Androgens Are Necessary for the Development of Fructose-Induced Hypertension

Dongzhe Song, Emi Arikawa, Denise Galipeau, Mary Battell, John H. McNeill

Abstract—Hyperinsulinemia and insulin resistance are closely associated with hypertension in humans and in animal models. Gender differences have been found in the development of hypertension in fructose-fed rats. The objectives of the present study were, first, to clarify whether androgens are required in the development of hyperinsulinemia, insulin resistance, and hypertension in fructose-fed rats, and second, to determine if cyclooxygenase-1 and cyclooxygenase-2 are also increased in the arteries of these rats. Male rats were gonadectomized or sham-operated and fed a 60% fructose diet beginning at age 7 weeks. Blood pressure was measured by a tail-cuff method, and an oral glucose tolerance test was performed to assess insulin sensitivity after 8 weeks of fructose feeding. Cyclooxygenase-1 and cyclooxygenase-2 mRNA expression was also assessed in the thoracic aortae and mesenteric arteries. Gonadectomy prevented hypertension from developing in the fructose-fed rats, but hyperinsulinemia and insulin resistance developed. There was an increase in cyclooxygenase-2 expression in the thoracic aortae and mesenteric arteries of the fructose-fed sham-operated rats while the expression of cyclooxygenase-1 remained unchanged. Gonadectomy prevented the mRNA overexpression of vascular cyclooxygenase-2 in the fructose-fed rats. These results suggest that the presence of androgens is necessary for the development of fructose-induced hypertension. Androgens apparently act as a link between hyperinsulinemia/insulin resistance and hypertension in fructose-hypertensive rats. Furthermore, an increase in the expression of cyclooxygenase-2 is implicated in the development of hypertension. The mechanisms involved require further study. (Hypertension. 2004;43:667-672.)

Key Words: fructose ■ insulin resistance ■ hyperinsulinemia ■ hypertension

Hyperinsulinemia and insulin resistance are closely linked to the development of hypertension in humans and in animal models.1–3 Several mechanisms have been proposed, including the sympathetic nervous system,4 renal abnormalities in handling sodium,5,6 and changes in endothelial function and vasoactive mediators such as endothelin-1, nitric oxide, and thromboxane A2 (TXA2).3,7,8

Interestingly, increased blood pressure (BP) was seen only in male rats. These results suggest that the association between hyperinsulinemia/insulin resistance and hypertension is gender-dependent and exists only in male rats. Based on these findings, we speculated that androgens might play an essential role in the relationship between hyperinsulinemia/insulin resistance and hypertension.

Gender-associated differences in BP have been widely observed and confirmed in humans. Women during their reproductive years are less prone to hypertension and hypertension-related diseases than men or postmenopausal women.9,10 Studies using 24-hour ambulatory BP monitoring have shown that BP is higher in men than in women at similar ages.10 The same phenomenon has also been observed in various animal models. Several investigations have demonstrated that male rats have higher BP than females of the same age group. This has been shown in spontaneously hypertensive rats (SHR),12,13 Dahl salt-sensitive rats,14 deoxycorticosterone-salt hypertensive rats,15 and in New Zealand genetically hypertensive rats.16 In fructose-hypertensive rats (FHR), we have shown that male rats had significant hypertension and hyperinsulinemia after 9 weeks of a high-fructose diet whereas female rats did not.17 These findings strongly suggest that androgens may also play a key role in the development of fructose-induced hypertension. Hence, the primary purpose of the present study was to determine whether male sex hormones are necessary in the development of fructose-induced hypertension and to evaluate the influence of androgens on hyperinsulinemia and insulin resistance in these fructose-fed rats.

