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SHORT COMMUNICATION

Down-regulation of Renal Glutathione Synthesis by Systemic Nitric Oxide Synthesis Inhibition in Spontaneously Hypertensive Rats

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ABSTRACT. Nitric oxide stimulates *in vitro* the synthesis of glutathione, an abundant thiol with a number of functions such as detoxification of xenobiotics and reactive oxygen species. In order to study this relationship in an animal model of hypertension, we treated spontaneously hypertensive rats (SHR) either with a nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) or with a nitric oxide donor isosorbide-5-mononitrate (IS-5-MN). Inhibition of nitric oxide synthesis led to malignant hypertension and to a marked decrease in glutathione synthesis through down-regulation of the rate-limiting enzyme γ-glutamylcysteine synthetase (GCS). The reduction in GCS activity was further augmented in SHR on a high sodium diet. Renal GCS activity in untreated SHR was 234 ± 14 and 240 ± 18 nmol/min/mg protein (mean ±SD) on a low and high sodium diet, respectively. When L-NAME was included in the diet, the activities dropped to 173 ± 28 and 123 ± 28 for the low and high sodium diets, respectively. IS-5-MN attenuated the rise in blood pressure induced by sodium chloride, but did not affect the GCS activity. The mechanism of GCS stimulation by nitric oxide is not known, but our results combined with the literature suggest that a relatively high concentration of nitric oxide is needed. BIOCHEM PHARMACOL **59**;4:441–443, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. glutamate–cysteine ligase; glutathione; hypertension; kidney; nitrate; sodium

NO¶ is a potent vasodilator synthesized by NOS (L-Arginine, NADPH: oxygen oxidoreductase, EC 1.14.13.39) from arginine [1]. In Sprague–Dawley rats, increasing dietary NaCl up-regulates endothelial, inducible, and neural NOS in renal medulla, indicating their importance in adaptation to dietary NaCl load [2]. An impaired ability to excrete sodium in relation to the renal perfusion pressure has been suggested to lead to hypertension in animals sensitive to dietary salt [3,4]. Furthermore, a long-term inhibition of NOS by L-NAME decreases renal medullary blood flow, resulting in sodium and water retention and subsequent hypertension not only in genetically hypertension-prone experimental animals, but also in other animals [5]. Interestingly, low-dose L-NAME treatment, insufficient to increase blood pressure, has been reported to transform dogs from salt-resistant to salt-sensitive [6].

GSH is an essential intracellular tripeptide involved in various biological phenomena including synthesis of DNA, proteins, and leukotrienes, as well as in detoxification of xenobiotics and reactive oxygen species [7]. It has been suggested that nitrosothiols such as *S*-nitrosoglutathione play a role in the storage and transport of NO, whose half-life is very short [8]. GSH is synthesized from cysteine, glutamate, and glycine by consecutive actions of two ATP-dependent enzymes, GCS (L-glutamate:L-cysteine γ-ligase, EC 6.3.2.2) and glutathione synthetase (γ-L-glutamyl-L-cysteine: glycine ligase, EC 6.3.2.3) [9]. GCS contains a catalytic heavy subunit and a regulatory light subunit, is the rate-limiting enzyme, and is feedback-inhibited by GSH [9–11].

Recently, inhibition of NO synthesis *in vitro* has been shown to down-regulate the transcription of GCS, which leads to a decreased GSH synthesis [12]. NO itself up-regulates GCS and increases GSH concentration [13]. Hypertension is associated with increased oxidative stress in SHR [14], a widely used animal model of hypertension. GSH is important in protecting the cells from oxidative stress, and thus its being synthesized adequately is of vital importance in hypertension. NO has been shown to play a role in both hypertension and GSH synthesis. This study was undertaken to examine these interactions between NO and GCS in kidneys of SHR.

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¶ *Abbreviations:* GCS, γ-glutamylcysteine synthetase; IS-5-MN, isosorbide-5-mononitrate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; and SHR, spontaneously hypertensive rat.

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MATERIALS AND METHODS

Forty-seven inbred nine-week-old male SHR rats (Harlan Sprague–Dawley Inc.) weighing 240–250 g were divided into six groups. The animals were kept for three weeks on diets containing 0.2, 1.1, or 6.0% NaCl (w/w of the diet) with or without L-NAME (0.025% in the diet, providing approximately 20 mg/kg body weight/day). In another experiment, 20 nine-week-old SHR, weighting 190–250 g, were divided into two groups, receiving 6.6% NaCl in diet with or without IS-5-MN (0.1% w/w of the diet, providing 60–70 mg/kg body weight/day) for eight weeks. The dose of IS-5-MN was based on the study of Ruskoaho [15].

The systolic blood pressures of unanesthetized rats were measured by a tail-cuff method as described previously [16]. At the end of the experiments the rats were killed by decapitation. The kidneys were removed and washed with ice-cold saline, frozen in liquid nitrogen, and kept at −70°. The procedures and protocols of the study followed our institutional guidelines and were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki.

Frozen kidneys were homogenized and filtered as in [17]. GCS activity was measured using the modified method of Nardi *et al.* [18]. After incubating 10 μL of sample with 300 μL of reaction mixture for 15 min at 37°, 50 μL was removed and added to 50 μL of 30 mM monobromobimane in 50 mM *N*-ethylmorpholine, pH 8.4. After derivatization for 5 min in the dark at room temperature, the reaction was stopped with 10 μL of 100% trichloroacetic acid. After centrifugation, 2 μL of supernatant was injected into a Waters Novapak C-18 HPLC column (4 mm, 3.9 × 150 mm). Isocratic elution was carried out with 4% acetonitrile, 0.25% acetic acid, and 0.25% perchloric acid pH 3.7, and fluorescent product γ-glutamylcysteine was detected with a Shimadzu RF-10A×L spectrofluorometer (excitation and emission wavelengths 394 nm and 480 nm, respectively). Protein concentrations were determined using the Biuret method.

