

Increased vasoconstriction to noradrenaline by 1400W, inhibitor of iNOS, in rats with streptozotocin-induced diabetes

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Abstract

There is evidence that inducible nitric oxide synthase (iNOS) is activated at the acute phase of diabetes. We examined if selective inhibition of iNOS by 1400W (*N*-3-aminomethyl-benzyl-acetamidine) increases vascular response to noradrenaline in rats with streptozotocin (60 mg/kg *i.v.*)-induced diabetes for a duration of 3 weeks. The effects of noradrenaline on mean arterial pressure (MAP; 6, 16, 45 and 122×10^{-9} mol/kg/min) and mean circulatory filling pressure (MCFP; 16 and 45×10^{-9} mol/kg/min) were obtained in conscious and unrestrained diabetic rats and control rats before as well as after treatment with 1400W (3 mg/kg followed by 3 mg/kg/h, *i.v.*). Rats with early streptozotocin-induced diabetes had decreased mean arterial pressure and mean circulatory filling pressure responses to noradrenaline. Treatment with 1400W did not affect responses in the control rats but increased maximum pressor response to noradrenaline (from 46 ± 3 to 63 ± 5) and mean circulatory filling pressure response to the high dose (45 nmol/kg/min) of noradrenaline (from 1.0 ± 0.2 to 3.8 ± 0.3 mmHg) in the diabetic rats. Thus, selective inhibition of iNOS by 1400W increases arterial and venous constriction to noradrenaline in conscious rats with streptozotocin-induced diabetes.

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1. Introduction

Diabetes is associated with functional abnormalities in the cardiovascular system. *In vitro* and *in vivo* studies have shown that pressor response and arterial constriction to vasoactive agents such as noradrenaline, angiotensin and endothelins are depressed in diabetic rats at 2 weeks after the injection of streptozotocin (Hebden *et al.*, 1987; Makino and Kamata, 1998; Bardell and MacLeod, 2001; Misurski *et al.*, 2001; Cheng *et al.*, 2003a). In addition, rats with streptozotocin-induced diabetes had depressed venoconstriction to noradrenaline *in vivo* at 2 weeks after injection of streptozotocin (Cheng *et al.*, 2003a).

It is unclear what was the primary cause of reduced vasoconstriction at the acute phase of diabetes. Several studies have shown that the inducible isoform of nitric oxide synthase (iNOS) is activated at both the acute and

chronic phases of diabetes. Indeed, iNOS was detected in the mesenteric artery of rats with streptozotocin-induced diabetes at 12–14 weeks (Bardell and MacLeod, 2001), cardiac myocytes from diabetic rats at 8 weeks after injection of streptozotocin (Smith *et al.*, 1997) and platelets from patients with type I and II diabetes (Tannous *et al.*, 1999). The total activities of iNOS and endothelium nitric oxide synthase (eNOS) and the level of mRNA encoding these isoforms of nitric oxide synthase are increased in the hearts of rats with streptozotocin-induced diabetes for a duration of 4 to 6 weeks after induction (Stockklauser-Färber *et al.*, 2000). Moreover, *in vitro* inhibition of iNOS by *S*-ethyl-isothiourea increased the potency (reduced EC₅₀) of contraction to noradrenaline in endothelium-denuded mesenteric arteries from rats rendered diabetic for 12–14 weeks (Bardell and MacLeod, 2001). There is as yet no published studies which examined if induction of iNOS contributes to the reduced arterial and venous constriction to noradrenaline at the acute phase of diabetes.

