Angiotensin II Induces Tumor Necrosis Factor Biosynthesis in the Adult Mammalian Heart Through a Protein Kinase C–Dependent Pathway

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Background—Previous studies suggest that angiotensin II (Ang II) upregulates the expression of tumor necrosis factor (TNF) in nonmyocyte cell types; however, the effect of Ang II on TNF expression in the adult mammalian heart is not known.

Methods and Results—To determine whether Ang II was sufficient to provoke TNF biosynthesis in the adult heart, we examined the effects of Ang II in isolated buffer-perfused Langendorff feline hearts. Ang II (10⁻⁷ mol/L) treatment resulted in a time- and dose-dependent increase in myocardial TNF mRNA and protein biosynthesis in the heart as well as in cultured adult cardiac myocytes. The effects of Ang II on myocardial TNF mRNA and protein synthesis were mediated through the angiotensin type 1 receptor (AT₁R), insofar as an AT₁R antagonist (AT₁a) blocked the effects of Ang II, whereas an angiotensin type 2 receptor (AT₂R) antagonist (AT₂a) had no effect. Stimulation with Ang II led to the activation of nuclear factor-κB and activator protein-1 (AP-1), two transcription factors that are important for TNF gene expression. Nuclear factor-κB activation was accompanied by phosphorylation of IκBα on serine 32 as well as degradation of IκBα, suggesting that the effects of Ang II were mediated through an IκBα-dependent pathway. The important role of protein kinase C (PKC) was suggested by studies in which a phorbol ester triggered TNF biosynthesis, and a PKC inhibitor abrogated Ang II–induced TNF biosynthesis.

Conclusions—These studies suggest that Ang II provokes TNF biosynthesis in the adult mammalian heart through a PKC-dependent pathway. (Circulation. 2002;105:2198-2205.)

Key Words: heart failure □ angiotensin □ genes □ growth substances □ proteins

One of the recent conceptual advances with regard to our understanding of the pathogenesis of heart failure has been the insight that heart failure may progress as the result of the overexpression of biologically active “neurohormonal” molecules, such as norepinephrine and angiotensin II (Ang II), which lead to disease progression by virtue of the “toxic effects” that they exert in the heart and the circulation.1–3 This insight has, in turn, provided the rational basis for using therapeutic antagonism of known neurohormonal targets.

Relevant to this discussion is the recent observation that a second class of biologically active molecules, termed cytokines, also has been identified in the setting of heart failure.7,8 Moreover, recent studies have shown that analogous to the situation with neurohormones, the overexpression of certain cytokines such as tumor necrosis factor (TNF) leads to disease progression in experimental models by virtue of the direct toxic effects that this protein exerts in the heart and the circulation.9,10 Whereas these neurohormonal and cytokine systems have been regarded heretofore as functionally distinct biological systems, 3 lines of observation foreshadowed the interesting possibility that Ang II might upregulate TNF gene expression in the adult mammalian heart. First, Ang II activates 2 transcription factors that are important in mediating TNF gene expression: nuclear factor-kB and activator protein-1 (AP-1).11–13 Second, experimental studies have shown that Ang II provokes TNF biosynthesis in various nonmyocyte cell types.14–16 Third, chronic blockade of the angiotensin type 1 receptor (AT₁R) results in a significant decrease in circulating levels of TNF in patients with hypertension and heart failure.17,18 To determine whether Ang II was sufficient to regulate myocardial TNF biosynthesis, we systematically examined the effects of pathophysiological concentrations of Ang II on TNF mRNA and protein biosynthesis in the adult mammalian heart.

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Methods

Myocardial TNF Biosynthesis Ex Vivo
Myocardial TNF biosynthesis was assessed ex vivo with the use of a modified Langendorff perfusion apparatus exactly as described, with the exception that the hearts were perfused for 180 minutes with Krebs-Henseleit buffer containing Ang II (10⁻⁷ to 10⁻⁵ mol/L) or diluent. To determine whether the AT,R and/or the angiotensin type 2 receptor (AT,R) was responsible for mediating the effects of Ang II, we pretreated hearts for 60 minutes with losartan (10⁻⁴ mol/L), a specific AT,R antagonist, or with PD123319 (10⁻⁴ mol/L), a specific AT,R antagonist, before stimulating the hearts with Ang II (10⁻⁷ mol/L) for 180 minutes.