A secondary purpose of the present study was to examine the role of cyclooxygenase (COX) enzymes in the develop-
ment of fructose-induced hypertension. This follows from our previous work in which we have shown that COX inhibition impairs vascular contraction in male FHR significantly more than in control rats (unpublished) and from studies implicating thromboxane (TXA2) in causing hypertension in this animal model. We have shown that plasma levels of thromboxane B2 (TXB2), a stable TXA2 metabolite, were significantly increased in FHR, and that treatment with dazmegrel, an inhibitor of thromboxane synthase, prevented this increase. The production of TXB2 by aorta was also significantly greater in FHR and was normalized by treatment with dazmegrel. We have hypothesized that hyperinsulinemia/insulin resistance may cause an increase in BP by stimulating an increase in the activity of endothelium-derived vasoconstrictors, such as TXA2. COXs are key enzymes that regulate the production of TXA2.19 There are 2 isoforms of COX: COX-1 and COX-2. Because there is an increase in TXA2 synthesis by vascular tissues of fructose-fed rats, the expression of COX-1 and/or COX-2 might also be increased in these rats. Therefore, in the present study, we examined the mRNA expression of COX-1 and COX-2 in the thoracic aorta and mesenteric arteries in a pilot study to determine if these enzymes were overexpressed in the presence of fructose-induced hypertension. We also examined whether androgens had any effect on COX-1 and COX-2 expression in addition to their influence on BP.

**Methods**

**Animals and Research Design**

Forty-eight male Wistar rats were obtained from Charles River Laboratories (St-Constant, Québec). Sixteen rats were gonadectomized; 32 rats were sham-operated at age 5 weeks and before shipment. The rats were divided into 4 experimental groups: sham-operated normal chow (C; n=16), sham-operated fructose-fed (F; n=16), gonadectomized normal chow (G; n=8), and gonadectomized fructose-fed (GF; n=8). At age 6 weeks, systolic BP, plasma glucose, insulin, and testosterone levels were measured. At age 7 weeks, the rats in the F and GF groups were started on a diet of 60% fructose for 9 weeks (Teklad Laboratory Diets, Madison, Wis), whereas control groups were maintained on normal laboratory rat chow for the same period of time. The rats were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. The rats were housed on a 12-hour light–dark cycle and received fructose or normal rat chow and water ad libitum. Food and fluid intake, body weight, and BP were monitored every 2 weeks. An oral glucose tolerance test was performed after 8 weeks of treatment. Final blood samples were collected for the measurement of plasma glucose, insulin, and testosterone levels. At the end of week 9 of treatment, rats from all the groups were euthanized with an overdose of pentobarbital. Thoracic aorta and mesenteric arteries were carefully isolated from each rat, cleaned of adherent connective tissue, and fixed in RNAlater. The tissues were stored at −70°C until reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to measure the mRNA content of COX-1 and COX-2.

**BP Measurement**

Systolic BP was measured in conscious rats using the indirect tail-cuff method without external preheating as previously described.18,20–22

**Oral Glucose Tolerance Test**

At the end of 8 weeks of fructose treatment, all the rats were fasted overnight (12 hours) and subjected to an oral glucose tolerance test. A 40% glucose solution was prepared and administered by oral gavage (1 g/kg) to conscious animals. Blood samples were taken at 0, 10, 20, 30, 60, and 90 minutes after glucose administration; the plasma was separated and stored frozen until glucose and insulin measurements.

**Insulin Sensitivity Index**

The insulin sensitivity index (ISI) was calculated from the data obtained during the oral glucose tolerance test using the formula of Matsuda and DeFronzo.23

**COX-1 and COX-2 mRNA Determination**

The RNA content of COX-1 and COX-2 in the thoracic aorta and mesenteric arteries was measured by extraction of mRNA followed by semiquantitative RT-PCR. A detailed description of the method, including the primers used, is available in an online data supplement at http://www.hypertensionaha.org

**Biochemical Analyses**

Plasma glucose levels were determined using the Beckman Glucose Analyzer II (Beckman, Fullerton, Calif). Plasma insulin and testosterone were determined with radioimmunoassay kits from Linco Research (St Charles, Mo) and ICN Pharmaceuticals (Irvine, Calif), respectively.

**Statistical Analysis**

Values were expressed as means±SE. For data with multiple time points, variables were analyzed by the general linear model ANOVA. Area under the curve values were calculated using the trapezoidal rule and ISI was calculated with a formula that correlates highly with results obtained from the euglycemic hyperinsulenic clamp technique.23 A 1-way ANOVA was used to compare area under the curve and ISI values. Mean differences were considered significant at *P*<0.05. When a mean difference was detected, a Newman-Keuls multiple-comparison test was used.

