cGMP phosphodiesterase inhibition improves the vascular and metabolic actions of insulin in skeletal muscle

A. J. Genders,* E. A. Bradley,* S. Rattigan, and S. M. Richards

Menzies Research Institute, University of Tasmania, Hobart, Tasmania, Australia

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Genders AJ, Bradley EA, Rattigan S, Richards SM. cGMP phosphodiesterase inhibition improves the vascular and metabolic actions of insulin in skeletal muscle. Am J Physiol Endocrinol Metab 301: E342–E350, 2011. First published June 7, 2011; doi:10.1152/ajpendo.00691.2010.—There is considerable support for the concept that insulin-mediated increases in microvascular blood flow to muscle impact significantly on muscle glucose uptake. Since the microvascular blood flow increases with insulin have been shown to be nitric oxide-dependent inhibition of cGMP-degrading phosphodiesterases (cGMP PDEs) is predicted to enhance insulin-mediated increases in microvascular perfusion and muscle glucose uptake. Therefore, we studied the effects of the pan-cGMP PDE inhibitor zaprinast on the metabolic and vascular actions of insulin in muscle. Hyperinsulinemic euglycemic clamps (3 mU·min⁻¹·kg⁻¹) were performed in anesthetized rats and changes in microvascular blood flow assessed from rates of 1-methylxanthine metabolism across the muscle bed by capillary xanthine oxidase in response to insulin and zaprinast. We also characterized cGMP PDE isoform expression in muscle by real-time PCR and immunostaining of frozen muscle sections. Zaprinast enhanced insulin-mediated microvascular perfusion by 29% and muscle glucose uptake by 89%, while whole body glucose infusion rate during insulin infusion was increased by 33% at 2 h. PDE2, -9, and -10 were the major isoforms expressed at the mRNA level in muscle, while PDE1B, -9A, -10A, and -11A proteins were expressed in blood vessels. Acute administration of the cGMP PDE inhibitor zaprinast enhances muscle microvascular blood flow and glucose uptake response to insulin. The expression of a number of cGMP PDE isoforms in skeletal muscle suggests that targeting specific cGMP PDE isoforms may provide a promising avenue for development of a novel class of therapeutics for enhancing muscle insulin sensitivity.

ENDOTHELIAL DYSFUNCTION ARISING FROM IMPAIRED NITRIC OXIDE (NO) SIGNALING UNDERLIES A RANGE OF CARDIOVASCULAR CONDITIONS INCLUDING ERECTILE DYSFUNCTION, HEART FAILURE, AND VASCULAR DISEASE IN DIABETES. AGENTS SUCH AS CINACIGUAT AND SILDENAFIL ENHANCE NO SIGNALING BY INCREASING THE FORMATION OF CYCLIC GUANOSINE MONOPHOSPHATE (cGMP) BY THE NO-SENSITIVE ENZYME SOLUBLE GUANYLATE CYCLASE OR BY REDUCING ITS HYDROLYSIS BY cGMP-DEGRADING PHOSPHODIESTERASES (cGMP PDEs), RESPECTIVELY. CINACIGUAT, OR BAY 58-2667, IMPROVES CARDIOVASCULAR FUNCTION IN PATIENTS WITH ACUTE DECOMPENSATED HEART FAILURE (19) AND PREFERENTIALLY VASODILATES BLOOD VESSELS CONTAINING A FORM OF SOLUBLE GUANYLATE CYCLASE AFFECTED BY OXIDATIVE STRESS FROM HUMAN TYPE 2 DIABETICS (36). IN ADDITION TO ITS USE FOR ERECTILE DYSFUNCTION, SILDENAFIL TREATMENT HAS BEEN SHOWN TO IMPROVE FLOW-MEDIATED DILATATION IN BRACHIAL ARTERIES OF TYPE 2 DIABETICS (14), AND ITS CHRONIC ADMINISTRATION ENHANCES THE METABOLIC ACTIONS OF INSULIN IN HIGH-FAT-FED MICE (2). SILDENAFIL IS SELECTIVE FOR PDE TYPE 5, BUT THE cGMP PDE FAMILY INCLUDES A LARGE NUMBER OF ISOFORMS THAT ARE EXPRESSED HETEROGENEOUSLY IN BLOOD VESSELS. LITTLE ATTENTION HAS THEREFORE BEEN PAID TO THE THERAPEUTIC POSSIBILITIES OF INHIBITION OF cGMP PDE ISOFORMS OTHER THAN PDE5 FOR THE TREATMENT OF CONDITIONS SUCH AS TYPE 2 DIABETES.