Myocardial TNF mRNA Biosynthesis
To assess Ang II–induced changes in TNF mRNA synthesis, a 500-ng sample of myocardium was excised from the suspended heart (carefully sparing the large epicardial vessels) immediately after exposure to buffer containing 10⁻⁷ mol/L Ang II (“time 0”). Myocardial samples were obtained every 30 minutes thereafter, for a total of 180 minutes. TNF mRNA was detected with a ribonuclease probe specific for feline TNF mRNA. Protected mRNA was resolved by electrophoresis with a 6% acrylamide/8 mol/L urea denaturing gel. Labeled unproctected riboprobe (1000 cpm) was run in a separate lane to serve as a size marker. After drying, the gel was exposed overnight to x-ray film at −70°C in the presence of intensifying screens.

Myocardial TNF Protein Biosynthesis
Starting at time 0, 0.5 mL of recirculating buffer was collected every 30 minutes for a total of 180 minutes. TNF protein levels were determined by ELISA with the use of a commercially available kit that recognizes both human and feline TNF (Human Ultrasensitive Cytoscreen, Biosource). Results are expressed as picograms of TNF per milliliter of superfusate.

Cellular Source for Myocardial TNF Biosynthesis
Previous studies from this laboratory have shown that endotoxin, nitric oxide, and hemodynamic overloading are sufficient to induce TNF biosynthesis in cultured adult cardiac myocytes. To determine whether Ang II stimulation was sufficient to provoke TNF biosynthesis in adult cardiac myocytes, we examined TNF mRNA and protein biosynthesis in diluent and Ang II–treated (10⁻⁷ mol/L) adult cardiac myocyte cultures. The methods for isolating adult feline cardiac myocytes, the characteristics and purity of the cell culture system (<2% contaminating cell types), and the serum-free cell culture conditions used in this study have been described in detail elsewhere. Total RNA was extracted at 0, 1, and 6 hours after addition of Ang II or diluent, and RNase protection assays were performed as described above. TNF cytosolic protein levels were determined at time 0 and 1 and 6 hours after stimulation with Ang II or diluent as described. TNF protein levels were determined by ELISA, with a commercially available kit that recognizes both human and feline TNF (Biosource). Results are expressed as picograms of TNF per milligram of total cellular protein.

Transcriptional Regulation of TNF
Previous studies have shown that Ang II is capable of activating NF-κB, as well as AP-1, in a variety of cell types. To determine whether Ang II–induced TNF biosynthesis in the heart was sufficient to activate NF-κB and/or AP-1 in the adult heart, we performed electrophoretic mobility shift assays (EMSA) with nuclear extracts from Ang II–treated hearts. Briefly, freshly isolated, buffer-perfused cat hearts were treated with 10⁻⁷ mol/L Ang II or diluent for a total of 180 minutes. Starting at time 0 and every 30 minutes thereafter, we obtained myocardial biopsy specimens (500 mg) from the Ang II–stimulated hearts. All myocardial samples were immediately frozen in liquid nitrogen and stored at −70°C. Nuclear isolations and EMSAs were performed exactly as described, with the use of a double-stranded consensus sequence for NF-κB (5'-GTTGAGGGACTTCCCCAGGCTCC'-3', Santa Cruz Inc, Santa Cruz, Calif) or AP-1 (5'-CGGCTGTAGCTACAGCGGAA-3', Santa Cruz Inc). The specificity of the binding was determined in “cold” competition experiments with either a 25-fold and 50-molar excess of the respective unlabeled oligonucleotides. To determine the components of the NF-κB and AP-1 DNA–protein binding complexes, we performed supershift assays by incubating the nuclear extracts with 2 μg of anti-human polyconal antibodies directed against various components of NF-κB, including p50, p52, p65 (Rel A), Rel B, and cRel (Santa Cruz Inc) or components of AP-1, including c-fos and c-jun (Santa Cruz Inc), as described.

