Endothelial Dysfunction in Chronic Myocardial Infarction Despite Increased Vascular Endothelial Nitric Oxide Synthase and Soluble Guanylate Cyclase Expression

Role of Enhanced Vascular Superoxide Production

Johann Bauersachs, MD; Anne Bouloumié, PhD; Daniela Fraccarollo, PhD; Kai Hu, MD; Rudi Busse, MD, PhD; Georg Ertl, MD

Background—Endothelial dysfunction of the peripheral vasculature is a well-known phenomenon in congestive heart failure that contributes to the elevated peripheral resistance; however, the underlying mechanisms have not yet been clarified.

Methods and Results—Dilator responses, the expression of protein and mRNA of the endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), and soluble guanylate cyclase (sGC), and superoxide anion (O$_2^-$) and peroxynitrite production were determined in aortic rings from Wistar rats 8 weeks after myocardial infarction and compared with those in sham-operated animals. In rats with heart failure, the concentration-response curve of the endothelium-dependent vasodilator acetylcholine (after preconstriction with phenylephrine) was significantly shifted to the right, and the maximum relaxation was attenuated. Determination of expression levels of the 2 key enzymes for NO-mediated dilations, eNOS and sGC, revealed a marked upregulation of both enzymes in aortas from rats with heart failure, whereas iNOS expression was not changed. Pretreatment with exogenous superoxide dismutase partially restored the acetylcholine-induced relaxation in aortas from rats with heart failure. Aortic basal and NADH-stimulated O$_2^-$ production assessed by use of lucigenin-enhanced chemiluminescence was significantly elevated in rats with chronic myocardial infarction. Peroxynitrite-mediated nitration of protein tyrosine residues was not different between the 2 groups of rats.

Conclusions—These results demonstrate that endothelial dysfunction in ischemic heart failure occurs despite an enhanced vascular eNOS and sGC expression and can be attributed to an increase in vascular O$_2^-$ production by an NADH-dependent oxidase. By inactivation of NO, O$_2^-$ production appears to be an essential mechanism for the endothelial dysfunction observed in heart failure. (Circulation. 1999;100:292-298.)

Key Words: endothelium • endothelium-derived factors • myocardial infarction • heart failure • free radicals

Endothelial dysfunction of the peripheral vasculature contributes to the elevated peripheral vascular resistance in patients with heart failure, as well as several animal models of cardiac dysfunction. However, the underlying mechanisms may be complex and have not yet been clarified. One attractive hypothesis appears to be a decrease in the production of endothelium-derived nitric oxide (NO). In a heart failure model of ventricular pacing in dogs, an endothelial hypersensitivity in the coronary circulation and an attenuated expression of the endothelial NO synthase (eNOS) in the aorta have been described. In contrast, other studies reported enhanced basal production of NO in heart failure, which might originate from the inducible NOS (iNOS) in the vasculature, because the expression of this high-output NO-generating enzyme has been shown in hearts from patients with dilated cardiomyopathy.

In other pathophysiological states, such as hypercholesterolemia and hypertension, compelling evidence suggests that endothelial dysfunction results from increased vascular production of superoxide anion (O$_2^-$). Because O$_2^-$ rapidly scavenges NO within the vascular wall, a reduction of bioavailable NO might occur despite an increased NO generation. In patients suffering from heart failure, elevated levels of plasma lipid peroxides as a marker of oxidative stress have been observed. This is further supported by the fact that the impaired flow-induced NO-mediated dilation in
patients with heart failure can be restored by short-term treatment with high doses of the antioxidant vitamin C.\textsuperscript{17}

In addition, more recently, alterations of the effector system of NO, in particular a reduced expression of the cGMP-forming soluble guanylate cyclase (sGC), were identified as an important mechanism of dilator dysfunction in hypertension.\textsuperscript{18}

