Peroxy nitrite Is a Major Contributor to Cytokine-Induced Myocardial Contractile Failure

Péter Ferdinandy, Hajira Danial, Ildikó Ambrus, Richard A. Rothery, Richard Schulz

Abstract—Proinflammatory cytokines depress myocardial contractile function by enhancing the expression of inducible NO synthase (iNOS), yet the mechanism of iNOS-mediated myocardial injury is not clear. As the reaction of NO with superoxide to form peroxynitrite markedly enhances the toxicity of NO, we hypothesized that peroxynitrite itself is responsible for cytokine-induced cardiac depression. Isolated working rat hearts were perfused for 120 minutes with buffer containing interleukin-1β, interferon-γ, and tumor necrosis factor-α. Cardiac mechanical function and myocardial iNOS, xanthine oxidoreductase (XOR), and NAD(P)H oxidase activities (sources of superoxide) were measured during the perfusion. Cytokines induced a marked decline in myocardial contractile function accompanied by enhanced activity of myocardial XOR, NADH oxidase, and iNOS. Cardiac NO content, myocardial superoxide production, and perfusate nitrotyrosine and dityrosine levels, markers of peroxynitrite, were increased in cytokine-treated hearts. The peroxynitrite decomposition catalyst FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), the NO synthase inhibitor Nω-nitro-arginine, and the superoxide scavenger tiron each inhibited the decline in myocardial function and decreased perfusate nitrotyrosine levels. Proinflammatory cytokines stimulate the concerted enhancement in superoxide and NO-generating activities in the heart, thereby enhancing peroxynitrite generation, which causes myocardial contractile failure. (Circ Res. 2000;87:241-247.)

Key Words: cytokines ■ nitric oxide synthase ■ xanthine ■ oxidoreductases ■ NADPH oxidase

Systemic inflammatory response syndrome and advanced heart failure syndrome resulting from diverse pathologic insults involve severe deterioration of cardiac mechanical function (see References 1 and 2 for review). Proinflammatory cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) produced by macrophages and endothelial and myocardial cells, have been shown to contribute to the evolution of myocardial dysfunction under these pathological conditions (see References 1 and 2 for review). The biochemical mechanism of proinflammatory cytokine-induced myocardial contractile failure is not entirely clear; however, this would be key information for the development of pharmacological tools to prevent or treat immune system–related cardiac dysfunction. We have previously shown that endotoxemia leads to the expression of inducible, Ca2+-independent NO synthase (iNOS) in cardiac muscle. This is mediated by proinflammatory cytokines, which increase iNOS activity in the cardiac myocyte, coronary vascular endothelium, and endocardium. The resultant excess production of NO in the intact heart contributes in part to the depression of cardiac mechanical function, as it can only be partially blocked by the nonselective NO synthase (NOS) inhibitor Nω-nitro-arginine methyl ester or the selective iNOS inhibitor mercaptoethylguanidine. How excess production of NO in the heart depresses myocardial function is unknown. The toxicity of NO is markedly enhanced by its reaction with superoxide to form peroxynitrite (see Reference 9 for review). We have shown that infusion of peroxynitrite into working rat hearts impairs cardiac contractile function by decreasing cardiac efficiency. Moreover, endogenous formation of peroxynitrite in the heart contributes to myocardial stunning in ischemia/reperfusion injury and to the spontaneous loss of cardiac function in the isolated working rat heart. Although some protective actions of peroxynitrite were found in myocardial ischemia-reperfusion injury in vivo, this was highly concentration-dependent and due to its reaction with free or protein-associated thiols to form nitrosothiols, and was lost at higher concentrations. Whether peroxynitrite in end-effect causes damage in the local environment depends on its concentration, site of formation, duration of exposure, and the concentration of antioxidants at that site.

