Oxidative Stress Mediates Tumor Necrosis Factor-α–Induced Mitochondrial DNA Damage and Dysfunction in Cardiac Myocytes

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Background—Tumor necrosis factor-α (TNF-α) and angiotensin II (Ang II) are implicated in the development and further progression of heart failure, which might be, at least in part, mediated by the production of reactive oxygen species (ROS). However, the cause and consequences of this agonist-mediated ROS production in cardiac myocytes have not been well defined. Recently, we demonstrated that increased ROS production was associated with mitochondrial DNA (mtDNA) damage and dysfunction in failing hearts. We thus investigated whether the direct exposure of cardiac myocytes to TNF-α and Ang II in vitro could induce mtDNA damage via production of ROS.

Methods and Results—TNF-α increased ROS production within cultured neonatal rat ventricular myocytes after 1 hour, as assessed by 2′,7′-dichlorofluorescin diacetate fluorescence microscopy. TNF-α also decreased mtDNA copy number by Southern blot analysis in association with complex III activity, which was prevented in the presence of the antioxidant α-tocopherol. A direct exposure of myocytes to H2O2 caused a similar decrease in mtDNA copy number. In contrast, Ang II did not affect mtDNA copy number, despite the similar increase in ROS production. TNF-α–mediated ROS production and a decrease in mtDNA copy number were inhibited by the sphingomyelinase inhibitor D609. Furthermore, N-acetylsphingosine (C2-ceramide), a synthetic cell-permeable ceramide analogue, increased myocyte ROS production, suggesting that TNF-α–mediated ROS production and subsequent mtDNA damage were mediated by the sphingomyelin-ceramide signaling pathway.

Conclusions—The intimate link between TNF-α, ROS, and mtDNA damage might play an important role in myocardial remodeling and failure. (Circulation. 2003;107:1418-1423.)

Key Words: myocytes ■ free radicals ■ heart failure ■ antioxidants ■ genes

Recent experimental and clinical evidence has suggested an excessive generation of reactive oxygen species (ROS) in heart failure.1 Mitochondrial electron transport is a potential source of ROS production within the heart.1 Furthermore, increased ROS can be associated with mitochondrial DNA (mtDNA) damage and dysfunction.2 ROS may result in the progressive destruction of the mtDNA, and such mtDNA damage can lead to a decline of mtRNA transcription and a loss of function, which might play an important role in the development of myocardial remodeling and failure.3 Tumor necrosis factor-α (TNF-α) is implicated in the pathophysiology of myocardial failure.4 TNF-α increases the production of ROS in cultured cardiac myocytes.5 If mitochondria are the principal source of ROS, it is quite conceivable to hypothesize that TNF-α directly impairs mitochondrial electron transport activity and produces ROS within cardiac myocytes. In fact, in tumor cells, hepatocytes, and vascular endothelial cells, mitochondria have been identified as a major source of TNF-α–induced ROS production.6–8 Furthermore, ROS production may cause mtDNA damage, decrease activity, and thereby contribute to the increase in oxidative stress. Similarly, angiotensin II (Ang II) is also increased in heart failure, Ang II increases ROS within cardiac myocytes.5 However, it has not been determined whether TNF-α and Ang II cause ROS production in association with mtDNA damage and dysfunction in cardiac myocytes. Even though TNF-α or Ang II may not be a single trigger or cellular mechanism leading to myocardial failure, an understanding of the processes leading to oxidative stress may help elucidate the common pathophysiology of heart failure.

The purpose of the present study was to determine whether TNF-α and Ang II could directly increase ROS production in association with mtDNA decline and dysfunction in cardiac myocytes...
myocytes. For this purpose, we determined, using cardiac myocytes in culture, whether (1) TNF-α and Ang II exposure causes the formation of ROS, (2) ROS can directly cause mitochondrial defects, and (3) TNF-α and Ang II, acting via ROS, cause mtDNA damage that has been implicated in the pathogenesis of myocardial remodeling and failure.2 We also assessed the role of sphingomyelin-ceramide in the signaling pathways responsible for TNF-α-mediated effects.

**Methods**

**Preparation of Neonatal Rat Ventricular Myocytes**

Primary cultures of cardiac myocytes were prepared from the ventricles of neonatal Wistar rats according to the methods of Simpson with slight modifications.3 Myocytes were maintained at 37°C in humidified air with 5% CO2. After dissociation of the myocardial tissue with trypsin, cells were plated for 1 hour into 100-mm culture dishes in DMEM with 10% FBS to reduce the number of nonmyocytes. Cells that were not attached to the dishes were plated into 6-well culture plates at a density of 10^3 cells/mm². The culture medium was replaced after 24 hours with serum-free medium consisting of DMEM, transferrin (5 μg/mL), insulin (1 μg/mL), and bromodeoxyuridine (0.1 mmol/L).

