Peroxy nitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts

PETER FERDINANDY, DONNA PANAS, AND RICHARD SCHULZ
Departments of Pediatrics and Pharmacology, Cardiovascular Research Group, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Ferdinandy, Peter, Donna Panas, and Richard Schulz. Peroxy nitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1861–H1867, 1999.—We examined the mechanism of the time- and protein synthesis-dependent decline in cardiac mechanical function in isolated working rat hearts. Hearts were perfused with Krebs-Henseleit buffer for 120 min in the presence or absence of the protein synthesis inhibitor cycloheximide (CX; 10 µM). Cardiac work remained stable for 60 min and then spontaneously decreased during 60–120 min of perfusion. This was accompanied by an increase in myocardial inducible nitric oxide synthase (iNOS) and xanthine oxidase (XO) activities and enhanced dityrosine formation in the perfusate, an indicator of peroxynitrite generation. CX markedly attenuated the loss in contractile function and prevented the increase in INOS and XO activities and dityrosine level. Despite the decline in cardiac work in control hearts, the coupling between tricarboxylic acid (TCA) cycle activity and oxygen consumption remained constant in both groups. ATP, creatine phosphate, and glycogen levels were not different between control and CX groups and did not differ over 120 min of perfusion. We concluded that the delayed and spontaneous loss in myocardial mechanical function in isolated working rat hearts is 1) attenuated by CX treatment, 2) accompanied by a concomitant increase in both iNOS and XO activities and peroxynitrite generation in the heart, and 3) not dependent on a direct impairment in myocardial ATP production, myocardial oxygen consumption, or TCA cycle acetyl-CoA production but may be due to an inefficiency of the heart to utilize ATP for contractile work.

cardiac function; inducible nitric oxide synthase; xanthine oxidase; cycloheximide; energy metabolism

SINCE THE REPORT of the original method of Neely et al. (22), the left atrially perfused, isolated working rat heart has become a well-established and sophisticated model for simultaneously measuring myocardial function and cardiac metabolism and is a favored approach for testing cardioactive agents (8, 11). However, a well-known limitation of this model is that cardiac mechanical performance gradually declines, especially beyond the first 60 min of perfusion (27, 33). The biochemical mechanism of this phenomenon is not known.

We have previously shown that endotoxemia stimulates the expression of inducible, Ca2+-independent nitric oxide (NO) synthase (iNOS) in cardiac muscle (26). This is mediated by proinflammatory cytokines, which increase iNOS activity in the cardiac myocyte (3, 26), coronary vascular endothelium (2), and endocardial endothelium (29). The resultant excess production of NO in the intact heart contributes to the depression of cardiac mechanical function and can be reduced by the NOS inhibitor Nω-nitro-arginine methyl ester and abolished by the protein synthesis inhibitor cycloheximide (CX) (27). How excess production of NO in the heart depresses myocardial function is unknown; however, this could involve several possibilities, including the ability of NO to inhibit mitochondrial respiration (35). The toxicity of NO is markedly enhanced by its reaction with superoxide to form peroxynitrite, which contributes, for example, to the impairment in vasoconstrictor tone during septic shock (see Ref. 31 for review). One of the possible sources of superoxide in the heart is the xanthine oxidoreductase system, which includes both xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (see Ref. 18 for review). We have shown that continuous infusion of peroxynitrite into isolated working rat hearts causes a delayed onset depression in cardiac contractile function by decreasing cardiac efficiency (25). Moreover, endogenous formation of peroxynitrite in the heart contributes to myocardial stunning in ischemia-reperfusion injury (20, 36).

We hypothesized that perfusion of the isolated heart would result in the induction of iNOS activity, which, in the presence of superoxide-generating XO activity, would increase the generation of peroxynitrite and contribute to the loss of myocardial function. Because both NO and peroxynitrite have inhibitory effects on mitochondrial respiration (35), we studied the decline in mechanical function in isolated working rat hearts, in the presence or absence of the protein synthesis inhibitor CX, in relation to changes in myocardial oxygen consumption (MV O2), energy substrate metabolism, and myocardial tricarboxylic acid (TCA) cycle activity as well as changes in iNOS and xanthine oxidoreductase activities and peroxynitrite formation.