Impairment of endothelial function in type 2 diabetes has typically been regarded to be a result of poor blood glucose control, but a number of studies have suggested that endothelium-dependent vasodilation is impaired prior to overt diabetes (11, 38). The impact of blood flow on muscle insulin action was for a long time uncertain, but recently, considerable support has emerged for the concept that insulin-mediated increases in microvascular blood flow significantly improve the metabolic actions of insulin in muscle by enhancing delivery of glucose and insulin to the skeletal myocytes (4, 8, 31). Changes in microvascular blood flow with insulin have been confirmed using multiple methodologies, including contrast-enhanced ultrasound (12), laser-doppler flowmetry (7), microdialysis (28), and metabolism of 1-methylxanthine (1-MX) by the capillary endothelial enzyme xanthine oxidase (33), as an indicator of the extent of capillary perfusion. The finding that microvascular responses to insulin are impaired in obese, insulin-resistant humans and animals (11) suggests that skeletal muscle insulin resistance may include a microvascular contribution. The microvascular action of insulin is likely to be NO dependent, since insulin enhances phosphorylation and activity of endothelial nitric oxide synthase in cultured endothelial cells (24), and systemic infusion of the nitric oxide synthase inhibitor Nω-nitro-d-arginine methyl ester (L-NAME) blocks insulin-mediated increases in microvascular blood flow and 50% of the insulin-mediated increase in glucose uptake by muscle, in rats (39). We have shown that augmenting microvascular flow in muscle with the acetylcholine cogerger methacholine enhances insulin-mediated glucose uptake in healthy rats (3, 22). Similarly, Murdolo et al. (25) have recently demonstrated improved microvascular and metabolic responses to physiological hyperinsulinemia in obese, insulin-resistant individuals during methacholine infusion. In view of the NO dependence on the microvascular actions of insulin, it is reasonable to predict that inhibition of cGMP PDEs would enhance insulin-mediated increases in microvascular perfusion and muscle glucose uptake. Therefore, in the present study we report the augmentation of the metabolic and vascular actions of insulin in muscle by the pan-cGMP PDE inhibitor zaprinast. To determine which cGMP PDE isoforms might be targeted by zaprinast we examined cGMP PDE expression in skeletal muscle and its constituent vasculature.

* A. J. Genders and E. A. Bradley contributed equally to this work.

Address for reprint requests and other correspondence: S. M. Richards, Menzies Research Institute, Private Bag 23, Univ. of Tasmania, Hobart, 7001, Tasmania, Australia (e-mail: stephen.richards@utas.edu.au).
MATERIALS AND METHODS

**Experimental animals.** Male hooded Wistar rats weighing 240 ± 15 g were bred in our own colony and raised on a commercial diet (Pivot, Australia) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamins and minerals together with water ad libitum. A total of 68 rats were used for the anesthetized rat experiments, and a further number of rats (~20) were used for the expression studies. Rats were housed at a constant temperature of 21 ± 1°C on a 12:12-h light-dark cycle. All procedures adopted and experiments undertaken were approved by the University of Tasmania Animal Ethics Committee (in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2004, 7th Ed.) approval no. A9894.

**Surgery for anesthetized rat experiments.** The anesthetized rat model as described previously (33) was used. Briefly, rats were anesthetized by injection of pentobarbital sodium (50 mg/kg body wt), and cannulas were surgically implanted into the carotid artery for arterial sampling, measurement of mean arterial blood pressure (MAP), and heart rate and into both jugular veins for continuous administration of the above-mentioned anesthetic and other intravenous infusions. Animals were allowed to spontaneously breathe room air through a tracheostomy tube. Femoral arterial blood flow (FBF) was measured by an ultrasonic flow probe (Transonic Systems, VB series 0.5 mm). Data for FBF, heart rate, and MAP were measured continuously using WINDAQ data acquisition software (DATAQ Instruments). The animals were maintained at 37°C under anesthesia for the duration of the experiment using a continual infusion of anesthetic. The total blood volume withdrawn from the animals before the final arterial and venous samples did not exceed 1.5 ml and was compensated by saline infusion.