**Evaluation of Myocyte Viability**

To examine the effects of various drugs on cellular viability, myocytes were treated with a drug, and then the counts of cells in 10 randomly chosen 1×1-mm fields and the percentage of cells excluding 0.4% trypan blue dye were assessed. To determine the induction of apoptosis, cardiac myocytes were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining.4 Staining with hematoxylin was carried out on the same section for visualization of nuclei. We also examined whether apoptosis is present in myocytes by the more sensitive ligase-mediated polymerase chain reaction (PCR) fragmentation assay (ApoAlert LM-PCR ladder assay kit).5

**Measurement of ROS Production**

A fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), was used for the assessment of intracellular ROS formation in cultured cardiac myocytes.6 This assay is a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H2O2), hydroxyl radical (OH·), and hydroperoxides (ROOH). DCFH-DA was dissolved in absolute ethanol at a concentration of 5 mmol/L. Cardiac myocytes were grown on collagen-coated glass coverslips in 6-well culture plates. On culture day 4, TNF-α (10 ng/mL), Ang II (10 μmol/L), or their diluent (control) was administered simultaneously with DCFH-DA (5 μmol/L) in culture medium. After incubation at 37°C for 30 minutes, cardiac myocytes were washed with PBS. Fluorescence images were acquired with a microscope (Olympus BX50), and its intensity on a region of interest was measured. Intensity values for treated cells were determined in comparison with control cells (diluent only; PBS containing 0.1% BSA without TNF-α or Ang II).

The amount of ROS was also quantified in myocytes (3×10⁶ cells per experiment) by electron spin resonance spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl, hydroxy-TEMPO.5 All measurements were performed in 2 parallel runs, in the presence and absence of the hydroxy-radical scavenger dimethylthiourea. In addition, the redox-sensitive fluorophore hydroethidium (dihydroethidium) was used to monitor intracellular ROS production.10

**Measurement of mtDNA Copy Number and Damage**

DNA was extracted from cultured myocytes, and a Southern blot analysis was performed to measure the mtDNA copy number.2 Primers for the mtDNA probe corresponded to nucleotides 2424 to 3605 of the mouse mitochondrial genome, and those for the nuclear-encoded mouse 18S RNA probe corresponded to nucleo-
hour after exposure, which persisted until 24 hours. The increase of DCF fluorescence induced by TNF-α and Ang II was inhibited with the addition of α-tocopherol (1 μg/mL), confirming the increased production of ROS.

The amount of ROS, expressed as the “dimethylthiourea-inhibitable” rate of electron spin resonance spectroscopy signal decay, was significantly greater in H₂O₂-treated (1.92±0.39×10⁻³/min, n=8) or TNF-α-treated (1.11±0.45×10⁻³/min, n=8) myocytes compared with control cells (−0.28±0.28×10⁻³/min, n=8). Furthermore, the intensity of dihydroethidium red fluorescence was increased significantly in the presence of TNF-α or Ang II (10-fold increase compared with control), indicative of enhanced ROS generation within cardiac myocytes.

To determine the enzymatic source of ROS production, the mitochondrial respiratory chain inhibitor rotenone was used to block the electron flow between mitochondrial complexes I and III. When rotenone (10 μmol/L) was added together with TNF-α, TNF-α–induced DCFH oxidation was inhibited significantly (Figure 2A), indicating that the mitochondria are the major source of TNF-α–induced ROS production. The NADPH-dependent oxidase inhibitor diphenylene iodonium (DPI, 10 μmol/L) partially inhibited the ROS production induced by TNF-α, which, however, did not reach statistical significance. The xanthine oxidase inhibitor allopurinol (10 μmol/L) did not affect TNF-α–induced DCFH oxidation (Figure 2A). Ang II–induced ROS generation was significantly inhibited by DPI (Figure 2B), indicating that NADPH oxidase is a major source within cardiac myocytes. Ang II–induced ROS production tended to be inhibited by rotenone and allopurinol, which, however, did not reach statistical significance.

