Tumor necrosis factor-α in mechanic trauma plasma mediates cardiomyocyte apoptosis

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Li S, Jiao X, Tao L, Liu H, Cao Y, Lopez BL, Christopher TA, Ma XL. Tumor necrosis factor-α in mechanic trauma plasma mediates cardiomyocyte apoptosis. *Am J Physiol Heart Circ Physiol* 293: H1847–H1852, 2007. First published July 6, 2007; doi:10.1152/ajpheart.00578.2007.—Mechanical traumatic injury causes cardiomyocyte apoptosis and cardiac dysfunction. However, the signaling mechanisms leading to posttraumatic cardiomyocyte apoptosis remains unclear. The present study attempted to identify the molecular mechanisms responsible for cardiomyocyte apoptosis induced by trauma. Normal cardiomyocytes (NC) or traumatic cardiomyocytes (TC; isolated immediately after trauma) were cultured with normal plasma (NP) or traumatic plasma (TP; isolated 1.5 h after trauma) for 12 h, and apoptosis was determined by caspase-3 activation. Exposure of TC to NP failed to induce significant cardiomyocyte apoptosis. In contrast, exposure of NC to TP resulted in a greater than twofold increase in caspase-3 activation (*P* < 0.01). Incubation of cardiomyocytes with cytomix (a mixture of TNF-α, IL-1β, and IFN-γ) or TNF-α alone, but not with IL-1β or IFN-γ alone, caused significant caspase-3 activation (*P* < 0.01). TP-induced caspase-3 activation was virtually abolished by an anti-TNF-α antibody, and TP isolated from TNF-α−/− mice failed to induce caspase-3 activation. Moreover, incubation of cardiomyocytes with TP upregulated inducible nitric oxide (NO) synthase (iNOS)/NADPH oxidase expression, increased NO/superoxide production, and increased cardiomyocyte protein nitration (measured by nitrotyrosine content). These oxidative/nitrative stresses and the resultant cardiomyocyte caspase-3 activation can be blocked by neutralization of TNF-α (anti-TNF-α antibody), inhibition of iNOS (1400W), or NADPH oxidase (apocynin) and scavenging of peroxynitrite (FP15) (*P* < 0.01). Taken together, our study demonstrated that there exists a TNF-α-initiated, cardiomyocyte iNOS/NADPH oxidase-dependent, peroxynitrite-mediated signaling pathway that contributes to posttraumatic myocardial apoptosis. Therapeutic interventions that block this signaling cascade may attenuate posttraumatic cardiac injury and reduce the incidence of secondary organ dysfunction after trauma.

apoptosis; signal transduction; heart; cytokines

IT IS WELL RECOGNIZED that blunt chest trauma causes direct heart damage (i.e., cardiac contusion and vascular lacerations) from mechanical force. However, several groups have reported that blunt chest trauma causes myocardial infarction (MI) even in the absence of coronary artery dissection or direct mechanical cardiomyocyte injury (10, 23, 24). Moreover, a recent multi-hospital clinical study (10) demonstrated that although blunt chest trauma creates the greatest risk (8-fold) for MI, abdominal or pelvic trauma also dramatically increases the risk for MI (6-fold). These results strongly suggest that besides the primary injury to the heart that occurs immediately after trauma, trauma may cause secondary heart injury through yet to be identified mechanisms.

In a recent study, we demonstrated that significant cardiomyocyte apoptosis, a cell death pathway that contributes to MI, occurs in mice subjected to a nonlethal mechanical trauma. Administration of a nonselective caspase inhibitor immediately after trauma not only blocked cardiomyocyte apoptosis but also attenuated posttraumatic cardiac dysfunction (22). These results demonstrated that traumatic injury causes cardiomyocyte apoptosis, which contributes to posttraumatic organ dysfunction. Since mechanical trauma may cause cardiomyocyte apoptosis by initiating a pathological signal locally within the heart or as a result of systemic injury, it is important to further investigate whether mechanical trauma causes cardiomyocyte apoptosis by cardiomyocyte-localized factors or by systemic circulatory mediators.

Peroxynitrite is the biradical reaction product of nitric oxide (NO) and superoxide and is a highly toxic species that can oxidize or nitrate a variety of molecules and alter their function (3, 19). We (22) have previously demonstrated that in the traumatic heart, NO and superoxide production were both significantly increased and peroxynitrite was formed. However, a causative link between peroxynitrite overproduction and posttraumatic cardiomyocyte apoptosis has not been established. Moreover, the molecular sources that are responsible for increased NO and superoxide production in the traumatized heart remain unknown. Finally, signaling mechanisms that cause upregulation of NO/superoxide producing enzymes have not been identified.