**Results**

**General Characteristics**

The gonadectomized rats had significantly lower body weight than the sham-operated rats, as noticed during week 2 of the study. Fructose feeding did not affect body weight throughout the experiment. There were no differences among the groups in food consumption when the food intake was calculated by body weight (Table) nor when expressed per rat (data not shown). The fluid intake of the G group was higher than that of the GF group, which indicates that fructose can decrease the fluid intake of gonadectomized rats but not that of the sham-operated rats. The fluid intake of the G group is also higher than that of the C and F groups when corrected for differences in body weight, likely because the body weight of G group is lower than that of the sham-operated groups (Table).

**Testosterone Levels**

The testosterone levels were not significantly changed in the sham-operated animals (C and F groups) whereas testosterone was undetectable in the gonadectomized animals (G and GF groups) at the beginning (data not shown) and the end of the experiment (Table). Fructose feeding had no effect on testosterone levels.

**Blood Pressure**

BP was significantly higher in the F group compared with the C group by week 6 of treatment and continued to increase

...
General Characteristics of the 4 Groups at Termination

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>C (n=16)</th>
<th>F (n=16)</th>
<th>G (n=8)</th>
<th>GF (n=8)</th>
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<tr>
<td>Body weight (g)</td>
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<td>519±11</td>
<td>460±11*</td>
<td>469±17*</td>
</tr>
<tr>
<td>Food intake (g/kg body weight per day)</td>
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<td>69±5</td>
<td>67±7</td>
<td>72±5</td>
</tr>
<tr>
<td>Fluid intake (mL/kg body weight per day)</td>
<td>136±3</td>
<td>141±7</td>
<td>163±4†</td>
<td>123±12</td>
</tr>
<tr>
<td>Plasma glucose (mM)</td>
<td>7.4±0.1</td>
<td>7.2±0.1</td>
<td>7.6±0.1</td>
<td>7.3±0.1</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
<td>2.63±0.23</td>
<td>3.05±0.43</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates not detectable, <0.1 ng/mL. *P<0.05, different from C and F groups; †P<0.05, different from C, F, and GF groups.

Throughout the study (129±4 mm Hg in F group versus 115±2 mm Hg in C group at week 8). There was no significant difference in BP between G and C group, and gonadectomy prevented the increase in BP caused by the fructose diet (Figure 1).

**Plasma Glucose and Insulin**

There were no differences in either glucose or insulin levels among all the experimental groups at the beginning of the experiment. Nine weeks of fructose treatment did not induce an increase in blood glucose in the fructose-fed groups (Table), consistent with the previous findings from our laboratory. However, the insulin levels of the fructose-fed rats in F and GF groups rats increased compared with that of the rats fed with normal chow (the C and G groups) (Figure 2).

**Oral Glucose Tolerance Test Responses**

After an oral glucose challenge, the plasma glucose profile was similar among all groups (Figure 3A). Both fructose-fed groups responded by secreting significantly more insulin, as indicated by a greater peak insulin response and area under the curve compared with control groups (Figure 3B). A comparison of the ISIs (calculated from oral glucose tolerance test data) shows that the fructose diet significantly impaired insulin sensitivity (F and GF groups) (Figure 4).

**Messenger RNA Expression of COX-1 and COX-2**

The COX-2 mRNA level of the F group was increased by 48% in thoracic arteries and 32% in mesenteric arteries when compared with that of the C group (Figure 5). Gonadectomy normalized the mRNA expression to an equal or slightly lower mRNA level compared with that of the C group in thoracic and mesenteric arteries. This indicates that gonadectomy prevented the increase in COX-2 mRNA expression induced by fructose feeding. Fructose feeding did not increase COX-1 mRNA expression.

