Adiponectin opposes endothelin-1-mediated vasoconstriction in the perfused rat hindlimb

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Adiponectin is one of a variety of metabolically active proteins secreted by adipocytes, collectively termed adipokines (39). Decreased levels of adiponectin are associated with a variety of disease states, including obesity, type 2 diabetes, hypertension, and coronary artery disease (1, 21, 23, 30, 48). Adiponectin administration causes weight loss in mice fed a high-fat diet and ameliorates insulin resistance in mouse models of obesity and type 2 diabetes (16, 52). Conversely, genetic deletion of adiponectin results in moderate insulin resistance in mice fed a high-fat diet and ameliorates insulin resistance in mouse models of obesity and type 2 diabetes (16, 52). Conversely, genetic deletion of adiponectin results in moderate insulin resistance in mice fed a high-fat diet and ameliorates insulin resistance in mouse models of obesity and type 2 diabetes (16, 52).

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hindlimb, at low doses, ET-1 stimulates metabolism [oxygen consumption rate and glucose uptake, in a similar manner to other “type A” vasoconstrictors such as angiotensin II or low doses of norepinephrine (NE) in the perfused hindlimb], whereas high doses lead to metabolic inhibition of oxygen consumption rate and glucose uptake (similar to the “type B” vasoconstrictors serotonin or high-dose NE) (24). These two different types of vasoconstriction are thought to be the result of constriction occurring at different locations in the vascular tree and thus affecting the extent of skeletal muscle perfusion (for review see Ref. 9). We then examined the action of adiponectin against low-dose NE, a vasoconstrictor with dose-dependent metabolic actions similar to those of ET-1 in the perfused hindlimb (12, 24), to assess the specificity of any vascular action of adiponectin. We found that adiponectin specifically inhibits ET-1-mediated vasoconstriction by an apparently NO-independent mechanism.

**METHODS**

**Adiponectin preparation.** The mouse full-length adiponectin cDNA excluding the NH2-terminal signal sequence (718 bp; GenBank accession no. NM_009605) was amplified from 3T3-L1 adipocytes, using the 5'-CACCAGTCATGCGAAGATGA-3' sense and 5'-ATGGGTAGTTGCAGTCAGTTGG-3' antisense primers. This sequence was incorporated into the pET100/D-TOPO bacterial expression vector (Invitrogen, Carlsbad, CA). Standard DNA sequencing and PCR analyses were performed to confirm plasmid fidelity and orientation. Addition of isopropyl β-D-1-thiogalactopyranoside to the growth medium of BL21(DE3) Escherichia coli (Invitrogen) induced expression of the histidine-tagged adiponectin, which was purified from bacterial extracts via elution from a nickel-nitrilotriacetic acid agarose column (Invitrogen). Samples were dialyzed using Vivaspin 20 ultrafiltration units (Sartorius, Göttingen, Germany) to remove contaminating imidazole, and protein concentrations were assessed using the Lowry method (DC Protein Assay; Bio-Rad, Hercules, CA) (26). The recombinant adiponectin was separated by 3–8% SDS-PAGE (Invitrogen) under nondenaturing, nonreducing conditions. This was followed by electrophoretic transfer to nitrocellulose membrane and immunoblotting with an anti-adiponectin antibody (AB3269P; Chemicon, Temecula, CA; dilution of 1:5,000). Blots were then probed with a secondary antibody linked to horseradish peroxidase (Cell Signaling, Danvers, MA), and immunoreactive proteins were visualized using an enhanced chemiluminescent method (Pierce Biotechnology, Rockford, IL). Adiponectin preparations were stored at 4°C for up to 30 days.

**Animals.** All experiments were approved by the University of Tasmania Animal Ethics Committee and were performed in accordance with the guidelines set out under the National Health and Medical Research Council of Australia Code of Practice for the care and use of animals for scientific purposes (7th edition). Male hooded Wistar rats (n = 85) were housed at 21 ± 1°C under a 12:12-h light-dark cycle. Rats were provided with rat chow (Gibson’s; Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber by weight, with added vitamins and minerals, and water ad libitum.