With regard to heart failure, no data are available on $O_2^-$ production within the vascular wall or on the potential alterations of the expression of smooth muscle sGC. Moreover, the influence of heart failure on vascular NOS expression is still controversial. Chronic myocardial infarction in the rat is considered to be a useful model to study the pathophysiological sequelae of heart failure. Indeed, the beneficial effects of ACE inhibitors were predicted from results obtained in this experimental model.\textsuperscript{19} In rats with heart failure due to myocardial infarction, endothelium-dependent relaxations, still normal at 1 week after coronary ligation, are reduced at 4 weeks and progressively worsen with time.\textsuperscript{20}

The aim of the present study was therefore to identify the potential mechanisms underlying endothelial dysfunction in heart failure by the simultaneous determination of endothelium-dependent dilator responses, the expression of the key enzymes of the NO/cGMP system, and $O_2^-$ formation in the aorta of rats 8 weeks after myocardial infarction.

### Methods

#### Myocardial Infarction: Hemodynamic Measurements

Left coronary artery ligations were performed in adult female Wistar rats (250 to 300 g) as previously described.\textsuperscript{21} Briefly, under ether anesthesia, the thorax was opened, the heart exteriorized, and a ligature placed around the proximal left coronary artery. Sham-operated rats were treated similarly except that the operative procedure did not produce a detectable infarction. Hemodynamic studies were performed 8 weeks after coronary artery ligation as described.\textsuperscript{21} Briefly, rats were anesthetized with ether, tracheotomized, and ventilated. Saline-filled catheters were advanced from the right carotid artery and jugular vein into the left ventricle and right atrium and connected to a Millar micromanometer and Statham transducer. Left ventricular systolic and diastolic pressures, mean arterial pressure, and heart rate were measured under light ether anesthesia and spontaneous respiration.

Infarct size was determined histologically by planimetry as described\textsuperscript{21} after formalin fixation, and only rats with large infarcts (>40%) were included in the study of vascular reactivity.

#### Measurement of Plasma Renin Activity

Plasma renin activity was measured as described previously\textsuperscript{21} with a commercial test kit (Sorin Biomedica Diagnostic).

#### Vascular Reactivity Studies

The descending thoracic aorta was dissected after removal of the heart, cleaned of connective tissue, and cut into 3 sections as described.\textsuperscript{18} The upper section (15 mm) was immediately frozen in liquid nitrogen for Western blot analysis. The lower section (10 mm) was used for measurement of $O_2^-$ production, and the remainder was cut into rings 3 mm long that were mounted in an organ bath (Föhr Medical Instruments) for isometric force measurement. The rings were equilibrated for 30 minutes under a resting tension of 2 g in oxygenated (95% $O_2$/5% $CO_2$) Krebs-Henseleit solution (pH 7.4, 37°C) of the following composition (mmol/L): NaCl 118, KCl 1.2, CaCl\textsubscript{2} 1.6, K\textsubscript{2}HPO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25, and glucose 12, and the cyclooxygenase inhibitor diclofenac 1 μmol/L. Rings were repeatedly contracted by KCl 50 mmol/L until reproducible responses were obtained. Thereafter, the rings were preconstricted with phenylephrine 0.3 to 1 μmol/L to comparable constriction levels, and the relaxant response to cumulative doses of acetylcholine and to sodium nitroprusside was assessed with or without prior inhibition of the endogenous superoxide dismutase (SOD) by use of diethyldithiocarbamate (DETC, 1 mmol/L, 40 minutes, followed by repeated washout). In additional experiments, the effect of exogenous SOD on relaxant responses was assessed.

