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Effect of green tea extract on cardiac hypertrophy following 5/6 nephrectomy in the rat

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Background. Left ventricular hypertrophy commonly complicates chronic renal failure. We have observed that at least one pathway of left ventricular hypertrophy appears to involve signaling through reactive oxygen species (ROS). Green tea is a substance that appears to have substantial antioxidant activity, yet is safe and is currently widely used. We, therefore, studied whether green tea supplementation could attenuate the development of left ventricular hypertrophy in an animal model of chronic renal failure.

Methods. Male Sprague-Dawley rats were subjected to sham or remnant kidney surgery and given green tea extract (0.1% and 0.25%) or plain drinking water for the next 4 weeks. Heart weight, body weight, and cardiac Na-K-ATPase activity were measured at the end of this period. To further test our hypothesis, we performed studies in cardiac myocytes isolated from adult male Sprague-Dawley rats. We measured the generation of ROS using the oxidant sensitive dye dichlorofluorescein (DCF) as well as (^{3}H)phenylalanine incorporation following exposure to cardiac glycosides with and without green tea extract.

Results. Administration of green tea extract at 0.25% resulted in attenuation of left ventricular hypertrophy, hypertension, and preserved cardiac Na-K-ATPase activity in rats subjected to remnant kidney surgery (all $P < 0.01$). In subsequent studies performed in isolated cardiac myocytes, both ouabain and marinobufagenin (MBG) were both found to increase ROS production and (^{3}H)phenylalanine incorporation at concentrations substantially below their inhibitor concentration (IC) 50 for the sodium pump. Addition of green tea extract prevented increases in ROS production as well as (^{3}H)phenylalanine incorporation in these isolated cardiac myocytes.

Conclusion. Green tea extract appears to block the development of cardiac hypertrophy in experimental renal failure. Some of this effect may be related to the attenuation of hypertension, but a direct effect on cardiac myocyte ROS production and growth was also identified. Clinical studies of green tea extract in chronic renal failure patients may be warranted.

Key words: sodium, potassium, ATPase, ventricular dysfunction, myocardial calcium.

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Patients with renal failure develop cardiac hypertrophy commonly and often to a remarkable degree. It is known that the sodium pump is abnormal in chronic renal failure and that a circulating inhibitor(s) can be demonstrated in the serum of uremic patients. [1–4]. Although it is still controversial as to exactly what this or these inhibitor(s) is (are), ouabain (or a closely related compound), which is derived from plants, and marinobufagenin, which has been isolated from the venom of the bufa toad (*Bufo Marinus*), are thought to be candidates [5–7].

Recently, we have observed that signal transduction leading to cardiac hypertrophy can proceed through sodium pump inhibition induced by cardiac glycosides. Moreover, we have observed that several of the genomic results of this signal transduction require increases in reactive oxygen species (ROS) within these cardiac myocytes [8–10]. Because of this, we postulated that inhibition of these ROS might attenuate the development of cardiac hypertrophy in a renal failure model.

Tea (*Camellia senesis*) is native to the East Asia region and is currently being investigated for a variety of putative health benefits, including cancer prevention and attenuation of aging [11–14]. Depending on the degree and method of fermentation, tea may be produced as green, black, and oolong varieties. Green tea, because it is the least fermented, is believed to have the greatest antioxidant properties [14]. The exact chemical(s) involved in this antioxidant effect are still unclear but are believed to include polyphenols such as epigallocatechin gallate and other green tea pigments [15].

As green tea is believed to have significant antioxidant properties and ROS may play a critical role in the devel-

opment of cardiac hypertrophy induced by cardiac glycosides, we performed the following studies in a rat model of renal failure and cardiac hypertrophy.