METHODS

The investigation conforms with the Guide to the Care and Use of Laboratory animals published by the Canadian Council on Animal Care (revised 1993). Isolated heart preparation. Male Sprague-Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). The hearts were rapidly excised, cannulated via the atrial, and initially perfused in a retrograde manner (Langendorff method) at 37°C with Krebs-Henseleit buffer gassed with 95% O2-5% CO2. During this initial perfusion, both the pulmonary artery and the left atrium were cannulated. After a 10-min equilibrium period, the perfusion of the heart was switched to the working heart.
mode as described previously (27). The working heart perfusate (100 ml recirculating Krebs-Henseleit solution containing 11 mM glucose, 5 mM pyruvate, 100 µU/ml insulin, 1.75 mM Ca²⁺, 0.5 mM EDTA, and 0.2% bovine serum albumin) was delivered from the oxygenator (supplied with 95% O₂–5% CO₂) into the left atrium at a hydrostatic preload pressure equivalent to 9.5 mmHg. The hydrostatic afterload pressure was set at a column height equivalent to 70 mmHg. Hearts were paced at 300 beats/min throughout the experiment with a Grass SD9 stimulator (regular stimuli; voltage 5 V; duration 0.6 ms; delay 0.4 ms) with leads placed on the aortic and left atrial cannulas. Heart rate and peak systolic pressure were measured with a TSD104 Grass pressure transducer in the aortic outflow line and recorded in real time using the AcoKnowledge III data-acquisition system (Biopac Systems, Goleta, CA). Cardiac output and aortic flow were measured using ultrasonic flow probes (Transonic Systems, Ithaca, NY) in the preload and afterload lines, respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. Cardiac work (cardiac output × peak systolic pressure) was used as an index of the global mechanical function of the heart and expressed as millimeters of Hg times milliliters per minute per gram of dry weight.

Experimental groups. After 10 min of equilibration in the working mode (referred to as 0 min), hearts were perfused for either 0, 60, or 120 min in the absence (control, n = 13) or presence of CX (10 µM; n = 16) in a closed recirculating perfusion system. CX was present in the perfusate from the start of the working perfusion. In separate experiments, hearts were perfused with glucose double labeled with 5-3H and U-14C in the absence (control, n = 7) or presence of CX (n = 6) for measurement of glycolysis and glucose oxidation. Pyruvate oxidation was measured in separate experiments with hearts perfused with [U-14C]pyruvate in the absence (control, n = 6) or presence of CX (n = 10). Peroxynitrite formation and NOS, XDH, and XO activities were determined in the presence or absence of CX at 0, 60, or 120 min of perfusion (n = 6 in each group at each time point).

Preparation of tissue samples. The ventricular portion of the heart was quickly frozen at the end of the perfusion with Wolfenberger clamps cooled to the temperature of liquid N₂. The atrial and ventricular tissue was then weighed and powdered in a mortar and pestle cooled to the temperature of liquid N₂. A preweighed portion of the frozen ventricular tissue powder was dried in an oven for 12 h at 100°C and then removed, dried, and weighed to obtain the total dry weight of the ventricle. The atrial tissue remaining on the cannula was dried and weighed to obtain the total dry weight of the atria. The atrial dry weight and the calculated ventricular dry weight were added together to obtain the total dry weight of the heart. A portion of the powdered frozen ventricular tissue was placed in 5 vols of ice-cold homogenization buffer (composition given in Ref. 26) and homogenized with an Ultra-Turrax disperser using three strokes of 10-s duration each. The homogenate was centrifuged (100,000 g for 35 min) at 4°C, and the supernatant (cytosolic fraction) was kept on ice for immediate assay of enzyme activities.

Measurement of myocardial metabolism. TCA cycle activity was calculated as the difference between cardiac output and aortic flow. Cardiac efficiency was determined as the ratio of cardiac work to O₂ consumption rate.