Mechanisms of NF-κB Activation by Ang II
Previous studies have examined the mechanisms for Ang II–induced activation of AP-1 DNA binding. Accordingly, in the present report, we studied potential mechanisms for Ang II–induced NF-κB activation. Previous studies have shown that phosphorylation of IκBα on the serine (Ser) 32 and 36 residues is a critical regulatory step that leads to the degradation of IκBα, with subsequent nuclear translocation of NF-κB. To determine whether Ang II stimulation was sufficient to phosphorylate IκBα on Ser 32 and to lead to the degradation of IκBα, we studied stimulated adult cardiac myocyte cultures with Ang II. Briefly, freshly isolated cardiac myocytes were cultured in Dulbecco’s Modified Eagle Medium (DMEM) for 24 hours, after which the DMEM was changed to labeling medium (9 parts methionine-free DMEM:1 part DMEM with methionine, 0.2% BSA) which contained 35S-methionine/cysteine (100 μCi/mL; Easy-Tag, DuPont NEN). After labeling for 10 hours, the cells were treated for 15 minutes or 30 minutes with diluent or Ang II (10⁻⁷ mol/L). The cells were then lysed, immunoprecipitated, analyzed by SDS-PAGE, soaked in a fluorographic solution, dried, and exposed to light-sensitive film as described.

Role of the Protein Kinase C Pathway in TNF Biosynthesis
Previous studies have shown that Ang II activates protein kinase C (PKC) in various cell types, including cardiac myocytes. To determine the role of the PKC pathway in Ang II–induced TNF biosynthesis, we performed 4 interrelated studies. First, we measured myocardial diacylglycerol (DAG) levels in response to Ang II stimulation. Second, we treated hearts with phorbol 12-myristate-13-acetate (PMA; 10⁻⁷ mol/L), a PKC agonist, for 180 minutes. Third, we pretreated another group of hearts for 60 minutes with chelerythrine (10⁻⁷ mol/L), a PKC inhibitor, before stimulation with Ang II (10⁻⁷ mol/L) for 180 minutes. At the end of the studies, hearts were assayed for TNF mRNA and protein levels, as described above. Fourth, to confirm the role of PKC in Ang II–mediated TNF biosynthesis, we directly measured PKC activity in Ang II–stimulated myocytes.

Myocardial DAG Assay
To determine whether Ang II treatment resulted in activation of the phospholipase C pathway with subsequent generation of DAG, we measured myocardial DAG mass in hearts that had been stimulated with diluent or Ang II. DAG was measured in myocardial biopsy specimens (∼100 mg) that were obtained at 0, 10, and 30 minutes after stimulation with diluent or Ang II (10⁻⁷ mol/L), either in the presence or absence of losartan (10⁻⁴ mol/L) or with PD123319 (10⁻⁶ mol/L). The biopsy specimens were snap-frozen and pulverized with a mortar and pestle under a stream of liquid nitrogen. Pulverized myocardial tissue was homogenized in 1 mL of buffer (135 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L MgSO₄, 5 mmol/L glucose, 1.5 mmol/L CaCl₂, 10 mmol/L Hepes, pH 7.2) with the use of a Polytron homogenizer (4 strokes, 20-second bursts). The pulverized myocardial tissue was extracted with 4 mL of chloroform:methanol (1:1 vol/vol), and the mixture was vortexed and allowed to separate on ice for 10 minutes. The aqueous phase was aspirated and used for determination of the protein concentration while the lipid phase was vacuum dried and used to determine DAG concentrations. The lyophilized myocardial extracts were resus-
PKC Activity Assay

To determine whether Ang II stimulation was sufficient to activate PKC, we measured PKC activity in cultured adult cardiac myocytes by using a commercially available assay (Amersham). In preliminary control studies, we determined that treatment (0 to 60 minutes) with either diluent (○) or ∼10⁻⁶ mol/L Ang II (●), AT₁a indicates AT₁ receptor antagonist (losartan); AT₂a, AT₂ receptor antagonist (PD123319). Data are representative of 3 separate experiments for each condition. Main panel of B shows dose-dependent effects of Ang II (10⁻¹⁰ to 10⁻⁵ mol/L; n=3 hearts for each dose); inset shows time course (0 to 180 minutes) for TNF protein synthesis after stimulation with either diluent (○) or ∼10⁻⁶ mol/L Ang II (●). Data are expressed as picomoles of phosphatidic acid per milligram of protein.

