Metformin reduces hepatic resistance and portal pressure in cirrhotic rats

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PORTAL HYPERTENSION IS A SERIOUS consequence of liver cirrhosis that results in life-threatening complications with elevated morbidity and mortality. The initial factor for its development is the presence of an increased vascular resistance in the cirrhotic liver, due to hepatic architectural distortion and increased vascular tone (5). Histologically, cirrhosis is mainly characterized by increased deposition and altered composition of the extracellular matrix, and by the development of regenerative nodules. In the fibrotic liver, hepatic stellate cells (HSC) become activated, and their phenotype change to be myofibroblast like, with contractile properties and elevated production of collagen (13, 38). The increased hepatic vascular tone in cirrhosis is considered to be mainly due to endothelial dysfunction, as a consequence of an insufficient nitric oxide (NO) bioavailability in sinusoidal endothelial cells (15). Low NO bioavailability has been related both to a decreased endothelial NO synthase (eNOS) activity, which occurs despite normal eNOS protein expression, and to increased NO scavenging by superoxide radicals (19, 20).

The biguanide drug metformin, besides having metabolic effects as an insulin sensitizer in type 2 diabetes mellitus, also confers vascular protection, improving the endothelial dysfunction in several cardiovascular diseases (7, 8, 10, 25, 31, 45). Indeed, previous studies in experimental models of arterial hypertension, diabetes, or fatty liver disease proposed that metformin-derived vasoprotection may be due to an increase in NO bioavailability, resulting from enhanced eNOS activity (42) and reduced oxidative stress (33, 34), as well as by an improvement in the phenotype of vascular smooth muscle cells (2, 24).

Then it is reasonable to propose that metformin treatment may have a potential role reducing intrahepatic resistance and, therefore, portal hypertension in cirrhosis. Interestingly, metformin has also been suggested to have an effect inhibiting the α-adrenergic tone, which was shown to be synergistic with the effect of propranolol controlling arterial hypertension (35, 37). Therefore, the primary purpose of the present study was to evaluate the hemodynamic and molecular effects of metformin administration on rats with liver cirrhosis. In addition, we also tested whether metformin influences the hemodynamic response to nonselective β-blockade.

MATERIALS AND METHODS

The study was carried out in male Wistar and Sprague-Dawley rats (Charles River Laboratories, Barcelona, Spain). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/ 609). The personnel who prepared and administered treatments and those that performed the experimental studies were different. Treatments’ codes were not open for interpretation of the results until the inclusion of all animals.

Experimental Models of Liver Cirrhosis and Metformin Administration

Induction of cirrhosis by carbon tetrachloride. Cirrhosis with intrahepatic portal hypertension was experimentally induced in Wistar rats by inhalation of carbon tetrachloride (CCl4) three times a week, and phenobarbital (0.3 g/l) was added to the drinking water (12). When cirrhotic rats developed ascites, after ~12–15 wk of CCl4 inhalation, administration of CCl4 and phenobarbital was discontin-
Instruments, Mountain View, CA). Hepatic vascular resistance (HVR, apparatus (4SP), and analyzed using the Chart v5.01 software (AD-

possible to the liver), and superior mesenteric artery blood flow

body temperature maintained at 37 °C, portal pressure (PP; mmHg; femoral artery), portal blood flow (PBF; ml/min; portal vein as close as possible to the liver), and superior mesenteric artery blood flow (SMABF; ml/min; superior mesenteric artery) were measured using perivascular ultrasonic transit-time flow probes connected to a flow meter (Transonic Systems, Ithaca, NY), recorded using a PowerLab apparatus (4SP), and analyzed using the Chart v5.01 software (AD-Instruments, Mountain View, CA). Hepatic vascular resistance (HVR, mmHg·ml⁻¹·min⁻¹) was calculated as PP/PBF.

In a subgroup of cirrhotic animals (n = 9 in CCl₄ and n = 6 in CBDL), after baseline hemodynamic data were obtained, rats received propranolol (5 mg/kg iv) for 10 min through the femoral vein catheter (39), and MAP and PP were recorded again.