The changes in the superoxide-generating ability of the heart during cytokine stimulation, necessary for peroxynitrite generation, have not been characterized. Possible sources of myocardial superoxide generation include the xanthine oxidoreductase (XOR) system, which comprises both xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (see Reference 10).
ere 16 for review) and NAD(P)H oxidoreductases. As NO itself is a cardioprotective and antioxidant molecule, we hypothesized that superoxide must also be upregulated in the heart as a consequence of cytokine exposure. Together with greater generation of NO through iNOS, this would result in enhanced peroxynitrite formation, which could contribute to the decline in myocardial function.

Materials and Methods

Hearts of male Sprague-Dawley rats (250 to 300 g) were perfused in working mode with recirculating Krebs-Henseleit solution containing (in mmol/L) glucose 11, pyruvate 5, Ca\(^2+\) 1.75, EDTA 0.5, and l-tyrosine 0.3 to measure peroxynitrite; 100 μmol/mL insulin; and 0.2% BSA. After 10 minutes of equilibration in the working mode (referred to as 0 minutes), hearts were perfused for either 0, 30, 60, 90, or 120 minutes in the absence (control, n=5 to 8 in each time point) or presence of 5 ng/mL human IL-1β, 9 ng/mL rat TNF-α, and 20 ng/mL human TNF-α (cycloxygenase, n=5 to 8, Genzyme). The ventricles were frozen and processed as described. Additional series of hearts were perfused with either 10 or 50 μmol/L FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III], a peroxynitrite decomposition catalyst; Calbiochem), its inactive form TPPS (50 μmol/L, Porphyrin Products), the NOS inhibitor N°-nitro-l-arginine (LNA, 3 μmol/L), or the superoxide scavenger tiron (100 μmol/L) in the presence or absence of cytokines.

NOS activities in ventricular homogenates were determined by the [\(^{14}\)C]citrulline assay. XDH and XO activities in the cytosolic fraction of ventricular tissue were determined by a fluorometric assay.

NADH and NADPH oxidase activities in ventricular homogenates were measured by a chemiluminescence assay in Krebs-Henseleit solution containing 0.25 mol/L HEPES (pH 7.4), 100 μmol/L lucigenin, and 50 μL of tissue homogenate in 1 mL of total volume using a Chrono-Log luminometer. The initial 15-second increase in luminescence signal was monitored after adding either 300 μmol/L NADH or NADPH. The presence of superoxide scavengers, 300 μmol/L nitroblue tetrazolium or 1 mmol/L tiron, or the flavoprotein inhibitor diphenylene iodonium (300 μmol/L), but not the XO inhibitor allopurinol (50 μmol/L), abolished the signal.

To exclude the possible contribution of XO-induced redox cycling of lucigenin, in some experiments NADH oxidase activity was measured in microsome-enriched fractions (29 000 g for 20 minutes followed by 100 000 g for 60 minutes) using 5 μmol/L lucigenin.

NO content of freshly minced ventricular tissue was measured using electron spin resonance spectroscopy after spin trapping with 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III], the NOS inhibitor N°-nitro-l-arginine (LNA, 3 μmol/L), or the superoxide scavenger tiron (100 μmol/L) in the presence or absence of cytokines.

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced luminescence. Approximately 150 mg of the heart apex was placed in 1 mL air-equilibrated Krebs-Henseleit solution containing 10 mmol/L HEPES-NaOH (pH 7.4) and 0.25 mmol/L lucigenin. Chemiluminescence was measured in a liquid scintillation counter (single photon identifier, out-of-coincidence mode).

We measured both perfusate dityrosine by spectrofluorometry and nitrotyrosine by ELISA as markers of myocardial peroxynitrite exposure. Together with greater generation of NO through iNOS, this would result in enhanced peroxynitrite formation, which could contribute to the decline in myocardial function.