**Discussion**

Previous experiments in our laboratory have shown that male rats had hypertension after 9 weeks of fructose feeding whereas female rats did not. These findings suggest that either the presence of estrogen or the lack of androgens protected against an increase in BP in female rats. If the latter is true, then androgens should be a necessary factor in the development of hypertension in this model. The present study was designed to further clarify how androgen affects the development of hypertension in FHR. The results demonstrate that gonadectomy prevented the development of fructose-induced hypertension in male rats, which indicates that the presence of testosterone is required for the development of hypertension in this hypertensive model. These results are consistent with the findings of other laboratories, which also showed that testosterone plays an important role in the development of hypertension in other hypertensive animal models. In these experiments, castration at a young age (3 to 5 weeks) attenuated the development of hypertension in SHR, in Dahl salt-sensitive hypertensive male rats, in male rats subjected to 2-kidney, 1-clip maneuver, and in male rats subjected to reduced renal mass. Moreover,
Reckelhoff et al reported that chronic blockade of the androgen receptor with flutamide attenuates BP in male SHR to the level found in female SHR, and testosterone treatment of ovariectomized females or castrated males promoted hypertension. Another interesting finding of this study is that the sham-operated rats and gonadectomized rats in the fructose-fed groups demonstrated an increase in insulin levels and an impairment in insulin sensitivity; that is, the absence of testosterone did not prevent the development of hyperinsulinemia and insulin resistance. This suggests that testosterone is not associated with the development of these metabolic abnormalities in the FHR. To our knowledge, this is the first report that shows the relationship between testosterone and the development of hyperinsulinemia and insulin resistance in FHR.

Although hyperinsulinemia and insulin resistance exist in the GF group, BP was not increased. This suggests two possibilities: first, hyperinsulinemia and insulin resistance are not related to the development of hypertension; second, testosterone acts as a link between the causal relationship of hyperinsulinemia/insulin resistance and hypertension. Although the causal relationship of hyperinsulinemia/insulin resistance and hypertension has been controversial, there is now evidence that suggests that hyperinsulinemia/insulin resistance can lead to hypertension in human and animal models. Our previous studies demonstrated that drugs that improve insulin sensitivity and decrease insulin levels prevented the development of hypertension in animal models such as SHR and FHR, which strongly suggests that hyperinsulinemia and insulin resistance are intrinsically linked to the pathogenesis of hypertension. In humans, the results of several prospective studies in which hyperinsulinemia has been used as a surrogate marker of insulin resistance support the view that insulin resistance/compensatory hyperinsulinemia are causally linked to the development of essential hypertension. A recent clinical trial conducted in 20 different centers also indicated that BP was directly related to insulin resistance and insulin concentration in essential hypertension. Because insulin resistance is not increased in patients with secondary forms of hypertension, it is more plausible to propose that insulin resistance/hyperinsulinemia leads to hypertension rather than vice versa. Based on these observations, we believe that the absence of testosterone in the GF group prevented the development of hypertension by cutting off the link between the causal relationship of hyperinsulinemia/insulin resistance and hypertension.

Thromboxane A_2 is a potent vasoconstrictor that can be produced by platelet cells and by the endothelium. Studies have shown that vascular production of TXA_2 was increased in several experimental models of hypertension. In hyperinsulinemic spontaneously hypertensive rats and in rats chronically infused with insulin, the development of hypertension can be prevented by treatment with a thromboxane synthase inhibitor. Previous results from our laboratory demonstrate that treatment with dazmegrel, an inhibitor of thromboxane synthase, prevents the development of fructose-induced hypertension in male rats. The vascular TXB_2 production observed in the fructose-fed rats was significantly greater and was normalized by treatment with dazmegrel. These data indicate that TXA_2 is involved in the development of hypertension in FHR. In the present study, we were
interested in finding out whether the mRNA expression of the key enzymes that regulate the production of TXA₂, namely COX-1 and COX-2, was increased in the vascular beds of FHR. Fructose feeding did not affect the mRNA expression of COX-1. However, the mRNA expression of COX-2 was increased by 42% in thoracic aorta and 32% in mesenteric arteries, respectively. These data support our previous findings and our hypothesis that alterations in COX synthesis and/or function are responsible for altered thromboxane activity in FHR. Interestingly, castration prevented the increase in COX-2 mRNA expression. It is now known that COX-1 is constitutively expressed in most tissues. It is the main isoform expressed in gastric mucosa, vascular endothelium, and renal tissues. In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels but is readily upregulated by inflammatory, mitogenic, and physical stimuli such as increased laminar shear stress. Because of the inducible nature of COX-2, it is possible that the increase in BP and, hence, the increase of laminar shear stress on vascular beds stimulated the expression of COX-2 in the thoracic and mesenteric arteries of the F group in the present study. It is also possible that hyperinsulinemia is responsible for the COX-2 induction. The exact role of COX-2 in the development of hypertension is not completely understood thus far. Inhibition of COX-2 in normotensive and hypertensive rats resulted in controversial effects on BP. However, because COX-2 regulates the synthesis of prostanooids from membrane-derived arachidonic acid, it is possible that the upregulation of COX-2 leads to an increase, specifically in the production of TXA₂, in the vascular beds of the fructose-fed rats. Our previous experiments have shown the there is a selective increase in thromboxane production by the vasculature, whereas pros-tacyclin levels remain unchanged. Changes in the balance among contracting and relaxing factors can result in endothelial dysfunction, which may be one of the mechanisms of increased BP in these fructose-fed rats.