#### Analysis of eNOS and sGC Expression by Reverse Transcription–Polymerase Chain Reaction

Total RNAs were extracted according to the method of Chomczynski and Sacchi.\textsuperscript{22} For the reverse transcription (RT), 2 μg total RNA was incubated with 200 U reverse transcriptase (Gibco), dNTP 125 μmol/L, oligo(dT) 200 ng, and reaction buffer in a final volume of 20 μL at 37°C for 60 minutes. After a final denaturation at 94°C for 7 minutes, 5 μL of cDNA was subjected to polymerase chain reaction (PCR) consisting of denaturation at 94°C for 1 minute, followed by 90 seconds of annealing at 52°C for eNOS/GAPDH or 81°C for α1- and 65°C for β2-subunits from the sGC and 90 seconds of elongation at 72°C for 25 to 30 cycles. The last cycle ended with 7 minutes of elongation at 72°C. The primers used (Table 1) were chosen as previously described.\textsuperscript{15,18,23} The PCR contained 0.4 μmol/L of each primer, dNTP 200 μmol/L, MgCl\textsubscript{2} 1 mmol/L reaction buffer, and 2.5 U Taq polymerase (Promega) in a final volume of 50 μL. The amplified cDNAs were size-fractionated by agarose gel electrophoresis, visualized under UV light by use of ethidium bromide staining, transferred to nylon membrane (Hybond-N, Amersham), and hybridized with a [\textsuperscript{32}P]-labeled eNOS fragment obtained from the cloned bovine eNOS cDNA, [\textsuperscript{32}P]-labeled GAPDH fragment isolated from PCR, and [\textsuperscript{32}P]-labeled human α1- and β2-subunit cDNAs. The cDNAs were quantified after autoradiography by scanning densitometry normalized by comparison with GAPDH cDNA.

#### Western Blot Analysis

After alcohol precipitation of the phenol phase obtained after the guanidine isothiocyanate/phenol/chloroform extraction method,\textsuperscript{22} crude protein extracts (100 μg) were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes as described.\textsuperscript{24} Proteins were detected by their respective antibodies and visualized by enhanced chemiluminescence; the autoradiographs were analyzed by scanning densitometry.
Determination of cGMP in Aortic Rings
For the determination of cGMP accumulation in rat aortic segments without confounding effects of endothelium-derived NO, cGMP levels were determined after denudation of the endothelium under basal conditions and after stimulation with sodium nitroprusside 1 mM for 2 minutes in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.3 mM) and freeze-clamped.

Measurement of Superoxide Anion and Peroxynitrite Formation
The \( \text{O}_2^- \) generation of the rings was assessed by lucigenin-enhanced chemiluminescence as described previously. Briefly, aortic segments (5 mm) were transferred into tubes containing 0.5 mL HEPES buffer and maintained at 37°C for at least 30 minutes before lucigenin 250 mM was added. The luminometer (LKB-Wallac 1251) was set to report arbitrary units of light emitted and integrated over a 30-second interval, and repeated measurements were made over 3 minutes and averaged. The specific chemiluminescence signal was calculated after subtraction of background activity and expressed as counts per minute per milligram dry weight of samples (cpm/mg). Peroxynitrite formation was assessed in a similar protocol using luminol 250 mM.

Materials
All biochemicals were obtained in the highest purity available from Sigma Chemical Co. The \([a-32P]dCTP\) was purchased from Hartmann Analytic. The cloned bovine eNOS cDNA was a gift from D.G. Harrison, Emory University, Atlanta, Ga. The monoclonal eNOS antibody was purchased from Transduction Laboratories (Affiniti), and the antibody against the sGC \( b_1 \)-subunit was kindly provided by Dr. Peter Yuen, Memphis, Tenn.

Statistics
Dilator responses were given as percentage dilatation relative to the preconstriction level. All data in the figures and in the text are expressed as mean ± SEM of experiments with aortic segments from \( n \) different animals. Statistical analysis was performed by 1-way ANOVA followed by a Bonferroni \( t \) test or by the 2-tailed Student’s \( t \) test for unpaired data, where appropriate, with probability values of \(<0.05\) considered statistically significant.

Results
Global Parameters
Global parameters of heart failure rats and sham-operated animals are shown in Table 2. Infarct size was 45±1%. Mean arterial blood pressure, left ventricular systolic pressure, and \( \text{dP/dt}_\text{max} \) were significantly lower in rats with chronic myocardial infarction, whereas left ventricular end-diastolic pressure was elevated. Plasma renin activity was significantly higher in rats with heart failure. Therefore, these rats demonstrated heart failure in a compensated stage.

