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Immediate Direct Peripheral Vasoconstriction in Response to Hyperinsulinemia and Metformin in the Anesthetized Pig

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Summary

Elevated levels of insulin have been reported to induce both an arterial vasodilation mediated by nitric oxide (NO), and vasoconstriction mediated by endothelin and reactive oxygen radicals. Metformin, used to control blood glucose levels in type 2 diabetes, has also been shown to cause NO-mediated dilation of conduit arteries. It is possible that these contradictory vascular effects are due to a non-direct action on arteries. Therefore, the direct effect of high levels of insulin and metformin infusion on resistance artery diameter was evaluated. Experiments were carried out on the anesthetized pig; blood flow and pressure were measured in the iliac artery. An adjustable snare was applied to the iliac above the pressure and flow measurement site to induce step decreases (3-4 occlusions at 5 min intervals were performed for each infusion) in blood flow, and hence iliac pressure, and the conductance (Δflow / Δpressure) calculated. Saline, insulin (20 and 40 mUSP/l/min), and metformin (1 μg/ml/min) were infused separately downstream of the adjustable snare and their effect on arterial conductance assessed. Insulin at both infusion rates and metformin caused a significant reduction in peripheral vascular conductance. In conclusion, hyperinsulinemia and metformin infusion constrict resistance arterial vessels *in vivo*.

Key words

Metformin • Insulin • Type 2 diabetes • Vascular resistance

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Introduction

The increase in the population of a group of conditions, insulin resistance, metabolic syndrome and type 2 diabetes mellitus associated with hypertension has focused attention on the effects of insulin and metformin upon blood vessels (Hardman *et al.* 1992, Dubrey *et al.* 1994, Stubbs *et al.* 1999a, b, Mather *et al.* 2001, Davis *et al.* 2006). It has been shown that insulin causes nitric oxide (NO) mediated vascular smooth muscle relaxation of the pig iliac, a conduit artery (Ruane-O'Hora *et al.* 2011, 2012). Hyperinsulinemia has also been shown to cause dilation of forearm vessels (Hermann *et al.* 2004), indicating a relaxation of resistance vessels, in addition insulin stimulates NO production from isolated umbilical vein endothelial cells (Zeng *et al.* 2000). However, insulin also releases endothelin and reactive oxygen species which are both vasoconstrictors (Ferri *et al.* 1995, Cardillo *et al.* 1999, Potenza *et al.* 2009). In fact, there are a number of published studies on peripheral resistance vessels that are not compatible with a dilatory effect of insulin (Sobrevia *et al.* 1998, Westerbacka *et al.* 2001, Westerbacka and Yki-Jarvinen 2002, Hansen *et al.* 2004, Jansson 2007, Kearney *et al.* 2008, Potenza *et al.* 2009). More difficult to interpret are the effects of insulin in addition to adrenaline or acetylcholine application (McNally *et al.* 1995, Mayhan *et al.* 2001), or contraction in response to electrical field stimulation (Garcia-Villalon *et al.* 2000) and inhibition of contraction by insulin-stimulated glucose transport (Kahn *et al.* 1995, Kahn and Song 1995).

It is possible that the contrary results in the literature on the effect of insulin on arterial diameter

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could be due to duration of exposure; specifically, indirect effects that could take place over longer time durations compared with acute exposure, as used in the iliac artery experiments (Ruane-O'Hora *et al.* 2011, 2012, 2013a, b). In the present study we set out to test this hypothesis by making direct acute exposures of the porcine iliac peripheral vascular bed to porcine insulin as well as metformin, which is the mainstay of treatment in type 2 diabetes, and has also been shown to cause NO-mediated dilation of the iliac artery (Davis *et al.* 2006, Ruane-O'Hora *et al.* 2012).

Methods

This investigation was carried out under licenses issued by the Department of Health Ireland as directed by the Cruelty to Animals Act Ireland and European Union and International Statutory Instructions.

