Accumulating evidence suggests that cardiovascular disease risk begins with insulin resistance, a silent condition that occurs long before diabetes.1 In man, insulin sensitivity and endothelial function are closely associated.2 Indeed, insulin resistance blunts vascular production of nitric oxide (NO), a factor crucial to the normal vasodilatory response and endothelial function.3 Moreover, it has been postulated that insulin resistance and the concomitant compensatory hyperinsulinemia contribute to the pathogenesis of hypertension.4 This may explain why hypertension is a major cardiovascular risk factor in patients with type 2 diabetes.5 However, the mechanisms by which insulin resistance affects blood pressure (BP) are unknown.

Many of clinical reports support oral phosphodiesterase type 5 (PDE5) inhibitors as safe and efficacious first-line on-demand agents for the treatment of erectile dysfunction.6 The concept of daily PDE5-inhibitor dosing has beneficial effects on endothelial function in men with increased cardiovascular risk.7 PDE5-inhibitors are known vasodilators, and given the contribution of endothelial function to BP in humans,8 may offer therapeutic potential as a treatment of essential hypertension.9 Indeed, it has been suggested that sildenafil or tadalafil, when taken on a daily basis, could exert prolonged beneficial effects on endothelial dysfunction in patients with increased cardiovascular risk.10,11 For tadalafil, the benefit of chronic dosing was sustained for at least 2 weeks after the discontinuation of treatment.11 However, the mechanism of action for such a prolonged benefit is yet unknown.

Oxidative stress has been suggested to both (i) contribute to insulin resistance,12 and (ii) play a crucial role in the pathogenesis of endothelial dysfunction.13 Moreover, it was
suggested that hyperglycemia was associated with both oxidative stress and an increase in vasoconstrictor thromboxane A₂ produced by COX, resulting in endothelial dysfunction. Given that NO can inhibit COX activity and peroxynitrite, a breakdown product of NO, promotes preferential thromboxane A₂ production by COX, and because chronic sildenafil administration upregulates the NO-cGMP pathway, it could be hypothesized that chronic PDE5 inhibition by sildenafil may affect COX metabolism. Moreover, since it was recently suggested that sildenafil could exert antioxidant effects, a chronic sildenafil administration may also directly modulate oxidative stress.

Therefore, we aimed to investigate whether chronic sildenafil could exert a beneficial effect on BP regulation in a pathophysiological setting associated with increased cardiovascular risk, that is, insulin resistance. Second, we attempted to correlate the effects of chronic sildenafil on basal BP and pressor response to norepinephrine in a conscious unrestrained model of insulin-resistant rat: the fructose-fed rat (FFR) with biological markers of COX metabolism and reactive oxygen species production, that is, thromboxane B₂ (TXB₂) and 8-isoprostan (IPT).

METHODS

Chronic treatment of the rats. A total of 50 male Wistar rats (Charles River, France, 180–220 g) were housed 7 days prior to the experiments with free access to standard rat chow and water. All procedures were performed in accordance with the legislation on the use of laboratory animals (NIH publication No. 85-23, revised 1996) and Animal Care Regulations in force in France as of 1988. After a 1-week acclimation period, rats were randomly placed on a purified control chow (TD.03102, CONT) or on an isocaloric fructose-enriched diet (TD.89247, FFR) for the following 9 weeks. All diets were prepared by Teklad Labs (Madison, WI). TD.89247 contained 18.3% protein, 60.3% fructose, and 5.2% lard expressed in % diet by weight.

From the beginning of week 6 of the experimental period, subcutaneous injections with saline (CONT, FFR) or sildenafil mesylate 20 mg/kg (FFR + SIL) were performed twice a day (40 mg/kg/day in total) during 3 weeks to achieve clinically relevant plasma exposures (~20 nmol/l unbound; Pfizer, data on file), using a previously described experimental paradigm. Then a 1-week washout period ensued, in order to ensure proper elimination of all circulating sildenafil and metabolites.

Radiotelemetry monitoring of BP and heart rate and pressor response to norepinephrine. At the end of the treatment period (week 8), the rats (n = 8/group) were implanted with a radio-telemetry transmitter (model TA11PA-C40, Data Sciences International, St Paul, MN) under inhaled isoflurane (2%). The attached catheter was tunneled through the abdominal wall over the left femoral artery, inserted centrally and secured in place with nylon sutures to be kept until BP monitoring. The right jugular vein was catheterized to allow subsequent intravenous perfusions. The animals were allowed to recover 1 week during the washout period after surgery before BP monitoring. On the day of recording, conscious unrestrained rats were allowed to acclimate to their new environment (30 min) before online recording of BP for 30 min. Resting systolic, diastolic, and mean arterial pressures were thus determined. Subsequently, increasing doses of norepinephrine (NE) were IV delivered for 5 min each (50, 100, 200, 400 ng/kg/min) and pressor responses determined for each dose as an average of the recorded response during the final minute.

