Homocysteine-dependent endothelial dysfunction induced by renal ischemia/reperfusion injury

Yagnik S. Bhalodia 1, Navin R. Sheth 2, Jitendra D. Vaghasiya 3, Nurudin P. Jivani 3

ABSTRACT

Background: Elevation of serum homocysteine is considered to contribute to endothelial dysfunction, which is considered to be the initial event in vascular disease following renal transplantation. We sought to investigate whether an association existed between serum homocysteine levels and endothelial dysfunction after renal ischemia/reperfusion (I/R) injury.

Materials and methods: Acetylcholine (Ach)-induced endothelium-dependent and sodium nitroprusside (SNP)-induced endothelial-independent relaxation responses were determined in thoracic aortas from different I/R groups. A correlation analysis was performed between Ach responses and homocysteine levels.

Results: Long-term I/R injury decreased the responses to acetylcholine and the pD2 values of the concentration response curves compared with controls. While vascular responses to SNP were unchanged among all groups. Homocysteine levels correlated with the pD2 values of acetylcholine among control and I/R groups, indicating that the increase in homocysteine was associated with decreased sensitivity to acetylcholine. In short-term I/R rats, no association was observed between these parameters.

Conclusion: These data suggest a possible link between serum homocysteine and decreased vascular reactivity to endothelium-dependent relaxation in I/R aorta.

Key words: Acetylcholine, Aorta, Homocysteine, Phenylephrine, Renal transplantation, Sodium nitroprusside

INTRODUCTION

Vascular diseases such as hypertension and atherosclerosis are becoming more and more common with renal transplant recipients (1), and their prevalence is even more in affluent societies (2). Renal ischemia/reperfusion (I/R) injury is a serious complication of this procedure, initiating a complex series of cellular events that eventually leads to renal cell death. Several factors contribute to the pathogenesis of I/R injury, including ATP depletion, phospholipase and protease activation, increased endothelin-1 formation and neutrophil infiltration (3). However, renal I/R injury impairs renal homocysteine metabolism leading to serum homocysteine accumulation (4). Homocysteine, at elevated levels, is associated with oxidative stress in extrarenal tissue (5), as well as in the kidney (6). Although the possible effect of renal transplantation on homocysteine levels has been investigated, the contribution of renal transplantation on homocysteine to the processes leading to posttransplant endothelial dysfunction have not been fully elucidated.

Thus, we sought to investigate the potential effects of renal I/R injury on serum homocysteine levels and to verify whether an association exists between serum homocysteine and endothelial dysfunction in rat thoracic aorta.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 180-200 g were placed in a quiet room where the temperature was 21°C ± 2°C and humidity was 60% ± 5%, and where a 12-hour light/12-hour
dark cycle was maintained. All of the experiments in this study were performed in accordance with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines, and were approved by our Institutional Animal Ethics Committee (IAEC) on animal research.

Chemicals

Phenylephrine (PE) and acetylcholine (Ach) were obtained from Sigma, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade. Stock solutions of PE, Ach and sodium nitroprusside (SNP) were prepared in double-distilled water.

Experimental design

Eighteen male Wistar albino rats were randomly assigned to 1 of the 3 experimental groups (n=5-6): (i) control; (ii) 60-minute ischemia, 1-day reperfusion (I/R1); and (iii) 60-minute ischemia, 5-day reperfusion (I/R5). Rats were placed on a warming pad and anesthetized with ketamine hydrochloride (60 mg/kg, intraperitoneally [i.p.]) and diazepam (5 mg/kg, i.p.). Body temperature was maintained at 37°C ± 1°C. A midline incision was performed, and then both renal arteries were carefully separated from the surrounding tissues. In the I/R groups, both renal arteries were occluded by nontraumatic microvascular clips for 60 minutes. After 60 minutes, the clamps were removed, and kidneys were observed to undergo reperfusion for 1 and 5 days (4). The muscle layer was closed with an interrupted suture, and the skin layer was closed with a continuous subcutaneous suture. For analgesia, rats received topical lignocaine jelly (2%) to the wound for the first 24 hours and 1 dose of diclofenac (25 mg/kg, orally [p.o.]) as deemed necessary by the animal care staff. All rats had free access to water and food. At various time points after kidney reperfusion (1 and 5 days), rats were killed, and thoracic aortas were rapidly removed for vascular reactivity study. Immediately before being killed, rats were anesthetized under light ether for collection of blood for assay of homocysteine.

