Age-related impairments in skeletal muscle PDH phosphorylation and plasma lactate are indicative of metabolic inflexibility and the effects of exercise training.

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Running Title: Aging and Metabolic Inflexibility

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Abstract
The purpose of this study was to determine if plasma lactate and skeletal muscle glucose regulatory pathways, specifically PDH dephosphorylation, are impaired during hyperinsulinemic conditions in middle- to older-aged individuals, and determine if exercise training could improve key variables responsible for skeletal muscle PDH regulation. Eighteen young (19-29 years, n=9/9, male/female) and twenty middle- to older-aged (57-82 years, n=10/10, male/female) underwent a 2-hr euglycemic-hyperinsulinemic clamp. Plasma samples were obtained at baseline, 30 min, 50 min, 90 min, and 120 min for analysis of lactate and skeletal muscle biopsies were performed at 60 min for analysis of protein associated with glucose metabolism. In response to insulin, plasma lactate was elevated in aged individuals when normalized to insulin action. Insulin-stimulated phosphorylation of skeletal muscle PDH on serine sites 232, 293, and 300 decreased in young individuals only. Changes in insulin-stimulated PDH phosphorylation was positively related to changes in plasma lactate. No age-related differences were observed in skeletal muscle phosphorylation of LDH, GSK3α, or GSK3β in response to insulin, or PDP1, PDP2, PDK2, PDK4 or MPC1 total protein. Twelve weeks of endurance- or strength-oriented exercise training, improved insulin-stimulated PDH dephosphorylation which was related to a reduced lactate response. These findings suggest that impairments in insulin-induced PDH regulation in a sedentary aging population contribute to impaired glucose metabolism and that exercise training is an effective intervention for treating metabolic inflexibility.
**Introduction**

Aging is associated with a number of detrimental health issues including insulin resistance and type 2 diabetes (T2D). Skeletal muscle dysfunction occurs during the aging process, involving impaired glucose uptake (10, 41, 46), increased intramuscular lipid (12), and reduced mitochondria oxidative capacity (21, 46), and is speculated to contribute to age-related whole-body insulin resistance. Metabolic flexibility is defined as the capacity of an organism to efficiently adjust substrate oxidation to match fuel availability (26) and is thought to protect individuals from insulin resistance (25). Recent research suggests aging is associated with the inability to switch from lipid to glucose oxidation during insulin stimulation (42), which could explain the increased incidence of type 2 diabetes in the elderly. Despite this knowledge, the role of skeletal muscle and the cellular mechanism(s) responsible for metabolic inflexibility, especially in the elderly, remain unclear and warrant further investigation.

A number of proteins are responsible for controlling the fate of glucose during insulin-stimulated uptake, which can be altered by age (41), disease (19), and energy demands (53). Insulin-stimulated glucose enters skeletal muscle and becomes phosphorylated to glucose-6-phosphate by the enzyme hexokinase and is either stored as intramuscular glycogen in response to increased GSK3 phosphorylation and glycogen synthase activity, or enters glycolysis (4, 13, 37, 47). The end-product of glycolysis, pyruvate, enters the mitochondria via the mitochondrial pyruvate carrier (MPC) (18) and is converted to acetyl CoA by pyruvate dehydrogenase (PDH) for oxidation (oxidative metabolism) (45), or is converted to lactate via lactate dehydrogenase (LDH) (anaerobic metabolism). It is thought that imbalances in any of these pivotal proteins may contribute to impairments in metabolic flexibility and the ultimate fate of glucose during insulin-stimulated glucose uptake.
Elevated plasma lactate is present in insulin resistant conditions including obesity and type 2 diabetes (2, 49), and has been shown to be a risk factor for the development of type 2 diabetes (23), suggesting that a diminished oxidative capacity may precede the onset of diabetes (47). Lactate has been reported to inhibit insulin-stimulated glucose uptake by inhibiting glycolysis and insulin signaling (7), indicating that the cellular mechanism(s) responsible for increasing lactate, especially during hyperinsulinemia, are unfavorable. Although not fully understood, one line of research suggests that lactate accumulation occurs as a result of pyruvate production exceeding pyruvate oxidation (2), possibly implicating the role of PDH.