Hearts were perfused with Krebs-Henseleit buffer containing 5 mM pyruvate and 11 mM glucose double labeled with 5-3H and U-14C for measurement of glycolysis by collection of 3H₂O and glucose oxidation by collection of 14CO₂, respectively, as described previously (25). Pyruvate oxidation was measured in hearts perfused with Krebs-Henseleit buffer containing glucose and [U-14C]pyruvate, and the perfusate was assayed for 14CO₂ production as described previously (25). Cumulative myocardial production of 14CO₂ and 3H₂O was determined at 20-min intervals during the perfusion by means of a scintillation counter, and the rates of glycolysis, glucose oxidation, and pyruvate oxidation were calculated for each 20-min interval of the perfusion. TCA cycle activity was calculated by determining the rate of acetyl-CoA entering the TCA cycle from both pyruvate and glucose oxidation [n = 6 in each group; pyruvate and glucose oxidation data were randomly chosen for calculation (19)]. For calculation of TCA cycle activity, it was assumed that 2 moles and 1 mole of acetyl-CoA were generated from each mole of glucose and pyruvate, respectively.

Determination of cardiac peroxynitrite generation. In separate experiments, hearts (n = 6 in both groups) were perfused in the presence or absence of 10 µM CX with Krebs-Henseleit buffer supplemented with 0.3 mM tyrosine. We have previously determined (36) that the measurement of dityrosine in heart perfusate, formed by the reaction of peroxynitrite with tyrosine, can be used as an estimate of peroxynitrite generation and that tyrosine at this concentration does not affect cardiac mechanical function. Samples of the perfusate were assayed by spectrofluorometry (34, 36) for dityrosine concentration at 0, 60, and 120 min of perfusion. Peroxynitrite-induced dityrosine formation was calibrated in Krebs-Henseleit buffer containing 0.3 mM tyrosine (37°C, equilibrated with carbogen), and a calibration curve of dityrosine fluorescence versus authentic peroxynitrite concentration was prepared. Peroxynitrite was synthesized as described previously (25). Control experiments showed that CX did not interfere with the formation or detection of dityrosine. Peroxynitrite formation was calculated from dityrosine fluorescence and expressed as nanomoles per hour per gram of dry weight.

Measurement of NO activities. NO activities in the cytosolic fraction of frozen ventricular tissue were determined from the conversion of L-[14C]arginine to L-[14C]citrulline as previously described (27). Samples were incubated for 25 min at 37°C in the presence or absence of either EGTA (1 mM) or EGTA plus N⁰-monomethyl-L-arginine (1 mM) to determine the level of Ca²⁺-dependent and -independent NO activities, respectively. The protein concentration of the ventricular cytosolic fraction was determined with the use of a biocinchoninic acid assay utilizing bovine serum albumin as a standard. NO activity was expressed in picomoles per minute per milligram of protein.

Measurement of XDH and XO activities. XDH and XO activities in the cytosolic fraction of frozen ventricular tissue were determined with the use of a fluorometric assay based on the conversion of pterine to isoxanthopterine in the presence and absence of the electron acceptor methylene blue, as described previously (6). The protein concentration of the cytosolic fraction was determined as described in Measurement of NOS activities. XDH and XO activities were expressed in picomoles per minute per milligram of protein.

Measurement of tissue ATP, creatine phosphate, and glyco- gen. ATP and creatine phosphate levels were determined after extraction of ~150 mg of ventricular tissue into 1 ml of ice-cold perchloric acid (6% wt/vol) by homogenization in an ice-cold mortar and pestle. The homogenate was centrifuged (10,000 g for 3 min at 4°C), and the supernatant was neutralized with 0.5 M K₂CO₃ and analyzed with high-
performance liquid chromatography, as described previously (1). Tissue glycogen content was determined with the use of an enzymatic-colorimetric glucose assay kit (Sigma Chemical, St. Louis, MO) after extraction and acidic hydrolysis (in 2 M \( \text{H}_2\text{SO}_4 \) at 100 °C for 3 h) of glycogen and was expressed as glycogen-derived glucose content.

Determination of perfusate endotoxin level. To test for possible endotoxin contamination, laboratory deionized water, Krebs-Henseleit buffer, and samples of perfusate at 0 and 120 min of perfusion were measured for endotoxin content with an E-tosylate kit (Sigma Chemical), which is based on the Limulus amoebocyte lysate assay. Endotoxin levels remained below 48 pg/ml in all samples, which is a concentration far below that required to stimulate iNOS expression in rat cardiac myocytes (37).