In an additional subset of animals similarly treated and equipped (CONT: n = 10, FFR: n = 10), BP was recorded during NE infusion as previously described, 30 min following intravenous injection of the COX inhibitor: indomethacin (7.5 mg/kg).

Biochemical measurements. After the 1-week washout period, blood samples were taken (n = 10 per experimental group) after a 5-h fasting period. Fasting glycemia was determined using a portable blood glucose meter on whole blood (Accuchek active, Roche diagnostics, France). Plasma triglycerides were determined using an enzymatic colorimetric assay kit on plasma samples (Sigma, St Louis, MO).

Two days later, rats were fasted overnight to perform an oral glucose tolerance test. The following morning, rats were gavaged with glucose (1 g/kg). Blood samples were taken at 0, 10, 20, 30, 60, and 90 min after the gavage for the determination of glycemia. The results were expressed as the percentage of increase in glycemia for each rat (normalized glycemia), and the total area under the curve was calculated.

After BP measurements, rats were deeply anesthetized (urethane IP 1.2 g/kg), and thoracic aorta samples were harvested, immediately frozen in liquid nitrogen, and stored at −80°C. After tissue homogenization, tissular cGMP was determined using a cGMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The concentration of tissular endothelin-1 (ET-1) was determined using a commercially available assay kit (Cayman Chemical, Ann Arbor, MI) after extraction using the method of Verma et al.

For the determination of urinary IPT and TXB₂ contents on 24-h urine samples, rats were fasted overnight and placed in metabolic cages at the end of the 1-week washout period to collect urine. Meanwhile, a blood sample was drawn from the tail vein. Urine and plasma creatinine were determined by the picric acid method. An affinity column purification procedure was followed before immunoenzymatic determination of IPT, according to the manufacturer’s instructions (Cayman chemical, Ann Arbor, MI). The concentration of TXB₂ was also determined in urines using a commercially available assay kit (Cayman Chemical, Ann Arbor, MI). Urinary IPT and TXB₂ levels were corrected by the clearance of creatinine to limit variability across the assays due to changes in renal excretory function.

Calculations and statistical analysis. Values are expressed as mean ± s.e.m. Pressor responses to NE were expressed as the absolute change in mean arterial pressure (in mm Hg). Comparisons of these curves were made using a two-way analysis of variance followed by Bonferroni’s post-test. The
was IV injected, the exaggerated pressor response to NE previously observed in FFR compared to CONT (Figure 1) was abolished, except at the highest concentration of NE (Figure 2c).

Biochemical determinations

The basal cGMP and ET-1 contents of aortic homogenates were determined after a 1-week washout from sildenafil treatment. Similar concentrations of tissular cGMP and ET-1 were present in all groups (not significant, Table 1).

On the other hand, creatinine clearance was slightly, but not significantly, reduced by the fructose diet, and ameliorated after 3 weeks of chronic sildenafil treatment followed by 1 week of washout period (Table 1). Urinary IPT levels were significantly increased following the fructose diet (FFR: 2.07 ± 0.36 vs. CONT: 0.88 ± 0.13 pg/ml/24 h, P < 0.01, Figure 3). Chronic sildenafil restored normal levels of urinary IPT excretion even 1 week after treatment cessation (FFR + SIL: 0.95 ± 0.14 pg/ml/24 h, P < 0.01 vs. FFR, Figure 3). Moreover, urinary TXB₂ excretion levels were also significantly increased in FFR, and this increase was prevented by chronic sildenafil even.