During insulin-stimulated conditions, PDH activity has been reported to increase in healthy, lean individuals (8, 27, 37, 39), but is impaired during lipid-induced insulin resistance (39), suggesting a link between insulin resistance and impaired PDH regulation. Exercise-induced PDH activity has been reported to be attenuated in elderly compared to young individuals (17), providing evidence that PDH activity may be compromised with aging, but it remains unclear if other physiological conditions that favor glucose oxidation are impacted. The regulation of PDH activity is under the control of post-translational modifications including phosphorylation/dephosphorylation on at least three different serine residues (232, 293, 300). Previous research suggests that phosphorylation on any of these serine sites has the ability to inhibit PDH activity (29). An inverse linear relationship has been demonstrated between PDH activity and PDH phosphorylation (43), with phosphorylation (inactivation) being regulated by PDH kinases (PDK1-4), and dephosphorylation (activation) under the control of PDPs (PDP1-2). In vitro research has demonstrated that PDK1, which is only modestly expressed in skeletal muscle, is capable of phosphorylating all three serine sites, whereas PDK2-4 may only
phosphorylate Ser293 and Ser300 (30). PDP1 has been reported to be expressed in higher concentrations in skeletal muscle compared to PDP2, but both isoforms appear capable of dephosphorylating the three PDH serine sites (24). To date, it remains unclear how human aging may influence the regulation of PDH dephosphorylation during hyperinsulinemia.

Collectively, these findings suggest that with aging, metabolic flexibility is attenuated in response to physiological stimuli, however, the cellular mechanisms remain unknown. The purpose of the current study was to determine if plasma lactate and skeletal muscle glucose regulatory pathways, including PDH dephosphorylation, are impaired during hyperinsulinemic conditions in middle- to older-aged individuals, and whether exercise training could improve key variables responsible for skeletal muscle PDH regulation.

**Methodology**

**Participants**

Eighteen young (19-29 years, n=9/9, male/female) and twenty middle- to older-aged (57-82 years, n=10/10, male/female) individuals were recruited from a larger study examining aging, exercise training, and insulin sensitivity (10). A detailed description of subject inclusion/exclusion criteria was published previously (10). Briefly, all participants were non-smokers and participated in less than 1h/week of organized physical activity. Individuals with diabetes, heart disease, metabolic disorders, and those taking lipid-altering medications were excluded. In an attempt to study a representative population reflective of the “normal’ aging process, BMI was between the 25th and 75th percentile of the United States population for the respective decade of age (38). Written informed consent was obtained and the protocol was in
accordance of the Declaration of Helsinki and approved by the East Carolina University Policy and Review Committee on Human Research.

**Euglycemic-hyperinsulinemic clamp**

Subjects reported to the laboratory at 0700 after a 12-h overnight fast. A 2-h euglycemic-hyperinsulinemic clamp was used to determine insulin sensitivity, as previously described (10, 20). Briefly, insulin was infused for 120 min at a rate of 100 mU/m2 per min. Blood samples were obtained every 5 min and autoanalyzed for glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer; YSI Inc., Yellow Springs, OH) with glucose infusion (20% dextrose) adjusted as needed to maintain euglycemia. A steady-state M-value was determined from the final 30 min of the clamp (14). Blood plasma was obtained prior to insulin infusion and at 30 min, 50 min, 90 min, and 120 min, and stored at -80°C for the subsequent analysis of lactate. A biopsy specimen was obtained from the vastus lateralis with the percutaneous muscle biopsy technique at baseline and at 60 min of the clamp. Tissue samples were immediately frozen in liquid nitrogen for subsequent analyses.

**Exercise Training**

A detailed description of exercise training has previously been reported (10). Briefly, subjects were randomized into a 12-week endurance (n=10, young; n=10, aged) or strength (n=8, young; n=10, aged) training program. Endurance training consisted of exercising on a graded treadmill, stationary cycle, or elliptical trainer at 70–75% VO2peak for a total of 180 min/week (three to four sessions per week). Strength training consisted of five upper body exercises (chest press, latissimus pull down, seated row, triceps pull down, and biceps curl) and three lower body
exercises (leg press, leg extension, and leg curl). Subjects performed 2-3 sets of 10-12 repetitions to failure, three times per week (~45 min/session). All subjects performed preliminary cardiovascular and body composition measures, which were repeated during the final week of training and were reported previously (10). VO2peak was measured with an incremental, maximal treadmill test (16), with expired gases analyzed continuously (TrueMax 2400; ParvoMedics, Sandy, UT). Body composition was measured by dual X-ray absorptiometry. The euglycemic-hyperinsulinemic clamp with muscle biopsies was performed before exercise training and ~40 h after the final exercise training session.

