—Original—

Effect of L-canavanine, an Inhibitor of Inducible Nitric Oxide Synthase, on Myocardial Dysfunction During Septic Shock

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Abstract

Overproduction of nitric oxide (NO) by inducible NO synthase (iNOS) plays a role in the pathophysiology of septic shock. The depression of cardiac contractility in such situations is mediated by proinflammatory cytokines, including interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). The effects of two NOS inhibitors with different isoform selectivity were compared in isolated working rat hearts. The depression of contractility by IL-1β and TNF-α was prevented by administration of a nonselective nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) or an inhibitor of inducible nitric oxide synthase, L-canavanine. In contrast, when L-NAME was administered in the absence of IL-1β and TNF-α, it depressed contractility over the 2h perfusion period by significantly reducing coronary flow. These results support current thinking that the depression of myocardial function by IL-1β and TNF-α is mediated, at least in part, by an intracardiac increase in inducible nitric oxide synthase, and that in contrast to L-NAME, the decline in coronary conductance seen in cytokine-treated is not prevented by L-canavanine hearts. L-canavanine shows selective inhibition of inducible nitric oxide synthase unlike the vasopressor action of L-NAME in cytokine-treated hearts.
(J Nippon Med Sch 2002; 69: 13–18)

Key words: nitric oxide (NO), nitric oxide (NO) synthase, working heart, L-canavanine, N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

Nitric oxide (NO) may be produced within the heart by either constitutive or inducible NO[1,2]. Depression of cardiac contractility by inflammatory conditions such as septic shock is mediated by proinflammatory cytokines, including interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)[3–5]. Inducible NO synthase expression contributes to depression of the contractile activity of isolated cardiac myocytes[6,7], papillary muscles[8], and the intact heart[9], as well as to the cytolysis of cardiac myocytes[10].

We have previously demonstrated that administration of L-canavanine or L-NAME attenuated the endotoxin-induced hypotension and vascular hyporeactivity to adrenaline, and the beneficial hemodynamic effects of L-canavanine are associated with inhibition of enhanced formation of NO by inducible NO synthase in a rat model of endotoxin shock[11]. L-canavanine is a selective inhibitor of inducible NO synthase[12–14]. It has been reported that L-canavanine ameliorates hypotension and vascular hyporeactivity to noradrenaline in rats with endotoxic shock, but does not affect blood pressure in normal anaesthetized rats[15]. However, these studies essentially

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focused on cardiovascular consequences of inhibitors, with only a limited interest towards their influence on heart. The present study was therefore designed to address this issue, by comparing the effects of the nonselective NOS inhibitor L-NAME to those of L-canavanine, a selective iNOS inhibitor, on myocardial depression induced by cytokine in the isolated working rat heart.

Methods

The Animal Experimental Ethical Review Committee at Nippon Medical School approved the experimental design.

Heart perfusion

Male Sprague-Dawley rats (250～300 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). The heart of each rat was rapidly excised, cannulated via the aorta, and initially perfused in a retrograde manner (Langendorff method) with Krebs-Henseleit bicarbonate buffer (KHB) that was continuously gassed with 95% O_2 −5% CO_2 and was maintained at 37℃. During this initial perfusion, the heart was trimmed of excess tissue, and the opening to the left atrium was cannulated. After a 10-min equilibration period, perfusion was switched to the working heart mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow and aortic outflow lines[16].

The standard perfusion medium was modified KHB containing the following (in mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; $CaCl_2$, 3; glucose, 10[5]. The medium was delivered into the left atrium at a hydrostatic preload equivalent to 9.5 mmHg. The hydrostatic afterload pressure was set at a column height equivalent to 70 mmHg. Each heart was paced at 300 beats/min throughout the experiment using a SEN-3301 stimulator (regular stimulus, duration 0.6 ms, delay 0.4 ms, Nihon Kohden, Tokyo, Japan) with leads placed on the aortic and left atrial cannula.

Aortic flow was measured by timed collection of perfusate from the overflow of the afterload column, and coronary flow was measured by timed collection of pulmonary artery effluent[5].