It should be pointed out that because of the limited tissues that could be acquired from each rat, we were only able to perform RT-PCR using homogenized tissue from a pooled sample; thus, a statistical analysis could not be performed to compare the COX-1 and COX-2 mRNA expression. However, there is convincing evidence in the literature to support the idea that COX-2 could be induced in this pathological condition, and the increase in COX-2 in thoracic aorta and mesenteric arteries of the F group is of interest. We are aware that the increase of COX-2 mRNA expression does not necessarily mean an increase of COX-2 enzyme production in these vascular beds. Further studies are required to explore whether COX-2 protein expression is also increased in these tissues.

The involvement of sex hormones in the development of hyperinsulinemia/insulin resistance and hypertension is obviously complex. Previous results from our laboratory demonstrated that ovariectomized female rats had a significant increase in systolic BP on fructose feeding compared with fructose-fed ovary-intact rats, indicating a modulatory role for estrogen and testosterone. How sex hormones are involved in the development of hyperinsulinemia/insulin resistance and hypertension in female rats and whether COX-2 mRNA is also upregulated in these conditions still need to be determined.

**Perspectives**

The role of hyperinsulinemia and insulin resistance in the development of hypertension has been an area of intense research in recent years. Although such research has firmly established that hyperinsulinemia/insulin resistance play a causal role in elevating BP, the exact mechanisms that link these pathological states has yet to be elucidated. This relationship is likely to be complex and multi-factorial. Recent results from our laboratory, and this study in particular, point toward sex hormones and defects in endothelium-derived vasoconstrictors as part of the link. This study demonstrated that androgens are necessary for male rats to have fructose-induced hypertension and, in addition, provide preliminary data on COX mRNA expression that support our previous studies showing that the COX/thromboxane pathway is upregulated in hyperinsulinemia, insulin resistance, and hypertension. The lack of change in COX-2 in the castrated fructose-fed rats that did not become hypertensive provides additional evidence suggesting that thromboxane or some product of the cyclooxygenase pathway might be a mechanistic link between insulin and BP, at least in rats. Further work is needed to fully understand whether androgens are directly involved in the interrelationship among...
these factors, or whether the sex hormones act indirectly on other important pathways, such as the production of thromboxane and/or other endothelium-derived vasoconstricting factors.

Acknowledgments

The present study was supported by a grant from the Heart and Stroke Foundation of British Columbia and Yukon (HSFBCY). We thank Dr Sterlivo Bandiera, An Ding, and Ashraf Abrahani for their technical advice and assistance. We also thank Dr Thomas Chang for the use of the PCR machine and technical support.

