THESIS

NITRIC OXIDE AND VASODILATING PROSTAGLANDINS CONTRIBUTE TO THE AUGMENTED SKELETAL MUSCLE HYPEREMIA DURING HYPOXIC EXERCISE IN HUMANS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ANNE RENEE CRECELIUS ENTITLED NITRIC OXIDE AND VASODILATING PROSTAGLANDINS CONTRIBUTE TO THE AUGMENTED SKELETAL MUSCLE HYPEREMIA DURING HYPOXIC EXERCISE IN HUMANS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

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Exercise hyperemia in hypoxia is augmented relative to the same level of exercise in normoxia. At mild exercise intensities, the augmented response is not explained by β-adrenoceptor mediated dilation. We hypothesized that elevated synthesis of nitric oxide (NO) and vasodilating prostaglandins (PGs) contribute to the augmented hyperemic response during hypoxic exercise. To test this hypothesis, in 10 healthy adults, we measured forearm blood flow (FBF; Doppler ultrasound) and calculated the vascular conductance (FVC) responses to rhythmic forearm handgrip exercise (20% maximal voluntary contraction) in normoxia and during systemic isocapnic hypoxia (85% arterial oxygen saturation; pulse oximetry) before and after local intra-brachial combined blockade of nitric oxide synthase (NOS; via N^G-monomethyl-L-arginine: L-NMMA) and cyclooxygenase (COX; via ketorolac) inhibition. All trials were performed during local blockade of α- and β-adrenoceptors to eliminate the sympathoadrenal effects on the forearm vasculature and isolate local vasodilation. A deep venous catheter was also placed in the experimental arm. Blood samples were taken from both the arterial and

venous catheters and analyzed to assess oxygen extraction and oxygen consumption of the exercising tissue. In control (saline) conditions, FBF after 5 minutes of exercise in hypoxia was greater than in normoxia (345 ± 21 ml min^{-1} *vs* 297 ± 18 ml min^{-1}; $P<0.05$). After NO/PG block, exercise hyperemia was significantly reduced in hypoxia (312 ± 19 ml min^{-1}; $P<0.05$), but not in normoxia (289 ± 15 ml min^{-1}; P=NS). The observed reduction in FBF during hypoxic exercise after NO/PG block resulted in a significant decrease in oxygen delivery (62 ± 5 ml min^{-1} *vs* 56 ± 4 ml min^{-1}; $P<0.05$). A compensatory increase in extraction was measured (59 ± 3% *vs* 64 ± 3%; $P<0.05$) which maintained oxygen consumption (36 ± 3 ml min^{-1} *vs* 36 ± 2 ml min^{-1}; $P<0.05$). We conclude that under the experimental conditions employed, NO and PGs have little role in normoxic exercise hyperemia whereas they significantly contribute to hypoxic exercise hyperemia at this intensity of exercise. The augmented response to hypoxia as compared to normoxia is reduced ~50% with combined NO/PG block. Additionally, during hypoxic exercise after combined NO/PG block, despite a decrease in oxygen delivery driven by attenuated blood flow, muscle oxygen extraction increases to maintain oxygen consumption. The factors contributing to the remaining augmentation of hypoxic exercise hyperemia (~50%) are yet to be determined.

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CHAPTER I

REVIEW OF THE LITERATURE

Metabolic autoregulation is a principal concept in the control of peripheral blood flow whereby oxygen supply to a tissue is appropriately matched to the level of oxygen demand. Oxygen supply is determined by the blood flow to the tissue and the content of oxygen within the blood. Oxygen demand is the metabolic consumption of oxygen by a given tissue. With an increase in oxygen demand, the oxygen supply will subsequently increase, generally by local vasodilation and increased blood flow to the tissue, as arterial oxygen content typically remains the same (Andersen & Saltin, 1985). Exercise hyperemia highlights the concept of metabolic autoregulation. Exercise significantly increases oxygen demand, which in healthy tissues, is matched by a tremendous increase in blood flow (Rowell, 2004). If arterial oxygen content is altered, as in acute systemic hypoxia, vasodilation ensues, and blood flow increases to match supply to the demand of the tissue (Heistad & Abboud, 1980). Previous work has established that when the two stimuli of exercise and acute hypoxia are present concurrently, the hyperemic response is greater than that to normoxic exercise, once again to appropriately match supply to demand of the tissue and maintain oxygen consumption given a set workload (Hartley *et al.*, 1973; Rowell *et al.*, 1986; Wilkins *et al.*, 2008). This augmentation of muscle blood flow and ability to maintain a constant workload differs at maximal exercise compared to

submaximal exercise and may also vary depending on the size of the muscle mass (Koskolou *et al.*, 1997; Calbet *et al.*, 2008). The mechanisms and obligatory factors by which vascular tone is regulated during exercise and acute hypoxia is a topic of much investigation and a combination of neural, mechanical, and local factors have all been implicated in the control of blood flow and oxygen delivery to these stimuli. However, vasomotor regulation in response to the combination of exercise and hypoxia is not as well understood and thus the mechanisms are largely unexplored.

The purpose of this review is to examine and summarize the current literature related to the control of peripheral vascular tone during exercise, acute hypoxia, and of most relevance to the current study, hypoxic exercise. A brief review of sympathoadrenal and mechanical influences will be given, followed by a discussion of potential locally-acting vasoactive substances, specifically nitric oxide (NO) and vasodilating prostaglandins (PGs).

Sympathoadrenal Activity - Influence on Skeletal Muscle Resistance Vessels

Skeletal muscle resistance vessels are innervated by sympathetic nerve fibers. Norepinephrine (NE) released from nerve endings binds to α_1- and α_2-adrenergic receptors located on the vascular smooth muscle, which causes smooth muscle cell depolarization and vasoconstriction. Endothelial cells also express α_2-adrenoceptors that can be bound by NE and cause vasodilation. However, the effect of these endothelial α_2-receptors is minimal and the net effect of α-adrenoceptor stimulation is an increase in vascular tone (Guimaraes & Moura, 2001). Basal vascular tone is highly influenced by tonic sympathetic nervous system activity, as α-adrenergic receptor blockade

significantly increases resting forearm blood flow and vascular conductance ~2-3 fold in young healthy humans (Dinenno *et al.*, 2002). Circulating epinephrine, released from the adrenal medulla, can bind to β_2-adrenergic receptors and cause vasodilation, but resting epinephrine levels are low so β-mediated dilation does not play a significant role in the control of vascular tone at rest in young healthy humans (Blauw *et al.*, 1995).

During moderate to intense exercise, sympathetic nervous system activity increases as measured directly by muscle sympathetic nerve activity (MSNA) and increased catecholamine levels (Seals *et al.*, 1988). The increase in sympathetic activity is dose-dependent with exercise intensity (Seals *et al.*, 1988). For rhythmic forearm exercise, light workloads of 10 or 20% maximal voluntary contraction (MVC) do not elicit a profound sympathetic response (Victor & Seals, 1989). Sympathetic stimulation of α-receptors serves to restrain blood flow to active muscle to some extent in order to preserve central blood pressure (Buckwalter & Clifford, 1999). Despite increased activation during exercise, β-mediated dilation does not appear to account for the profound vasodilation observed during this stimulus (Buckwalter *et al.*, 1997).

Systemic hypoxia has also been shown to significantly increase sympathetic activity as measured by MSNA and systemic increases in heart rate and blood pressure (Rowell *et al.*, 1989; Leuenberger *et al.*, 2005). Despite the increase in sympathetic activity, vasodilation still occurs in response to acute hypoxia (Dinenno *et al.*, 2003). It has been experimentally shown that this vasodilation is not due to a hypoxic-blunting of α-mediated constriction (Dinenno *et al.*, 2003). When an α-receptor antagonist is administered concomitant with systemic hypoxia, vasodilation occurs to a greater extent than prior to blockade, suggesting that increased sympathetic activity and α-mediated

constriction actually 'masks' the degree of magnitude of hypoxic vasodilation (Weisbrod *et al.*, 2001). This 'unmasked' dilation to hypoxia was shown to be reduced by 50% after β-adrenergic blockade, suggesting a role of β-mediated dilation to the hypoxic vasodilatory response (Weisbrod *et al.*, 2001).

As might be expected, hypoxic exercise results in a further increase in sympathetic nervous activity beyond that of hypoxia or exercise alone (Seals *et al.*, 1991). However, there is still vasodilation and an increase in blood flow that, in order to maintain appropriate oxygen delivery is greater than during normoxic exercise (Wilkins *et al.*, 2008). The augmented response to hypoxic exercise, like the response to hypoxia at rest, is not explained by blunted α-receptor vasoconstriction (Wilkins *et al.*, 2006), suggesting greater vasodilatory signaling in this augmented response. Recently, Wilkins et al., examined the influence of the sympathetic nervous system on forearm blood flow and vasodilation during hypoxic exercise (Wilkins et al., 2008). Rhythmic handgrip exercise at 10% and 20% of maximal voluntary contraction was performed during intra-arterial infusion of saline (control), phentolamine (non-selective α-receptor antagonist), and the combination of phentolamine and propranolol (non-selective β-receptor antagonist). Similar to resting hypoxic conditions, α-receptor blockade revealed an enhanced vasodilatory response to hypoxic exercise compared to normoxic exercise. At 10% MVC, the addition of propranolol resulted in a significant decrease in hypoxic exercise vasodilation from the phentolamine alone condition. The effect of propranolol was not significant for 20% MVC exercise. The authors conclude that β-mediated dilation occurs during hypoxic exercise at a low workload (10% MVC) but that this contribution is intensity-dependent and does not play a role in hypoxic exercise

vasodilation during 20% MVC exercise (Wilkins *et al.*, 2008). Some caution is advised when interpreting these data from the Wilkins *et al.* study. The experimental design employed by these investigators did not include a normoxic rest period prior to hypoxic exercise onset. Thus, when calculating the response to hypoxic exercise, the investigators used resting hemodynamic measurements from the normoxic trials. That said, it appears the authors' general conclusion of an intensity-dependent role of β-mediated dilation is valid. The extent of the β-mediated contribution for each of the exercise intensities may not be as clear as their interpretation would suggest. To summarize the findings of Wilkins *et al.*, hypoxic exercise-induced increases in α-adrenoreceptor stimulation mask much of the vasodilatory response, which, at low intensities, is partially mediated by β-receptors. Therefore, at 20% MVC forearm handgrip, if the augmented response to hypoxic exercise is not regulated by sympathetic influences, local factors are likely the primary mediators influencing the increased hypoxic exercise hyperemia, as compared to normoxic exercise.

In addition to the direct α- and β-adrenergic receptor effects of the sympathoadrenal system on the vasculature, sympathetic engagement via moderate- to severe-intensity exercise, systemic hypoxia, or the combination of the two elicits systemic hemodynamic changes. In healthy humans, removal of parasympathetic restraint along with increased sympathetic activity will increase heart rate, stroke volume, and therefore cardiac output. Changes in systemic hemodynamics can result in significant alterations in regional muscle blood flow that are independent of local mechanisms. Considering this, in experiments that are designed to investigate *local* vascular regulation, exercise modes and intensities should be chosen that do not elicit a

profound sympathetic response. It is difficult to safely and selectively deoxygenate arterial inflow to only one region of the human body; therefore, when investigating the role of hypoxia, limb isolation is not possible. Hypoxia is generally induced systemically, and subsequently elicits a sympathetic response. If interest lies in the local mechanism of hypoxic dilation, sympathoadrenal blockade is warranted, to inhibit both the local and systemic influences of increased sympathetic activity.

Mechanical Forces – Influence on Skeletal Muscle Resistance Vessels

In the past few decades, it has been proposed that the skeletal 'muscle pump' and other mechanical forces of contracting skeletal muscles can contribute to the onset and maintenance of exercise hyperemia (Laughlin, 1987). Recent evidence suggests that mechanical forces, independent of the muscle pump may also contribute to the initial rise in forearm blood flow via rapid vasodilatory mechanisms (Kirby *et al.*, 2007). In regard to steady-state exercise hyperemia, the muscle pump likely facilitates cardiac output during whole-body exercise through increased venous return (Wang *et al.*, 1960; Sheriff *et al.*, 1993). However, for a small muscle mass, like the forearm, the muscle pump is not obligatory for exercise-induced increases in muscle blood flow (Tschakovsky *et al.*, 1996), suggesting there are redundant vasodilating pathways that are able to regulate arterial inflow even if the muscle pump is unable to contribute to venous outflow.

When hypoxia is induced at rest via a breathing system in a normobaric environment, there are no additional mechanical forces acting on the vasculature compared to normoxia. Subsequently, the role of mechanical forces related to the augmented hypoxic exercise response would appear to be minimal. An interaction could

exist between the hypoxic environment and the vasodilating mechanisms of mechanical stimulation whereby hypoxia increased sensitivity to the mechanical stimulus. However, this proposition has yet to be studied.

Local Vasoactive Substances - Influence on Skeletal Muscle Resistance Vessels

In addition to the effects of sympathoadrenal activity and the mechanical stimulus of exercise, vascular tone is highly regulated at the local tissue level. Substances released by exercising muscle, produced within the endothelium, and carried in the blood have all been examined for their role in vascular regulation. Some of the more commonly studied substances are muscle metabolites such as lactate, H^+, and K^+, adenosine triphosphate (ATP) released from red blood cells, adenosine, endothelium-derived hyperpolarizing factors, NO and vasodilating PGs (Clifford & Hellsten, 2004). It appears that many of these local vasodilatory mechanisms are not 'obligatory' for exercise hyperemia and hypoxic dilation. That is, if one of the substances is independently inhibited, either in formation or receptor binding, another pathway may be upregulated and vasodilation and hyperemia are not compromised. This redundancy of vasodilatory signaling pathways may be a protective mechanism for pathological vascular dysfunction (Clifford & Hellsten, 2004). The exact mechanisms by which these vasoactive molecules are synthesized and released as well as the potential interaction between various local and systemic influences are not well described. Further, there is little consensus on what functions as the local 'oxygen sensor' regulating the vascular response to exercise or hypoxia. The red blood cell (Ellsworth *et al.*, 1995), endothelial cells (Frisbee *et al.*, 2001b), and smooth muscle cells themselves (Coburn *et al.*, 1986) have all been shown to

mediate the response to an alteration in the partial pressure of oxygen (PO_2) *in vitro*. Using various pharmacological manipulations, the independent or combined role of some of the potential vasodilating substances these 'sensors' may stimulate has been assessed related to the responses to exercise and acute systemic hypoxia. The combined stimulus of hypoxic exercise is a relatively unexplored area in this regard.