Vasodilator Responses in Aortic Rings
In phenylephrine-preconstricted aortic rings, acetylcholine elicited a concentration-dependent relaxation that was blunted in aortas from rats with cardiac dysfunction (Figure 1A). Acetylcholine-induced relaxations were mediated by NO because they were abolished after incubation with the NOS inhibitor \( \text{N}^G \)-nitro-\( L \)-arginine 0.3 mM for 30 minutes (data not shown). Endothelium-independent relaxations induced by sodium nitroprusside were slightly but not significantly attenuated at lower concentrations in rats with heart failure, and maximum relaxation was not different (100%) in the 2 groups of rats (Figure 1B).

NO- and cGMP-Generating Enzymes in the Aorta
To elucidate whether the attenuation of endothelium-dependent relaxation is the result of an alteration in the expression of the key enzymes of NO-mediated dilation, the

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<th>Sham (( n=13 ))</th>
<th>Infarct (( n=25 ))</th>
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<td>Infarct size, %</td>
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<td>MAP, mm Hg</td>
<td>112±5</td>
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<tr>
<td>LVSP, mm Hg</td>
<td>147±6</td>
<td>128±3*</td>
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<tr>
<td>LVEDP, mm Hg</td>
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<td>14.8±2.3*</td>
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<tr>
<td>( \text{dP/dt}_\text{max} ), mm Hg/s</td>
<td>14 762±835</td>
<td>12 020±525*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>414±17</td>
<td>392±9</td>
</tr>
<tr>
<td>PRA, ng ( \text{Ang I} ) · mL⁻¹ · h⁻¹</td>
<td>42±4</td>
<td>65±9*</td>
</tr>
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\( \text{MAP} \) indicates mean arterial pressure; \( \text{LVSP} \), left ventricular systolic pressure; \( \text{LVEDP} \), left ventricular end-diastolic pressure; \( \text{PRA} \), plasma renin activity; and \( \text{Ang} \), angiotensin.

\( ^*P<0.05 \) vs sham.
expression of protein and mRNA of both eNOS and iNOS as well as sGC was determined in aortic segments from rats with heart failure and sham-operated animals by Western blot and RT-PCR. As shown in Figure 2, eNOS mRNA and protein levels were found to be significantly increased in aortas from rats with heart failure compared with sham-operated animals (2.9- and 2.1-fold increase, respectively, $P < 0.05$, $n = 4$), whereas the iNOS expression in the thoracic aorta, hardly detectable by Western blot analysis (Figure 3A), remained unchanged.

In addition, Western blot analysis performed on whole aortic protein extracts showed that the protein level of the $\beta_1$-subunit of the sGC was markedly enhanced in rats with myocardial infarction (Figure 3B, 2.5-fold increase, $P < 0.05$, $n = 4$), whereas the RT-PCR analysis failed to detect significant differences between infarcted and sham-operated animals (data not shown).

**Effects of Radical Scavengers on Vascular Reactivity and cGMP Production**

Because NO- and cGMP-generating enzymes were found to be upregulated in rats with heart failure, we investigated the potential involvement of reactive oxygen species in the alteration of the endothelial function. The effects of radical scavengers were studied on the vascular reactivity and cGMP production.

![Figure 2](image1)

**Figure 2.** Comparison of eNOS expression in aortas from rats with heart failure 8 weeks after myocardial infarction vs sham-operated animals. Left, Western blot and densitometric analysis showing aortic eNOS protein levels. Right, Southern blot and densitometric analysis showing aortic eNOS mRNA levels. Values are mean±SEM from 4 separate experiments.

![Figure 3](image2)

**Figure 3.** Comparison of protein expression of iNOS (A) and $\beta_1$-subunit of sGC (B) in aortas from rats with heart failure 8 weeks after myocardial infarction vs sham-operated animals. A, Representative Western blot showing aortic iNOS protein levels. B, Western blot and densitometric analysis showing aortic sGC $\beta_1$-subunit protein levels. Values are mean±SEM from 4 separate experiments.
In phenylephrine-constricted rings, addition of exogenous SOD 600 U/mL elicited a relaxation that was significantly enhanced in aortic rings from rats with heart failure (83±3% versus 56±4%, P<0.01). Furthermore, in the presence of exogenous SOD 200 U/mL, the relaxation induced by submaximal concentrations of acetylcholine in aortas from rats with chronic cardiac dysfunction was significantly enhanced (Figure 4).

