Insulin-induced endothelin release and vasoreactivity in hypertriglyceridemic and hypertensive rats

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Nava, Pilar, Verónica Guarnier, Rosalinda Posadas, Israel Pérez, and Guadalupe Banos. Insulin-induced endothelin release and vasoreactivity in hypertriglyceridemic and hypertensive rats. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H399–H404, 1999.—Insulin-elicited endothelin release in hypertriglyceridemic, hypertensive, hyperinsulinemic (HTG) rats was shown. Weaning male Wistar rats were given 30% sucrose in their drinking water for 20–24 wk. In vitro contractions of aorta and femoral arteries were elicited with 40 mM KCl. Endothelin release induced with KCl plus 50 μU/ml insulin increased in response to KCl plus 40% HTG aortas and femoral arteries. The endothelin ETB-receptor blocker BQ-788 decreased responses by 10.2 ± 0.3 % in HTG aortas and femoral arteries, respectively. The ETα-receptor antagonist PD-151242 inhibited these responses by 12 ± 1% and 9 ± 1% in control and by 51.5 ± 9 and 58.5 ± 1% in HTG aortas and femoral arteries, respectively. These results suggest that endothelin may contribute to the hypertension in this model.

IN OUR INSTITUTION we have developed a variant of fructose-induced hypertension by giving sucrose to rats. The fructose-fed rat becomes hypertensive, hypertriglyceridemic, hyperinsulinemic and has insulin resistance (HTG) (10); in other words, it exhibits what is known as “syndrome X.” Some characteristics of our model have already been described (1), and they coincide with those of fructose-fed animals, i.e., our rats also develop hypertension, hypertriglyceridemia, and hyperinsulinemia.

With regard to humans, it has been shown that subjects with syndrome X have levels of endothelin-1 (ET-1) that are higher than normal when stimulated by both insulin and triglycerides (22). On the other hand, in vitro ET-1 release from rat femoral arteries stimulated by insulin has been found in normal animals (18). Endothelins participate in many functions both physiological and pathological; among the latter, the role of endothelins in hypertension has been a subject of study, on account of their properties as vasoconstrictors and promoters of vascular hypertrophy (23).

The ET-1 system is activated in some forms of hypertension but not in others, which poses an interesting subject for investigation (24). The effect of bosentan (an ET-1 antagonist) to lower fructose-induced hypertension shown by Verma et al. (30) suggests an important participation of ET-1 in the sustained high blood pressure characteristic of this model. In the same work, an increased content of ET-1 in the mesenteric tissue of fructose-fed rats was found, although this was not altered by the antagonist.

Therefore, we undertook to investigate whether arteries from HTG rats could be stimulated with insulin and whether the release of ET-1 was increased. Because HTG animals have elevated circulating insulin, it can be expected that hyperinsulinemia would contribute to such an ET-1 increase. For comparison between vessels of different caliber and sensitivity to both peripheral and local factors, aorta and femoral arteries were chosen for testing. Because resistance to insulin has been found in comparable models, we also obtained curves of glucose tolerance in both control and HTG animals at the end of the sucrose treatment period.

MATERIALS AND METHODS

Animals. Weaning male Wistar rats aged 28 days and weighing ~45 ± 2 g were separated into two groups: group I, control rats given tap water for drinking and group II, HTG rats given 30% sucrose in drinking water for periods of ~20–24 wk. All animals were fed commercial rat chow ad libitum. The end of the sucrose treatment period was taken as the time when blood pressure was significantly increased in at least 80% of the animals in group II. Monthly measurements of systolic arterial pressure were taken by the tail-cuff method; the cuff was connected to a pneumatic pulse transducer (Narco Bio-systems, Healthdyne, Houston, TX) and a programmed electrosphygmomanometer (Narco Bio-systems). The recordings were taken in triplicate by means of a Grass polygraph (model 79, Grass Medical Instruments, Quincy, MA).

Blood samples. At the end of the treatment period, the animals were fasted overnight and then killed by decapitation. Blood samples were collected with and without anticoagulant (EDTA), taking care to avoid hemolysis. The blood was spun, and either serum or plasma was separated. Triglycerides were measured by means of an enzymatic technique (Boehringer Mannheim, Mannheim, Germany) in an Abbott VP Series II Autoanalyzer (Irvine, TX), according to the method described by Nägele et al. (17). Insulin was measured by immunoassay (Boehringer Mannheim).