Results

In control hearts, cardiac performance was stable in the first 90 minutes of perfusion, but at 120 minutes there was a statistically significant decline in cardiac work (Figure 1). Cytokine treatment, in contrast, markedly decreased cardiac work between 60 and 120 minutes of perfusion (Figure 1). Heart rate and coronary flow did not differ between groups, nor did they change over time (data not shown).

In ventricular tissue from hearts perfused for 0, 30, 60, 90, or 120 minutes, the Ca\(^2+\)-independent NOS activity (between 1 and 2 pmol/min×mg\(^{-1}\) protein) did not change over time, nor did it differ between the control and cytokine groups (Figure 2). Ca\(^2+\)-independent (iNOS) activity was near the detection limit of the assay in control hearts for the first 90 minutes of perfusion and was slightly elevated at 120 minutes (Figure 2). In contrast, cytokine treatment caused a time-dependent increase in Ca\(^2+\)-independent activity that was first elevated at 90 minutes of perfusion and that continued to rise until 120 minutes up to \(\approx5\) pmol/min×mg\(^{-1}\) protein (Figure 2).

We also investigated the activities of enzymes in ventricular tissue that generate superoxide, XOR (XO and XDH), and NAD(P)H oxidases. In control hearts, a significant basal elevation in XDH activity was observed (Figure 3).
XO activity (\(\sim 1.5\) pmol/min/mg protein) remained unchanged during perfusion (Figure 3A). Cytokine treatment, however, stimulated a rapid, \(\sim 2\)-fold increase in XO activity that was statistically significant at \(60\) minutes of perfusion. Basal activity of XDH, the precursor enzyme of XO, was \(\sim 5\)-fold greater than XO activity in control hearts and did not significantly change during the 120-minute perfusion (Table). Cytokine treatment also caused a concomitant increase in XDH activity that was significantly enhanced at \(\geq 60\) minutes of perfusion and was \(\sim 2\)-fold greater than that seen at the start of perfusion (Table). XO activity expressed as a percentage of the total XOR activity did not change significantly over time in either group (Table).

Basal myocardial NADH oxidase activity stayed near its baseline level over the entire perfusion period in control hearts (Figure 3B). Cytokine treatment, however, caused a near \(2.5\)-fold increment in NADH oxidase activity at \(90\) and \(120\) minutes (Figure 3B). NADPH oxidase activity remained very low as compared with NADH oxidase activity in both groups of hearts and was not changed over the 120-minute perfusion time (Figure 3B). To exclude the possibility of XDH-induced redox cycling of lucigenin, the NADH oxidase assay was repeated using \(5\) \(\mu\)mol/L lucigenin in microsome-enriched fractions from control and cytokine-treated hearts perfused for 120 minutes. A \(2\)-fold increase in activity was still observed with cytokine treatment (16.3 \(\pm\) 3.0 versus 8.6 \(\pm\) 2.3 arbitrary luminescence/min/mg protein; \(n = 5\) in each group).

After 120-minute perfusion, ventricular electron spin resonance signal intensity of NO increased \(5\)-fold in cytokine-treated hearts as compared with controls (Figure 4A), whereas myocardial superoxide generation determined by lucigenin chemiluminescence was \(\sim 2\)-fold higher in the cytokine group (Figure 4B). The level of dityrosine, a marker of peroxynitrite formation in the perfusate at the end of the 120-minute perfusion, was significantly increased by \(\sim 70\%\) in the cytokine group (Figure 4C).

As cytokine treatment enhanced the production of NO, superoxide, and peroxynitrite with a concomitant decline in cardiac mechanical function, we then tested the effect of a peroxynitrite decomposition catalyst, FeTPPS. Because FeTPPS interfered with the fluorometric determination of dityrosine, we measured the perfusate nitrotyrosine level at 120 minutes of perfusion as another index of peroxynitrite generation. In control hearts, FeTPPS (10 \(\mu\)mol/L [data not shown] or 50 \(\mu\)mol/L [Figure 5A]) did not significantly change myocardial function over 120 minutes of perfusion. FeTPPS markedly attenuated the loss of cardiac mechanical function in a concentration-dependent fashion in cytokine-treated hearts (Figure 5A) without affecting the increase in myocardial iNOS activity (data not shown).

