Testosterone-dependent increase in blood pressure is mediated by elevated Cyp4A expression in fructose-fed rats

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Abstract Endothelial dysfunction and increased blood pressure following insulin resistance play an important role in the development of secondary cardiovascular complications. The presence of testosterone is essential for the development of endothelial dysfunction and increased blood pressure. Testosterone regulates the synthesis of vasoconstrictor eicosanoids such as 20-hydroxyeicosatetranoic acid (20-HETE). In a series of studies, we examined: (1) the role of the androgen receptor in elevating blood pressure and (2) the effects of Cyp4A-catalyzed 20-HETE synthesis on vascular reactivity and blood pressure in fructose-fed rats. In the first study, intact and castrated male rats were made insulin resistant by feeding fructose for 9 weeks following which their superior mesenteric arteries (SMA) were isolated and examined for changes in endothelium-dependent relaxation in the presence and absence of 1-aminobenzotriazole (ABT) and N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), which are inhibitors of 20-HETE synthesis. In another study, male rats were treated with either ABT or the androgen receptor blocker, flutamide, following which changes in insulin sensitivity, blood pressure, and vascular Cyp4A expression were measured. In the final study, HET0016, which is a more selective inhibitor of 20-HETE synthesis, was used to confirm our earlier findings. Treatment with HET0016 or ABT prevented or ameliorated the increase in blood pressure. Gonadectomy or flutamide prevented the increase in both the Cyp4A and blood pressure. Furthermore, both ABT and DDMS improved relaxation only in the intact fructose-fed rats. Taken together our results suggest that in the presence of testosterone, the Cyp4A/20-HETE system plays a key role in elevating the blood pressure secondary to insulin resistance.

Keywords Insulin resistance · Blood pressure · Testosterone · Cyp4A · 20-HETE

Introduction

Hypertension and impaired endothelial relaxation are secondary complications associated with insulin resistance [1]. In the long term, these factors play key roles in the increased incidences of mortality and morbidity associated with the metabolic syndrome. Insulin resistance impairs vascular endothelium-dependent relaxation, which contributes to increasing the blood pressure in both humans and rodents [1]. The attenuated endothelial relaxation in fructose-fed rats is caused by impaired endothelial nitric oxide [1, 2] and endothelium-derived hyperpolarizing factor (EDHF) [3] signaling. Differences in gender and sex hormones play a key role in regulating endothelium-dependent vascular homeostasis and blood pressure. The loss of testosterone by castration [1, 4, 5] or by chemically blocking the androgen receptor [6] prevented endothelial dysfunction and hypertension.

Testosterone has been reported to regulate the synthesis and actions of several vasoconstrictor systems such as the renin–angiotensin system (RAS) in various models of hypertension such as the spontaneously hypertensive and the Dahl salt sensitive rats [7, 8]. Testosterone also influences the synthesis of several arachidonic acid metabolites, which constrict blood vessels and present potential pathways in the induction of endothelial dysfunction and hypertension [9, 10].

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The cyclooxygenase-2/thromboxane A2 (COX-2/TXA2) system plays an important role in the development of vascular abnormalities and hypertension secondary to insulin resistance [11–13]. Both insulin resistance and testosterone upregulated the COX-2-dependent responses to phenylephrine along with COX-2 expression, at the protein [12] and mRNA levels, [4] in the thoracic aorta of male fructose-fed rats in addition to increasing the participation of COX-2 in mediating responses to phenylephrine [14].

The second vasoactive cascade involving arachidonic acid as the starting substrate involves metabolism of arachidonic acid by monooxygenases belonging to the cytochrome P450 (Cyp) family [15]. Androgens upregulate Cyp4A expression, which catalyzes the synthesis of the prohypertensive agent, 20-hydroxyeicosatertanoic acid (20-HETE) [16–18]. 20-HETE blocks the calcium-activated potassium (K_{Ca}) channels, which mediate hyperpolarization [19]. Recently, Cheng et al. [20] have reported that 20-HETE uncouples eNOS in endothelial cell cultures. Additionally, other papers have also indicated the counteracting effects of 20-HETE and nitric oxide on vascular tone [21, 22]. The attenuation of endothelial relaxation and subsequent increase in intracellular calcium in the vascular smooth muscle may explain the contribution of 20-HETE to impaired relaxation. Several endogenous vasoconstrictors such as norepinephrine and angiotensin-2, whose responses are altered in insulin resistance, recruit 20-HETE as a downstream target [23, 24]. However, the role of 20-HETE in insulin resistance is unclear. The effects of insulin on Cyp4A activity and thereby 20-HETE synthesis vary depending on the organ. Diabetes increases Cyp4A activity in the liver [25, 26], while it is reduced in the kidney [27]. Studies on a high fat fed rat model show decreased 20-HETE levels in the kidney despite obesity and an increase in blood pressure [28, 29]. Although studies in human obese subjects have attempted to ascertain the association between circulating insulin and 20-HETE levels, the findings were inconclusive [30, 31]. In experimental models of hypertension such as the spontaneously hypertensive rat (SHR), stroke-prone SHR [32], and in androgen-infused rats, 20-HETE is elevated [17]. We hypothesized that in the presence of testosterone, insulin resistance increases Cyp4A expression and therefore 20-HETE synthesis in the mesenteric vasculature, which contributes to endothelial dysfunction and hypertension.