Statistics. Data were expressed as means ± SE and analyzed with one-way analysis of variance (ANOVA) between groups or ANOVA for repeated measurements within a group as appropriate. If a significant difference was established, Tukey’s test was used to compare data from each time point to 0-min values. The Student-Newman-Keuls test was applied to test differences in NOS, XDH, and XO activities between groups. Differences were considered significant at \( P < 0.05 \).

RESULTS

Cardiac function and efficiency. Whereas cardiac performance was stable during the first 60 min of perfusion, there was a marked decline in cardiac work over the next 60 min in the control group (Fig. 1A). CX significantly reduced the loss in cardiac performance, because within this group the decline in cardiac work compared with that at 0 min of perfusion was statistically significant only after 120 min of perfusion.

Cardiac oxygen consumption did not change significantly during the 120 min of perfusion in either the control or CX group (Fig. 1B). Cardiac efficiency tended to increase in both groups for the first 60 min of perfusion and was significantly higher in the CX group when 60- and 0-min values were compared. However, during the second hour, there was a marked decline in efficiency in control hearts that was attenuated in the CX-treated group (Fig. 1C).

Cardiac NOS activity. In ventricular tissue from hearts perfused for 60 or 120 min, \( \text{Ca}^{2+} \)-dependent NOS activity was not different between control and CX groups and also was not different from that of 0-min controls (Fig. 2). In contrast, \( \text{Ca}^{2+} \)-independent (iNOS) activity was significantly higher after 120 min, but not 60 min, of perfusion compared with 0-min control values. CX prevented the increase in iNOS activity caused by 120 min of perfusion (Fig. 2).

Cardiac XDH and XO activity. Ventricular XDH activity was significantly increased at 120 min, but not at 60 min, of perfusion in both control and CX-treated hearts compared with 0-min control values, and there was no difference between CX and control hearts after 120 min of perfusion (Fig. 3A). Ventricular XO activity was significantly increased only after 120 min of perfusion in control hearts. CX prevented the increase in XO activity (Fig. 3B).

Cardiac peroxynitrite formation. In control hearts, generation of peroxynitrite, estimated from the rate of formation of dityrosine in the perfusate, remained below the detection limit in the first hour of perfusion and was significantly increased during the second hour of perfusion (Table 1). In the CX-treated group, generation of peroxynitrite stayed below the detection limit throughout the 120-min perfusion period (Table 1).

Cardiac energy substrate metabolism. In control hearts, the rate of pyruvate oxidation showed a tendency to decline in the second hour of perfusion, and the 120-min value was significantly reduced compared with the initial rate (Fig. 4A). CX prevented the time-dependent decline in pyruvate oxidation rate (Fig. 4A). Rates of glucose oxidation and glycolysis were stable during the first 60 min in both groups (Fig. 4, B
From 60 to 120 min of perfusion, a marked stimulation of glucose oxidation and glycolysis was observed in either the control or CX group, with no significant difference between the groups. The calculated rate of acetyl-CoA entering the TCA cycle remained unchanged over the 120-min perfusion period, and no differences were observed between control and CX groups (Fig. 5A). The ratio of acetyl-CoA production and cardiac oxygen consumption did not change over the 120 min of perfusion in either the control or CX group (Fig. 5B). Ventricular ATP, creatine phosphate, and glycogen contents were not different between control and CX groups at the end of the perfusion and were not significantly changed compared with 0-min values (Table 2).

**DISCUSSION**

Our present results show that the time-dependent loss in myocardial contractile function and efficiency in isolated working rat hearts is accompanied by increased iNOS, XDH, and XO activities and by cardiac peroxynitrite generation and that these changes (except for XDH) can be prevented by the protein synthesis inhibitor CX. The loss in myocardial performance is not dependent on the impairment in myocardial ATP production, because mitochondrial oxidation, acetyl-CoA production entering the TCA cycle, and high-energy phosphate levels were not changed, but this loss may be due to an inefficiency in utilizing ATP for contractile work. Our data show that induction of iNOS and XO activities leads to an increased formation of peroxynitrite, which, in turn, may decrease the ability of the heart to convert ATP into mechanical work.