**Experimental procedures.** Hyperinsulinemic euglycemic clamps were performed in fasted anesthetized rats. Figure 1 describes the protocol. Following surgery, there was a 60-min equilibration to allow for the blood pressure and FBF to stabilize. Rats were then allocated to one of four groups: saline, saline + zaprinast, insulin clamp (3 mU·kg⁻¹·min⁻¹ Humulin R; Eli Lilly, Indianapolis, IN) or insulin clamp + zaprinast. Zaprinast (Sigma-Aldrich, St. Louis, MO) was given as a 1.6-mg bolus at the start of the insulin infusion (0 min in protocol) and then as a constant infusion of 10 μg·min⁻¹·kg⁻¹ for the course of the experiment into the jugular vein. Samples were taken from the carotid artery and femoral vein at the end of the experiment for determination of blood and plasma glucose, plasma zaprinast concentration, and 1-MX (Sigma-Aldrich) metabolism. Zaprinast concentrations were determined by reverse-phase HPLC as generally used to resolve nucleoside mixtures (32, 41). 1-MX metabolism was used to measure microvascular perfusion (perfused capillary surface area) as described previously (33). Briefly, a bolus of allopurinol was given 5 min prior to commencing 1-MX infusion, to reduce the rate of whole body 1-MX metabolism. 1-MX was infused at a rate established previously to maintain a constant saturating arterial concentration, and arterial and venous blood samples taken at the end of the clamp where indicated. Arteriovenous extraction of 1-MX [see Supplementary Fig. S3 (supplementary materials are found with the online version of this paper at the Journal website)] was multiplied by femoral arterial blood flow to calculate 1-MX disappearance by the Fick principle. Muscle glucose uptake was measured in a subset of animals by 2-deoxy-D-[1-¹⁴C]glucose ([¹⁴C]2-DG, Amersham Life Science) uptake. At 75 min, a 0.37-MBq bolus of [¹⁴C]2-DG in saline (154 mmol/l NaCl) was administered, and plasma clearance of radioactivity was measured. At the conclusion of the experiment, the lower leg muscles [soleus, plantaris, gastrocnemius white, gastrocnemius red, EDL (extensor digitorum longus) and tibialis muscles] were removed and freeze-clamped in liquid nitrogen and assayed for [¹⁴C]2-DG-6-phosphate content. Insulin levels at the end (120 min) of the euglycemic insulin clamp (Fig. 1) were determined from arterial plasma samples by ELISA assay (Mercodia, Sweden).

2-DG uptake assay. Individual frozen muscles from the clamps were ground under liquid nitrogen and homogenized. Free and phosphorylated [¹⁴C]2-DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8) (17, 18). Biodegradable Counting Scintillant-BCA, (Amershams USA) was added to each radioactive sample, and radioactivity was determined using a scintillation counter (Beckman LS3801). From this measurement and a knowledge of plasma glucose and the time course of plasma 2-DG disappearance, R’g, which represents glucose uptake into the muscle, was calculated as previously described by others (18).

**Cell culture of rat aortic vascular smooth muscle cells.** Aortic vascular smooth muscle cells (VSMCs) were isolated from rat thoracic aorta as described previously (29) and grown for four to five passages in DMEM containing 10% fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin.

**Reverse transcription-PCR.** RNA was extracted from rat gastrocnemius-planteris-soleus skeletal muscle and cultured rat aortic VSMCs by using the Tri Reagent method (Sigma-Aldrich). mRNA was extracted from total RNA using the Dynabeads mRNA DIRECT kit (Dynal Biotech Australia).

The GeneAmp Gold RNA PCR Reagent kit was used for both reverse transcription and PCR. The reverse transcription incubation conditions were 21°C for 10 min followed by 42°C for 15 min. For PCR the following conditions were used: 95°C for 10 min followed by 40 cycles of 30 s at 95°C, 1 min at 60°C, and 2 min at 72°C followed by a final extension step at 72°C for 8 min. Samples were size fractionated on a 2% agarose gel containing ethidium bromide in comparison with a 100-bp DNA ladder (New England Biolabs, GeneSearch). The identity of PCR products was confirmed by sequencing and/or restriction enzyme digestion. Primers were designed