NO and PGs: Synthesis, Mechanisms, and Role in Basal Vascular Control

Nitric oxide is a gaseous biological messenger that participates in a variety of signaling cascades. Nitric oxide is formed from the conversion of L-arginine to L-citrulline, a reaction catalyzed by the enzyme nitric oxide synthase (NOS). There are two constitutive forms of NOS, endothelial NOS (eNOS) and neuronal NOS (nNOS), which are localized in vascular endothelial cells and skeletal muscle, respectively. Generation of NO in the endothelium via eNOS can increase due to a variety of stimuli: β-adrenergic stimulation, mechanical forces such as shear stress, and receptor binding of multiple local vasoactive molecules (Furchgott & Martin, 1985; Pohl *et al.*, 1986; Dawes *et al.*, 1997). Generation of NO in skeletal muscle via nNOS may be stimulated by increases in intracellular calcium (Fleming & Busse, 2003). In addition to NO production by NOS, the red blood cells can bind NO and release it concurrent with oxygen off-loading (Stamler *et al.*, 1997). Deoxygenated hemoglobin has also been shown to reduce nitrite to NO within the vasculature (Cosby et al., 2003). Regardless of the source, it is thought that NO diffuses to the vascular smooth muscle, increases intracellular cyclic guanosine monophosphate (cGMP), a second messenger, which increases the production of protein kinase G, decreasing intracellular calcium concentrations, leading to smooth muscle

relaxation (Murad, 1986). Acute NOS inhibition reduces resting forearm blood flow and vascular conductance, suggesting a role for NO in the regulation of basal vascular tone (Radegran & Saltin, 1999; Dinenno & Joyner, 2003).

Prostaglandins are a group of eicosanoids that, like NO, have a variety of physiological functions. The enzyme cyclooxygenase (COX), of which there are two isoforms (1 and 2), oxidizes arachidonic acid to form the intermediate PGH_2. From PGH_2, vasodilating (PGE_2 and prostacyclin) and vasoconstricting (thromboxane A_2) PGs are formed. Greater availability of arachidonic acid, through increased endothelial cell concentrations of calcium can increase PG synthesis (Brotherton & Hoak, 1982). In normal, healthy, young humans, the vasodilating PGs predominate, with prostacyclin (PGI_2) being most potent (Davidge, 2001). Like NO, PGs can also be formed within skeletal muscle (Symons *et al.*, 1991). Vasodilation mediated by PGs is thought to occur through increased cyclic adenosine monophosphate (cAMP), another second messenger, which decreases intracellular calcium concentration leading to vascular smooth muscle relaxation (Vanhoutte & Mombouli, 1996). While the role of NO in the regulation of basal vascular tone is well established, there is some disagreement regarding PGs in this same regard. Depending on experimental design and pharmacological inhibitor used, evidence exists both for and against PG-mediated regulation of basal tone (Duffy *et al.*, 1999; Boushel *et al.*, 2004; Schrage *et al.*, 2004). What does appear clear is that when both COX and NOS inhibitors are administered, there is an increase in vascular tone at rest (Dinenno & Joyner, 2004; Schrage *et al.*, 2004).

Role of NO and PGs in Vascular Control – Exercise

Human studies investigating the role of NO in exercise hyperemia have produced contradictory results depending on experimental design, inhibitor efficacy and the method of blood flow measurement. When a NOS inhibitor is administered *prior* to exercise, some groups have shown no change in exercise hyperemia (Radegran & Saltin, 1999; Frandsen *et al.*, 2001; Dinenno & Joyner, 2003) while those employing the technique of venous occlusion plethysmography (VOP) have shown a significant reduction in exercise limb blood flow (Gilligan *et al.*, 1994; Duffy *et al.*, 1999). When VOP is used, muscle contractions must cease at the time of measurement; thus, using this method actually measures flow during muscle recovery as opposed to active contraction. Since NO has been shown to play a significant role in muscle blood flow during the recovery from exercise (Radegran & Saltin, 1999; Frandsen *et al.*, 2001), this method has the potential to overestimate the contribution of NO to exercise hyperemia. In some of the studies that report no change in exercise blood flow, there is a significant reduction in vascular conductance, driven by an increase in MAP (Frandsen *et al.*, 2001; Dinenno & Joyner, 2003). This increase in MAP is likely due to NOS inhibitor administration spillover into the systemic circulation and an increase in total peripheral resistance, due to the contribution of NO to resting vascular tone. When a NOS inhibitor is administered *during* exercise, as in rhythmic forearm handgrip, a modest reduction in forearm blood flow (~20-30%) has been observed (Dyke *et al.*, 1995; Schrage *et al.*, 2004).

The role of PGs in exercise hyperemia is equally confounded by methodological differences in the existing studies. When VOP is used, and a COX inhibitor is administered *prior* to exercise, there is a significant reduction in exercise limb blood flow

(Kilbom & Wennmalm, 1976; Wilson & Kapoor, 1993; Duffy *et al.*, 1999). However, using Doppler ultrasound (Shoemaker *et al.*, 1996), and thermodilution (Mortensen *et al.*, 2007) there were no observed effects of COX blockade. When a COX inhibitor was administered *during* exercise, Schrage and colleagues, using Doppler ultrasound, report a transient reduction of ~12% in forearm blood flow, that returned to pre-infusion levels by the end of the 5 minute infusion period (Schrage *et al.*, 2004).

The combined role of NO and PG appears to differ from the individual effects of either NOS or COX blockade alone. In one of the studies that showed no difference in exercise hyperemia with COX inhibition, there was a significant reduction when NOS was also inhibited (Mortensen *et al.*, 2007). Two other studies, using a similar model of single-leg extension at moderate workloads and inhibition *prior* to exercise support the finding that combined blockade of NOS and COX reduces exercise hyperemia (Boushel et al., 2002; Kalliokoski et al., 2006). However, at the most mild workload tested (15W) there was no effect of NO/PG blockade on exercise hyperemia (Boushel *et al.*, 2002). When inhibition occurred *during* 10% MVC forearm exercise, the reduction in hyperemia due to combined blockade of NOS and COX was ~20% (Schrage *et al.*, 2004). In this study a 5 minute exercise bout was performed after double blockade that, in contrast to the aforementioned studies in the leg, revealed no difference in steady-state exercise hyperemia (Schrage *et al.*, 2004). Another study in the forearm at a similar workload also demonstrated no change in exercise blood flow after NOS and COX inhibitors were administered *prior* to exercise (Dinenno & Joyner, 2004). Potential limb-specific and intensity-dependent responses make these data regarding double blockade of NOS and COX somewhat difficult to interpret. That said, it appears that in the human forearm

circulation, NO and PGs likely play a role in exercise hyperemia; however, redundant mechanisms allow for similar levels of hyperemia to be reached if the production of these vasodilating substances has been pharmacologically inhibited prior to exercise onset.

Role of NO and PGs in Vascular Control – Systemic Hypoxia

Few studies have been performed in humans examining the role of NO in hypoxic vasodilation of resistance vessels. The first study to address this question in healthy humans was performed by Blitzer and colleagues (Blitzer *et al.*, 1996). In this study, subjects breathed a hypoxic gas mixture that reduced arterial PO_2 before and after intra-brachial administration of a NOS inhibitor. Forearm blood flow was measured by VOP. A significant increase in FBF and reduction in forearm vascular resistance (FVR) was observed in response to hypoxia, and the decrease in FVR was attenuated after NOS inhibition. As a control for the effect of NOS inhibition on baseline FBF, phenylephrine (α_1-adrenoceptor agonist) was given to a subset of subjects in place of the NOS inhibitor. There was no effect of phenylephrine on hypoxic vasodilation, suggesting that there is a role for endothelial-derived NO in hypoxic dilation of forearm resistance vessels (Blitzer *et al.*, 1996). Another study in humans administered a NOS inhibitor following α- and β-adrenoceptor blockade (via phentolamine and propranolol, respectively) (Weisbrod *et al.*, 2001). There was no change in the vascular response to hypoxia when NOS was inhibited after α- and β-blockade. While these results contradict those of the study by Blitzer *et al.*, the authors point out that in addition to the lack of α-adrenoceptor blockade in the Blitzer *et al.* study, there are data which suggest dilation from β-adrenoceptor agonism is 50-60% mediated through NO (Dawes *et al.*, 1997). Thus, Weisbrod et al.

concluded that any contribution of NO is mediated through β-receptor stimulation (Weisbrod *et al.,* 2001).

A thorough search of the literature reveals no published studies that directly address the role of PGs in human hypoxic vasodilation. There are however, studies performed in animal models and isolated vessels that suggest prostaglandins may contribute to the vasodilatory response to hypoxia. Ray et al. demonstrated *in vivo* that inhibition of COX reduced femoral vascular conductance during systemic hypoxia in rats (Ray *et al.,* 2002). When skeletal muscle arterioles are isolated and subjected to hypoxic baths, administration of a COX inhibitor nearly abolishes hypoxic dilation (Messina *et al.,* 1992; Frisbee *et al.,* 2001b; Frisbee *et al.,* 2002). Release of 6-keto-$PGF_{1\alpha}$, a stable PG analogue, increased in hypoxic conditions in rat tail arteries, and canine femoral and coronary arteries (Messina *et al.,* 1992). Given the existing animal data, it is reasonable to hypothesize a potential, yet unidentified, role for PGs in human hypoxic vasodilation.

As there is a lack of human data related to the independent role of PGs in hypoxic vasodilation, subsequently there are no published studies performed in humans to examine the combined role of NO and PGs in the vascular response to hypoxia. Our laboratory has observed that individual inhibition of NOS or COX does not significantly reduce forearm vascular conductance to a hypoxic stimulus (~85% SaO_2) but combined blockade of NOS and COX nearly abolishes hypoxic vasodilation (Unpublished observations, Markwald RR and Dinenno FA). Published studies support a significant interaction between NO and PGs during hypoxia and other vasodilatory stimuli. The aforementioned work by Ray and colleagues suggests that PG synthesis increases NO production which would link these two vasodilating pathways (Ray *et al.,* 2002).

Interactions of NO and PGs in a redundant and compensatory manner in response to stimuli other than hypoxia have also been previously demonstrated. In male eNOS knockout mice, PG-mediated dilation was increased in response to a flow stimulus (Sun et al., 1999). In the human forearm, the combined inhibition of NOS and COX significantly reduced total excess flow during reactive hyperemia more so than individual inhibition alone (Engelke *et al.*, 1996). While neither of these latter studies address hypoxic vasodilation directly, in agreement with exercise data, they argue for a significant interaction between NO and PGs in the regulation of vascular tone to a given stimulus. Based on published findings and our laboratory's observations, it appears the combined influence of NO and PGs on hypoxic vasodilation may be significant.

Role of NO and PGs in Vascular Control – Hypoxic Exercise

Relatively few studies have been performed to examine potential mechanisms by which hypoxia augments muscle blood flow during exercise of the same intensity level. While the initial observations of this phenomenon were made over thirty years ago (Hartley *et al.*, 1973), subsequent studies investigating the mechanism of regulation of vascular control to this stimulus have been few (Wilkins *et al.*, 2006; Wilkins *et al.*, 2008). The majority of studies performed have been somewhat descriptive in nature, employing differing hypoxic and exercise stimuli while measuring various systemic and limb-specific cardiovascular responses, blood gases, and blood metabolites (Raynaud *et al.*, 1986; Rowell *et al.*, 1986; Yoshida *et al.*, 1989; Mazzeo *et al.*, 1995; Koskolou *et al.*, 1997; Calbet *et al.*, 2003; Hanada *et al.*, 2003; DeLorey *et al.*, 2004; Lundby *et al.*, 2008). Likely, the lack of research in this area stems from a non-definitive understanding

of neither exercise hyperemia regulation in normoxia nor hypoxic vasodilation at rest. Recent work that addresses the regulation of vascular tone in response to hypoxic exercise has shown that the response is significantly restrained by increased sympathetic outflow, as revealed by α-adrenoceptor blockade. At low intensities of exercise, this 'unmasked' dilation is partially mediated by β-adrenoceptors; however the β-mediated contribution was not significant at greater exercise intensity (Wilkins et al., 2008). The remaining augmented response to hypoxic exercise after combined α-/β-receptor blockade remains in question. Since the influence of mechanically-related vasodilatory mechanisms likely does not change in hypoxia, it is thought that local vasodilating factors may play a significant role. Data from studies investigating normoxic exercise suggest a partial role of NO and PGs in exercise hyperemia (Schrage *et al.*, 2004; Mortensen *et al.*, 2007). Some human studies and animal data suggest that NO and PGs also partially mediate hypoxic vasodilation (Blitzer *et al.*, 1996; Ray *et al.*, 2002). Given these data, it is possible that NO and PGs significantly contribute to the augmented hypoxic exercise response.

STATEMENT OF PROBLEM

To date, the role of nitric oxide and vasodilating prostaglandins in hypoxic exercise hyperemia is unclear. Whether or not local blockade of NO and PGs will influence the hyperemic response to hypoxic exercise is not known. Further, the influence of NO and PG inhibition on oxygen delivery, oxygen extraction, and oxygen consumption during hypoxic exercise has not been tested.

HYPOTHESIS

Inhibition of NO and PGs will significantly reduce the augmented hyperemia observed with hypoxic exercise as compared to normoxic exercise. Despite reduced forearm blood flow during hypoxic exercise after the inhibition of NO and PG synthesis, we hypothesize that oxygen consumption will be maintained through a compensatory increase in extraction.