Therefore, the aims of the present study were to 1) determine the origin [i.e., traumatic cardiomyocytes (TC) vs. traumatic plasma (TP)] of the pathological mediators that cause cardiomyocyte apoptosis after trauma; 2) identify specific factor(s) responsible for initiating cardiomyocyte apoptosis after trauma; and 3) delineate the intracellular signaling pathway by which cardiomyocyte apoptosis is initiated following traumatic injury.

**MATERIALS AND METHODS**

*Induction of sham traumatic or traumatic injury in adult male mice.* Nonlethal mechanical trauma was induced as previously described (22). In brief, male adult Swiss Webster mice were anesthetized with pentobarbital sodium (40 mg/kg). Mice were placed in a Noble-Collip drum, a 12-in.-diameter plastic wheel with internal shelves on which a mouse is traumatized as the wheel is rotated (200 revolutions at a rate of 40 rpm). Sham traumatic mice were subjected to the same...
revolved, but animals were taped to the inner wall of drum, thus
avoiding traumatic injury. After completion of the procedure, mice
were either immediately killed (for cardiomyocyte culture as de-
scribed below) or allowed to recover in a warmed chamber and killed
at the time points specified in RESULTS (for plasma collection). Exper-
iments were performed in adherence with National Institutes of Health
guidelines on the use of laboratory animals and were approved by the
Thomas Jefferson University Committee on Animal Care.

Cardiomyocyte culture and treatments. Hearts from sham trauma-
tized or traumatized mice were removed and perfused at 37°C for −3
min with a Ca2+-free bicarbonate-based buffer. The enzymatic diges-
tion was initiated by adding collagenase type B/D and protease type
XIV to the perfusion solution. When the hearts became swollen and
hard after −3 min of digestion, 50 μM Ca2+ was added to the enzyme
solution. Approximately 7 min later, the left ventricle was removed,
cut into several chunks, and further digested in a shaker for 10 min at
37°C in the same enzyme solution. The supernatant containing the
dispersed myocytes was filtered into a sterilized tube and centrifuged
at 800 g for 1 min. The cell pellet was then resuspended in bicarbon-
ate-based buffer containing 125 μM Ca2+. After the myocytes had
been pelleted by gravity for −10 min, the supernatant was aspirated,
and myocytes were resuspended in bicarbonate-based buffer contain-
ing 250 μM Ca2+. Myocytes were plated at 0.5–1 × 10^6 cells/cm² in
culture dishes precoated with mouse laminin. After 1 h of culture in
a 5% CO2 incubator at 37°C, the medium was changed to FBS-free
MEM.

To determine the origin of the pathological mediators that cause
cardiomyocyte apoptosis after trauma, cardiomyocytes were isolated
from either sham traumatic mice (normal cardiomyocytes (NC)) or
traumatic mice (TC) immediately after the trauma procedure and
cultured in vitro for 12 h with medium containing 10% plasma (4)
obtained from sham trauma (normal plasma (NP)) or trauma animals
(TP; isolated from a different group of animals 1.5 h after trauma). To
identify the specific factor(s) responsible for initiating cardiomyocyte
apoptosis after trauma, cardiomyocytes isolated from traumatized
mice were incubated with one of the following factors for 12 h: 1) NP;
2) TP; 3) Cytomix (a mixture of 1,000 U/ml IFN-γ, 1 ng/ml IL-1β,
and 10 ng/ml TNF-α) (18); 4) individual components of Cytomix at
to one three times the concentration used in the mixture; or 5) TP plus
anti-TNF-α antibody (1 μg/ml) (11). To delineate the role of oxidative/
nitrosative stress in cardiomyocyte apoptosis initiated by traumatic
injury, cardiomyocytes isolated from traumatized mice were subjected
to one of the following treatments: 1) NP; 2) TP; 3) TP plus 1400W
[a highly selective inducible NO synthase (iNOS) inhibitor, 400 μM
(9)]; 4) TP plus apocynin [a NADPH oxidase inhibitor, 100 μM (11)];
or 5) TP plus FP15 [a peroxynitrite decomposition catalyst, 100 μM
(20)]. Each experimental condition listed above was examined in
triplicate using cardiomyocytes isolated from the same animal.
Results from the same animal were averaged and counted as one sample.
At least seven to nine animals were used in each group. At the end of
the experiments, cardiomyocytes were lysed in lysis buffer, and
apoptosis was determined by caspase-3 activity assay as described in
our previous study (21).