The present study examined if attenuated *in vivo* arterial and venous vasoconstriction to noradrenaline at the early phase (3 weeks) of streptozotocin-induced diabetes is due to

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activation of iNOS. Pressor and mean circulatory filling pressure (MCFP; index of body venous tone) responses to noradrenaline were measured in vehicle-treated control rats and streptozotocin-induced diabetic rats prior to and following the administration of 1400W (*N*-3-aminomethyl-benzyl-acetamidine), a selective inhibitor of iNOS (Garvey et al., 1997; Alderton et al., 2001). Mean circulatory filling pressure is the driving force of venous return and is experimentally the equilibrium pressure that exists in the circulation immediately after an abrupt cessation of blood flow (Guyton, 1963; Guyton et al., 1954; Rothe, 1993; Pang, 2000). It has been shown that 1400W is >1000-fold (in vitro) and >50-fold (in vivo) more selective in inhibiting iNOS than eNOS (Garvey et al., 1997) and is able to reverse hypotension (Wray et al., 1998) and augment cardiac output (Cheng et al., 2003b) in rats with *Escherichia coli* lipopolysaccharide-induced sepsis, a condition associated with the activation of iNOS.

2. Materials and methods

2.1. Experimental animals and induction of diabetes

Male Wistar rats (300–350 g) were obtained from Charles River Canada. The rats were maintained under 12:12 h light:dark cycle (lights on from 7 a.m. to 7 p.m.) and supplied with standard laboratory chow diet (PMI Feeds) and water ad libitum.

The rats were injected with streptozotocin (60 mg/kg, 1 ml/kg, i.v.) or an equal volume of vehicle (0.9% NaCl) via the tail vein under light halothane anaesthesia. The rats were considered to be diabetic and used for the study if they had hyperglycemia (>15 mM) at 48 h after injection of streptozotocin as detected by AccuSoft (Hoffmann-La Roche) test strips (Sambandam et al., 2000; McNeill, 1999). Plasma glucose was measured by the glucose oxidase method (Sigma, Trinder 100 kit) via the use of a Spectrarainbow (ART F039039, Austria). The rats were studied at 3 weeks after injection of streptozotocin or the vehicle.

2.2. Surgical preparation

The streptozotocin-induced diabetic and control rats were surgically prepared under halothane anaesthesia. A saline-filled, balloon-tipped catheter was inserted into the right atrium through the right external jugular vein. Cannulae were also inserted into an iliac artery for the measurement of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, Oxnard, CA), into the right iliac vein for the withdrawal of blood (0.1 ml) for the measurement of plasma glucose and infusion of drugs, and into the inferior vena cava via the left iliac vein for the measurement of central venous pressure by another pressure transducer (P23DB, Gould Statham). All cannulae were tunnelled subcutaneously to the back of the neck and exteriorised.

The rats were recovered from surgery and anaesthesia for at least 6 h prior to the study.

2.3. Experimental protocol

The diabetic and control rats ($n=6$ or 7 each) were placed in a small cage and allowed to wander freely during the study. After equilibration for 1 h, the rats were first pretreated with propranolol (8×10^{-7} mol/kg i.v. bolus followed by continuous infusion at 3.4×10^{-7} mol/kg/min) to block β -adrenoceptors. At 15 min after the start of administration of propranolol, dose–response curves of single doses of noradrenaline (6, 16, 45 and 122×10^{-9} mol/kg/min) were constructed in the diabetic and control rats. Mean arterial pressure and heart rate (HR) measurements were taken prior to and at 10 min after the start of infusion of propranolol, and at the plateau phase of response to noradrenaline (3 to 10 min after the start of infusion), whereas mean circulatory filling pressure readings were taken at the baseline and the plateau phase of response to the 2nd and 3rd doses of noradrenaline (16 and 45×10^{-9} mol/kg/min). Each dose of noradrenaline was followed by a recovery period of 10–15 min. After recovery of the response to the last dose of noradrenaline, the rats were given 1400W (3 mg/kg followed by 3 mg/kg/h, i.v.). At 1 h later, a 2nd dose–response curve of noradrenaline was constructed in both groups of rats. Haemodynamic measurements were again taken at the baseline condition (5 min prior to the infusion of noradrenaline) and at the plateau phase of response to noradrenaline.