Surgery and instrumentation

Ten female landrace pigs (20-25 kg) were sedated with ketamine (14 mg/kg) and xylazine (2.7 mg/kg) i.m., not all ten were subjected to all the interventions, the half-life of ketamine is 10-15 min and so the dose used for sedation would not affect the measured physiological parameters. A cannula was inserted into an ear vein and the animal was anesthetized with a bolus, followed by a continuous infusion of sodium pentobarbital (induction 30 mg/kg; maintenance 6 mg/kg/h i.v.). The continuous infusion was maintained *via* a catheter inserted into the jugular vein, using an infusion pump (Harvard). End-tidal carbon dioxide ($ETCO_2$), pulse oximetry and core temperature were monitored using SurgiVet AdvisorVital Signs Monitor (Smiths Medical, Dublin, OH). Arterial pH, PCO_2 and PO_2 were assessed using a hand held i-STAT blood gas analyzer (Abbot Point of Care Inc, Princeton, NJ) and maintained within their normal ranges. Blood glucose was monitored with a Bayer Glucometer. Following tracheotomy animals were ventilated with 40 % O_2 in room air using a Harvard ventilation pump at a rate adjusted to keep end-tidal and arterial PCO_2 within a normal range. A cannula attached to a pressure transducer (Grass; Grass Technologies, West Warwick, RI) was inserted into the left carotid artery for measurement of arterial blood pressure. The iliac artery was prepared as previously described (Kelly *et al.* 2006). Briefly, the left or right iliac artery was dissected from the aortic bifurcation to the deep femoral branch, and a cannula attached to a 3-way tap was inserted into the deep femoral artery for infusions of saline, insulin, or metformin downstream of the flow and pressure measurement sites in the iliac. During certain experiments, ultrasonic piezoelectric crystals were placed on diametrically opposite sides of the iliac artery for continuous measurement of the diameter using a sonomicrometer (Sonometrics Corporation, London, Ontario, Canada). An ultra-sonic transit time flow transducer (Transonic Systems Inc, Ithaca, New York, NY) was placed around the artery to measure blood flow. Pressure within the iliac was measured with a second catheter tipped manometer (Millar, Houston, USA) placed in a side branch of the iliac. An adjustable snare was placed just below the iliac-aorta bifurcation, above the pressure and flow measurement sites, to produce controlled restrictions to blood flow. Hemodynamic signals were recorded using Power lab pre-amplifiers and software (AD Instruments Ltd, Oxford, UK) and a Dell computer. Electronic measurements of hemodynamic variables were taken off-line using Chart 7 software (AD Instruments). Following experimental procedures, animals were killed using a lethal intravenous injection of pentobarbitone and KCl.

Experimental procedure

Following instrumentation, all variables inscribed on the electronic display of the Powerlab system were monitored until they remained constant. This steady state was maintained for half an hour before proceeding with the following interventions.

The proximal snare was used to restrict blood flow 3-4 times control levels as recently described (Ruane-O'Hora *et al.* 2013a, b). Close intra-arterial infusions *via* the deep femoral artery were made of: (a) saline at the same flow rate as the insulin solution infusion rates, (b) insulin at an infusion at a rate of 20 or 40 mUSP/l/min, (c) metformin at an infusion rate of 1 μg/ml/min. The infused concentrations of insulin and metformin were chosen because they cause NO mediated dilatation of the iliac conduit artery (O'Hora *et al.* 2012). For each infusion, changes in flow and arterial pressure were observed in the absence of any occlusion of the test segment below the snare. Then, while continuing the infusion for 4-5 min, the occluders were tightened in distinct steps of restriction of blood flow and flow and test segment pressure recorded.

Measurements and statistical analysis

Chart records were analyzed off line in accordance with accepted principles (Braakman *et al.* 1983). Group values were expressed as mean ± S.E.M. The instantaneous peripheral vascular conductance was assessed for individual step changes at each step as the immediate drop flow (ΔF), divided by the instantaneous drop in pressure (ΔP). In the data illustrated in Figure 2, the lines connect the saline to the intervention values in each pig and were normally distributed and analyzed by paired t test. The slopes were subjected to homogeneity of regressions analysis (SPSS). The F variance ratios were calculated for differences in slopes (Drake-Holland *et al.* 1984).

All data were tested for normal distribution. Normally distributed data were subjected to unpaired or paired Student's t test as appropriate. Non-normally distributed data were subjected to Mann Whitney or Wilcoxon paired sign test, or paired chi square (with correction for continuity) as appropriate. Comparison of linear regression data were carried out by ANCOVA.

