Stimulatory Effect of Ginseng Saponin on Endogenous Production of Nitric Oxide

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ABSTRACT

Ginseng saponin (GS) purified from *Panax ginseng*, increase renal blood flow in rats. Nitric oxide (NO) is thought to be a substance endogenously released by GS in preconstricted lungs and cultured endothelial cells. The present study aims to determine whether GS could stimulate endogenous release of NO in rat kidney and urine levels of the stable NO metabolites, nitrite (NO_2) and nitrate (NO_3) and urinary cGMP levels were measured 8 hr after a single intraperitoneal injection of GS (200 mg/kg) into rats. The effects of the NO synthesis inhibitor, Nω-nitro-L-arginine methyl ester, and the NO precursor, L-arginine, on the GS-induced changes were also determined. The activity of NO synthase, as determined by conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline, in whole kidney, glomeruli and cortical tubules were also investigated. A single injection of GS resulted in endogenous production of NO as reflected by increase in serum and urine levels of NO_2/NO_3 and urinary cGMP levels, which were inhibited by the addition of N-nitro-L-arginine methyl ester and restored by L-arginine. GS also stimulated the activity of NO synthase in whole kidney as well as glomeruli and cortical tubules, and this increase was significantly prevented by Nω-nitro-L-arginine methyl ester. In conclusion, GS stimulates endogenous NO production and thus, may play a protective role in the kidney by modulating renal blood flow.

Introduction

L-arginine-derived nitric oxide (NO) is produced by several cells within the kidney, including glomerular mesangial *(Schultz et al., 1990a)* and endothelial cells *(Marsden et al., 1990)*, and a renal epithelial cell line *(Ishii et al., 1990)*, but its integrative role in the control of renal function is not clear. In the isolated or intact kidney, the L-arginine-NO pathway determines basal vascular resistance and mediates vasodilator responses to acetylcholine and bradykinin (Lahera *et al.*, 1990). In addition to regulating vascular tone, endothelial cell-derived NO inhibits sodium transport by cortical collecting tubule cells *(Stoos et al., 1992)* and inhibits renin release in kidney cortical slices *(Vidal et al., 1988)*. During tubular-fluid reabsorption, arginine-derived NO is generated in macula densa, as an intercellular signalling molecule mediating a vasodilating component to the tubuloglomerular feedback response by countering the vasoconstriction of the afferent arteriole *(Wilcox et al., 1992)*.

This series of recent findings suggests that arginine-derived NO has an important role in body fluid-volume and blood-pressure homeostasis in kidney.

Panax ginseng has been used for more than 2000 yr as a general tonic in traditional Oriental medicine. Recently it was reported that ginsenosides (GS), saponins extracted from *Panax ginseng*, protected renal function from ischemia and contralateral nephrectomy (Zhang, 1992) and exerted an antinephritic action via increased renal blood flow in rats (Hattori *et al.*, 1991). The relation between GS and renal blood flow was also reported by Chen *et al.* (1984); GS decreased renal vascular resistance by controlling blood flow. From our previous study (Kim *et al.*, 1992), GS significantly increased NO production as reflected by conversion of arginine to citrulline in bovine aortic endothelial cells. In addition, GS vasodilated preconstricted lungs, which was inhibited by nitro-L-arginine, an inhibitor of NO synthase. This suggests that NO itself could be the substance endogenously released by GS.

The purpose of present study was to determine whether GS stimulates endogenous production of NO in rat kidney by measuring the serum and urine levels of the stable NO metabolites, nitrite (NO_2) and nitrate (NO_3), and by measuring urinary cGMP levels. We have assessed the effects of the NO synthesis inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), and the NO precursor, L-arginine (L-ARG), on the GS-induced changes in these biochemical measurements. GS-induced alterations in the activity of NO synthase, as reflected by conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline, in whole kidney, glomeruli and cortical tubules were also compared.