**Hindlimb perfusions.** Hindlimb surgery was performed on rats (180–200 g) as described previously (37), with additional details as outlined elsewhere (11). Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and allowed to freely breathe room air. Once the animal was unresponsive to a tail pinch, ligatures were positioned around the tarsus of the experimental leg and the tail to restrict blood flow. Vessels supplying the testes, bladder and seminal vesicles, colon, large intestine, and duodenum were ligated to allow removal of the organs. Ligatures were placed around the superficial epiepigastric vessels, iliofemoral vessels, and the common iliac artery supplying the contratraleral hindlimb, since only one hindlimb was perfused. The descending aorta and vena cava were cannulated between the origins of the renal and iliolumbar vessels, and the cannulas were connected to the perfusion line. The entire procedure was completed within 25 min, with care taken to ensure perfusion of the experimental hindlimb was interrupted for no more than 2 min. Once the hindlimb was connected to the perfusion network, the animal was killed with an intracardial overdose of pentobarbital sodium and a further ligature was positioned around the abdomen at approximately the level of the L3 vertebra.

The hindlimb was perfused with Krebs-Henseleit bicarbonate buffer containing 2.5 mM CaCl2, 8.3 mM glucose, and 4% (wt/vol) bovine serum albumin at a constant flow rate of 5 ml/min unless otherwise stated. The perfusate was gassed with 95% O2-5% CO2 in a Silastic tube oxygenator. The experiment was conducted at 32°C in a temperature-controlled cabinet, with the buffer temperature maintained by passage through a heat exchanger coil. Perfusion pressure was constantly monitored via a pressure transducer located in the arterial line. Venous effluent oxygen content was measured using a flow-through Clark-type oxygen electrode assembly, also maintained at 32°C. Oxygen uptake was calculated as described previously (12). The venous effluent was periodically sampled and the remainder discarded. Glucose and lactate determinations were made using a glucose analyzer (model 2300 Stat Plus; Yellow Springs Instruments), and hindlimb uptake or release data, respectively, were derived from the product of flow and arteriovenous (A-V) concentration difference. The hindlimb was allowed to equilibrate for 30–40 min to reach a steady state before any experimental protocols were commenced.

Adiponectin solutions were diluted to 0.65 mg/ml in saline and infused at 50 µl/min, equivalent to 1/100th of the pump flow rate, to give a final concentration of 6.5 µg/ml. Insulin (Humulin R; Eli Lilly, Indianapolis, IN) was diluted in saline and infused at 50 µl/min, to provide 15 nM to the hindlimb. ET-1 (Calbiochem, San Diego, CA) was dissolved in acetic acid and made up to volume in saline. NE (Arterenol) was obtained from Sigma-Aldrich (St. Louis, MO). Both...
Vasoconstrictors were infused at 25 μl/min, 1/200th of the pump flow rate, providing final concentrations of 1 or 20 nM ET-1 and 50 nM NE. Sodium nitroprusside (SNP; Sigma-Aldrich) was infused at 1/200th of the flow rate to achieve a final concentration of 50 μM. All were dissolved in 0.9% NaCl before infusion. Substances were infused into a small magnetically stirred bubble trap located in the arterial perfusion line.

**Statistics.** Two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science, Chicago, IL). Differences between conditions were assessed using the Student-Newman-Keuls post hoc test. Significance was assumed at the level of $P < 0.05$. Data are means ± SE; if error bars are not visible, they are within the symbol.

**RESULTS**

Native state of recombinant adiponectin. Similarly to that found in human plasma, rat adiponectin circulates as low-,
Despite the observed increase in glucose uptake during adiponectin infusion, there was no concomitant increase in lactate release, unlike that seen with insulin treatment, suggesting that the transported glucose is either oxidized or stored as glycogen (Fig. 2D). In addition, no significant adiponectin-induced change in oxygen consumption was seen under basal conditions (Fig. 2B).