At the end the hemodynamic study, serum samples from cirrhotic rats were collected by cardiac puncture to subsequently evaluate alanine aminotransferase, aspartate aminotransferase, and bilirubin, all by standard protocols.

Evaluation of the hepatic endothelial phenotype. NO BIOAVAILABILITY. Cyclic guanosine monophosphate, a marker of NO bioavailability, was determined in liver homogenates from cirrhotic rats treated with metformin or vehicle using an enzyme immunoassay (Cayman Chemical, Taelinn, Estonia), as previously described (1). In addition, real-time levels of NO were determined using the 4-amino-5-methyl-amino-2',7'-difluorofluorescein diacetate staining in liver sinusoidal endothelial cell (LSEC) freshly isolated from cirrhotic rats treated with metformin or vehicle, and in isolated LSEC from cirrhotic livers and treated in vitro with metformin (1 mM; 24 h) or its vehicle (PBS) (n = 3 per condition) (18, 19).

NO MOLECULAR PATHWAY. Protein expression of eNOS, phosphorylated eNOS (p-eNOS), AMP-activated protein kinase (AMPK), and phosphorylated AMPK (p-AMPK) were assessed by Western blot in livers from cirrhotic rats treated with metformin or vehicle (17). Primary antibodies comprehended a mouse antibody recognizing eNOS (BD Transduction Laboratories, Lexington, KY), a rabbit anti-p-eNOS at Ser1176 (Cell Signaling Technology, Beverly, MA), a rabbit anti-AMPK (Cell Signaling Technology), or a rabbit p-AMPK at Thr172 (Cell Signaling Technology). Protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film, Düsseldorf, Germany). After being stripped, blots were assayed for GAPDH content as standardization of sample loading. Quantitative densitometry values of eNOS and AMPK were normalized to GAPDH. The degree of eNOS phosphorylation and AMPK phosphorylation was calculated as the ratio between the densitometry readings of p-eNOS/eNOS and p-AMPK/AMPK.

NO SCAVENGING BY SUPEROXIDE. Superoxide (O₂⁻) levels were quantified with the oxidative fluorescent dye dihydroethidium (10 μM; Molecular Probes, Eugene-OR) in liver slices and LSEC isolated from metformin- or vehicle-treated cirrhotic animals, as described (19, 21). Specificity of the assay was ensured using SOD (200 U/ml) as negative control. Nitrotyrosine content, as secondary marker of NO scavenging by O₂⁻ to form peroxynitrite, was analyzed in liver sections (10 μM) through fluorohistochemistry (22) and in liver tissue through Western blot (1:1,000, Sigma) (19). Fluorescence images were obtained with a fluorescence microscope (Olympus BX51, Tokyo, Japan), and quantitative analysis of at least 10 images per slide was performed with Image J 1.44m software (National Institutes of Health, Bethesda, MD).

SOD ACTIVITY. Total SOD activity was measured in liver homogenates using a commercially available immunooassay (Sigma, Tres Cantos, Madrid, Spain), according to the manufacturer’s instructions (22).

Evaluation of hepatic fibrosis. QUANTIFICATION OF HEPATIC FIBROSIS. Semiquantitative analysis of hepatic fibrosis was performed in liver tissue blocks fixed in 10% formalin, embedded in paraffin, sectioned, and stained with 0.1% Sirius red. Eight fields from each slide were randomly selected and photographed, and the red-stained area per total area was measured using AxioVision software (Carl Zeiss MicroImaging) (41). Additionally, fibrosis was further quantified determining hepatic hydroxyproline content using a commercial kit (BioVision, Milpitas, CA), as previously described (4). Briefly, 40 mg of snap-frozen livers were hydrolyzed in HCl (12 N) at 120°C for 3 h and centrifuged to remove precipitates. Aliquots (10 μl) from each sample were evaporated to dryness and incubated with chloramine T (2.5 mM) for 5 min and Ehrlich’s reagent (410 mM) for 90 min at 60°C. Absorption was measured at 560 nm and referred to a standard curve. Results are expressed as micrograms per milligram liver tissue.