### Total XOR Activity, XDH Activity, and XO (Expressed as a Percentage of Total XOR) in Cytosolic Fraction of Ventricular Homogenates From Isolated Working Hearts Perfused With Cytokines

<table>
<thead>
<tr>
<th>Activity</th>
<th>Treatment</th>
<th>Perfusion Time, Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0*  30  60  90  120</td>
</tr>
<tr>
<td>XOR, pmol/min/mg protein (^{-1})</td>
<td>Control</td>
<td>9.4(\pm)1.2  9.9(\pm)1.0  8.9(\pm)0.7  11.2(\pm)1.3  11.6(\pm)1.8</td>
</tr>
<tr>
<td></td>
<td>Cytokine</td>
<td>14.3(\pm)2.0  16.4(\pm)1.1†‡  15.8(\pm)2.4†‡  19.3(\pm)1.3†‡</td>
</tr>
<tr>
<td>XDH, pmol/min/mg protein (^{-1})</td>
<td>Control</td>
<td>7.8(\pm)0.9  8.1(\pm)0.7  7.4(\pm)0.6  9.2(\pm)1.0  9.6(\pm)0.8</td>
</tr>
<tr>
<td></td>
<td>Cytokine</td>
<td>11.6(\pm)1.5  13.2(\pm)0.8†‡  12.6(\pm)2.0†  15.6(\pm)1.0†‡</td>
</tr>
<tr>
<td>XO, percentage of XOR</td>
<td>Control</td>
<td>16.5(\pm)1.1%  17.8(\pm)1.0%  16.0(\pm)0.9%  17.1(\pm)1.1%  16.5(\pm)1.1%</td>
</tr>
<tr>
<td></td>
<td>Cytokine</td>
<td>18.5(\pm)0.9%  19.4(\pm)0.6%‡  20.9(\pm)1.4%  19.0(\pm)0.8%</td>
</tr>
</tbody>
</table>

Data are mean \(\pm\) SEM; \(n = 5\)–6 in each group.

*The 0-minute group is combined for both Control and Cytokine groups.

†\(P < 0.05\) vs 0 minutes.

‡\(P < 0.05\) vs corresponding control.
The perfusate nitrotyrosine level increased significantly by \( \approx 80\% \) in the cytokine group as compared with control hearts (Figure 5B). FeTPPS (10 \( \mu \text{mol/L} \) [data not shown] or 50 \( \mu \text{mol/L} \) [Figure 5B]) did not significantly change nitrotyrosine concentration in control perfusate; however, it attenuated the accumulation of nitrotyrosine in a concentration-dependent fashion in the cytokine-treated group (Figure 5B). Data combined from all groups of perfused hearts show a significant inverse correlation between nitrotyrosine concentration in the perfusate and cardiac work at 120 minutes of perfusion (Figure 5C).

TPPS (50 \( \mu \text{mol/L} \)), an inactive form of FeTPPS, did not prevent the cytokine-induced loss in cardiac work as measured at 120 minutes (cytokine, 42\( \pm \)10; cytokine+TPPS, 64\( \pm \)7 \( \text{mm Hg} \times \text{mL/min\times g}^{-1} \) dry weight; \( n=6 \) in both groups, \( P>0.05 \)) or perfusate nitrotyrosine levels (cytokine, 18.4\( \pm \)1.9; cytokine+TPPS, 16.5\( \pm \)2.2 \( \text{nmol/L} \); \( n=6 \) in both groups, \( P>0.05 \)).

Either the NOS inhibitor LNA (3 \( \mu \text{mol/L} \)) or the superoxide scavenger tiron (100 \( \mu \text{mol/L} \)) attenuated the loss of myocardial function (Figure 6A) and nitrotyrosine accumulation in the perfusate (Figure 6B) at 120 minutes of perfusion in cytokine-treated hearts.