CHAPTER II

MANUSCRIPT

Nitric oxide and vasodilating prostaglandins contribute to the augmented skeletal muscle hyperemia during hypoxic exercise in humans

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ABSTRACT

Exercise hyperemia in hypoxia is augmented relative to the same level of exercise in normoxia. At mild exercise intensities, the augmented response is not explained by β-adrenoceptor mediated dilation. We hypothesized that elevated synthesis of nitric oxide (NO) and vasodilating prostaglandins (PGs) contribute to the augmented hyperemic response during hypoxic exercise. To test this hypothesis, in 10 healthy adults, we measured forearm blood flow (FBF; Doppler ultrasound) and calculated the vascular conductance (FVC) responses to rhythmic forearm handgrip exercise (20% maximal voluntary contraction) in normoxia and during systemic isocapnic hypoxia (85% arterial oxygen saturation; pulse oximetry) before and after local intra-brachial combined blockade of nitric oxide synthase (NOS; via N^G-monomethyl-L-arginine: L-NMMA) and cyclooxygenase (COX; via ketorolac) inhibition. All trials were performed during local blockade of α- and β-adrenoreceptors to eliminate the sympathoadrenal effects on the forearm vasculature and isolate local vasodilation. A deep venous catheter was also placed in the experimental arm. Blood samples were taken from both the arterial and venous catheters and analyzed to assess oxygen extraction and oxygen consumption of the exercising tissue. In control (saline) conditions, FBF after 5 minutes of exercise in hypoxia was greater than in normoxia (345 ± 21 ml min^{-1} *vs* 297 ± 18 ml min^{-1}; $P<0.05$). After NO/PG block, exercise hyperemia was significantly reduced in hypoxia (312 ± 19 ml min^{-1}; $P<0.05$), but not in normoxia (289 ± 15 ml min^{-1}; P=NS). The observed reduction in FBF during hypoxic exercise after NO/PG block resulted in a significant decrease in oxygen delivery (62 ± 5 ml min^{-1} *vs* 56 ± 4 ml min^{-1}; $P<0.05$). A compensatory increase in extraction was measured (59 ± 3% *vs* 64 ± 3%; $P<0.05$) which

maintained oxygen consumption (36 ± 3 ml min^{-1} *vs* 36 ± 2 ml min^{-1}; $P<0.05$). We conclude that under the experimental conditions employed, NO and PGs have little role in normoxic exercise hyperemia whereas they significantly contribute to hypoxic exercise hyperemia at this intensity of exercise. The augmented response to hypoxia as compared to normoxia is reduced ~50% with combined NO/PG block. Additionally, during hypoxic exercise after combined NO/PG block, despite a decrease in oxygen delivery driven by attenuated blood flow, muscle oxygen extraction increases to maintain oxygen consumption. The factors contributing to the remaining augmentation of hypoxic exercise hyperemia (~50%) are yet to be determined.

INTRODUCTION

In healthy humans, skeletal muscle blood flow is primarily regulated by the matching of oxygen supply to oxygen demand, termed metabolic autoregulation. In cases where demand is greater, such as exercise, blood flow to the active skeletal muscle increases substantially. If the supply of oxygen is challenged, such as with exposure to acute systemic hypoxia, peripheral vasodilation and an increase in skeletal muscle blood flow is also observed. Hypoxic exercise presents a unique challenge to the tissues; a significant increase in oxygen demand coinciding with decreased arterial oxygen content.

It is well established that during hypoxic exercise, muscle blood flow is augmented relative to the same level of exercise in normoxia in order to maintain oxygen delivery to the active tissue (Hartley *et al.*, 1973; Rowell *et al.*, 1986; Wilkins *et al.*, 2008). This augmentation of blood flow occurs in spite of an increase in sympathetic activity and α-adrenoceptor-mediated vasoconstriction (Hanada *et al.*, 2003; Wilkins *et*

al., 2008). During exercise, hypoxia does not alter the responsiveness of directly stimulated α-adrenoceptors as compared to normoxia, suggesting that the augmented response is not due to a blunting of the vasoconstrictor signal (Wilkins *et al.*, 2006). Instead, it is reasoned that an enhanced vasodilatory signal must be present during hypoxic exercise that serves to augment blood flow.

Recently, Wilkins et al. demonstrated β-adrenoceptor-mediated dilation during hypoxic exercise (Wilkins *et al.*, 2008). At 10% maximal voluntary contraction (MVC) rhythmic forearm exercise during systemic isocapnic hypoxia (arterial oxygen saturation ~80%), β-mediated dilation accounted for ~50% of the augmented response 'unmasked' by local α-adrenoceptor blockade (via phentolamine; non-selective α-adrenoceptor antagonist). At an intensity of 20% MVC, the contribution of β-mediated dilation was not significant (Wilkins *et al.*, 2008). These data suggest a role for other vasodilator signals beyond β-mediated dilation to the enhanced hypoxic exercise response at greater intensities of exercise.

Beyond sympathoadrenal influences on vascular tone, a variety of substances produced locally by the endothelium, released by skeletal muscle, or found within in the blood have been investigated for their respective contributions to exercise hyperemia and hypoxic dilation. Adenosine, K^+, ATP released from red blood cells, endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO), and vasodilating prostaglandins (PGs) are such commonly studied substances. During hypoxic exercise at mild intensities, cardiac output does not limit the hyperemic response (Hartley *et al.*, 1973). Thus, if the sympathoadrenal influences on the vasculature in a contracting skeletal muscle are blocked, local vasodilatory mechanisms can be isolated.

Nitric oxide (synthesized via nitric oxide synthase; NOS) and PGs (synthesized via cyclooxygenase; COX) have been shown to contribute to moderate-intensity knee extensor exercise hyperemia when inhibition occurred *prior to* exercise onset (Boushel *et al.*, 2002; Kalliokoski *et al.*, 2006; Mortensen *et al.*, 2007). In the forearm vasculature, it appears that acute inhibition of NOS and COX *during* low-intensity exercise reduces hyperemia, but subjects maintain the ability to achieve the same hyperemic response when NO and PGs synthesis is blocked *prior to* exercise onset (Schrage *et al.*, 2004). The discrepancy in those studies where inhibition occurred *prior to* exercise may be related to an intensity-dependent nature of the contribution of NO and PGs to limb blood flow during exercise (Boushel *et al.*, 2002). In regards to acute systemic hypoxia, acute inhibition of NOS attenuated the vasodilatory response in healthy humans (Blitzer *et al.*, 1996). Evidence from *in vivo* rat models and isolated vessel studies implicate PGs as mediators of hypoxic vasodilation (Messina *et al.*, 1992; Frisbee *et al.*, 2001a; Ray *et al.*, 2002). Recently, we have observed combined inhibition of NOS and COX, after local α-/β-adrenoreceptor blockade, significantly reduces vasodilation in the forearm during systemic hypoxia (Unpublished observations, Markwald RR and Dinenno FA). However, the combined role of NO and PGs in hypoxic exercise hyperemia is currently not known.

With this information as background, the purpose of the present investigation was to test the hypothesis that NO and PGs contribute to the augmented muscle blood flow observed during hypoxic exercise as compared to normoxic exercise at the same mild workload. Further, we hypothesized that even with potential reductions in muscle blood flow and thus oxygen supply, oxygen extraction would increase in a compensatory

manner to maintain oxygen consumption of the active skeletal muscle tissue. To test these hypotheses, we measured forearm hemodynamics (Doppler ultrasound) and arterial and venous blood gases during rhythmic forearm handgrip exercise in normoxia and hypoxia before and after local combined NOS and COX inhibition. All experiments were performed during local α- and β-adrenergic blockade to eliminate sympathoadrenal effects on the forearm vasculature and isolate local vasodilatory mechanisms. Our findings indicate that NO and PGs contribute to the enhanced local vasodilatory response to hypoxic exercise, however oxygen consumption of the tissue is unchanged due to increased extraction at the level of the muscle.

METHODS

Subjects

With Institutional Review Board approval and after written informed consent, a total of 10 young healthy adults (8 men, 2 women; age = 21 ± 1 years; weight = 72.5 ± 2.3 kg; height = 178 ± 2 cm; body mass index = 22.7 ± 0.6 kg m^{-2}; means ± S.E.M.) participated in the present study. All subjects were non-smokers, non-obese, normotensive (resting blood pressure <140/90), and not taking any medications. Studies were performed after a 4 h fast and 24 h abstention from caffeine and exercise with subjects in the supine position. Female subjects were studied during the early follicular phase of their menstrual cycle to minimize any potential cardiovascular effects of sex-specific hormones. All studies were performed according to the *Declaration of Helsinki*.

Arterial and venous catheterization

A 20 gauge, 7.6 cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml hr^{-1} with heparinized saline. The two side ports were used for drug infusions. In addition, an 18 gauge, 5.1 cm catheter was inserted in retrograde fashion into an antecubital vein of the experimental arm for deep venous blood samples. Heparinized saline was continuously infused through this catheter at a rate of approximately 3 ml min^{-1} for the duration of the study to keep it patent.

Forearm blood flow and vascular conductance

A 12MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to determine brachial artery mean blood velocity (MBV) and brachial artery diameter. The probe was securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described (Dinenno & Joyner, 2003). For blood velocity measurements, the probe insonation angle was maintained at <60 degrees and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analyzed via a Multigon 500V TCD (Multigon Industries, Mt Vernon NY, USA) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter measurements were made in duplex mode at end-diastole and between contractions during steady-state conditions.

Forearm blood flow (FBF) was calculated as:

$$FBF = MBV \times \pi \text{ (brachial artery diameter/2)}^2 \times 60,$$

where the FBF is in ml min^{-1}, the MBV is in cm s^{-1}, the brachial diameter is in cm, and 60 was used to convert from ml s^{-1} to ml min^{-1}. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) × 100, and expressed as ml min^{-1} (100 mmHg)$^{-1}$.

Cardiac output

A finger pressure cuff was placed around the middle phalanx of the middle finger on the non-experimental arm. Beat-to-beat pressure waveforms were obtained and stroke volume was estimated using the Modelflow method (Finometer 1, Finapres Medical Systems BV, Amsterdam, The Netherlands) (Wesseling *et al.*, 1993; Bogert & van Lieshout, 2005; Gonzalez-Alonso *et al.*, 2006). Cardiac output (Q) was calculated as the product of heart rate and stroke volume. The Modelflow method has been shown to reliably track changes in cardiac output during exercise in healthy young humans (Sugawara *et al.*, 2003).

Blood gas analysis

Brachial artery and deep venous blood samples were immediately analyzed with a clinical blood gas analyzer (Siemens Rapid Point 400 Series Automatic Blood Gas System, Los Angeles, CA, USA) for partial pressures of oxygen and carbon dioxide (PO_2 and PCO_2), hemoglobin concentration ([Hb]), oxygen content (ctO_2), pH, and oxygen saturation (SO_2).

Rhythmic handgrip exercise

Maximal voluntary contraction (MVC; mean 44.1 ± 2.8 kg, range 28.3 – 57.3 kg) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other. Forearm exercise during the trials was performed with weight corresponding to 20% MVC (mean 8.6 ± 0.6 kg, range 5.6 – 11.5 kg) attached to a pulley system and lifted 4–5 cm over the pulley at a duty cycle of 1s contraction–2 s relaxation (20 contractions per minute) using both visual and auditory feedback to insure the correct timing as described previously (Dinenno & Joyner, 2003). We chose this workload based on recent data that suggests a greater potential role for local vasodilators at this workload (Wilkins *et al.*, 2008). We also aimed to minimize increases in sympathetic activity due to exercise (Victor & Seals, 1989).

Systemic isocapnic hypoxia

To isolate the effects of systemic isocapnic hypoxia, we used a self-regulating partial rebreathe system developed by Banzett *et al.* (Banzett *et al.*, 2000). This system allows for constant alveolar fresh air ventilation independent of changes in breathing frequency or tidal volume (Banzett *et al.*, 2000; Dinenno *et al.*, 2003; Wilkins *et al.*, 2008). Using this system we were able to clamp end-tidal CO_2 levels despite the hypoxia-induced increases in ventilation. The level of oxygen was manipulated by mixing nitrogen with medical air via an anesthesia gas blender. For the hypoxic trials, inspired oxygen was titrated to achieve arterial oxygen saturations of ~85% as assessed via pulse oximetry. For normoxic trials, subjects were placed on the rebreathe system but

inspired room air. Subjects breathed through a scuba mouthpiece with a nose-clip to prevent nasal breathing. An anesthesia monitor (Cardiocap/5, Datex-Ohmeda Louisville, CO, USA) was used to determine heart rate (HR; 3-lead ECG) and expired CO_2 sampled at the mouthpiece. Ventilation was measured via a turbine pneumotachograph (model 17125 UVM, Vacu-Med, Ventura, CA, USA).

Pharmacological infusions

Regional Sympathoadrenal Blockade: Phentolamine mesylate (Bedford Laboratories, Bedford, OH, USA), a non-selective α-adrenoceptor antagonist and propranolol hydrochloride (Baxter, Deerfield, IL, USA), a non-selective β-adrenergic receptor antagonist, were administered via brachial artery catheter to eliminate the effects of sympathoadrenal influences on vascular tone. A loading dose totaling 1000 μg (200 μg min^{-1} for 5 min) of each drug was infused prior to all experimental trials and a maintenance dose (50 μg min^{-1}) was infused throughout the entire study to ensure continuous blockade. The dose of phentolamine used was twice as great as those previously documented to effectively block α-adrenergic receptors (Eklund & Kaijser, 1976; Dietz *et al.*, 1997; Halliwill *et al.*, 1997). The dose of propranolol used has been shown to inhibit forearm vasodilation in response to isoproterenol (non-selective β-adrenergic receptor agonist) (Johnsson, 1967) as well as reduce vasodilation in the resting forearm during contralateral isometric handgrip exercise (Eklund & Kaijser, 1976).

Regional NOS Inhibition: N^G-monomethyl-L-arginine (L-NMMA; Clinalfa/Bachem, Weil am Rhein, Germany), a NOS inhibitor, was administered intra-arterially to inhibit the production of NO. A loading dose totaling 25 mg (5 mg min^{-1} for

5 minutes) and a maintenance dose (1.0 mg min^{-1}) was infused for the duration of the study to ensure continuous blockade. This dose of L-NMMA has been previously shown to significantly reduce basal tone and also the vasodilatory effects of acetylcholine (Dietz *et al.*, 1994; Dinenno & Joyner, 2003), consistent with effective NOS inhibition (Vallance *et al.*, 1989).

Regional COX Inhibition: Ketorolac (KET; trade name Toradol, Hospira, Lake Forest, IL, USA), a non-selective COX inhibitor, was administered intra-arterially to inhibit the production of PGs. A loading dose totaling 6 mg (600 μg min^{-1} for 10 min) and a maintenance dose (120 μg min^{-1}) was infused for the duration of the study to ensure continuous blockade. This dose of KET is twice that which was previously demonstrated to reduce circulating $PGF_{1\alpha}$ (a stable breakdown product of PGs) at rest and during handgrip exercise (Dinenno & Joyner, 2004).

Experimental protocol

A timeline for the overall study and each trial is depicted in Figure 1. After catheter setup, resting FBF was determined. Loading doses of phentolamine and propranolol to inhibit sympathetic stimulation of α- and β-adrenergic receptors, respectively, were then administered and all experimental trials followed. Normoxic trials consisted of a 3 minute baseline and a 5 minute rhythmic handgrip exercise period. Hypoxic trials consisted of a 3 minute baseline, 5 minutes of steady-state hypoxia of ~85% oxygen saturation (monitored via pulse oximeter; transition ~2 min), and 5 minutes rhythmic handgrip exercise. Each trial was performed under control condition (saline infusion) and with combined NO/PG block (L-NMMA and KET, respectively). Twenty

minutes of rest separated each experimental trial. The order of normoxic and hypoxic trials was counter-balanced between subjects. Arterial and venous blood samples were taken at the end of rest, normoxic exercise, steady-state hypoxia, and hypoxic exercise (Fig. 1).

