The effect of nitric oxide inhibition on the renin response to frusemide, in man

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Aims We wished to see if renin release in man was inhibited by nitric oxide blockade, suggesting a role for nitric oxide in renin release. Evidence from animal studies has shown variable effects on renin release depending on the model and stimulus used.

Methods Ten normal male volunteers, received either L-NMMA as a front loaded infusion (4 mg kg^{-1} bolus, with 4 mg kg^{-1} infusion), or placebo, followed by an intravenous bolus of 5 mg frusemide to stimulate renin. To investigate whether any alteration in renin release was due to the pressor effect of the L-NMMA, the experiment was repeated using an equipressor dose of phenylephrine ($0.5 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$).

Results L-NMMA caused the expected increase in mean arterial pressure (96 ± 2.6 vs $89 \pm 3.3 \text{ mmHg}$ $P < 0.05$ [mean $\pm$ s.e.mean]), and a reduction in heart rate (59 ± 3.6 vs $67 \pm 2.5 \text{ beats min}^{-1}$ $P < 0.05$). L-NMMA completely blocked the renin rise following the bolus of frusemide (1.18 ± 0.196 vs $1.96 \pm 0.333 \text{ ng ml}^{-1} \text{ h}^{-1}$ $P < 0.01$). Phenylephrine $0.5 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$ produced very similar haemodynamic effects to L-NMMA, and also suppressed the renin response to frusemide (1.43 ± 0.290 vs $2.67 \pm 0.342 \text{ ng ml}^{-1} \text{ h}^{-1}$ $P < 0.01$).

Conclusions In man, the renin inhibition seen with NO synthesis inhibition is similar to that seen with a standard pressor stimulus, hence inhibition of renin in man by L-NMMA, may be due to both direct effects on macula densa cells and indirect haemodynamic effects.

Keywords: blood pressure, frusemide, nitric oxide, phenylephrine, renin

Introduction

There has recently been much experimental evidence to suggest that nitric oxide is important in control of the secretion of a variety of hormones, including pituitary secretion of growth hormone [1], insulin from pancreatic islet cells [2], and hypothalamic secretion of CRF [3]. This evidence would suggest that nitric oxide may be important as a signalling molecule in these situations, possibly working in a paracrine fashion. More recently, experiments have been undertaken to examine the possibility that nitric oxide also mediates renin release.

This suggestion first arose when isoforms of nitric oxide synthase were found in the macula densa cells around the renal tubules [4], i.e. the same cells that are believed to control the secretion of renin from the

juxtaglomerular cells [5]. Nitric oxide synthase has also been localized in a variety of other sites within the kidney including vascular and tubular elements [6]. Furthermore, activation of nitric oxide synthase in the macula densa appears to occur under conditions which would be expected to increase renin secretion, e.g. frusemide stimulation [7]. In whole animal experiments, mainly in rats, nitric oxide antagonists can be shown to inhibit the release of renin in response to frusemide [8], which suggests a permissive role for nitric oxide in renin release [7, 9, 10]. However with regard to pressure-dependent renin release the results are conflicting. Sigmon *et al.* [11] found that the inhibition of renin by nitric oxide antagonists was due to their effects on renal perfusion pressure, and that if this was controlled, the inhibition of renin could be reversed. However more recently, Reid *et al.* using a rabbit model, have demonstrated that the effect is not pressure dependent, but is, in contrast, a direct effect of nitric oxide inhibition, as renin was not suppressed by an equipressor dose of phenylephrine [7].

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The above controversy arises from a series of animal studies, and we felt it appropriate to examine the situation in man. We therefore investigated the effect of nitric oxide blockade on renin secretion in response to a small intravenous bolus of frusemide. We extended our study to examine whether any observed effect of nitric oxide blockade on renin release was due to its pressor effect *per se*, or whether it was a specific and direct effect of nitric oxide, independent of pressure, as suggested by Reid *et al.* [7].

Methods

The study has two separate limbs. For both limbs written informed consent was obtained for the study protocols, which had been previously approved by the Tayside Committee for Medical Research Ethics and this investigation conformed with the principles outlined in the Declaration of Helsinki.

Protocol 1

Ten healthy, nonobese, male volunteers, age 26 ± 1.6 years (mean $\pm$ s.e.mean) were studied. All had normal clinical history and examination, 12-lead electrocardiogram, echocardiogram and haematological and biochemical profiles. Subjects had taken no medications for at least 1 month before the study.

