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Short communication

Rapid increase in inducible nitric oxide synthase gene expression in the heart during endotoxemia

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Abstract

Inducible, Ca^{2+}-independent nitric oxide (NO) synthase activity in the heart is elevated during endotoxemia and the resulting excess release of NO depresses cardiac contractile function. We show here that this is due to an extremely rapid induction of inducible NO synthase gene expression. Following injection of endotoxin (bacterial lipopolysaccharide) in rats we detected increased inducible NO synthase mRNA levels in the left ventricular wall within 30 min which then peaked at 3 h. This was followed by an increase in myocardial inducible NO synthase enzyme activity and plasma levels of NO metabolites, nitrate and nitrite, which peaked at 6 and 12 h, respectively. The extremely rapid induction of inducible NO synthase may serve to protect the heart against microbial infection and concomitantly alter myocardial mechanical function.

Keywords: Endotoxin; Nitric oxide (NO) synthase; mRNA; Septic shock; Plasma nitrate; Heart, rat

1. Introduction

Considerable evidence shows that nitric oxide (NO) modulates cardiac muscle contractile function. NO and NO donors exert a negative inotropic effect in isolated papillary muscles and cardiac myocytes (Shah and Lewis, 1993; Brady et al., 1993). Ca^{2+}-dependent NO synthase activity has been characterized in cells of the normal heart, including cardiac myocytes (Schulz et al., 1992) and endocardial endothelial cells (Schulz et al., 1991). These cells, along with NO released from coronary endothelium, account for a significant basal production of NO from the heart.

Immunological stimulation of the heart can increase NO production to levels which depress cardiac contractile function. We have shown that inducible, Ca^{2+}-independent NO synthase activity increases in the myocardium after lipopolysaccharide treatment of rats and after the exposure of isolated cardiac myocytes, from untreated animals, to interleukin-1β and tumour necrosis factor-α (Schulz et al., 1992). Furthermore, exposure of isolated working rat hearts to these cytokines causes a delayed depression of cardiac mechanical function. This is associated with enhanced inducible NO synthase activity that can be prevented by cycloheximide and attenuated by the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (Schulz et al., 1995). Cardiac myocytes isolated 4 h after treating guinea pigs with lipopolysaccharide (Brady et al., 1992) or incubated with pro-inflammatory cytokines in vitro (Balligand et al., 1994) show depressed contractile function which is attenuated by NO synthase inhibitors.

While these and other studies show that inducible NO synthase may be quickly induced in the heart, all have only determined NO production or NO synthase activity at least 2 h after immunological challenge. No study to date has characterized the early phase of the induction of inducible NO synthase, either at the level of enzyme activity or gene expression, which we address here.

2. Materials and methods

2.1. Experimental protocol

Male Sprague-Dawley rats (275–325 g) were injected i.p. with 4 mg kg^{-1} lipopolysaccharide (*Salmonella ty-*

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phosa 0901, Difco) or pyrogen-free saline vehicle (control). Animals were killed by pentobarbital overdose at 6 h following injection (control) or at the times indicated in Fig. 1. A blood sample was immediately taken by cardiac puncture with a heparinized syringe and the plasma fraction was obtained following centrifugation (10000 × *g*, 5 min) and was stored at −20°C for later determination of plasma NO metabolites, nitrate and nitrite. The plasma was deproteinized by centrifugal ultrafiltration (Ultrafree-MC, Millipore), diluted with distilled water (1:1, v/v) and total nitrate and nitrite levels were determined according to the method of Green et al. (1982). The heart was rapidly removed, rinsed and placed into ice-cold Krebs' buffer. A segment of myocardium from the left ventricular wall (3–4 mm) was excised, avoiding any major blood vessels, rinsed with Krebs' buffer, blotted and freeze-clamped with tongs at liquid nitrogen temperature. Ventricular tissue was homogenized and centrifuged (100000 × *g* for 35 min) at 4°C as described (Schulz et al., 1995) and the cytosolic (supernatant) fraction was kept on ice for immediate assay of NO synthase activities. NO synthase activity (Ca^{2+}-dependent and -independent) in the cytosolic fraction was measured by the formation of [^{14}C]citrulline from L-[U-^{14}C]arginine (305 mCi mmol^{-1}, Amersham) as described (Schulz et al., 1995). Cytosolic protein was determined by the bicinchoninic acid method using bovine serum albumin as a standard.