Plasma lactate and western blot procedures

Plasma lactate was analyzed in duplicate using the Sigma Lactate II colorimetric assay (Sigma, St. Louis, MO). Skeletal muscle was homogenized and protein content was determined as previously described (9, 10). Twenty to twenty-five μg of cellular protein was separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and probed overnight with Cell Signaling Technology (Beverly, MA) antibodies for pGSK3α-Ser21, pGSK3β-Ser9, GSK3α, GSK3β, pGS-Ser641, GS, pLDH-Tyr10, LDH-A, Hexokinase II, MPC1, Calbiochem (Darmstadt, Germany) antibodies for pPDH-E1α-Ser232, pPDH-E1α-Ser293, pPDH-E1α-Ser300, Abcam (Cambridge, MA) antibodies for PDP1, PDP2, PDK2, as well as, Sigma antibody for PDK4, and Santa Cruz Biotechnology (Santa Cruz, CA) antibody for PDH-E1a. Samples were normalized to a control sample on each gel, and phosphorylation levels were additionally normalized to their corresponding total protein after membranes were stripped, as previously reported (10). Non-phosphorylated total protein was normalized to Actin (Santa Cruz Biotechnology).
**PDH activity**

PDH activity (in the active form) was determined using the PDH Activity Colorimetric Assay Kit (Biovision, Mountain View, CA) according to manufacturer’s instructions with minor modifications. Briefly, PDH activity was determined using 10mg of skeletal muscle tissue homogenized in buffer containing NaF (phosphatase inhibitor) and dichloroacetic acid (kinase inhibitor). PDH activity was measured by the kinetic reduction of NAD⁺ to NADH, which resulted in a colorimetric (450 nm) product proportional to the enzymatic activity present.

**Statistics**

Analyses were performed using SPSS version 21.0 software (SPSS Inc., Chicago, IL). Pearson correlation coefficients and stepwise regression analysis were used to determine associations. Comparisons between young and aged individuals under basal and insulin stimulated conditions, as well as, before and after exercise training were performed with repeated-measures ANOVA. Significant main effects and interactions were further analyzed using unpaired (age group) and paired (pre- vs. post-training and baseline vs. insulin-stimulated) contrast-contrast comparisons. Data are presented as means ± SEM. Statistical significance was defined as P < 0.05.

**Results**

**Subject Characteristics and Plasma Lactate**

Aged individuals had lower whole-body insulin action (M-value), aerobic capacity, and higher percent body fat as we previously published (10). There was a significant negative relationship between fasting plasma lactate and whole-body insulin action (Figure 1A, r=-0.35, P<0.05),
however, fasting plasma lactate did not differ between the two age groups (Figure 1B, P=0.62). In response to the hyperinsulinemic-euglycemic clamp, plasma lactate levels were significantly increased by 30 min (~25%, P<0.0005) and were further increased at 50 min (~50%, P<0.0001), irrespective of age (Figure 1C). Plasma lactate AUC during the hyperinsulinemic-euglycemic clamp did not differ between the age groups, however, when expressed relative insulin action, lactate AUC was greater (51%, P<0.05, Figure 1D) in the aged group.

Skeletal muscle PDH activity in response to the hyperinsulinemic-euglycemic clamp and relationship with PDH phosphorylation status

Due to limited skeletal muscle tissue, PDH activity was only determined during basal and insulin-stimulated conditions in five young (mean age: 24 years) and five aged (mean age: 71 years) individuals (Figure 2A). In the fasted state, PDH activity did not differ between the two age groups. In response to insulin, PDH activity increased in the young (~34%, P<0.05, Figure 2A), but not the aged individuals. Skeletal muscle PDH activity (all age and conditions pooled) was negatively associated with PDH phosphorylation on serine sites 232 (r=-0.47, P<0.05, Figure 2B), 293 (r=-0.37, P=0.10, Figure 2C), and 300 (r=-0.49, P<0.05, Figure 2D).

Skeletal muscle signaling response during hyperinsulinemic-euglycemic clamp

In the fasted state, skeletal muscle GSK3β phosphorylation was greater in aged compared to young individuals (~55%, P<0.05, Figure 3E), whereas fasting skeletal muscle phosphorylation of PDH (Figure 3A-C), GSK3α (Figure 3D), GS (Figure 3F), and LDH (Figure 3G), did not differ based on age or gender.
There was a significant interaction between age and the insulin response of PDH Ser232, Ser293 and Ser300 as the young individuals decreased phosphorylation by -43\% (P<0.01, Figure 3A), -26\% (P<0.01, Figure 3B), and -31\% (P<0.05, Figure 3C), respectively, whereas PDH phosphorylation did not change in aged individuals. There was a tendency for PDH Ser293 phosphorylation to increase in aged individuals (49\%, P=0.09, Figure 3B). Insulin infusion increased skeletal muscle phosphorylation of GSK3α (~50\%, P<0.005, Figure 3D), GSK3β (~115\%, P<0.0001, Figure 3E), and LDH-A (~30\%, P<0.05, Figure 3G), and decreased phosphorylation of GS (~-55\%, P<0.005, Figure 3F), regardless of age or gender. Insulin-stimulation had no effect on total protein content. Significant changes with insulin stimulation were similar whether phosphorylation status was expressed relative to total protein (Figure 3) or relative to actin.