HSC AND PORTAL MYOFIBROBLASTS PHENOTYPES. Hepatic protein expression of α-smooth muscle actin (α-SMA; surrogate marker of HSC activation) and desmin (structural marker of HSC) were analyzed by immunohistochemistry. Immunostaining of paraffin-embedded liver sections was performed with a mouse anti-α-SMA antibody (1:1,000, Sigma) and a mouse anti-desmin antibody (1:50, DAKO) or, as a negative control, with phosphate-buffered saline. Bound antibodies were visualized using Daco Real Envision Detection System Peroxidase/DBA+, and slides were then counterstained with hematoxylin. α-SMA and desmin relative volume was determined by point-counting morphometry using a point grid to obtain the number of intercepts over α-SMA- and desmin-positive cells over the tissue. Twelve fields were counted in each liver. All measurements were performed by two blinded observers (12).

Fibrosis was further characterized in livers determining the expression of α-SMA, and platelet-derived growth factor receptor β polypeptide (PDGFRB), and the Rho kinase activity by Western blot in hepatic samples using a mouse antibody against α-SMA (1:1,000, Sigma), a goat antibody against PDGFRB (1:500, Santa Cruz Biotechnology), a mouse antibody recognizing moesin (1:200, Santa Cruz Biotechnology), and a mouse anti-phosphorylated moesin at Thr558 antibody (1:200 Santa Cruz Biotechnology). Rho kinase activity was calculated as the ratio of p-moesin/moesin (41). Taqman expression assays were used to determine hepatic mRNA expression of procollagen I, marker of fibrosis, collagen 15A1, marker of portal myofibroblasts (30), and metalloproteinases (MMP)-2, MMP-9, and MMP-13 and its inhibitors TIMP-1 (tissue inhibitor of metalloprotei

case-1) and TIMP-2, as markers of fibroinolysis.

Specific effects of metformin on HSC phenotype was investigated using the human activated HSC cell line LX-2, kindly provided by Dr. Bataller. Cells were treated with 1 mM metformin for 24 h, and the expression of α-SMA, PDGFRB, transforming growth factor
control rats (Table 1). CCl4 cirrhotic rats receiving metformin exhibited portal hypertension compared with animals receiving vehicle (Fig. 1). Metformin-treated cirrhotic animals exhibited significantly higher NO levels than those treated with vehicle (1.27 ± 0.12 vs. 2.06 ± 0.13 U/ml; +61%; P < 0.05).

Metformin does not modify eNOS and AMPK pathways but exerts an antioxidant effect within the cirrhotic liver. No effects of metformin administration were found analyzing eNOS, p-eNOS, AMPK, or p-AMPK protein expression (data not shown). However, livers from cirrhotic rats treated with metformin exhibited markedly lower O2− levels compared with the vehicle group (−76% in tissue, −71% in isolated LSEC; Fig. 1, C and D), which was associated with marked diminished levels of nitrotyrosinated proteins (−43% by fluorochemistry and −28% by Western blot; Fig. 1, E and F), surrogate marker of NO scavenging by O2−. Liver SOD activity was significantly higher in metformin-treated cirrhotic rats, indicating greater liver antioxidant capacity than those treated with vehicle (1.27 ± 0.12 vs. 2.06 ± 0.13 U/ml; +61%; P < 0.05).