**Mitochondrial DNA Copy Number and Complex Enzyme Activity**

Exposure of cardiac myocytes to TNF-α for 24 hours caused a dose-dependent decrease in mtDNA copy number, with a 30±8% decrease at 10 ng/mL (Figure 3). In contrast, TNF-α did not cause a decrease in the 18S rRNA gene, coded by nuclear DNA. The TNF-α–induced decrease in mtDNA copy number was statistically significant 12 hours after exposure. Furthermore, QPCR showed a decrease in 13-kb mtDNA products in TNF-α–treated cardiac myocytes, indicating increased mtDNA damage. Cells were viable at the times of mtDNA level assessment as described above, verifying that the decrease in mtDNA copy number was not caused by cell death. α-Tocopherol (1 μg/mL) abolished the decrease in mtDNA copy number in response to TNF-α (Figure 3), suggesting that TNF-α–induced ROS production is involved in mtDNA damage. To determine whether ROS can directly damage mtDNA, myocytes were exposed to exogenous H₂O₂, H₂O₂ (5 to 100 μmol/L, 10 minutes) caused a dose-dependent
decrease in mtDNA copy number, which was completely inhibited in the presence of the H$_2$O$_2$ scavenger catalase (500 U/mL) (Figure 4). In contrast, no significant nuclear DNA damage was observed. In addition, QPCR showed a decrease in 16-kb mtDNA products in H$_2$O$_2$-treated cardiac myocytes. Under these experimental conditions, there was no significant decline in the number of myocytes (99.3/110.0 1.7% of baseline values) after exposure to H$_2$O$_2$. In addition, the percentage of viable myocytes excluding trypan blue dye did not change with H$_2$O$_2$ (from 97.2/110.0 0.5% to 97.0/110.0 0.7%, P NS), and myocyte apoptosis was not induced by H$_2$O$_2$. Therefore, the exposure of cardiac myocytes to H$_2$O$_2$ could mimic the TNF-α-mediated decrease in mtDNA copy number, confirming that ROS can directly damage mtDNA.

Role of Sphingomyelin-Ceramide Signaling Pathway in TNF-α-induced Effects

To explore the potential involvement of the ceramide-activated intracellular pathways in TNF-α-induced ROS production and mtDNA damage, we used D609, an inhibitor of the phosphatidylcholine-specific phospholipase C pathway and hence an indirect inhibitor of acid sphingomyelinase. Pretreatment of myocytes with D609 (25 μg/mL) inhibited both TNF-α-induced ROS production (Figure 7A) and decrease in mtDNA copy number (Figure 7B). The inhibition of TNF-α-induced ROS production by D609, even though partial at 1 hour of exposure, resulted in almost complete protection of mtDNA after 24 hours. Furthermore, ROS were increased to levels similar to those of TNF-α by N-acetylsphingosine (C$_2$-ceramide; 10 μmol/L, 30 minutes), a synthetic cell-permeable ceramide analogue (Figure 8). In contrast to TNF-α, ceramide-induced ROS production was transient and the increase in ROS production. In contrast, complex II activity was not altered by TNF-α (Figure 6B). A decrease in complex III activity induced by TNF-α was followed by a decline in mtDNA copy number.
returned to control levels after 24 hours of exposure (Figure 8B). Ceramide did not affect the mtDNA copy number after 24 hours of exposure (data not shown).

**Discussion**

The present study demonstrates that TNF-α directly induces mitochondrial ROS production within cardiac myocytes and causes mtDNA damage and dysfunction. This effect occurs via a ceramide-dependent signaling pathway initiated by sphingomyelinase. In contrast, Ang II did not affect mtDNA copy number despite increased ROS generation via NADPH oxidase.

TNF-α produced a significant increase in ROS as early as 1 hour of exposure, which persisted until 24 hours. The decrease in complex III activity induced by TNF-α was also recognized as early as 1 hour of exposure. Importantly, the TNF-α-induced decrease in mtDNA copy number was statistically significant 12 hours after exposure. On the basis of these results, TNF-α inhibited complex III activity and thereby produced mitochondrial ROS first, probably because of the direct inhibition of mitochondrial electron transport, as has been suggested by previous studies.6–8 TNF-α–induced ROS production would be followed by a decrease in mtDNA copy number. The decrease of mtDNA and complex III activity was abolished in the presence of antioxidants. Furthermore, addition of •O₂− similarly caused the decline of mtDNA copy number. The concentration of TNF-α used in this study (10 ng/mL) was consistent with the range previously used on cardiac myocytes in vitro.9 On the basis of these results, we concluded that TNF-α could damage mtDNA and cause mitochondrial dysfunction in cardiac myocytes via the increase of intracellular oxidative stress. These results add to a growing literature suggesting that ROS can act as signaling mediators of TNF-α.5,6 TNF-α–induced effects were observed without any cytotoxicity because appreciable myocyte death was not evidenced by cell viability assays. In addition, we could not detect significant apoptotic cells after exposure to TNF-α.