Determination of homocysteine level

Homocysteine levels were then measured in the serum samples according to the instructions of the manufacturer of the homocysteine reagent kit (Chromsystems, Germany).

Vascular reactivity study

Isolated thoracic aortas of rats were carefully cleaned of fat and connective tissues and prepared in spiral strips of 2-cm segments. Extreme care was taken not to stretch or damage the luminal surface of the aorta, to ensure the integrity of endothelium. Aortic strips were suspended in organ bath containing 20 ml Krebs bicarbonate solution (pH 7.4) maintained at 37°C ± 0.5°C and continuously aerated with 95% O₂ and 5% CO₂. The composition of the Krebs solution was NaCl 118 mM/L, KCl 4.7 mM/L, CaCl₂ 2.5 mM/L, MgSO₄ 1.2 mM/L, KH₂PO₄ 1.2 mM/L, NaHCO₃ 22.0 mM/L and glucose 11.0 mM/L. The strips were connected to a force displacement transducer for measurement of isometric force, which was continuously recorded online on a personal computer via a 4-channel transducer data acquisition system (Physiopac; Medicaid Systems, Chandigarh, India) using software analysis. The strips were maintained under a tension of 2 g and equilibrated for 90 minutes before initiating the experimental protocol. During this period, the Krebs solution was changed at 15-minute intervals. After the equilibration period, concentration response curves to increasing concentrations of PE (1 nM-10 µM) were performed in strips with intact endothelium. Endothelium-mediated relaxation was measured as a concentration response curve to Ach (1 nM-10 µM) in strips precontracted with PE. Endothelium-independent aortic relaxation to SNP (0.001 nM-10 µM) was also measured on the strips.

Statistical analysis

All values are expressed as means ± standard error mean (SEM). Statistical significance between more than 2 groups was tested using 1-way analysis of variance (ANOVA) followed by the Bonferroni post test. The agonist pD₂ value was calculated from concentration response curve by nonlinear regression analysis of the curve, and associations between serum homocysteine and the pD₂ values for Ach concentration response curves were determined by linear regression analysis using a computer-based fitting program (Prism 5; Graphpad). Differences were considered to be statistically significant when the p value was <0.05.

Results

Serum homocysteine

Long-term renal I/R injury resulted in significant increases in serum levels of homocysteine as compared with the control group, while short-term renal I/R injury did not show any significant differences when compared with control groups (Tab. I).
Relaxation response to Ach and SNP on aorta

Addition of Ach to all aortic strips with intact endothelium resulted in concentration-dependent relaxation of strips that were precontracted with PE. Ach-induced relaxation in aortas obtained from the I/R5 group was significantly lower as compared with the control group (Fig. 1). The pD2 value of Ach in the I/R5 group was significantly lower as compared with the control group (Tab. I). While no significant differences were found between the I/R1 and control groups. Addition of SNP completely relaxed aortic strips of all groups. There was no significant change in SNP-induced relaxation on aortic strips in any of the groups (Fig. 2).

Correlation between serum homocysteine levels and vascular function

Serum homocysteine was positively correlated with the pD2 values of Ach-induced concentration response curves in the control (Fig. 3A), I/R1 (Fig. 3B) and I/R5 (Fig. 3C) groups, indicating that the increased homocysteine was associated with decreased sensitivity of aortic strips to endothelium-dependent relaxation.

