The effect of intravenous insulin infusion on renal blood flow in conscious sheep is partially mediated by nitric oxide but not by prostaglandins

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ABSTRACT: To test the effect of insulin on renal perfusion and the participation of NO and PG as mediators of this response, renal blood flow (RBF) was measured in sheep (n = 8) implanted with ultrasonic flow probes around renal arteries and with a systemic arterial pressure (SAP, n = 4) telemetry device. Three protocols were performed: 1) RBF and SAP were recorded (0800 to 1800 h) in fed and fasted sheep, with the latter receiving intravenous (i.v.) infusions (0.5 mL/min) of insulin at 2 or 6 mU/(kg·min); 2) fasted sheep received i.v. infusions of either an inhibitor of NO synthesis (N[G]-nitro-L-arginine methyl ester, L-NAME) alone [0.22 mg/(kg·min), 1000 to 1200 h] or L-NAME (1000 to 1200 h) + insulin during the second hour (6 mU/(kg·min), 1100 to 1200 h); and 3) the same protocol was followed as in protocol 2, substituting L-NAME with ketoprofen [0.2 mg/(kg·min)], a cyclooxygenase inhibitor. In all protocols, plasma insulin and glucose were determined. During insulin administration, euglycemia was maintained and hypokalemia was prevented by infusing glucose and KCl solutions. After the onset of meals, a long-lasting 18% increase in RBF and a 48% insulin increase were observed (P < 0.05), without changes in SAP. Low- and high-dose insulin infusions increased RBF by 19 and 40%, respectively (P < 0.05). As after meals, the increases in RBF lasted longer than the insulin increase (P < 0.05). The L-NAME infusion decreased RBF by 15% (P < 0.05); when insulin was added, RBF increased to preinfusion values. Ketoprofen decreased RBF by 9% (P < 0.05); when insulin was added, RBF increased to 13% above preinfusion values (P < 0.05). In no case was a modification in SAP or glucose noted during the RBF changes. In conclusion, insulin infusion mimics the meal-dependent increase in RBF, independent of SAP, and lasts longer than the blood insulin plateau. The RBF increase induced by insulin was only partially prevented by L-NAME. Ketoprofen failed to prevent the insulin-dependent RBF increase. Both facts suggested that complementary vasodilatory agents accounted for the insulin effect on sheep renal hemodynamics.

Key words: arterial pressure, insulin, nitric oxide, prostaglandin, renal blood flow, sheep

INTRODUCTION

Renal hypoperfusion is a common condition in critical care medicine and often results in kidney function impairment. To propose treatments to restore renal blood flow (RBF), it is necessary to investigate the role of vasoactive mediators involved in renal hemodynamic regulation. An insulin-induced vasodilatory response has been reported in different species (Muniyappa et al., 2007), and plasma insulin increases after meals in ruminants (Trenkle, 1978; Mineo et al., 1990). In a sheep model, we observed an increase in RBF after meals, independent of blood pressure (Denis et al., 2004; Tebot et al., 2009). Consequently, a causal relationship between insulin and the meal-associated RBF changes could be proposed. The cardiovascular effects of insulin are partly mediated by the release of NO (Muniyappa et al., 2007). In lambs, the administration

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of \(N^\text{G}\)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, increased renal vascular resistance, reducing RBF (Sener and Smith, 2001). Therefore, it is reasonable to propose that NO could be the mediator of the hypothetical insulin-induced changes of RBF in sheep. In nonruminant animals, vasodilatory PG have been widely invoked to account for RBF changes in response to pancreatic hormones, protein load, or AA infusion. Nevertheless, some results indicate that inhibition of PG synthesis with ketoprofen or indomethacin does not suppress this response (Woods, 1993). Hence, the participation of the PG system in the insulin-induced changes in RBF is controversial.

The hypothesis tested was that insulin would increase RBF in sheep and that this effect would be mediated by NO, PG, or both. Therefore, the aim was to describe the effect on RBF of insulin infusions alone and after blockade of NO synthesis by L-NAME, or of PG synthesis by ketoprofen, in sheep chronically implanted with ultrasonic flow probes around both renal arteries.