Materials and methods

Animals

The fructose hypertensive rat model was used in the studies outlined below. Six-week-old male Wistar rats were obtained from Charles River, Montreal, Canada. In separate studies 1a, 1b, and 1c, half of the rats were castrated surgically at 5 weeks of age, prior to shipment. The rats were cared for as per the guidelines outlined by the Canadian Council on Animal care (CCAC) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Subsequent to arriving, the rats were acclimatized in the animal facility at the Faculty of Pharmaceutical Sciences, University of British Columbia, for 1 week prior to treatment. The starch present in normal laboratory rat chow was replaced with 60% fructose, which was obtained commercially as a pre-formulated diet (Teklad Labs, Madison, WI). Feeding this fructose-enriched diet has been shown in previous studies to induce insulin resistance, hyperinsulinemia, hypertriglyceridemia, and an increase in the systolic blood pressure by 15–30 mm Hg, which is independent of obesity [1, 33].

Study design

Study 1

In three separate studies 1a, 1b, and 1c we used the same experimental design to answer different questions. In each study, 6-week-old intact and castrated rats were divided into four groups of six rats each (intact control (C), intact fructose-fed (F), gonadectomized control (G), and gonadectomized fructose-fed (GF)) and were fed with either standard rodent chow or 60% fructose diet for 9 weeks. Rats were matched for age with identical housing, husbandry, and experimental conditions. Following measurement of blood pressure and confirmation of impaired insulin sensitivity, the superior mesenteric arteries were isolated from euthanized rats and evaluated for changes in endothelium-dependent relaxation to increasing concentrations of acetylcholine (ACh; 10^{-9}–10^{-4} mol/l).

In study 1a, subsequent to determining baseline relaxation to ACh, the SMA was treated with the 20-HETE synthesis inhibitor 1-aminobenzotriazole (ABT; 10^{-5} mol/l).

In study 1b, the SMA was incubated with the selective 20-HETE synthesis inhibitor N-methylsulfonyl-12, 12-dibromododec-11-enamide (DDMS; 10^{-5} mol/l, Cayman Chemicals, MI), following which changes in relaxation to ACh were examined. In both the studies, 1a and 1b, following the establishment of functional endothelium, the tissues were equilibrated and incubated with ABT or DDMS for 20 min after which the tissues were contracted with the ED_{70} dose of PE and changes in relaxation to increasing concentrations of ACh were measured.

In study 1c, the SMA was homogenized and changes in Cyp4A expression were determined by western blotting as described below.

Study 2

To determine the involvement of the androgen receptor and 20-HETE in elevating blood pressure, 6-week-old male Wistar rats (210–240 g) were divided into six groups of 12 rats (control (C), fructose-fed (F), control + flutamide (anti-androgen) (CF), fructose + flutamide (FF), control + 1-aminobenzotriazole (ABT; Cyp4A inhibitor) (CA) and fructose + ABT (FA)). Following determination of baseline systolic blood pressure, the animals were kept on either 60% fructose diet or normal rat chow for 9 weeks after which blood pressure was again measured and treatment regimes were started. Drug treatment was based on data obtained from the literature. Two groups (CF and FF) were injected subcutaneously (s.c.) with a suspension of flutamide in castor oil (8 mg/kg/day) [6], and two groups (CA and FA) were injected intraperitoneally (i.p.) with ABT (25 mg/kg/day) [34]. At the end of 3 weeks of drug treatment, changes in blood pressure and insulin sensitivity index were measured and the animals were sacrificed. Blood was collected at termination by cardiac puncture to obtain plasma for measuring testosterone levels.

Study 3

Since ABT is a non-selective inhibitor of Cyp action, we chose to obtain further evidence with a more potent and Cyp4A selective inhibitor. We used HET0016, which is the most selective inhibitor of Cyp4A and 20-HETE synthesis [35–37] as compared to ABT and DDMS. Thirty-two rats were divided into four groups of eight rats (control + chow-fed (C), control + fructose-fed (F), control + HET0016 (CH) and fructose-fed + HEET0016 (FH). Rats were fed with the normal or fructose 60% chow for 9–11 weeks to induce insulin resistance. Following 8 weeks of fructose feeding, rats in the CH and FH groups were treated with HET0016 (10 mg/kg/day, i.p. injection) [38, 39] for 2 weeks until termination. Induction of insulin resistance was confirmed at the end of 9 weeks (post 1 week of HET0016 treatment). Systolic blood pressure was measured prior to and following 9 weeks of fructose feeding. Rats were sacrificed by an overdose of pentobarbital (60 mg/kg, i.p.; Euthanyl™). Blood was collected by cardiac puncture for measuring 20-HETE.

Blood pressure

Systolic blood pressure was measured in conscious rats using the tail cuff method as previously reported [11, 40].