2.2. Determination of inducible NO synthase mRNA steady-state levels

Total RNA was isolated from pulverised frozen heart tissue (approx. 0.3 g) using TRIzol reagent (Gibco-BRL). Preliminary Northern blot experiments were conducted which demonstrated that a 300-bp *Eco*RI fragment of the murine macrophage inducible NO synthase cDNA (a kind gift from Dr. Cunningham, Harvard Medical School, Boston, MA) recognized the same transcript in rat heart as did a 45-base synthetic oligonucleotide complementary to the 3′-untranslated region of the rat vascular smooth muscle inducible NO synthase mRNA (Nunokawa et al., 1993) (data not shown). We, therefore, used this oligonucleotide to quantify steady-state inducible NO synthase levels using a sensitive multiprobe solution hybridisation S1 nuclease protection assay (O'Donovan et al., 1991). A 37-base synthetic oligonucleotide, complementary to rat β-actin mRNA, was used as control. Protected oligonucleotides were separated by PAGE and gels were apposed to a Fuji BAS imaging plate for 2–3 days. To control for possible run-to-run variability, a positive control (mRNA from the heart of a 3 h lipopolysaccharide-treated rat) and a negative control (yeast tRNA substituted for mRNA) were common to every gel. Radioactivity present in each band was determined on a BAS-IIIS Bio Imaging Analyzer (Fuji). For each sample, inducible NO synthase mRNA levels were expressed as a ratio of the radioactivity present in the bands corresponding to the protected inducible NO synthase β-actin oligonucleotides. Data points between gels were normalized to the positive control value.

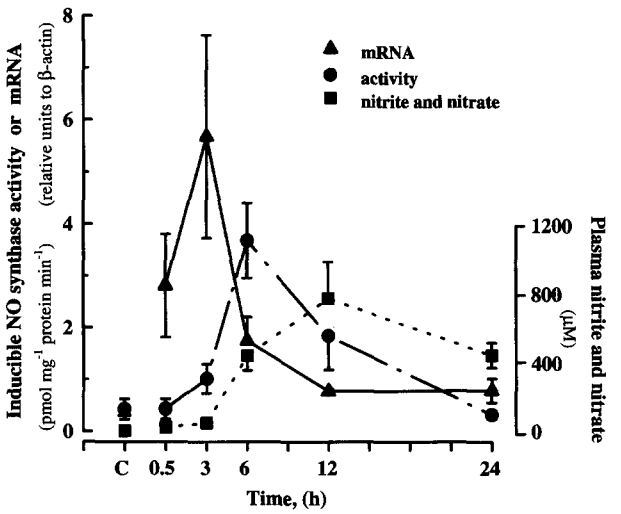

Fig. 1. Time course of change in inducible NO synthase mRNA levels and enzyme activity in the left ventricular wall of rats, and plasma nitrate and nitrite levels, following injection of lipopolysaccharide (4 mg kg^{-1}) or pyrogen-free saline vehicle (C, 6 h). Inducible NO synthase mRNA levels are expressed as relative radioactivity compared to β-actin mRNA in the same heart (see Materials and methods). Each point represents the mean from 4-11 animals. If no error bar is shown it lies within the symbol. $P < 0.05$ compared to the respective control value for time points ≥ 0.5 h for inducible NO synthase mRNA, for 6 and 12 h points for inducible NO synthase, and for ≥ 3 h for plasma nitrate and nitrite.

2.3. Statistics

Results are expressed as the mean ± S.E.M. for *n* hearts. For statistical analysis, inducible NO synthase mRNA data were transformed to their arcsine values in order to provide a normal distribution. Student's unpaired *t*-test was used for statistical analysis using $P < 0.05$ as the criterion for statistical significance.

3. Results

The cytosolic extracts obtained from the left ventricular wall of control rats 6 h following injection with saline vehicle showed negligible inducible NO synthase mRNA levels. Within 0.5 h of lipopolysaccharide treatment, there was a rapid and significant increase in inducible NO synthase mRNA in the heart which reached a peak at 3 h. By 12 h, mRNA levels dropped to near baseline levels (Fig. 1).