Figure 1. Ang II–induced myocardial TNF biosynthesis in adult heart. A, TNF mRNA expression (RNase protection assay) was assessed ex vivo in diluent-treated and Ang II–treated (10⁻⁷ mol/L) (0 to 180 minutes) buffer-perfused Langendorff hearts, in the presence or absence of 10⁻⁶ mol/L PD123319, an AT₂ receptor antagonist (AT₂a), or 10⁻⁶ mol/L losartan, an AT₁ receptor antagonist (AT₁a). Data are representative of 3 separate experiments for each condition. B, Myocardial TNF protein production was assessed in superfusates of Ang II–treated hearts with ELISA in the presence or absence of PD123319 (10⁻⁶ mol/L) or losartan (10⁻⁶ mol/L) pretreatment. Data are representative of 3 separate experiments for each condition. Main panel of B shows dose-dependent effects of Ang II (10⁻¹⁰ to 10⁻⁵ mol/L; n=3 hearts for each dose); inset shows time course (0 to 180 minutes) for TNF protein synthesis after stimulation with either diluent (○) or ∼10⁻⁶ mol/L Ang II (●). Data are expressed as picomoles of phosphatidic acid per milligram of protein.

Statistical Analysis

Data are expressed as mean±SEM. One-way ANOVA was used to test for differences between group means. When appropriate, post hoc multiple comparisons were performed to test for differences between control and experimental groups (Dunnett’s test) or between experimental groups (Newman-Keuls test). Two-way ANOVA was used to evaluate overall differences in the means between different groups as a function of time. Significant differences were said to exist at a value of P<0.05.
Myocardial TNF Biosynthesis Ex Vivo

In preliminary control experiments that used $10^{-10}$ to $10^{-3}$ mol/L Ang II, we determined that the EC$_{50}$ for Ang II–induced effects was between $10^{-8}$ and $10^{-7}$ mol/L; therefore, we used $10^{-7}$ mol/L Ang II, a pathophysiologically relevant concentration of Ang II, for the majority of the experiments detailed below.

Myocardial TNF mRNA Biosynthesis

The salient finding shown by Figure 1A is that treatment with $10^{-7}$ mol/L Ang II resulted in a time-dependent increase in TNF mRNA biosynthesis in the adult heart. TNF mRNA expression was detectable within 30 minutes and increased strikingly within 180 minutes. Similar qualitative findings with regard to TNF mRNA expression were observed in 2 additional Ang II–treated hearts. In contrast, TNF mRNA was not detectable in the diluent-treated hearts. Pretreatment of hearts with losartan, an AT$_1$ R antagonist (AT$_1$a) completely abrogated the Ang II–induced increase in TNF mRNA levels, whereas pretreatment with PD123319, an AT$_2$ R antagonist (AT$_2$a), had no effect on Ang II–induced TNF mRNA biosynthesis.

Myocardial TNF Protein Biosynthesis

Figure 1B also shows that Ang II ($10^{-10}$ to $10^{-3}$ mol/L) provoked a concentration-dependent increase in TNF biosynthesis. The inset of Figure 1B shows that TNF protein synthesis was detectable as early as 60 minutes after treatment with $10^{-7}$ mol/L Ang II and that TNF protein synthesis increased by $\approx$6-fold within 180 minutes. In contrast, TNF protein levels were not detectable in the diluent-treated hearts. Pretreatment of hearts with losartan (AT$_1$a) completely abrogated the Ang II–induced increase in TNF protein biosynthesis, whereas pretreatment with PD123319 (AT$_2$a) had no effect on Ang II–induced TNF protein biosynthesis. One-way ANOVA indicated that Ang II treatment led to a significant overall increase in TNF protein synthesis ($P<0.001$); post hoc multiple comparison testing (Dunnett’s test) showed that there were significant differences ($P<0.05$) from control values for Ang II concentrations $>10^{-7}$ mol/L.

Cellular Source for Myocardial TNF Biosynthesis

The important finding shown by Figure 2A is that TNF mRNA levels were barely detectable in the diluent-treated cultures, whereas TNF mRNA levels increased in a time-dependent manner in the Ang II–treated ($10^{-7}$ mol/L) myocyte cultures. Similar qualitative results were observed in two additional primary myocyte isolations. Figure 2B shows that TNF protein levels were negligible in diluent-stimulated myocyte cultures, whereas Ang II provoked a significant 15-fold increase in TNF protein levels by 24 hours. Two-way ANOVA indicated that there was a statistically significant ($P<0.001$) difference between groups with respect to Ang II–induced TNF protein biosynthesis.