Recent studies suggest that the harmful effects of NO are due to the formation of peroxynitrite, the reaction product of NO and superoxide (5), rather than the possible toxic actions of NO itself. The cytotoxic effects of peroxynitrite include lipid peroxidation, nitration of tyrosine residues, oxidation of sulphydryl groups, and DNA-strand breakage (see Refs. 5 and 24 for reviews).

**Table 1. Rates of cardiac peroxynitrite generation estimated from the formation of dityrosine in perfusate in control and cycloheximide-treated hearts**

<table>
<thead>
<tr>
<th></th>
<th>Rate of Peroxynitrite Generation, ( \text{nmol} \cdot \text{h}^{-1} \cdot \text{g dry wt}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>ND</td>
</tr>
<tr>
<td>60–120 min</td>
<td>461 ± 67</td>
</tr>
<tr>
<td><strong>CX</strong></td>
<td></td>
</tr>
<tr>
<td>0–60 min</td>
<td>ND</td>
</tr>
<tr>
<td>60–120 min</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE; CX, cycloheximide; ND, not detectable.
Our present results show increased activities of both iNOS and XO, sources of NO and superoxide, respectively, and increased formation of peroxynitrite during the second hour of perfusion. CX prevented the increase in iNOS and XO activities and the generation of peroxynitrite. These results suggest that the increase in iNOS and XO activities is due to de novo protein synthesis. However, the stimulation of XDH activity is independent of de novo protein synthesis and may be due to other possible biochemical modulations of this enzyme, i.e., limited proteolysis and modification of sulfhydryl groups (see Ref. 14 for review). Nevertheless, other enzymatic or nonenzymatic sources of superoxide may also contribute to peroxynitrite formation in the heart (18).

Proinflammatory cytokines and bacterial endotoxins are well-known inducers of iNOS, thereby leading to increased formation of NO-derived peroxynitrite (38). In endothelial cells and cardiac myocytes, proinflammatory cytokines were shown to stimulate XDH and XO activities and enhance the formation of superoxide (9, 12), which is necessary for peroxynitrite generation. Because endotoxin contamination was negligible in our experiments, the stimulus causing enhanced iNOS, XDH, and XO activities remains to be elucidated. However, the endogenous synthesis of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1α and -1β, interleukin-6, interleukin-8, and interferon-γ in the heart as a consequence of its isolation and ex vivo perfusion may account for the induction of iNOS (16, 17). The cellular sources of cardiac peroxynitrite are Ambiguity (i.e., cytokines, endotoxins, other unknown factors).

Our present results show increased activities of both iNOS and XO, sources of NO and superoxide, respectively, and increased formation of peroxynitrite during the second hour of perfusion. CX prevented the increase in iNOS and XO activities and the generation of peroxynitrite. These results suggest that the increase in iNOS and XO activities is due to de novo protein synthesis. However, the stimulation of XDH activity is independent of de novo protein synthesis and may be due to other possible biochemical modulations of this enzyme, i.e., limited proteolysis and modification of sulfhydryl groups (see Ref. 14 for review). Nevertheless, other enzymatic or nonenzymatic sources of superoxide may also contribute to peroxynitrite formation in the heart (18).

Proinflammatory cytokines and bacterial endotoxins are well-known inducers of iNOS, thereby leading to increased formation of NO-derived peroxynitrite (38). In endothelial cells and cardiac myocytes, proinflammatory cytokines were shown to stimulate XDH and XO activities and enhance the formation of superoxide (9, 12), which is necessary for peroxynitrite generation. Because endotoxin contamination was negligible in our experiments, the stimulus causing enhanced iNOS, XDH, and XO activities remains to be elucidated. However, the endogenous synthesis of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1α and -1β, interleukin-6, interleukin-8, and interferon-γ in the heart as a consequence of its isolation and ex vivo perfusion may account for the induction of iNOS (16, 17). The cellular sources of cardiac peroxynitrite are Ambiguity (i.e., cytokines, endotoxins, other unknown factors).