Conversely, after inhibition of the endogenous SOD by use of DETC 1 mmol/L for 40 minutes, the acetylcholine-induced relaxation in aortic rings was markedly depressed in aortas from sham-operated rats and abolished in animals with chronic myocardial infarction (Figure 4).

Basal levels of cGMP in aortas from rats with heart failure (2.4±0.4 pmol/mg protein) were not different from those in sham-operated animals (1.5±0.3 pmol/mg protein, n=6, Figure 5). Stimulation with sodium nitroprusside induced a marked increase in cGMP formation, and cGMP levels were lower in rats with cardiac dysfunction than in sham-operated animals. However, in the presence of the radical scavenger Tiron 10 mmol/L, sodium nitroprusside–induced cGMP formation was significantly enhanced in aortas from rats with heart failure (Figure 5).

Production of Superoxide Anions and Peroxynitrite in Aortic Segments
Finally, we assessed the production of O$_2^-$ and of peroxynitrite generated by aortic rings by lucigenin- and luminol-enhanced chemiluminescence, respectively. O$_2^-$ release was greater in aortas from rats with chronic myocardial infarction (Figure 6A). Removal of the endothelium slightly but not significantly reduced radical production in both groups (Figure 6A). After addition of NADH 100 μmol/L, O$_2^-$ formation was markedly stimulated and significantly higher in aortas from rats with heart failure (Figure 6B).

The luminal–mediated luminescence as indicator of peroxynitrite formation was hardly detectable and not different in aortas from rats with chronic myocardial infarction and those from sham-operated animals. Moreover, Western blot analysis of aortic proteins with a specific antibody against nitrotyrosine to detect peroxynitrite-mediated nitration of tyrosine residues showed no increase in nitrotyrosine in rats with heart failure compared with sham-operated animals (data not shown).

Discussion
In the present study, we observed a pronounced endothelial dysfunction in rats with chronic myocardial infarction despite a marked upregulation in the expression of 2 key enzymes of vasorelaxation: eNOS, regulating the synthesis of the most important vasodilator, NO, and its target enzyme in smooth muscle cells, sGC. Our data suggest that even this upregulation is not sufficient to compensate for the increased formation of O$_2^-$, which rapidly inactivates NO.

Expression of eNOS, iNOS, and sGC
Heart failure is associated with an endothelial dysfunction of coronary arteries as well as large conductance and peripheral arteries, with considerable implications for myocardial perfusion, cardiac workload, and peripheral vascular resistance.1,7,26 From these functional studies, the mechanism underlying the reduction of agonist-stimulated dilator responses in heart failure has been proposed to be a defective production of endothelium-derived NO, and in a heart failure model of ventricular pacing in dogs as well as in monocrotaline-induced cardiac failure, a reduction of endothelial...
thelial NO release was associated with an attenuated expression of eNOS.\textsuperscript{8-27} Data on basal production of NO in heart failure have been controversial: using the amount of constriction in response to an NOS inhibitor as an indirect measure for basal NO release, some investigators found an increase\textsuperscript{6,9} and speculated that expression of iNOS in the vasculature may be induced, as has been shown in hearts from patients with dilated cardiomyopathy.\textsuperscript{19} However, other reports found no difference or even a decrease of basal NO formation in patients with heart failure.\textsuperscript{28,29}

Our results for the first time provide insights into the mechanisms of the alteration of endothelial function in heart failure after myocardial infarction, which represents the most important cause for cardiac failure in patients. Although in agreement with the results obtained in monocrotaline-induced heart failure,\textsuperscript{27} iNOS expression was hardly detectable in rats with chronic myocardial infarction, we observed an unexpected marked increase in the expression of eNOS. The association of an increased eNOS expression with a marked attenuation of endothelium-dependent relaxation adds to the mounting evidence that enhanced NO formation or NOS expression does not necessarily imply improved dilator function but rather may even be detrimental\textsuperscript{15} or at least a failed counterregulatory mechanism. In parallel with our results obtained in the aorta, in the myocardium of spontaneously hypertensive genetically heart failure–prone rats, an upregulation of cardiac eNOS expression has been observed; however, the functional consequences of this were not investigated.\textsuperscript{30}