References

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Hypertension. 2004;43:667-672; originally published online February 2, 2004;
doi: 10.1161/01.HYP.0000118018.77344.4e
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/43/3/667

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ANDROGENS ARE NECESSARY FOR THE DEVELOPMENT OF FRUCTOSE-INDUCED HYPERTENSION

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ON-LINE SUPPLEMENT: “Androgens are Necessary for the Development of Fructose-induced Hypertension”. By Dongzhe Song, Emi Arikawa, Denise Galipeau, Mary Battell, and John H. McNeill

**Methods**

**COX-1 and COX-2 mRNA Determination**

Chemicals and solvents were obtained from Sigma Chemical Co, St. Louis MO unless otherwise stated. The RNA content of COX-1 and COX-2 in the thoracic aorta and mesenteric arteries was measured by extraction of RNA followed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, tissues were powdered in a mortar filled with liquid nitrogen and homogenized. Total RNA was extracted from aorta and mesenteric arteries using TRIzol® Reagent (Invitrogen, Burlington ON), according to the manufacturer’s instructions. Tissues (thoracic aorta or mesenteric arteries) from each of the groups were pooled (8 rats per pool). The tissues were homogenized together in 1 ml ice-cold TRIzol® Reagent. RNA was extracted from the homogenates by adding chloroform (1 vol homogenate + 0.2 vol chloroform). The tubes were shaken for 15 seconds and then incubated for 2-3 min (15-30°C). The suspensions were centrifuged at 12,000 × g (4°C, 15 min) and RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol. Samples were incubated (4°C, 15 min) and centrifuged at 12,000 x g (4°C, 10 min). The supernatant was removed and the RNA was washed once with 75% ethanol and centrifuged at 7,500 x g (4°C, 5 min). After removing the supernatant, the RNA pellet was dried briefly and dissolved in 50μl diethylpyrocarbonate (DEPC)-treated distilled water. RNA was quantified by measuring absorbance at 260 nm, and its
integrity was assessed after electrophoresis on nondenaturing 1.5% agarose gel stained with ethidium bromide (5 µg/ml).

**Semiquantitative RT-PCR**

Invitrogen (Burlington, ON) was the source of all of the reagents used for RT-PCR except as noted. For each pooled tissue, reverse transcription of 2 µg total RNA was performed in a 20 µl reaction volume containing 200 U of Moloney murine leukemia virus reverse transcriptase, 20 U RNase inhibitor, 1 × RT buffer, 0.5 µg random primers, 1 µμ 10M dNTP and 2 µμ 0.1M DTT for 60 min at 42°C. This process was repeated three to four times. The PCR amplifications were performed in 50 µl reaction mixture. The PCR mixture contained 2 µl 10mM dNTP, 2.5U REDTaq™ DNA polymerase (Sigma, St Louis), 1 μl of sense and antisense COX-1/COX-2 primers, 1 μl of sense and antisense β-actin primers, 2 μl of the RT product, and 5µl REDTaq™ 10 × PCR buffer. The oligonucleotide primers for COX-1 and COX-2, shown in Table 1 were designed from published reports,1-3 and were obtained from Biotechnology Laboratory, Nucleic Acid Protein Services Unit, University of British Columbia, Vancouver, BC.

In a preliminary study, we found that 28 PCR cycles for COX-1 and 32 cycles for COX-2 were necessary to obtain a visible product on a 1.5 % agarose gel and that the quantity of the product was in proportion to the amount of cDNA used. After an initial denaturation step at 94°C for 2 min, cycles of annealing at 56°C (for COX-1) and 62°C (for COX-2) for 45 seconds, elongation at 72°C for 1.5 min, and denaturation at 94°C for 45 s were performed with 2 µl of the cDNA as described above. The PCR amplification was completed with a final extension step (10 minutes at 72°C). The RT-PCR product of the gene encoding β-actin served as a quantity control, and it required 22 cycles to obtain a visible product. PCR products (12µl) were electrophoresed on 1.5 % agarose-Tris-acetate-EDTA gels (75V for 2 hours). The resulting gel was stained with
ethidium bromide (0.5 µg/ml), visualized by ultraviolet trans-illuminator, and photographed. The photographs were scanned, and the PCR products were analyzed by densitometry using a computer with Quantity One® software (PDI Inc., Huntington Station, NY). The densitometric values of the amplified products of COX-1 and COX-2 were normalized against those of the housekeeping gene β-actin. The values shown are the mean of the repeated samples.

References
TABLE 1. Oligonucleotide primers used for PCR in the study

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<th>Gene</th>
<th>Primer</th>
<th>Accession number</th>
<th>Primer sequences</th>
<th>Size (bp)</th>
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