![Fig. 1. Study design. Arterial and venous samples were collected at the end of the experiment for HPLC analysis and plasma glucose determination. Arterial samples for glucose and lactate determination indicated by Δ. Jugular vein infusions of zaprinast (10 μg·min⁻¹·kg⁻¹), insulin (3 mU·min⁻¹·kg⁻¹), 30% glucose, and 1-MX (0.4 mg·min⁻¹·kg⁻¹), ALP, allopurinol (10 μmol/kg), 1-MX, 1-methylxanthine.](http://ajpendo.physiology.org/)

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so that only certain splice isoforms would be amplified and are described in Supplementary Table S1 of the SUPPLEMENTARY METHODS.

Real-time-PCR. RNA was extracted from rat soleus and EDL skeletal muscle, aorta, and cultured aortic VSMC as detailed above. Before reverse transcription, each RNA sample was DNase treated using DNase I (Sigma-Aldrich). Reverse transcription was performed using SuperScript III RNase H- reverse transcriptase (Invitrogen Australia) and random hexamers (Applied Biosystems, Foster City, CA). Real time-PCR was performed using the Quant iTech SYBR Green PCR Kit (Qiagen) to quantify PCR product formation. Primers used are described in Supplementary Table S2. Specificity of the primers was checked by DNA sequencing of the purified PCR products. All results are expressed relative to the number of ribosomal 18S (r18S) copies.

Western blotting. Tissues were prepared as described in Ref. 6, with the exception that protease inhibitor cocktail (Sigma) was added to the solubilization buffer. Protein (25 μg) was then heated with 125 mM Tris·HCl, pH 6.8, 20% glycerol, 4% 2-mercaptoethanol, 0.2% bromophenol blue, and 4% SDS for 10 min at 90°C. Heat-treated samples were then subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes. All gels were 10% Bis-Tris polyacrylamide (Invitrogen) except where the PD1A-101AP, PD1B-201AP, and PD1C-301AP antibodies were used to probe the blots from 3–8% Tris-acetate polyacrylamide gels (Invitrogen). After transfer, the membranes were blocked and then incubated with the different primary antibodies. Membranes were then washed and incubated with an HRP-linked secondary antibody (anti-mouse and anti-rabbit secondary antibodies; Cell Signaling, GeneSearch). The enhanced chemiluminescence method (SuperSignal West Pico Chemiluminescent substrate, Thermo Scientific) was used to develop the membranes. The membranes were then exposed to X-ray film (HyperFilm; Amersham Biosciences, UK) to detect bound antibodies.

Immunofluorescence. Soleus muscle was dissected out, mounted for transverse sectioning, and frozen in liquid nitrogen-cooled isopentane, and 7-μm sections were cut. A standard immunofluorescence procedure was followed. Briefly, sections were fixed in 4% paraformaldehyde, permeabilized with 0.3% triton X-100, and blocked with 5% goat serum (Sigma-Aldrich) before incubation with the primary antibodies, smooth muscle α-actin (diluted 1:400; DAKO), PD1A-101AP, PD1B-201AP, PD1C-301AP, and PD11A-112AP (all diluted 1:100; FabGennix International), and PDE9A (1:100) and PDE10A (1:400) (Scottish Biomedical) overnight at 4°C. Double labeling was performed whereby smooth muscle α-actin primary antibody was added to all sections and different PDE antibodies to different sections. Following this, the sections were washed and incubated with the fluorescent secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1:1,000) and Alexa Fluor 594 goat anti-mouse IgG (1:2,000) (Molecular Probes/Invitrogen) for 1 h at 37°C, washed, and then dried before mounting in Permafluor (Immunotech). Secondary antibody controls were performed for each experiment to discount background staining.

