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Mechanisms involved in the blunted nitric oxide–cGMP pathway in hypertensive TGR(mREN2)27 rats

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Abstract

In hypertensive TGR(mREN2)27 rats (TGR), the subsensitivity of vascular guanylyl cyclase to nitric oxide could depend on oxidized heme, reduced heme content, or decreased expression of the enzyme. In this study, enzyme activity was stimulated by protoporphyrin-IX, which acts independently of heme, and expression was assessed by Western blot analysis. In TGR aorta, maximum stimulation of soluble guanylyl cyclase by protoporphyrin-IX was 40% lower than in Sprague–Dawley controls, and expression of the β1-subunit of the enzyme was reduced by 50% ($P < 0.05$, *t*-test). In conclusion, decreased expression of soluble guanylyl cyclase leads to a blunted response of the nitric oxide–cGMP (guanosine 3′,5′-cyclic monophosphate) pathway in TGR aorta.

Keywords: Guanylyl cyclase; Protoporphyrin-IX; TGR(mREN2)27 rat; Aorta

1. Introduction

Soluble guanylyl cyclase, a five-coordinated ferrous heme-containing heterodimer, is the major receptor for nitric oxide (NO), which activates this enzyme by forming a nitrosyl–heme complex. The stimulated heterodimer catalyzes the formation of the vasodilating second messenger guanosine 3′,5′-cyclic monophosphate (cGMP). Thus, soluble guanylyl cyclase represents an important enzyme involved in vasorelaxation and in the regulation of blood pressure.

In previous studies, it has been shown that the activity (Kojda et al., 1998) and the mRNA and protein expression of soluble guanylyl cyclase (Ruetten et al., 1999; Klöß et al., 2000) are lower in spontaneously hypertensive rats than in normotensive controls.

Transgenic hypertensive TGR(mREN2)27 rats (TGR), originally developed by Mullins et al. (1990), are characterized by an overactive renin–angiotensin system (Véniant et al., 1995), and may serve as a model of human secondary hypertension (Lemmer et al., 1993). In a recent study, we could demonstrate that vascular soluble guanylyl cyclase in TGR was less responsive to stimulation by NO, and that the sensitivity of the enzyme could be restored by prolonged inhibition of the renin–angiotensin system (Jacke et al., 2000). However, we were unable to define the molecular mechanisms involved in the subsensitivity of the enzyme toward NO in this model of renin-dependent hypertension. Three principal mechanisms might play a role: (i) an oxidized heme iron, (ii) a reduced heme content, or (iii) a decreased enzyme expression in TGR aorta. Therefore, in the present study we measured the expression of soluble guanylyl cyclase in abdominal aortae from TGR and Sprague–Dawley controls by Western blot analysis. Vascular enzyme activity was measured in thoracic aortae in vitro under basal conditions and after NO-independent stimulation by protoporphyrin-IX (10^{-8}–10^{-3} M). Protoporphyrin-IX is the iron-free precursor of heme and is a potent activator of soluble guanylyl cyclase (Ignarro et al., 1982). It stimulates soluble guanylyl cyclase by mimicking the conformation of the NO-heme complex (Koesling, 1999) and is active even if the enzyme is heme-deficient (Ignarro and Wood, 1986).

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2. Methods

2.1. Animals

Six-week-old male Sprague–Dawley ($n = 6$) and transgenic TGR(mREN2)27 rats ($n = 5$) were obtained from M&B (Ry, Denmark). Animals were kept singly in plastic cages under a 12:12-h light/dark regimen with lights on at 07:00 h, under constant environmental conditions. They had free access to food and water.

At the age of 10 weeks, the animals were killed by decapitation under enflurane anesthesia. The thoracic and abdominal aorta were dissected, freed from connective tissue, rinsed in ice-cold isotonic saline solution, dried on filter paper, frozen in liquid nitrogen, and stored at −60°C.

2.2. Guanylyl cyclase assay

The activity of soluble guanylyl cyclase was measured in thoracic aorta, while its protein expression was determined in abdominal aorta. Methodological experiments in 10-week-old TGR rats demonstrated that basal cGMP formation as well as the concentration-dependent stimulation by diethylamineNONOate (2-(*N*,*N*-diethylamino)-diazenolate-2-oxide) and protoporphyrin-IX were not different in thoracic and abdominal aorta (*t*-test, not significant). Thus, the maximum increase (±S.D.) in cGMP formation by protoporphyrin-IX was 415.3 ± 91.2 and 449.5 ± 59.0 pmol mg^{-1} min^{-1} in thoracic and abdominal aorta, respectively.

Each thoracic aorta was homogenized in 2 ml ice-cold assay buffer (Tris 50 mM, $MgCl_2$ 5 mM, DL-dithiothreitol 1 mM, pH 7.4) with a Potter S glass homogenizer (Braun, Melsungen, Germany). The suspension was centrifuged for 10 min at 25,000 × *g* and 4°C. The resulting supernatant was used for measurement of soluble guanylyl cyclase activity. Basal and stimulated soluble guanylyl cyclase activity was determined as described (Witte et al., 1995).