Our present results show increased activities of both iNOS and XO, sources of NO and superoxide, respectively, and increased formation of peroxynitrite during the second hour of perfusion. CX prevented the increase in iNOS and XO activities and the generation of peroxynitrite. These results suggest that the increase in iNOS and XO activities is due to de novo protein synthesis. However, the stimulation of XDH activity is independent of de novo protein synthesis and may be due to other possible biochemical modulations of this enzyme, i.e., limited proteolysis and modification of sulfhydryl groups (see Ref. 14 for review). Nevertheless, other enzymatic or nonenzymatic sources of superoxide may also contribute to peroxynitrite formation in the heart (18).

Proinflammatory cytokines and bacterial endotoxins are well-known inducers of iNOS, thereby leading to increased formation of NO-derived peroxynitrite (38). In endothelial cells and cardiac myocytes, proinflammatory cytokines were shown to stimulate XDH and XO activities and enhance the formation of superoxide (9, 12), which is necessary for peroxynitrite generation. Because endotoxin contamination was negligible in our experiments, the stimulus causing enhanced iNOS, XDH, and XO activities remains to be elucidated. However, the endogenous synthesis of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1α and -1β, interleukin-6, interleukin-8, and interferon-γ in the heart as a consequence of its isolation and ex vivo perfusion may account for the induction of iNOS (16, 17). The cellular sources of cardiac peroxynitrite are Ambiguity (i.e., cytokines, endotoxins, other unknown factors).

Our present results show increased activities of both iNOS and XO, sources of NO and superoxide, respectively, and increased formation of peroxynitrite during the second hour of perfusion. CX prevented the increase in iNOS and XO activities and the generation of peroxynitrite. These results suggest that the increase in iNOS and XO activities is due to de novo protein synthesis. However, the stimulation of XDH activity is independent of de novo protein synthesis and may be due to other possible biochemical modulations of this enzyme, i.e., limited proteolysis and modification of sulfhydryl groups (see Ref. 14 for review). Nevertheless, other enzymatic or nonenzymatic sources of superoxide may also contribute to peroxynitrite formation in the heart (18).

Proinflammatory cytokines and bacterial endotoxins are well-known inducers of iNOS, thereby leading to increased formation of NO-derived peroxynitrite (38). In endothelial cells and cardiac myocytes, proinflammatory cytokines were shown to stimulate XDH and XO activities and enhance the formation of superoxide (9, 12), which is necessary for peroxynitrite generation. Because endotoxin contamination was negligible in our experiments, the stimulus causing enhanced iNOS, XDH, and XO activities remains to be elucidated. However, the endogenous synthesis of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1α and -1β, interleukin-6, interleukin-8, and interferon-γ in the heart as a consequence of its isolation and ex vivo perfusion may account for the induction of iNOS (16, 17). The cellular sources of cardiac peroxynitrite are Ambiguity (i.e., cytokines, endotoxins, other unknown factors).

Table 2. Myocardial levels of ATP, creatine phosphate, and glycogen in control and CX-treated hearts

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATP (µmol/g dry wt)</th>
<th>Creatine Phosphate (µmol/g dry wt)</th>
<th>Glycogen (µmol/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-min Control</td>
<td>34.3 ± 2.5</td>
<td>24.8 ± 3.4</td>
<td>51.4 ± 2.6</td>
</tr>
<tr>
<td>120-min Control</td>
<td>28.2 ± 1.8</td>
<td>17.2 ± 1.9</td>
<td>59.8 ± 6.8</td>
</tr>
<tr>
<td>120-min CX</td>
<td>30.0 ± 1.4</td>
<td>21.2 ± 3.3</td>
<td>62.0 ± 4.9</td>
</tr>
</tbody>
</table>

Data are means ± SE.
Cardiac efficiency were not determined in the present study. Coronary and endocardial endothelial cells, cardiac myocytes, and cardiac nerves may all potentially contribute to peroxynitrite formation, because all of these tissues are able to synthesize NO and superoxide (10, 18, 28).