Insulin sensitivity

Following an overnight fast, rats were challenged with a 1 g/kg oral gavage dose of a 40% glucose solution. Blood was collected immediately prior to and at 10, 20, 30, 60, and 90 min following the glucose load for measuring plasma glucose and insulin. Using the formula developed by Matsuda and DeFronzo [41], we determined the changes in insulin sensitivity index.

Cyp4A1, 2/3 expression

The mesenteric bed was used in study 3, while SMA was used in study 1c and study 2 for measuring changes in the tissue Cyp4A protein levels (arachidonic acid ω-hydroxylase) by western blotting. A rabbit polyclonal antibody against Cyp4A1, 2/3 was used in our studies (Acris, Herford, Germany) [42]. As the initial western blots were inconclusive due to the high noise and non-specific binding of the antibody, we switched to measuring protein expression in immunoprecipitated samples. Briefly, 500 μl of 1 μg/μl protein lysate was prepared with RIPA buffer to which 1.1 μl of antibody was added and rotated overnight at 4°C. The next day 20 μl of protein A Sepharose (Sigma, MO) slurry was added and rotated for 1.5 h at 4°C. The suspension was centrifuged and washed twice with RIPA buffer. After discarding the supernatant, the final pellet was resuspended in 60 μl of resuspension buffer. 20 μl of sample loading buffer was added and boiled at 95°C for 5 min. The immunoprecipitated samples thus obtained were separated on 7.5% SDS-PAGE gels and transferred to PVDF membranes. Subsequent to blocking with 5% non-fat milk in TBS, the membranes were incubated with 1:3000 dilution of antibody in 5% BSA. Following washes in TBS-T, the membranes were incubated with HRP conjugated anti rabbit secondary antibody and then developed with ECL detection reagent (GE Life Sciences).

Plasma

Blood was collected by cardiac puncture into polypropylene tubes containing 20 IU heparin (0.2 ml of 100 units/ml strength). The blood was centrifuged at 4500 rpm, 4°C in a Beckman Tabletop centrifuge for 25 min. The plasma obtained was then aliquoted and stored at −70°C until further use.

Biochemical parameters

Plasma glucose was estimated with an automatic Beckman Glucose Analyzer II. Plasma insulin was measured using commercially available radioimmunoassay kits from Linco Diagnostics Inc, USA.

Drugs

HET0016 was obtained as a gift sample from Taisho Pharma, Japan and prepared as a suspension in lecithin as

advised by the company. Briefly, the drug was added to a 20% lecithin suspension, warmed to 70°C and sonicated in an ice bath. The resulting suspension was injected i.p. at a dose of 10 mg/kg daily. Both flutamide and ABT were purchased from Sigma. All other chemicals, unless specified, were obtained from Sigma Chemical Co. (MO).

Statistical analyses

All data are presented as mean ± the standard error of the mean (SEM). In studies involving multiple time points, intergroup comparisons of the dependent variables in each study were performed by general linear model analysis of variance (GLM-ANOVA). The Newman–Keuls multiple comparison test was used for post hoc comparisons upon detection of difference in the means. All statistical analyses were performed using the number cruncher statistical system (NCSS) software package. Mean values were considered significant at $P < 0.05$.

Results

Study 1a

Insulin sensitivity and blood pressure

Fructose feeding decreased the insulin sensitivity and elevated the blood pressure in the sham-operated rats. Gonadectomy prevented the increase in blood pressure but not the decrease in insulin sensitivity. These results have been previously reported as part of Vasudevan et al. [5]. Endothelial relaxation was attenuated in the SMA of intact fructose-fed rats [1], which was ameliorated by ABT. Endothelial relaxation was significantly higher in the gonadectomized groups compared to F and was unaffected by inhibiting 20-HETE (Fig. 1).

Study 1b

#### Insulin sensitivity and blood pressure

At the end of 9 weeks, fructose feeding decreased insulin sensitivity in both sham-operated and gonadectomized rats (F: 8.5 ± 1 and GF: 8.5 ± 1) as compared to normal chow-fed controls (C: 11 ± 1 and G: 14.6 ± 1; $P < 0.05$ F and GF vs. C and G).

Blood pressure was elevated in intact male rats after 9 weeks of fructose feeding (F: 134 ± 4 mmHg) compared to controls (C: 116 ± 3 mmHg; $P < 0.05$ F vs. C), which was prevented by gonadectomy (G: 109 ± 2 and GF: 115 ± 2 mmHg; $P < 0.05$ G and GF vs. F). These data confirm data previously published for study 1a [5] and study 1c [13].

Plasma testosterone

Testosterone was undetectable in gonadectomized rats. Fructose feeding did not affect testosterone levels (C: 1.3 ± 0.3 vs. F: 1.6 ± 0.5 ng/ml).