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. Forearm blood flow, HR, MAP, oxygen saturations (pulse oximeter), and Q represent an average of the last 30 seconds of the appropriate time period. Minute ventilation and end-tidal CO_2 were determined from an average of the data over the last minute of each trial. The sampling timeframe used for averaging was greater for respiratory variables than hemodynamic variables in order to insure an adequate number of sampling points. Blood gas values were determined from blood samples obtained during each condition. From the blood gas data, arteriovenous oxygen difference (a-vO_2) was calculated as the difference between arterial and venous oxygen content. Oxygen consumption across the forearm ($\dot{V}O_2$) was calculated as: (FBF × a-vO_2 difference) and expressed in ml min^{-1}. Oxygen delivery was calculated as: (FBF × arterial oxygen content) and expressed in ml min^{-1}. Oxygen extraction, reported as a percent, was calculated as: (arterial oxygen content - venous oxygen content) / arterial oxygen content × 100.

As an alternative way of expressing the data, we calculated the 'hypoxic augmentation' as the difference between the absolute FBF during hypoxic exercise and

during normoxic exercise. The difference between steady-state hypoxic FBF and resting FBF was also calculated. In order to examine a potential relation between systemic hemodynamics and FBF, the changes in Q and changes in FBF from rest were calculated for each exercising condition as: Exercise FBF or Q – Rest FBF or Q.

Statistics

Data are presented as mean ± S.E.M. Differences within and between trials and conditions were determined via 2-way repeated measures analysis of variance (ANOVA). Due to large differences in the magnitude of values of exercise and rest, for comparisons of FBF, FVC, a-vO_2, oxygen extraction, oxygen delivery, and oxygen consumption, two ANOVAs were completed, one including both exercise conditions (normoxic and hypoxic) and one including both rest periods and hypoxia. For all other variables collected, all time points were included in the ANOVA analysis. Specific hypothesis testing was performed using 2-tailed Student's *t* test for paired data and when appropriate, *post hoc* comparisons were made with Tukey's HSD test. Linear regression analysis was performed to determine the relationship between changes in Q and FBF for individual trials and pooled data. Significance was set *a priori* at $P < 0.05$.

RESULTS

Effect of regional α- and β-adrenergic blockade

Prior to all experimental trials, combined blockade of α- and β-adrenoreceptors significantly increased resting FBF (40 ± 4 *vs* 87 ± 10 ml min^{-1}) and FVC (43 ± 5 *vs* 92 ± 10 ml min^{-1} $(100\ mmHg)^{-1}$) by ~100% ($P < 0.05$). Mean arterial pressure (93 ± 2 *vs* 95 ±

2 mmHg; P=0.30) and HR (57 ± 2 *vs* 56 ± 2 beats min^{-1}; P=0.24) did not change due to α- and β-receptor blockade.

Systemic hemodynamic and respiratory responses

Systemic hemodynamic and respiratory responses are presented for each trial and condition in Table 1. Mean arterial pressure was not different during exercise between all trials (P=0.34-0.92). As expected, hypoxia resulted in significant increases in HR and ventilation (P<0.05). Ventilation data were not obtained for one subject; therefore, all ventilation data represent an average of 9 subjects. The targeted oxygen saturation of ~85% was achieved in all hypoxic conditions. Subjects remained isocapnic across all experimental trials, as no significant differences were observed in end-tidal CO_2 (P=0.18-0.67).

Cardiac output was not acquired for one subject resulting in 9 subjects for all cardiac output data analysis. Cardiac output was reduced after NO/PG block for all time points within the hypoxic trial (P<0.05).

Forearm blood flow and vascular conductance responses

Absolute values of FBF and FVC for each trial are presented in Table 3 and Figure 2. No difference was observed in resting FBF or FVC between normoxic and hypoxic trials (P=NS). Resting FBF and FVC was lower following NO/PG block for both the normoxic and hypoxic trials (P<0.05). Steady-state hypoxia increased both FBF and FVC in the control condition (P<0.05), but both FBF and FVC were significantly lower than control with NO/PG block (P<0.05) and no longer different than resting

values (both NS). The reduction in the FBF response to hypoxia due to combined NO/PG inhibition was ~70% (Fig. 3; 29 ± 6 *vs* 9 ± 4 ml min^{-1}; $P<0.05$).

In the control condition, both FBF and FVC were significantly greater during hypoxic exercise than normoxic exercise (Table 3 and Fig. 2; $P<0.05$). Combined blockade of NO/PG did not change FBF ($P=.33$) nor FVC ($P=.18$) during normoxic exercise. During hypoxic exercise in the NO/PG block condition, both FBF and FVC were significantly reduced from control hypoxic exercise ($P<0.05$). Combined blockade of NO/PG reduced the hypoxic augmentation of exercise FBF by ~50% (Fig. 4; 48 ± 6 *vs* 23 ± 9 ml min^{-1}; $P<0.05$). Despite this reduction, hypoxic exercise FBF and FVC were still significantly greater than normoxic exercise FBF and FVC in the NO/PG block condition.

In the control condition, there was a slight positive correlation (normoxic exercise r=0.14, $P=0.68$; hypoxic exercise r=0.33, $P=0.39$; data not shown) between the change in Q and change in FBF. In the NO/PG block condition, there was a moderate negative correlation between the changes in Q and FBF (normoxic exercise r=0.57, $P=0.11$; hypoxic exercise r=0.37, $P=0.31$; data not shown). When the data was pooled for all exercise trials, the correlation was nearly zero (r=0.09, $P=0.60$; data not shown).

Blood gases and forearm oxygen delivery, extraction, and consumption

Blood gas data are presented in Table 2. As anticipated, hypoxia reduced arterial oxygen saturation, arterial PO_2, and arterial oxygen content as compared to normoxia ($P<0.05$). There was no difference in arterial PCO_2 between normoxic exercise and hypoxic exercise in control or NO/PG conditions (P=NS). Exercise, both in normoxia

and hypoxia, significantly reduced venous pH, venous PO_2, and venous oxygen content (P<0.05).

Oxygen delivery, presented in Figure 4*A*, represents the product of arterial oxygen content and FBF. In the control condition, oxygen delivery was increased during steady state hypoxia, as compared to rest (20 ± 3 *vs* 17 ± 2 ml min^{-1}; P<0.05). In the NO/PG block condition, oxygen delivery was no longer greater during hypoxia than at rest (11 ± 2 *vs* 11 ± 1 ml min^{-1}; P=NS). During normoxic exercise, there was no change in oxygen delivery between control and NO/PG block conditions (60 ± 4 *vs* 58 ± 4 ml min^{-1}; P=NS). For hypoxic exercise, oxygen delivery was lower following NO/PG block (62 ± 5 *vs* 56 ± 4 ml min^{-1}; P<0.05).

Oxygen extraction for all trials and conditions is presented in Figure 4*B*. At rest and in hypoxia, oxygen extraction was greater after NO/PG block compared to control (Rest 1: 21 ± 3 *vs* 41 ± 4%, P<0.05; Rest 2: 22 ± 3 *vs* 34 ± 5%, Hyp: 21 ± 4 *vs* 28 ± 5%; P<0.05). Similarly, for hypoxic exercise, oxygen extraction was greater in the NO/PG block condition than control (59 ± 3 *vs* 64 ± 3%; P<0.05). There was no change in normoxic exercise oxygen extraction as a result of NO/PG blockade (60 ± 4 *vs* 63 ± 2%; P=0.20).

Oxygen consumption across the forearm is presented in Figure 4*C* for all trials and conditions. There were no differences in oxygen consumption at rest or during hypoxia within or between condition (P=0.15-0.94). Similarly, there were no differences in oxygen consumption during exercise as a result of hypoxia or NO/PG blockade (P=0.64-0.99).

DISCUSSION

The purpose of the present study was to investigate the combined role of NO and PGs to hypoxic exercise hyperemia. The primary novel findings from this study are as follows. First, under sympathoadrenal blockade, acute inhibition of NO and PGs reduces exercise hyperemia in hypoxia but not in normoxia (Table 3; Fig. 2). In other terms, the augmentation of the hyperemic response to hypoxic exercise, as compared to normoxic exercise, is significantly reduced by NO/PG block (~50%; Fig. 4). Secondly, following NO/PG inhibition, despite a reduction in hypoxic exercise FBF and therefore oxygen delivery, a significant increase in oxygen extraction was observed, and oxygen consumption was maintained at control levels (Fig. 4). Finally, data from the present study support previous unpublished observations from our laboratory that combined blockade of NO and PGs abolishes the hyperemic response in skeletal muscle observed during systemic hypoxia at rest in healthy humans (~70%; Fig. 3).

Limb blood flow responses to hypoxic exercise

Many investigators have reported that during hypoxic exercise, muscle blood flow is augmented relative to the same intensity level of exercise in normoxia (Hartley *et al.*, 1973; Rowell *et al.*, 1986; Koskolou *et al.*, 1997; Wilkins *et al.*, 2006). What factors regulate hypoxic exercise hyperemia in comparison to normoxic exercise has been a topic of recent interest. One idea, tested by Wilkins et al., was that low- to mild-intensity hypoxic exercise blunted post-junctional α-receptor-mediated vasoconstriction (Wilkins *et al.*, 2006). However, the vasoconstrictor response to endogenously released norepinephrine (via tyramine) was similar in normoxic and hypoxic exercise conditions.

The finding of no difference in the ability to blunt α-mediated constriction led the same group to hypothesize that an enhanced vasodilator signal accounts for the augmented muscle blood flow observed during hypoxic exercise. Thus, in a follow-up study, using local pharmacological α-receptor blockade (via phentolamine) to 'unmask' the hyperemic response to hypoxic exercise normally restrained by increased muscle sympathetic nervous activity, β-receptor-mediated dilation was assessed by administration of the non-selective β-receptor antagonist propranolol (Wilkins *et al.*, 2008). Based on the data presented, it appeared that β-mediated dilation was playing a partial role in the hyperemic response to hypoxic exercise at a low intensity (10% MVC), but not at a greater intensity (20% MVC). Thus, at mild intensity hypoxic exercise, the contributors to the enhanced vasodilator signal are not yet identified. Our data from the present study suggest a combined role NO and PGs in this capacity.

NO and PGs: Role in exercise, hypoxia, and hypoxic exercise hyperemia

The finding that there was no effect of NO/PG blockade on normoxic exercise hyperemia (Table 3 and Fig. 2) is in agreement with previous work from our group that demonstrated NO and PGs do not play a significant role in normoxic exercise hyperemia when inhibition occurs prior to forearm exercise onset (Dinenno & Joyner, 2004; Schrage *et al.*, 2004). While this lack of an effect is in contrast to some of the findings within the leg vasculature (Kalliokoski *et al.*, 2006; Mortensen *et al.*, 2007), the contributions of NO and PGs to exercise hyperemia appear to be intensity-dependent (Boushel *et al.*, 2002). The workload utilized in the present study (20% MVC), while higher than that in previous forearm studies (Dinenno & Joyner, 2004; Schrage *et al.*, 2004), may not be

great enough to elicit a major contribution from NO and PGs during normoxic exercise hyperemia.

Relatively little data exists that examines the combined role of NO and PGs in human hypoxic vasodilation. Previously, NO was shown to contribute to human hypoxic vasodilation (Blitzer *et al.*, 1996) and it was demonstrated that PGs contribute to hypoxic vasodilation in rats (Ray *et al.*, 2002). Our results from the present study indicate that combined inhibition of NO and PGs eliminate hypoxia-induced hyperemia (Fig. 3). The present findings are in agreement with observations from our laboratory that combined blockade of NO and PGs significantly reduces hypoxic vasodilation in the human forearm (Unpublished observations, Markwald RR and Dinenno FA).

Our data under control conditions is consistent with previous work that demonstrates an augmented hyperemic response to hypoxic exercise as compared to normoxic exercise at the same workload (Hartley *et al.*, 1973; Rowell *et al.*, 1986; Wilkins *et al.*, 2008). Inhibition of NO and PGs significantly reduced hypoxic exercise hyperemia, whereas it had no effect on normoxic exercise hyperemia. In turn, the augmented response of hypoxic exercise was significantly lower following NO/PG blockade (~50%; Fig. 4). Thus, we conclude that NO and PGs, in combination, significantly contribute to the augmented hyperemia during hypoxic exercise. After NO/PG block, hypoxic exercise FBF remains somewhat greater than normoxic exercise FBF suggesting other local NO/PG-independent vasodilator mechanisms also contribute to the augmented response.

Oxygen delivery, extraction, and consumption

In the present study, oxygen consumption did not differ between any of the exercise trials (Fig. 5*C*). It has been proposed that NOS inhibition can independently reduce oxygen consumption during normoxic exercise (Mortensen *et al.*, 2007). Indeed, there is some experimental evidence suggesting that NOS inhibition can reduce oxygen consumption for a given force production; however, these studies were done on isolated muscles subjected to electrical stimulus (King-VanVlack *et al.*, 2002; Baker *et al.*, 2006). Other studies performed *in vivo* with contracting skeletal muscle have shown no effect of NOS inhibition on oxygen consumption (O'Leary *et al.*, 1994; Frandsen *et al.*, 2001). Taking this into consideration, we do not believe that L-NMMA infusion had any direct effect on oxygen consumption in the present study. We interpret our findings related to oxygen delivery, extraction and consumption to suggest that at relatively mild workloads, oxygen extraction can be enhanced to maintain oxygen consumption, if delivery is compromised due to a reduced hyperemic response.

Potential mechanisms for the stimulus of NO and PG synthesis during hypoxic exercise

The underlying mechanism for the augmented stimulus of NO and PGs during hypoxic exercise is not well understood. Given that our experimental trials were performed with α- and β-adrenergic blockade, there should not have been any neural (norepinephrine/epinephrine) or adrenal (epinephrine) contribution to forearm vascular tone. Thus, the stimulus for NO and PG synthesis likely occurred at the local tissue level. There was no effect of NO and PG block on normoxic exercise hyperemia, and there was a significant reduction in the hyperemic response to both hypoxia at rest and hypoxic

exercise. These data suggest that the stimulus for NO and PG synthesis may be linked with the oxygen status of the blood or vessel. In this context, several hypotheses have been proposed for what may function as an 'oxygen sensor' to regulate peripheral blood flow.