We previously showed that the continuous infusion of peroxynitrite at 40 µM into working rat hearts caused a marked depression in cardiac work that began within 30 min and was irreversible after 45 min (25). At a higher concentration (200 µM), peroxynitrite has been shown to inhibit contractile function within 5 min in isolated cardiac myocytes (15). Given that peroxynitrite causes cumulative oxidative damage, its cytotoxic actions in biological systems can be best understood as the product of both concentration and exposure time. The direct measurement of peroxynitrite concentration is not yet attainable. Indirect methods such as the determination of dityrosine formation used in this study greatly underestimate peroxynitrite concentration (36). Moreover, the biologically effective concentration of peroxynitrite is unknown because of its rapid breakdown at physiological pH. Therefore, the estimated rate of peroxynitrite generated by the heart in our present study (Table 1) is not directly comparable with concentrations of exogenously administered peroxynitrite in the aforementioned studies.

Our present results show that in the second hour of working heart perfusion the rate of glucose oxidation was significantly stimulated, whereas pyruvate oxidation was decreased. As a result, the rate of acetyl-CoA entering the TCA cycle remained constant. TCA cycle activity was the major source of energy production in the heart because the rate of glycolysis remained markedly lower throughout the perfusion. It is well known that a variety of myocardial stresses stimulate cardiac glucose metabolism and glycogen depletion (see Ref. 30 for review), possibly due to the accumulation of intracellular Ca²⁺ (21). At the time when cardiac work and efficiency were depressed, the activity of the TCA cycle and cardiac oxygen consumption remained unchanged. In addition, cardiac high-energy phosphate and glycogen levels at the beginning and at the end of the perfusion were not significantly different. These findings show that mitochondrial respiration and the rate of acetyl-CoA entering the TCA cycle were not affected by long-term perfusion; consequently, the rate of ATP synthesis did not decrease. The loss of cardiac function could also not be explained by the depletion of glycogen. In fact, in the second hour of perfusion, the total rate of myocardial ATP synthesis may have increased slightly due to the increased rate of glycolysis, although the contribution of glycolysis to the total rate of ATP production in our study is minor compared with that provided by TCA cycle activity. However, we observed no change in high-energy phosphate content. A minor increase in ATP breakdown may account for this. We conclude that the loss in cardiac efficiency may be due to the uncoupling of ATP synthesis from its utilization for mechanical work. CX prevented the loss of cardiac efficiency without affecting metabolic parameters, which further supports this assumption. In fact, Furchgott and de Gubareff (13) observed more than 40 years ago that "experimental failure," seen as a steady loss of contractile force in the electrically driven, isolated guinea pig atria preparation, was independent of change in myocardial high-energy phosphate content.

A major limitation of the isolated working heart model is the time-dependent decline in cardiac mechanical performance (4, 23, 27, 33). This is the first study providing data on the possible biochemical mechanism of this phenomenon. On the basis of our results we suggest that the spontaneous decline in cardiac mechanical performance can be reduced by inhibition of de novo protein synthesis or, possibly, by treatment with specific iNOS inhibitors or peroxynitrite scavengers. In accordance with this finding, urate, a putative scavenger of peroxynitrite (32), has been shown to attenuate the time-dependent loss in myocardial function in isolated working guinea pig hearts (4).

We conclude that an increase in cardiac iNOS and XO activities due to extended ex vivo perfusion of the heart leads to an increased generation of peroxynitrite, which results in an uncoupling of ATP synthesis from mechanical work, thereby leading to the loss of cardiac efficiency and performance.

We thank Dr. Gary Lopaschuk for helpful discussions and Dr. Wilma Suarez-Pinzon for measuring endotoxin levels.

This study was funded by a grant from the Medical Research Council of Canada (MT-11563). P. Fendinandy is a Fellow of the Medical Research Council of Canada and of the Alberta Heritage Foundation for Medical Research. R. Schulz is a Senior Scholar of the Alberta Heritage Foundation for Medical Research and was a Scholar of the Medical Research Council of Canada.

Address for reprint requests and other correspondence: R. Schulz, Cardiovascular Research Group, 4-62 Heritage Medical Research Centre, Univ. of Alberta, Edmonton, Alberta, Canada T6G 2Z2 (E-mail: richard.schulz@ualberta.ca).

Received 28 September 1998; accepted in final form 8 February 1999.

REFERENCES


7. Davies, N. J., J. J. McVeigh, and G. D. Lopaschuk. Effect of TA-3090, a new calcium channel blocker, on myocardial sub-