Subjects were studied at the same time of day on two occasions in a randomised, double-blind, cross-over design. Subjects lay supine for 1 h prior to the start of the study to establish baseline haemodynamics, and baseline renin levels. Baseline readings were taken 10 min before (–10 min) and at the start of the infusion (time 0).

After the rest period an infusion of either placebo or N^G -monomethyl L-arginine, [4 mg kg^{-1} (S & G Analyticals, London) dissolved in 25 ml 0.9% saline], was commenced and ran over 50 min. At the start of the infusion, a priming bolus of either N^G -monomethyl L-Arginine (L-NMMA) 4 mg kg^{-1} in 25 ml 0.9% saline, or placebo was given over 2 min. The placebo was a volume matched infusion and bolus of 0.9% saline. Treatment was randomly assigned, by a second investigator, before the start of the experiment. Fourteen minutes into the infusion, an i.v. injection of frusemide (Antigen Pharmaceuticals, Roscrea, Ireland) 5 mg i.v. was given to stimulate renin release.

Measurements

Haemodynamic Blood pressure, as mean arterial pressure, systolic and diastolic pressure, and heart rate were measured by semiautomatic sphygmomanometer (Vital Signs Monitor, Critikon, Tampa, FL, USA) and taken as

the mean of three readings at each timepoint. Readings were taken at –10, 0, 5, 10, 15, 20, 30, 40, and 50 min. **Renin** Renin samples were taken at –10, 0, 5, 10, 20, 30, 40, and 50 min. Venous blood was taken into EDTA and centrifuged at $3000 \text{ rev min}^{-1}$ for 15 min, before plasma was taken off and stored at -20°C until assayed. Renin levels were analysed by radioimmunoassay (Sorin Biomedica, Italy) and expressed as the generation of angiotensin I as $\text{ng ml}^{-1} \text{h}^{-1}$.

Data analysis

Comparisons were made between active and placebo treatments by analysis of variance (ANOVA). Where the overall ANOVA was significant, multifactorial analysis of variance was used to determine differences at individual time points. A *P* value of less than 0.05 was considered significant and results are expressed as means $\pm$ s.e.mean.

Protocol 2

Five of the initial 10 subjects underwent an identical protocol, using phenylephrine (Tayside Pharmaceuticals, Dundee) to mimic the pressor effects of L-NMMA to assess the effect this might have on renin release. This protocol was not blinded.

Subjects again rested supine for 1 h prior to baseline haemodynamic and renin levels. Phenylephrine was commenced at $0.5 \mu\text{g kg}^{-1} \text{min}^{-1}$, as an infusion, with a 25 ml bolus of normal saline at the start of the infusion. Frusemide 5 mg i.v. was administered as a bolus 14 min into the infusion as in protocol 1. The phenylephrine dose produced the appropriate haemodynamic changes in four volunteers, the fifth had the dose titrated up to $0.75 \mu\text{g kg}^{-1} \text{min}^{-1}$ after 4 min to achieve an adequate haemodynamic response. Haemodynamic and renin measurements were collected at the same intervals as above.

Results

Protocol 1

Haemodynamic The doses of L-NMMA used caused a significant pressor effect prior to the bolus of frusemide, with an average increase at 10 min of 7.5 mmHg (95% CI 3.9–11.1) ($P < 0.05$) in mean arterial pressure (MAP) (Figure 1). Similarly, diastolic pressure increased by 6.8 mmHg [95% CI 4.1–9.5] ($P < 0.01$), and there was a small but non significant rise in systolic pressure of 1 mmHg (Figure 1). Heart rate was suppressed by 7.2 beats min^{-1} (95% CI 3.0–11.4) ($P < 0.05$) (Figure 1). The bolus of frusemide increased the blood pressure in both the active and treatment days, but had no effect on heart rate.

Figure 1 The haemodynamic effects of nitric oxide synthesis inhibition with L-NMMA (●, ■) in normal subjects ($n=10$), compared with volume matched placebo (○, □); frusemide injection at 14 min. * $P<0.05$ vs placebo, ** $P<0.01$ vs placebo.