**Discussion**

Our results show that cytokines, via concerted stimulation of cardiac iNOS, XO, and NADH oxidase activities, enhance the formation of NO and superoxide, and their reaction product, peroxynitrite, thereby leading to the loss of myocardial function, which can be prevented by a peroxynitrite decomposition catalyst, FeTPPS.

Many studies suggest that NO itself is a cardioprotective and antioxidant molecule\(^{18,19}\) and that the harmful effects of excess NO are due to peroxynitrite formed by the extremely rapid, diffusion rate–limited reaction between NO and superoxide.\(^9,28\) However, little is known about oxygen free radical generation in the heart during systemic inflammatory disease states (see Reference 29 for review). Major sources of superoxide in the rat heart include the XOR system\(^{16}\) and NAD(P)H oxidoreductases.\(^{17}\) Our results show that cytokines rapidly increased myocardial activities of XO and its precursor enzyme XDH after 30 minutes and NADH oxidase activity within 60 minutes of perfusion. In correlation with these increased enzyme activities and accompanied by a
progressive decline in cardiac function after 60 minutes of perfusion, myocardial superoxide production was also significantly enhanced. Proinflammatory cytokines can induce the biosynthesis of mRNA for XO in rat cardiac myocytes and that for NADH oxidase in vascular smooth muscle cells. No one to date has characterized changes in myocardial superoxide-generating activities stimulated by cytokines and related this to changes in the mechanical function of the heart.

Proinflammatory cytokines increase iNOS activity in the heart, and the resultant enhanced production of NO contributes in part to depressed cardiac mechanical function. However, the time course of these changes has not been studied. Cytokine treatment time-dependently increased myocardial iNOS activity after 60 minutes, and myocardial NO content was significantly increased. LNA partially reduced the cytokine-induced loss of myocardial function, confirming previous ex vivo and in vivo studies with nonselective and iNOS-selective inhibitors. These results indicate that induction of iNOS is only partially responsible for cytokine-induced myocardial failure. A limitation of our study is that myocardial superoxide and NO generation were directly measured only at 120 minutes of perfusion; however, this was only a means to confirm the enhanced activities of XO, NADH oxidase, and iNOS.

We have previously found that isolated working rat hearts show a spontaneous loss of cardiac function at 60 to 120 minutes of perfusion, which was accompanied by an increase in iNOS and XO activities. In that study, in addition to the aorta and the left atrium, the pulmonary aorta was also cannulated, and hearts were continuously paced. In the present study, however, spontaneously beating hearts were used and without pulmonary aorta cannulation. Cardiac function in the control group tended to decrease and became statistically different at 120 minutes. XO, XDH, and iNOS activities were slightly but not significantly elevated at 120 minutes of perfusion time, which followed the pattern seen in our previous study, albeit less pronounced. Electrical pacing and pulmonary aorta cannulation apparently enhance the loss in cardiac performance.

Our results provide the following compelling evidence that peroxynitrite acts as a major contributor to cytokine-induced myocardial dysfunction in this in vitro system: (1) 2 different markers of peroxynitrite, dityrosine and nitrotyrosine, accumulated in the perfusate over the course of the heart perfusion, (2) FeTPPS abolished the cytokine-induced loss in cardiac function accompanied by a reduction in perfusate nitrotyrosine concentration; (3) there was a significant inverse correlation between myocardial function and perfusate nitrotyrosine content; and (4) LNA or tiron attenuated the cytokine-induced loss in cardiac function and nitrotyrosine accumulation. Although myeloperoxidase activity in the presence of nitrate may lead to nitrotyrosine formation, both myeloperoxidase activity and nitrite concentration are very low in granulocyte-free, Krebs-perfused hearts. Furthermore, biochemical data that suggested that peroxynitrite does not cause tyrosine nitration have been recently refuted.