METHODS

Animals

Male Sprague-Dawley rats (200 to 250 g) were subjected to either 5/6 nephrectomy produced by removal of the right kidney and segmental infarction of two thirds of the remaining kidney with silk ligatures, suprarenal aortic constriction [produced by tying a silk ligature (4-0) around a 21 gauge needle and the suprarenal abdominal aorta], and then removing the needle or performing sham surgery, and allowing recovery for 4 to 6 weeks. The surgical approaches have been described in detail in previous publications from our laboratory [8, 16]. At this point, the animals were anesthetized and the blood pressure was determined by placing a catheter in the carotid artery prior to removal of the heart for subsequent studies.

Determination of cardiac weight and Na-K-ATPase activity

Hearts were removed with sharp scissors cutting through aorta and vena cava. Freshly removed hearts were stripped of adherent noncardiac tissues and blotted on dry gauze prior to weighing with a quick balance. In some cases, cardiac tissue was homogenized, allowing for the formation of vesicles and determination of Na-K-ATPase activity as we have previously reported [8].

Measurement of digitalis-like substances

Ouabain, marinobufagenin, and digoxin dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) immunoassays were performed as described previously [6, 7]. The assays are based on a competition between immobilized conjugated antigens [MBG-bovine serum albumin (BSA), ouabain-ovalbumin, and digoxin-ovalbumin] and digitalis-like substances within the sample for a limited amount of binding sites on polyclonal rabbit antiserums raised against MBG (1:100,000), ouabain (1:200,000, Chemicon International, Inc., Temecula, CA, USA) and digoxin (1:20,000, Sigma Chemical Company, St. Louis, MO, USA). Secondary (goat antirabbit) antibody (1:2,000, Sigma Chemical Company) was labeled with europium using a labeling kit (Perkin-Elmer, Boston, MA, USA). The limit of detection for each of these assays was approximately 10 pmol/L. The cross-immunoreactivity of these assays with a panel of other, known digitalis-like substances and steroid hormones, including ouabain, MBG, digoxin, digitoxin, fufalin, cinofagin, prednisone, spironlactone, proscillaridin, and progesterone, was less than 5% except for the digoxin assay where digitoxin displayed 10% cross-immunoreactivity [6, 7].

Isolation and culture of cardiac myocytes

Details of the method of isolation and culture of calcium-tolerant adult myocytes may be found in several recent reports from our laboratory [10, 17]. This method of isolation produced a good yield of rod-shaped (70% to 80%) myocytes in each of the experimental groups presented in this paper. In some experiments, ouabain-sensitive ^{86}Rb uptake was determined as previously described [8].

Determination of cardiac myocyte ROS production and amino acid incorporation

Measurement of ROS was performed using the oxidant sensitive dye, CMD-CFH (Molecular Probes, Eugene, OR, USA), and monitoring fluorescence induced by excitation at 490 nm and emission at 520 nm as we have described previously [9]. This was accomplished with an Attoflor Ratioarc imaging spectrofluorimeter equipped with a Zeiss inverted microscope and a 40× Fluar objective (Zeiss Instruments, Zurich, Switzerland). In other experiments, amino acid incorporation was measured using 3H-labeled phenylanalanine incorporation over a 12-hour period as we have previously reported [8].

Preparation of green tea extract

Longjin green tea leaves were extracted with hot water. Aqueous extracts were then filtered, concentrated, and extracted twice with chloroform to remove caffeine. The remaining aqueous phase was extracted again with ethyl acetate to remove tea polyphenol and residual chloroform. The extracts were then air-dried under vacuum, dissolved in ethanol, and filtered. The final extract was obtained after ethanol was evaporated under vacuum. This was provided by Dr. Han of the Institute of Nutrition and Food Hygiene Chinese Academy of Preventive Medicine, Beijing, China. This extract, which consists primarily of tea pigments, has a dark brown color and is readily dissolved in water. The preparations used in our studies were devoid of caffeine and contained about 20% theaflavins and thearubigin [18].

Statistical analysis

Data obtained were compared using the unpaired or paired Student *t* test with Scheffe's correction for multiple comparisons depending on the unpaired or paired nature or the data [19]. Statistical analysis was performed using Sigmastat™ software. All animal experimentation described in the manuscript was conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical College of Ohio Institutional Animal Use and Care (IACUC) Committee.