Renin Baseline renin was unaffected by the infusion of L-NMMA, but there was a significant suppression of the renin response to frusemide ($P<0.01$) at all timepoints after the frusemide bolus (Figure 2). Mean differences in renin in $\text{ng ml}^{-1} \text{h}$ between placebo and L-NMMA at timepoints 20, 30, 40 and 50 min, respectively, were 0.85

Figure 2 Plasma renin activity during nitric oxide synthesis inhibition with L-NMMA, and following an injection of frusemide to stimulate renin. ○ placebo, ● L-NMMA, $n=10$.

(95%CI 0.18–1.51), 0.79 (95%CI 0.32–1.23), 0.44 (95% CI 0.08–0.8) and 0.42 (95% CI 0.11–0.72).

Protocol 2: (five volunteers only)

Haemodynamic The phenylephrine doses were aimed to mimic the pressor effects noted with the L-NMMA infusion. A similar reduction in heart rate, and increase in mean arterial pressure were noted. Heart rate, became significantly lower than placebo at the final two timepoints (Figure 3).

Renin The renin response to frusemide was suppressed to the level noted during the L-NMMA infusion. This difference between placebo and phenylephrine was statistically significant (Figure 4). Mean differences for the timepoints after frusemide, 20, 30, 40, and 50 min, respectively, in $\text{ng ml}^{-1} \text{h}^{-1}$.

Saline vs L-NMMA 1.42 (95%CI 0.13–2.95), 1.11 (95%CI 0.3–1.91), 0.68 (95% CI 0.25–1.12) 0.54 (95% CI 0.09–0.97).

Saline vs phenylephrine 1.33 (95% CI 0.51–3.16), 1.24 (95% CI 0.16–2.32), 0.81 (95% CI 0.06–1.56), 0.76 (95% CI 0.08–1.43).

Discussion

Haemodynamics

The dose of L-NMMA chosen was expected to be a pressor dose, and was between the second and third doses given by Stamler *et al.* [12], in their dose ranging study, and the alterations in MAP and diastolic pressure are comparable. We used a front loaded infusion, with an initial 4 mg kg^{-1} bolus to ensure adequate inhibition of nitric oxide at 14 min, when the frusemide stimulus was given. Previous work has shown that the haemodynamic

Figure 3 Haemodynamic effects, in five volunteers, of an infusion of phenylephrine (■), compared with those due to L-NMMA (●), and placebo (○). * $P < 0.05$ vs placebo, ** $P < 0.01$ vs placebo, + $P < 0.05$ phenylephrine vs placebo only.

Figure 4 Plasma renin activity in five volunteers, during infusions of L-NMMA (●), and phenylephrine (■), compared with placebo (○), and following an injection of frusemide to stimulate renin. * $P < 0.05$ vs placebo, ** $P < 0.01$ vs placebo.

effects of an L-NMMA bolus take 15 min to reach peak effect [13], and we felt that the combination of a bolus with an infusion, would give the best overall profile of nitric oxide inhibition over the whole timecourse of the study.

Following the injection of frusemide, the MAP and diastolic pressure rose on both the active and placebo days. This effect has been observed previously, in normal [14] and cardiac failure patients [15]. Frusemide provokes an initial venodilation, which causes a fall in blood pressure, followed by a subsequent rise in MAP, which is believed to be due to an increase in systemic vascular resistance. This late pressor effect may be due to activation of the renin angiotensin system [16], as the elevation in blood pressure could be blocked by captopril [17]; however, we observed a dissociation between renin and blood pressure in this study.

Renin

In this protocol our volunteers were salt replete, hence baseline renin levels were very low in all subjects. We did not observe suppression of baseline renin by L-NMMA in contrast to the literature where NO synthase inhibitors reduced baseline renin within 15 min in rats [11], rabbits [7] and man [18]. This may reflect our shorter observation time of 10 min.

L-NMMA did suppress the renin response to frusemide, but this did not appear to be a specific effect, because phenylephrine, in equipressor doses, had a similar effect in inhibiting the renin response to frusemide. This is the first demonstration of the effect of nitric oxide synthesis inhibition on stimulated renin release in man, and in many ways agrees with much of the published animal data [7, 9]. It is however, difficult to reconcile our data with that of Reid *et al.* [7], who performed essentially this experiment in rabbits but did not find any phenylephrine induced inhibition of frusemide induced renin release, despite producing similar haemodynamic effects to the nitric oxide inhibition. One reason for this may be the large difference in frusemide dose between the studies. On a mg kg^{-1} basis Reid *et al.* gave an equivalent of 2 mg kg^{-1} bolus in contrast to 0.07 mg kg^{-1} used here. Despite the 30 fold difference mg kg^{-1} dosage, the percentage increase in renin was similar suggesting a species difference, which is known to exist in renin physiology [19]. Our frusemide dose was chosen to allow effective renin secretion without the desire to void [20].