Briefly, the enzyme reaction was carried out in assay buffer containing GTP 0.5 mM, phosphocreatine 10 mM, creatine phosphokinase 0.1 g/l (Roche, Mannheim, Germany), and 3-isobutyl-1-methylxanthine 1 mM (Sigma, Deisenhofen, Germany). For NO-independent stimulation of guanylyl cyclase, the heme precursor protoporphyrin-IX (Sigma) was added to the assay buffer in concentrations of 10^{-8} to 10^{-3} M. The reaction was started by the addition of 100 μl of the supernatant containing 30–70 μg of protein. After a 10-min incubation at 37°C, the enzyme reaction was stopped by heating at 120°C and samples were centrifuged at 10,000 × *g*. The amount of cGMP formed was measured by radioimmunoassay (TRK 500, Amersham Life Science, Germany), with [^{3}H]cGMP as tracer.

2.3. Expression of soluble guanylyl cyclase

Western blots were carried out according to the methods of Laemmli (1970) and Towbin et al. (1979) with minor modifications. The abdominal aorta was homogenized as described above. After centrifugation at 25,000 × *g* (10 min, 4°C), concentrated denaturation buffer was added to the supernatant (final concentration: Tris 125 mM, pH 6.8, sodium dodecyl sulfate (SDS) 1%, glycerol 10%, DL-dithiothreitol 0.5%, bromphenol blue 0.01%), and the samples were boiled for 5 min. Denaturated proteins (1.8 μg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. After transfer to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Freiburg, Germany), blots were incubated in TBS-T buffer (Tris 10 mM, pH 7.4, NaCl 0.9%, Tween-20 0.1%) with 3% bovine serum albumin for blocking non-specific binding sites. Thereafter, blots were washed three times with TBS-T, incubated with a polyclonal antibody against the β1-subunit of soluble guanylyl cyclase (cat # 371712, Calbiochem, Bad Soden, Germany), washed three more times, and finally incubated with a horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG) (SC 2317, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized by using the ECL-Plus system (RPN 2132, Amersham Pharmacia Biotech), and the 70-kDa band, representing the β1-subunit of soluble guanylyl cyclase, was quantified by transmission densitometry, using a preparation from rat lung tissue as standard.

2.4. Protein concentration

Protein content in the supernatant was measured by using the Coomassie® Plus assay (Pierce, Rockford, IL, USA). Bovine serum albumin (Sigma) was dissolved in assay buffer and used as standard.

2.5. Statistics

The non-linear fitting program PHARMFIT (Mattes et al., 1991) was used to analyze the concentration–response curves for protoporphyrin-IX. The pD_2- (negative logarithm of EC_{50}) and E_{max}-values (maximum increase in cGMP formation) were obtained from the fitted curves. Differences between the strains were tested by *t*-test. BiAS 6.0 (Ackermann, 1997) was used as statistics software.

3. Results

Basal soluble guanylyl cyclase activity in the thoracic aorta was slightly, but not significantly, lower in 10-week-old TGR than in aged-matched Sprague–Dawley rats (Table 1). In the presence of 10^{-5} and 10^{-4} M protoporphyrin-IX, the enzyme activity in TGR aortic tissue was

Table 1
Activity and expression of soluble guanylyl cyclase in aorta from 10-week-old Sprague–Dawley (SPRD) and transgenic TGR(mREN2)27 rats (TGR)

Strain	Basal cGMP formation (pmol mg^{-1} min^{-1})	PP-IX E_{max} (pmol mg^{-1} min^{-1})	PP-IX pD_2 ($-\log M$)	Protein (% standard)
SPRD ($n = 4–6$)	42.9 ± 8.0	791.9 ± 352.1	5.34 ± 0.24	96.2 ± 17.2
TGR ($n = 5$)	30.1 ± 10.6	395.2 ± 112.2[a]	5.62 ± 0.35	48.7 ± 14.9[a]

Mean values ± S.D.; E_{max} = maximum increase in cGMP formation; pD_2 = negative logarithm of EC_{50}; PP-IX = protoporphyrin-IX; cGMP = guanosine 3′,5′-cyclic monophosphate; cGMP formation was measured in thoracic aorta; expression of soluble guanylyl cyclase protein was determined in abdominal aorta.

[a] $P < 0.05$, *t*-test.

significantly reduced compared to that in Sprague–Dawley tissue. The maximum effect of protoporphyrin-IX (E_{max}), calculated from individual concentration–response curves over a range of 10^{-8} to 10^{-4} M, was significantly lower in TGR aorta than in controls, while the pD_2-values were not different (Table 1). In both strains, the concentration of 10^{-3} M protoporphyrin-IX led to a decrease in cGMP formation (Fig. 1) and, therefore, data for this concentration were excluded from the concentration–response analysis.