Transcriptional Regulation of TNF

Figure 3A shows the temporal increase in NF-κB–DNA complexes in Ang II–treated ($10^{-7}$ mol/L) hearts. As shown,
was added to the nuclear extract. Consistent with our results regarding the role of the AT,R and the AT,R in Ang II–induced TNF mRNA and protein synthesis, there was no NF-κB nor AP-1 activation in the hearts that had been pretreated with losartan before Ang II stimulation. In contrast, pretreatment with PD123319 did not inhibit Ang II–induced NF-κB or AP-1 activation. Similar results for each of the above observations were obtained for 2 additional hearts.

**Mechanisms for Ang II–Induced NF-κB Activation**

As shown in Figure 4A, Ang II (10⁻⁷ mol/L) stimulation led to the rapid (<30 minutes) degradation (≈60% decrease) in the level of IκBα protein in cultured adult cardiac myocytes and led to the phosphorylation of IκBα on Ser32 (Figure 4B), consistent with the rapid degradation of the phosphorylated IκBα protein reported in nonmyocyte cell types.26

**Role of the PKC Pathway in TNF Biosynthesis**

**Myocardial DAG Levels**

Figure 5A shows a representative thin-layer chromatograph of myocardial extracts from Ang II–stimulated (10⁻⁷ mol/L) hearts; Figure 5B shows the results of group data. Ang II stimulation led to a rapid (<10 minutes) 3-fold increase in myocardial DAG levels. The Ang II–induced increase in DAG mass was completely inhibited by losartan (10⁻⁶ mol/L), whereas pretreatment with PD123319 (10⁻⁶ mol/L) had no effect on Ang II–induced DAG mass. ANOVA indicated that the Ang II–induced overall changes in DAG mass were statistically significant (P<0.001); post hoc analysis ANOVA testing (Dunnett’s) showed that DAG levels were significantly different (P<0.05) from control values at 10 and 30 minutes after Ang II stimulation as well as after pretreatment with PD123319; however, the DAG levels were not significantly different from control values after pretreatment with losartan.

**PKC Activity in Cardiac Myocytes**

Figure 5C shows that Ang II stimulation led to a rapid 3-fold increase in PKC activity within 15 minutes in cultured cardiac myocytes. In contrast, there was no significant change in PKC activity in diluent-treated myocytes. Furthermore, when myocytes were pretreated with chelerythrine (10⁻⁶ mol/L)
mol/L) before Ang II stimulation, PKC activity was completely abrogated. Stimulation of myocytes with the PKC agonist PMA (10^{-7} mol/L) also resulted in a 3.8-fold increase in PKC activity within 15 minutes. One-way ANOVA indicated that there were significant differences in the values for PKC activity in Ang II–treated myocytes (P<0.001); post hoc ANOVA testing (Dunnett’s) indicated that PKC activity was significantly different from control at 5 and 15 minutes after Ang II stimulation as well as after stimulation with PMA (P<0.01), whereas there was no significant difference from control values after pretreatment with chelerythrine (P>0.05). Finally, the Ang II–induced increase in PKC activity was completely inhibited by 10^{-6} mol/L losartan (P>0.05 compared with diluent), whereas pretreatment with 10^{-6} mol/L PD123319 had no effect on Ang II–induced PKC activity (P<0.01 compared with diluent). Taken together, these results suggest that Ang II increases PKC activity in the adult feline heart through activation of AT1R.

**Effect of Inhibition and Activation of PKC on TNF Biosynthesis**

To determine the role of the PKC pathway in Ang II–mediated TNF biosynthesis, we used two interrelated approaches. First, as shown in Figure 6, A and B, when hearts were stimulated with 10^{-7} mol/L PMA, a PKC agonist, we observed a robust induction of TNF mRNA and protein synthesis, respectively. Second, when hearts were pretreated with the PKC inhibitor chelerythrine (10^{-6} mol/L) before stimulation with 10^{-7} mol/L Ang II, we were able to completely abrogate TNF mRNA and protein synthesis. Taken together, these results suggest that Ang II–induced biosynthesis of TNF occurs through a PKC-dependent pathway.