Table 1. Effect of green tea extract on mean arterial pressure (MAP) and heart size in sham-treated and remnant kidney bearing rats

	Control	0.1%	0.25%
Sham surgery *number*	5	5	5
Weight *g*	418 ± 10	452 ± 8	440 ± 11
Weight gain *g/week*	36 + 3	44 + 2[a]	42 + 2
Water consumption *mL/day*	46 ± 2	44 ± 2	43 ± 2
MAP *mm Hg*	118 ± 4	123 ± 3	110 ± 3
Heart weight/body weight ratio $\times 10^3$	2.71 ± 0.06	2.95 ± 0.17	2.81 ± 0.10
Partial nephrectomy surgery *number*	10	8	10
Weight *g*	435 ± 16	419 ± 18	462 ± 8
Weight gain *g/week*	41 ± 4	37 ± 4	47 ± 4
Water consumption *mL/day*	62 ± 1	66 ± 1	64 ± 1
MAP *mm Hg*	156 ± 8	178 ± 5	112 ± 9[b]
Heart weight/body weight ratio $\times 10^3$	3.20 ± 0.09	3.31 ± 0.19	2.71 ± 0.08[b]

Data presented as mean ± SEM.
[a] $P < 0.05$, [b] $P < 0.01$ vs. control

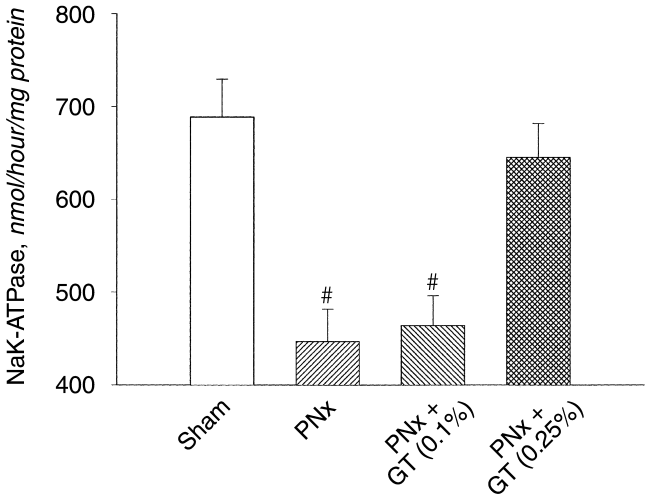

Fig. 1. Na-K-ATPase activity in hearts isolated from sham-operated rats ($N = 8$) as well as rats subjected to partial nephrectomy (PNx) given drinking water alone ($N = 8$) or supplemented with 0.1% ($N = 8$) and 0.25% ($N = 8$) green tea extract (GT). #$P < 0.01$ vs. sham-operated animals.

RESULTS

Effect of green tea extract on heart size and blood pressure

The production of 5/6 nephrectomy as well as aortic constriction both resulted in considerable increases in both blood pressure and heart size. Rats subjected to 5/6 nephrectomy drank substantially more water than sham-treated rats, but administration of green tea extract in the drinking water at 0.1% and 0.25% did not alter this water intake in either the sham operated or 5/6 nephrectomy rats. Green tea extract administered in the drinking water at 0.25% but not 0.1% substantially decreased blood pressure and cardiac hypertrophy in the 5/6 nephrectomy rats (Table 1).

Effect of green tea extract on cardiac Na-K-ATPase activity

Rats subjected to 5/6 nephrectomy demonstrated marked decreases in Na-K-ATPase activity compared with sham-treated rats. Addition of green tea extracts to the drinking water at 0.25% markedly attenuated this decrease in Na-K-ATPase activity (Fig. 1).

Effect of partial nephrectomy on serum levels of digoxin-like compound (DLC), MBG, and ouabain-like compound (OLC)

We measured the concentrations of DLC, MBG, and OLC using an immunoassay. We found that 4 weeks following 5/6 nephrectomy, rats had significant elevations of DLC and MBG, but not OLC. The MBG increase was more than twofold. These data are presented in Figure 2.

