Vitamin E supplementation reverses renal altered vascular reactivity in chronic bile duct-ligated rats

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Vitamin E supplementation reverses renal altered vascular reactivity in chronic bile duct-ligated rats. Am J Physiol Regul Integr Comp Physiol 292: R1486–R1493, 2007. First published December 7, 2007; doi:10.1152/ajpregu.00309.2006.—An altered vascular reactivity is an important manifestation of the hemodynamic and renal dysfunction during liver cirrhosis. Oxidative stress-derived substances and nitric oxide (NO) have been shown to be involved in those alterations. In fact, both can affect vascular contractile function, directly or by influencing intracellular signaling pathways. Nevertheless, it is unknown whether oxidative stress contributes to the impaired systemic and renal vascular reactivity observed in cirrhosis. To test this, we evaluated the effect of vitamin E supplementation (5,000 IU/kg diet) on the vascular reactivity of isolated perfused kidneys and aortic rings of rats with cirrhosis induced by bile duct ligation (BDL), and on the expression of renal and aortic phospho-extracellular regulated kinase 1/2 (p-ERK1/2). BDL induced a blunted renal vascular response to phenylephrine and ACh, while BDL aortic rings responded less to phenylephrine but normally to ACh. Cirrhotic rats had higher levels of oxidative stress-derived substances (measured as thiolbarbituric acid-reactive substances (TBARS)] and NO (measured as urinary nitrite excretion) than controls. Vitamin E supplementation normalized the renal hyporesponsivity to phenylephrine and ACh in BDL, although failed to modify it in aortic rings. Furthermore, vitamin E decreased levels of TBARS, increased levels of NO, and normalized the increased kidney expression of p-ERK1/2 of the BDL rats. In conclusion, BDL rats showed a blunted vascular reactivity to phenylephrine and ACh, more pronounced in the kidney and reversed by vitamin E pretreatment, suggesting a role for oxidative stress in those abnormalities.

Chronic Bile Duct Ligation (BDL) induces intense changes in the circulatory and renal function, such as a fall in blood pressure and sodium retention, leading to the development of ascites and the hepatorenal syndrome with the advance of the disease (21, 24) At this point, the therapeutic options are limited and reversed by vitamin E pretreatment, suggesting a role for oxidative stress in those abnormalities.

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not ligated or cut. All rats were maintained under comparable conditions with ad libitum diet and free access to drinking water. All of the experimental protocols were performed 21 days after surgery.

Study Design

Rats were randomized into three groups: 1) untreated control rats (control group; n = 19), 2) untreated BDL rats (BDL group; n = 24), and 3) BDL rats treated with vitamin E (BDL+VitE group; n = 22). A time control group of rats treated with vitamin E (Sham+VitE group; n = 4) was also included in the study. Both untreated groups were maintained on standard rat chow containing a normal essential amount of vitamin E (50 IU/kg diet). Group 3 received a diet supplemented with vitamin E (5,000 IU/kg diet, dry vitamin E acetate; Panlab S. L., Barcelona, Spain) starting 1 wk before surgery (day 0). All of the rats were acclimated to individual metabolic cages to collect 24-h urine samples, to determine urinary nitrites excretion. These were obtained during two consecutive days before surgery (days −2 and −1) and 2 days just before the experimental day (days 20 and 21 after surgery).