**MATERIALS AND METHODS**

The experiments were performed in accordance with international ethical guidelines, and the protocol was approved by the Ethical Committee of VetAgro Sup (Campus Vétérinaire de Lyon), France.

**Animals**

Eight adult nonpregnant, nonlactating Ile de France ewes (60 ± 4 kg of BW) were housed in individual pens in temperature-controlled rooms (18 to 22°C) with a natural lighting cycle. Sheep, 2 to a room to promote social interaction, were fed alfalfa pellets and hay once daily (0900 to 1100 h). During eating time, food was available for ad libitum ingestion, and food was added as needed to ensure an unrestrained voluntary ingestion. At the end of the eating time, food was always removed. The amount ingested was monitored (by weighing the food), and data from days of reduced ingestion (<60% of the current food consumption) were not taken into account. Access to water was provided ad libitum. The animals were adapted to pens and the feeding routine for 10 d before surgery.

**Surgical Preparation and Recordings**

After 24 h of fasting, sheep were premedicated with xylazine (0.2 mg/kg of BW; Rompun, Bayer, Puteaux, France), and general anesthesia was induced with intravenous (i.v.) sodium thiopentone (15 mg/kg of BW; Nesonal, Mérail, Lyon, France) and maintained with isoflurane (0.5 to 2%; Aerrane, Baxter, Maurepas, France) in \(O_2\) (6 L/min). Transit-time ultrasonic flowmetering probes (4 mm, R-series, Transonic Systems, Ithaca, NY) were bilaterally implanted around the renal arteries for continuous measurement of RBF, as described previously (Tebot et al., 2009). After surgery, the sheep were given amoxicillin [15 mg/kg of BW intramuscularly (i.m.), Clamoxyl, Beecham, St. Brieuc, France] for 5 d and ketoprofen (3 mg/kg of BW i.m.; Ketofen, Mérial, Lyon, France) for 3 d. No postsurgical disturbances were observed. When optimal ultrasonic signals were obtained (1 wk after surgery, required for probe encapsulation), 4 of the sheep were fitted with a telemetry measurement system (model TL11M3-D70-CCP Physiotel Transmitter, Data Sciences International, St. Paul, MN) into 1 carotid artery (same anesthetic protocol) for systemic arterial pressure (SAP) monitoring, as also described previously (Tebot et al., 2009). This monitoring was necessary because the autonomic nervous system was reported to take part in the skeletal muscle vasomotion induced by insulin (Muniyappa et al., 2007), and variations in renal hemodynamics were partly attributed to changes in arterial pressure (Millar-Craig et al., 1978). Finally, both jugular veins were fitted with chronic catheters for infusions and blood sampling.

After experimental procedures, the implanted probes were validated for zero blood flow and calibrated in situ to verify the identity between the real (pumped) and the measured flows, according to the method of D’Almeida et al. (1995) and described in a previous work (Denis et al., 2003). If necessary, measured flows were corrected for the slight difference between the true and the read flow rate during calibration.

Flow probes were connected to a dual-channel flowmeter (model T208, Transonic Systems). Flowmeter and telemetry outputs were transmitted to a processing system (Acqknowledge III for MP150WSW, Biopac Systems Inc., Santa Barbara, CA). Rate of data acquisition for RBF and SAP was 200 samples per second. Mean values of RBF (right + left flows) and SAP were calculated every 10 min.

**Experimental Protocols**

**Exp. 1.** The first protocol was designed to determine the plasma insulin changes during the reported (Tebot et al., 2009) RBF increase associated with meals, and to evaluate the effect of insulin infusions on RBF. On 3 alternate days, RBF and SAP were continuously recorded between 0800 and 1800 h, and blood samples were taken every 30 min. Subsequently, all sheep with 23 h of fasting received 1) a primed constant-rate i.v. infusion (0.5 mL/min, 1000 to 1200 h) of insulin (Caninsulin, Intervet SA, Beaucouzé, France) at 6 mU/(kg·min) [high dose (HI) primed with 70 mU/kg] or 2 mU/(kg·min) [low dose (LO) primed with 30 mU/kg] in 0.9% saline; 2) saline only (same infusion rate); and 3) no infusion. Insulin doses were similar to those used previously by others (Sano et al., 1990, in sheep; Grossini et al., 2004, in pigs). Infusions were once daily on 3 alternate days, in random order. During insulin administration, euglycemia was maintained and hypokalemia was prevented by perfusing (0.5 mL/min) a 40% (HI) or 30% (LO) glucose and 2.5 (HI) or 1.5% (LO) KCl.
solution. On the infusion days, RBF and SAP were recorded between 0800 and 1600 h, and blood samples were taken every 30 min between 0900 and 1500 h.