Metformin stimulates reduction of fibrosis in CCl4 cirrhotic rats. As expected, CCl4 cirrhotic rats exhibited marked distortion of hepatic parenchyma with abundant fibrosis, as evaluated by Sirius red staining. As shown in Fig. 2, metformin produced a significant reduction in hepatic fibrosis compared with vehicle administration (−41% in Sirius red; −17% in hydroxyproline; −34% in pro-collagen I); however, it did not reach normal values. Importantly, fibrosis improvement was associated with significant diminutions in the expression of α-SMA (−74% by Western blot; −61% by immunohistochemistry), desmin (−46%), and PDGFRB (−39%), and in Rho kinase activity (−55% in p-moesin-to-total moesin ratio), altogether suggesting decreased activation and abundance of HSC. Fibrinolysis characterization revealed no changes in the mRNA expression of MMP-2, MMP-9, and TIMP-2 (data not shown). However, we observed a trend to reduction in MMP-13 (−50%; P = 0.3) and TIMP-1 (−30%; P = 0.2).

In addition, livers from metformin-treated animals showed reduced levels of the recently proposed marker of portal myofibroblasts, collagen 15A1, although it did not reach statistical significance (Fig. 2F).

As shown in Fig. 3, cellular studies confirmed the effects of metformin improving HSC phenotype. Indeed, LX-2 cells treated with metformin showed decreased expression of pro-collagen I (−50%), α-SMA (−48%), PDGFRB (−41%), and TGF-β1 (−16%), without significant differences in TGF-β expression (data not shown).

### Table 2. Effects of metformin on biochemical parameters in CCl4-cirrhotic and CBDL-cirrhotic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Metformin</th>
<th>P Value</th>
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<tr>
<td>CCl4</td>
<td>AST, U/l</td>
<td>269 ± 120</td>
<td>264 ± 65</td>
</tr>
<tr>
<td></td>
<td>ALT, U/l</td>
<td>148 ± 41</td>
<td>103 ± 37</td>
</tr>
<tr>
<td></td>
<td>Bilirubin, mg/dl</td>
<td>0.20 ± 0.07</td>
<td>0.18 ± 0.06</td>
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<tr>
<td>CBDL</td>
<td>AST, U/l</td>
<td>486 ± 222</td>
<td>460 ± 201</td>
</tr>
<tr>
<td></td>
<td>ALT, U/l</td>
<td>106 ± 19</td>
<td>67 ± 36</td>
</tr>
<tr>
<td></td>
<td>Bilirubin, mg/dl</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.2</td>
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</table>

Values are means ± SD; n = 12 CCl4 and 9 common bile duct ligation (CBDL) rats. AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Metformin reduces hepatic inflammation in CCl4 cirrhotic rats. Analysis of metformin effects on hepatic inflammation showed that M1 subtype macrophages (CD68+) were decreased (−31%; \( P = 0.04 \)) compared with vehicle-treated cirrhotic rats. No differences in the expression of M2 subtype (CD163+) macrophages were observed.

Effects of Metformin in CBDL Cirrhotic Rats

Metformin ameliorates portal hypertension in CBDL cirrhotic rats. Animals with cirrhosis of the liver caused by CBDL were used as a validation group. In this experimental model, animals treated with metformin also exhibited signifi-
cantly lower PP than the vehicle-treated group (17.2 ± 2.3 vs. 19.1 ± 2.7 mmHg; -10%; \( P = 0.009 \)), with no differences in MAP or HR (Table 3).

Similar to what was observed in CCl\(_4\)-cirrhotic rats, intravenous propranolol produced a significant reduction in PP in CBDL rats treated with either metformin or vehicle. As a consequence, the final PP in the metformin + propranolol group was markedly lower than that in the vehicle + propranolol group (14.8 ± 1.7 vs. 17.5 ± 1.4 mmHg; -15%; \( P = 0.01 \)). No differences in biochemical parameters were found comparing CBDL-cirrhotic rats receiving metformin or vehicle (Table 2).

**Metformin improves eNOS activity but does not affect liver fibrosis in CBDL-cirrhotic animals.** Metformin treatment enhanced the phosphorylation of eNOS, suggesting improvement in its enzymatic activity, without modifying eNOS, AMPK, or p-AMPK (Fig. 4, A and B). Fibrosis evaluation using Sirius red staining revealed no differences between CBDL cirrhotic rats receiving metformin or vehicle (Fig. 4C). For quantification of portal myofibroblasts using collagen 15A1, although 18% lower in the metformin-treated rats, the difference did not reach statistical significance between both groups (data not shown).