Results

Insulin

Infusion of saline or insulin was carried out during step decreases in blood flow and test segment pressure, as described recently (Ruane-O'Hora *et al.* 2013a, b). The recovery of flow and test segment pressure from the initial value after the step was due to autoregulation and was seen in both saline and insulin infusion runs.

In order to enumerate the change in peripheral vascular conductance more precisely, the linear regression lines for the change in flow versus the change in diameter for the experiments in which saline or the same infusion rate to achieve delivery of 20 and 40 mUSP/l/min insulin in blood (Fig. 1). Both infusion rates of insulin, 40 and 20 mUSP/l/min, caused a significant decrease in conductance, the saline slope for the lower infusion rate (Fig. 1A) fell from 1.69±0.16 (slope ± s.e. slope) to 1.27±0.43 ml/min/mm Hg ($P<0.00001$, homogeneity of slopes test); the saline slope for the insulin 40 mUSP/l/min was 1.58±0.17 and this fell to 0.67±0.32 ml/min/mm Hg ($P<0.00001$, homogeneity of slopes test). There was no significant difference between the slopes of the insulin infusion ($P=0.514$, homogeneity of slopes test).

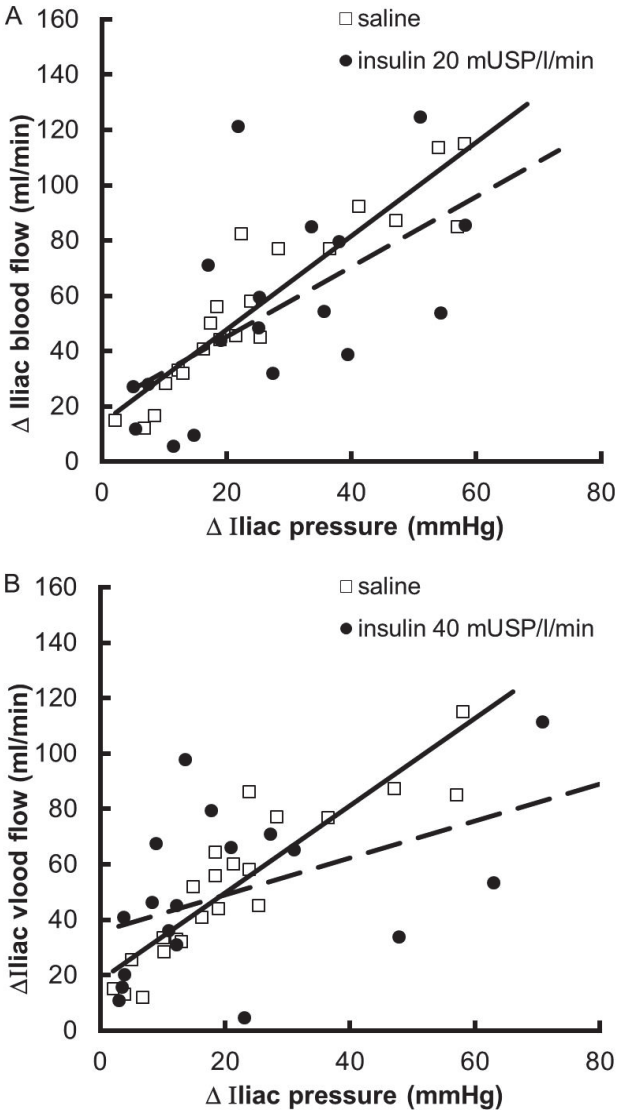

Fig. 1. The effect of insulin infusion on arterial conductance. Insulin infusion at 20 mUSP/l/min (**A**; n=6; dashed line) and 40 mUSP/l/min (**B**; n=6, dashed line) both caused a significant decrease ($P<0.0001$, test homogeneity of slope) in arterial conductance when compared to saline infusion (solid line).