Statistical analysis. All data are expressed as means ± SE. Mean FBF, mean heart rate, and MAP were calculated from 5-s subsamples of the data, representing ~500 flow and pressure measurements every 15 min. Repeated-measures two-way ANOVA was used to determine whether there was a difference between the treatment groups for throughout the time course, or two-way ANOVA was used for single point measurements. When a significant difference was found (P < 0.05), pairwise comparisons by the Student-Newman-Keuls test were used to determine at which individual time points there was a significant difference. To test the effect of insulin on muscle glucose uptake in the presence and absence of zaprinast, the variance of the means was calculated. From this the standard deviation of each of the treatments was calculated (insulin alone or insulin with zaprinast). A t-test was then used to determine whether there was a significant difference between the two groups of data. The SigmaStat statistical program (Jandel Software v. 2.03) was used to perform these tests.
of the experiment in order to rapidly attain and maintain a constant concentration of zaprinast (21, 35). Plasma concentrations of zaprinast achieved in both treatment groups were not significantly different (saline + zaprinast 34.9 ± 2.3, insulin + zaprinast 34.6 ± 2.9 μM). Zaprinast also had no significant effect on plasma insulin concentrations during the clamps (insulin 432 ± 20, insulin + zaprinast 420 ± 39 pmol/l).

Zaprinast infusion during the insulin clamp caused small but significant decreases in MAP at 90, 105, and 120 min (up to 10 mmHg at 120 min, Fig. 2A) but had no significant effects on FBF. Zaprinast alone did not have a significant effect on MAP or FBF (Fig. 2). Zaprinast did not have any significant effects on heart rate (Supplementary Fig. S1) alone or in the presence of insulin. Zaprinast alone gave rise to a small but significant reduction in vascular resistance at the 75- and 120-min time points (Supplementary Fig. S2). Insulin alone slightly reduced VR as previously reported (33, 44), but this was not altered by the further addition of zaprinast.

Insulin significantly increased microvascular perfusion over saline (51%), measured by 1-MX metabolism, and these changes were similar to those previously reported (33, 44). Coinfusion of insulin with zaprinast significantly enhanced the insulin-mediated increase in microvascular perfusion (29%; Fig. 2C), as indicated by increased 1-MX extraction across the leg without a change in femoral blood flow (Supplementary Fig. S3).

Effects of zaprinast on metabolism. Glucose infusion rate (GIR) for all groups is shown in Fig. 3A. Insulin + zaprinast significantly enhanced the insulin-mediated increase in GIR from 75 min onwards (33% at 120 min).

Fig. 3. Effects of zaprinast on glucose metabolism. Glucose infusion rate (GIR; A), blood glucose concentration (B), muscle glucose uptake (R’g) for individual muscles (C), change in muscle glucose uptake (ΔR’g) for combined calf muscles (D), and blood lactate (E) for saline (○ or open bars, n = 10), saline + zaprinast (● or hatched bars, n = 8), 3 mU·min⁻¹·kg⁻¹ insulin (● or filled bars, n = 8), and 3 mU·min⁻¹·kg⁻¹ insulin + zaprinast (▲ or cross-hatched bars, n = 10) -treated rats. GR, red gastrocnemius; GW, white gastrocnemius; EDL, extensor digitorum longus. Values are means ± SE from a subset of experiments in which muscle glucose uptake measurements were performed. Significant values: P < 0.05 saline vs. insulin are indicated by *, saline vs. saline + zaprinast by †, insulin vs. insulin + zaprinast by #, and ΔR’g saline vs. zaprinast by #.
The glucose uptake (R’g) for combined muscles showed a significant increase with insulin infusion (saline 2.88 ± 0.44, insulin 7.28 ± 0.78 μg·g⁻¹·min⁻¹). Insulin infusion alone also significantly increased the R’g for plantaris, gastrocnemius red, EDL, and tibialis muscles compared with saline controls (Fig. 3C). The effect of zaprinast infusion alone was not significantly different from that of saline, although there was a trend toward reduced R’g in all muscles. The coinfusion of zaprinast with insulin showed a significant increase in glucose uptake in soleus (110%) and gastrocnemius red muscles (43%). The effect of insulin to increase muscle glucose uptake was significantly (P < 0.005) higher overall in the combined muscles in the presence of zaprinast (89%; Fig. 3D) than when insulin was used alone.