Experimental Procedure

Preparation of isolated perfused kidneys and functional procedures

The rats were anesthetized and placed on a heated table to maintain body temperature at 37°C. A polyethylene cannula (PE-50) was placed in the right femoral artery for blood collection and for measuring blood pressure. Subsequently, we proceeded to isolate and perfuse the kidney, as described previously (19). Briefly, the left kidney was exposed by a midline laparotomy, and the renal artery was cannulated via suprarenal aorta to prevent or minimize interruption of blood flow. The kidney was perfused in situ with warm oxygenated Krebs’ buffer (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 KH2PO4, 0.026 edetate calcium disodium, and 5.6 glucose, pH 7.4, at 37°C, blood-free and at a constant rate of 5 ml/min/kg kidney weight. The renal vessels were cannulated and tied with a peristaltic pump (Master-flex 7518–00, Coler-Parmer Instrument, Niles, IL). The left renal vein was then cut and the ureter transected to allow exit of the perfusate. Finally, the kidney was excised from the surrounding tissues, decapsulated (to minimize any influence of interstitial pressure changes on renal vascular responses), and placed in a chamber containing Krebs solution at 37°C and the perfusate lost from the renal vein, both renal vascular responses), and placed in a chamber containing Krebs solution. Aortic rings were placed in 10-ml organ chambers to analyze vascular reactivity, as previously described (39). After equilibration, a cumulative concentration-response curve for PHE (10⁻⁷ to 10⁻⁵ M) was obtained. After repeated washing, rings were again constricted at 75% of the PHE maximal contraction to perform the cumulative concentration-response curve to ACH (10⁻⁸ to 10⁻⁴ M). L-NAME (10⁻⁴ M) was also used in a subgroup of rats from groups 1, 2, and 3. The presence of functional endothelium was confirmed after obtaining at least a 70% relaxation to ACh. In the l-NAME-treated rings, SNP (10⁻⁴ M) was used to test the functionality of aortic smooth muscle. Relaxation to ACh was expressed as a percentage of the maximal effect obtained with SNP. To inhibit NO synthesis with these drugs were prepared in distilled water and maintained frozen. Working solutions were prepared daily in Krebs solution. Drug concentrations are expressed as final bath concentrations.

Analytical Measures

Thiobarbituric acid reactive substances (TBARS) were determined in plasma and kidney tissue, as a measure of lipid peroxidation, by a colorimetric method used previously (41). Urinary excretion of nitrites was determined by using the Griess reaction (40) in 24-h urine samples.

Renal and Aortic Expression of Phospho-ERK1/2

Western blot analysis of aortas and kidneys were performed following standard procedures, as described previously (20). Frozen aortas and kidneys were homogenized in a lysis buffer containing 150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl, supplemented with detergents (0.5% Igepal CA-630 and 1% Triton-X) and a cocktail of protease inhibitors (aprotinin 2 µg/ml, pepstatin A 1 µg/ml, leupeptin 10 µg/ml, 500 µM Na3VO4, and 1 mM PMSF). The homogenate tissue was spun down (10 min, 20,000 rpm) at 4°C, the pellet discarded, and the supernatant kept at −80°C until analyses. The protein concentration was measured in the lysates using a bicinchoninic acid method (Sigma, St. Louis, MO). Protein samples (50–100 µg) were mixed (1:1) in 2× sample buffer (2% 2-beta-mercaptoethanol, 4% SDS, 20% glycerol, 0.001% bromophenol blue, and 500 mM Tris-HCl, pH 7.4) and boiled for 5 min. Then, proteins were separated by electrophoresis at constant voltage (100 V) on a polyacrylamide-SDS gel (11%) in a 2-mM Tris Base/192-mM Glycine/0.1% SDS buffer. Proteins were transferred to a 0.4-µm polyvinylidene difluoride membrane (Millipore) by wet electrobloating in a 25 mM-Tris Base/192-mM Glycine/M appearance. SDs buffer. Nonspecific binding was blocked for 2 h in a Tris-buffered saline-Tween (TBS-T) blocking buffer (20 mM Tris Base, 500 mM NaCl, pH 7.5, 0.1% Tween 20) with 3% BSA. Western blot analysis was performed with specific monoclonal antibody anti-phospho-ERK1/2 (p-ERK1/2; BD Transduction). Blots were incubated in TBS-1% BSA with the primary antibodies (1:1,000) overnight at 4°C, the pellet discarded, and the supernatant kept at −80°C until analyses. The protein concentration was measured in the lysates using a bicinchoninic acid method (Sigma, St. Louis, MO). Protein samples (50–100 µg) were mixed (1:1) in 2× sample buffer (2% 2-beta-mercaptoethanol, 4% SDS, 20% glycerol, 0.001% bromophenol blue, and 500 mM Tris-HCl, pH 7.4) and boiled for 5 min. Then, proteins were separated by electrophoresis at constant voltage (100 V) on a polyacrylamide-SDS gel (11%) in a 2-mM Tris Base/192-mM Glycine/0.1% SDS buffer. Proteins were transferred to a 0.4-µm polyvinylidene difluoride membrane (Millipore) by wet electrobloating in a 25 mM-Tris Base/192-mM Glycine/M buffer. Nonspecific binding was blocked for 2 h in a Tris-buffered saline-Tween (TBS-T) blocking buffer (20 mM Tris Base, 500 mM NaCl, pH 7.5, 0.1% Tween 20) with 3% BSA. Western blot analysis was performed with specific monoclonal antibody anti-phospho-ERK1/2 (p-ERK1/2; BD Transduction). Blots were incubated in TBS-1% BSA with the primary antibodies (1:1,000) overnight at 4°C and washed. Then, the blots were incubated for 1 h in TBS-T-1% BSA with the secondary antibody (anti-mouse IgG, HRP-linked; 1:1,000) and washed. Preincubated protein markers (Bio-Rad, Hercules, CA) were used for molecular weight determination. Lysates from A431 cells stimulated by EGF were used as positive control for p-ERK1/2. Both protein markers and positive control were run in parallel to the samples. Detection of specific proteins (42/44 kDa) was carried out by enhanced chemiluminescence (ECL; Western blotting analysis system, Amersham, Little Chalfont, Buckinghamshire, UK). The bands corresponding to the different proteins in the ECL films were scanned, and the relative expression of protein was quantified by densitometric analysis. Densitometric results are reported as integrated values (area × times density of the band, corrected by protein loading) and expressed as a percentage.