Metformin

The recovery of flow and test segment pressure from the initial value after the step was due to autoregulation and was seen in both saline and metformin infusion runs. Exposure of the iliac peripheral vasculature to 1 μg/ml/min compared with saline controls resulted in the values presented in Figure 2. The mean peripheral vascular conductance decreased with metformin from 2.38±0.15 control to 1.72±0.17 ml/min/mm Hg ($P=0.001$, paired t test). Metformin infusion caused significant decreased conductance, i.e. an increase in vascular resistance, the saline slope fell from 1.69±0.16 (slope ± s.e. slope) to 1.25±0.3 ml/min/mm Hg ($P<0.00001$, homogeneity of slopes test).

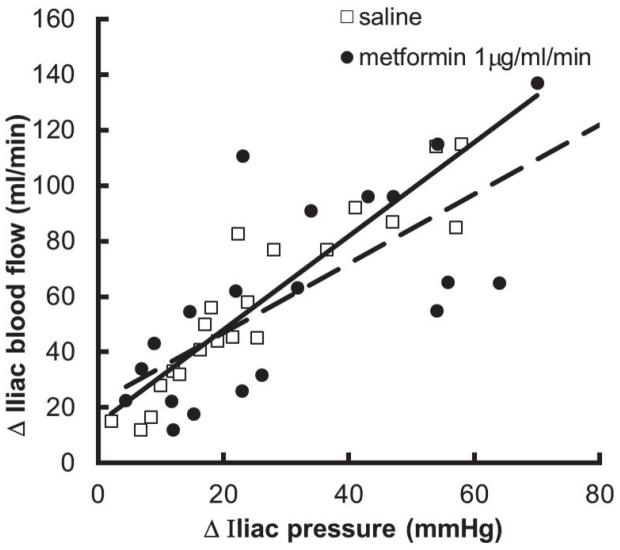

Fig. 2. The effect of metformin infusion on arterial conductance. Metformin infusion at 1 μg/ml/min (n=6, dashed line) caused a significant decrease (P<0.0001, test homogeneity of slope) in arterial conductance when compared to saline infusion (solid line).

Discussion

Insulin

We previously examined the direct effect of applying raised insulin doses to the endothelial luminal surface of the pig iliac artery, and recorded a NO dependent dilatation (Ruane-O'Hora *et al.* 2011, 2012). The results were compatible with those from un-anesthetized human studies, because our experiments could be influenced by species variation and the effects of sedation and anesthesia, as could the other animal studies referenced. There were no other studies available on the effects of insulin on conduit artery, but there was considerable published data on the effect of insulin on resistance vessels. Insulin is thought to be a NO dependent dilator of resistance vessels (Steinberg *et al.* 1994, Muniyappa *et al.* 2007) and to increase NO synthase (Kawaguchi *et al.* 2001). This effect was thought to be indirect, since it was claimed to occur when insulin is administered systemically, but not following direct arterial injection (Cardillo *et al.* 1998). The absence of a direct effect of insulin on peripheral resistance was accompanied by mechanistic data showing an increase in endothelin-1, leading to vasoconstriction (Cardillo *et al.* 1999, Muniyappa *et al.* 2007) and data showing activation of the sympathetic nervous system (Muniyappa *et al.* 2007). In order to exclude indirect effects of systemic hyperinsulinemia (Baron 1994), in the present study a method involving step changes in pressure and flow into the peripheral iliac vascular bed was used (Ruane-O'Hora *et al.* 2013a, b).

Interest in the effect of insulin resistance associated with high insulin levels in patients with acute coronary syndromes (Stubbs *et al.* 1999a, b) was re-aroused by the findings that the effect of insulin on vascular endothelium is impaired in insulin resistant subjects (Sobrevia *et al.* 1998, Jansson 2007, Kearney *et al.* 2008, Potenza *et al.* 2009). The effect of insulin on arterial stiffness is also impaired in insulin resistant subjects (Westerbacka *et al.* 2001, Westerbacka and Yki-Jarvinen 2002, Hansen *et al.* 2004). These effects were clearly contrary to our findings of NO dependent smooth muscle relaxation in conduit artery wall (Ruane-O'Hora *et al.* 2011, 2012), and led to the hypothesis that there was a direct dilatory effect of insulin on peripheral resistance vessels, and that the contrary effects in the literature were secondary, occurring over a longer time span.