Determination of total NOx content in cardiomyocyte culture me-
dium. At the end of in vitro experiments, cardiomyocyte culture
medium was collected. NO and its metabolic products (NO2 and
NO3), collectively known as NOx, were determined in culture medium.
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Immunoblot analysis. Proteins from cardiomyocytes were sepa-
rated on SDS-PAGE gels, transferred to nitrocellulose membranes,
and Western blotted with monoclonal antibodies against gp91phox or
iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) followed by
secondary antibody incubation. The blot was developed with Super
Signal Femto Reagent (Pierce Biotechnology, Rockford, IL) and
visualized with a Kodak Image Station 400. Blot densities were
analyzed with Kodak 1D software.

Statistical analysis. All values in the text and figures are presented
as means ± SE of n independent experiments. All data (except
Western blot density) were subjected to ANOVA followed by Bon-
ferroni correction for post hoc t-test. Western blot densities were
analyzed with the Kruskal-Wallis test followed by Dunn post hoc test.
Probabilities of 0.05 or less were considered to be statistically signif-
icient.

RESULTS

Proapoptotic mediators present in TP. Noble-Collip drum
exposure is a well-accepted traumatic model that results in a
whole body nonpenetrative mechanical trauma (6). Proapop-
totic mediators can thus be produced either within TCs them-
selese or by remote organs and circulate to the heart. In a
recent study, Carlson et al. (4) reported that the addition of
10% plasma harvested from rats subjected to burn trauma in
culture medium caused significant cardiomyocyte apoptosis,
suggesting that plasma from burn trauma animals contains
proapoptotic factors. However, whether mechanical trauma
may also result in the accumulation of proapoptosis factors in
the plasma, thus stimulating postraustra cardiomyocyte apo-
ptosis, remains unknown. Moreover, whether traumatic injury
may activate apoptotic machinery within cardiomyocytes
themselves has never been previously investigated. As shown
in Fig. 1, the addition of TP to TCs caused a 2.2-fold increase
in caspase-3 activity. No significant caspase-3 activation was
observed when TCs were cultured with NP. However, significant
caspase-3 activation (1.9-fold) was observed when TP
was added to NCs. These results provide clear evidence that
proapoptotic mediators are present in the plasma of traumatic
animals, not within TCs themselves.

Time course of cytokine production in traumatic animals.
Substantial evidence exists that cytokines, including TNF-α,
IFN-γ, and IL-1β, are proapoptotic factors that are responsible
for cardiomyocyte apoptosis under many pathological condi-
tions (2, 5, 15). Having demonstrated that proapoptotic medi-
ators in traumatic animals are present in plasma, we wanted to
determine whether plasma levels of cytokines are elevated in
traumatic animals. Animals were killed at different time points
after trauma, blood samples were obtained, and plasma IFN-γ,
IL-1β, and TNF-α concentrations were determined using
ELISA assay kits. As shown in Fig. 2, plasma IL-1β, IFN-γ,
and TNF-α levels were all significantly elevated in traumatic
animals, although individual cytokines exhibited different time
courses after trauma. Noticeably, all three cytokines peaked at
or before 1.5 h after traumatic injury, a time point that is −2 h
ahead of significant cardiomyocyte apoptosis occurring after
trauma (22).

Identification of a specific cytokine responsible for the in-
duction of cardiomyocyte apoptosis. Since levels of TNF-α,
IFN-γ, and IL-1β were all significantly increased in traumatic
animals (Fig. 2) and a mixture of these three cytokines, termed
Cytomix, has been previously shown to stimulate apoptotic cell
death in fibroblasts (17) and in β-cells (16), we then deter-
mined whether these three cytokines (Cytomix) may cause
significant apoptosis in TCs and, if so, to further identify the
specific cytokine(s) responsible for trauma-induced cardiomyocyte apoptosis. As shown in Fig. 3, incubation of TCs with NP supplemented with Cytomix (a mixture of 1,000 U/ml IFN-γ, 1 ng/ml IL-1β, and 10 ng/ml TNF-α) (18) for 12 h resulted in significant caspase-3 activation that was comparable with that seen in cardiomyocytes treated with TP, suggesting that cytokines are likely to be responsible for TP-induced cardiomyocyte apoptosis. The addition of IFN-γ or IL-1β alone (at the same concentration used in the mixture, as shown in Fig. 3, or up to 3 times of the concentrations used in the mixture, data not shown) had no significant effect on caspase-3 activity. However, the addition of TNF-α alone at the concentra-

tion used in the Cytomix significantly increased cardiomyocyte caspase-3 activity (Fig. 3), indicating that TNF-α is the cytokine that is responsible for the cardiomyocyte apoptosis induced by Cytomix.