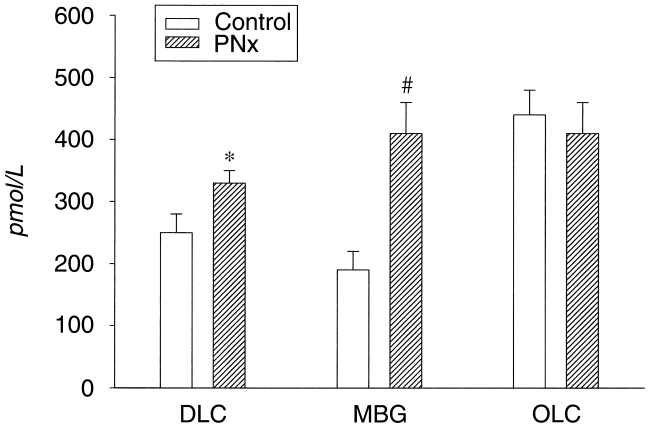

Fig. 2. Effect of partial nephrectomy (PNx) on the plasma concentrations of substances that crossreact with digitalis, marinobufagenin (MBG), and ouabain antibodies. We considered these substances, digitalis-like compound (DLC), MBG, and ouabain-like compound (OLC). *$P < 0.05$; #$P < 0.01$ vs. control.

Effect of cardiac glycosides on ^{86}Rb uptake in isolated cardiac myocytes

Cells isolated from adult cardiac myocytes were subjected to increasing amounts of ouabain and MBG, and ^{86}Rb uptake was measured. These data are summarized in Table 2. In separate studies ($N = 5$), we observed no effect of green tea extract upon the inhibition of ^{86}Rb uptake by either ouabain (100 μmol/L) or MBG (1 μmol/L).

Effect of green tea extract and cardiac glycosides on cardiac myocyte growth and ROS generation

Cells isolated from adult rats were subjected to increasing amounts of ouabain and MBG, and (^{3}H) phenylalanine incorporation was measured after 24 hours. These

Table 2. Effects of ouabain and marinobufagenin on ^{86}Rb uptake in cultured adult cardiac myocytes

Ouabain	Ouabain-sensitive ^{86}Rb uptake *% control*	Marinobufagenin	Ouabain-sensitive ^{86}Rb uptake *% control*
1×10^{-6} M	81.5 ± 1.9	1×10^{-8} M	78.6 ± 6.4
1×10^{-5} M	54.7 ± 2.3	1×10^{-7} M	67.0 ± 6.4
1×10^{-4} M	19.6 ± 1.2	1×10^{-6} M	49.9 ± 3.5
2×10^{-4} M	6.3 ± 2.2	1×10^{-5} M	25.2 ± 1.5

Ouabain-sensitive refers to that ^{86}Rb uptake inhibitable by 10^{-3} M Ouabain. Data presented as mean ± SEM of five determinations.

Table 3. Effects of ouabain and marinobufagenin on (^{3}H) phenylalanine incorporation in cultured adult cardiac myocytes

Ouabain	Phenylalanine incorporation *% control*	Marinobufagenin	Phenylalanine incorporation *% control*
Control	100.0 + 1.2		
1×10^{-6} M	100.1 ± 1.1	1×10^{-8} M	101.0 ± 0.7
1×10^{-5} M	105.5 + 1.7[a]	1×10^{-7} M	107.5 ± 2.1[a]
1×10^{-4} M	107.7 ± 1.8[a]	1×10^{-6} M	100.4 ± 0.9

Data presented as mean ± SEM of 15 determinations in each group.
[a] $P < 0.01$ vs. control