The results of the present study clearly disprove this hypothesis, since the direct acute effect of insulin upon peripheral vascular conductance actually found was one of vasoconstriction, not the expected vasodilatation. There is a clear separation of the data for changes in flow during step changes in pressure in the negative direction. The results of the insulin infusions suggest that there may be a positive concentration-response relationship, but full delineation of this would require a very large number of experiments, which were beyond our resources.

This leads us to conclude that the dilatory effects of insulin on peripheral resistance vessels previously found by Cardillo *et al.* (1998, 1999) were correctly concluded by those authors to be indirect. Their failure to detect a direct constrictor effect could be attributed to the lesser accuracy of measurement possible in human subjects, compared to an invasive animal method. The results also seem compatible with the conclusions indicating that high insulin concentrations in insulin resistance subjects cause a constricting effect upon the vasculature (Cardillo *et al.* 1999, Potenza *et al.* 2009). In addition to a possible contribution to increased arterial stiffness and hypertension found in such patients; no doubt there are other contributing factors to this hypertension. Our results are also compatible with the insulin-induced vasoconstriction associated with protein kinase C activation (Bakker *et al.* 2008). In isolated cerebral vessels, an initial vasoconstriction was seen, followed by vasodilation (Katakam *et al.* 2009),

suggesting compatibility with our observations of initial responses only; the vasoconstriction in these experiments was abolished by removal of reactive oxygen species. When insulin-mediated vasorelaxation is the dominant response, as in isolated aortic rings, the relaxation is enhanced by endothelin antagonism (Elgebaly *et al.* 2008). It is claimed that endothelin ET-1 and ETA receptors are activated by hyperglycemia and alter vascular structure in Type 2 diabetes (Sachidanandam *et al.* 2009).

There are clear associations between diabetes mellitus and hypertension, a condition of high peripheral resistance (Sowers 2013) and between diabetes mellitus and endothelial dysfunction (Cosentino and Luscher 1998). Of particular concern is the prevalence of insulin resistance (Park *et al.* 2005), a predictor of future diabetes mellitus in young subjects identified this as a cardiovascular risk in young Finnish subjects (Park *et al.* 2005). The "SEARCH" for diabetes in youth is a multicentre study of the prevalence of this condition from early reports (SEARCH for Diabetes in Youth Study Group 2006, Writing Group for the SEARCH for Diabetes in Youth Study Group 2007). Of particular relevance to our present interest is the endothelial dysfunction found in insulin resistance (Kearney *et al.* 2008) and the concept of endothelial insulin resistance (Kanter and Bornfeldt 2013).

Metformin

This drug is accepted by many as first line treatment in diabetes mellitus which is not insulin dependent (Type 2) (United Kingdom Prospective Diabetes Study 1995, Holman 2007) and is resistant to dietary control (Brehm *et al.* 2003, Samaha *et al.* 2003). It is widely recommended as having a hypoglycemic action, a beneficial effect on endothelial dysfunction (Mather *et al.* 2001) and to induce reversal of insulin resistance (Verma *et al.* 2000, Wiernsperger 2000, Clarke *et al.* 2005). The drug is not thought to affect blood pressure (Wulffele *et al.* 2004). Most relevant to the present study is the finding of improved peripheral vascular flow (Sirtori *et al.* 1984) over longer duration of metformin exposure than our experimental period (20 min). Nevertheless we postulated that we would find a peripheral vasodilatation within 20 min but this was disproved in the present series of experiments.

There are few accounts of metformin inducing increased vascular resistance deleted, in accordance with our present findings. The main mechanism claimed for the effect of metformin on vascular function, is activation of AMP-activated protein kinase. However, this would not explain a short term vasoconstrictive effect, for which no mechanism has appeared in the literature. It is possible that our observations have been missed in existing studies, in which the duration of metformin application is much longer than in our experiments.

Conclusions

It would appear from our results *in vivo*, that the vasoconstrictor effects of insulin upon peripheral resistance, attributed to endothelin and reactive oxygen species, out-weigh the vasodilator effect attributed to NO production. The finding of short term vasoconstriction in response to metformin infusion appears to be at variance with all other reports of the effects of metformin on the vasculature. Both these findings were unexpected, as our previous studies on conduit artery revealed NO-mediated dilatation with both agents!

Conflict of Interest

There is no conflict of interest.

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