To obtain more evidence to support a causative role of TNF-α in TP-induced cardiomyocyte apoptosis, two additional experiments were performed. In the first series of experiments, an anti-TNF-α antibody was added to the TP at 1 μg/ml, a concentration that blocks TNF-α-induced cardiomyocyte apoptosis (11). As shown in Fig. 4, neutralization of TNF-α almost completely blocked TP-induced cardiomyocyte caspase-3 activation. In the second series of experiments, TNF-α−/− mice were subjected to traumatic injury as described above, and their plasma was isolated 1.5 h after trauma and added to cardiomyocytes isolated from wild-type mice subjected to traumatic injury. As shown in Fig. 4, the addition

**Fig. 1.** In vitro experimental evidence showing that proapoptotic mediators are present in trauma plasma (TP). Normal cardiomyocytes (NC) or traumatic cardiomyocytes (TC) were cultured with normal plasma (NP) or TP for 12 h, and cardiac caspase-3 activation (A) and DNA fragmentation (B) were determined by Cell Death Detection ELISAplus (Roche Applied Science, Indianapolis, IN). Results were normalized against the mean value of caspase-3 activity and nucleosomes content in the NC + NP group. To determine whether incubation of cardiomyocytes with TP may also cause necrosis, the nucleo-
some content in culture medium (supernatant) was also measured (C). n = 7–9 mice/group. **P < 0.01 vs. the NC + NP group.

**Fig. 2.** Time course of cytokine production in traumatic mice. Pentobarbital-anesthetized mice (n = 10 mice/group) were subjected to sham trauma or trauma, and their plasma samples were obtained at the time points specified. *P < 0.05 and **P < 0.01 vs. the sham trauma group.
of TP from TNF-α−/− mice failed to induce significant caspase-3 activation. These results provide firm evidence that the increased TNF-α concentration in traumatic animals is the primary cause for the cardiomyocyte apoptosis observed in traumatic animals.

Downstream signaling pathway linking TNF-α overproduction and cardiomyocyte apoptosis. In a recent study, we (22) demonstrated that NO and superoxide production were markedly increased in traumatic hearts. Having demonstrated that TNF-α is the primary cytokine responsible for posttrauma cardiomyocyte apoptosis, we further tested the hypothesis that TNF-α may initiate cardiomyocyte apoptosis by upregulating iNOS/NADPH oxidase expression with resultant oxidative/nitrative stress. As shown in Figs. 5–7, iNOS and gp91phox (a major component of NADPH oxidase) protein expression were upregulated, NO and superoxide production were increased, and the production of peroxynitrite, a proapoptotic/cytotoxic molecule, was significantly increased in cardiomyocytes exposed to TP. More importantly, neutralization of TNF-α with anti-TNF-α antibody almost completely blocked TP-induced oxidative and nitrative stress (Figs. 4–6). These results provide clear evidence that TNF-α present in the TP is the primary factor that causes significant oxidative/nitrative stress in TCs.

To further dissect the cause-effect relationship between TP-initiated oxidative/nitrative stress and TP-induced cardiomyocyte apoptosis, cardiomyocytes were treated with 1400W (a selective iNOS inhibitor), apocynin (a NADPH oxidase inhibitor), or FP15 (a peroxynitrite decomposition catalyst) immediately after the addition of TP. As shown in Fig. 8, treatment with 1400W significantly reduced NO production (A) without affecting superoxide production (B). In contrast, treatment with apocynin significantly reduced superoxide production without affecting NO production (Fig. 8B). Treatment with FP15 affected neither NO nor superoxide production (Fig. 8, A and B). However, all three treatments significantly reduced myocardial nitrotyrosine content (Fig. 8C) and reduced TP-induced caspase-3 activation (Fig. 8D). Taken together, our data show that blockade of iNOS/NADPH oxidase activity with 1400W/apocynin or increasing peroxynitrite decomposition with FP15 markedly reduced posttraumatic cardiomyocyte apoptosis. These results demonstrated that TNF-α-initiated...
oxidative/nitrative stress plays a causative role in TP-induced cardiomyocyte apoptosis.