FeTPPS catalyzes the isomerization of peroxynitrite to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radicals. It does not have any direct NO-scavenging effect. FeTPPS protected against peroxynitrite-induced cell death stimulated by cytokines or by its exogenous supply in macrophages and in mixed neuron-glia cultures. Some iron porphyrins related in structure to FeTPPS may also have a superoxide-scavenging effect, which could have contributed to its beneficial effect. Peroxynitrite inhibited contractile function in cardiac myocytes and in isolated hearts. Nitrotyrosine was found in hearts from rats with autoimmune myocarditis and in dogs with IL-1β–induced myocardial failure. Extensive protein tyrosine nitration was also found in human myocardial inflammation. The relationship between tissue and perfusate nitrotyrosine or dihydroxyl content is unknown; nevertheless, the results of this study and another suggest that the latter is also a useful index of peroxynitrite generation.

NO derived from iNOS as a result of exposing the heart to proinflammatory cytokines or from endothelial NOS (in endothelial cells and cardiac myocytes) in the setting of acute reperfusion of the ischemic myocardium results in excess generation of NO and myocardial injury. Clearly, the time courses of these phenomena are different; in the former, this occurs after a lag phase and results in a continuous upregu-
lation of NO generation, whereas in the latter, there is a short-lived burst of NO of only a few minutes’ duration. It is interesting to note that superoxide generation also peaks within the first minutes of reperfusion of the ischemic myocardium, whereas in this study the upregulation of superoxide occurred over a longer time scale. Thus, the formation of peroxynitrite might be from eNOS-derived NO in acute ischemia-reperfusion injury but from both endothelial NOS and iNOS in proinflammatory cytokine-induced injury. The resultant detrimental effects of peroxynitrite in biological systems are proportional to the area under the curve represented by its concentration-versus-exposure time.9

The high mortality associated with systemic inflammatory response syndrome urges the development of pharmacological tools to prevent depressed cardiac contractile function. NO, via cytokine-induced upregulation of iNOS, was shown to be involved in these pathological heart conditions in both animals and humans (see References 1 and 2 for review). However, NOS inhibitors could not fully recover myocardial function in this syndrome and caused severe pulmonary hypertension and further depression of right ventricular function.42 Antioxidant therapy with superoxide dismutase in canine hemorrhagic shock43 or with ascorbate and α-tocopherol in septic shock patients44 showed only limited beneficial effects on cardiac function. Our present study is the first demonstration that targeting peroxynitrite specifically abolishes cytokine-induced loss in cardiac mechanical function, whereas blocking either NO7,8 or superoxide45 synthesis alone is less effective. Understanding when the generation of both NO and superoxide is enhanced is important in developing treatment strategies, as an increase of NO generation in and around the postischemic heart is independently associated with improved cardiac function.9

In summary, proinflammatory cytokines stimulate peroxynitrite generation in the heart, leading to deterioration of myocardial contractile function. Whether inhibition of peroxynitrite will treat or prevent the development of cardiac dysfunction in diverse immune system–related cardiac diseases requires further studies.

Acknowledgments

This study was funded by a grant from the Medical Research Council of Canada (MT-11563) and by Hungarian research funds (OTKA-T029843 and FKFP-0340(2000)). At the time this research was done, P.F. was a Fellow of the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research (AHFMR) and H.D. was a summer student of the AHFMR. R.S. is a Senior Scholar of the AHFMR. We thank Marek Radomski and Alex Rabiniroivich for helpful comments.

References


Peroxnitrite Is a Major Contributor to Cytokine-Induced Myocardial Contractile Failure
Péter Ferdinandy, Hajira Danial, Ildikó Ambrus, Richard A. Rothery and Richard Schulz

Circ Res. 2000;87:241-247
doi: 10.1161/01.RES.87.3.241
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/3/241

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/