Materials and Methods

Chemicals

[^{14}C]-L-arginine menohydrochloride (300 mCi/mmol) and RIA kit for cGMP, Amersham International plc (Buckinghamshire, England); Dowex AG 50WX-8, Bio-Rad Laboratories (Richmond, CA); Nω-nitro-L-arginine methyl ester (L-NAME), L-arginine, creatinine diagnostic kit, and all other chemicals, Sigma Chemical Co. (St Louis, MO). *Escherichia coli* (ATCC 25922), American Type Culture Collection (Rockville, MD). Ginsenosides (GS) purified from *Panax ginseng* was kindly provided from the Korea Ginseng & Tobacco Research Institute (Taejon, Korea).

Animals

Male Sprague-Dawley rats, weighing between 250 and 300 g, were used for all experiments.

Experimental protocols

Serum and urine samples from the rats were obtained on two consecutive days in the following manner: animals were placed in metabolic cages and 8-hr urine collections were obtained for mea-

surement of NO_2/NO_3, creatinine, and cGMP. Animals were allowed free access to water but were not given food during the urine collections. At the end of this period, serum was obtained from a tail incision for NO_2/NO_3 and creatinine. On the first day, no treatment was given and these samples are labeled as baseline. On the next day, animals were given i.p. injections following the protocols outlined below and experimental serum and urine samples were obtained in the same manner as the baseline samples. Four different groups of animals were studied:

1. Group I, GS. Rats (n=8) in this group received a single i.p. injection of GS at a dose of 200 mg/kg at time 0.
2. Group II, GS+L-NAME. These rats (n=8) also received a single i.p. dose of GS (200 mg/kg) at time 0, but in addition the rats were given the competitive inhibitor of NO synthesis, L-NAME 75 mg/kg i.p., 30 min before and simultaneously with the GS.
3. Group III, GS+L-NAME+L-ARG. These rats (n=8) were given GS and L-NAME as above and in addition the rats were given 300 mg/kg i.p. L-ARG concomitantly with each injection of L-NAME. L-ARG (1% wt/vol) was also given in the drinking water of these animals, beginning 12 hr before the experimental period.
4. Group IV, L-NAME. These rats (n=9) were given L-NAME in the same times as described above; however, 1 ml/kg i.p. of sterile normal saline was given instead of GS at time 0.

All substances were dissolved in saline and prepared fresh each day. In our preliminary study, i.p. injections of saline in the same volume and times as described above had no effect on the parameters measured in this study. At the end of the 8-hr experimental period, the rats were anesthetized and killed by exsanguination, and the kidney tissue was removed for NO synthase assay and protein content. In addition to the groups outlined above, to determine the appropriate experimental period and the dose of GS, rats received a single i.p. injection of GS at a dose of each 50, 100, 150, and 200 mg/kg at time 0 and at the end of the 8-hr experimental period the rats were killed and serum NO_2/NO_3 was analyzed. In other experiments, GS, 200 mg/kg i.p., was given to rats and serum NO_2/NO_3 was analyzed. In other experiments, GS, 200 mg/kg i.p., was given to rats and serum NO_2/NO_3 was determined at 2, 4, 6 and 8 hr.

Creatinine and urinary cGMP assays

Serum and urine creatinine obtained from the experiment were measured colorimetrically by the method of Tobias *et al.*, (1962) using a commercial diagnostic kit. cGMP was assayed on 8-hr urine collections from the three groups of animals (Saline, GS, GS+L-NAME) using a radioimmunoassay (Shultz *et al.*, 1990b). Urinary excretion of these substances was calculated by multiplying the measured concentration in the urine by the total urine volume during the 8-hr collection. Creatinine clearance was calculated as the urinary excretion of creatinine divided by the plasma creatinine.

NO_2/NO_3 assay

Serum and urine samples were assayed for the stable end products of NO, NO_2 and NO_3. Samples were first incubated with *E. coli* nitrate reductase to convert the NO_3 in the samples to NO_2 as described by Bartholomew (1984) and Granger *et al.*, (1990). To prepare this enzyme, *E. coli* (ATCC 25922) were grown for 18 hr under anaerobic conditions, washed, resuspended in PBS, and frozen at -70℃ until use. The samples were incubated with enzyme in a ratio of 50:1 (sample:enzyme) in Hepes ammonium formate buffer for 1 hr at 37℃. This ratio and time were found to give maximum and virtually complete reduction of NO_3 without any significant effect on NO_2 levels of standards as well as samples *(Shultz and Raij, 1992)*. After the enzyme incubation, the total NO_2 in the samples (representing both NO_2 and NO_3) was measured using Griess reagent, as described by *Green et al.*, (1982).

