Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment

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1Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia Health System, Charlottesville, Virginia; 2Department of Medicine, Shandong University Jinan Central Hospital, Shandong, China; and 3Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina

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Dong Z, Chai W, Wang W, Zhao L, Fu Z, Cao W, Liu Z. Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment. Am J Physiol Endocrinol Metab 304: E222–E228, 2013. First published November 27, 2012; doi:10.1152/ajpendo.00473.2012.—Glucagon-like peptide 1 (GLP-1) causes vasodilation and increases muscle glucose uptake independent of insulin. Recently, we have shown that GLP-1 recruits muscle microvasculature and increases muscle glucose use via a nitric oxide (NO)-dependent mechanism. Protein kinase A (PKA) is a major signaling intermediate downstream of GLP-1 receptors. To examine whether PKA mediates GLP-1’s microvascular action in muscle, GLP-1 was infused to overnight-fasted male rats for 120 min in the presence or absence of H89, a PKA inhibitor. Hindleg muscle microvascular recruitment and glucose use were determined. GLP-1 infusion acutely increased muscle microvascular blood volume within 30 min without altering microvascular blood flow velocity or blood pressure. This effect persisted throughout the 120-min infusion period, leading to a significant increase in muscle microvascular blood flow. These changes were paralleled with an approximately twofold increase in plasma NO levels and hindleg glucose extraction. Systemic infusion of H89 completely blocked GLP-1-mediated muscle microvascular recruitment and increases in NO production and muscle glucose extraction. In cultured endothelial cells, GLP-1 acutely increased PKA activity and stimulated endothelial NO synthesis, and PKA inhibition abolished these effects. In ex vivo studies, perfusion of the distal saphenous artery with GLP-1 induced significant vasorelaxation that was also abolished by pretreatment of the vessels with PKA inhibitor H89. We conclude that GLP-1 recruits muscle microvasculature by expanding microvascular volume and increases glucose extraction in muscle via a PKA/NO-dependent pathway in the vascular endothelium. This may contribute to postprandial glycemic control and complication prevention in diabetes.

MATERIALS AND METHODS

Animal preparations and experimental protocols. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 220–320 g were studied after an overnight fast. Rats were housed at 22 ± 2°C on a 12:12-h light-dark cycle and fed standard chow ad libitum. Rats were randomly assigned to the following experimental groups: control (Group C; n = 8), GLP-1 (Group GLP; n = 8), and sitagliptin (Group SIG; n = 8). Sitagliptin was administered subcutaneously at a dose of 100 mg/kg immediately before the experimental procedure. The control group received an equal volume of saline. Each treatment group consisted of three rats that were euthanized at each time point (20, 60, and 120 min). The animals were anesthetized with sodium pentobarbital, and the hearts were perfused retrogradely with saline and finally with 10% formalin. Posterior descending arteries or left anterior descending arteries were then removed, and the adventitia was left intact. The size of the arteries was measured using ImageJ software (National Institutes of Health, Bethesda, MD). Aortic conduit segments were analyzed using transmission electron microscopy to confirm the structural integrity of the arterial wall. The degree of medial thickening was estimated by measuring the ratio of medial thickness to the external diameter of the arterial wall. Finally, the arterial media were mounted on glass slides and stained with hematoxylin and eosin for histological examination. The presence of nuclear labeling for PKA catalytic subunit (PKAc) was determined using immunohistochemistry. The results are expressed as the mean ± SE. The statistical significance of differences among the groups was determined using one-way ANOVA followed by Bonferroni’s post hoc test. p < 0.05 was considered statistically significant.
laboratory chow and water ad libitum before the study. After being anesthetized with pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL), rats were placed in a supine position on a heating pad to ensure euthermia and intubated to maintain a patent airway. Polyethylene cannulae (PE-50; Fisher Scientific, Newark, DE) were inserted into the carotid artery and jugular vein for arterial blood pressure monitoring, arterial blood sampling, and various infusions.