The left ventricular pressure was measured with a transducer (Abbott Ireland, Sligo, Ireland) that was connected to a thin 18-gauge catheter (Argyle Intramedicut Catheter, Sherwood, Tokyo, Japan) inserted into the left ventricle through the mitral valve from the angled steel cannula in the left atrium. The left ventricular pressure was recorded continuously using a MacLab 8-channel data acquisition unit connected to an Apple computer. The ventricular pressure signal was digitally processed to yield the heart rate and dp/dt[17].

Experimental protocol

After 20 min of equilibration in the working mode, cardiac output, aortic pressure, and coronary flow were measured. Cardiac work, the product of cardiac output (ml/min) × peak systolic pressure (mmHg), was used as an index of contractile function[16]. A combination of IL-1β (5 ng/ml), and TNF-α (20 ng/ml), with or without L-canavanine (1 mM), was added to the perfusate of some hearts (referred to as t = 0h), and the heart was perfused for 2h. Other hearts were treated with L-NAME (1 mM) at the beginning of perfusion in the working mode. A dose of 1 mM of L-canavanine reversed the LPS-induced vascular hyporeactivity[14], and there were similar effects whereby L-canavanine (1 mM) or L-NAME (1 mM) inhibited the endotoxin-induced increases in venous levels of NO-hemoglobin[11]. Therefore, a dose of 1 mM of L-canavanine or L-NAME was used in this study. An additional group of hearts was perfused without cytokines in the presence of L-canavanine (1 mM) or L-NAME (1 mM).

Materials

L-canavanine and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan)

Statistics

Data are expressed as the mean ± S.D. Comparisons among groups were performed by one-way repeated-measures analysis of variance (ANOVA)

followed by Fisher's protected least significant difference (Fisher's PLSD) test. Statistical significance was defined as $p<0.05$. All analyses were performed with StatView II software.

Results

Time course of cardiac depression by IL-1β and TNF-α

Fig. 1A shows the time course of changes in cardiac function, measured as cardiac work, in control hearts and cytokine-treated hearts. In contrast to control hearts, hearts treated with IL-1 β and TNF-α showed a significant reduction in cardiac work after 1h of perfusion (73.5±14.5%, 49.5±8.1%, $p<0.05$). After 2h, cardiac work was 48.9±8.3% in the control hearts and was markedly reduced in the cytokine-treated hearts (13.2±1.1%, $p<0.05$).

Effects of L-canavanine and L-NAME on hearts perfused without cytokines

Additional hearts were perfused without cytokines, and the actions of L-canavanine or L-NAME were assessed by comparison with control hearts (**Fig. 1B**). The hearts perfused with L-canavanine alone showed no deterioration of cardiac function over the 2h perfusion period. Interestingly, the L-NAME perfused hearts showed significant depression of cardiac function compared with the control hearts. This depression of cardiac function was significantly more severe after 2h of perfusion.

Effects of L-canavanine and L-NAME on the cardiac depressant action of IL-1 β and TNF-α

After 1h of perfusion with IL-1β and TNF-α plus L-NAME, significantly better cardiac function was seen compared with hearts perfused using only IL-1β and TNF-α (64.3±14.1%, 49.5±8.1%, $p<0.05$, **Fig. 1C**). Moreover, after 2h of perfusion, the progressive cardiac depression seen in the cytokine-treated hearts was prevented by L-NAME and the cardiac function curve of this group could be superimposed on that of the control hearts. Interestingly, pretreatment with L-canavanine prevented the loss of function in the cytokine-treated hearts over the entire 2h perfusion period.