Skeletal muscle total PDH was significantly higher in young compared to aged individuals (~70\%, P<0.05, Figure 4A), but did not differ by gender. The increased PDH content in young individuals resulted in a trend for the overall phosphorylation of all PDH three sites to be increased under basal and insulin-stimulated conditions compared to aged individuals (but did not affect the age x insulin interaction, Figure 3). Skeletal muscle total LDH (not shown), GSK3α (not shown), GSK3β (not shown), GS (not shown), hexokinase II (not shown), PDP1 (Figure 4B), PDP2 (Figure 4C), MPC1 (Figure 4D), PDK2 (Figure 4E), and PDK4 (Figure 4F) protein content did not differ with age or gender. PDH phosphorylation in the fasted state was not related to skeletal muscle PDKs or PDPs.
Univariate correlational analyses indicated that the change in phosphorylation of PDH Ser232 and PDH Ser293 with insulin were inversely related with whole body insulin action ($r=-0.50$, $P<0.005$, Figure 5A, and $r=-0.42$, $P<0.05$, Figure 5B, respectively) and VO2peak ($r=-0.49$, $P<0.01$, Figure 5D, and $r=-0.75$, $P<0.0001$, Figure 5E, respectively). There was a positive relationship between the change in phosphorylation of PDH Ser232, and PDH Ser293 with insulin and the change in plasma lactate AUC relative to insulin action ($r=0.79$, $P<0.0001$, Figure 5G, and $r=0.41$, $P<0.05$, Figure 5H, respectively). Fasting plasma lactate was positively related to the change in PDH phosphorylation on Ser232 in response to insulin ($r=0.48$, $P<0.05$, not shown). Changes in the phosphorylation of PDH on serine site 300 during insulin were negatively related with VO2peak ($r=-0.43$, $P<0.05$, Figure 5F), and skeletal muscle PDP2 content ($r=-0.42$, $P<0.05$, not shown). There were no significant relationships between skeletal muscle PDK2 or PDK4 and insulin-induced changes in PDH phosphorylation.

Stepwise linear regression was performed using variables linked to metabolic flexibility (age, gender, insulin sensitivity, aerobic capacity and percent body fat), pyruvate transport into the mitochondria (skeletal muscle MPC1), as well as, the dephosphorylation/phosphorylation of PDH (skeletal muscle PDP1, PDP2, PDK2, and PDK4) to determine variables that independently predicted the site specific phosphorylation response of PDH to insulin (Table 1). Chronological age and skeletal muscle PDP1 content were determined to be independent predictors of the insulin-induced change in PDH phosphorylation on serine site 232 ($R^2=0.41$, $P<0.005$). Aerobic capacity was determined to be the sole predictor of insulin-induced changes in the phosphorylation of PDH Ser293 ($R^2=0.50$, $P<0.001$), whereas chronological age and skeletal muscle PDP2 content were determined to be the best predictors of insulin-induced changes in PDH Ser300 ($R^2=0.36$, $P<0.005$).
Table 1. Stepwise linear regression analysis for predictors of insulin-stimulated changes in PDH phosphorylation.

<table>
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<th>Dependent Variable</th>
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<tr>
<td></td>
<td>Age</td>
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Age was coded (0,1) with the higher number indicating aged individuals.

Exercise Training

Differences in whole-body insulin action (M-value) (young: 8.6 ± 0.7 mg/kg/min, aged: 5.8 ± 0.5 mg/kg/min, P<0.005), total percent body fat (young: 24 ± 3%, aged: 38 ± 2%, P<0.0005), and VO2peak (young: 37 ± 2 ml/kg/min, aged: 21 ± 1 ml/kg/min, P<0.0001), were evident between the young and aged groups before initiating 12 weeks of endurance or strength training, as previously published (10). In response to exercise training, whole-body insulin action and aerobic capacity increased (28% and 11%, respectively, P<0.01), regardless of age or mode of training. Percent body fat decreased (-5%, P<0.01) with endurance training only.

In response to the hyperinsulinemic-euglycemic clamp, plasma lactate AUC relative to insulin action decreased after endurance (-23%, P<0.05, Figure 6A) but not strength training (-10%, P=0.17, Figure 6B), regardless of age. After 12 weeks of training, the plasma lactate response
remained higher in aged individuals (P<0.05), regardless of the type of exercise training (Figure 6).