The red blood cell has been proposed to regulate vascular tone by release of ATP in response to deoxygenation (Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995; Gonzalez-Alonso *et al.*, 2001). Some investigators have even demonstrated an obligatory role for RBCs in hypoxic vasodilation (Dietrich *et al.*, 2000). Related to exercise, plasma ATP levels tended to be higher during hypoxic leg extension exercise compared to normoxic exercise (Gonzalez-Alonso *et al.*, 2002). Recently, inhibition of NOS and COX was shown to significantly reduce the vasodilatory response to infused ATP (Mortensen *et al.*, 2008), suggesting that ATP-induced dilation may be partially mediated through NO and PGs. Thus, red blood cell release of ATP and subsequent binding to endothelial P_{2y} receptors could possibly be one stimulus for increased NO and PG production during hypoxic exercise as compared to normoxic exercise.

Another potential mechanism for NO and PG stimulus in response to changes in vessel oxygen tension and content is intrinsic to the endothelial cell layer itself. In vessel preparations perfused with physiological saline solution (no red blood cells), hypoxic vasodilation has been observed, and endothelium removal abolishes this response (Frisbee *et al.*, 2002). Hypoxia may act directly on endothelial cells and cause calcium release from the endoplasmic reticulum (Aley *et al.*, 2005). Increased intracellular calcium subsequently may activate phospholipase A_2, increasing intracellular arachidonic acid availability and thus initiating the production of PGs (Brotherton & Hoak, 1982).

Further, in response to hypoxia, endothelial cells can release adenosine, which can cause vasodilation through increased synthesis of NO and PGs (Marshall, 2000; Ray *et al.*, 2002). Additional studies are necessary to determine the specific mechanisms for increased NO and PG synthesis during hypoxic exercise and what other mechanisms may contribute to the augmented hyperemic response that remains after NO/PG blockade.

Experimental Considerations and Limitations

We did observe some unexpected systemic changes that might appear to affect the interpretation of our results. Interestingly, cardiac output was reduced during the hypoxic trial in the NO/PG block condition (Table 1). Since it could be argued that reduced cardiac output may explain the attenuated response during the NO/PG block hypoxic exercise trial, we correlated the change in blood flow to the change in cardiac output in this trial and all other exercise trials (see *Results*). Based on the lack of a significant correlation in these changes, we do not attribute the reduction in hyperemia to reduced cardiac output.

Our experimental approach of combined inhibition of NO and PG does not allow us to make conclusions on the individual contributions of these vasodilators, or determine whether any compensatory interactions exist. We chose the approach of combined inhibition based on our laboratory's observations that individual blockade of NO or PG did not influence hypoxic vasodilation at rest and combined inhibition significantly reduced hypoxic vasodilation (Unpublished observations, Markwald RR and Dinenno FA). Subsequent studies would need to be designed to specifically address the effects of independent blockade on hypoxic exercise hyperemia. Due to the long half-lives of the

inhibitors used (L-NMMA and ketorolac) we did not balance the order of control and blockade conditions. We did however, counter-balance the normoxic and hypoxic exercise trials and therefore any effect of time would not change our conclusions related to the differential effect of NO/PG blockade on hypoxic exercise hyperemia compared to normoxia.

One limitation of the present study is that we did not directly test the efficacy of any of our pharmacological blockers. However, based on the use of similar doses in previous studies (see *Methods*), and maintenance administration of drugs throughout the protocol, we are confident that we achieved successful inhibition. Further, based on our findings that NO/PG block did in fact reduce hypoxic exercise hyperemia, had we not achieved full inhibition of these vasodilator pathways, our results would *underestimate* the contribution of NO and PGs. It has been suggested that for studies involving exercise, N^G-nitro-L-arginine methyl ester (L-NAME) is the preferred NOS inhibitor for its ability to inhibit nNOS more sufficiently than L-NMMA (Frandsen *et al.*, 2001). However, in a similar exercise model, L-NMMA and L-NAME produced congruent results (Dinenno & Joyner, 2003). Again, since we did observe a significant reduction, if nNOS was not sufficiently blocked, we would be underestimating the contributions of NO. Additionally, as many of the studies related to the role of NO in hypoxic vasodilation have used L-NMMA, there is little reason to suggest that our choice of inhibitors would affect the hypoxic exercise response any differently than the normoxic exercise response.

Another potential limitation of this study is the use of mild intensity exercise with a small muscle mass. It is important to note that our conclusions related to the role of NO

and PGs in hypoxic exercise hyperemia are limited to small muscle mass mild exercise during moderate systemic hypoxia. As the role of NO and PGs has been shown to vary according to either exercise intensity (Boushel *et al.*, 2002) or severity of hypoxia (Frisbee *et al.*, 2002), it would not be surprising if the role of these vasodilators in hypoxic exercise also was stimulus-specific. Further, the conclusions related to the ability for changes in extraction to compensate for decreased blood flow and thus maintain oxygen consumption are only appropriate for this given level of exercise and hypoxia.

Conclusions

The results from the present investigation demonstrate that during local sympathoadrenal blockade, acute inhibition of NOS and COX significantly reduces the hyperemic response during mild intensity handgrip exercise with systemic isocapnic hypoxia. Inhibition of NO and PG synthesis does not affect normoxic exercise hyperemia. Therefore, we conclude NO and PGs play a significant role in the observed augmented hyperemic response to hypoxic exercise as compared to normoxic exercise. Oxygen consumption during hypoxic exercise was maintained after NO/PG block, despite a reduction in blood flow, due to a compensatory increase in oxygen extraction. Collectively, in control conditions, blood flow is regulated to match oxygen delivery with oxygen demand. When vasodilating mechanisms are altered, as with NO/PG block, this regulatory mechanism can be dissociated; however, oxygen consumption can be maintained by increased extraction. After NO/PG blockade, ~50% of the augmented hyperemia during hypoxic exercise remains, suggesting that other local factors also play a role in mediating this response.

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Table 1: Systemic Hemodynamic and Ventilatory Responses					
	Rest	**Normoxic Exercise**	**Rest**	**Hypoxia**	**Hypoxic Exercise**
Control					
MAP (mmHg)	95 ± 2	98 ± 3	94 ± 2	98 ± 4	100 ± 3†
HR (beats min^{-1})	56 ± 2	58 ± 2	57 ± 3	70 ± 3†	72 ± 3†◊
Minute Vent. (l min^{-1}; BTPS)	9.0 ± 1.2	9.6 ± 0.9	9.7 ± 1.3	15.0 ± 2.2†	18.1 ± 2.5†◊
End Tidal CO_2 (%)	6.2 ± 0.3	6.4 ± 0.2	6.2 ± 0.2	6.3 ± 0.2	6.5 ± 0.2
SpO_2 (%)	97 ± 0	97 ±0	98 ± 0	84 ± 1†	84 ± 1†◊
Cardiac Output (l min^{-1})	4.04 ± 0.38	4.55 ± 0.36	4.91 ± 0.51‡	5.78 ± 1.37†	6.03 ± 0.51†◊
NO/PG Block					
MAP (mmHg)	97 ± 2	99 ± 2	97 ± 2	100 ± 3	101 ± 3
HR (beats min^{-1})	55 ± 2	58 ± 2	53 ± 2*	64 ± 3*†	70 ± 3†◊
Minute Vent (l min^{-1}; BTPS)	11.0 ± 1.2	11.0 ± 0.5	10.9 ± 1.1	20.1 ± 3.2*†	21.2 ± 2.0*†◊
End Tidal CO_2 (%)	6.1 ± 0.2	6.4 ± 0.2	6.2 ± 0.2	6.3 ± 0.2	6.4 ± 0.2
SpO_2 (%)	97 ± 0	97 ± 0	98 ± 0	84 ± 1†	84 ± 1†◊
Cardiac Output (l min^{-1})	3.80 ± 0.27	4.38 ± 0.27	3.86 ± 0.31*	4.40 ± 0.38*	4.81 ± 0.42*†

*$P < 0.05$ *vs* control condition; † $P < 0.05$ *vs* rest (within trial and condition); ‡ $P < 0.05$ *vs* normoxic rest (within condition); ◊ $P < 0.05$ *vs* normoxic exercise (within condition).

Table 2: Blood Gases					
	Rest	**Normoxic Exercise**	**Rest**	**Hypoxia**	**Hypoxic Exercise**
Control					
pH_a	7.428 ± 0.010	7.410 ± 0.007	7.415 ± 0.011	7.428 ± 0.009	7.408 ± 0.009
pH_v	7.399 ± 0.011	7.314 ± 0.008†	7.390 ± 0.009	7.403 ± 0.009	7.324 ± 0.010†
P_aCO_2 (mmHg)	35.8 ± 1.4	36.0 ± 1.1	36.7 ± 1.5	35.5 ± 1.2	36.5 ± 1.0
P_vCO_2 (mmHg)	39.8 ± 1.7	55.0 ± 1.4†	42.0 ± 1.9	38.2 ± 1.4	52.1 ± 1.8†
$[Hb]_a$ (g dl^{-1})	15.2 ± 0.5	15.1 ± 0.5	15.2 ± 0.5	15.0 ± 0.5	15.0 ± 0.5
S_aO_2 (%)	96.4 ± 0.2	96.3 ± 0.2	95.9 ± 0.4	85.1 ± 1.0†	85.7 ± 1.0†◊
P_aO_2 (mmHg)	84.2 ± 1.8	84.0 ± 1.3	81.0 ± 2.1	49.5 ± 1.5†	51.7 ± 1.6†◊
P_vO_2 (mmHg)	44.3 ± 2.8	26.3 ± 1.4†	43.4 ± 2.3	37.5 ± 2.0†	24.3 ± 0.7†
ct_aO_2 (ml l^{-1})	203 ± 6	201 ± 7	202 ± 6	177 ± 6†	178 ± 6†◊
ct_vO_2 (ml l^{-1})	161 ± 8	82 ± 8†	159 ± 9	141 ± 10	74 ± 5†
a-v O_2 (ml l^{-1})	43 ± 6	120 ± 8†	43 ± 7	36 ± 7	104 ± 6†◊
NO/PG Block					
pH_a	7.425 ± 0.012	7.419 ± 0.011	7.429 ± 0.011	7.426 ± 0.008	7.411 ± 0.007
pH_v	7.384 ± 0.008	7.317 ± 0.008†	7.388 ± 0.009	7.401 ± 0.006	7.320 ± 0.006†
P_aCO_2 (mmHg)	34.1 ± 1.3	33.8 ± 1.2	33.0 ± 1.2*	32.1 ± 1.1*	32.8 ± 1.8*
P_vCO_2 (mmHg)	40.0 ± 1.3	51.9 ± 1.5†	39.5 ± 1.9	36.5 ± 1.5	50.1 ± 1.9†
$[Hb]_a$ (g dl^{-1})	14.8 ± 0.5*	14.9 ± 0.4	14.9 ± 0.4*	14.8 ± 0.4	14.9 ± 0.4
S_aO_2 (%)	96.3 ± 0.3	96.5 ± 0.2	96.3 ± 0.3	85.6 ± 1.0†	84.7 ± 1.0*†◊
P_aO_2 (mmHg)	84.4 ± 1.5	85.2 ± 1.0	85.7 ± 2.0*	52.5 ± 1.6*†	51.0 ± 1.5†◊
P_vO_2 (mmHg)	33.9 ± 2.0*	25.0 ± 0.9†	38.9 ± 3.3*	36.1 ± 2.2	24.8 ± 1.8†
ct_aO_2 (ml l^{-1})	199 ± 6	199 ± 0.6	199 ±5	176 ± 5†	181 ± 8†◊
ct_vO_2 (ml l^{-1})	118 ± 9*	74 ± 0.3†	131 ± 10*	126 ± 9*	64 ± 4†
$_{a\text{-}v}O_2$ (ml l^{-1})	81 ± 9*	126 ± 0.6†	68 ± 9*‡	49 ± 8*†	117 ± 9†

*P < 0.05 *vs* control condition; † P < 0.05 *vs* rest (within trial and condition); ‡ P < 0.05 *vs* normoxic rest (within condition); ◊ P < 0.05 *vs* normoxic exercise (within condition).

Table 3: Absolute FBF and FVC Responses					
	Rest	**Normoxic Exercise**	**Rest**	**Hypoxia**	**Hypoxic Exercise**
FBF (ml min^{-1})					
Control	87 ± 10	297 ± 18†	81 ± 10	110 ± 15†	345 ± 21†◊
NO/PG Block	50 ± 8*	289 ± 15†	52 ± 6*	61 ± 8*	312 ± 19*†◊
FVC (ml min^{-1} (100 mmHg)$^{-1}$)					
Control	92 ± 10	303 ± 17†	87 ± 11	113 ± 17†	345 ± 22†◊
NO/PG Block	52 ± 9*	291 ± 13†	54 ± 6*	61 ± 9*	310 ± 17*†◊

*P < 0.05 *vs* control condition; † P < 0.05 *vs* rest (within trial and condition); ◊ P < 0.05 *vs* normoxic exercise (within condition).