Cytosolic extracts from control hearts showed significant Ca^{2+}-dependent NO synthase activity (1.89 ± 0.45 pmol min^{-1} mg^{-1} protein, $n = 10$) but no significant Ca^{2+}-independent NO synthase activity (0.43 ± 0.20 pmol min^{-1} mg^{-1} protein, $n = 11$). Lipopolysaccharide treatment of rats caused a rapid and progressive rise in myocar-

dial inducible NO synthase activity to a maximum at 6 h of 3.68 ± 0.72 pmol min^{-1} mg^{-1} protein ($n = 11$) which then declined to the control level by 24 h ($P < 0.05$ vs. control at 6 and 12 h, Fig. 1). The time course of the rise and fall in myocardial inducible NO synthase activity coincided with the onset and disappearance of endotoxemia-like symptoms in the rats, namely a hunched appearance, ruffled coat and lethargy, all of which were resolved by 24 h. There were no significant changes in Ca^{2+}-dependent NO synthase activity following lipopolysaccharide treatment (data not shown).

Control rats had a plasma nitrate and nitrite concentration of 21 ± 2 μM ($n = 5$). Injection of lipopolysaccharide caused a time-dependent increase in the concentration of plasma nitrate and nitrite which was significantly higher than the control value by 3 h (47 ± 8 μM, $n = 6$, $P < 0.05$) and rose markedly to a peak level of 768 ± 212 μM at 12 h ($n = 4$). By 24 h, these levels remained significantly higher than control (439 ± 73 μM, $n = 4$, Fig. 1).

4. Discussion

These results show that inducible NO synthase mRNA steady-state levels rapidly increase within 30 min in the myocardium of lipopolysaccharide-treated rats and reach a maximum prior to attaining maximal activity of inducible NO synthase. This rapid expression of inducible NO synthase mRNA was followed by the sequential rise in both inducible NO synthase activity and plasma nitrate and nitrite, indicating the increased production of NO.

This is, to our knowledge, the most rapid increase in inducible NO synthase mRNA levels in any cell or tissue to date. It, therefore, appears that lipopolysaccharide-induced stimulation of inducible NO synthase activity in rat heart occurs via an increase in expression of the inducible NO synthase gene. While we cannot distinguish between an increase in transcription rate from an increase in mRNA stability, inducible NO synthase gene expression in most cells is regulated primarily at the transcriptional level, although post-transcriptional and post-translational regulatory mechanisms have also been described (see Nathan and Xie, 1994, for review).

As we studied a portion of the left ventricular myocardium, a number of different cells could contribute to the expression of inducible NO synthase, including cardiac myocytes (Schulz et al., 1992), endocardial endothelial cells (Smith et al., 1993), coronary microvascular endothelial cells (Balligand et al., 1995) and vascular smooth muscle cells, all of which express inducible NO synthase activity following exposure to lipopolysaccharide and/or pro-inflammatory cytokines. In situ hybridization and immunohistochemical studies will be necessary to resolve this. The time course of expression of inducible NO synthase mRNA and activity we observed in the intact heart during endotoxemia is far more rapid than that seen in various isolated and purified cell cultures (Nunokawa et al., 1993; Balligand et al., 1994). We suggest that an optimal complement of inducers and cofactors of inducible NO synthase are present during endotoxemia which stimulate its rapid expression in the heart. In their native environment, the cells which comprise the myocardium may have a more rapid (and possibly differential) time course of expression of inducible NO synthase activity in response to lipopolysaccharide.

Lipopolysaccharide treatment will stimulate the production of NO from a number of tissues, thus the plasma levels of nitrate and nitrite we observed reflect a balance between both the synthesis of NO from multiple sources and the excretion of nirate and nitrite. The long half-life of nitrate and nitrite (Zeballos and Bernstein, 1995) likely reflects the fact that after 24 h the levels remain significantly higher in the plasma although inducible NO synthase activity has declined to baseline values.

In summary, we have shown that the induction of inducible NO synthase enzyme activity in the myocardium during endotoxemia is preceded by an extremely rapid increase in inducible NO synthase mRNA. This in turn may be caused by an increase in transcription of the inducible NO synthase gene. Investigation of myocardial inducible NO synthase expression in other models of sepsis, which are also associated with depressed myocardial performance (see Abel, 1989, for review), is necessary. Given the emerging role of inducible NO synthase and the resultant production of excess NO in the pathophysiology of cytokine-mediated cardiac depression (Schulz et al., 1995), it seems rational to pursue therapeutic paradigms to specifically inhibit the activity or expression of inducible NO synthase.

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