Vascular tissue preparation and tension recording. After decapitation, the upper thoracic aorta and femoral arteries were immediately excised and kept in an oxygenated standard Tyrode solution of the following composition (mM): 136.9 NaCl, 5.4 KCl, 1.0 CaCl2, 1.05 MgCl2, 1.19 NaHCO3, 0.33
NaH₂PO₄, and 5.5 glucose. Under a dissecting microscope, we cleaned the vessels of surrounding tissue, avoiding damage to the endothelium. Arterial segments of 2–3 mm and 5 mm were cut from the aorta and femoral arteries respectively, and two 250-µm-diameter S-shaped silver hooks (Medwire) were inserted into the lumen to measure the tension developed transversally. One of the silver hooks was fixed to the bottom of a double-walled organ chamber, and the other was attached to an isometric force-displacement transducer (FT03, Grass Medical Instruments). The transducers were connected to a Grass polygraph model 79 D. A basal passive tension of 2 g or 500 mg was applied to the aortic and femoral artery rings, respectively, because these were found previously to be the optimal resting tensions for both control and HTG vessels under our experimental conditions (1, 18). The Tyrode fluid was bubbled continuously with a gas mixture of 95% O₂-5% CO₂. The liquid in the chamber was kept warm (37°C) and was changed every 20 min. The rings were allowed to equilibrate for 1 h. Contraction were induced by a Tyrode solution containing 40 mM KCl; this concentration was chosen after concentration-response curves were obtained because it gave submaximal responses (data not shown). After developed tension was recorded, the rings were washed with normal Tyrode solution and were allowed to return to their basal tension level. The procedure was repeated, and the mean value of the force developed was taken as a 100% response.

Endothelin release from the rings was stimulated by adding the 40 mM KCl solution plus 50 µU/ml human insulin (Eli Lilly). These concentrations were chosen because they gave the largest responses when different doses were tested under our experimental conditions, as already reported (18).

The proportion of ET-1 participating in the basal and insulin-stimulated contractile responses was estimated by adding specific blockers to the above solution. Either 10 mM PD-151242 (ET₃-receptor antagonist) or 10 mM BQ-788 (ET₅-receptor antagonist) was added to the Tyrode solution, and the insulin-stimulated responses were obtained. The rings were washed with normal solution, and a final test was carried out with 40 mM KCl solution alone to confirm that the vessels had recovered the level of the initial contraction. The percentage of the response to insulin and of the inhibition induced by the antagonists for each ring was calculated from the respective basal value.

Glucose tolerance test. Control and HTG rats were fasted overnight (n = 10/group). Anesthesia was induced by pentobarbital sodium (30 mg/kg ip) and maintained by infusion of the same (63 mg/dl saline, 2 drops/min ip). Artificial respiration was applied by an endotracheal catheter. Blood sampling and infusion of glucose solution (1 g/kg in 0.6 ml saline) were carried out by inserting a polyethylene catheter filled with a solution of heparin (200 U/ml saline) into a femoral artery. Blood samples (0.25 ml) were taken as follows: two basal samples before glucose infusion and samples at 15, 30, 60, 90, and 120 min after infusion. Plasma glucose was measured by the glucose oxidase method with a Beckman glucose analyzer (Fullerton, CA). The basal plasma glucose values obtained from control and HTG rats were normalized to 100%, and the corresponding changes in percentages were calculated for the rest of the data.

Statistical analysis. Mean and standard errors of 6–10 results from different arteries were calculated. When values are expressed as percentages, the percentage in each experiment was calculated and then the mean and standard error were determined. The statistical significance of differences between groups was analyzed by paired Student’s t-test and ANOVA. The differences were considered statistically significant when P < 0.05.

RESULTS

Previous observations (1) showed that HTG animals have a larger caloric intake than normal ones, although neither body weight nor blood level of glucose was significantly different between the two groups. The results from different arteries were calculated. When values are expressed as percentages, the percentage in each experiment was calculated and then the mean and standard error were determined. The statistical significance of differences between groups was analyzed by paired Student’s t-test and ANOVA. The differences were considered statistically significant when P < 0.05.

RESULTS

Figure 1 shows representative traces of the KCl-induced responses of aortas and femoral arteries (Fig. 1A and B, respectively) in control and HTG rats in the presence and absence of insulin. Endothelin release induced with Tyrode-40 mM KCl plus 50 µU/ml insulin resulted in increases in contractile responses: 41 ± 5.9 and 57 ± 6% for control and 65.5 ± 6 and 95 ± 9% for HTG aortas and femoral arteries, respectively.
slope with which the vessels acquired such tension was steeper in aortas than in femoral arteries. The contractions elicited in HTG vessels were significantly larger than those in control arteries ($P < 0.0001$) (Fig. 3). In aortas, although significant, the difference between control and HTG values was not as large ($P < 0.01$; Fig. 2).