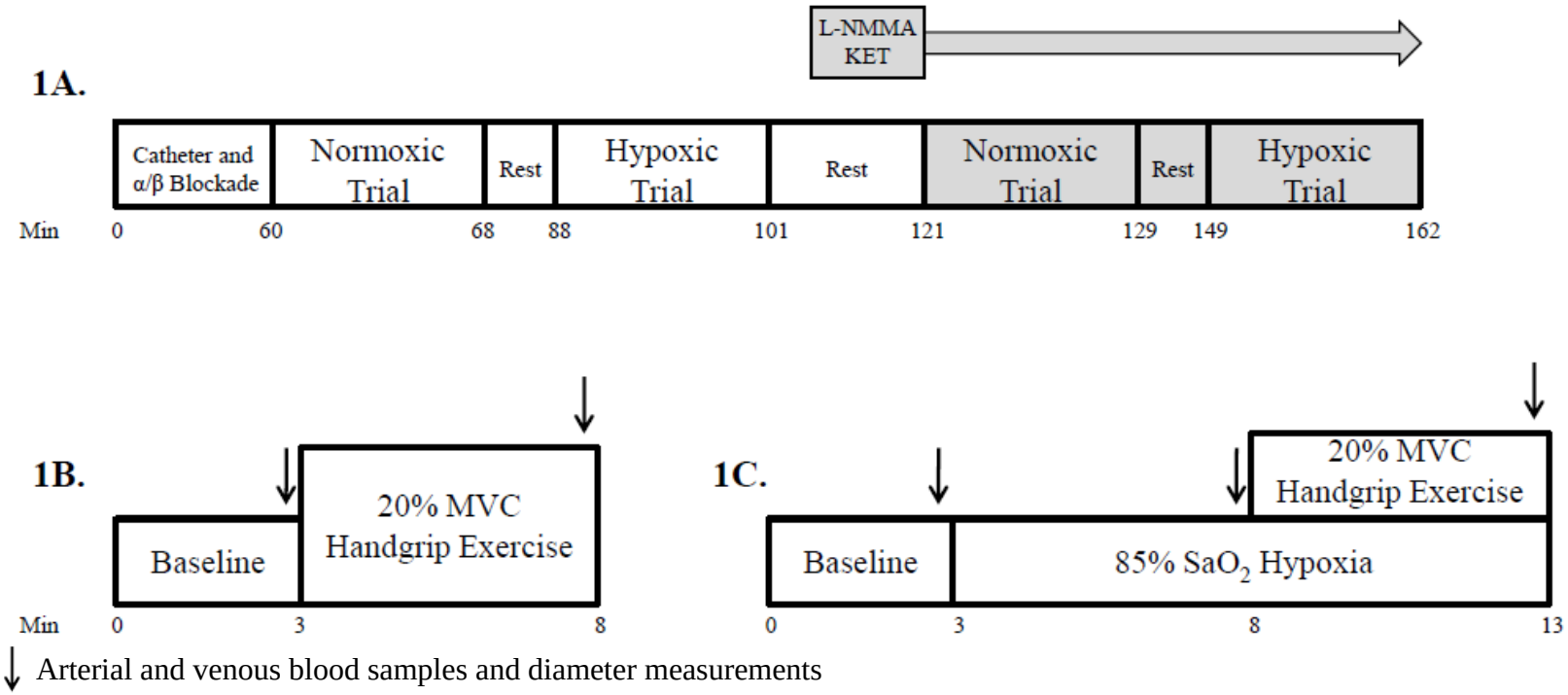

Figure 1. Timeline and experimental protocol

A: Overall experimental protocol; subjects' non-dominant arm was instrumented with a brachial catheter and a deep venous catheter. Phentolamine and propranolol were administered intra-arterially to block α- and β-adrenergic receptors, respectively, before the experimental trials. Twenty minutes of rest separated each trial. After both normoxic and hypoxic trials were performed in the control (saline) condition, L-NMMA and ketorolac were administered intra-arterially to block NO and PG synthesis, respectively. Normoxic and hypoxic trials were repeated under the blockade condition (shaded boxes). The order of the normoxic and hypoxic trials was counter-balanced. *B:* Normoxic trial timeline; baseline measurements were made for 3 minutes, followed by 5 minutes of 20% MVC rhythmic handgrip exercise. *C:* Hypoxia trial timeline; baseline measurements were made for 3 minutes, oxygen saturations were then reduced to ~85% (within first 2 minutes) and maintained for duration of trial. After 3 minutes of steady-state hypoxia, subjects performed 5 minutes of 20% MVC rhythmic handgrip exercise.

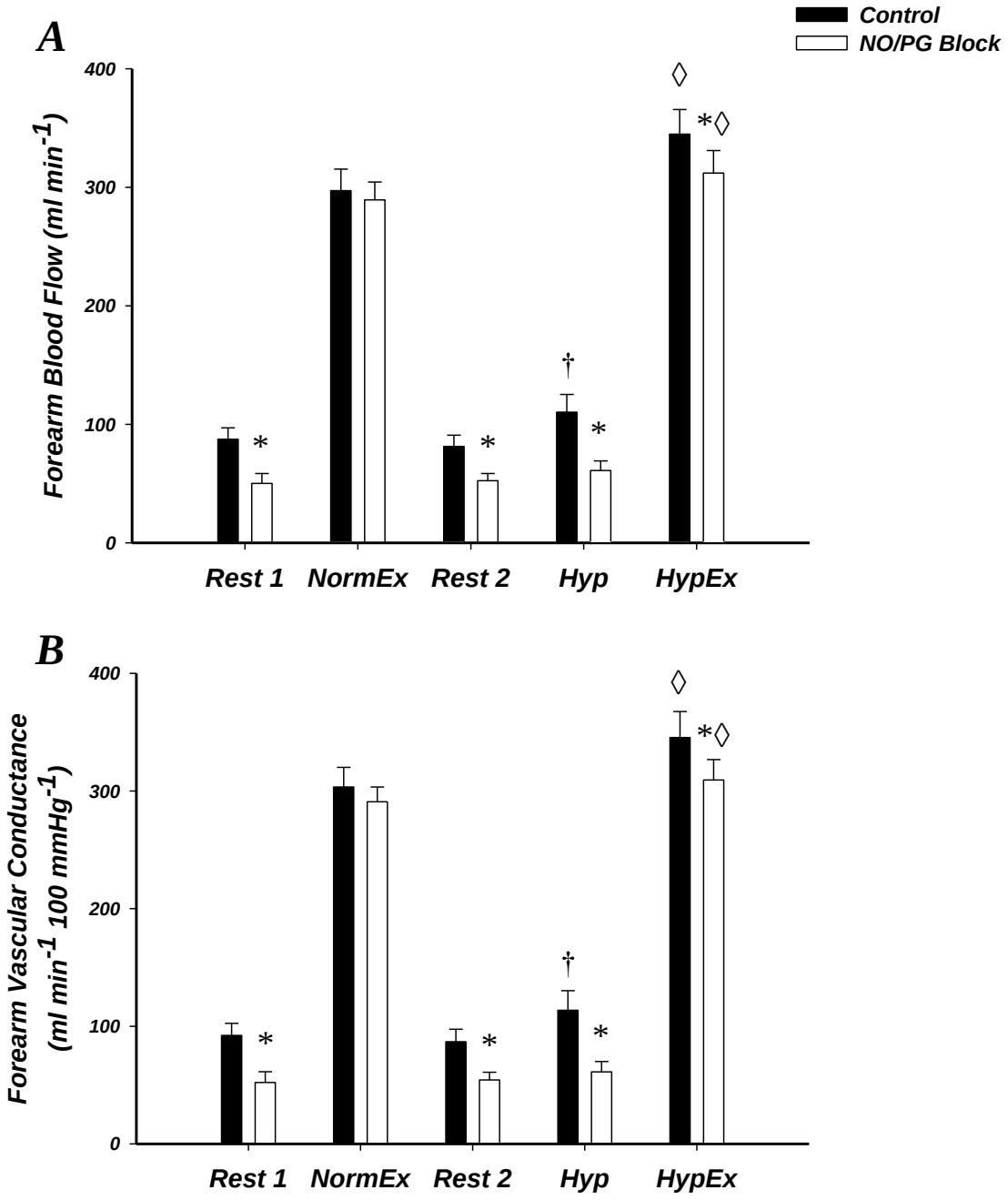

Figure 2. Forearm hemodynamics at rest, during normoxic exercise, hypoxia, and hypoxic exercise.

Forearm blood flow (*A*) and forearm vascular conductance (*B*) across all trials. Combined inhibition of NO/PG significantly reduced FBF and FVC at all time points except for normoxic exercise (NormEx). In control conditions, hypoxic exercise (HypEx) FBF and FVC was significantly greater than NormEx FBF and FVC. In NO/PG block conditions, this augmentation remained. Hypoxic Rest (Hyp) was significantly greater than Rest in control conditions, but not with NO/PG inhibition. **P*<0.05 *vs* control condition; † $P < 0.05$ Hyp *vs* Rest 2; ◊ $P < 0.05$ *vs* normoxic exercise (within condition)

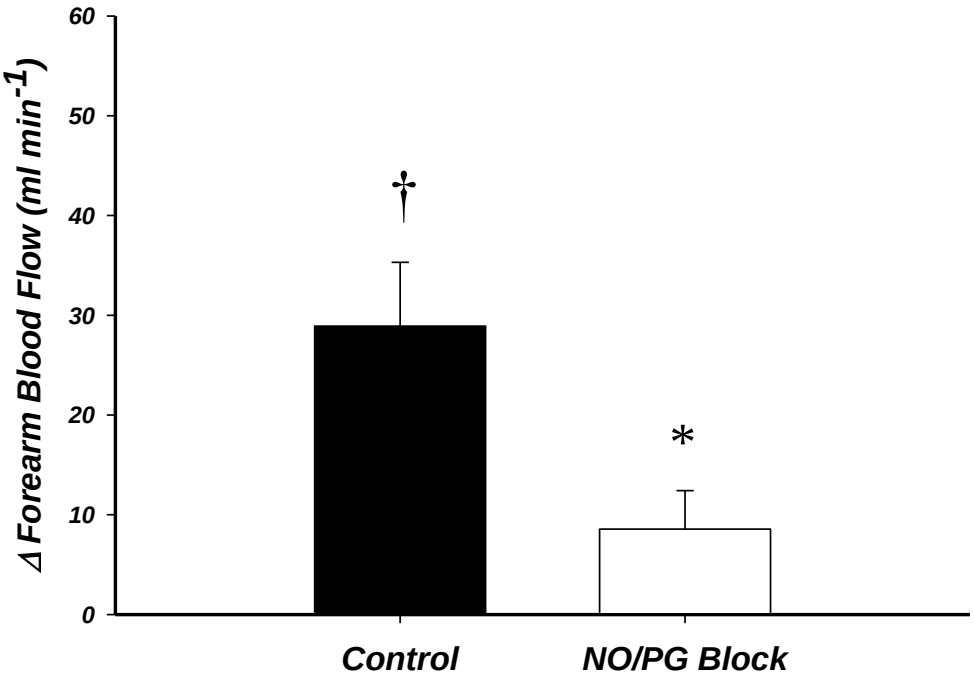

Figure 3. Forearm blood flow response to hypoxia at rest.
The hyperemic response to steady-state hypoxia (~85% SaO_2; pulse oximetry) from normoxia was calculated. Inhibition of NO and PG synthesis (L-NMMA and ketorolac, respectively) significantly reduced (~70%) the response to hypoxia. **P* < 0.05 *vs* control condition; † *P* < 0.05 *vs* zero.

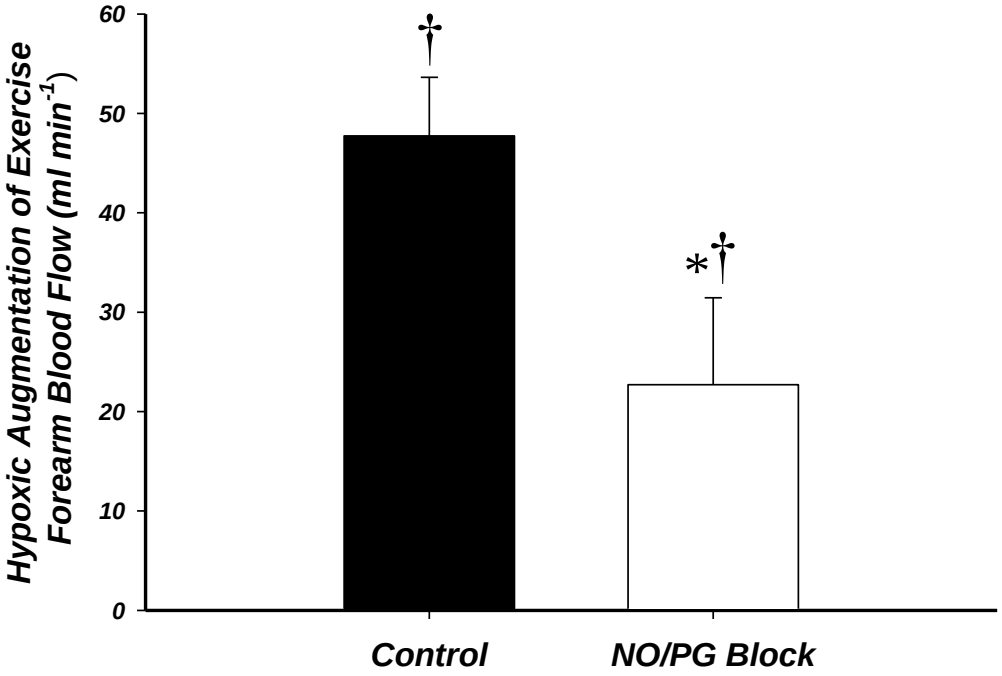

Figure 4. Effect of NO/PG blockade on exercise hyperemia.
Hypoxic augmentation was calculated for each condition as the difference in absolute forearm blood flow between hypoxic exercise and normoxic exercise. Inhibition of NO and PG synthesis (L-NMMA and ketorolac, respectively) significantly reduced (~50%) the hypoxic augmentation of exercise hyperemia. *$P < 0.05$ *vs* control condition; † $P < 0.05$ *vs* zero.

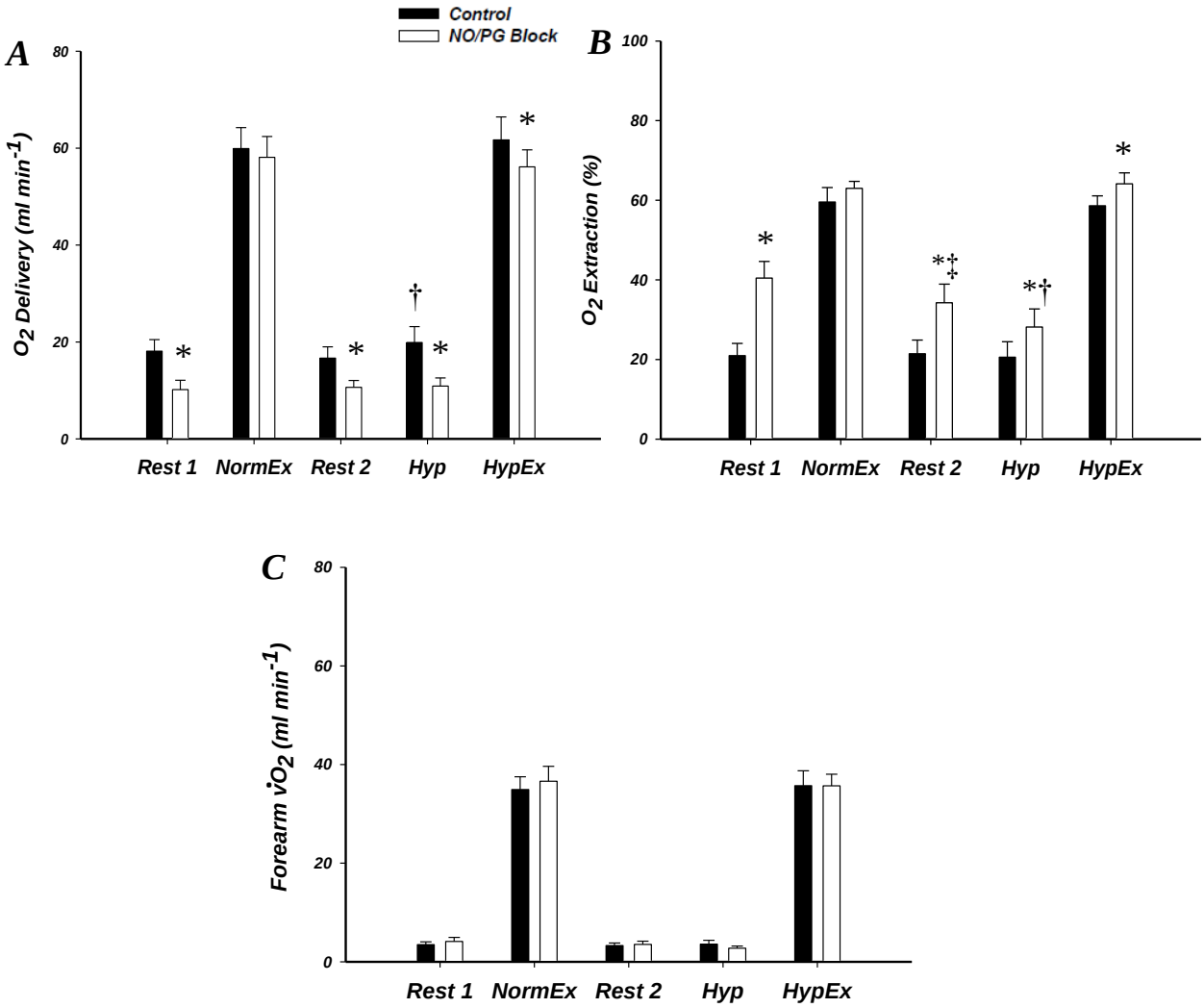

Figure 5. Forearm oxygen delivery, extraction and consumption for control and NO/PG block conditions.