After a 30- to 45-min baseline period to ensure hemodynamic stability and a stable level of anesthesia, rats were randomly studied in one of the following four groups (Fig. 1). Each rat received an intravenous infusion of either saline (control) or GLP-1-(7–36) amide (30 pmol·kg⁻¹·min⁻¹; Bachem Americas, Torrance, CA) for 120 min in the presence or absence of systemic infusion of H89 (160 pmol·kg⁻¹·min⁻¹; Sigma-Aldrich, St. Louis, MO). At the doses selected, GLP-1 has been shown to potentiate the release of insulin (25, 26). After serum starvation for 14 h, cells culture were purchased from Lonza (Walkersville, MD) and cultured as described previously (8). Cells between passages 3 and 8 were exposed to GLP-1-(7–36) amide (1 ng/ml; Bachem Americas, Torrance, CA) for 20 min in the absence of presence of H89 (10 μM; Sigma-Aldrich) and then used for either Western blotting, measurement of NO production, or PKA activity.

Measurement of plasma NO and insulin levels and endothelial NO production. Plasma NO levels were measured using a 280i Nitric Oxide Analyzer (GE Analytical, Boulder, CO) according to the manufacturer’s instructions and as described previously (8, 9). Plasma insulin concentrations were determined using a rat insulin ELISA assay kit (Mercodia, Uppsala, Sweden). For endothelial NO production, bAECs were exposed to GLP-1-(7–36) amide (1 ng/ml) for 20 min in the absence or presence of H89 (10 μM). NO levels in media were measured using the Nitric Oxide Synthase Detection System (Sigma-Aldrich) according to the manufacturer’s instructions. Insulin (100 nM) with or without L-NAME (10 nM) was used as positive and negative control for NO production.

Quantification of PKA activity. PKA activities in tissue and ECs were quantified using a PKA assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, skeletal muscle, heart, aorta, or ECs (5 × 10⁶) were homogenized in cold PKA extraction buffer, and the lysate was centrifuged for 5 min at 4°C at 14,000 g. For aorta, samples from four rats were pooled together. The supernatant was mixed with assay mixture and incubated for 30 min at room temperature, and the

**Fig. 1.** Experimental protocols. CEU, contrast-enhanced ultrasound; GLP-1, glucagon-like peptide 1.

**Fig. 2.** Inhibition of protein kinase A (PKA) abolishes GLP-1-mediated muscle microvascular recruitment. GLP-1 was infused continuously at 30 pmol·kg⁻¹·min⁻¹ in the absence or presence of H89. A: changes in microvascular blood volume (MBV). B: changes in microvascular blood flow velocity (MFV). C: changes in microvascular blood flow (MBF). *P < 0.05 compared with −30 or 0 min; n = 5–11.
MAP, mmHg

Table 1. Changes in MAP and plasma insulin concentrations

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Values are means ± SE. MAP, mean arterial blood pressure; GLP-1, glucagon-like peptide 1. *P < 0.05 compared with −30 min; #P < 0.05 compared with 0 min.

Myograph experiments. Distal saphenous artery was dissected from overnight-fasted rats immediately after the rats were euthanized. After the adhering connective tissue was trimmed off, each artery was cut into three ring segments ~2 mm in length. Each segment was mounted in a Multig Myograph System (Danish Myo Technology, Aarhus, Denmark). The organ chamber was filled with 6 ml of physiological salt solution buffer (130 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl₂, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 14.9 mM NaHCO₃, 0.026 mM EDTA, and 5.5 mM glucose, pH 7.4), which was constantly bubbled with 95% O₂-5% CO₂ and maintained at 37°C. Each ring was stretched initially to 5 mm, an optimal tension, and then allowed to stabilize at baseline tone. Relaxant responses to phenylephrine (300 nM) or acetylcholine (1 μM) were performed to test for vessel viability and integrity of endothelium. After precontraction with phenylephrine, GLP-1 (1 ng/ml) was added to the incubation bath with or without H89 (1 μM) pretreatment for 1 h. The relaxant response was expressed as percentage of the contraction induced by phenylephrine.

Statistical analysis. All data are presented as means ± SE. Statistical analyses were performed with SigmaStat 3.1.1 software (Systat Software, Chicago, IL), using either Student’s t-test or analysis of variance (ANOVA) with post hoc analysis as appropriate. A P value of <0.05 was considered statistically significant.