Fig. 1 Inhibition of 20-HETE synthesis by 1-aminobenzotriazole (ABT) improves relaxation to acetylcholine (ACh) in intact fructose-fed rat mesenteric arteries. Male Wistar rats were divided into 4 group: *C* intact control rats, *F* fructose-fed intact rats, *G* gonadectomized, and *GF* gonadectomized fructose-fed rats ($n = 4$–6*/ group). The response to ACh in the absence (*open square*) and presence of ABT (*filled inverted triangle*). Endothelium-dependent relaxation to acetylcholine was unaffected by ABT in superior mesenteric arteries of C, G and GF rats. All groups $n = 4$–6. *$P < 0.05$ F (PE + ACh) versus F (PE + ACh + 1-ABT). All values are presented as mean ± SEM
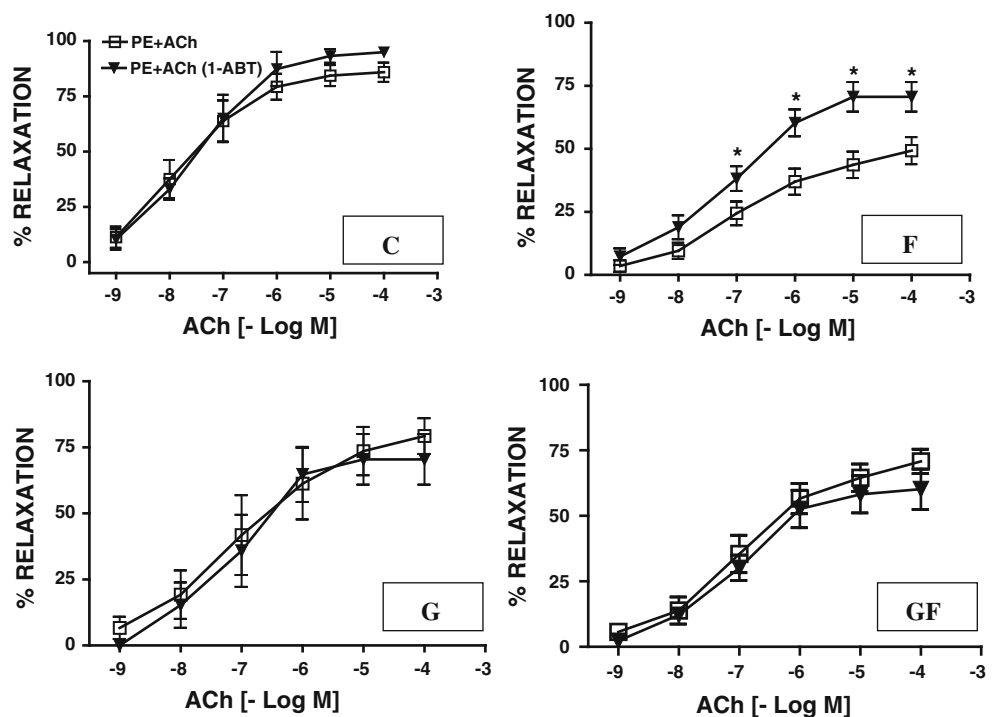
Vascular reactivity studies

Since ABT was a non-selective inhibitor of eiconsanoid synthesis, DDMS, a more selective inhibitor of 20-HETE synthesis, was used in this study. In the isolated superior mesenteric arteries, relaxation to acetylcholine was impaired in F but not GF as previously reported [1]. Inhibition of 20-HETE synthesis with DDMS (10^{-5} M) improved the relaxation to ACh in the SMA of F but not GF (Fig. 2). This demonstrates the involvement of androgen-dependent 20-HETE synthesis in impairing endothelium-dependent vasodilation.

Study 1c

The changes in insulin sensitivity and blood pressure in intact and gonadectomized rats were similar to that observed in Study 1a. These results were previously published in another manuscript [13].

Cyp4A1, 2/3 expression in the SMA

In the SMA, Cyp4A expression was increased in F compared to C in the SMA, which was prevented by gonadectomy (Fig. 3).
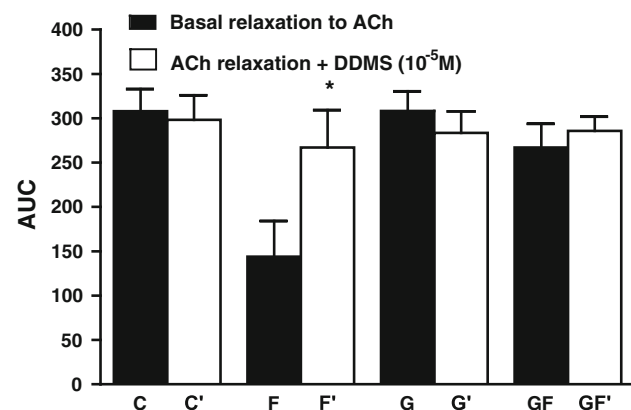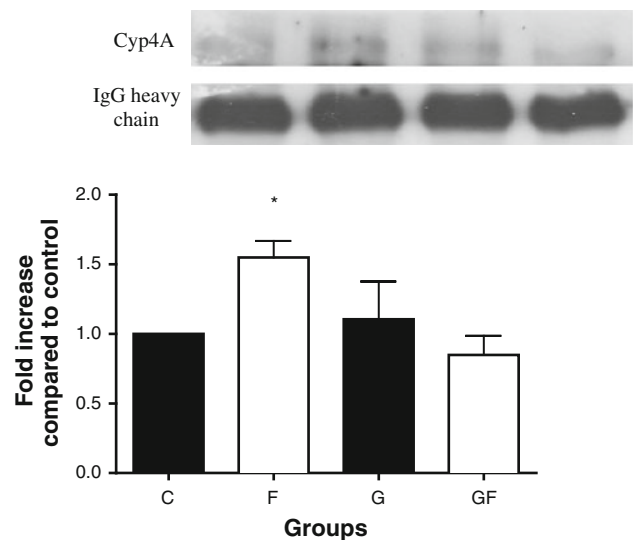