**Exp. II.** A second series of protocols was carried out to describe the effect of insulin infusions on RBF after blockade of NO synthesis with L-NAME. On 3 alternate days and in random order, 23-h fasted sheep received constant-rate i.v. infusions (once daily, same rate and time as in Exp. I) of 1) L-NAME [0.22 mg/(kg·min); Sigma-Aldrich, St. Quentin Fallavier, France] in saline, and 2) L-NAME + insulin (dose same as HI of Exp. I) or saline during the second hour (1100 to 1200 h) of L-NAME infusion. Solutions of 40% glucose and 2.5% KCl were infused with insulin. On infusion days, RBF and SAP were recorded between 0900 and 1500 h, and blood samples were taken every 30 min.

**Exp. III.** In a third series of protocols, the effect of insulin infusions on RBF was studied after blockade of PG synthesis by ketoprofen. The same protocol as in Exp. II was carried out, substituting ketoprofen [0.2 mg/(kg·min); Ketofen, Merial, Lyon, France] for L-NAME. On infusion days, RBF and SAP were recorded between 0900 and 1500 h, and blood samples were taken every 30 min.

**Analytical Procedures**

Blood samples (2 mL on heparin iodoacetate) were immediately centrifuged (1,200 × g for 10 min at 4°C), and plasma was stored in Eppendorf tubes and frozen at −20°C. Plasma insulin concentration was determined by RIA (Insik 5 Kit, DiaSorin, Antony, France). The sensitivity of the method was 3 mU/L. The intraassay CV ranged between 5.5 and 10.6%, and the interassay CV ranged between 6.2 and 10.8%. A specific validation was done for sheep. When 12.5, 25, 50, 100, 150, and 187.5 μL of sheep serum (10.5 mU/L insulin) were added to, respectively, 187.5, 175, 150, 100, 50, and 12.5 μL of sheep serum (242.5 mU/L of insulin), the expected concentrations were 25.5, 33.0, 59.0, 113.5, 148.0, and 225.0 mU/L, respectively. The regression of the measured on the expected concentrations after dilution had a slope of 0.937 (R² = 0.976). Plasma glucose was determined by the enzymatic method (GOD POD Kit, Thermo Fisher Scientific Oy, Vantaa, Finland).

**Statistics**

Data were expressed as means ± SD over 10-min periods. The RBF, SAP, and blood data were analyzed with an intragroup ANOVA for repeated measures, except when there was inequality of variance (P < 0.01; StatView, SAS Inst. Inc., Cary, NC). The a posteriori significance of the intragroup contrast difference was analyzed using a Student’s paired t-test. When there was inequality of variance (P < 0.01), Friedman and Wilcoxon post hoc tests for intragroup comparison were used. The significance threshold was set at α = 0.05 with a power of analysis of 80% for bilateral comparisons.

**RESULTS**

**Exp. I**

After the onset of meals, a long-lasting increase (P < 0.05) in RBF was observed (Figure 1) without any change in SAP. The maximum RBF mean value reached was 759 ± 49 mL/min, 18% greater than 1-h mean prefeeding values (P < 0.05). Insulin concentration also increased (22.2 ± 1.6 mU/L maximum mean value, 48% greater than 1-h mean prefeeding values, P < 0.05) and remained increased for 7 h. During HI and LO infusions (Figure 2), RBF increased to maxima of 895 ± 59 (HI) and 759 ± 43 mL/min (LO), without changes in SAP. These values corresponded to 40% (HI) and 19% (LO) of the corresponding preinfusion mean values (P < 0.05). During infusions, the mean plasma insulin concentrations were 214 ± 7 (HI) and 93 ± 5 mU/L (LO), meaning 6.6 and 2.9 times greater than preinfusion values, respectively (P < 0.05). As observed after meals, in both infusions, the increase in RBF lasted longer than the insulin increase (P < 0.05), and the maximum flow values were reached after the onset of the decline in plasma insulin. The magnitude and time course of the RBF increase under LO infusion were similar to those found after meals. A progressive RBF reduction during saline or no infusion was observed (P < 0.05). No changes were found in plasma glucose concentration during and after feeding (2.89 ± 0.16 and 3.03 ± 0.20 mmol/L, respectively), compared with 2.77 ± 0.18 prefeeding values (P > 0.05). Owing to the euglycemic clamp, no changes in plasma glucose during insulin infusions were observed: 2.90 ± 0.15 (HI) and 2.92 ± 0.13 (LO) mean values vs. 2.85 ± 0.20 mmol/L before infusions (P > 0.05).