Blockade of NO/PG resulted in a significant reduction in oxygen delivery (*A*) at rest, hypoxia (Hyp), and hypoxic exercise (HypEx). Subsequently, oxygen extraction (*B*) was significantly increased after NO/PG blockade for Rest, Hyp and HypEx. NO/PG blockade had no effect on oxygen delivery or extraction for normoxic exercise (NormEx; $P>0.05$). Oxygen consumption (*C*) was not different during NormEx and HypEx for control or NO/PG block conditions. All values for NormEx and HypEx in both conditions are significantly greater than Rest and Hyp ($P<0.05$ within condition). *$P<0.05$ *vs* control condition; † $P < 0.05$ *vs* rest (within trial and condition); ‡ $P < 0.05$ *vs* normoxic rest (within condition).

APPENDIX A

Colorado State University

Research Integrity & Compliance Review Office
Office of Vice President for Research
Fort Collins, CO 80523-2011
(970) 491-1553
FAX: (970) 491-2293

Notice of Approval for Human Research

Principal Investigator: Frank Dinenno, HES, 1582

Title: Regional Blood Flow Control & Vascular Function: Effects of Aging & Regular Physical Activity

Protocol #: 04-151H **Funding Source:** NIA

Number of Participants/Records: Remaining 18 + 100 additional = 118 remaining participants

Board Action: **Approval Date:** November 20, 2008 **Expires:** November 19, 2009

IRB Administrator: Janell Barker Janell Barker

Consent Process:
The above-referenced project was approved by the Institutional Review Board with the condition that the attached consent form is signed by the subjects and each subject is given a copy of the form. *NO changes may be made to this document without first obtaining the approval of the IRB.*

Investigator Responsibilities:

- It is the PI's responsibility to obtain this consent form from all subjects.
- It is the responsibility of the PI to immediately inform the IRB of any serious complications, unexpected risks, or injuries resulting from this research.
- It is also the PI's responsibility to notify the IRB of any changes in experimental design, participant population, consent procedures or documents. This can be done with a memo describing the changes and submitting any altered documents.
- Students serving as Co-Principal Investigators must obtain PI approval for any changes prior to submitting the proposed changes to the IRB for review and approval.
- The PI is ultimately responsible for the conduct of the project.
- A status report of this project will be required within a 12-month period from the date of review. Renewal is the PI's responsibility, but as a courtesy, a reminder will be sent approximately two months before the protocol expires. The PI will be asked to report on the numbers of subjects who have participated this year and project-to-date, problems encountered, and provide a verifying copy of the consent form or cover letter used. The necessary continuation form (H-101) is available from the RICRO web page http://ricro.research.colostate.edu.
- Upon completion of the project, an H-101 should be submitted as a close-out report.
- If approval did not accompany a proposal when it was submitted to a sponsor, it is the PI's responsibility to provide the sponsor with the approval notice.
- **Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.**

This approval is issued under Colorado State University's OHRP Federal Wide Assurance 00000647.
Please direct any questions about the IRB's action on this project to me for routing to the IRB.

Attachment Date of Correspondence: 12/1/08

APPENDIX B

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: *Regional Blood Flow Control and Vascular Function: Effects of Aging and Regular Physical Activity*

PRINCIPAL INVESTIGATOR: *Frank A. Dinenno, Ph.D. 491-3203*
CO-PRINCIPAL INVESTIGATORS: *Matt Hickey, Ph.D. 491-5727*
Wyatt Voyles, M.D. 663-3107

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? *You are a man or woman between the ages of 18-35 or 55-90 years. You are either 1) not exercising vigorously and regularly, or 2) have exercised vigorously and regularly for a number of years. Our research is looking at the effect of aging and exercise on regional blood flow control and how your blood vessels work.*

WHO IS DOING THE STUDY? *This research is being performed by Frank Dinenno, Ph.D., and Matt Hickey, Ph.D. of the Health and Exercise Science Department, and also by Wyatt Voyles, M.D., of the Heart Center of the Rockies. Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Institute on Aging, a part of the US Government.*

WHAT IS THE PURPOSE OF THIS STUDY? *The way in which blood flow (and oxygen delivery) and blood vessels are regulated by local factors and nerves during exercise and during changes in the composition of air you breathe is being studied. Importantly, cardiovascular regulation under these conditions might change in older people, it might be different between men and women, and it might be affected by regular physical exercise. The purpose of the research is to understand differences in how blood vessels work in various groups of adults, in different muscle groups (forearm, thigh, calf), as well as in the neck. The makeup of muscle fibers is also being studied.*

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
This whole research project will take place over a period of approximately five years. However, your part of this study will be either:
__X___ 1) one or two visits over a several day period, or _____ (your initials)
_____ 2) several visits over a few to several weeks. _____ (your initials)

WHAT WILL I BE ASKED TO DO? *This consent form applies to a large research project. You are only being asked to participate in one part of the total project. Depending on the part of the research project that you are involved in, you will be asked to participate in some of the following procedures. Many potential procedures are described in the section below. However, the procedures that you will be asked to do for*

this part of the study have a check mark next to them. The check marks were put there by one of the researchers. The time associated with each procedure reflects the amount of time you will spend performing or undergoing the procedure, not the total time of the study. A member of the research team will fully explain each checked procedure that applies to your participation and specifically how long each session (total time) in the laboratory will be.

__X___ ***Health and Physical Activity Questionnaire.*** *You will be asked to answer some questions about your health and exercise habits to determine if you can participate in the study. (~20 minutes)*

_____ ***(your initials)***

_____ ***Pregnancy Test.*** *If you are female you may be required to have a sample of your urine tested for the presence of human chronic gonadotropin (HCG), a hormone which indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. (~10 minutes)*

_____ ***(your initials)***

__X___ ***Heart Rate and Blood Pressure.*** *Heart rate will be measured by placing three sticky electrodes on your chest and reading the electrocardiogram (ECG) signal. Blood pressure will be measured with an automated machine that requires the placement of a cuff around your upper arm (bicep), or a small cuff on your finger. (continuous monitoring throughout study)*

_____ **(*your initials*)**

_____ ***Graded Exercise Test.*** *If you are in the 55-90 yr-old age group, you will be asked to perform a maximal exercise test on a treadmill under the supervision of a physician. This test will occur in the Human Performance Clinical/Research Laboratory in the Department of Health and Exercise Science on the CSU campus. Sticky electrodes will be placed on your chest, and you will walk briskly or jog while the steepness of the treadmill is increased. Your blood pressure and heart beat will be closely measured during and immediately after the test.*
(~1 hour) _____ ***(your initials)***

_____ ***Maximal Oxygen Consumption.*** *VO_{2max} testing will be performed on a treadmill while you are walking or running and the steepness of the treadmill is increased until you can't exercise any more. You will be asked to put your mouth around a scuba-like mouthpiece and wear a nose clip to prevent breathing through your nose. The amount of oxygen your body uses for energy will be determined from the oxygen and carbon dioxide you breathe in and out during the exercise. Your heart rate will be measured using a heart rate monitor. Body mass and height will be measured on a medical beam scale. (~30 - 45 min)*

_____ ***(your initials)***

__X__ ***Body Composition.*** *The fat, muscle, and bone in your body will be measured using an x-ray device (dual-energy x-ray absorptiometer) that will scan you from head to toe while you lie quietly on a special table for approximately 20 minutes. The amount of x-ray radiation you will receive is extremely low. (~20 minutes)*

_____ ***(your initials)***

_____ ***Forearm Volume.*** *The volume of your forearm will be measured via water displacement.*
You will place your forearm in a large water-filled cylinder, and the spillover of this water is collected in a large graduated cylinder to determine the volume. (~5 minutes)
_____ ***(your initials)***

__X___ ***Forearm Exercise****. You will lay flat on a bed and squeeze your hand and forearm muscles using a handgrip device while your hand and arm are comfortably secured. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (1 – 2 hours)*
_____ ***(your initials)***

_____ ***Calf Exercise.*** *You will sit in a special chair and squeeze your calf muscles (similar to standing on your toes) while your arms, hips, and shoulders are comfortably secured. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials.*
(1 – 2 hours)
_____ ***(your initials)***

_____ ***Knee Extensor Exercise.*** *You will sit in a special chair and squeeze your thigh muscles while your hips and shoulders are comfortably secured. Your feet will be secured in specially designed boots and you will be asked to extend your leg against resistance until your ankle is about at the height of your knee, relax back to a regular seated position, and then repeat. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (1 – 2 hours)*
_____ ***(your initials)***

__X___ ***Maximum Voluntary Contraction.*** *This will consist of 3-4 trials where you will squeeze your muscles (either forearm, calf, or thigh) and generate as much force as you can. You will be asked to generate as much force over the course of ~3 seconds and hold this force another 5 seconds. After a 2-3 minute rest period, you will be asked to do this again. This is typically used to determine how heavy of exercise you perform so everybody is exercising at similar percentages of their maximum. (~ 20 minutes)*
_____ ***(your initials)***

_____ ***Exercise Training.***
<u>Forearm</u>: You will be instructed to exercise five times per week, for a total of four weeks. You will be given a special exercise device and will be instructed to exercise with your non-dominant forearm squeezing your muscles 12 times per minute at 30-35% of your maximum until you can't exercise any more. When you are able to exercise at this initial workload for 30 minutes, the workload will be increased. You will need to visit the laboratory once per week to adjust the training workload as your performance improves.
_____ ***(your initials)***

Calf: You will be instructed to exercise five times per week, for a total of four weeks. You will be instructed to exercise with your calf muscles and squeeze this muscle 12 times per minute at 30-35% of your maximum until you can't exercise any more. You will be instructed to perform calf extension exercise in the upright position with added weight (if necessary) to achieve the pre-determined workload. When you are able to exercise at this initial workload for 30 minutes, the workload will be increased. You will need to visit the laboratory once per week to adjust the training workload as their performance improves.

_____ ***(your initials)***

Knee extensor: You will be instructed to exercise 3 times per week, for a total of eight weeks. You will be required to perform the training studies in the laboratory under supervision. Each training session will be 60 minutes. The first two weeks will consist of short (5-10 min) high intensity exercise bouts, whereas the second two weeks will consist of long (15-45 min) low intensity exercise bout. This pattern of training will be repeated to attain a total training period of eight weeks. As your exercise performance improves, the training workload will be adjusted accordingly.

_____ ***(your initials)***

Whole-body: You will be instructed to exercise 5 times per week, 40-50 minutes per exercise session at 60-85% of your maximum heart rate, for a total of twelve weeks. You will be asked to cycle, walk, jog, or run during this training period. You will be taught how to use heart rate monitors (provided by the lab) in order to train at the proper intensity as well as to record your exercise sessions.

_____ ***(your initials)***

____ ***Ischemic Exercise.*** *You will exercise your calf or forearm with a blood pressure cuff on your thigh or upper arm that is inflated very tightly to temporarily block the blood flowing to your muscle. You will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (20 – 30 minutes)*

_____ ***(your initials)***

____ ***Cold Pressor Test.*** *You will place your hand or foot in ice water for 2-3 minutes on several occasions. (~10 minutes)*

_____ ***(your initials)***

____ ***Lower Body Negative Pressure.*** *You will be placed in a sealed wooden chamber while you are laying flat on a bed. The chamber is sealed at your waist. Using a standard vacuum that is attached to the chamber, suction will be applied to mimic what happens when you go from laying to standing up. This will occur several times throughout the study for about 15 minutes at a time. (~ 1 hour)*

_____ ***(your initials)***

____ ***Up-right or head-down tilting.*** *You will be laying on a bed that is specially designed to be tilted ~60 degrees upright, or tilted downward ~10 degrees. This mimics what happens when you go from laying to standing up, and vice versa. (~ 1 hour)*

_____ ***(your initials)***

____ ***Forearm Negative/Positive Pressure.*** *You will place your forearm in a sealed chamber up to your elbow. Application of suction (like a vacuum) increases blood flow to your arm, whereas the opposite pressure reduces blood flow to your arm. (1-2 hours)*

_____ ***(your initials)***

____ ***Brachial Artery Compression.*** *A special device that is mounted to a frame above your forearm will be placed over your brachial artery at the elbow. When this device presses down on your arm, it will temporarily reduce the amount of blood to your forearm. This will be performed for approximately 5 minutes at a time, and will occur several times throughout the study. (1-2 hours)*

_____ ***(your initials)***

__X__ ***Breathing a low Oxygen or high Carbon Dioxide Gas Mixture.*** *The purpose of this test is to mimic what happens when you go up to altitude. You will be asked to place your mouth around a scuba mouthpiece while wearing a nose clip to prevent breathing through your nose. The amount of oxygen or carbon dioxide you are breathing will be changed carefully with a specially designed system, and you will breathe this for a maximum of 20 minutes at a time. You will be asked to do this several times throughout the study, with plenty of time in-between each trial. The amount of oxygen that is in your blood will be measured with a light sensor on your fingertip or earlobe. (1-1.5 hours)*