DISCUSSION

We have made several novel observations in the present study. First, we demonstrated, for the first time, that proapoptotic mediators that trigger cardiomyocyte apoptosis in traumatic animals are present in the TP rather than the cardiomyocytes themselves. Second, we identified that TNF-α is the most critical factor that contributes to posttraumatic cardiomyocyte apoptosis. This result is consistent with a recent study (12) demonstrating that TNF-α plays a critical role in myocardial dysfunction and apoptosis during hindlimb ischemia and reperfusion. However, our result is different from that reported by Carlson et al. (4) in a burn trauma model in which endotoxin, not TNF-α, was shown to be responsible for cardiomyocyte apoptosis, indicating that proapoptotic factors differ in different traumatic models. Finally, we provided clear evidence that peroxynitrite, the biradical reaction product between NO and superoxide, is the downstream molecule that mediates TC apoptosis. Taken together, our present study demonstrates that there exists a TNF-α-initiated, cardiomyocyte iNOS/NADPH oxidase-dependent, and peroxynitrite-mediated signaling pathway that contributes to posttraumatic myocardial apoptosis.

In a recent study, we (22) demonstrated that the mild trauma model used in our study causes cardiomyocyte death by apoptosis, not by necrosis. Our present study further demonstrated that pathological factors that mediate cardiomyocyte apoptosis are not formed by cardiomyocytes themselves. The proapoptotic effect of TNF-α has been extensively investigated in a variety of in vitro and in vivo models (7, 13). TNF-α causes apoptotic cell death by two different yet interrelated mechanisms (14). First, by binding to its cell surface receptors, TNF-α triggers apoptosis signaling through the activation of caspase-8 and subsequent caspase-3 activation (extrinsic pathway). Second, TNF-α is a powerful proinflammatory factor that upregulates the expression of many inflammatory genes, including genes encoding iNOS and NADPH oxidase (14). Therefore, TNF-α can also stimulate apoptosis through the activation of caspase-9 (which, in turn, activates caspase-3) as a result of overproduction of reactive oxygen/nitrogen species and mitochondrial injury (intrinsic pathway). Our present results demonstrated that although multiple cytokines are significantly increased in traumatic animals and the addition of Cytomix induced significant cardiomyocyte apoptosis, only TNF-α, not IFN-γ or IL-1β, induced significant cardiomyocyte apoptosis when added alone. Moreover, TP-induced oxidative/nitrative stress and cardiomyocyte apoptosis were significantly reduced when TNF-α was neutralized by an anti-TNF-α antibody. In addition, TP isolated from TNF-α−/− mice failed to induce significant caspase-3 activation. These results clearly demonstrated that TNF-α is the most important proapoptotic molecule that causes posttraumatic cardiomyocyte apoptosis.

Fig. 7. TP-induced peroxynitrite formation (as measured by nitrotyrosine content) and its inhibition by anti-TNF-α antibody. n = 7–9 mice/group. **P < 0.01 vs. the NP group; ##P < 0.01 vs. the TP group.

Fig. 8. Effect of iNOS inhibition (1400W), NADPH oxidase inhibition (apocynin), and peroxynitrite scavenging (FP15) on TP-induced NO production (A), superoxide generation (B), peroxynitrite formation (C), and caspase-3 activation (D). n = 7–9 mice/group. **P < 0.01 vs. the NP group; ##P < 0.01 vs. the TP group.
It is well recognized that TNF-α is the primary cytokine responsible for the upregulation of iNOS and NADPH oxidase gene expression under inflammatory conditions (8). Moreover, considerable evidence exists that increased NO and superoxide production results in the formation of peroxynitrite, a highly cytotoxic molecule that causes significant cardiomyocyte apoptosis (2). Our present study demonstrated that treatment with an iNOS inhibitor, a NADPH oxidase inhibitor, or a peroxynitrite scavenger dramatically attenuated posttraumatic cardiomyocyte apoptosis. These results strongly suggest that the TNF-α-induced overproduction of reactive oxygen/nitrogen species is the primary mechanism by which TNF-α results in cardiomyocyte apoptosis in this traumatic injury model.

In conclusion, our study demonstrated that cardiomyocyte apoptosis caused by mechanical trauma is initiated by TNF-α produced by injured peripheral tissues and mediated by overproduction of cytotxic reactive oxygen/nitrogen species within cardiomyocytes. As posttraumatic multiorgan failure is becoming a critical factor determining the overall mortality and quality of life after trauma, those therapeutic interventions that are capable of blocking this signaling cascade may attenuate post-traumatic cardiac injury and reduce the incidence of secondary organ dysfunction after trauma.

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