RESULTS

PKA inhibition abolishes GLP-1-induced increase in muscle microvascular perfusion. We have demonstrated previously that GLP-1 recruits muscle microvasculature and increases glucose use via a NO-dependent mechanism and that incubation of bAECs with GLP-1 increases endothelial PKA activity significantly (8). To examine the potential role of PKA in GLP-1’s microvascular action in muscle, we infused rats with the selective PKA inhibitor H89 prior to beginning GLP-1 infusion. Control rats received saline or H89 infusion only. Similarly to our previous report (8), GLP-1 potently recruited muscle microvasculature by increasing muscle MBV (~2-fold, P < 0.001, ANOVA) without affecting MFV (Fig. 2). This led to an approximately twofold increase in muscle MBF (P < 0.001, ANOVA). This was associated with a significant increase in cardiac muscle and aorta PKA activity (Fig. 3). Skeletal muscle PKA activity did not change significantly across any of the groups. Inhibition of PKA activity by coinfusion of H89 completely prevented a GLP-1-induced increase in PKA activity (Fig. 3) and abolished GLP-1-induced microvascular recruitment (Fig. 2). H89 infusion alone did not alter muscle microvascular parameters despite a brief decrease in MAP either alone or with GLP-1 (Table 1).
**PKA inhibition blocks GLP-1-induced NO production and muscle glucose extraction.** Because microvascular endothelial surface area determines substrate exchange between plasma and muscle interstitium and we have shown previously that GLP-1 increases muscle microvascular recruitment and glucose extraction via NO-dependent mechanism (8), we next assessed whether inhibition of PKA also prevented GLP-1-induced NO production and muscle glucose extraction. As shown in Fig. 4A, GLP-1 infusion increased plasma NO levels approximately threefold within 30 min, which remained elevated for 90 min ($P < 0.001$, ANOVA). This was associated with a two- to threefold increase in muscle glucose extraction ($P < 0.001$ and $P < 0.02$, respectively; Fig. 4B). Concurrent H89 infusion completely abolished GLP-1-stimulated increase in both NO production and muscle glucose utilization and prevented a GLP-1-induced decrease in arterial plasma glucose levels ($P = 0.169$, ANOVA). Plasma insulin concentrations remained stable during the experiments for all groups (Table 1).

**GLP-1 effects on Akt1, eNOS, and ERK1/2 phosphorylation in skeletal muscle in vivo.** Because we have shown that GLP-1 increased the phosphorylation of Akt1 and eNOS in cultured endothelial cells, we further examined whether GLP-1 also stimulates phosphorylation of Akt1, eNOS, and ERK1/2 in skeletal muscle. As shown in Fig. 5, GLP-1 and H89 had no effect on either Akt1 or eNOS phosphorylation in the skeletal
induced increases in PKA activity, eNOS phosphorylation, and NO production, confirming that GLP-1-mediated endothelial NO production is PKA-dependent.

PKA mediates GLP-1-induced vasodilation in isolated saphenous artery communicating branch. Because we observed increased PKA activity in heart and aorta but not in the skeletal muscle, we finally used an ex vivo approach to examine the role of PKA activation in isolated rat saphenous (Fig. 7). Incubation of the artery rings with GLP-1 potently increased vasorelaxation (~30%, P < 0.001, ANOVA), and this effect was once again prevented by pretreatment of the vessels with H89. Thus, GLP-1-mediated PKA activity within the vessel wall is indeed responsible for GLP-1-induced vasorelaxation.

DISCUSSION

The current study demonstrates that GLP-1-induced PKA activity plays a pivotal role in GLP-1-mediated microvascular recruitment and glucose use in muscle. Whereas systemic infusion of GLP-1 increased tissue PKA activity in vivo and incubation of ECs with GLP-1 potently increased PKA activity in vitro, administration of H89, a selective inhibitor of PKA, completely abolished GLP-1-induced NO production, microvascular recruitment, and glucose use in muscle and ex vivo vasodilation. Thus, our data strongly suggest that PKA signaling is required in GLP-1’s microvascular actions and the associated metabolic consequences.

Although ex vivo studies using rat arterial rings or pulmonary arteries have shown that vascular endothelium and NO production are essential in the vasorelaxant effect of GLP-1 (16, 38), and incubation of ECs with GLP-1 analog liraglutide dose and time dependently increases NO production (19), our recent report demonstrated a direct stimulatory effect of GLP-1 on eNOS phosphorylation and that endothelial production of NO is crucial in GLP-1-induced microvascular recruitment and glucose use in muscle, as inhibition of NO production completely abolished the microvascular effects of GLP-1 (8). The current study further extends our previous findings by demonstrating that these effects are mediated via the PKA signaling pathway. This is consistent with the fact that activation of the GLP-1 receptor triggers the generation of second-messenger

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cAMP via a direct action on the adenylate cyclase (18) and leads to the activation of PKA (14) and that eNOS is colocalized with the catalytic subunit of PKA in EC junctions (20).