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compared with controls (100%). To determine protein loading, we
stained the blots with a 0.1% of Ponceau Red solution. All drugs were
purchased from Sigma Chemical (Madrid, Spain) except where indicated.

**Histologic Analysis**

Liver tissue samples were fixed in 10% buffered formaldehyde and
then processed and embedded in paraffin and sectioned (~5 μm), as
previously reported (15, 41). These sections were then stained with
hematoxylin and eosin and viewed by light microscopy. A pathologist
then performed morphologic evaluation in blinded randomized sec-
tions of the liver tissue.

**Statistical Methods**

Data are presented as means ± SE. Differences between groups
were compared by one-way, repeated-measures ANOVA. For the
dose-response curves, we applied a two-way repeated-measures
ANOVA, and when significantly different, means were further com-
pared by the Newman-Keuls test. The ED50 (concentration of agonist
producing 50% of the maximal response) is derived from logarithm
transformation and regression analysis of each individual concentra-
tion response curves. Statistical significance was considered as $P < 0.05$.

**RESULTS**

**General Parameters**

BDL rats had significant lower body weight (244 ± 11 vs.
322 ± 6 g) and hematocrit (39.2 ± 1.2 vs. 45.6 ± 0.8%) than
controls and higher spleen (1.81 ± 0.11 vs. 0.86 ± 0.03 g) and
kidney-to-body wt ratio (0.42 ± 0.01 vs. 0.32 ± 0.01), although
similar kidney weights (1.04 ± 0.03 vs. 1.01 ± 0.03 g),
respectively. Vitamin E treatment significantly increased body
weight, hematocrit, and kidney weight (288 ± 8 g, 43 ± 1%,
and 1.13 ± 0.03 g, respectively) in the BDL animals, without
altering spleen weight and kidney ratio (1.81 ± 0.11 g and
0.40 ± 0.02 g, respectively). The Sham + VitE group exhibited
differently altered values only in the body weight (344 ± 4 g)
but similar hematocrit (45.8 ± 0.6%), spleen weight (0.80 ±
0.07 g), kidney-to-body wt ratio (0.35 ± 0.02), and kidney
weight (1.19 ± 0.08 g) than the untreated control group.