**Discussion**

The major conclusion to be drawn from this study is that Ang II is sufficient to provoke TNF biosynthesis in the adult mammalian heart. Three distinct but mutually complementary lines of evidence support this statement. First, treatment of isolated, buffer-perfused hearts with Ang II resulted in a rapid and robust increase in TNF mRNA (Figure 1A) and protein synthesis (Figure 1B). Consistent with previous observations from this laboratory, neither TNF mRNA nor protein was observed in diluent-treated hearts.\(^{19-21}\) Second, these findings were evident at the level of the intact ventricle as well as in the isolated cardiac myocyte itself. That is, stimulation of nonmyocyte cell types. Finally, the effects of Ang II on TNF mRNA and protein synthesis and NF-κB and AP-1 activation were mediated exclusively through the AT1 receptor, insofar
TNF GAPDH

Figure 6. Effects of Ang II on TNF biosynthesis are mediated by PKC. Buffer-perfused Langendorf hearts were treated for 180 minutes with 10⁻⁷ mol/L Ang II in the presence or absence of chelerythrine (CE; 10⁻⁶ mol/L) or with PMA (10⁻⁷ mol/L); TNF (A) mRNA and (B) protein levels were determined (n=3 hearts for each condition tested).

as pretreatment with losartan (an AT₁ receptor antagonist) completely abolished the effects of Ang II on TNF biosynthesis, whereas pretreatment with an AT₂ receptor antagonist (PD123319) had no effect on Ang II–induced TNF biosynthesis (Figures 1 and 2).

A second important finding of these studies was that the effects of Ang II on TNF biosynthesis were PKC dependent. That is, we were able to mimic the effects of Ang II on TNF mRNA (Figure 6A) and protein biosynthesis (Figure 6B) by using a concentration of phorbol ester that was sufficient to activate PKC (Figure 5C). Moreover, we were able to completely abrogate TNF mRNA and protein biosynthesis (Figure 6, A and B) when the hearts were pretreated with a concentration of a PKC inhibitor (chelerythrine) that completely blocked Ang II–induced PKC activation (Figure 5C).

A third interesting finding of the present study was that stimulation of isolated adult cardiac myocytes with Ang II led to the degradation of IκBα (Figure 4B) and to the phosphorylation of IκBα on the critical serine 32 residue (Figure 4B). These findings are consistent with previous studies that have shown that phosphorylation of IκBα on the serine 32 and 36 residues is a critical regulatory step that leads to the degradation of IκBα, with subsequent nuclear translocation of NF-κB.²⁶,²⁷ Taken together, the results of the present study suggest that Ang II provokes TNF biosynthesis in the adult heart through a pathway that involves the sequential activation of PKC, followed by activation of NF-κB and possibly AP-1. Nonetheless, although the results of these studies suggest an important role for Ang II–induced NF-κB and AP-1 activation with respect to TNF mRNA biosynthesis, the results of these studies should not be viewed as conclusive, insofar as NF-κB and AP-1 were not shown to be necessary for Ang II–induced TNF mRNA biosynthesis. Finally, the differences between the present report in adult feline myocytes and a previous report in neonatal rat myocytes,²¹ in which Ang II had no effect on TNF biosynthesis, may be related to the very low level of expression of AT receptors that has been reported in neonatal cardiac myocytes.³²

Conclusions

This study constitutes the initial demonstration that Ang II induces TNF biosynthesis in the adult mammalian heart through a PKC-dependent pathway. Although the exact biological significance of these findings is not known, the results of this study may provide potential insight into one of the mechanisms that is responsible for disease progression in the failing heart. That is, the results of this study suggest that there may be biologically important cross-talk between the renin–angiotensin system and proinflammatory cytokines in the heart. Functionally, this cross-talk may lead to self-sustaining or self-amplifying autocrine/paracrine feedback circuits in the failing heart, with the result that TNF and/or Ang II production becomes sustained or increases to the extent where the toxic effects of these molecules are sufficient to contribute to disease progression in the failing heart. Whether this mechanism provides a satisfactory explanation for the sustained expression of TNF and/or increased activation of the renin–angiotensin system that have been observed in hemodynamic pressure overloading³³ and or the failing human heart will require further study.

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