**Exp. II**

During the 2-h infusion of L-NAME alone (Figure 3), a 15% decrease in RBF (from 635 ± 38 to 538 ± 35 mL/min, P < 0.05) was observed, which recovered to preinfusion values 1 h after the end of the infusion. When insulin, but not saline, was added during the second hour, RBF increased to preinfusion values in spite of the continuous administration of L-NAME. No SAP changes were evident during L-NAME or insulin administration (P > 0.05). As in Exp. I, no changes in plasma glucose during insulin infusions were observed (P > 0.05).

**Exp. III**

During the infusion of ketoprofen (Figure 4), a 9% decrease in RBF (from 670 ± 11 to 608 ± 43 mL/min, P < 0.05) was observed. When insulin, but not saline,
was added, RBF rapidly increased from 620 ± 29 to 750 ± 54 mL/min (13% above preinfusion values, *P* < 0.05), in spite of the continuous infusion of ketoprofen and without changes in SAP. The significant increase in RBF lasted 100 min after the end of insulin infusion and was similar to that observed after meals (Figure 1). As in Exp. I, no changes were observed in plasma glucose during insulin infusions (*P > 0.05*).

**DISCUSSION**

The transit-time flow-metering system was successfully used previously for chronic RBF measurements in adult sheep over many months (Bednarik and May, 1995; Tebot et al., 2009). The technique provided reliable and accurate data, allowing a long-lasting, continuous, and bilateral recording of RBF without stressing the animal and enabling the recording of transient and rapid flow changes.

Plasma insulin concentrations before meals and infusions were within the range of 10 to 50 mU/L reported in the literature for nonportal blood (reviewed by Trenkle, 1972). Even if plasma insulin concentrations during infusions in the 3 experiments were greater than those elicited by meals, they could be considered physiological values. In fact, Bassett (1974) found plasma insulin concentrations of 80 to 90 mU/L in sheep fed twice daily, and Sano et al. (1990) increased the plasma insulin of sheep to almost 200 mU/L by means of the hyperglycemic clamp technique. Leuvenink et al. (1997), perfusing Na propionate at 2 mmol/min into a mesenteric vein, reported plasma insulin concentrations of 297 ± 40 (portal vein) and 244 ± 22 mU/L (jugular vein). Moreover, Sano et al. (1995), by means of increasing i.v. infusions of n-butyrate at physiological doses in sheep, increased the plasma insulin dose-dependently to 496 mU/L.

The long-lasting increase in RBF associated with meals was reported in a previous work (Tebot et al., 2009). This increase showed 2 phases: a progressive increase for about 6 h and then a slight decline. The meal-associated increase in plasma insulin also showed 2 phases: an effective increase for 3 h after the onset of meals and then a progressive decrease. A similar 3-h increase and posterior decline of plasma insulin were reported by Sasaki et al. (1984) and Mineo et al. (1990) in sheep fed once daily. When insulin was infused in fasted sheep, a dose-dependent increase in RBF com-