In accordance with our previous studies in failing hearts,2 mtDNA was more susceptible to ROS-mediated damage than nuclear DNA. It is possible that the concentrations of ROS achieved in the nucleus are lower than those in the mitochondria. However, it is more likely that differences in structure, protein content, and repair system make the nuclear DNA more resistant to oxidative damage. The mtDNA lacks both protective histone and nonhistone proteins and has a limited DNA repair capacity. For example, the mtDNA accumulates significantly higher levels of the DNA oxidation product 8-hydroxydeoxyguanosine than does the nuclear DNA.12 In addition, the high lipid-to-DNA ratio in the mitochondria makes them especially susceptible to lipophilic species, whereas the attachment of the mtDNA to the matrix side of the inner membrane also increases sensitivity to membrane disturbances and makes mitochondria a target for ROS. In addition, differences in antioxidant defense system activities may contribute to the sensitivity of these organelles to ROS-mediated DNA damage. Because the amount of intact DNA present at any time point reflects a balance between damage and repair, it is possible that the extensive and persistent mtDNA damage is a result of increased generation of secondary ROS caused by mitochondrial dysfunction.

The involvement of mitochondrial electron transport in TNF-α–induced ROS production has been demonstrated in various cell types6,8 and in isolated mitochondria from hepatocytes.13 In the present study, a major site of ROS generation during TNF-α stimulation was distal to complex I (Figure 2A). These results are consistent with those obtained from mouse fibrosarcoma cells6 and liver mitochondria.7 In addition, DPI also tended to inhibit TNF-α–induced ROS production, even though this inhibition did not reach statistical significance (Figure 2A). This might be a result of the known inhibitory effect of DPI on flavoproteins within complex I.14 Furthermore, DPI is not a specific inhibitor of NADPH oxidase because it inhibits flavin-containing enzymes in general. In contrast, Ang II produced ROS via an NAD(P)H oxidase (Figure 2B). Ang II–induced
ROS production was also inhibited, even though statistically insignificantly, by rotenone. These results indicate that TNF-α and Ang II have overlapping sources of ROS production. Interestingly, although TNF-α and Ang II increased ROS production similarly, only TNF-α decreased mtDNA copy number, but not Ang II (Figures 3 and 5). The differences in the source and/or target of ROS between these 2 stimuli might be responsible for different responses in mtDNA between these 2 agonists. TNF-α generates ROS at the mitochondrial inner membrane, which may easily result in the progressive destruction of the mtDNA, possibly because of its proximity to the site of ROS production. In contrast, Ang II induces ROS generation primarily via NADPH oxidase, which is located within the plasma membrane. In addition, differential activation of ROS-dependent signaling pathways might be also involved in this phenomenon.

The signal transduction pathways responsible for TNF-α-mediated cellular events are not yet fully elucidated in cardiac myocytes. Previous studies in various cell types have demonstrated that TNF-α exerts a direct effect on mitochondrial function and ROS generation via involvement of sphingomyelin hydrolysis to produce ceramide as a lipid second messenger. Our findings support the predominant role of sphingomyelin-ceramide signaling pathways in TNF-α-induced ROS generation and mtDNA decline in cardiac myocytes as well. This is in line with the observation by Oral et al that TNF-α may cause a rapid activation of sphingomyelinase, with subsequent sphingomyelin hydrolysis and sphingosine production in cardiac myocytes. Ceramide has been shown to result in an inhibition of electron transport at the level of complex III followed by an increased generation of ROS.

In the present study, generation of ROS, mtDNA decline, and loss of complex activity were observed in vitro when cardiac myocytes were exposed to TNF-α. TNF-α decreased complex III activity but did not affect complex II, which might be because of its higher susceptibility to ROS-induced damage. In addition, the decrease of the mtDNA copy number leads to a decrease in complex III activity. These results are consistent with recent studies using an animal model of heart failure and mice with heart-specific inactivation of mitochondria. This work was supported in part by grants 09670724 and 12670676 from the Ministry of Education, Science, and Culture, Tokyo, Japan. Part of this study was conducted at Kyushu University Station for Collaborative Research.

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