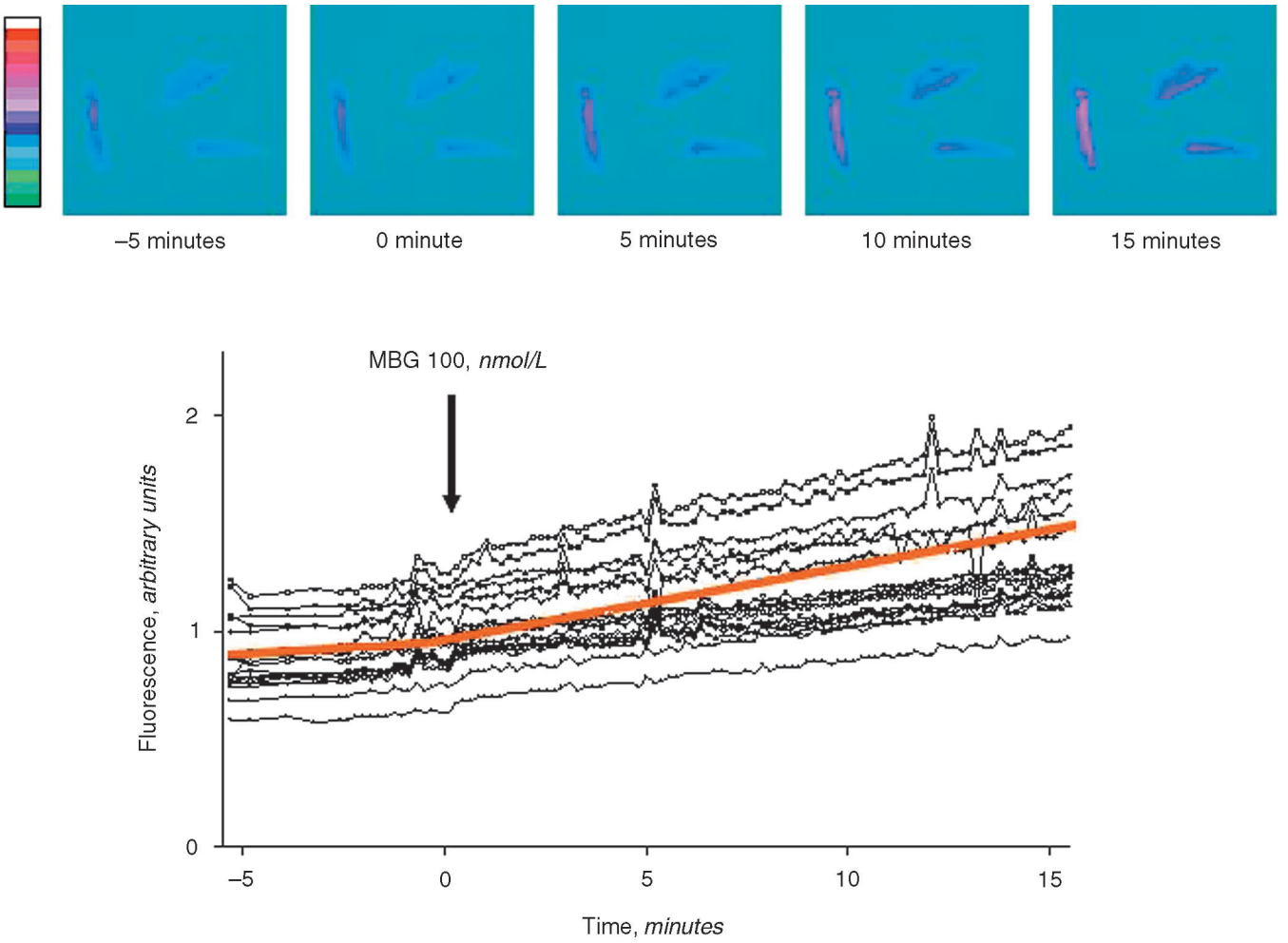

Fig. 3. Representative fluorescence imaging spectroscopy experiment obtained from adult cardiac myocytes loaded with 5 and 6 chloromethyl 2H, 7H dichlorofluorescein (CMD-CFH). Representative images shown with pseudo color intensity from −5, 0, 5, 10, and 15 minutes relative to administration of marinobufagenin (MBG) (100 nmol/L). We have also displayed a plot of fluorescence intensity over time in each of the 24 voxels picked within the three myocytes imaged. Average fluorescence values were fit with linear regression before and following addition of the MBG (red lines on graph).

results are summarized in Table 3. We found that both ouabain and MBG increased amino acid incorporation compared with control. Green tea extract alone did not significantly affect amino acid incorporation.