Effect of adiponectin on the hemodynamic and metabolic effects of ET-1. Low-dose ET-1 (1 nM) significantly increased perfusion pressure within 10 min of administration, with an increase of 29.3 ± 4.4 mmHg following a 40-min infusion (P < 0.001; Fig. 3A). The increased perfusion pressure was accompanied by the enhanced metabolism typical of type A vasoconstrictors (9), indicated by increases in oxygen consumption (1.3 ± 0.2 μmol·g\(^{-1}\)·h\(^{-1}\)), glucose uptake (7.4 ± 0.9 μmol·g\(^{-1}\)·h\(^{-1}\)), and lactate release (15.8 ± 2.7 μmol·g\(^{-1}\)·h\(^{-1}\)), similar to the effects we have previously reported (24). Pretreatment and coinfusion with physiological levels of adiponectin led to a significant attenuation of ET-1-mediated vasoconstriction from 15 min after ET-1 infusion commenced until the end of the protocol (Fig. 3A). Perfusion pressure was increased by 14.3 ± 2.1 mmHg, representing a 51% reduction compared with ET-1 alone (P < 0.001). In conjunction, the associated metabolic stimulation was also diminished, with the increases in oxygen consumption, glucose uptake, and lactate release blunted by 44% (0.7 ± 0.1 μmol·g\(^{-1}\)·h\(^{-1}\)), 57% (3.2 ± 0.6 μmol·g\(^{-1}\)·h\(^{-1}\)), and 36% (10.0 ± 1.9 μmol·g\(^{-1}\)·h\(^{-1}\)), respectively (Fig. 3). SNP similarly blocked ET-1-mediated metabolic stimulation along with the vasoconstriction, indicating that this is likely to be secondary to the vascular relaxation (24). The attenuated response to ET-1 was maintained after adiponectin coinfusion ceased, and indeed, the differences between the two groups continued to increase for the duration of the experiment, indicating that the vascular actions of adiponectin are not rapidly reversible.

We next examined whether adiponectin was able to oppose established ET-1-mediated vasoconstriction. The previous experiments were repeated with the adiponectin infusion commencing 10 min after the ET-1 infusion. Once ET-1 vasoconstriction had developed, a physiological dose of adiponectin was unable to attenuate the increase in perfusion pressure or the associated stimulation of oxygen uptake (Fig. 4). Similarly, there was no significant difference in glucose uptake between rats treated with adiponectin and those without, with an increase of 5.5 ± 1.2 μmol·g\(^{-1}\)·h\(^{-1}\) following coinfusion com-
pared with 4.8 ± 0.8 µmol·g⁻¹·h⁻¹ after 40 min of ET-1 alone. Adiponectin did cause a small but significant diminution in lactate release by the end of the experiment, reducing it to 11.6 ± 1.4 µmol·g⁻¹·h⁻¹ compared with 15.6 ± 2.4 µmol·g⁻¹·h⁻¹ after ET-1 infusion alone (P < 0.05). In contrast to the effect of adiponectin, SNP was effective at reducing ET-1-mediated vasoconstriction, even when it had already developed (Fig. 5). This effect was dramatic and rapid, with perfusion pressure returning to a level not significantly different from basal within 2 min of the SNP infusion commencing (P > 0.05). Perfusion pressure continued to rise again from this point, once again becoming significantly different from basal 5 min into the SNP infusion but persisting at a significantly lower level than that seen with ET-1 alone for the remainder of the experiment (P < 0.05).

The biphasic dose-response to ET-1 that we have previously reported was again demonstrated (24). A 20 nM ET-1 infusion elicited a marked increase in perfusion pressure of 185.8 ± 13.7 mmHg (P < 0.001; Fig. 6A), but unlike the 1 nM ET-1 dose, the high dose caused a transient metabolic stimulation before shifting toward an inhibition of metabolism. After a 40-min infusion of high-dose ET-1, oxygen consumption was significantly inhibited compared with basal, with a net decrease in oxygen uptake of 2.9 ± 0.7 µmol·g⁻¹·h⁻¹ (P < 0.001; Fig. 6B). Glucose uptake and lactate release showed a similar trend, with significant (P < 0.001) increases of 10.6 ± 0.8 and 40.2 ± 3.0 µmol·g⁻¹·h⁻¹, respectively, after 10 min of ET-1 infusion and a return to near basal levels by 40 min (Fig. 6). Thus high doses of ET-1 were again seen to display a type B vasconstrictor response with an overall metabolic inhibition (9). There was no overall significant difference in any of the parameters measured between rats treated with 20 nM ET-1 alone or in conjunction with adiponectin. Therefore, physiological adiponectin was unable to overcome the effects of high-dose ET-1-mediated vasoconstriction (Fig. 6).