Exercise Training and Skeletal Muscle

There was a significant interaction (P<0.05) between age and exercise training for the change in phosphorylation of skeletal muscle PDH on serine site 293. In response to insulin, the change in the phosphorylation of PDH Ser293 decreased in the aged group only after endurance (-60%, P<0.05, Figure 7C) and resistance (-71%, P<0.05, Figure 7D) training. Similarly, there was a tendency (age x training interaction P=0.07) for only aged individuals to suppress PDH serine 300 phosphorylation in response to strength training (-62%, interaction P=0.07, Figure 7F) during insulin infusion. There was a trend for endurance training to decrease the phosphorylation of PDH on sites 232 (-35%, P=0.06, Figure 7A) and 300 (-23% P=0.09, Figure 7E) during insulin infusion, regardless of age. Exercise training had no effects on the insulin-induced phosphorylation of GSK3α, GSK3β and LDH (not shown).

Endurance training increased PDH (89%, P<0.05), PDP1 (98%, P<0.05), PDP2 (57%, P<0.05), PDK2 (92%, P=0.05), PDK4 (62%, P<0.05), hexokinase II (147%, P<0.01), and MPC1 (105%, P<0.05) protein content with a tendency to increase GSK3α (54%, P=0.08), GSK3β (39%, P=0.07) protein content, regardless of age or gender (Figure 8A). Twelve weeks of strength training increased PDK2 (84%, P<0.05), PDK4 (68%, P<0.05), MPC1 (86%, P<0.05), and PDP1 (82%, P<0.05), regardless of age or gender (Figure 8B).

When endurance and strength training data was pooled, changes in insulin-induced PDH phosphorylation on sites Ser232 and Ser300 were positively related with changes in plasma
lactate AUC relative to insulin action (r=0.39, P<0.05, and r=0.40, P<0.05, respectively, not shown). Training induced changes in whole-body insulin action were negatively related to changes in insulin-stimulated PDH Ser300 phosphorylation (r=-0.36, P<0.05, not shown). Exercise-induced changes in skeletal muscle PDP2 content were negatively related to insulin-stimulated changes in phosphorylation of PDH on site Ser293 (r=-0.39, P<0.05, not shown).

Discussion

In the current study, we demonstrate for the first time that the insulin-stimulated dephosphorylation of skeletal muscle PDH, indicative of PDH activity, is impaired with the aging process (Figure 3A-C) and associated with an enhanced plasma lactate response (Figure 5G-I). Increased PDH activity during hyperinsulinemic conditions has been observed in healthy, lean individuals (37, 40, 52), providing evidence of metabolic flexibility, whereas PDH activity has been reported to be impaired during lipid infusion (37). Collectively, the current findings in aged individuals and the previous findings with insulin resistance, provide evidence of a link between conditions of insulin resistance and the desensitization of PDH during physiological conditions, indicative of metabolic inflexibility.

To our knowledge, this is the first study to demonstrate an augmented plasma lactate response relative to insulin action during hyperinsulinemia in aged individuals (Figure 1D), suggestive of impaired oxidative capacity or inefficient substrate utilization in insulin responsive tissue. Despite having a lower glucose uptake, our aged individuals had similar absolute lactate concentrations during the hyperinsulinemic clamp (Figure 1C), suggesting an exaggerated anaerobic response relative to glucose uptake. We acknowledge that our measured plasma
lactate levels were the net effect of lactate production and uptake, and that a number of tissue are responsible for lactate production, however, skeletal muscle is a primary site for insulin-stimulated glucose uptake and lactate production during fasting (44) and hyperinsulinemic conditions (11) and could contribute to the elevated lactate response. Recent findings by Vigelso et al. (54) suggest the augmented lactate response with aging is not limited to insulin-stimulation. Compared to their younger counterparts, aged individuals had an increased lactate response during acute exercise, and similar to the present study (Figure 3G-I), this increased lactate response was associated with an attenuated PDH response. These findings suggest that when modest amounts of glucose are taken into the skeletal muscle of aged individuals, inefficiencies in glucose regulatory pathways within the tissue are accentuated, likely as a result of impaired PDH activity, resulting in the preferential shuttling of glucose towards lactate.