Untreated BDL rats had lower blood pressure (BP) com-
pared with controls (84 ± 3 vs. 122 ± 6 mmHg), and treatment
with vitamin E significantly increased it (BDL + VitE, 100 ± 6
mmHg, Fig. 1, top). Basal RPP was also lower in the BDL rats
than in controls (37 ± 3 vs. 66 ± 11 mmHg) and vitamin E
normalized it (65 ± 8, Fig. 1, bottom). A similar pattern in
basal RPP was observed in the L-NAME subgroup (BDL, 29 ±
5; Control, 63 ± 10; and BDL + VitE, 48 ± 5 mmHg), in which
nonstatistical differences were observed after L-NAME
administration (43 ± 9, 72 ± 13, and 73 ± 12
mmHg, respectively). Chronic vitamin E administration did not
modify either BP (115 ± 4 mmHg) or RPP (54 ± 4 mmHg) to
the control rats.

Finally, histological analysis of all the BDL rat livers re-
vealed an altered pattern of liver tissue structure compared with
the control groups with a marked bile-duct proliferation and
patchy necrosis.

**Renal Vascular Responses in the Isolated and
Perfused Kidneys**

The administration of PHE induced dose-dependent in-
creases in RPP (Fig. 2, top) that were significantly lower in the
BDL rats, and normalized by vitamin E supplementation (left).
The ED50 value (Table 1) was higher in the BDL group and
also normalized by vitamin E. L-NAME administration (right)
eliminates the differences between groups in response to PHE,
and the BDL + VitE group reached higher values than the
untreated BDL, as well as greater ED50 and maximal response
values (Table 1).

The response to ACh (Fig. 2, bottom, and Table 1) was
reduced in the BDL kidneys, and treatment with vitamin E
restored it (left bottom and Table 1). No differences were found
between groups after L-NAME administration (right bottom).
The ED50 values for ACh responses were similar in all groups
(Table 1). The Sham + VitE group showed a similar pattern of
renal vascular responses to PHE and ACh than the control
group, even though the PHE-ED50 value was a little different.
SNP induced similar responses in all groups before and after
L-NAME treatment.

**Vascular Responses in Aortic Rings**

The response to PHE in aortic rings (Fig. 3, top and Table 2)
was significantly lower in BDL (treated or not with vitamin E)
than in controls, and the addition of L-NAME to both BDL
groups abolished the hypo responsiveness to PHE. The ED50
value (Table 2) was similar in control and BDL rings and lower

Fig. 1. Blood pressure (BP; top) and basal renal perfusion pressure (RPP; bottom) in the different experimental groups. Data are expressed as the
means ± SE. *$P < 0.05$ vs. control group; †$P < 0.05$ vs. bile duct ligation (BDL) group.
in the BDL+VitE group. L-NAME significantly decreased ED_{50} in control and BDL rings. In contrast, ACh induced similar relaxations without differences between groups (except for BDL vs. BDL+VitE) before or after L-NAME administration (Table 2). Both ED_{50} values and the aortic vascular responses to PHE and ACh in the Sham+VitE group were comparable to the control group (Table 2). Finally, treatments or manipulation did not alter smooth muscle function, as SNP relaxed evenly and 100% of all groups of rings.

### Systemic and Renal Levels of ROS and NO (TBARS and 24-h Urinary Nitrites)

Plasma levels of TBARS were increased in the BDL group and remained elevated after vitamin E supplementation (Fig. 4, top). Conversely, in the kidney tissue (Fig. 4, middle), vitamin E treatment decreased the elevated renal levels of TBARS in the BDL group. BDL rats had elevated 24-h urinary nitrite excretion, and administration of vitamin E further increased this value in BDL rats (Fig. 4, bottom).