**Table 1** | Physiological parameters after 3 weeks of chronic treatment with sildenafil and 1-week washout

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CONT (mg/dl)</th>
<th>FFR (mg/dl)</th>
<th>FFR + SIL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>418±7</td>
<td>400±9</td>
<td>397±10</td>
</tr>
<tr>
<td>Fasting glycemia</td>
<td>1440±5.8</td>
<td>1500±5.9</td>
<td>1518±5.3</td>
</tr>
<tr>
<td>AUC of normalized glycemia following OGTT</td>
<td>10649±520</td>
<td>12892±718*</td>
<td>14081±803**</td>
</tr>
<tr>
<td>Triglycerides (equivalent triolein, mmol/l)</td>
<td>1.13±0.16</td>
<td>1.97±0.40*</td>
<td>1.01±0.13†</td>
</tr>
<tr>
<td>Resting mean arterial pressure (mm Hg)</td>
<td>106.5±4.5</td>
<td>103.2±5.6</td>
<td>109.4±8.8</td>
</tr>
<tr>
<td>Resting heart rate (beats per minute)</td>
<td>407±19</td>
<td>380±22</td>
<td>423±9</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>0.50±0.05</td>
<td>0.39±0.05</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>Aortic cGMP content (pmol/mg prot)</td>
<td>0.414±0.097</td>
<td>0.414±0.107</td>
<td>0.551±0.107</td>
</tr>
<tr>
<td>Aortic ET-1 content (pmol/mg prot)</td>
<td>2350±53.3</td>
<td>1769±39.3</td>
<td>2207±33.6</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. One-way ANOVA followed by Newman–Keuls analysis. AUC, area under the curve; CONT, control chow; FFR, fructose-fed rat; OGTT, oral glucose tolerance test; SIL, sildenafil.

*P < 0.05; **P < 0.01 vs. CONT; †P < 0.05 vs. FFR.
after 1 week of washout (CONT: 7.25 ± 0.59 pg/ml/24 h; FFR: 12.86 ± 1.91 pg/ml/24 h, P < 0.01 vs. CONT; FFR + SIL: 7.88 ± 0.68 pg/ml/24 h, P < 0.01 vs. FFR, Figure 3).

**DISCUSSION**

In the present study, we demonstrated that (i) the FFR, an animal model of hypertriglyceridemia and insulin resistance, presented an exaggerated BP response to NE and (ii) that daily sildenafil administration to these FFR corrected hypertriglyceridemia and hyper-responsiveness to NE. Similar effects could be induced by direct inhibition of COX by indomethacin in FFR. In addition, the elevation of the COX-derived vasoconstrictor product, TXB₂, and the oxidative stress marker, IPT, were prevented by daily sildenafil administration. Altogether, these results suggest a pivotal role for COX regulation by chronic sildenafil leading to its beneficial effects on vascular tone regulation in FFR.

Even though the fructose-enriched diet did not induce obesity nor fasting hyperglycemia in our experimental conditions, the FFR presented impaired glucose tolerance and hypertriglyceridemia as previously reported. Although 3 weeks of chronic sildenafil treatment followed by the 1-week washout period did not significantly affect glucose metabolism in FFR, this treatment totally prevented hypertriglyceridemia, even after its complete elimination due to the 1-week washout period attributed by the absence of significant change in tissue cGMP content in FFR + SIL compared to untreated FFR. The modulation of COX pathway by sildenafil may constitute a valid explanation for its triglycerides-lowering effects in FFR. Indeed, in hypertriglyceridemic Min mice, the chronic inhibition of COX by indomethacin reduces dramatically circulating triglycerides. These observations may be in direct line with our results. Moreover, oxidative stress has recently been suggested to upregulate the COX pathway. It is noteworthy that, IPT levels, a marker of oxidative stress, were enhanced in FFR. Thus, a plausible hypothesis would be that chronic sildenafil exerted its antihypertriglyceridemic effects by reducing oxidative stress and in turn preventing COX upregulation.

In contrast with other studies, we were not able to detect any modification of BP in FFR. However, it is to be noted that all of these studies measured BP using tail-cuff plethysmography which imposed both thermal and contention stress to animals and could lead to the measurement of pressor response rather than baseline BP, as previously suggested. Our results showing unchanged BP in FFR are in fact perfectly in agreement with those previously reported using the same method of measurement, that is, telemetry recording. Indeed, we recently showed that after 9 weeks of diet, FFR are in an early stage of the pathology, thus showing vascular dysfunction rather than a declared hypertensive stage. In this context, it is not expected to observe an antihypertensive effect of chronic sildenafil administration because such a treatment only exerts an antihypertensive effect in hypertensive animals.