Participation of endothelin in basal and insulin-induced responses. KCl-induced contractions in the absence of insulin but in the presence of ET$_A$- and ET$_B$-receptor antagonists were tested in vessels from control and HTG rats. Both blockers significantly reduced the basal response in control aortas by $\sim 20\%$ (Fig. 2). In femoral arteries the basal response was not significantly reduced by the ET$_A$-receptor blocker PD-151242, but it was diminished by 30% in the presence of the ET$_B$-antagonist BQ-788 (Fig. 3). In contrast, in the HTG group only the ET$_B$ antagonist reduced the response of aortas, whereas the response of the femoral arteries was diminished by both the ET$_A$ and ET$_B$ antagonists (Figs. 2 and 3). The decreases in both arteries from the HTG group were larger than in the control group, reaching $\sim 50\%$ of the basal response. Between control and HTG femoral arteries, statistically significant differences in the degree of inhibition of basal contractions were found using PD-151242 ($P = 0.0004$) and BQ-788 ($P = 0.006$). The increased response to KCl in the presence of insulin in aortas from control rats was significantly reduced by both blockers to nearly basal contraction level (Figs. 2 and 3), whereas in the HTG group, the response was reduced to levels well below the basal response. The ET$_B$-Receptor blocker BQ-788 decreased responses to Tyrode-40 mM KCl-insulin by 39 $\pm 8$ and 53 $\pm 5\%$ in control and by 48 $\pm 13$ and 79 $\pm 3.5\%$ in HTG aortas and femoral arteries, respectively. The ET$_A$-receptor antagonist PD-151242 inhibited these responses by 12 $\pm 10$ and 1 $\pm 9\%$ in control and by 51.5 $\pm 9$ and 58.5 $\pm 1\%$ in HTG aortas and femoral arteries, respectively. Figures 4 and 5 show representative traces of the effect of the ET$_A$ (PD-151242)- and ET$_B$ (BQ-788)-receptor blockers on the insulin-stimulated KCl response in aortas and femoral arteries. There were statistically significant differences between the control and HTG groups in aortas with the ET$_A$ antagonist PD-151242 and in femoral arteries with both blockers.

Glucose tolerance curves. Basal plasma glucose concentration was 139 $\pm 5$ ($n = 10$) and 130 $\pm 18$ ($n = 10$) mg/dl for control and HTG, respectively. After these values were normalized to 100%, the corresponding percentages at 15, 30, 60, 90, and 120 min after glucose infusion were 218.8 $\pm 29$, 105.3 $\pm 12$, 96.8 $\pm 8$, 115.1 $\pm 6.3$, and 115.1 $\pm 6.3$ mg/dl, respectively. Figures 6 and 7 show representative traces of the effect of the ET$_A$ (PD-151242)- and ET$_B$ (BQ-788)-receptor blockers on the insulin-stimulated KCl response in aortas and femoral arteries. There were statistically significant differences between the control and HTG groups in aortas with the ET$_A$ antagonist PD-151242 and in femoral arteries with both blockers.

**Figure 2.** Effects of Ins and endothelin-receptor antagonists BQ-788 and PD-151242 on KCl-induced contractions in presence ($n = 6$) and absence ($n = 10$) of Ins in aortas from control and HTG rats. *$P < 0.05$ between treatments; †$P < 0.05$ control vs. HTG rats.

**Figure 3.** Effects of Ins and endothelin-receptor antagonists BQ-788 and PD-151242 on KCl-induced contractions in presence ($n = 6$) and absence ($n = 10$) of Ins in femoral arteries from control and HTG rats. *$P < 0.05$ between treatments; †$P < 0.05$ control vs. HTG rats.

**Figure 4.** Traces from representative experiment showing contraction of vascular smooth muscle by 40 mM KCl in presence of 50 µU/ml Ins and of endothelin-receptor antagonists BQ-788 and PD-151242 in aortas from control and HTG rats.
with insulin resistance is quite frequent both in hypertension, dyslipidemias, and hyperinsulinemia by the work of several authors. The association of hyperinsulinemia to hypertension has been suggested but not in hypertensive rats (8).

Insulin is also associated with modify the blood pressure in the treated animals (12). azone, in spontaneously hypertensive rats did not drug that improved insulin sensitivity, CS-045 or troglitazone. ET-1 release has been included as a possible damage to the endothelium. A defective endothelium might be a major component. Our research in this paper was aimed at analyzing whether the endothelin release induced by insulin was altered in our model of HTG rats. The larger response of HTG vessels in the presence of insulin and the important inhibition of KCl-induced contractions by endothelin blockers suggest that the endothelial cells in these rats have increased endothelin storage and releasing mechanisms, being capable of a very considerable secretory response. This increased capability may overcome the reduced sensitivity to ET-1, by a down-regulation process, as reported by Verma et al. (31).