The second key enzyme for endothelium-dependent dilation, the sGC in smooth muscle cells, is activated after binding of endothelium-derived NO to generate large amounts of cGMP. Recently, an attenuation of aortic sGC expression was recognized as a potential mechanism of reduced dilator response in aged spontaneously hypertensive rats.\textsuperscript{18} In rats with chronic myocardial infarction, however, we observed an upregulation of sGC expression that was associated with a blunted cGMP formation in response to sodium nitroprusside. Because sGC activity is susceptible to superoxide\textsuperscript{25} and cGMP production was restored by prior treatment with the radical scavenger Tiron, enhanced production of superoxide anions may be responsible for the reduced activity of sGC despite the increase in its expression. Enhanced degradation of cGMP due to increased phosphodiesterase activity\textsuperscript{31} is not likely, because our experiments were performed in the continuous presence of a high concentration of a phosphodiesterase inhibitor. An enhanced \(O_2^-\) formation in rats with heart failure appears to account for the paradoxical attenuation of cGMP accumulation despite increased sGC expression.

**Oxidative Stress in Heart Failure and Vascular \(O_2^-\) Formation**

Elevated levels of plasma lipid peroxides in patients suffering from heart failure provide clear evidence of an enhanced oxidative stress under this condition.\textsuperscript{16,32} In addition, the transition from hypertrophy to heart failure in coarctation-induced hypertension was associated with an increased oxidative stress and could be prevented by treatment with the antioxidant vitamin E, thus indicating a pathophysiological role for oxidative stress in the pathogenesis of heart failure.\textsuperscript{33} High doses of vitamin C were able to restore the impaired NO-mediated dilatation in patients with heart failure,\textsuperscript{17} and in line with these observations, our results provide the first direct experimental evidence for an enhanced release of reactive oxygen species from the vasculature in chronic ischemic cardiac dysfunction. The source of superoxide formation appears to be vascular smooth muscle cells, because removal of the endothelium did not significantly attenuate radical production. Cultured and native vascular smooth muscle cells are able to generate superoxide in response to the vasoconstrictor peptide angiotensin II, which stimulates the expression of an NAD(P)H-dependent oxidase.\textsuperscript{13,34} Plasma renin activity as well as tissue ACE activity is markedly elevated in heart failure.\textsuperscript{35} Therefore, an enhanced formation of angiotensin II may lead to an enhanced vascular superoxide formation through the expression of an NAD(P)H-dependent oxidase in aortic smooth muscle cells.\textsuperscript{13,34} Indeed, the observed upregulation of NADH-dependent \(O_2^-\) formation in aortas from rats with chronic myocardial infarction suggests that this mechanism may be operative in ischemic heart failure.

The deleterious role of \(O_2^-\) formation for endothelial function in ischemic cardiac dysfunction is further strengthened by the observation that exogenous SOD exerted a significantly greater relaxation in rats with chronic myocardial infarction and by the partial restoration of the acetylcholine-induced relaxation in the presence of SOD.

An imbalance between NO and superoxide production with enhanced inactivation of NO, leading to a reduction of bioactive NO despite a normal or even increased generation of NO, has been associated with endothelial dysfunction and appears to be a common feature of many cardiovascular diseases, such as hypercholesterolemia and hypertension.\textsuperscript{11-13,15,18} In addition, depending on the pathophysiological circumstances, NO and superoxide may react to the powerful oxidant peroxynitrite, which can form hydroxyl radicals and nitrate protein tyrosine residues.\textsuperscript{15} However, we detected neither enhanced luminal chemiluminescence nor tyrosine nitration in rats with heart failure, so there was no hint for the formation of peroxynitrite.

In conclusion, our data indicate that an increased NADH-dependent vascular \(O_2^-\) generation represents an important mechanism for the endothelial dysfunction in heart failure by enhancing the inactivation of NO. Even a presumably counterregulatory upregulation of eNOS and sGC is not sufficient to restore endothelium-dependent relaxations.

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