Notably, blood lactate was significantly increased in the presence of zaprinast from the 10-min time point onward and continued to increase for the duration of the experiment (Fig. 3E). This lactate was not derived from muscle, as the muscle arteriovenous difference in plasma lactate indicated muscle uptake rather than release when zaprinast was present (Supplementary Fig. S4). This suggests that zaprinast may alter lactate handling by other tissues such as liver.

cGMP PDE mRNA expression. Upon examination of cGMP PDE isoform mRNA expression in cultured aortic VSMC and skeletal muscle, it was apparent that most isoforms were present (Fig. 4). The exceptions were PDE10A14 and PDE11A3 in skeletal muscle and PDE11A2 in both skeletal muscle, it was apparent that most isoforms were present, whereas PDE1C was barely detectable (Table 1). The major isoforms in skeletal muscle appeared to be PDE2A, -9A, and -10A, although PDE10A expression was dramatically different between soleus and EDL muscle. We also noted a striking downregulation of PDE mRNA expression during culture of aortic VSMC compared with freshly isolated aorta. A range of primer sets complementary to PDE11A covering the length of the sequence common to all three splice isoforms was used alone.

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<th>Table 1. PDE mRNA expression by real-time PCR</th>
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All values are expressed as copy numbers relative to rp 18S and have been multiplied by 100,000; n = 3–4 for each tissue. PDE, phosphodiesterase; ASMC: aortic smooth muscle cells, EDL: extensor digitorum longus.

expression. Real-time-PCR was used to quantify relative PDE isofrom mRNA expression (Table 1).

PDE5A was the most abundant mRNA isoform in aorta, with lesser albeit significant amounts of PDE2A and PDE9A present, whereas PDE1C was barely detectable (Table 1). The major isoforms in skeletal muscle appeared to be PDE2A, -9A, and -10A, although PDE10A expression was dramatically different between soleus and EDL muscle. We also noted a striking downregulation of PDE mRNA expression during culture of aortic VSMC compared with freshly isolated aorta. A range of primer sets complementary to PDE11A covering the length of the sequence common to all three splice isoforms reported from rats was tested, but no specific product was able to be quantified.

cGMP PDE protein expression. Western blotting was used to assess the specificity of the antibodies and demonstrated that each antibody detected only the appropriate cGMP PDE iso-

Fig. 4. cGMP-degrading phosphodiesterase (PDE) mRNA expression in skeletal muscle and cultured aortic vascular smooth muscle cells (VSMC) by RT-PCR. A: skeletal muscle. B: cultured aortic VSMC. PCR products were electrophoresed on an agarose. Left lanes, molecular weight ladders (each band represents 100 bp). PCR product sizes (in bp): PDE1A = 596, 1B = 466, 1C = 512, 2A = 309, 5A1 = 195, 5A2 = 467, 9A = 242, 10A2 = 176, 10A3 = 232, 10A11 = 172, 10A12 = 188, 10A13 = 177, 10A14 = 247, 11A2 = 184, 11A3 = 210, and 11A4 = 223. Isoforms marked in italics are not expressed in that tissue.
form (Fig. 5). PDE1C appeared to be expressed only in skeletal myocytes; however, PDE1B, -9A, -10A, and -11A appeared also to be expressed in the vasculature within skeletal muscle (Fig. 6). Although a single protein of the correct size was detected on Western blots in brain by using a PDE1A antibody, levels of PDE1A in skeletal muscle were too low to be detected by Western blotting, and faint immunoreactivity around the borders of myocytes in muscle sections would support this conclusion. Unfortunately, a primary antibody directed against PDE2A with sufficient specificity for immunofluorescence could not be found from commercial sources. PDE5A also appeared to be expressed in the blood vessels of skeletal muscle (data not shown). Negative control immunofluorescence images with secondary antibody alone (i.e., omitting primary antibody) were uniformly black (i.e., no detectable fluorescence) at the exposure times used in Fig. 6.

Greater smooth muscle actin expression was found in soleus than in EDL, reflecting the larger amount of vascular tissue present. S6 ribosomal protein, which served as a loading control, indicated that an equal amount of total protein was loaded in all wells (Fig. 5A).

**DISCUSSION**

The chief finding of the present report was that acute administration of zaprinast was able to enhance insulin-mediated microvascular perfusion and that this was accompanied by an increase in insulin-mediated muscle glucose uptake. The latter contributed to a significant increase in whole body glucose infusion rate by 2 h. However, zaprinast has been reported to competitively inhibit cGMP PDE isoforms 1, 5, 9, 10 and 11 (15, 16, 40). Since immunofluorescence showed that PDE1B, -9A, -10A, and -11A are expressed at the protein level in skeletal muscle vasculature, it is likely that zaprinast mediates its effects via inhibition of one or more of these isoforms.