The role of PKA in GLP-1-mediated glucose-stimulated insulin secretion is well characterized in pancreatic β-cells (3, 14). Binding of GLP-1 to its receptors activates adenylate cyclase and increases intracellular cAMP levels, leading to activation of PKA (21). Our observations that PKA inhibition completely prevented GLP-1-induced NO production, microvascular recruitment, and muscle extraction of glucose confirm that PKA signaling plays a key role in GLP-1’s microvascular actions. That administration of PKA inhibitor H89 completely blocked GLP-1-induced PKA activity in the aorta and abolished GLP-1-induced vasodilatation ex vivo in the rat saphenous artery strongly suggests that PKA mediates GLP-1 actions in conduit artery and resistance arterioles as well.

In the current study, we observed that GLP-1 infusion increased the phosphorylation of ERK1/2 but not Akt1 in muscle. Although the increased phosphorylation of ERK1/2 was consistent with a previous report (1), the same study showed that incubation of isolated rat soleus muscles with GLP-1 for 3 min potently increased phosphatidylinositol 3-kinase (PI 3-kinase) activity and Akt phosphorylation. This discrepancy may be explained by differences in GLP-1 exposure time (2 h vs. 3 min), variations in Akt isoforms (the antibody we used did not cross-react with other Akt isoforms), and study setting (in vivo vs. in vitro). Indeed, in isolated rat hearts GLP-1 markedly increased cardiac muscle glucose uptake without altering Akt1 phosphorylation (41).

Inasmuch as both PKA and the PI 3-kinase are able to phosphorylate eNOS and increase NO production and GLP-1 has been shown to activate the PI 3-kinase pathway in muscle (1) and β-cells (7), our study findings suggest that the PI 3-kinase pathway is likely uninvolved in GLP-1-induced NO production and microvascular recruitment since inhibition of PKA alone with H89 completely blocked GLP-1-induced NO production and vascular responses both in vivo and ex vivo. Our findings are in agreement with prior reports in cultured ECs that PKA but not PI 3-kinase mediates GLP-1-induced attenuation on ROS-induced senescence (36).

H89 is a potent and selective inhibitor of PKA (11, 23) and has been widely used to inhibit PKA in biochemical studies. It blocks PKA actions through competitive inhibition of the adenosine triphosphate site on the PKA catalytic subunit. A limitation of the current study is that H89 at high concentrations also inhibits several other kinases, such as p70S6K, MSK1, ROCK11, and MAPKAP-K1b (28, 29). However, it is less likely that these kinases contributed to GLP-1-mediated NO production since inhibition of PKA activity with another specific inhibitor, Rp-cAMPS, also abolished exendin-4-induced activation of eNOS and cell proliferation (15). Rp-cAMPS is a cell-permeable cAMP analog that specifically inhibits PKA by interacting with cAMP-binding sites on the regulatory subunits (39).

It seems contradictory that we observed that GLP-1 increased muscle microvascular perfusion via the PKA-dependent pathway but not PKA activity in the skeletal muscle. However, PKA activity did increase significantly in both heart and aorta after GLP-1 infusion. This is not surprising since skeletal muscle per se expresses much lower GLP-1 receptors than heart (8-fold less) and aorta (4-fold less) (17). Together with our findings in cultured cells and ex vivo saphenous artery study, our findings are entirely consistent with a direct action of GLP-1 on its receptors to activate PKA in the vasculature.

In conclusion, GLP-1 recruits muscle microvasculature, which leads to expansion of the microvascular blood volume and increased glucose use in muscle through a process that involves PKA activation and NO production. Given that in the resting state only ~30% of muscle capillaries are perfused (22) and an increase in muscle endothelial surface area could significantly increase insulin delivery and action and substrate exchange in muscle (4, 12), GLP-1’s microvascular actions in muscle may contribute significantly to postprandial glycemic control and diabetes complication prevention in diabetes.

REFERENCES