Preparation of kidney tissues

Rats were anesthetized with pentobarbital (30 mg/kg, i.p.), and a midline incision was performed to expose the aorta at the level of the renal arteries. A 22-gauge needle was placed in the aorta with the tip positioned between the left and right renal artery. The aorta was clamped above the level of the renal arteries and both renal veins were cut to facilitate drainage. The kidneys were perfused gently with sterile PBS until they blanched and were then removed. One kidney from each rat was homogenized in 50 ml of homogenizing buffer containing 0.32 M sucrose, 20 mM Hepes, 0.5 mM EDTA, 1mM dithiothreitol and 5 mg of phenylmethylsulfonyl fluoride, pH 7.4 *(Rengasamy and Johns, 1991)*. Glomeruli were isolated from the other kidney using a series of nylon sieves *(Schultz et al., 1990a)* and homogenized in the above buffer. The cortical tissue suspension remaining after harvesting the glomeruli, containing mostly fragments of tubules, was also homogenized for NO synthase assay. Tissue protein was determined by the method of Lowry et al. (1951).

Assay of NO synthase

NO synthase was measured by determining the production of [^{14}C]-L-citrulline, which is a byproduct of the enzyme reaction from [^{14}C]-L-arginine. The reaction was initiated by adding 80 μl tissue homogenate and 20 μl [^{14}C]-L-arginine (final 20 nCi/ml) to 900 μl assay buffer (0.32 M sucrose, 20 mM Hepes, 0.5 mM EDTA, 1 mM dithiothreitol, 0.45 mM CaC_{12}, 1 mM NADPH) containing 90μg phenylmethylsulfonyl fluoride. After 30 min incubation at 37℃, the reaction was stopped by the addition of 1.5ml ice-cold Hepes/EDTA buffer (20/2 mM, pH 6.0). A quantity of 0.75 ml of the reaction mixture was applied to a 1-ml column of Dowex AG 50x-8 cation exchange column. [^{14}C]-L-citrulline in the effluent and a subsequent 1-ml water wash was quantified by liquid scintillation spectrometry (Kim *et al.*, 1992). Background [^{14}C]-L-citrulline (dpm) in medium without tissue homogenate was subtracted from the sample results which were then factored for the pro-

tein content of the kidney tissues.

Statistics

All values represent mean ± SE. Differences among groups were determined by one-way ANOVA with Newman-Keuls test (Zar, 1984). Values were considered significantly different if $P < 0.05$.

Results

Rats were assigned to the groups at random and the baseline values for all the parameters measured were not different between any of the groups. There were no deaths among animals receiving any of the experimental protocols.

Urine volume and creatinine clearance

There was no significant effect of any of the experimental protocols on body weight. Urine volume was significantly increased in all experimental groups compared with baseline, which may be partially related to the saline in the injections. Animals that received L-NAME, either with GS, with GS + L-ARG, or alone, had more significant diuresis than animals that received only GS without L-NAME. This effect is likely due to the increase in blood pressure induced by L-NAME, leading to a pressure natriuresis. The mean creatinine clearance during the baseline period was 1.03 ± 0.05 ml/min and did not change significantly during the experimental period in the group that received GS. In contrast, GS + L-NAME rats had a significant reduction in creatinine clearance compared with both baseline and rats that received GS alone ($P < 0.01$ vs baseline and GS alone). When L-ARG was given in addition to GS + L-NAME, partial restoration of the creatinine clearance towards baseline was observed. L-NAME alone also reduced creatinine clearance ($P < 0.01$ vs baseline).