Values are presented as the means ± SD. Comparisons between L-NAME-treated and non-treated animals in each salt group were carried out by Student's *t*-test. The relationship between GCS activity and blood pressure was assessed by Pearson's linear correlation coefficient. Statistical analyses were performed with SPSS 8.0 program (SPSS Inc.).

RESULTS AND DISCUSSION

L-NAME treatment lead to a significant down-regulation of GCS activity (Fig. 1). The reduction was largest in the high sodium group. This finding is in agreement with the study by Kuo *et al.*, which showed reduced enzymatic activity and steady-state levels of mRNA for GCS in cultured rat hepatocytes after NOS inhibition [12]. Other factors may partly contribute to the reduced activity of renal GCS, because L-NAME treatment leads to a generalized endothelial dysfunction in these animals. We have previously reported that long-term L-NAME treatment markedly increases systolic blood pressure and induces cardiac and renal hypertrophies and damage [16]. The GCS activity correlated negatively with the systolic blood pressure at the end of the follow-up period ($r = -0.60$, $P < 0.001$) and with the change in systolic blood pressure during the study ($r = -0.52$, $P < 0.001$). Dietary salt did not have a significant effect on blood pressure whether L-NAME was given or not. This is probably due to the brevity of the experiment. Previous studies indicate that the hypertensive effects of increased dietary NaCl take at least 2–4 weeks to develop [19]. However, NaCl in the diet did increase the target organ damage [16].

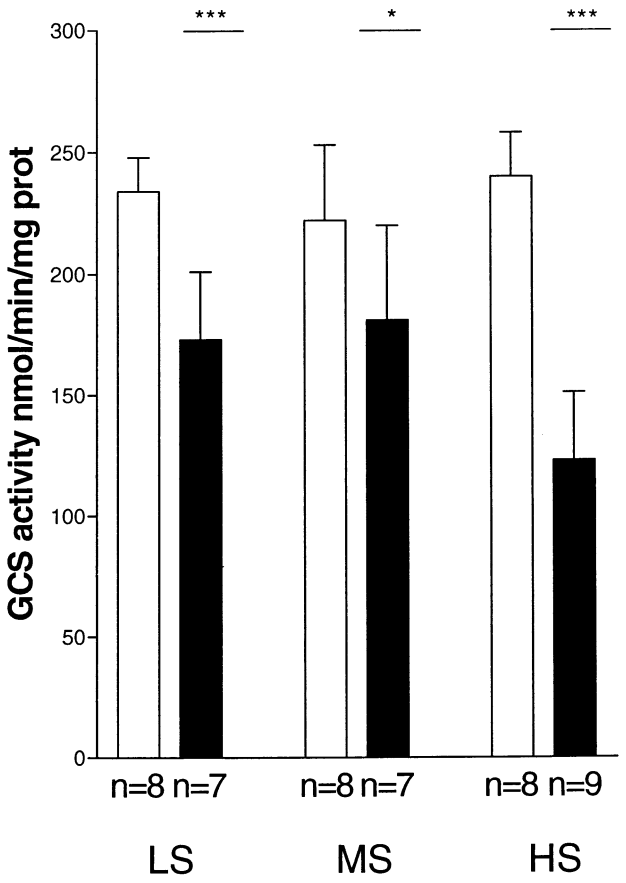

FIG. 1. γ-Glutamylcysteine synthetase activity in kidneys of spontaneously hypertensive rats treated with (solid bars) or without (open bars) N^{ω}-nitro-L-arginine methyl ester and receiving a diet of variable NaCl content (low sodium (LS) NaCl 0.2%, medium sodium (MS) NaCl 1.1%, high sodium (HS) NaCl 6.0%). N indicates the number of animals in each group. Mean ± SD is given. *$P < 0.05$, *** $P < 0.001$.

In the second part of this study, IS-5-MN treatment did not have an effect on the renal GCS activity (190 ± 35 and 191 ± 36 nmol/min/mg protein with and without IS-5-MN, respectively) in the SHR. This treatment attenuated, but did not completely reverse, the sodium-induced increase in systolic blood pressure, as reported earlier [20]. It is noteworthy that the flux of NO obtained with the dose of

IS-5-MN used in this study is far smaller than that used by Moellering *et al.* [13]. It seems unlikely that GCS activity would already be fully stimulated by NO under basal conditions *in vivo*. Therefore, GCS synthesis may be up-regulated only in sites where high NO production occurs.

Little is known about the correlation of the level of GCS down-regulation and glutathione content *in vivo*. In a study by Drew *et al.* [21], the effect of the GCS inhibitor buthionine sulfoximine on kidney GCS activity and GSH levels was studied in mice. From buthionine sulfoximine dose–response curves, it could be estimated that down-regulation of GCS by 50%, the level which was achieved in our study in the high salt group receiving L-NAME, resulted in a 50% reduction of GSH. Although the kidneys of adult mice appear to be unaffected by this degree of GSH deficiency over a 3-week period [22], GSH depletion may render tissues susceptible to oxidative stress. Further studies are needed to elucidate the role of GCS down-regulation in the end-organ damage caused by chronic inhibition of NOS.

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