The possibility of such an increased capacity to secrete endothelin agrees with the initial description of our model (1) in which we reported that, in the isolated vessels, responses observed in full concentration-response curves to vasoconstrictors such as norepinephrine were increased and endothelin-dependent vasodilation by acetylcholine was reduced, indicating possible damage to the endothelium. A defective endothelium can produce larger quantities of vasoressors and smaller quantities of vasodilators (27); among the former, endothelin might be a major component.

From our experiments we can deduce that the expression of endothelin receptors and their capacity to alter the contractile response are modified in HTG rats. This is shown by experiments in which endothelin blockers modified responses in different ways in control and HTG rats.

In the absence of insulin, aortas from control rats were sensitive to both ET A and ET B blockers, but their response to the antagonists was small. In contrast, in HTG rats, the sensitivity to the ET B blocker was lost but the participation of the ET B receptor was increased, because the contractile response was reduced to a

DISCUSSION

For several years the participation of hyperinsulinemia and hypertriglyceridemia in the pathogenesis of hypertension has been a subject of investigation. More recently, ET-1 release has been included as a possible factor contributing to the sustained high blood pressure found in some types of hypertension. The body of evidence provides controversial results; the multifactorial origin of cardiovascular diseases makes it particularly difficult to establish the degree of participation of each one of the risk factors involved.

Concerning hyperinsulinemia on the one hand, it is known that insulin has vasodilator properties by reducing α-adrenergic vasoconstriction and intracellular calcium levels in vascular smooth muscle (11). The mechanism of this action has been attributed to insulin enhancing α2-adrenergic vasorelaxation through a pertussis toxin-sensitive mechanism that stimulates the production of endothelial nitric oxide. This mechanism is defective in hypertensive animals (13). The use of a drug that improved insulin sensitivity, CS-045 or troglitazone, in spontaneously hypertensive rats did not modify the blood pressure in the treated animals (12). Insulin is also associated with β-adrenergic-mediated vasodilation; it enhances this responsiveness in normal but not in hypertensive rats (8).

On the other hand, the possible contribution of hyperinsulinemia to hypertension has been suggested by the work of several authors. The association of hypertension, dyslipidemias, and hyperinsulinemia with insulin resistance is quite frequent both in humans and in experimental animal models. In fructose-fed rats or in humans and rodents with similar pathological characteristics, the rise in blood pressure can be reduced if hyperinsulinemia and insulin resistance are prevented by treatment with drugs such as metformin (29), vanadium (2), or thiazolidinediones (21). In several other animal models of hypertension or in patients, insulin reduction has lowered blood pressure (32).

Furthermore, high blood pressure has been induced in rats by elevating circulating insulin either through glucose feeding or exogenous insulin injection, although this type of experiment has given disparate results, depending on the conditions applied by the authors (16). Other reports suggest that insulin increases endothelin synthesis and release under tissue culture conditions and could thereby contribute to hypertension (19).

We recently reported (18) an in vitro hypercontractile response that is mediated by insulin in femoral arteries from normal rats. The best results, i.e., the largest and most significantly different from basal values, were obtained when normal concentrations of glucose (5.5 mM) were assayed. The level of insulin that elicited the largest contractile response, 50 µU/ml, is below that which would normally be found after the stimulus of a meal (5- to 10-fold increase of basal levels) and can be expected in the sucrose-fed animals.

Our research in this paper was aimed at analyzing whether the endothelin release induced by insulin was altered in our model of HTG rats. The larger response of HTG vessels in the presence of insulin and the important inhibition of KCl-induced contractions by endothelin blockers suggest that the endothelial cells in these rats have increased endothelin storage and releasing mechanisms, being capable of a very considerable secretory response. This increased capability may overcome the reduced sensitivity to ET-1, by a down-regulation process, as reported by Verma et al. (31).

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In the absence of insulin, aortas from control rats were sensitive to both ET A and ET B blockers, but their response to the antagonists was small. In contrast, in HTG rats, the sensitivity to the ET B blocker was lost but the participation of the ET B receptor was increased, because the contractile response was reduced to a...
larger extent than in control rats. In femoral arteries from the control group, only the ET\textsubscript{A} receptors seem to participate in the KCl response, whereas both receptors participate in the response of femoral arteries from HTG rats. Thus it should be noted that the response to KCl alone suggests that, by itself, it induces release of endothelin, and therefore this effect might influence the response of vessels from diabetic animals contracted with KCl.