### Aorta and Kidney Protein Expression of Phospho-ERK1/2

The aortic expression of pERK1/2 (Fig. 5, top) had a tendency to be greater in BDL groups, although we did not find statistical differences between them. On the contrary, the renal expression of this protein (Fig. 5, bottom) was increased in the BDL group, and vitamin E treatment returned the expression to control values.

### DISCUSSION

The main findings in the present study are that vitamin E administration to BDL rats corrects the lower RPP and renal vascular responses to PHE and ACh. These effects were associated with a decrease in kidney TBARS and increased excretion of urinary nitrites, suggesting a better availability of NO in the renal vascular bed. We also found that the increased renal expression of pERK1/2 return to control values in the group of BDL treated with vitamin E. These results are consistent with our previous study, in which we reported that vitamin E prevents renal dysfunction and improved hypotension induced by experimental BDL using in vivo anesthetized rats (41) and, furthermore, brings to light additional mechanisms by which vitamin E may exert this beneficial effect.
Prolonged obstruction of the bile duct in the rat usually causes a cholestatic liver disease with hepatocellular damage and complicated by portal hypertension, ascites, and the presence of a hyperdynamic circulation that leads to progressive hypotension, renal dysfunction, and death. The evolution is variable and the rats died usually within 5–8 wk, depending on the strain used and even on the supplier (13, 15–16, 31–32, 41). After 21 days of BDL (4th wk), the animals used in the present study showed typical signs of cirrhosis, such as decreased growth, jaundice, and coluria, and a lower hematocrit. After death, the abdominal inspection revealed a small amount of ascites only in one or two rats of the BDL groups, but there was mesenteric edema, as well as an enlarged liver and spleen. Death, the abdominal inspection revealed a small amount of ascites only in one or two rats of the BDL groups, but there was mesenteric edema, as well as an enlarged liver and spleen (indirect evidence of portal hypertension) in all of them. Morphologically, the livers had marked ductal proliferation, fibrosis, and patchy necrosis, and as we previously found (41), vitamin E treatment had small or no effect on the liver morphology, portal hypertension, or spleen weight. That is not entirely surprising since the vitamin E therapy is not addressing the primary insult, which is the surgical cholestasis. In addition, analyzing previous studies using different antioxidants that found a recovery in the systemic hemodynamic changes, we have observed that the improvement in liver function is minimal (17, 32, 41). In fact, there was no improvement in serum bilirubin or albumin, and very minor decreases in plasma aspartate aminotransferase levels or portal pressures. Thus, we think that the data overall suggest that the antioxidant therapies employed in the present and past studies are not effective (or at best only marginally effective) in preventing the progression of the liver disease.

Chronic obstructive jaundice is also associated with severe hypotension and a predisposition to acute renal failure that may be related to changes in renovascular responsiveness (14, 27). In this regard, reduced vascular responses to vasoconstrictors in different vascular beds have been reported in experimental models of portal hypertension with or without cirrhosis (3, 13, 19, 39). In this study, we also found a lower renal vascular reactivity to PHE in isolated kidneys from rats subjected to BDL compared with their controls. Moreover, basal RPP of the kidneys from BDL rats was also lower than in the control animals, suggesting a basal dilation of the renal vasculature of the BDL group. This decrease of basal RPP in the BDL kidneys cannot be due to the systemic hypotension exhibited by BDL rats because 1) the kidney was isolated from the animal and 2) the perfusion rate was the same as in the control kidneys. Likely, this lower than normal renal vascular resistance is due to intrarenal factors.