2.4. Mean circulatory filling pressure measurements

Central venous pressure was measured after transiently stopping the circulation through injection of a small volume of fluid into the right atrial balloon. Within 5 s following inflation of the balloon, mean arterial pressure decreased to a plateau value (referred to as final arterial pressure), while central venous pressure increased to a plateau value (referred to as venous plateau pressure). Mean circulatory filling pressure was calculated as follows: mean circulatory filling pressure = venous plateau pressure + $1/60$ (final arterial pressure – venous plateau pressure), using $1/60$ as the ratio of arterial to venous compliance (Yamamoto et al., 1980).

2.5. Drugs

N-3-aminomethyl-benzyl-acetamidine (1400W) was obtained from Calbiochem (San Diego, CA). Noradrenaline and propranolol were from Sigma (USA). All drugs were dissolved in normal saline (0.9% NaCl).

2.6. Statistical analyses

Data were log-transformed prior to statistical analysis to obtain ED_{50} values to noradrenaline using the GraphPad

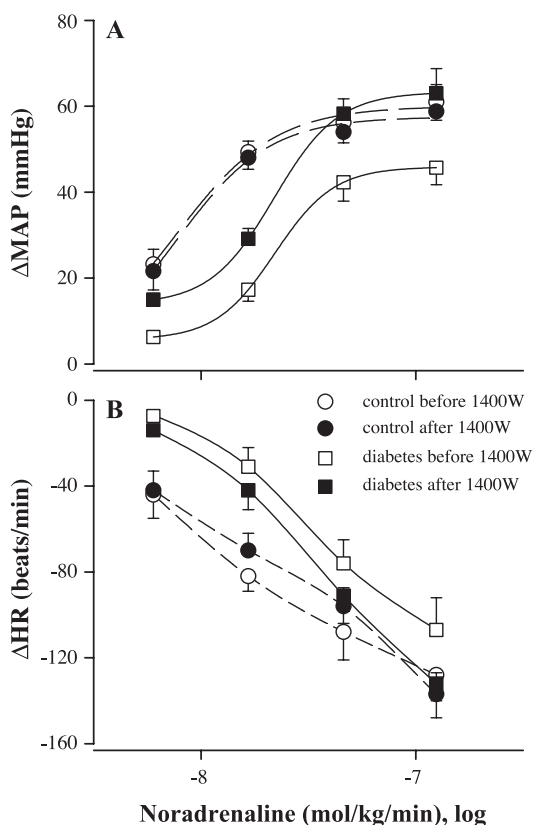


Fig. 1. Dose–response curves for the effects (mean \pm S.E.M., $n=6-7$ per group) of noradrenaline on (A) mean arterial pressure (Δ MAP), and (B) heart rate (Δ HR) before [(○) and (□)] and after [(●) and (■)] the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in conscious rats injected with streptozotocin [60 mg/kg, (□) and (■)] or the vehicle [0.9% NaCl, (○) and (●)] at 3 weeks prior to these experiments.

Prism program. ED_{50} and E_{max} values before and after the administration of 1400W were analysed by one way analysis of variance (ANOVA) followed by the Tukey Test. Dose–response relationships of mean arterial pressure (Fig. 1) were analysed by one-way (between groups) and two-way (within the same group) repeated measures ANOVA followed by the Tukey test (SigmaStat statistical software). In all cases, a probability of error ($P<0.05$) was selected as the criterion for statistical significance.

3. Results

3.1. Baseline values

The rats had higher plasma concentration of glucose at 48 h after i.v. injection of streptozotocin relative to the vehicle-treated control rats (21.0 ± 1.5 vs. 5.7 ± 0.2 mM, respectively).