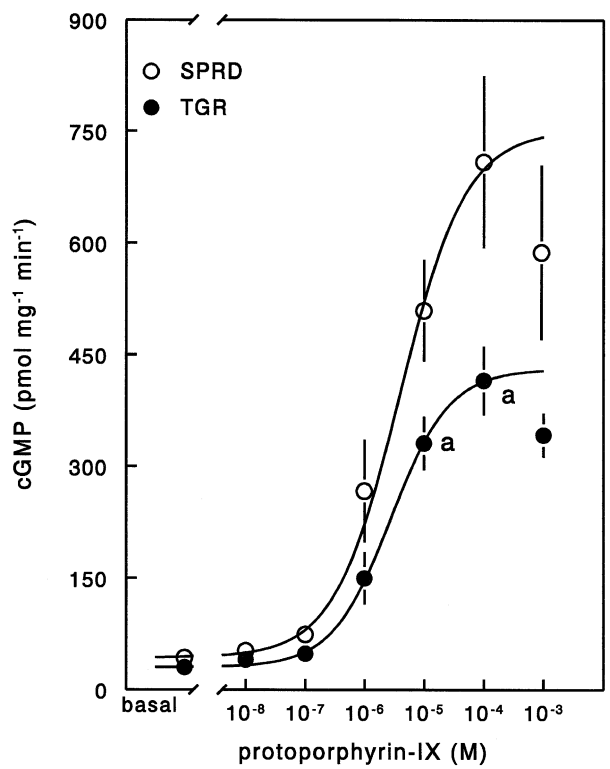

Fig. 1. Formation of guanosine 3′,5′-cyclic monophosphate (cGMP) under basal conditions and after stimulation by protoporphyrin-IX in aortic tissue from normotensive 10-week-old Sprague–Dawley (SPRD) and transgenic hypertensive TGR(mREN2)27 rats (TGR). In the presence of 10^{-5} and 10^{-4} M protoporphyrin-IX, the enzyme activity in TGR aorta was significantly lower than in SPRD control aorta. In both strains, the concentration of 10^{-3} M protoporphyrin-IX led to a decrease in cGMP formation, and, therefore, was not used for the concentration–response curve. Means ± S.E.M., $n = 4–6$ (SPRD) and $n = 5$ (TGR), [a] $P < 0.05$, *t*-test.

Protein expression of the β1-subunit of soluble guanylyl cyclase was significantly decreased by about 50% in aortae from transgenic rats compared to Sprague–Dawley controls (Table 1).

4. Discussion

The present study demonstrates that NO-independent stimulation by protoporphyrin-IX is blunted in transgenic hypertensive TGR(mREN2)27 rats compared to normotensive Sprague–Dawley controls. This finding extends our previous observation that aortic soluble guanylyl cyclase in TGR rats is less responsive to stimulation by NO (Jacke et al., 2000). Since activation of soluble guanylyl cyclase by NO requires a functional heme group, we hypothesized that the reduced NO-dependent cGMP formation in TGR aorta could be due to a loss of the prosthetic heme group, or to oxidation of the central Fe^{2+}. However, protoporphyrin-IX has been shown to activate the heme-containing and heme-depleted soluble guanylyl cyclase to a similar extent (Serfass and Burstyn, 1998). The present finding that protoporphyrin-IX was less effective in TGR aorta suggests that the amount of enzyme available for stimulation could be reduced in vascular tissue from TGR rats. Our additional observation, that the protein expression of the β1-subunit of soluble guanylyl cyclase was also significantly lower in TGR, is clearly in support of our conclusion that the impaired stimulation of vascular soluble guanylyl cyclase in TGR rats is not due to changes in its prosthetic heme group, but reflects a downregulation of soluble guanylyl cyclase itself.

Our observation that the highest concentration of protoporphyrin-IX resulted in a small decrease in cGMP formation is in agreement with previous findings reported by Serfass and Burstyn (1998), who demonstrated that low concentrations of protoporphyrin-IX activate while high concentrations inhibit soluble guanylyl cyclase.

The present results for 10-week-old TGR rats are in good agreement with those of an earlier study by Ruetten et al. (1999), who demonstrated a decreased expression of aortic soluble guanylyl cyclase in spontaneously hypertensive rats. Therefore, it is plausible to assume that a high blood pressure per se leads to downregulation of vascular

soluble guanylyl cyclase. In contrast to spontaneously hypertensive rats, a model of primary polygenic hypertension, transgenic hypertensive TGR(mREN2)27 rats are a model of secondary hypertension characterized by an overactive tissue renin–angiotensin system, due to the expression of the additional mouse renin gene. In a previous study with TGR, we observed that prolonged antihypertensive treatment with the Ca^{2+} channel antagonist amlodipine did not increase aortic soluble guanylyl cyclase activity to the same extent as treatment with enalapril, despite a comparable reduction in blood pressure (Jacke et al., 2000). The greater effectiveness of the angiotensin-converting enzyme inhibitor suggests that the overactive renin–angiotensin system contributes independently to downregulation of soluble guanylyl cyclase in TGR aorta.

In conclusion, NO-independent stimulation of vascular soluble guanylyl cyclase is blunted in transgenic hypertensive TGR(mREN2)27 rats. A decreased expression of the β1-subunit, leading to a reduced amount of the functional guanylyl cyclase heterodimer, is likely to be responsible for the reduced cGMP formation in TGR aorta.

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