Table 2. ED50 values and maximum contraction or relaxation to phenylephrine and ACh in aortic rings of the different experimental groups

<table>
<thead>
<tr>
<th>Phenylephrine</th>
<th>ACh</th>
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<tr>
<td>Phenylephrine</td>
<td>ACh</td>
</tr>
<tr>
<td>Aortic Rings</td>
<td>ED50, 10−5 mol/l</td>
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<tr>
<td>Control (6)</td>
<td>5.62±0.49</td>
</tr>
<tr>
<td>Sham+VitE (4)</td>
<td>4.18±0.57</td>
</tr>
<tr>
<td>BDL (7)</td>
<td>4.40±0.33</td>
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<tr>
<td>BDL+VitE (5)</td>
<td>3.52±0.42*</td>
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<tr>
<td>L-NAME</td>
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<tr>
<td>Control (4)</td>
<td>1.96±0.13*</td>
</tr>
<tr>
<td>BDL (5)</td>
<td>2.23±0.13</td>
</tr>
<tr>
<td>BDL+VitE (5)</td>
<td>3.29±0.79</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Numbers in parentheses are numbers of animals per group. *P < 0.05 vs. control group; †P < 0.05 vs. BDL group. a,b,P < 0.05 with respect to the control, BDL, and BDL+VitE basal groups, respectively.
responses in isolated kidneys (19) of rats with portal hypertension, as well as we found in the BDL model, indicating that elevated levels of NO are contributing to the renal vasodilation. In this regard, an interesting finding of our study is that the response to ACh in the BDL kidneys was severely attenuated, and inhibiting NO synthesis with L-NAME further blunted this response. These results suggested that a factor different than NO is affecting the kidney; alternatively, it is possible that the excess of NO or some NO-derived substance is being harmful to the endothelium relaxing function. In this way, Inan et al. (27) obtained similar results, and they suggested that the overproduction of NO may cause endothelial damage and/or desensitization of guanylate cyclase, which, in turn, may result in loss of endothelium-dependent and -independent vasorelaxation. This is important because an altered renal endothelial vasodilatation may contribute to explain previous in vivo studies in cirrhotic animals showing a defective renal vascular and excretory response to maneuvers aimed at vasodilating the kidney (2, 4–5).

To further investigate this, we analyzed oxidative stress between other factors contributing to the altered vascular responses in cirrhosis. Oxidative stress is increased in patients and animals with cirrhosis, and its levels correlate with the...
diminishing the interaction between ROS (O$_2^-$) and NO, as suggested by the increased levels of urinary excretion of nitrites in this group. Superoxide anion (O$_2^-$) can rapidly react with NO, thus decreasing the availability of NO and resulting in peroxynitrite formation (OONO$^-$), which can subsequently initiate again lipid peroxidation (7), thus completing a vicious circle where NO is continuously consumed. Then, antioxidant administration will break that circle by diminishing the interaction between ROS (O$_2^-$) and NO, thus increasing NO bioavailability.

However, vitamin E treatment alone did not affect the aortic BDL hyporesponsiveness, and only after NOS inhibition, the aortic response to PHE was normalized in the cirrhotic rats and further increased if they were pretreated with vitamin E. These responses in the aortic rings may be related to the different contribution of NO in the vascular responsiveness, depending on the vascular bed studied (42, 46), as well as the different patterns of oxidative stress that may be found in different tissues (31, 38). Actually, the levels of TBARS in kidney tissue are higher than in plasma, suggesting that kidneys are specially affected by oxidative stress, and thus the effect of vitamin E supplementation is more pronounced in this tissue than systematically. In fact, during cirrhosis, several substances that are able to induce oxidative stress are particularly elevated or affected by oxidative stress, and thus the effect of vitamin E supplementation is more pronounced in this tissue than systematically. In fact, during cirrhosis, several substances that are able to induce oxidative stress are particularly elevated or affected by oxidative stress, and thus the effect of vitamin E supplementation is more pronounced in this tissue than systematically.

In conclusion, we have shown that vitamin E supplementation restores the abnormal renal vascular responses to PHE and ACh in BDL-induced cirrhotic rats. These effects were due to a decrease in oxidative stress-derived substances levels in the kidney and to a better availability of renal vascular NO. The changes in pERK1/2 expression suggest that this intracellular signaling pathway may be implicated in the contractile dysfunction of VSMC during chronic BDL and can also contribute to explaining part of the beneficial effects of vitamin E on renal vasculature.

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