At 3 weeks after the induction, the diabetic rats had lower body weight and higher plasma glucose than the control rats (Table 1). At this time, the diabetic rats had slightly (insignificant) lower baseline mean arterial pressure,

Table 1

Baseline values of body weight, plasma glucose, mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in conscious, diabetic rats and control rats pretreated with propranolol at 3 weeks following the administration of streptozotocin (60 mg/kg i.v.) or the vehicle (0.9% NaCl), respectively

	Control	Diabetic
Body weight (g)	436 ± 21	332 ± 10^a
Plasma glucose (mM)	6.1 ± 0.3	24.4 ± 2.4^a
<i>MAP (mmHg)</i>		
1st baseline	118 ± 6	109 ± 2
2nd baseline	113 ± 6	105 ± 2
<i>HR (beats/min)</i>		
1st baseline	369 ± 5	339 ± 10^a
2nd baseline	365 ± 4	336 ± 13^a
<i>MCFP (mmHg)</i>		
1st baseline	7.3 ± 0.3	6.8 ± 0.4
2nd baseline	6.7 ± 0.2	6.8 ± 0.5

All values are means \pm S.E.M. ($n=6-7$ per group).

^a Denotes the significant difference from controls ($P<0.05$). Baseline values of MAP, HR and MCFP were obtained at 5 min prior to the start of noradrenaline infusion, both before (1st baseline) and after (2nd baseline) the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.).

mean circulatory filling pressure and heart rate (110 ± 2 , 6.3 ± 0.1 mmHg and 374 ± 13 beats/min, respectively) relative to the control rats (119 ± 5 , 6.5 ± 0.2 mmHg and 402 ± 7 beats/min).

Pretreatment with propranolol decreased the heart rate in the diabetic (-35 ± 7 beats/min) as well as control (-33 ± 6 beats/min) rats at 10 min after the start of infusion but did not significantly alter the mean arterial pressure and mean circulatory filling pressure. After treatment with propranolol, the heart rate was slightly lower in the diabetic rats relative to the control rats, but mean arterial pressure and mean circulatory filling pressure remained slightly but insignificantly lower in the diabetic rats relative to the controls (1st baseline, Table 1). The administration of 1400W did not significantly affect the mean arterial pressure, heart rate and mean circulatory filling pressure in either the diabetic or control rats (2nd baseline, Table 1).

Table 2

ED_{50} and E_{max} for dose–response curves of the changes in mean arterial pressure (MAP) to noradrenaline before and after the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in diabetic rats and control rats ($n=6-7$ per group) at 3 weeks following i.v. injection of streptozotocin (60 mg/kg) and the vehicle (0.9% NaCl), respectively

	ED_{50} (nmol/kg)		E_{max} (mmHg)	
	Control	Diabetic	Control	Diabetic
MAP before 1400W	6.8 ± 0.2	23.6 ± 3.7^a	61 ± 3	46 ± 3^a
MAP after 1400W	6.3 ± 0.6	16.3 ± 6.5^a	62 ± 2	63 ± 5^b

All values are means \pm S.E.M. ($n=6-7$ per group). All data of ED_{50} were log-transformed prior to statistical analysis.

^a Denotes significant difference from control ($P<0.05$).

^b Denotes significant difference from before the administration of 1400W ($P<0.05$).

3.2. Response to noradrenaline

Noradrenaline had caused a dose-dependent increase in mean arterial pressure and decreases in heart rate in the control and diabetic rats. Curve analyses show that the changes in mean arterial pressure and decreases in heart rate in the diabetic rats were significantly smaller than those of the controls (Fig. 1A,B). The diabetic rats had lower potency (higher ED_{50}) and lower efficacy (E_{max}) of response to noradrenaline than the controls (Table 2). Noradrenaline had (16 and 45 nmol/kg/min) increased the mean circulatory filling pressure in the diabetic and control rats, and both changes were significantly and markedly less in the diabetic than the controls rats (Fig. 2A,B).