Furthermore, when the response to KCl was stimulated by the presence of insulin in the superfusing medium, the increased participation of the receptors was evidenced by a higher degree of inhibition reaching, in the femoral arteries, levels of tension well below the basal contractions. ET\textsubscript{A} receptors are usually expressed in smooth vascular muscle, and they mediate vasoconstriction and mitogenesis (20). ET\textsubscript{B} receptors are also expressed in muscle cells, eliciting contraction, and in endothelial cells, in which they mediate nitric oxide release (20).

With regard to ET-1 release and the cardiovascular system, particularly its relationship with hypertension, in the spontaneously hypertensive rat the ET-1 system does not appear to be activated, and its contribution to the blood pressure in this model seems a minor one (14).

We did not measure the levels of circulating endothelin, because several authors reported that the levels are not altered in this and other comparable models, although the release of ET-1 by endothelium is abnormally elevated in response to stimulation (9, 24, 31). This phenomenon characterizes some aspects of endothelin behavior, i.e., abluminal release and paracrine effects. Conversely, the role of endothelin in other models was found to be more relevant, such as in the case of the salt-dependent hypertensive rats, i.e., the DOCA-salt hypertensive or the DOCA-salt-treated spontaneously hypertensive rat (3, 25).

In the fructose-fed hypertensive rat, a model comparable to the one we have developed, Verma et al. (31) investigated the constrictor response to ET-1 of isolated mesenteric arteries from rats with and without bosentan treatment. They found a reduced sensitivity to ET-1, and this effect was reversed in the bosentan-fructose-treated rats. The endothelin-dependent relaxation of hypertensive animals was decreased compared with that of normal animals, and bosentan did not modify the response. The authors suggest a downregulation to ET-1 resulting from its overproduction by the endothelium and the increase in its gene expression. The relationship with the hyperinsulinemia present in this model is possibly caused by the modulating role of hyperinsulinemia and insulin resistance as initiating factors, which would stimulate the release of ET-1 followed by a downregulation.

The results we obtained from our model of HTG animals are somewhat different from those previously published by other groups in other models. The explanation for such findings might be, in the first place, the rat strain [Wistar in our case and Sprague-Dawley as used by Reaven and co-workers (10)]. Another source of differences is the experimental protocol carried out to develop the pathological alterations. The animals received the sucrose (30%) in their drinking water; therefore, the doses were small and the evolution time was long (20–24 wk). This period might have allowed some compensatory mechanisms to evolve, which would explain the finding that although the increase in blood pressure, triglycerides, and insulin were significant compared with initial values, they were not pronouncedly high and the condition can be considered mild.

Our model does not show insulin resistance as in fructose-fed rats. Empirically, the ratio plasma glucose (mg/dl)/plasma insulin (µU/ml) < 6 is characteristic of individuals with obesity, glucose intolerance, and hypertension. This ratio was higher in our animals, both control and HTG, indicating no overt insulin resistance. Glucose tolerance tests confirmed that glucose handling was normal in HTG rats, although it took 15 min longer than in control rats for the levels to descend to basal values.

Dyslipidemias are often associated with blood pressure increase. In the sucrose-fed model we have described (1), there is a significant alteration in the plasma fatty acid composition and an elevation in the level of triglycerides (5). Our animals are not obese but do accumulate an excess of retroperitoneal fat. This feature has also been observed by another group of investigators (28). Thus these characteristics have been identified as risk factors linked to cardiovascular pathology (4, 6). Nevertheless, plasma fatty acid composition, independently from insulin action, can influence blood pressure (15, 26). The participation of an altered lipid profile in the hypertension of HTG rats cannot be dismissed. As previously reported (5), the analysis of plasma fatty acid composition showed, among other abnormalities, an increase in the content of oleic acid, which has pressor effects when infused (7), as well as a reduced level of arachidonic acid. Both changes are significant with respect to the control values (5). The diminished level of circulating arachidonic acid suggests that its metabolism is enhanced, probably in favor of the production of vasoactive prostaglandins. Furthermore, hypertriglyceridemia has been found to increase endothelin levels independently from hyperinsulinemia (22).

In summary, insulin-stimulated contraction in vessels is significantly increased in HTG animals compared with normal animals, probably because of endothelin release, alterations in the endothelin receptors, or both. It is likely that this event takes place in vivo, and it could contribute to the hypertension found in this model. Other factors, such as altered lipid profile and degree of lipoperoxidation, might modulate the blood pressure also, but their exact participation in the modified contractile responses of the vessels cannot be defined at present.

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