One conclusion from our findings is that the inhibition of renin by nitric oxide inhibition is pressure dependent, and an indirect effect. Haemodynamic factors are well known to be important in renin release [21]: below a specific level of renal perfusion pressure, renin secretion appears to double for every 2–3 mmHg reduction [5].

This pressure dependence complicates any attempt to unravel the role of nitric oxide in renin release since nitric oxide donors reduce perfusion pressure, and nitric oxide inhibitors increase perfusion pressure. However there is good evidence that both frusemide induced renin release [6, 9, 22] and renin gene [22] expression are inhibited by nitric oxide synthase inhibitors, provided that renal perfusion pressure is stable.

As nitric oxide synthase has been identified in the macula densa, the direct effect of nitric oxide on juxtaglomerular cells has been studied. In pure isolated juxtaglomerular cell culture, nitric oxide application initially produces inhibition of renin release, followed by stimulation [23, 24]. In isolated perfused juxtaglomerular apparatus, He *et al.* [25] showed that if nitric oxide was applied directly to the macula densa via the tubular lumen, renin release was stimulated, but if nitric oxide was applied to the whole preparation, it was found to be inhibit renin release. This suggests that nitric oxide acts at several sites within the tubule to alter renin secretion.

Therefore the data from isolated cells suggests that the macula densa does indeed use nitric oxide as a signal for renin release, with much of the data suggesting that nitric oxide is inhibitory on renin release at the JG cells. Since inhibiting nitric oxide synthesis would be expected to enhance renin release, the observed reduction suggests that, in more complex models, haemodynamic factors interfere with the basic cellular effects.

Using an isolated perfused kidney model, with falling perfusion pressure used as the stimulus for renin release, Sholtz and Kurtz [21] demonstrated that renin secretion directly parallels renal perfusate flow, and that inhibition of renin by L-NAME is pressure dependant; it has a limited effect at normal perfusion pressure, but a more marked effect at artificially low perfusion pressure. This led to the suggestion that the effect of nitric oxide on renin secretion has two components: a stimulatory effect of nitric oxide on renin release, but at higher perfusion pressures baroreceptor function can suppress the stimulation of renin by nitric oxide. Furthermore, in whole animal studies where alterations in renal perfusion pressure have been used to stimulate renin secretion the results have tended to show that haemodynamic factors are more important than changes in nitric oxide levels in determining renin release [11, 26]; these observations are consistent with our results.

One potential explanation for the complexity of the results, is that nitric oxide inhibition affects not only the cells of the macula densa in the preparation, but also renal blood flow and mean arterial pressure, particularly in systemic application studies, causing baroreceptor activation, and alterations in tubuloglomerular feedback. In our study we have shown that a small renin stimulus can be inhibited by the application of a nitric oxide

inhibitor, which increased arterial pressure and via baroreceptor reflex reduced heart rate. A similar pressure increase using a non specific pressor agent also abolished the renin response indirectly. The nitric oxide inhibitor may have caused its effect via several mechanisms.

The effect of phenylephrine on renin release must also be considered, as it is an α_1 -adrenoreceptor agonist which could also affect renin release. In dogs, α -adrenoceptor blockade reduces renin release in response to haemorrhage [27], suggesting the phenylephrine might stimulate renin release. In a study employing controlled hypotension in man, phenylephrine had no effect on renin secretion increases in renin following adrenaline [28]. Furthermore, where phenylephrine is applied directly to the renal arteries, stimulation of renin, not inhibition is seen [29], suggesting that the renin inhibition shown in our study was purely a pressure effect.

This study is the first to demonstrate, in man that nitric oxide inhibition reduces renin release. From the present data it is difficult to delineate the mechanism further but we have demonstrated using an equipressor dose of phenylephrine, that a major part of the suppression is due to indirect stimulation of the baroreceptor inhibition of renin.

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