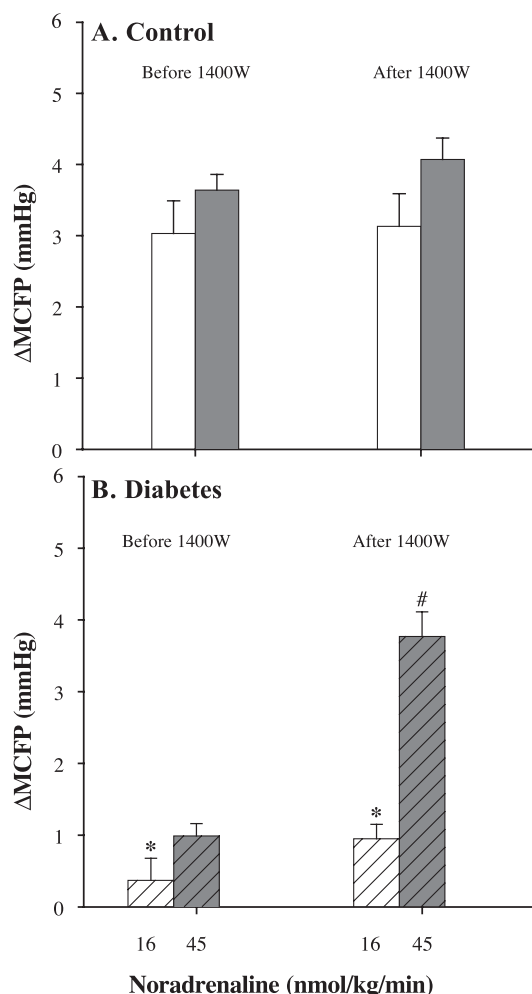


Fig. 2. Effects (mean \pm S.E.M., $n=6-7$ per group) of noradrenaline (16 and 45 nmol/kg/min) on the changes in mean circulatory filling pressure (Δ MCFP) before and after the administration of 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) in conscious rats injected with (A) vehicle (0.9% NaCl), or (B) streptozotocin (60 mg/kg) at 3 weeks prior to these experiments. *Significantly different ($P<0.05$) from corresponding responses (in panel A) in the control rats given the vehicle. #Significantly different from responses in the diabetic rats before the administration of 1400W.

3.3. Effects of pretreatment with 1400W on responses to noradrenaline

Pretreatment (60 min) with 1400W did not significantly affect the mean arterial pressure, heart rate (Fig. 1) and mean circulatory filling pressure (Fig. 2A) responses to noradrenaline in the control group. The 1400W treatment in the diabetic rats augmented the noradrenaline-induced increases in mean arterial pressure (Fig. 1A), such that the maximum mean arterial pressure was restored, and potency of response to noradrenaline was slightly (insignificantly) increased (Table 2). Pretreatment with 1400W slightly (insignificantly) increased the mean circulatory filling pressure response to the low dose of noradrenaline but restored the mean circulatory filling pressure response to the high dose of noradrenaline relative to the corresponding response in the control rats (Fig. 2B).

4. Discussion

At 3 weeks after the induction of diabetes with streptozotocin, the rats had reduced potency (higher ED_{50}) and efficacy (E_{max}) of pressor response as well as attenuated the mean circulatory filling pressure response to noradrenaline. These results were similar to those reported previously for rats with streptozotocin-induced diabetes for a duration of 2 weeks (Cheng et al., 2003a) and are indicative of generalised depression of α -adrenoceptor-mediated vasoconstriction at the acute phase of diabetes. Both the diabetic and control rats had dose-dependent bradycardic response to noradrenaline that was likely due to reflex parasympathetic activation and not sympathetic withdrawal because the rats were pretreated with propranolol to block the chronotropic and inotropic (β -adrenoceptor-mediated) effects of noradrenaline. Reduced pressor response to noradrenaline has been reported in pithed rats at 2 weeks (Foy and Lucas, 1976; Lucas, 1985), and in conscious rats at 4 to 5 weeks (Jackson and Carrier, 1983) and 6 weeks (Yu and McNeill, 1992) following the induction with streptozotocin. However, unlike the present study, these rats were not pretreated with β -adrenoceptor antagonist.