Fig. 3 Changes in Cyp4A1, 2/3 expression in the superior mesenteric artery of intact and gonadectomized rats. Male Wistar rats were divided into four groups: *C* intact control rats, *F* fructose-fed intact rats, *G* gonadectomized rats, and *GF* gonadectomized fructose-fed rats ($n = 4$/group). Western blotting was performed on immunoprecipitated protein lysates. Controls are bands from the IgG fraction. All groups $n = 4$. *$P < 0.05$ F versus C, and GF. All values are presented as mean ± SEM

Study 2

Body weights

Treatment with both flutamide and ABT did not affect body weight in control or fructose-fed rats (data not shown).

Insulin sensitivity

Fructose feeding induced insulin resistance, which was observed by hyperinsulinemia and delayed glucose clearance. Interestingly, treatment with ABT for 3 weeks improved the insulin sensitivity in fructose-fed rats (FA) whereas flutamide had no effect on insulin sensitivity (FF) (Fig. 4c). This was demonstrated by insulin levels similar to C in the FA group (Fig. 4a, inset). Fructose-fed rats were hyperglycemic compared to C or the CA rats (Fig. 4b, inset).

Blood pressure

Treatment with either flutamide or ABT restored the blood pressure to control values suggesting the involvement of both testosterone and Cyp4A (Fig. 5).
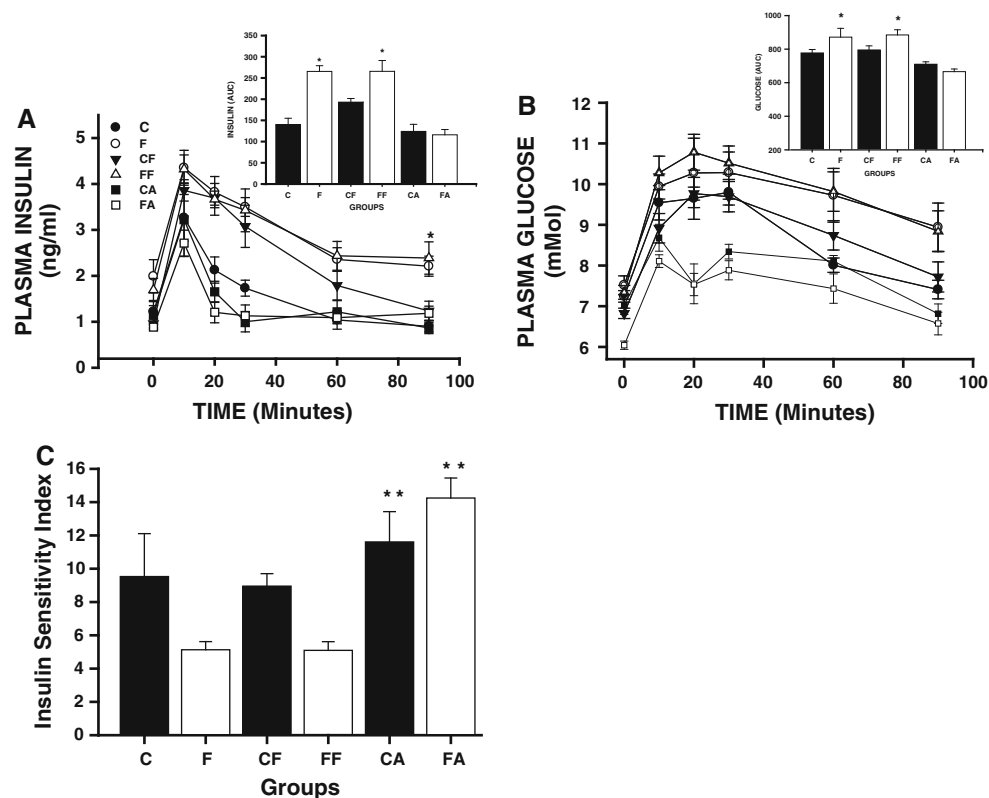

Fig. 2 Inhibition of 20-HETE synthesis by DDMS improves relaxation to acetylcholine (ACh) in intact fructose-fed rat mesenteric arteries. Male Wistar rats were divided into four groups: *C* intact control rats, *F* fructose-fed intact rats, *G* gonadectomized, and *GF* gonadectomized fructose-fed rats ($n = 4$/group). The response to ACh in absence *(filled square)* and presence of DDMS *(open square)*. Endothelium-dependent relaxation to acetylcholine was unaffected by DDMS in superior mesenteric arteries of C, G and GF rats. Relaxation is shown as AUC values. All groups $n = 4$. *$P < 0.05$ F versus F′. All values are presented as mean ± SEM