**DISCUSSION**

In liver cirrhosis, increased HVR, due to an increased hepatic vascular tone and to architectural abnormalities of the liver parenchyma, is the main player in the development of portal hypertension. Different studies have evaluated the possibility of reducing HVR by enhancing hepatic NO bioavailability and reducing hepatic vascular tone using several experimental strategies (14, 21, 29). Although these studies showed beneficial effects, novel therapeutic strategies based on European Medicines Agency/US Food and Drug Administration approved drugs with no systemic adverse effects are required to improve treatments for patients with portal hypertension.

The present study shows that 1-wk metformin administration decreases PP in two different models of liver cirrhosis. In fact, in CCl\(_4\)-cirrhotic rats treated with metformin, PP was 27% lower than in those receiving vehicle. The decrease in PP was not associated with modifications in PBF, pointing to a decreased HVR. Importantly, the beneficial effects of metformin reducing portal hypertension were confirmed, although of less magnitude, in CBDL-cirrhotic rats.

Interestingly, metformin appears to ameliorate HVR differently in the two cirrhotic models used. Indeed, in CCl\(_4\)-cirrhotic animals treated with metformin, a marked amelioration in fibrosis was observed, which was associated with an improvement in HSC phenotype and reductions in the hepatic content of HSC and probably, although to a less extent, in portal myofibroblasts. This finding is in agreement with previous studies suggesting that long-term treatment with metformin ameliorates mild fibrosis in liver and heart (6, 40, 50); nevertheless, our report describes for the first time the effects of metformin reducing fibrosis in a pathology where exaggerated collagen deposition exists. Characterization of the underlying molecular mechanisms leading to fibrosis diminution in terms of fibrosis regression revealed a decrease in the expression of...
the MMP-13 and MMP inhibitor TIMP-1, although it did not achieve significance, with no differences in MMP-2, MMP-9, and TIMP-2 compared with vehicle-treated cirrhotic rats. Overall, these data suggest that matrix degradation, at the dose and duration of treatment, may not play a major role in the reduction of fibrosis. Metformin-mediated HSC deactivation may result from its capability to reduce oxidative stress, a well-known profibrogenic stimulus, but also from the inhibition of the proliferative and profibrogenic pathways PDGF, TGF-β, and Rho kinase (38, 47). Indeed, our study agrees with previous data demonstrating that inhibition of Rho kinase results in HSC phenotype amelioration and senescence (27). Metformin-derived inhibition of Rho kinase may occur through, at least, two different mechanisms: increment in NO bioavailability, and enhancement of the Rac1-cdc42 signaling pathway. Both of them have been described as Rho kinase inhibitors (28, 32).

By contrast, we were unable to demonstrate an effect of metformin on liver fibrosis in the CBDL model. This discrepancy may be due to the different characteristics of fibrogenesis in the two models; the CBDL model is characterized by a very rapid progression of fibrosis with no possibility of spontaneous regression due to the persistence of the bile obstruction, while the CCl4 is much slower and susceptible to regression upon stopping administration of the toxic. Importantly, discrepancies in the mechanisms explaining the beneficial effects of a certain drug when comparing different experimental models of cirrhosis are not new. In fact, previous works from our team and others using the thromboxane A2 receptor antagonist terutroban or the farnesoid X receptor agonist obeticholic acid already demonstrated such phenomena (41, 44).