Effect of adiponectin on the hemodynamic and metabolic effects of NE. Infusion of low-dose NE caused rapid vasoconstriction, demonstrated by an increase in perfusion pressure of 13.0 mmHg after 5 min (P < 0.01; Fig. 7A). A low NE dose was used to stimulate type A vasconstriction (9), similar to that observed with low-dose ET-1. Enhanced metabolism was demonstrated by a significant increase in oxygen consumption of 1.7 ± 0.2 µmol·g⁻¹·h⁻¹ after 40 min of treatment (P < 0.001; Fig. 7B). Glucose uptake and lactate release were similarly stimulated, with maximal increases of 5.8 ± 0.9 and 8.2 ± 1.4 µmol·g⁻¹·h⁻¹, respectively, after 5 min of NE infusion. After ~10 min, glucose uptake and lactate release began to decline, illustrating the short-acting nature of NE in contrast to the long-lasting effects of ET-1. Pretreatment and coinfusion with adiponectin, using the same protocol as the original ET-1 experiments, had no effect on any measure of NE action under these conditions (Fig. 7).

DISCUSSION

This study demonstrated that adiponectin opposes ET-1-mediated vasoconstriction, although no change in perfusion pressure was observed with adiponectin alone. Adiponectin was unable to overcome vasoconstriction due to ET-1 once it had developed or that caused by high doses of ET-1. In addition, under the same conditions, adiponectin infusion had no effect on NE-induced vasoconstriction, suggesting a specific interaction between adiponectin and ET-1.

The attenuation of ET-1 by adiponectin observed in this study appears to be due to a specific action of adiponectin on ET-1-mediated vasoconstriction and its associated metabolic effects. No hemodynamic changes were observed during the infusion of adiponectin alone. This is probably due to the dilated state of this denervated, blood-free preparation under basal conditions. Insulin infusion alone similarly causes no
that adiponectin may not, in fact, be acting as a NO-dependent dilator. This is because the local nature of ET-1 production means that the effects are high in the area where ET-1 is produced, but lower in areas where NO is produced. However, it is unknown whether in vivo ET-1 levels are high. Therefore, the reported insulin-sensitizing effects of adiponectin, and the ability of insulin to modulate ET-1 action, may provide an avenue for investigation.

Several groups have proposed that the hemodynamic effects of insulin are regulated by the balance between the vasodilator and vasoconstrictor effects of NO and ET-1, respectively (4, 13, 45). Thus the ability of adiponectin to oppose ET-1-mediated vasoconstriction is an important aspect of its insulin-sensitizing actions, leading to enhanced insulin action in muscle due to improved glucose and insulin supply to myocytes (10). In addition, the actions of ET-1 and NO are mutually antagonistic, allowing any imbalance, such as those seen in obesity or insulin resistance, to be amplified. The hypoadiponectinemia present in obesity, and the consequent reduction in the limiting effects of adiponectin on ET-1-mediated vasoconstriction, is a potential activator of this positive-feedback cycle. Progressive dysregulation of the relationship between ET-1 and NO may then contribute to the endo-feedback cycle. Progressive dysregulation of the relationship between ET-1 and NO may then contribute to the endothelial dysregulation and thus not require pretreatment as demonstrated in the present study. The finding that adiponectin did not diminish NE-mediated vasoconstriction, whereas the endothelial NO-dependent dilators UTP and ATP both rapidly dilate against NE (35), further points to a NO-independent process. In fact, there is a precedent for this in a recent study by Fesus et al. (15), who reported adiponectin-mediated relaxation of denuded aortic rat rings preconstricted with serotonin. Thus it is possible that the hemodynamic effects of adiponectin described in the present study may be due to a specific inhibitory action on the ET-1 signaling pathway in vascular smooth muscle cells. ET-1 and NE elicit vasoconstriction via similar pathways, acting via inositol trisphosphate to stimulate calcium release from the sarcoplasmic reticulum of vascular smooth muscle cells (47). The considerable overlap between the ET-1 and NE contractile pathways leaves few targets for the specific action of adiponectin, although differences in the ion channels activated for extracellular calcium entry, such as the L-type voltage-operated calcium channels reported to be activated by NE but not ET-1 (18), may provide an avenue for investigation. Alternatively, the requirement for adiponectin to be present before ET-1 exposure suggests a mechanism early in the pathway, probably operating between the receptor and inositol trisphosphate formation, before the signaling pathways of the two vasoconstrictors merge. One possibility suggested by reports of direct binding of growth factors by adiponectin (46a) is the attenuation of ET-1 action by its sequestration by adiponectin. This would explain why adiponectin cannot reverse vasoconstriction by ET-1 when added after ET-1, if ET-1-mediated vasoconstriction is irreversible once established. However, removal of ET-1 led to an immediate, albeit slow, reversal of perfusion pressure, suggesting that adiponectin does not act by sequestering ET-1 (Supplemental Fig. S1). (Supplemental material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.) Further studies are needed to determine the mechanism via which adiponectin is able to modulate ET-1 action.