The stimulation of insulin-mediated glucose uptake by zaprinast is likely to have been a result of the increased microvascular perfusion, increasing glucose supply to myocytes, rather than a direct enhancement of myocyte glucose uptake. Thus, the lack of vascular effects from zaprinast alone was not unexpected. Although it might initially seem surprising that an agent that raises cGMP would not vasodilate, substantial rises in intracellular cGMP in muscle arterioles would not be expected until there was a stimulus for cGMP synthesis (e.g., insulin-mediated NO generation). This seems to be borne out by the significantly greater microvascular hemodynamic effects of zaprinast in the presence of insulin (Fig. 2C). Also, the absence of significant differences in vascular resistance (as measured from MAP divided by FBF; data not shown) compared with control in either saline- or insulin-infused animals, concomitant with increased microvascular perfusion (as measured from 1-MX disappearance) suggests that zaprinast is quite selective in the microvessels it dilates. This is reminiscent of the behavior of sildenafil, which appears to dilate selected vessels in the arterial tree. Indeed, the selective dilation of vessels, avoiding major systemic dilation, is why cGMP PDE inhibitors such as sildenafil and zaprinast are so attractive as potential therapeutics or lead-in compounds.

Similar reasoning can also be applied to explain the effects of zaprinast on muscle glucose uptake. Muscle glucose uptake is increased by zaprinast only when in the presence of a stimulus for cGMP (insulin), which causes the greater microvascular perfusion leading to greater insulin and glucose delivery to the muscle myocytes.

Although zaprinast acutely enhances muscle microvascular perfusion, this does not occur via inhibition of PDE5A, at least in rodents. In a previous study, we demonstrated that acute

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**Fig. 5.** Protein expression by Western blotting. **A**: PDE1A, -B, and -C protein expression. PDE1A expected size 65–72 kDa, PD1A-101AP primary antibody diluted 1:250; PDE1B expected size 65–72 kDa, PD1B-201AP primary antibody diluted 1:250; PDE1C expected size 70–87 kDa, PD1C-301AP primary antibody diluted 1:1,000 (FabGennix International). **B**: protein expression in soleus and EDL skeletal muscle and aorta. PDE9A primary antibody diluted 1:2,000 (Scottish Biomedical), expected size 75 kDa; PDE10A primary antibody diluted 1:10,000 (Scottish Biomedical); expected sizes of splice isoforms between 74 and 100 kDa; PDE11A primary antibody diluted 1:1,000 (FabGennix International) expected size 78 kDa; smooth muscle α-actin primary antibody diluted 1:2,000 (DAKO), expected size 42 kDa; S6 ribosomal protein primary antibody diluted 1:1,000, expected size 32 kDa.
infusion of T-1032, a more specific inhibitor of PDE5A, actually blocks insulin-mediated microvascular perfusion in rats (23) rather than enhancing it. The reduction in microvascular perfusion was accompanied by a reduction in muscle 2-deoxyglucose uptake. A number of studies have similarly demonstrated that blockade of insulin-mediated changes in microvascular perfusion (32, 39, 42) are accompanied by a ~50% reduction in insulin-mediated glucose uptake by muscle. Ayala et al. (2) demonstrated that acute treatment of high-fat-fed mice with sildenafil, a PDE5A inhibitor, also failed to enhance insulin-mediated muscle glucose uptake.

Chronic sildenafil treatment had the opposite effect, increasing muscle insulin action. It is possible that when administered chronically sildenafil acts via other pathways to exert its positive effects, such as changes in transcription, for example, reducing superoxide production by downregulating gp91phox (34). Although Ayala et al. did not assess microvascular perfusion of muscle in their study, others have reported improvements in endothelial function in animal models of insulin resistance following chronic sildenafil treatment (5). However, it seems that, although PDE5A is expressed in muscle vasculature (data not shown), PDE5A is less relevant as a target for
inhibition for acutely enhancing muscle microvascular perfusion.