Fig. 4 **a–c** Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Male Wistar rats were divided into six groups: *C* intact control rats, *F* fructose-fed intact rats, *CF* control flutamide-treated rats, *FF* fructose-fed flutamide-treated rats, *CA* control 1-aminobenzotriazole (ABT)-treated rats and FA-fructose-fed ABT-treated rats (*n* = 6/group). Flutamide (FF) did not alter ISI values in fructose-fed rats. Treatment with ABT improved the insulin sensitivity as demonstrated by high ISI value (4C). **a** Plasma insulin levels following a 1 g/kg glucose challenge. *Inset* shows area under the curve values (AUC) for each group. **b** Plasma glucose concentration levels following a 1 g/kg glucose challenge. *Inset* shows area under the curve values (AUC) for each group. **c** Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula [41]. All groups *n* = 6. *$*P < 0.05$ F and FF versus C, CF, CA, and FA (**a**, **b**). **$**P < 0.05$ F and FF versus CA and FA. All values are presented as mean ± SEM

Plasma testosterone levels

Blocking the androgen receptor increased plasma testosterone levels in both CF and FF groups (9.33 ± 1 ng/ml and 13.6 ± 1.5 ng/ml) compared to untreated groups (C: 3.19 ± 0.4 ng/ml and F: 3.05 ± 0.6 ng/ml). Fructose diet, by itself, did not affect testosterone levels.

Study 3

Body weights

Body weight at termination was unaffected by either diet or HET0016 treatment (C: 579 ± 14, F: 584 ± 10, CH: 563 ± 21 and FH: 576 ± 8 gm).

Insulin sensitivity

Fructose feeding induced insulin resistance as indicated by the reduced insulin sensitivity index (ISI) (Fig. 6c). Treatment with HET0016 was inconclusive as the ISI

values were not different from either C or F groups. HET0016-treated rats did not vary in their total insulin levels as indicated by the area under curve values (Fig. 6a, inset). Fructose feeding and/or HET0016 did not alter plasma glucose levels subsequent to the glucose challenge (Fig. 6b).

Blood pressure

Fructose feeding elevated the blood pressure in F compared to C at the end of 9 weeks. Treatment with HET0016 for 1 week prevented the increase in blood pressure following insulin resistance in FT rats (Fig. 7a).

Cyp4A protein expression

Insulin resistance elevated Cyp4A1, 2/3 expression in the mesenteric bed of untreated fructose-fed rats (F). Treatment with HET0016 decreased Cyp4A expression and prevented 20-HETE synthesis (Fig. 7b).
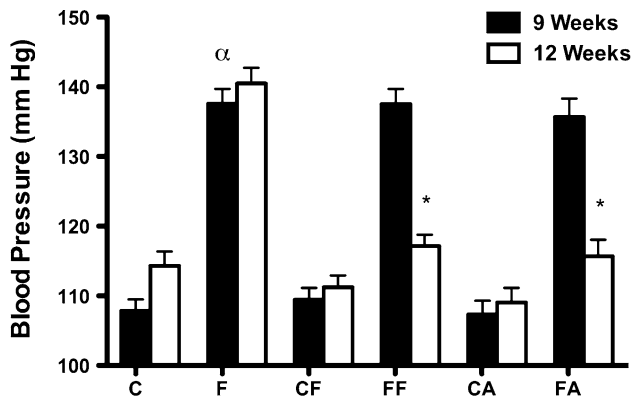
Fig. 5 Systolic blood pressure values at the end of 9 and 12 weeks of fructose feeding. Male Wistar rats were divided into six groups: *C* intact control rats, *F* fructose-fed intact rats, *CF* control flutamide-treated rats, *FF* fructose-fed flutamide-treated rats, *CA* control 1-aminobenzotriazole (ABT)-treated rats and *FA* fructose-fed ABT-treated rats (*n* = 8/group). Treatment with flutamide (8 mg/kg/day, subcutaneously) or ABT (25 mg/kg/day, intraperitoneally) was started at the end of 9 weeks. Blood pressure was measured 3 weeks post treatment. All groups *n* = 8. *$*P < 0.05$ FF and FA (treated) versus FF and FA (untreated) and F (untreated and treated); $^{+}P < 0.05$ F (treated and untreated) vs. C, CF and CA. All values are expressed as mean ± SEM