The inability of adiponectin to moderate the actions of ET-1 when introduced after vasoconstriction is established suggests that adiponectin may not, in fact, be acting as a NO-dependent vasodilator as hypothesized. In contrast to adiponectin, SNP, a direct NO donor, rapidly reduced perfusion pressure when administered during ET-1-mediated vasoconstriction. Chen et al. (6) studied adiponectin-mediated NO production in endothelial cells and found that this effect was rapid, reaching its peak within a few minutes of exposure to adiponectin. This suggests that were adiponectin-mediated inhibition of ET-1 occurring via endothelial NO release, it would be a rapid effect and thus not require pretreatment as demonstrated in the present study. The finding that adiponectin did not diminish NE-mediated vasoconstriction, whereas the endothelial NO-dependent dilators UTP and ATP both rapidly dilate against NE (35), further points to a NO-independent process. In fact, there is a precedent for this in a recent study by Fesus et al. (15), who reported adiponectin-mediated relaxation of denuded aortic rat rings preconstricted with serotonin. Thus it is possible that the hemodynamic effects of adiponectin described in the present study may be due to a specific inhibitory action on the ET-1 signaling pathway in vascular smooth muscle cells. ET-1 and NE elicit vasoconstriction via similar pathways, acting via inositol trisphosphate to stimulate calcium release from the sarcoplasmic reticulum of vascular smooth muscle cells (47). The considerable overlap between the ET-1 and NE contractile pathways leaves few targets for the specific action of adiponectin, although differences in the ion channels activated for extracellular calcium entry, such as the L-type voltage-operated calcium channels reported to be activated by NE but not ET-1 (18), may provide an avenue for investigation. Alternatively, the requirement for adiponectin to be present before ET-1 exposure suggests a mechanism early in the pathway, probably operating between the receptor and inositol trisphosphate formation, before the signaling pathways of the two vasoconstrictors merge. One possibility suggested by reports of direct binding of growth factors by adiponectin (46a) is the attenuation of ET-1 action by its sequestration by adiponectin. This would explain why adiponectin cannot reverse vasoconstriction by ET-1 when added after ET-1, if ET-1-mediated vasoconstriction is irreversible once established. However, removal of ET-1 led to an immediate, albeit slow, reversal of perfusion pressure, suggesting that adiponectin does not act by sequestering ET-1 (Supplemental Fig. S1). (Supplemental material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.) Further studies are needed to determine the mechanism via which adiponectin is able to modulate ET-1 action.

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thelial dysfunction observed in hypertension and type 2 diabetes (27). Therefore, in the absence of adiponectin resistance, therapeutic targeting of adiponectin may redress the imbalance between ET-1 and NO in insulin resistance and hypertension and improve disease outcomes in these states.

In summary, this study has identified a specific activity of adiponectin to oppose the vasoconstrictor effects of ET-1 and its associated metabolic effects. The lack of such an effect once vasoconstriction is established or against NE indicates that adiponectin is unlikely to be acting via a NO-dependent mechanism. These findings suggest that an interaction between adiponectin and ET-1 may contribute to the maintenance of normal vascular tone. Imbalance in this relationship in obesity may contribute to the development of insulin resistance and cardiovascular disease.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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