It is unclear what was the primary cause of attenuated vasoconstriction. There is evidence that iNOS is activated at the acute phase of diabetes (see Introduction). Furthermore, iNOS induction in diabetes may be a consequence of hyperglycemia. The exposure of aortic endothelial cells to high glucose for 7–10 days was reported to increase the release of peroxynitrite, a highly reactive and cytotoxic oxidant derived from the interaction of NO with the free radical superoxide (Zou et al., 2002). Moreover, 2 h perfusion of hearts with a solution containing a high concentration of glucose in vitro was associated with an increase in the expression of the iNOS (but not eNOS) gene, the generation of nitric oxide and superoxide, and the apoptosis

of cardiac cells (Ceriello et al., 2002). The expression of eNOS, in contrast to that of iNOS, is decreased in retinal vascular endothelial cells incubated with high glucose and glycated proteins (Chakravarthy et al., 1998). There is also an indication that, in addition to causing cellular damage, an overproduction of nitric oxide through iNOS induction can cause cardiovascular depression. Indeed, hypotension in lipopolysaccharide-induced septicaemia is associated with iNOS induction, and the blockade of iNOS by 1400W in this condition reversed hypotension (Wray et al., 1998) and augmented the peripheral vascular resistance and cardiac output (Cheng et al., 2003b). It is therefore imperative to determine if the blockade of iNOS restores vasoconstriction in diabetes.

It is well known that a small amount of nitric oxide, as synthesized by the constitutive enzymes eNOS and nNOS, is important for normal physiological function. To elucidate the vascular role of iNOS in diabetes, it is therefore important to selectively inhibit the activity of iNOS. Among the inhibitors of nitric oxide synthases, 1400W is by far the most selective for inhibiting the activity of iNOS; its ratio of selectivity for iNOS vs. eNOS is >4000-fold, in contrast to those of aminoguanidine (11-fold), *N*⁵-iminoethyl-L-ornithine (49-fold; Alderton et al., 2001) and isothioureas (two- to sixfold; Garvey et al., 1994). In addition, the *in vitro* potency of 1400W in inhibiting iNOS is 135 and 19 times those of aminoguanidine and *N*⁵-iminoethyl-L-ornithine, respectively (Alderton et al., 2001). There is also evidence that 1400W is effective in reversing vascular abnormalities in pathophysiological conditions known to be associated with the induction of iNOS. Indeed, a single injection (0.3 mg/kg s.c., ED₅₀ dose) of 1400W reversed the delayed lipopolysaccharide (iNOS)-induced vascular injury in rats (Garvey et al., 1997). Furthermore, 1400W (3 mg/kg followed by 3 mg/kg/h i.v.) restored the blood pressure of rats with lipopolysaccharide-induced endotoxic shock, and the drug did not affect the blood pressure of control rats (Wray et al., 1998; Cheng et al., 2003b).

In the present study, 1400W treatment restored the efficacy (maximum response) and insignificantly increased the potency (reduced ED₅₀) of mean arterial pressure response to noradrenaline, and it also significantly increased the mean circulatory filling pressure response to a high dose of noradrenaline. Furthermore, 1400W did not affect any measured variables in the control rats. This ability of 1400W to selectively restore vascular response to noradrenaline in the diabetic rats shows that overproduction of nitric oxide by iNOS plays an important role in depressing α -adrenoceptor-mediated arterial and venous constriction at the acute phase of diabetes. This interpretation is in line with well-known clinical and experimental observations that nitric oxide is a potent and efficacious dilator of arterial resistance as well as capacitance vessels. Furthermore, the exogenous supply of nitric oxide through the administration of nitrovasodilators, such as acetylpenicillamine or sodium nitroprusside, has been shown to reduce mean arterial pressure

and mean circulatory filling pressure of anaesthetised rats (Ng and Pang, 1998). An important mechanism by which nitric oxide mediates vasodilatation is through the inhibition of noradrenaline-mediated vasoconstriction (Zanzinger et al., 1994).

In summary, the present study is the first to demonstrate that the selective inhibition of iNOS by 1400W increases α -adrenoceptor-mediated constriction in arterial resistance as well as capacitance vessels at the acute phase of diabetes. Our findings suggest that selective inhibitors of iNOS may be a viable therapeutic option in restoring vascular contraction in diabetes.

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