Of particular interest, the insulin-induced phosphorylation of PDH on serine sites 232, 293, and 300 all decreased in our young, but not aged individuals (Figure 3A-C), suggesting the capacity for glucose oxidation during hyperinsulinemia is impaired in the skeletal muscle of aged individuals. This findings was further supported by our data demonstrating that insulin increased PDH activity in our subgroup of young individuals but not our aged individuals (Figure 2A). The current study adds to previous studies (5, 17, 42) by suggesting a specific cellular impairment, attenuated PDH dephosphorylation, contributes to the metabolic inflexibility with aging. Despite our previous research documenting reduced insulin-stimulated glucose uptake with aging, likely the result of diminished skeletal muscle AS160 phosphorylation and reduced GLUT4 protein, we do not feel the reduced PDH responsiveness was the sole result of diminished glucose uptake. While insulin action was related to some site-specific changes in
PDH phosphorylation (Figure 5A-B), regression analysis determined it was not an independent predictor of any of these PDH sites (Table 1). Our exercise training data provides further support since 12 weeks of either endurance or strength training did not abolish age-related discrepancies in insulin action but did normalize PDH responsiveness between the two age groups. Furthermore, it has been reported that aged individuals have diminished PDH activity in response to acute exercise, despite having a similar glucose uptake during exercise as their younger counterparts (54). Taken together, these findings provide evidence that a mechanism besides diminished glucose uptake contributed to the PDH desensitization in our aged individuals.

The current research adds to previous research demonstrating PDH activity is attenuated in response to exercise in the elderly (17) and highlights the severity of this impairment based on its presence in more than one physiological condition (exercise and hyperinsulinemia). Based on our findings (Figures 2A) and others (54), PDH activity in the resting state does not appear to be compromised with age but instead the capacity for PDH to become activated under conditions favoring glucose oxidation is diminished with aging. It remains unclear if the cellular mechanism(s) that contribute to the age-related reduction in PDH activity under hyperinsulinemia and exercise are similar since the two conditions stimulate independent and dependent signaling pathways. The use of hyperinsulinemia in the current study highlights the specific role that PDPs may have in age-related PDH responsiveness since insulin-stimulation is thought to increase PDP activity, but not PDK activity (6, 51), whereas exercise is expected to impact both PDK and PDP activity.
In contrast to the age-dependent response of PDH, the insulin-stimulated response of non-oxidative pathways (GSK3, GS, and LDH) were similar between the two age groups (Figure 3D-G). Although we did not measure glycogen content, or glycogen synthase (GS) activity, we do not expect that this pathway was increased to compensate potential decrements in pyruvate oxidation since we did not observe age-related differences in insulin-stimulated GSK3 or GS phosphorylation (Figure 3D-F). A paucity of research exists regarding the effects of aging and non-oxidative pathways in response to insulin. Insulin-induced GSK3 phosphorylation and glycogen synthase activity have been reported to be reduced with type 2 diabetes (19, 55), suggesting this pathway is impaired with other etiologies of insulin resistance. A novel finding in the current study was that skeletal muscle LDH-A, the enzyme responsible for the preferential conversion of pyruvate to lactate, increased phosphorylation in response to insulin, and there was a tendency for increased LDH phosphorylation (∼60%, Figure 3G) in the aged individuals.

In an attempt to gain an understanding of factors that regulate site specific dephosphorylation of PDH during insulin, we performed regression analyses using factors directly linked to skeletal muscle pyruvate transport into the mitochondria and PDH phosphorylation/dephosphorylation, as well as, factors known to influence metabolic flexibility. In addition to being young (under the age of 35), increased skeletal muscle PDP1 and PDP2 levels were independent predictors of insulin-stimulated dephosphorylation of PDH on serine sites 232 and 300, respectively (Table 1). To date, PDP1 and PDP2 are the only identified PDPs known to regulate PDH dephosphorylation, therefore, it was not totally unexpected that these would be predictors of PDH dephosphorylation, however, the fact that each was predictive of different serine sites is of significance since it suggests that each phosphatase could have a preferential site of action. In
contrast to PDPs, neither PDK2 nor PDK4 were predictors of PDH phosphorylation changes which supports previous research indicating insulin has an effect on PDPs and not PDKs (6, 51).

Aerobic capacity was determined to be the lone independent predictor of PDH dephosphorylation. This relationship was also not surprising since aerobic capacity was strongly correlated with PDH dephosphorylation during insulin stimulation (Figure 5D-F) and Love et al. (35) previously determined that skeletal muscle citrate synthase activity (a measure of skeletal muscle aerobic capacity) was related with PDP activity, and speculated that individuals with a greater aerobic capacity would also have a greater capacity for PDH dephosphorylation, PDH activity, and glucose oxidation, depending on cellular needs.