In the present study, FFR rats were normotensive; however, they developed hyper-responsiveness to chemical stress induced by NE. Interestingly, in human with insulin-resistant states such
as type 2 diabetes and obesity, pressor responses to exogenous NE and angiotensin II are also potentiated. Moreover, similar results were observed in the spontaneously hypertensive rat at prehypertensive stages. Therefore, it seems that this hyperresponsiveness to chemical stress could constitute a harbinger for the subsequent development of hypertension.

Strikingly, chronic sildenafil administration totally corrected the enhanced pressor response to NE in FFR even after its complete elimination from the plasma provided by the 1-week washout period. These results are perfectly in accordance with our previous study carried out in the same model, demonstrating that the endothelial dysfunction displayed by FFR was totally corrected after a 3-week chronic administration of sildenafil followed by a 1-week washout period. This effect seems to result from other mechanisms of action than direct inhibition of PDE5 because we could not detect any significant accumulation of vascular cGMP in vascular rings from these rats as already reported. In fact, we and others have uncovered the fact that despite this washout, one could await other long-lasting specific effects on endothelial NO synthase activity from a chronic treatment with sildenafil. Moreover, other factors may also be responsible for the enhanced vascular reactivity (or sensitivity) following NE challenge in FFR, such as a decreased liberation of vasodilators or an increased production of vasoconstrictors.

NO constitutes the major vasodilator mediator released by the endothelium. In FFR, the modulation of NO bioavailability may result not only from a decreased synthesis as previously reported, but also from an increased degradation which is highly dependent on NO scavenging by free radicals such as superoxide anions. Increased generation of superoxide anions, the hallmark of oxidative stress, has recently been implicated in the pathogenesis of endothelial NO synthase dysfunction in a number of disease states including insulin resistance and was shown in FFR. These observations perfectly corroborate our findings because urinary IPT, a validated marker of oxidative stress, is highly elevated in FFR. Moreover, chronic sildenafil treatment corrected IPT overproduction which is in line with the recently reported properties of sildenafil inhibiting superoxide production, thus increasing NO bioavailability. Moreover, the upregulation of the NO-cGMP pathway following chronic sildenafil has been previously described, with endothelial NO synthase activation by phosphorylation, independently from the direct PDE5 inhibition. Even though enhancement of the production of NO in FFR following a chronic treatment with sildenafil still needs to be confirmed, this may constitute a valid hypothesis for the attenuated hypertensive responses to a pressor challenge in the present study. Therefore, chronic sildenafil could both enhance NO production by endothelial NO synthase and reduce oxidative stress, preventing thus the decrease in NO bioavailability in FFR and yielding restored normal responses to NE.

However, other participants to the regulation of the vascular tone may also be evoked; for example, an enhanced production of contractile vasoactive substances such as TXA2 or ET-1 could also occur. Interestingly, in the present experiments, the aortic tissue levels of ET-1 were unchanged contrasting with a previous report. However, this does not exclude the hypothesis that sensitivity to ET-1 may be modified in vessels from FFR as previously suggested, but this aspect was not investigated in the present study.

On the other hand, we showed that the increase in urinary levels of TXB2 in FFR was prevented by chronic sildenafil. Studies have shown that renal and/or vascular production of TXA2 may participate in the development of hypertension associated with hyperinsulinemia and insulin resistance in the FFR. Moreover, insulin potentiates the response of coronary blood vessels to TXA2 in vitro, suggesting that in vivo, hyperinsulinemia could lead to hypertension via the potentiation of the effects of TXA2. In the present study, TXB2 urinary level in FFR was increased but probably not enough to cause hypertension. However, our results were obtained in unstimulated conditions, and this might not preclude an increased production of ROS and TXA2 in response to a pressor stimulus. This hypothesis is further reinforced by the present results showing that the enhanced pressor response to NE in FFR was totally abolished when COX was inhibited by indomethacin. Because chronic treatment with sildenafil is able to restore normal levels of both TXB2 and IPT, the unifying hypothesis for this would thus be that chronic sildenafil administration in FFR could act as a regulator of free-radical generation and/or of COX pathway.

To conclude, it was shown that the modulation of COX activity and/or ROS generation are involved in the beneficial effects of chronic sildenafil in an experimental model of insulin resistance and hypertriglyceridemia. It is important to note that in the present study, the 3 weeks of chronic sildenafil administration were followed by a 1-week washout period indicating that these observed effects were beyond PDE5 inhibition. Finally, the added benefits of chronic sildenafil compared to acute dosing may be of particular importance in the maintenance of vascular endothelial integrity during the metabolic syndrome development in view of preventing or delaying the possible associated cardiovascular complications.

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