_____ ***(your initials)***

____ ***Venous Occlusion Plethysmography.*** *The blood flow in your forearm or calf will be measured by the use of blood pressure cuffs around your upper arm or thigh, and around your wrist or ankle. These cuffs will be inflated and deflated periodically. A sensitive gauge (similar to a rubber band) will also be placed around the maximum circumference of your forearm or calf. (2-3 hours)*

_____ ***(your initials)***

_X___ ***Doppler Ultrasound.*** *The blood flow in your arm, leg, neck, or brain will be measured using an ultrasound machine which produces sound waves to measure your blood vessel size and the speed of your blood. This also provides information about how elastic or stiff your blood vessels are. (2-3 hours)*

_____ ***(your initials)***

____ ***Reactive Hyperemia.*** *A blood pressure cuff will be placed on your upper arm or thigh and inflated really tight to temporarily block the blood to your forearm or calf. After 5, 10, or 15 minutes, the cuff will be released and the blood flow in your forearm or calf will be measured. This test is a measure of how much your blood vessels can relax and will be repeated several times throughout the study. (1- 1.5 hours)*

_____ ***(your initials)***

____ ***Flow-Mediated Vasodilation.*** *A blood pressure cuff will be placed on your forearm or your calf and inflated really tight to temporarily block the blood to your hand or foot. After 5, 10, or 15 minutes, the cuff will be released and the diameter changes of the blood vessels in your arm or leg will be measured using Doppler ultrasound. In some cases, your hand or foot will be warmed up for 15 minutes and the changes in blood vessel diameter will be measured. This will be repeated several times throughout the study. (1-1.5 hours)*

_____ ***(your initials)***

____ ***Sympathetic Nervous System Activity.*** *The measurement of sympathetic nervous system activity involves measuring the activity of one of your nerves on the side of your knee. Two small microelectrodes (small needles) will be placed through your skin. The position of one of the electrodes will be moved back and forth through your skin while a very small electrical impulse (1-2 volts) is passed through the electrode. This search procedure will continue until the electrode being moved causes your foot to twitch. This procedure will take between 5-60 minutes. When a foot or hand twitch is observed, measurement of the activity of the sympathetic nervous system will begin. (2-3 hours)* _____ ***(your initials)***

__X__ ***Blood Sample.*** *Up to 100 ml (approximately 7 tablespoons) of your blood will be drawn from a vein on the front of your elbow or artery in a standard fashion using a sterilized hypodermic needle. (~15 minutes)*

_____ ***(your initials)***

***** For Arterial Catherization, Venous Catheterization, or Muscle Biopsy:*** *<u>If you are allergic to lidocaine or novacaine, or have had a negative reaction to medicines injected while at the dentist, you should notify us immediately and not have any of these procedures done.</u>*

__X__ ***Venous Catheterization.*** *Your skin will be cleaned and a catheter (plastic needle) will then be inserted on the front side of your elbow and secured to the skin. In some cases, a local anesthetic might be used to reduce any discomfort. (~2-4 hours)*

_____ ***(your initials)***

__X__ ***Brachial Artery Catheterization.*** *Your skin will be cleaned and a local anesthetic will be given with a small needle to numb the area where the catheter will be placed (front side of your elbow). The catheter (plastic needle) will then be inserted and secured to the skin. (~2-4 hours)*

_____ ***(your initials)***

____ ***Femoral Artery Catheterization.*** *Your skin will be cleaned and a local anesthetic will be given with a small needle to numb the area where the catheter will be placed (about half way between your hip bone and groin on the front side of your leg). The catheter (plastic needle) will then be inserted and secured to the skin. (~2-4 hours)*

_____ ***(your initials)***

____ ***Drug Administration (~ 2 - 4 hours).*** *The administration of one of more of the following drugs might occur several times throughout the study.*

Vasoconstrictors – cause temporary narrowing of the blood vessels (minutes)
___ *Tyramine*
___ *Norepinephrine*
___ *Phenylephrine*
___ *Clonidine*
___ *Dexmedetomidine*
___ *L-NAME*
_X__ *L-NMMA*
___ *Aspirin*
_X__ *Ketorolac*

Vasodilators – temporarily relax the blood vessels (minutes)
___ Acetylcholine
___ Adenosine
___ Sodium Nitroprusside
___ L-Arginine
_X__ Phentolamine
___ Adenosine Triphosphate (ATP)

No major effects
___ Ascorbic Acid (Vitamin C)
_X__ Propranolol
___ Aminophylline

_____ ***(your initials)***

_____ ***Muscle Biopsy.*** *A sample of muscle will be taken from a muscle on the outside of your thigh. This will take place under the supervision of a medical doctor in the Hartshorn Health Center on the CSU campus. Your skin will be temporarily numb using lidocaine, a medicine similar to novacaine. After deadening the skin, a ¼ inch incision, or cut, is made in the skin over the muscle using a sterilized scalpel. The sample is obtained using a sterilized sampling needle. The muscle sample obtained is usually about ½ the size of the eraser on the end of a pencil. You will not have to reduce your activity afterwards, but should not perform any unusual or extremely vigorous activity for a few days. You will receive written instructions regarding care of the incision, and a telephone number to contact if you have any questions. (30 - 45 minutes)*

_____ ***(your initials)***

FUTURE USE OF BLOOD OR MUSCLE SAMPLES
It is possible that we may want to use any extra blood or muscle tissue for future research not described in this consent form. For example, this may include determination of certain gene expressions that relate to various measures of cardiovascular function measured as part of this study. This information will remain private as will all of the data collected from the study.
Only choose one of the following:

_____ *I give permission for the use of my blood or muscle tissue collected as part of <u>the current study only</u>.* _____ ***(your initials)***

_____ *I give permission for the use of my blood or muscle tissue for the current study <u>as well as for future studies.</u>* _____ ***(your initials)***

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
If you are not 18-35 or 55-90 years of age, are pregnant, are a regular smoker, or have any diseases that would affect our measurements or significantly increase the risks associated with this study, we will not be able to include you in the research.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?
(The procedures that apply to your proposed participation are checked)

- _X____ *<u>Health and Physical Activity Questionnaire</u> – there are no known risks associated with answering health questions. All information is kept strictly confidential.* _____ ***(your initials)***

- _____ *Graded Exercise Test – there is a risk of fatigue (temporary muscle tiredness), muscle strain, heart beat abnormalities (arrhythmias), a 0.01% chance of death (in people who have heart problems), a 0.02% risk of cardiac arrhythmias that would require you to go to a hospital (in people who have heart problems), and a risk of an increase or decrease in blood pressure.*

  _____ ***(your initials)***

- _____ *Maximal Oxygen Consumption – There is the possibility of fatigue, muscle strains, heart rhythm abnormality, and change in blood pressure. There is the possibility of falling off of the treadmill. Incidence of myocardial infarction (MI) is also a risk. 1 in 10,000 individuals with cardiovascular disease may die and 4 in 10,000 may have abnormal heart rhythms or chest pain.*

  _____ ***(your initials)***

- __X___ *Body composition (DEXA) scan – the risks associated with the DEXA are very low. The radiation you will receive is less than 1/3000th of the Food and Drug Administration (FDA) limit for annual exposure. The FDA is a government organization responsible for medical safety. In other words, you could receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation exposure. In this study you will receive one scan. The more radiation you receive over the course of your life, the greater the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.* _____ ***(your initials)***

- __X___ *Muscle contractions (Exercise) – There is a slight risk of muscle strain and muscle soreness resulting from brief strong muscle contractions. Soreness should not last more than two days or affect your normal function.*

  _____ ***(your initials)***

- _____ *Exercise training – There is a slight risk of muscle strain and muscle soreness resulting from brief strong muscle contractions. Soreness should not last more than two days or affect your normal function and should get progressively less as training continues.*

  _____ ***(your initials)***

- _____ *Ischemic Exercise – There is a risk of temporary discomfort and possible cramping in the forearm or calf during and after the exercise. These symptoms will be relieved when the exercise stops.*

  _____ ***(your initials)***

- _____ *Cold Pressor Test – There is a risk of temporary discomfort of the hand or foot. In rare cases, subjects might feel light-headed or nauseous. These symptoms will be relieved when the hand or foot is removed from the ice water and wrapped in a blanket.* _____ ***(your initials)***

- _____ *Lower Body Negative Pressure- There is a small risk of feeling nauseous or fainting. These symptoms will be relieved when the vacuum is turned off.*

  _____ ***(your initials)***

_____ *Up-right or head-down tilting – Small risk of feeling nausea or fainting during up-right tilt. These symptoms will be relieved when the table is tilted back and the subject is lying supine. There are no known risks for head-down tilt.*

_____ ***(your initials)***

- _____ *Forearm Positive/Negative Pressure – There is a small risk of slight discomfort or cramping if performing forearm exercise at the same time.*

  _____ ***(your initials)***

- _____ *Brachial Artery Compression – There is a risk of slight discomfort at the site of compression (elbow). There is also a risk of slight discomfort or cramping if performing forearm exercise at the same time.*

  _____ ***(your initials)***

- __X___ *Breathing a low oxygen or high carbon dioxide content gas mixture- The risks associated with this include light-headedness, headache and fainting. However, we will be monitoring all of your vital signals and will stop the procedure if this occurs. Symptoms will end momentarily after breathing normal room air.*

  _____ ***(your initials)***

- _____ *Venous Occlusion Plethysmography- There is a risk of temporary discomfort of the hand or foot when the blood pressure cuffs are inflated.*

  _____ ***(your initials)***

- _____ *Reactive Hyperemia/Flow-Mediated Vasodilation- There is a risk of temporary discomfort of the upper arm or thigh when the blood pressure cuffs are inflated. The discomfort might be greater the longer the cuffs are inflated.*

  _____ ***(your initials)***

- _____ *Sympathetic Nervous System Activity – Some subjects experience a temporary (seconds) pain and discomfort while the microelectrodes are being inserted. After the procedure there is a small risk of numbness, pins and needles type sensations, or pain which lasts 1-3 days. In very rare cases, numbness, pins and needles type sensations, or pain in the leg or arm has lasted several weeks or months (1-3 in 1000). These problems can be minimized by only having experienced individuals perform this technique. In addition, by minimizing the time to find the nerve to less than 60 minutes, the risk of unpleasant after-effects is reduced even more.*

  _____ ***(your initials)***

- __X___ *Blood sample – The risks associated with blood drawing include bruising, slight risk of infection, soreness, and fainting. These are minor risks which usually do not last more than one day if they occur.*

  _____ ***(your initials)***

- __X___ *Venous Catheterization- The risk of allergic reaction to lidocaine is extremely low. There is a risk of bruising, slight risk of infection, local soreness, and fainting.*

  _____ ***(your initials)***

- __X___ *Arterial Catheterization – The risk of allergic reaction to lidocaine is extremely low. There is a risk that pain or discomfort may be experienced when the catheter is*

inserted in the artery, and local soreness after the study. In about 1 in 10 cases a small amount of bleeding under the skin will cause a bruise. There is about a 1 in 1,000 risk of infection or significant blood loss. In about 1 in 4,000 damage may occur to the artery requiring surgery.

_____ ***(your initials)***

- *__X___ Drug Administration - The risks associated with drug administration include temporary increases or decreases in blood pressure and heart rate. In the case of clonidine and dexmedetomidine, you might experience mild drowsiness. These symptoms should resolve when the drug stops. With any of the vasoconstrictor drugs, there is a slight risk that ischemia (lack of blood to the tissues) could occur. Risks of these effects are minimized by calculating the amount of drug given relative to the size of your forearm or leg, and not the entire body. Finally, there is a potential risk of an allergic reaction to vasoactive drug administration. If you are allergic to aspirin, you should not participate.*

  _____ ***(your initials)***

- *_____ Muscle Biopsy – The risks associated with the muscle sample procedure include discomfort, soreness in that muscle, bruising, infection, and minor scarring. The discomfort and localized soreness are likely, but generally last only 24-48 hours. Temporary scarring is also expected. How wounds heal over time is different between people. The scar will only be about ¼ inch long, and is usually difficult to distinguish 8-12 months after the procedure. The risk of bruising is low, and infections are extremely rare.*

  _____ ***(your initials)***

- It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

WILL I BENEFIT FROM TAKING PART IN THIS STUDY? *There are no direct benefits to you for participating in this study beyond receiving information on your body composition and cardiovascular risk factors.*

DO I HAVE TO TAKE PART IN THE STUDY? *Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.*

WHAT WILL IT COST ME TO PARTICIPATE? *There is no cost to you for participating except that associated with your transportation to our facilities.*

WHO WILL SEE THE INFORMATION THAT I GIVE? *We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.*

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court or to the Human Research Committee at CSU.

CAN MY TAKING PART IN THE STUDY END EARLY? *Your participation in the study could end in the rare event of muscle strain, if you become pregnant, or if you miss an excessive number of appointments.*

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? *For experiments that involve the blood sample, muscle sample, fine wire electrodes, and arterial or venous catheterization, you will be paid $15/hour.*

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? *Please be aware that for this study the University has made special arrangements to provide initial medical coverage for any injuries that are **directly related** to your participation in this research project. The research project will provide for the coverage of reasonable expenses for emergency medical care related to the treatment of research-related injuries, if necessary.*

LIABILITY:
Because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries incurred as a result of participation in this study under a Colorado law known as the Colorado Governmental Immunity Act (Colorado Revised Statutes, Section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury.

In light of these laws, you are encouraged to evaluate your own health and disability insurance to determine whether you are covered for any physical injuries or emotional distresses you might sustain by participating in this research, since it may be necessary for you to rely on your individual coverage for any such injuries. Some health care coverages will not cover research-related expenses. If you sustain injuries, which you believe were caused by Colorado State University or its employees, we advise you to consult an attorney.

WHAT IF I HAVE QUESTIONS? *Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the principal investigator, Frank Dinenno, Ph.D., at (970)491-3203, or via email at fdinenno@cahs.colostate.edu. If you would like to ask a medical doctor about your participation in the study, you may contact Wyatt Voyles, M.D. at 663-3107. If you have any questions about your rights as a volunteer in this research, contact Janell Barker, Human Research Administrator, at 970-491-1655. We will give you a copy of this consent form to take with you.*

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 9 pages.

_____ _____

Signature of person agreeing to take part in the study Date

_____

Printed name of person agreeing to take part in the study

_____ _____

Name of person providing information to participant Date

_____

Signature of Research Staff

**** List of Contact Numbers in Case of Medical Emergency**

Wyatt Voyles, M.D.	Work: 970-221-1000 (24 hours a day)
Poudre Valley Hospital Emergency	970-297-6250
Frank A. Dinenno, Ph.D.	Work: 970-491-3203 Home: 970-266-1719