It might seem surprising that vasodilation does not always enhance glucose and insulin delivery to muscle, but there is considerable evidence that not all blood flow through muscle is necessarily “nutritive” for (i.e., in close contact with) the skeletal myocytes. That is to say that not all blood flow through muscle is able to enhance metabolism in the myocytes. For example, Mahajan et al. (22) reported that, although vasodilation with methacholine infusion during an insulin clamp augmented insulin-mediated muscle glucose uptake in rats, a similar degree of vasodilation with bradykinin failed to alter muscle metabolism due to insulin. Increases in total blood flow to muscle with vasodilators such as adenosine (26), nitroprusside (27), low doses of IGF-I (30), and epinephrine (33) also does little to influence glucose uptake in healthy subjects or improve insulin sensitivity in animals. A separation between changes in total blood flow and microvascular perfusion changes suggests redistribution of blood flow between two different routes in muscle. This concept is supported by extensive experiments in constant-flow perfused rat hindleg (reviewed in Refs. 9, 10, 31), in which some vasoconstrictors result in reduced muscle metabolism (measured as insulin-mediated muscle glucose uptake or contraction-mediated muscle glucose or oxygen uptake), whereas others appear to divert flow to the myocytes and enhance metabolism. It is also clear that there is some variation of cGMP PDE expression throughout the vascular tree. For example, human mesenteric artery expresses PDEs 1–5 inclusive, whereas saphenous vein expresses only 1, 4, and 5 (40). In the current study, although not examined in detail, there was some indication of heterogeneity in PDE expression between muscle blood vessels (arrows in Fig. 6). The site(s) of augmented vasodilation by cGMP PDE inhibition, in the presence of insulin, may therefore be crucial in determining whether muscle glucose delivery is enhanced or inhibited.

Since PDE5A inhibition is unlikely to be involved in the enhanced microvascular perfusion in this study, our attention was turned to the other cGMP PDE isoforms expressed in muscle vasculature. Indications for vascular PDE1B, -9A, -10A, and -11A expression at the protein level were obtained from immunofluorescence. We were unable to quantify skeletal muscle PDE11A mRNA expression by real-time PCR, but nonquantitative RT-PCR confirmed its presence in skeletal muscle and cultured aortic VSMC, and PDE11A protein was indicated by Western blotting of muscle and by immunofluorescence in the blood vessels of skeletal muscle. Several studies have detected PDE11A mRNA in skeletal muscle (13, 43); however, Loughney et al. (20) failed to detect PDE11A mRNA in blood vessels or skeletal muscle in humans. PDE1B and PDE9A appear to be expressed in both myocytes and vasculature, while PDE10A protein expression in muscle sections appeared to be predominantly vascular, with weaker staining of skeletal myocytes. It was notable that PDE10A mRNA expression was ~10 times higher in soleus than in EDL and that a similar difference in PDE10A protein levels exists between the two muscles by Western blotting. Although this might be related to the different fiber type composition of these muscles (1), it seems more likely to be due to the much higher volumes of vascular smooth muscle in soleus compared with EDL, apparent from smooth muscle actin protein content, since there were no obvious differences in fiber type PDE staining. Although aortic PDE10A protein levels were low, aorta may not be representative of the microvessels within the skeletal muscle. Similarly, culturing of aortic vascular smooth muscle cells revealed a marked decrease in overall cGMP PDE mRNA expression (Table 1), which may have been associated with the change from a contractile to a synthetic, proliferative phenotype that occurs during culture [reviewed in Thyberg et al. (37)]. Whatever the reason for the altered expression, it indicates that cGMP PDE expression in cultured cells can be misleading, as it is not representative of expression patterns in vivo. However, the accumulation of large amounts of plasma lactate (Fig. 3E) may preclude the use of zaprinast as an insulin-sensitizing agent. However, our results suggest that studies similar to these performed with PDE isoform-specific inhibitors (e.g., to PDE9A or -10A) to enhance microvascular perfusion and thus muscle insulin sensitivity would be of great interest.

In conclusion, coinfusion of zaprinast with insulin enhanced whole body insulin sensitivity and significantly increased insulin-mediated muscle glucose uptake. It is likely that the increased muscle glucose uptake is a result of the enhanced microvascular perfusion observed with zaprinast infusion. The expression of multiple cGMP PDE isoforms in muscle vasculature, taken together with the failure of acute PDE5A inhibition to augment insulin-mediated microvascular perfusion, indicates that inhibition of an alternative cGMP PDE isoform (e.g., PDE1B, -9A, -10A, or -11A) may provide an attractive and novel therapeutic target for ameliorating muscle insulin resistance.

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DISCLOSURES

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

REFERENCES

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PDE INHIBITION IMPROVES INSULIN ACTION