In the next study, we saw that both ouabain and MBG caused an increase in ROS formation (as measured by DCF fluorescence) above that seen in control adult cardiac myocytes. Green tea extract alone did not affect ROS formation compared with control (N = 10, data not shown). However, the addition of green tea extract

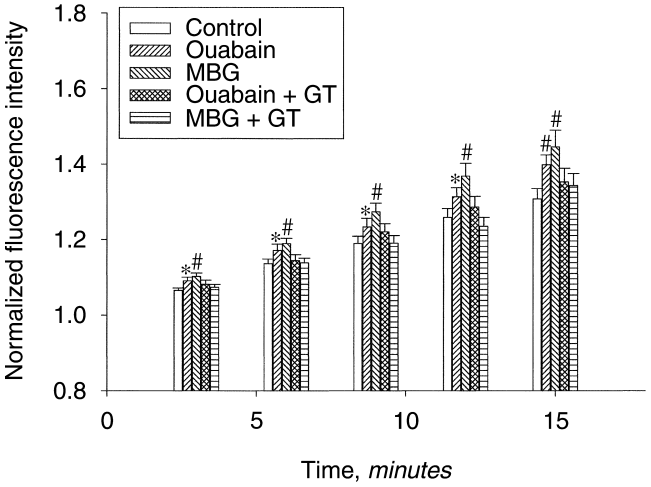

Fig. 4. Effect of green tea extract (GT) on CMD-CFH fluorescence in adult cardiac myocytes. Data shown as mean ± SEM of 15 determinations at 3, 9, 12, and 15 minutes following addition of either ouabain (10 μmol/L) or marinobufagenin (MBG) (100 nmol/L) alone or in combination with GT (50 μg/mL). Data normalized to pretreatment value of 1.0. Note that time control samples showed increase in fluorescence over time. Fluorescence in cells treated with GT alone increased at a rate comparable to control (data not shown). $*P < 0.05$; $\#P < 0.01$ vs. control.

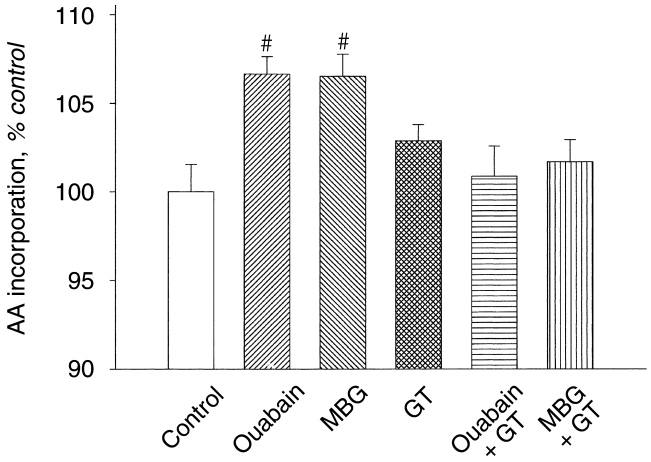

Fig. 5. Effect of green tea extract (GT) (50 μg/mL) on (^{3}H)phenylalanine incorporation following exposure to ouabain (10 μmol/L) or marinobufagenin (MBG) (100 nmol/L). Data shown as mean ± SEM of 15 determinations. $\#P < 0.01$ vs. control.

to ouabain and MBG prevented increases in ROS compared with control conditions (Figs. 3 and 4).

In the last study, we saw that the dose of green tea extract, which prevented increases in ROS from ouabain and MBG, also prevented increases in amino acid incorporation seen with these agents (Fig. 5).