Differences in nutrient and metabolite ratios during hyperinsulinemia may have contributed to the age-related PDH response. During insulin-stimulation, we have previously observed a blunted decline in plasma NEFA levels with the aging process (LAC, unpublished data) suggesting that lipolysis is elevated and desensitized to insulin in older individuals. Based on previous research demonstrating that elevated FFA levels were responsible for the inhibition of PDH activity (43), it is conceivable that elevated FFA levels during hyperinsulinemia in the aged individuals played a role in the impaired ability to dephosphorylate PDH. Increased reliance on beta-oxidation in the aged individuals would also increase the ratios of NADH:NAD and acetyl-CoA:CoA which have been shown to inhibit PDH activity (48). Although not measured in the present study, it is also possible that age-related differences in skeletal muscle fiber type composition played a role in the phosphorylation status of PDH. It was recently reported in humans that insulin-stimulated PDH phosphorylation was decreased in type II fibers only (1), and in a rodent study, it was determined that PDP activity was higher in fast-twitch oxidative
muscle compared to slow-twitch oxidative muscle (31). Despite no differences in PDP content between the age groups (Figure 4B-C), the well-recognized age-associated decline in cross-sectional area of type II, but not type I fibers (28, 34) could have played a role in decreasing PDP activity, resulting in the loss of PDH responsiveness. Insulin has been shown to increase mitochondrial calcium (15), a known activator of PDP1, and conditions of insulin resistance have been associated with decreased mitochondria retention of calcium (50). Therefore, it is plausible that during insulin-stimulation, less calcium was available in the mitochondria of aged individuals to activate PDP1, and this contributed to the inability to dephosphorylate PDH. Taken together, it is evident that a number of age-related mechanisms have the potential to contribute to the desensitization of PDH to insulin, and warrant further investigation.

Exercise has long been recognized as a method to improve insulin sensitivity and substrate utilization in individuals, including the elderly (36). In general, there was a tendency for enhanced dephosphorylation of PDH with insulin in both young and aged individuals in response to exercise training (Figure 7), which was associated with a depressed lactate response, providing evidence that improving PDH responsiveness may reduce the flux of glucose towards anaerobic metabolism. Of particular importance, only aged individuals experienced exercise-induced improvements in insulin-stimulated dephosphorylation of PDH on site 293 (Figure 7C-D), suggesting that either endurance or strength training can offset initial age-related deficits on this site. For the most part, changes in phosphorylation were due to increased PDH phosphorylation in the fasted state, which was likely a result of exercise-induced increases in PDKs, however, there was an inclination for greater dephosphorylation during insulin-stimulation, particularly for aged individuals on serine site 300 after endurance and strength training. Of interest, we
determined that PDP2 content, which is known to be activated by insulin (22), was the best predictor of PDH 300 phosphorylation. These findings suggest that this site may be more receptive to insulin after exercise training in aged individuals in an effort to compensate for the initial age-related deficit. It could also explain differences between our findings and the study by Bienso et al. (3) that reported that PDH phosphorylation in response to an OGGT did not change after 8 weeks of endurance training in the elderly. Since insulin levels during an OGGT are variable and are generally lower after exercise training, it is difficult to measure the specific responsiveness of PHD relative to insulin levels during this procedure.

Our finding that exercise training increased both PDK2 and PDK4 expression has been reported previously with respect to PDK2 but not PDK4 (33). To our knowledge this is the first study to investigate the response of PDK expression to exercise training in older adults and provides evidence of continued muscle plasticity with aging. To date, no known data has been reported in humans regarding the effect of exercise training on PDP content. Our finding that both endurance and resistance training increases PDP1 content, regardless of age, supports previous research reporting the PDP1 content and activity was increased in obese rats in response to 8 weeks of endurance training (32), suggesting this isoform is responsive to exercise training even in conditions of metabolic inflexibility and insulin resistance. Since PDP2 is believed to be activated by insulin (22), the increase in PDP2 content that occurred in the aged individuals in response to endurance training (and trend to increase with resistance training) may have contributed to the increased PDH dephosphorylation that occurred in response to insulin after training. Collectively, our data in sedentary and exercise trained individuals highlight the importance of PDP content in the ability to dephosphorylate PDH and suggests that
interventions to increase skeletal muscle PDP content may be important in decreasing variables that inhibit insulin signaling, including lactate (7).

In response to exercise training, especially endurance training, there was an upregulation of glucose (10) and pyruvate transport proteins (Figure 8). To our knowledge, this is the first study to examine the effects of age and exercise training on skeletal muscle MPC1, one of two proteins responsible for the transport of pyruvate into the mitochondria. Whereas, MPC1 protein content did not appear to be affected by age, it was interesting to note that protein content increased in response to both endurance and strength training, which could increase the import of pyruvate into the mitochondria during physiological conditions requiring increased glucose oxidation.