Serum concentration and urinary excretion of NO_2/NO_3

Serum levels of NO_2/NO_3 increased with the doses of GS injected at 8hr experimental period from $155 \pm 45\mu M$ at baseline to $300 \pm 60\mu M$ at a single i.p. injection of GS (200 mg/kg), which was sustained up to a dose of GS, 300 mg/kg. To determine the appropriate experimental period, serum NO_2/NO_3 levels were measured at 2, 4, 6 and 8 hr after a single i.p. injection of GS (200 mg/kg). With the experimental period, serum levels of NO2/NO3 increased and this increment tended to be reduced after the 8 hr experimental period. Thus, we decided to use a dose of GS as 200 mg/kg for the 8-hr experimental period as a stimulated state of NO production by GS. The baseline urinary excretion of NO_2/NO_3 was not different between any of the four groups, so this data was pooled and is shown as a single baseline value. GS injection (200 mg/kg) significantly increased the serum NO_2/NO_3 levels ($P < 0.01$). Treatment of the animals with the NO synthesis inhibitor L-NAME sig-

nificantly inhibited the increase in serum NO_2/NO_3 induced by GS [$P < 0.01$ GS + L-NAME vs GS alone]. Mean serum NO_2/NO_3 levels in rats receiving GS + L-NAME + L-ARG was not different than the GS + L-NAME groups. L-NAME given alone, without GS, had no effect on serum NO_2/NO_3. The mean value for serum levels of NO_2/NO_3 in this latter group was $189 \pm 48 \mu M$, which was not significantly different than the values obtained during the baseline period. The values for the baseline urinary NO_2/NO_3 excretion was 2125 ± 211 nmol/8 hr. GS injection significantly increased this value to 3879 ± 560 ($P < 0.01$). This increase was significantly prevented in rats given GS + L-NAME ($P < 0.01$). When animals were treated with L-ARG in addition to L-NAME and GS, significant reversal of this inhibition could be seen, which was different from the result reflected as the serum levels of NO_2/NO_3. Lastly, L-NAME alone did not significantly alter urinary NO_2/NO_3 levels compared with baseline.

Urinary cGMP excretion

We measured urinary cGMP excretion on 8hr urine collections obtained from rats treated with normal saline, GS or GS + L-NAME. Rats injected with saline excreted 21 ± 2.0 nmol cGMP/8 hr whereas rats treated with a single injection of GS excreted 32 ± 4.5 nmol cGMP/8 hr ($P < 0.05$ vs saline). Lastly, the rats that received L-NAME + GS excreted 22 ± 4.0 nmol cGMP/8 hr, a value that is significantly lower than rats receiving GS alone ($P < 0.05$ vs GS alone) and no different than rats given saline.

NO synthase activity in kidney tissue

NO synthase activities of rats injected with saline showed 1802 ± 145 dpm/mg protein in whole kidney, 3100 ± 300 in glomeruli and 3000 ± 256 in cortical tubules. These values were significantly increased by the treatment of a single injection of GS to 4500 ± 500 in kidney, 5890 ± 560 in glomeruli and 8700 ± 600 in tubules. The tubules showed the most dramatic increase in NO synthase activity after exposure to GS. The rats receiving L-NAME + GS had 2998 ± 320 in whole kidney, 3908 ± 300 in glomeruli and 6200 ± 540 in cortical tubules. Those were significantly lower than rats receiving GS alone ($P < 0.01$ vs GS alone).

Discussion

Our study demonstrates that a single i.p. injection of GS increases the levels of the NO end products, NO_2 and NO_3, in the serum and urine of rats. The changes in urinary NO_2/NO_3 were completely inhibited by the NO synthesis inhibitor, L-NAME and significantly restored when rats were given excess L-ARG in addition to GS + L-NAME. This confirms that the changes in urinary NO_2/NO_3 that we detected were due to changes in NO production. The serum NO_2/NO_3 levels were also signif-

icantly, although not completely, inhibited in rats given GS + L-NAME. L-ARG failed to reverse this inhibitory effect, but this could be due to the fact that the serum NO_2/NO_3 levels represent a single point in time whereas the urine NO_2/NO_3 levels, measured on a time collection, represents a more integrated value, reflecting the accumulated NO_2/NO_3 production during the 8-hr experimental period. From present results, we found that GS given *in vivo* to rats stimulate endogenous production of NO. *Hibbs et al., (1992) and Schultz and Raij (1992)* reported that urinary NO_2/NO_3 excretion, and perhaps to a lesser extent serum concentration of NO_2/NO_3, can be used as markers of NO production *in vivo* and present results support their reports.