Discussion

The present results indicate that long-term I/R injury (5-day reperfusion period) significantly attenuated the relaxant responses to Ach in rat thoracic aortas when compared with controls, with a significant decrease in pD2 values, indicating a decreased sensitivity of the aortic strips to endothelium-dependent relaxation. Endothelial-dependent relaxation responses were consistent with elevated serum homocysteine levels. In terms of endothelium-independent relaxation response, no significant changes were observed in SNP-induced responses between the groups. Endothelium-dependent relaxation response is related to the production of nitric oxide (NO) from endothelial cells, which in turn acts on smooth muscle cells (7). Thus, the finding that endothelium-dependent relaxation response to Ach was di-

**TABLE I**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum homocysteine, µmol/L</th>
<th>pD2 value of Ach</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.48 ± 0.20</td>
<td>7.05 ± 0.09</td>
</tr>
<tr>
<td>I/R1</td>
<td>4.18 ± 0.35</td>
<td>6.76 ± 0.11</td>
</tr>
<tr>
<td>I/R5</td>
<td>6.87 ± 0.30*</td>
<td>6.70 ± 0.06*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
Ach = acetylcholine; I/R1 = 60-minute ischemia, 1-day reperfusion; I/R5 = 60-minute ischemia, 5-day reperfusion; pD2 = negative logarithm of molar concentration of Ach required to produce a 50% response of its actual response or negative logarithm to base 10 of the EC50 values.
*δ<0.05, vs. control group.
minimized in the aortas from the I/R5 group without a significant change in the endothelium-independent relaxation to the NO donor, SNP, suggests that endothelial function was selectively impaired without a loss in the ability of smooth muscle cells to respond to vasoactive agents. Indeed, it has previously been demonstrated that ischemic renal injury attenuated Ach-induced relaxation without a significant change in endothelium-independent relaxation to SNP (8, 9). Our results are in accordance with previous finding that long term reperfusion period induces endothelial dysfunction. In a clinical trial investigating the effects of transplantation procedure on endothelial function among renal transplant patients, Oflaz et al (10) reported significant endothelial dysfunction in the renal transplant recipients. Because elevated levels of homocysteine are an important risk factor for premature atherosclerosis, we investigated the possible effects of I/R-induced elevated serum homocysteine level on endothelial dysfunction, observing that the short-term I/R injury caused no significant changes in this parameter. Moreover, a correlation was observed between serum concentrations of homocysteine levels and Ach-induced endothelial-dependent relaxation response. These data provide additional support for the hypothesis that the long-term reperfusion period interferes with serum homocysteine level. These data are in accord with the results of previous studies, which have reported elevated serum homocysteine levels in renal transplant patients (11, 12). Also, recent studies demonstrated that elevated homocysteine level during I/R is attributed to reduced activity of cystathionine-β-synthase (CBS) that catalyzes the rate-limiting step in renal homocysteine metabolism (4). It was found that decreasing the NO level prevented the inhibition of CBS enzyme in the kidneys subjected to I/R. This led to the conclusion that excess NO production in the reperfusion phase is the factor most probably responsible for the reduced CBS activity (13). This can be considered as a possible mechanism for the elevation of homocysteine during renal I/R. However, this needs to be clarified by further investigation.

We used the pD2 values for Ach concentration response curves to perform a correlation analysis between homocysteine levels and vascular endothelial function. The pD2 value of Ach (negative logarithm of molar concentration of Ach required to produce a 50% response of its actual response or negative logarithm to base 10 of the EC50 values) represents the sensitivity of endothelial cells to Ach. The finding that decreased sensitivity (indicated by decreased pD2 values) of aortic strips to endothelium-dependent relaxation was associated with increased homocysteine in I/R groups suggested a possible association between high homocysteine and decreased endothelial cell function in rats. These results take into consideration that hyperhomocysteinemia is an independent risk factor for endothelial dysfunction in rat aorta.

In conclusion, our data suggest a possible link between long-term I/R and decreased vascular sensitivity to endothelium-dependent relaxation response in rats. Moreover, renal I/R-induced vascular endothelial dysfunction was associated with elevated serum homocysteine levels.

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Conflict of interest statement: None declared.

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