![Figure 1. Renal blood flow (RBF), systemic arterial pressure (SAP, mean value), and plasma insulin (Pins) during (bracketed with dotted lines) and after meals. The RBF and SAP data are 10-min means ± SD; n = 8 (for RBF and Pins) and n = 4 (for SAP) sheep. *P* < 0.05 vs. 1-h prefeeding mean values.](image)
parable with that associated with meals was observed, independent of blood pressure and lasting longer than the increase in plasma insulin. This fact suggests the appearance of an insulin-induced mediator responsible for the RBF increase. The progressive RBF reduction during saline or no infusion was similar to that described for fasted sheep (Tebot et al., 2009). The RBF increases induced by feeding and by insulin infusion were similar, although the insulin plasma concentrations reached with infusions were much greater than those achieved with feeding. A possible explanation for this is that the meal-associated increase in RBF could be determined not only by insulin, but also by other vasoactive substances with possible additive effects, such as glucagon, another important glucoregulatory hormone in ruminants. It has been shown that in sheep fed 1 to 3 times daily, plasma glucagon increased after meals (Bassett, 1972; Mineo et al., 1990), and in a previous work, we found that glucagon infusions at physiological doses elicited dose-dependent increases in sheep RBF (Denis et al., 2003). In spite of the known insulin-induced sympathetic activation in both animals and humans (reviewed by Scherrer and Sartori, 1997), HI infusion did not increase blood pressure, probably because the sympathetic pressor effects were offset by peripheral vasodilatation, as proposed in dogs (Reikeras and Gunnes, 1986) and humans (Scherrer et al., 1994).

The 0.22 mg/(kg·min) dose of L-NAME infused [13.2 mg/(kg·h)] was greater than that infused by Junot (2008) in sheep [7 mg/(kg·h)] and is widely recommended in the literature (reviewed by Junot, 2008). The observed reduction in RBF during the first hour of the NO synthesis inhibition with L-NAME, disclosing

Figure 2. Renal blood flow (RBF), systemic arterial pressure (SAP, mean value), and plasma insulin (Pins) during (bracketed with dotted lines) and after infusions of high (solid diamonds) and low (solid triangles) insulin doses and saline (open circles); open squares = no infusion. The SAP values correspond to a high insulin dose [6 mU/(kg·min)]. The RBF and SAP data are 10-min means ± SD: n = 8 (for RBF and Pins) and n = 4 (for SAP) sheep. *P < 0.05 vs. 1-h preinfusion mean values.
the existence of a NO-dependent vasodilatory tone, is widely reported in the literature for nonruminant animals (Lahera et al., 1997; Gabbai and Blantz, 1999). In sheep, the same effect was reported after an i.v. bolus injection of 10 mg/kg (total dose of approximately 400 mg) of the arginine analog \(N\)-nitro-l-arginine methyl ester (L-NAME; bracketed with dotted lines), alone (open triangles) or with insulin (solid circles) or saline (open circles) during the second hour. The SAP values correspond to L-NAME + insulin infusion. The RBF and SAP data are 10-min means ± SD; \(n = 8\) (for RBF and Pins) and \(n = 4\) (for SAP) sheep. *\(P < 0.05\) vs. 1-h preinfusion mean values.

On the contrary, Booke et al. (2000), perfusing sheep for 3 h with \(N\)-monomethyl-l-arginine (L-NMMA), another competitive inhibitor of NO synthase, did not observe any changes in RBF. Nevertheless, their dose of L-NMMA was about one-half the amount used here for L-NAME; in addition, L-NAME was found to be approximately 20-fold more potent than L-NMMA in terms of actions on renal hemodynamics (Broere et al., 1998). The absence of an effect of L-NAME on SAP