In addition, our data also show that metformin enhances liver NO bioavailability, which contributes to reduce HVR and PP. In the CCl4-cirrhotic model, we did not detect any increase in eNOS phosphorylation, a marker of increased eNOS activity, but we observed an increased endothelial NO bioavailability that was mainly derived from an upregulated SOD activity, thus reducing NO scavenging and formation of peroxynitrite. The use of antioxidants to increase liver NO was primarily described by our group and validated using different antioxidants (11, 12, 14, 19, 48, 49). So, our results agree with previous reports demonstrating the antioxidant effects of metformin in other vascular beds (33, 34). On the other hand, in the CBDL-cirrhosis model, we found that metformin induces the activation of the NO-generating enzyme eNOS. Previous in

Table 3. Effects of metformin on hepatic and systemic hemodynamics in CBDL-cirrhotic rats

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<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Metformin</th>
<th>P Value</th>
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<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>98 ± 11</td>
<td>100 ± 19</td>
<td>1.00</td>
</tr>
<tr>
<td>PP, mmHg</td>
<td>19.1 ± 2.7</td>
<td>17.2 ± 2.3</td>
<td>0.009</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>340 ± 35</td>
<td>335 ± 49</td>
<td>0.83</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>336 ± 4</td>
<td>331 ± 5</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats.
vitro studies showed that metformin is able to increase eNOS-dependent NO production in endothelial cells (11–13). The reason for such discrepancy between experimental models of cirrhosis is not clear. However, this has been previously reported by our group and others (16, 41, 44), emphasizing the need of extending studies to different experimental models to increase the chances of translating to human beings the findings observed at the bench-side. It is important to note that the reduction in PP due to metformin was observed in both models of cirrhosis, although the magnitude of the reduction, probably because of the observed reduction in fibrosis, was greater in the CCl4 than in the CBDL model.

Although NO certainly plays a central role modulating vascular tone within the cirrhotic liver (46), we cannot discard that other mechanisms that have been implicated in metformin-derived vasoprotection, such as inhibition of proinflammatory responses (31, 40), suppression of vasoconstrictor prostanoids (34), or modulation of calcium flux within endothelial cells (23, 34), may also contribute to improve the intrahepatic vascular tone in cirrhosis.

It is worthy to note that no detrimental systemic effects, analyzed as changes in MAP, SMABF, or in HR, were observed in cirrhotic animals receiving metformin. This observation is in agreement with a recently published study demonstrating no contraindications of metformin when administered to patients with liver cirrhosis (51). Such differential effects of metformin, according to the vascular bed, could be attributed to the known affinity of metformin for the damaged/dysfunctional endothelium, evidenced by previous studies demonstrating no effect of metformin on vascular function in normal rats (25, 33, 35, 45). In fact, our results support this hypothesis, since no significant changes in PP or in systemic hemodynamics were observed when metformin was administered to control rats (Table 4).

Considering that β-blockers are accepted therapeutic agents for primary and secondary prophylaxis in portal hypertensive patients, we further evaluated the possible synergistic effects of an acute administration of propranolol in animals under metformin treatment. Metformin-receiving animals exhibited a further decrease in PP after propranolol administration, reaching much lower levels than in animals treated with placebo, with no differences in systemic hemodynamics, thus demonstrating that metformin may add a significant advantage to the established treatment with propranolol. The additive effects of both treatments reducing PP may result from the capability of metformin to reduce the HVR, together with the reduction in portal blood inflow due to nonselective β-blockade. These results are in accordance with previous reports describing the combined effect of metformin and propranolol ameliorating arterial hypertension (35, 37).

In conclusion, the present study provides novel information showing that metformin administration to cirrhotic animals decreases PP, thus ameliorating portal hypertension. Moreover, the metformin-derived improvement has a synergistic effect with β-blockers reducing PP, suggesting a new therapeutic approach to treat portal hypertensive patients.

Table 4. Effects of metformin on hepatic and systemic hemodynamics in control rats

<table>
<thead>
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<th>Parameter</th>
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<th>Metformin</th>
<th>P Value</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>124 ± 18</td>
<td>135 ± 17</td>
<td>0.23</td>
</tr>
<tr>
<td>PP, mmHg</td>
<td>7.8 ± 1.1</td>
<td>7.9 ± 1.5</td>
<td>0.72</td>
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<td>HR, beats/min</td>
<td>346 ± 42</td>
<td>337 ± 43</td>
<td>0.89</td>
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</table>

Values are means ± SD; n, no. of rats.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES