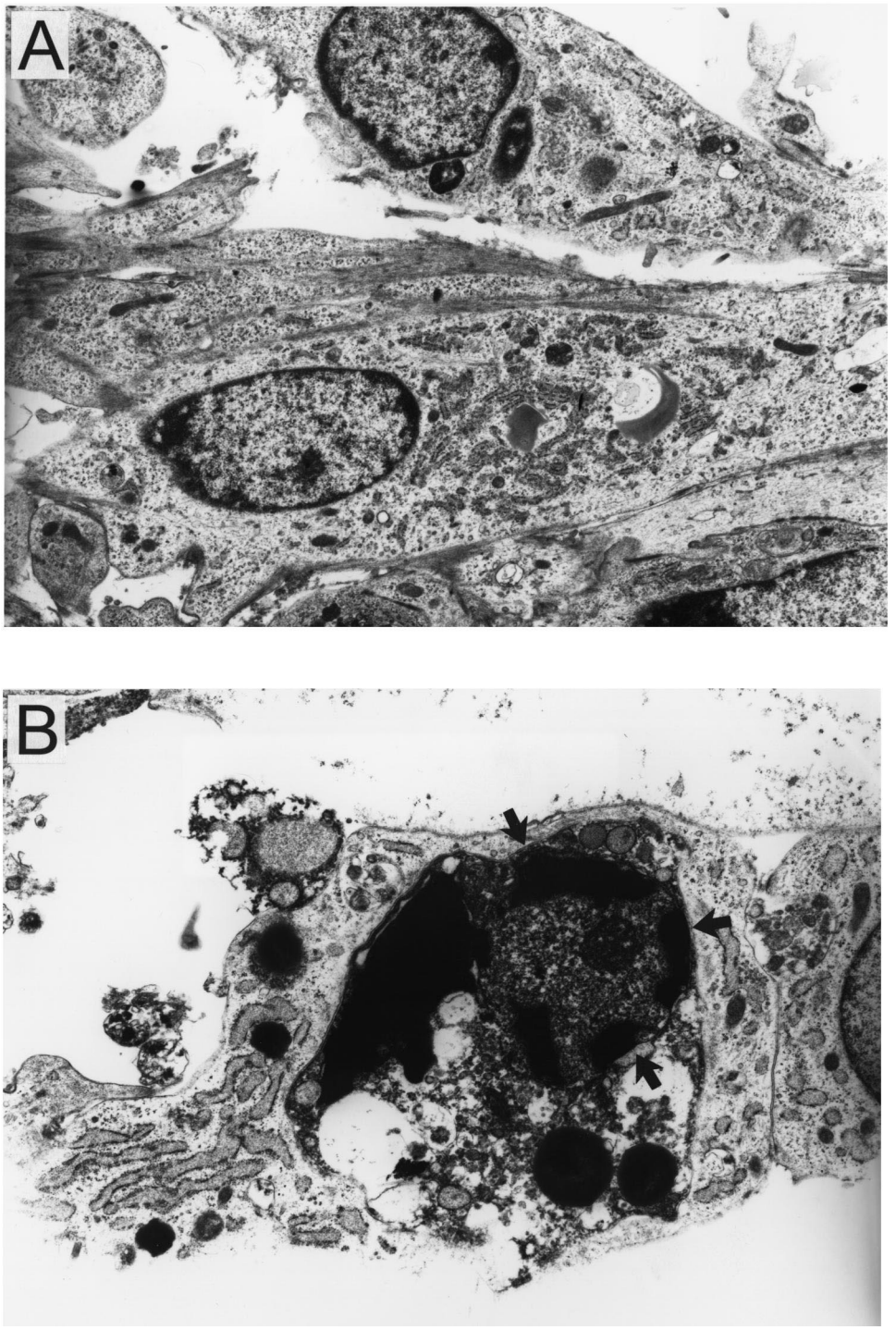

Fig. 2. Electron micrographs of cardiomyocyte apoptosis. (A) Normal cellular morphology of a cardiomyocyte (original magnification×5400). (B) Cardiomyocyte treated with 100 μM *S*-nitroso-N-acetyl-penicillamine (SNAP) for 18 h displays characteristic features of apoptosis, including condensation and margination of nuclear chromatin and cellular shrinkage (original magnification×6900).

Cardiomyocytes constitutively express eNOS which has been demonstrated to play an important role in the regulation of myocardial function [4]. Under pathophysiological conditions or upon cytokine stimulation, iNOS is induced in cardiomyocytes. iNOS expression associated with excessive NO production has been shown to induce apoptosis in neonatal rat cardiomyocytes [17]. Furthermore, the ability of cytokines to induce NO production in cardiomyocytes exactly parallels their ability to activate apoptosis [17]. TNFα in combination with other cytokines such as IL-1β and interferon-γ has been shown to induce iNOS expression in rat cardiomyocytes [17,19].

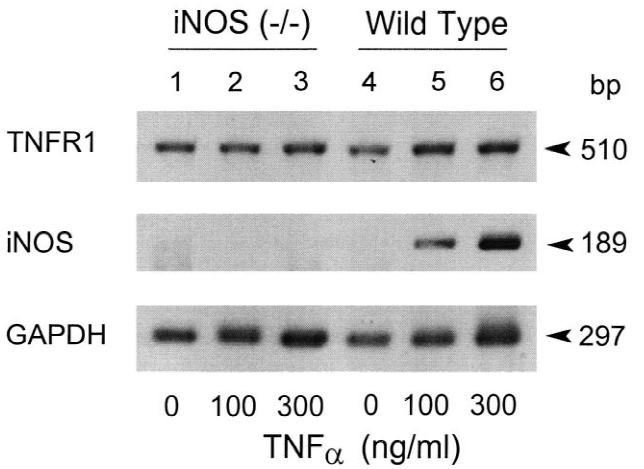

Fig. 3. Expression of TNFR1, iNOS and GAPDH mRNA in cardiomyocytes determined by RT-PCR determination. PCR products for TNFR1, iNOS, and GAPDH were 510, 189 and 297 bp, respectively. GAPDH expression was used to confirm equal loading of RNA in each lane. Cardiomyocytes from iNOS (−/−) mutant and wild type mice were treated with TNFα for 18 h with the concentrations indicated. A representative of three independent experiments is shown.

Surprisingly, these studies were not able to show TNFα-induced iNOS expression in neonatal rat cardiomyocytes [17,19]. This is most likely due to the fact that the concentration of TNFα (25 ng/ml) used in these studies is too low to stimulate iNOS expression and NO production, although this low concentration of TNFα may enhance the actions of other cytokines. In the present study, higher concentrations of TNFα (100 and 300 ng/ml) were employed and the effects of TNFα on iNOS expression and NO production were clearly demonstrated in the neonatal mouse cardiomyocytes from the wild type mice. Furthermore, TNFα-induced NO production paralleled cardiomyocyte apoptosis in both concentration- and time-dependent manners. The effects of TNFα were completely abrogated in cardiomyocytes from iNOS (−/−) mutant

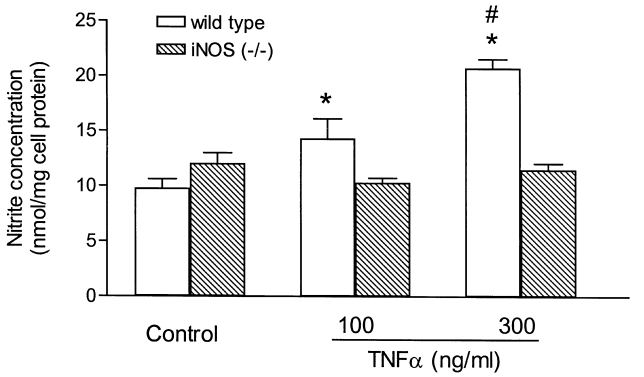

Fig. 4. Effects of tumor necrosis factor-α (TNFα) on nitrite concentration in cultured neonatal mouse cardiomyocytes from wild type and iNOS (−/−) mutant mice. n=4–7 in each group. Each experiment was performed on a litter of five to eight mice. *P<0.05 vs. wild type control; #P<0.05 vs. TNFα treatment in iNOS (−/−) mice.

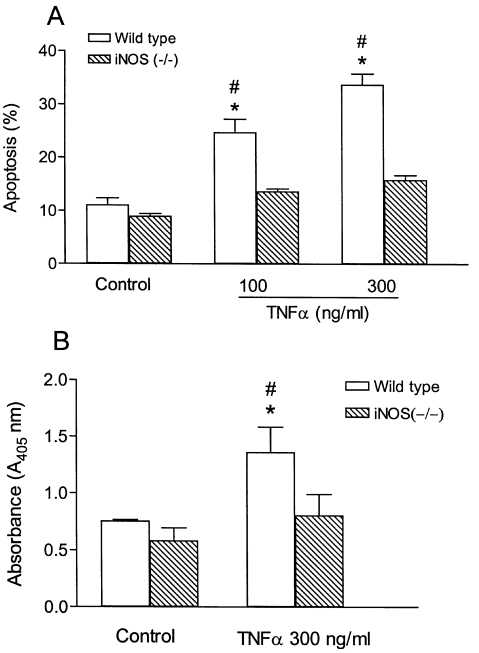

Fig. 5. Effects of tumor necrosis factor-α (TNFα) on apoptosis in cultured neonatal mouse cardiomyocytes from wild type and iNOS (−/−) mutant mice. (A) Apoptosis determined by TUNEL; (B) apoptosis determined by cell death detection ELISA, n=4–7 in each group. Each experiment was performed on a litter of five to eight mice. *P<0.05 vs. wild type control; #P<0.05 vs. TNFα treatment in iNOS (−/−) mice.

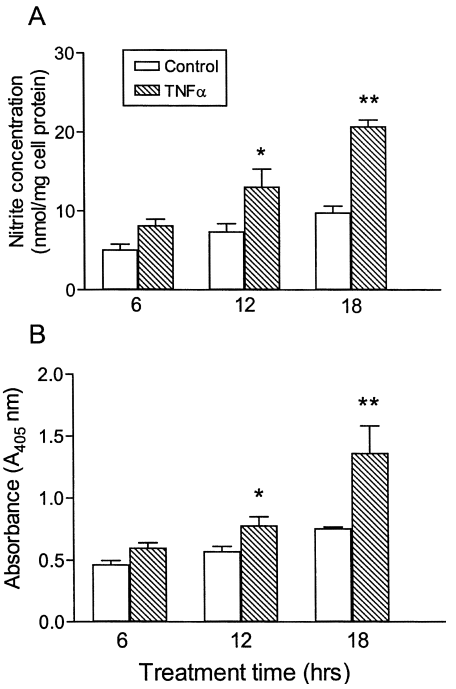

Fig. 6. Time course of tumor necrosis factor-α (TNFα)-induced NO production and apoptosis in cultured neonatal mouse cardiomyocytes from wild type mice. (A) Nitrite concentration in the culture medium; (B) apoptosis determined by cell death detection ELISA, n=5 in each group. Each experiment was performed on a litter of five to eight mice. *P<0.05, **P<0.01 vs. respective control.

mice. The results suggest that TNFα induces apoptosis by iNOS expression and NO production in neonatal mouse cardiomyocytes.

TNFα induces apoptosis by activation of TNFR1 in many cell types, including cardiomyocytes [3,13]. In the present study, we demonstrated that this receptor is present in neonatal mouse cardiomyocytes. In a recent study, Krown et al. demonstrated that TNFα induced cardiomyocyte apoptosis by a sphingosine-dependent mechanism in adult cardiomyocytes [16]. In neonatal rat cardiomyocytes however, similar concentrations of TNFα (1–5 nmol/ml equivalent to 17–85 ng/ml) did not induce apoptosis, while sphingosine did induce apoptosis. Furthermore, TNFR1 mRNA was not detectable by RT-PCR in neonatal rat cardiomyocytes. Based on these results, the authors concluded that TNFR1 was not present in neonatal rat cardiomyocytes [16]. Upon close examination, there was about a 10°C difference in the melting temperature and a 45% difference in GC content between the forward and reverse primers used to amplify TNFR1 [16]. This may be the reason that TNFR1 mRNA was not detected in the neonatal rat cardiomyocytes. The reason that TNFα did not induce apoptosis in the neonatal rat cardiomyocytes is most likely due to the fact that neonatal rat cardiomyocytes are less sensitive to TNFα stimulation compared to adult cardiomyocytes. Thus, higher concentrations of TNFα are needed to induce apoptosis in the neonatal cardiomyocytes. Indeed, by using 100 and 300 ng/ml of TNFα in the present study, we were able to demonstrate the pro-apoptotic effects of TNFα in the neonatal mouse cardiomyocytes. The fact that TNFR1 is present in the neonatal mouse cardiomyocytes may suggest a receptor-mediated process in TNFα-induced apoptosis. However, further experiments are required to prove this TNFR1-mediated apoptosis pathway in the neonatal mouse cardiomyocytes.

In summary, we demonstrated that TNFR1 was present in neonatal mouse cardiomyocytes. TNFα induced apoptosis with parallel increases in iNOS mRNA expression and NO production in wild type mice cardiomyocytes while these effects of TNFα were abrogated in cardiomyocytes from iNOS (−/−) mutant mice. We conclude that TNFα induces apoptosis via iNOS expression and NO production in cultured neonatal mouse cardiomyocytes. Myocardial TNFα and NO production has been demonstrated to be increased in acute myocardial infarction and heart failure [14,15,31,32]. Elevated TNFα and NO levels may induce cardiomyocyte apoptosis which may contribute to the progression and outcome of these diseases.

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