Figure 3. Renal blood flow (RBF), systemic arterial pressure (SAP, mean value), and plasma insulin concentration (Pins) before, during, and after infusions of \(N\)-nitro-l-arginine methyl ester (L-NAME; bracketed with dotted lines), alone (open triangles) or with insulin (solid circles) or saline (open circles) during the second hour. The SAP values correspond to L-NAME + insulin infusion. The RBF and SAP data are 10-min means ± SD; \(n = 8\) (for RBF and Pins) and \(n = 4\) (for SAP) sheep. *\(P < 0.05\) vs. 1-h preinfusion mean values.
could be attributed to the reduced sensitivity of systemic vessels to NO synthesis inhibition compared with that of the renal vasculature (Lahera et al., 1991). Tresham et al. (1994) reported an increase in sheep SAP, owing to an increase in total peripheral resistance, after an i.v. bolus injection of NOLA but at doses 10- or 30-fold greater than our L-NAME minute-infused dose. Under our experimental conditions, the addition of insulin during the second hour of L-NAME infusion increased RBF to preinfusion values, but not to the values reached when insulin was infused in the absence of NO synthesis inhibition. Nevertheless, the delta in RBF when insulin was infused alone at the HI dose was approximately 100 mL/min (from roughly 550 to roughly 750 mL/min), and the same delta was observed when insulin was infused at the same dose in the presence of L-NAME (from roughly 550 to roughly 650 mL/min). However, only in the first case was RBF still increased after the end of insulin infusion. As observed in the L-NAME alone and L-NAME + saline infusions, the effect of the NO blockade lasted at least 1 h after stopping the infusions, which suggests that inhibition of NO release by L-NAME prevented, to a certain extent, the full RBF response to insulin. This idea was reinforced by recent results in our laboratory (unpublished data), showing that in sheep, the inhibition of NO release by L-NAME infusion only partially prevented the increase in RBF induced by an i.v. infusion of a solution of mixed AA that stimulates insulin release. Although there is conclusive evidence that inhibition of NO release by specific inhibitors of NO synthase abolishes insulin-induced vasodilatation in nonruminant species

**Figure 4.** Renal blood flow (RBF), systemic arterial pressure (SAP, mean value), and plasma insulin concentration (Pins) before, during, and after infusions of ketoprofen (bracketed with dotted lines) with insulin (solid circles) or saline (open circles) during the second hour. The SAP values correspond to ketoprofen + insulin infusion. The RBF and SAP data are 10-min means ± SD; n = 8 (for RBF and Pins) and n = 4 (for SAP) sheep. *P < 0.05 vs. 1-h preinfusion mean values.
(reviewed by Scherrer and Sartori, 1997), the present results suggest that the renal vasodilatatory effect of infused insulin is only partially mediated by NO in sheep. Yu et al. (2002) also suggested, in fetal sheep, that when NO synthesis is chronically blocked by NOLA, other vasodilator pathways take over the role of NO in the maintenance of renal vascular tone.

The dose of ketoprofen infused [0.2 mg/(kg-min)] primed with 6 mg/kg was greater than the 3 mg/kg used as a single dose in goats by Arifah et al. (2003) and in sheep by Junot (2008). In both cases, the authors obtained significant reductions in PG concentrations. The slight RBF decrease during the first hour of ketoprofen infusion disclosed a PG-dependent vasodilatatory tone. Similarly, an increase in renal vascular resistance was reported in lambs, although transient, using indomethacin as a cyclo-oxygenase inhibitor (Ebenezar et al., 2007). Insulin infusions elicited similar increases in RBF with, as well as without, ketoprofen pretreatment, indicating that inhibition of PG synthesis was unable to prevent the RBF response to insulin. This idea was strengthened by recent protocols in our laboratory (unpublished data) showing, in sheep, that the inhibition of PG release by ketoprofen failed to prevent the increase in RBF consecutive to an AA load. In humans, the renal vasodilatation caused by insulin infusion seems independent of renal PG (Stenvinkel and Alvestrand, 1994). However, in the isolated infused kidney, the inhibition of PG synthesis with indomethacin prevented the vasodilatatory effect of insulin (Cohen et al., 1989). We have no satisfactory explanation for the slight, although significant, reduction in SAP during the declining phase of RBF consecutive to ketoprofen + insulin administration. The present results suggest that the insulin-induced renal vasodilatation in sheep is not dependent on the PG pathway. The same evidence was given in dogs, in which the renal vasodilatation induced by an acute arginine load was not mediated by PG (Napathorn et al., 1992). Further work is needed to explore other hypothetical mediators of the renal hemodynamic effect of insulin.

In conclusion, i.v. insulin infusion mimics the meal-dependent increase in RBF, independent of blood pressure and lasting longer than the blood insulin plateau. Blocking NO synthesis with L-NAME reduced renal infusions in sheep but only partially prevented RBF increases induced by insulin. Blocking PG synthesis with ketoprofen reduced renal infusions but failed to prevent the insulin-dependent RBF increase. Both facts suggest the existence of complementary vasodilatatory agents accounting for the effect of insulin on renal hemodynamics in sheep.

**LITERATURE CITED**