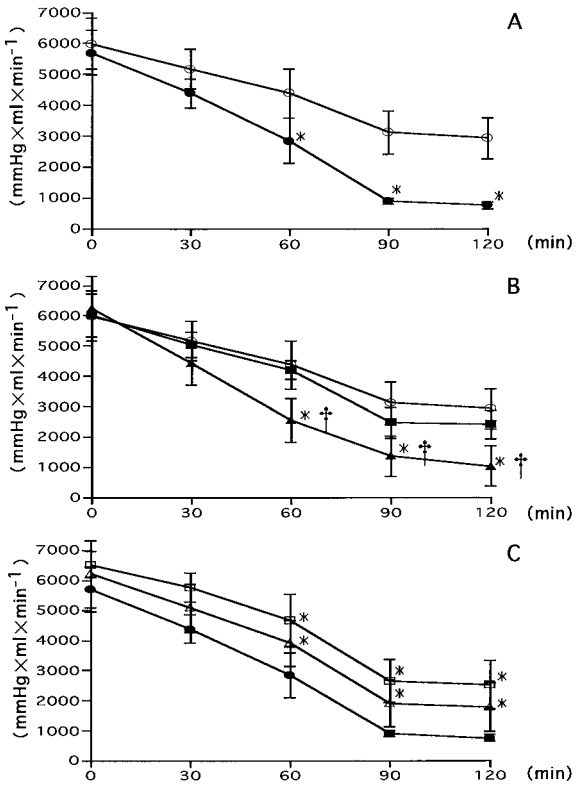

Fig. 1 A: Effects of interleukin-1β and tumor necrosis factor-α (cytokine) on the time course (min) of the changes in cardiac work (mmHg × m*l* × min^{-1}) in isolated working rat hearts. ○, control hearts (n=6); ●, hearts treated with cytokines (n=6) at t=0h. ＊$p<0.05$ vs. control, ANOVA. B: Time course (min) of the changes in cardiac work (mmHg × m*l* × min^{-1}) in isolated working rat hearts perfused without cytokines. ○, control hearts; ▲, L-NAME (n=6); ■, L-canavanine (n=6). ＊$p<0.05$ vs. control, † $p<0.05$ vs. L-canavanine, ANOVA. C: Time course (min) of the changes in cardiac work (mmHg × m*l* × min^{-1}) in isolated working hearts treated with cytokines. ●, cytokine-treated hearts; △, cytokines + L-NAME (n=6); □, cytokines + L-canavanine (n=6). ＊$p<0.05$ vs. cytokines, ANOVA.

Effect of treatment on coronary flow

Table 1 summarizes the coronary flow data after 0, 1 and 2h of perfusion. After 2h, the coronary flow of the control hearts was 55.2±9.1% of the initial value measured at 0h (n=6, $p<0.05$), and it was markedly lower in the cytokine-treated hearts (31.4±16.9%, n=6, $p<0.05$ vs. control at 2h). Both L-canavanine and L-NAME abolished the decrease

Table 1 Effects of each treatment protocol on coronary flow

Group	n	Coronary Flow, ml/min		
		0h	1h	2h
Control	6	15.4±1.5	12.7±1.9	8.4±1.1
L-canavanine	6	14.2±2.0	11.4±0.9	10.3±2.2
L-NAME	6	15.7±1.7	7.7±2.1	4.7±0.9*†
Cytokines	6	17.6±2.7	10.6±2.3	5.2±2.2*†
Cytokines+L-canavanine	6	17.3±2.8	12.1±1.9	8.5±2.0
Cytokines+L-NAME	6	16.5±2.5	11.4±1.9	7.9±2.4

Values are the mean±SD. Cytokines, interleukin-1β plus tumor necrosis factor-α.
*$p<0.05$ vs. control. †$p<0.05$ vs. L-canavanine (one-way ANOVA).

in coronary flow induced by cytokine treatment. Although L-canavanine did not prevent the spontaneous decrease in coronary flow during 2h of perfusion, coronary flow was markedly reduced by perfusion with L-NAME (29.9±4.7%, n=6, $p<0.05$ vs. control or L-canavanine at 2h).

Discussion

This study shows that the proinflammatory cytokines IL-1β and TNF-α caused time-dependent and progressive depression of cardiac function in isolated working rat hearts. It has been demonstrated that an enhanced formation of NO contributes to circulatory failure in animals[18] with endotoxic shock. According to the results of many experimental studies[11,19], NO is partly responsible for the hypotension and catecholamine hyporesponsiveness observed in septic states several hours after lipopolysaccharide injection. However, a relatively rapid onset of the effects of IL-1β and TNF-α was also noted, which contrasts with the results of many studies on inducible NO synthase activity[20]. It seems to be too soon for expression of new NO synthase, but a recent study[2] showed a rise in myocardial Ca^{2+}-independent NO synthase activity within 30 minutes of the injection of endotoxin into rats. It has been reported that inducible NO synthase mRNA levels in the left ventricular wall increased within 30 min and then peaked at 3h after injection of endotoxin into rats[21]. In this study, exposure of hearts to IL-1β and TNF-α resulted in greater depression of cardiac function within 60 minutes of the start of perfusion when compared with control hearts. This depression was accompanied by Ca^{2+}-independent NO synthase activity, suggesting that the early loss of cardiac contractility caused by IL-1β and TNF-α may precede the induction of iNOS.