DISCUSSION

The cardiac hypertrophy seen with chronic renal failure presents a tremendous clinical problem. Mortality rates in end-stage renal disease (ESRD) patients are approximately 20% per year, and more than 50% of this mortality is cardiac [20]. Although a number of factors have been implicated in the pathogenesis of this cardiac hypertrophy, including anemia and hypertension, it is suspected that some uremic factors are involved in this process [21–24]. Our laboratory has been interested in the cardiac hypertrophy, which can be induced by sodium pump inhibition, and we have observed that this process appears to involve signal transduction through the sodium pump [10, 17]. We have also observed that the genomic consequences of signal transduction through this system appear to require an increase in myocyte ROS [9].

In the present study, we observed that the administration of green tea extract essentially blocked the development of cardiac hypertrophy in the 5/6 nephrectomy model. When we designed this study, we did not expect to see that the green tea extract would also attenuate the increase in blood pressure seen with the 5/6 nephrectomy model, but this, in fact, occurred. However, perhaps this should have been anticipated as Yokozawa et al [25] reported that green tea protected against progression of renal failure in a similar model. We should also stress that we measured anesthetized blood pressure in these animals, and it is possible that conscious blood pressure values might have differed. Previously, we have observed that sodium pump inhibition induced by dietary potassium restriction can increase cardiac hypertrophy seen with suprarenal aortic constriction; however, rats treated with dietary potassium restriction alone did not develop measurable cardiac hypertrophy [8]. We concluded that, in vivo, hypertension appeared to be a necessary cofactor to allow for pump inhibition to induce measurable hypertrophy.

Because the attenuation of hypertension might explain all of the green tea extract effect on cardiac hypertrophy, we chose to directly examine the effects of green tea extracts on the cardiac hypertrophy process and moved to the isolated cardiac myocyte system where other factors (e.g., blood pressure, anemia) could be easily controlled. As we demonstrated that digitalis-like substances, specifically substances that react with digitalis and MBG but not ouabain antibodies were increased in the 5/6 nephrectomy model, this was the pertubation on which we chose to focus. Unfortunately, we did not examine whether the green tea extract might affect these elevations in digitalis-like substances in this model. In the isolated myocyte system, we saw that ouabain and MBG caused cardiac hypertrophy as assessed by radioactive amino acid incorporation at levels substantially below the observed IC 50 for these substances. We also saw that doses of these cardiac glycosides, which caused hypertrophy, also resulted in substantial increases in ROS production as measured with the oxidant sensitive dye, DCF. Finally, we observed that administration of

green tea extracts blocked both the increases in production of ROS and amino acid incorporation seen with the administration of ouabain and MBG.

We chose to use green tea extract as a method of interfering with ROS production in the current study despite it not being a pharmacologically "pure" preparation. Although much of the antioxidant effect of green tea has been ascribed to polyphenols such as epigallocatechin gallate, other chemicals are believed to be important. From a practicality standpoint, green tea extract has substantial antioxidant properties and was (is) extremely easy to administer this material in drinking water. As reported, we saw that rats drank water containing green tea extract at 0.25% without any reservations whatsoever.

We think that these results are interesting for a number of reasons. On one level, these data support the concept that sodium pump inhibition is important in the cardiac hypertrophy seen with chronic renal failure, and that the generation of ROS is an essential step in this signal transduction cascade. However, the practical application of these results is even more appealing. Green tea is a beverage that is enjoyed by people around the world. If administration of such a beverage to patients with ESRD had the same or even some of the beneficial effects seen in the animals we studied, this would be preferable to the use of another "medication" as these patients currently consume, on average, more than 10 different medications each day [26].

CONCLUSION

We found that administration of green tea extract in the drinking water of rats subjected to partial nephrectomy resulted in marked attenuation of hypertension and cardiac hypertrophy. In an isolated adult cardiac myocyte preparation, we found that green tea extract prevented increased generation of ROS and amino acid incorporation following exposure to cardiac glycosides, which circulate at elevated levels in renal failure. If these data are confirmed in humans, green tea extract may ultimately prove to be a useful dietary supplement in patients with chronic renal failure.

ACKNOWLEDGMENTS

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