Discussion

We have demonstrated for the first time that in the presence of testosterone, Cyp4A expression is elevated in the mesenteric vessels following insulin resistance, which contributes to the development of endothelial dysfunction and increased blood pressure. Both ABT (Fig. 1) and DDMS (Fig. 2) improved the relaxation to acetylcholine only in intact fructose-fed rat SMA. The robust relaxation to ACh in control tissues (C) in spite of Cyp4A expression suggests the presence of functional endothelial nitric oxide synthase (eNOS). The selectivity of drug action towards blood vessels from intact insulin resistant rats supports our previous findings where the loss of functional endothelial nitric oxide system contributes to attenuated relaxation and endothelial dysfunction, which in turn may unmask the effects of vasoconstrictors [1]. The equilibrium between endothelial nitric oxide and endothelial vasoconstrictors such as eicosanoids is further supported by the decrease in vascular Cyp4A expression and its salutary effects on blood pressure in both gonadectomized (G & GF) and flutamide-treated rats (FF) compared to untreated and
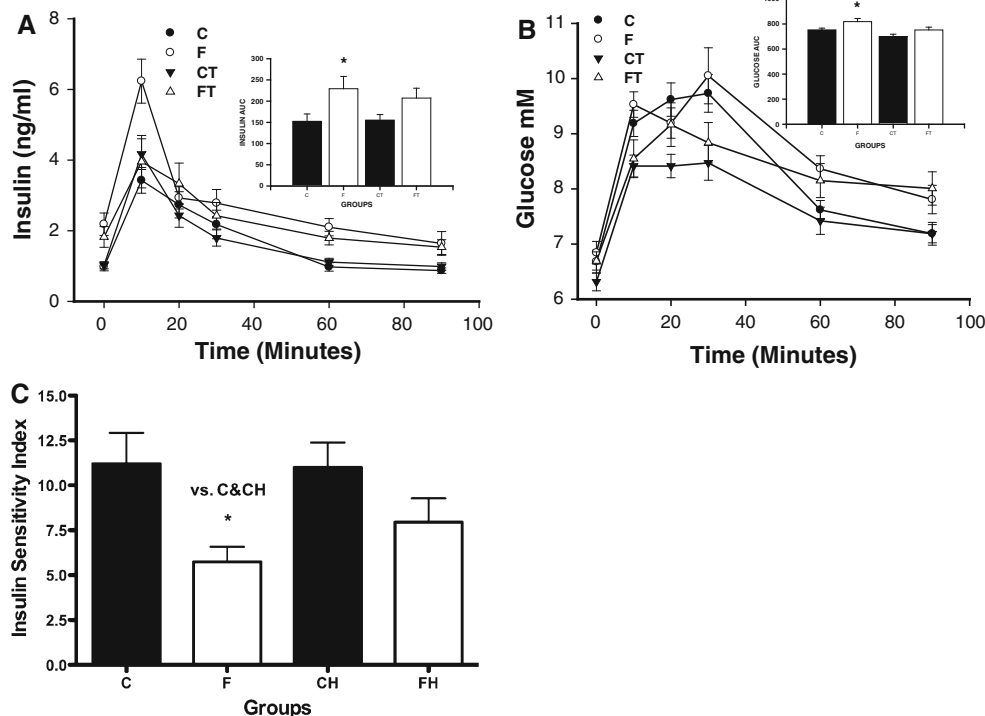

Fig. 6 a–c Plasma insulin and glucose profiles and insulin sensitivity index (ISI) following an oral glucose challenge. Intact male Wistar rats were divided into four groups: *C* control, *F* fructose-fed, *CT* control HET0016-treated, and *FT* fructose-fed and HET0016 treated (*n* = 7/group). HET0016 treated rats did not exhibit statistically different ISI values. **a** Plasma insulin levels following a 1 g/kg glucose challenge. Inset shows area under the curve values (AUC) for each group. **b** Plasma glucose levels following a 1 g/kg glucose challenge. *Inset* shows area under the curve values (AUC) for each group. **c** Insulin sensitivity index values for all groups as calculated by the Matsuda and DeFronzo formula [41]. All groups *n* = 7. *$*P < 0.05$ F versus C and CT. All values are presented as mean ± SEM
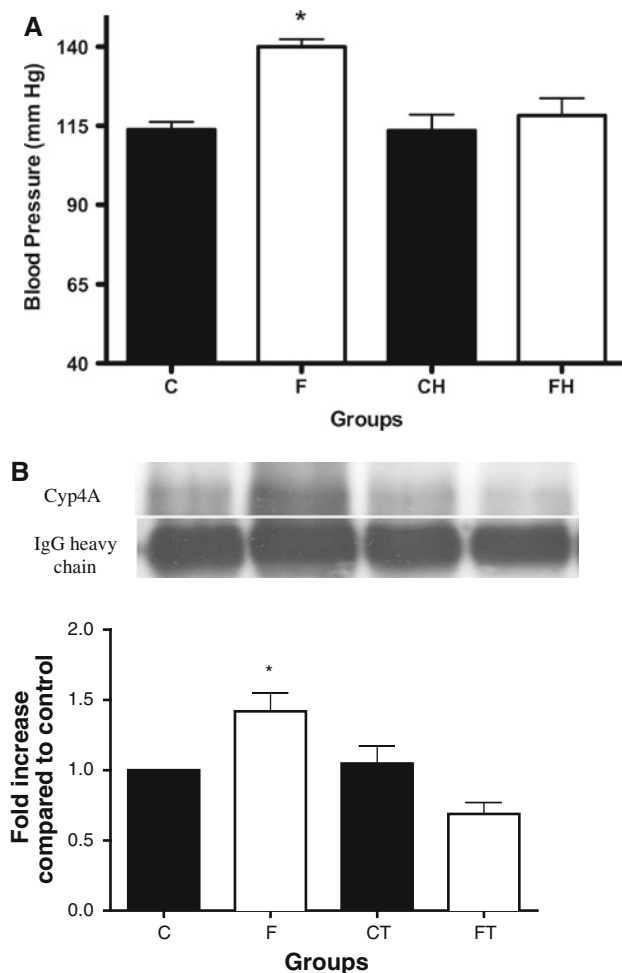
Fig. 7 a Systolic blood pressure values at the end of 9 weeks of fructose feeding. Intact male Wistar rats were divided into four groups: *C* control, *F* fructose-fed, *CT* control HET0016-treated, *FT* fructose-fed and HET0016 treated ($n = 7$/group). HET0016 (10 mg/kg/day, intraperitoneally) was started at the end of 8 weeks. Blood pressure was measured 1 week post treatment. All groups $n = 7$. *$P < 0.05$ F versus C, CT, and FT. All values are presented as mean ± SEM. **b** Changes in Cyp4A in the mesenteric bed of control and fructose-fed rats treated with HET0016 (10 mg/kg/day, intraperitoneally). Intact male Wistar rats were divided into four groups: *C* control, *F* fructose-fed, *CT* control HET0016-treated, and *FT* fructose-fed and HET0016 treated ($n = 4$/group). Western blotting was performed on samples immunoprecipitated with Cyp4A1, 2/3 Ab. Controls are bands from antibody IgG used in IP. All groups $n = 4$. *$P < 0.05$ F versus C, CT and FT. All values are presented as mean ± SEM