Urinary excretion of cGMP has previously been shown by Tollins (1990) and Schultz and Raij (1992) to be a marker of NO synthesis. In the study of Tollins et al. (1990), acetylcholine was thought to stimulate NO production via the constitutive NO synthase in the vascular endothelium while Schultz and Raij (1992) reproted that LPS induced NO production *in vivo* and increased urinary cGMP. Both studies suggest that NO produced is biologically active since most cellular actions of NO occur via stimulation of intracellular soluble guanylate cyclase, leading to increase in cGMP. In the present study, we show that urinary cGMP excretion increases after GS and that this rise is prevented when L-NAME is given with the GS. These changes in urinary excretion of cGMP are evidence that GS stimulate NO production in vivo.

Serum and urine levles of NO_2/NO_3 and urinary excretion of cGMP do not allow us to determine precisely the relative contributions of different tissues to the changes in these biochemical measurements after i.p. injected GS. Thus, we measured NO synthase activities of whole kidney, glomeruli and cortical tubules. GS increased the production of [^{14}C]-L-citrulline from [^{14}C]-L-arginine in whole kidney as well as glomeruli and tubules. Of all tissues tested, the tubules showed the most dramatic increase in NO synthase activity after exposure to GS. Moreover, the increased production of [^{14}C]-L-citrulline was inhibited in tissues taken from animals treated with GS + L-NAME.

Glomerular endothelial cells possess an inducible cytokine-stimulated NO synthase, in addition to the well-studied constitutive isoforms *(Lamas et al., 1991).* An inducible NO synthase in vascular smooth muscle and glomerular mesangial cells were reported (Beasley *et al.,* 1991; Schultz *et al.,* 1991). In addition, NO is also produced in the renal epithelial cell (Ishii *et al.,* 1989) and the macula densa at the junction of the ascending limb of the loop of Henle and the distal convoluted tubule (Wilcox *et al.,* 1992). Although there are several different cell types within the kidney, glomeruli and cortical tubules may contribute to the GS-induced changes in NO_2/NO_3 that we measured. It is clear that the kidney is one of the possible sources of the GS-stimulated NO production *in vivo* in our study.

NO significant changes in renal function were detected after the single injection of GS. However, in the rats given the NO synthesis inhibitor L-NAME, alone or in addition to a single injection of GS, we found a significant reduction in creatinine clearance. Partial restoration of NO production by

L-ARG significantly reversed the decrease in creatinine clearance seen with GS + L-NAME. The effect of L-ARG was specific, presumably by overcoming the competitive inhibitory effect of L-NAME. We conclude from these results that inhibition of NO production may lead to renal dysfunction.

NO is one of the endothelium-derived relaxing factors and it plays a role in basal vascular tone, as well as in disease states, such as hypertension and acute renal failure (Tollins *et al.*, 1990; Schultz *et al.*, 1990b). Most of these works have studied alterations in the constitutive enzyme within the vascular endothelium. In present study, GS stimulated endogenous production of NO in rat kidney. These results were also shown in cultured aortic endothelial cells and in situ pulmonary artery endothelium (Kim *et al.*, 1992). Our results may support one of the mechanisms of an antinephritic action of GS by increasing renal blood flow *(Hattori et al., 1991)* as well as a protective role of GS on renal function from ischemia and contralateral nephrectomy (Zhang, 1992). It is possible that GS may regulate transcriptional or translational levels of NO synthase or it may affect the stability or the activity of the enzyme for NO production. Further studies should be done to investigate the mechanism of action of GS for endogenous production of NO.

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