Potentiation of the depression of contractility by IL-1β and TNF-α was prevented by both of the NO synthase inhibitors, L-NAME and L-canavanine. It has been reported that there are some differences between the effectiveness of L-NAME and L-canavanine for reversing hypotension and vascular hyporeactivity during endotoxic shock. In the present study, L-NAME was more effective than L-canavanine in reversing both hypotension and vascular hyporeactivity, but L-NAME markedly reduced pulse pressure and heart rate, possibly due to the inhibition of both constitutive NO synthase and inducible NO synthase[11].

L-NAME is a nonselective inhibitor of both Ca^{2+}-dependent NO synthase, such as that found in the vascular endothelium, and the inducible Ca^{2+}-independent enzyme. In the absence of IL-1β and TNF-α, L-NAME still caused a significant reduction in coronary flow in control hearts. This direct and early impairment of coronary flow by L-NAME may cause the depression of cardiac function during perfusion of hearts not exposed to cytokines. There have been a growing number of recent publications addressing the influence of NO on the contractile (inotropic) and relaxant (lusitropic) properties of cardiac myocytes and the heart[22-24]. Endothelial shear stress has been shown to increase NO by stimulating constitutive NO synthase[25,26]. However, to date, there has been no evidence indicating that altering the cardiac load independent of coronary blood flow

affects NO synthesis in vivo[27]. In conscious and chronically instrumented dogs, N^G-monomethyl-L-arginine (L-NMMA) was shown to induce a dose-related (L-arginine-reversible) decrease in coronary flow[28]. The coronary vasodilation that follows vagal stimulation has also been suggested to be NO dependent. Three inhibitors of NO synthase, L-NMMA, N-iminoethyl-L-ornithine (L-NIO), and L-NAME cause a concentration-dependent increase in the resting coronary perfusion pressure, confirming previous in vitro reports[29-31] that basal NO synthesis plays a role in the regulation of resting coronary vascular tone. Similar conclusions have also been drawn from studies with these compounds in anesthetized dogs[32] and conscious dogs[28,33]. On the other hand, L-canavanine did not prevent the spontaneous decline in coronary flow during 2h of perfusion, and coronary flow was markedly lower in L-NAME-treated hearts.

L-canavanine has been previously shown to inhibit the synthesis of NO by macrophages[12] and neutrophils. In contrast, it was reported to be a less effective inhibitor of cytosolic NO synthase in endothelial cells than in macrophages[14] and did not augment phenylephrine-induced contraction of endothelium intact or denuded rabbit aortas at concentrations up to 10 mM[34]. In addition, prolonged (2h) incubation with a high (>2 mM) concentration of L-canavanine was shown to only partially inhibit agonist-stimulated endothelial NO release[34].

In marked contrast to L-NAME, L-canavanine did not inhibit baseline coronary flow; nor did it reduce cardiac work compared with control hearts, thus adding further support to its selective iNOS inhibitory profile. This difference between L-canavanine and L-NAME also implies that some of the NO derived from iNOS expression may counteract vasoconstrictor substances released in response to cytokines.

In conclusion, this study demonstrates that both L-NAME and L-canavanine prevent the depression of contractility in cytokine-treated hearts, and that in contrast to L-NAME, the decline in coronary conductance seen in cytokine-treated hearts is not prevented in L-canavanine hearts. L-canavanine showed selective inhibition of inducible nitric oxide synthase unlike the vasopressor action of L-NAME (a nonselective nitric oxide synthase inhibitor) in cytokine-treated hearts. Selective inhibitors of iNOS should be useful in assessing how enhanced NO production contributes to cardiac dysfunction in experimental models of heart failure.

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(Received, June 5, 2001)
(Accepted, June 28, 2001)