intact fructose-fed rats (F). Finally treatment with either ABT or HET0016 decreased or prevented, respectively, the increase in blood pressure in fructose-fed rats. In conjunction with previously published data from our laboratory, we suggest that the absence of testosterone prevents endothelial dysfunction and increases in blood pressure by reducing the responses to vasoactive prostanoids and eicosanoids [1, 4, 13]. Our findings are in agreement with the existing evidence in other models to support the role

of androgens in regulating Cyp4A/20-HETE synthesis [16, 18].

It has been previously demonstrated by various laboratories that fructose feeding induced insulin resistance and endothelial dysfunction as early as 2 weeks and as long as 9 weeks for observing elevated blood pressure [1, 2, 43]. Currently no studies have investigated the changes in Cyp4A expression/20-HETE synthesis during the development of insulin resistance and high blood pressure in fructose-fed rats. Although no direct evidence exists, our findings along with literature reports, where insulin resistance has been shown to precede and induce hypertension, it may be speculated that elevated Cyp4A expression follows insulin resistance.

We initially used the Cyp4A suicide substrate inhibitor ABT as it was the agent of choice to study the role of 20-HETE. However, the inability of ABT to selectively inhibit Cyp4A [44] and the advent of more potent and Cyp4A selective drugs such as HET0016 [35, 37] led us to revisit our studies. HET0016 inhibited Cyp4A expression (Fig. 7b) and prevented the increase in blood pressure (Fig. 7a). The work published to date has only measured changes in 20-HETE levels and not Cyp4A expression in HET0016 treated rats [18, 45]. The limited availability of HET0016 as a gift sample was a key factor in our starting prophylactic treatment after 8 weeks of fructose feeding. The rationale for the decision was based on data from our laboratory, which showed a significant blood pressure elevation only at the end of 9 weeks of feeding fructose. Compared to untreated fructose-fed rats, HET0016 treatment did not increase the ISI value in FH (Fig. 4c). However, it was also not different from C suggesting there maybe a high variability in the effects of HET0016 on glucose disposal, which warrants future study. Interestingly ABT normalized the glucose disposal profile in fructose-fed rats (Fig. 4a–c). We have no mechanism to support this finding but we believe that ABT reduces blood pressure by improving insulin sensitivity independent of Cyp4A. Studies are required at the molecular level to investigate the effects of ABT on insulin sensitivity and the effects of HET0016 on individual Cyp4A isoforms at the protein and message levels.

Plasma testosterone levels were unaffected by ABT. HET0016-treated samples were not assayed for testosterone, since there are no previous reports that have examined changes in testosterone levels following treatment with HET0016.

Alterations in vascular arachidonic acid metabolism may play an important role in the development of vascular abnormalities and high blood pressure following insulin resistance. Increased vascular Cyp4A expression following insulin resistance increases 20-HETE synthesis, which contributes to the increase in blood pressure. As 20-HETE

has been shown to decrease NO synthesis and function by blocking the K_{Ca} channels, the above findings could, in part, explain the concomitant decrease in endothelial vasodilator levels and function. Thus, it may be possible that 20-HETE impairs vascular homeostasis by both decreasing endothelial relaxation and increasing vascular smooth muscle $(Ca^{+2})_i$ as a possible common downstream target of angiotensin-2, norepinephrine and endothelin-1, which induces vasoconstriction and increased blood pressure. Furthermore angiotensin-2, norepinephrine and endothelin-1 also induce vascular remodeling by activating various matrix metalloproteinases (MMPs 2/9 and 7), whose activity is elevated in insulin resistant blood vessels following stimulation by phenylephrine [46]. Examining the effects of 20-HETE on MMP activation and therefore remodeling could implicate 20-HETE as a key target in altering hemodynamics following insulin resistance. Further studies need to be conducted to understand the intricate association between insulin resistance and sex hormones in determining the contributions of arachidonic acid metabolites to regulating blood pressure.

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