In summary, the findings from the current study indicate for the first time that insulin-stimulated dephosphorylation of PDH is impaired on multiple serine sites with the aging process, indicative of metabolic inflexibility. The impaired PDH response was associated with an augmented lactate response, suggesting that under insulin-stimulated conditions, pyruvate is shuttled towards anaerobic metabolism with aging. With respect to twelve weeks of exercise training, impairments in PDH phosphorylation were rescued and were associated with a blunted lactate response, providing evidence that exercise training is an effective option to counteract metabolic inflexibility with aging.
REFERENCES


FIGURE LEGENDS

Figure 1. Plasma lactate during fasting and hypersinsulinemic conditions. Relationship of fasting plasma lactate and whole-body insulin action in the whole group (n=32), squares represent response from young individuals and circles represent response from aged individuals (A). Absolute fasting plasma lactate in young (n=16) and aged (n=16) individuals (B). Plasma lactate (C) and plasma lactate relative to insulin action (D) in response to a hyperinsulinemic-euglycemic clamp in young (n=16) and aged individuals (n=16). *P<0.05 vs. young, **P<0.005 and ***P<0.0001 vs baseline.

Figure 2. Skeletal muscle insulin-stimulated (1-hr) PDH activity is impaired in aged individuals and PDH activity is negatively associated with PDH phosphorylation. Skeletal muscle PDH activity under basal and insulin-stimulated conditions in a subset of young (n=5) and aged individuals (n=5) (A). Skeletal muscle PDH activity and skeletal muscle PDH phosphorylation on serine site 232 (B), 293 (C), and 300 (D). For B-D, squares represent data points for young individuals in the basal state, circles represent data points for aged individuals in the basal state, triangles represent data points for young individuals in the insulin-stimulated state, and diamonds represent aged individuals in the insulin-stimulated state.

Figure 3. Phosphorylated protein responsible for skeletal muscle glucose metabolism under basal and insulin-stimulated (1-hr) conditions. Phosphorylation levels of skeletal muscle PDH on sites Ser232 (n=15, young, n=16, aged) (A), 293 (n=16, young, n=16, aged) (B), 300 (n=14, young, n=15, aged) (C), GSK3α (n=16, young, n=16, aged) (D), GSK3β (n=16,
young, n=16, aged) (E), GS (n=6, young, n=6, aged; due to limited tissue) (F) and LDH (n=16, young, n=16, aged) in response to basal and hyperinsulinemic conditions. Phosphorylation data are normalized to total protein. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001 vs. basal condition; † P<0.05 vs. young individuals under the same condition.

**Figure 4.** Skeletal muscle total protein responsible for regulating PDH. Skeletal muscle PDH (n=16, young, n=16, aged) (A), PDP1 (n=16, young, n=16, aged) (B), PDP2 (n=16, young, n=16, aged) (C), MPC1 (n=15, young, n=15, aged) (D), PDK2 (n=12, young, n=12, aged) (E), and PDK4 (n=15, young, n=15, aged) (F). Protein analyzed with less than n=16 in each group or only under basal conditions (PDKs) is reflective of limited skeletal muscle tissue. Total protein data are presented relative to β-actin. *P<0.05 vs. basal condition.

**Figure 5.** Relationships among insulin-stimulated phosphorylation levels of skeletal muscle PDH and whole-body and plasma lactate variables. Changes in skeletal muscle PDH Ser232 and whole body insulin sensitivity (A), VO2peak (D), and plasma lactate AUC/M (G). Changes in skeletal muscle PDH Ser293 and whole body insulin sensitivity (B), VO2peak (E), and plasma lactate AUC/M (H). Changes in skeletal muscle PDH Ier300 and whole body insulin sensitivity (C), VO2peak (F), and plasma lactate AUC/M (H). Squares represent data points for young individuals and circles represent data points for aged individuals.
Figure 6. **Plasma lactate in response to exercise training.** Plasma lactate AUC relative to insulin action in response to Endurance (n=8, young; n=8, aged) (A) and Strength training (n=8, young; n=8, aged) (B). Plasma lactate AUC was normalized to insulin action *P<0.05 vs. pre-training, † P<0.05 vs. young at the same time point.

Figure 7. **Change in insulin-induced PDH phosphorylation with exercise training.** Fold change in the phosphorylation levels of skeletal muscle PDH Ser232 in response to insulin before and after 12 weeks of endurance (n=7, young; n=8, aged) (A) and strength training (n=8, young; n=8, aged) (B). Fold change in the phosphorylation levels of skeletal muscle PDH Ser293 in response to insulin before and after endurance (n=7, young; n=8, aged) (C) and strength training (n=8, young; n=8, aged) (D). Fold change in the phosphorylation levels of skeletal muscle PDH Ser300 in response to insulin before and after endurance (n=7, young; n=7, aged) (E) and strength training (n=8, young; n=8, aged) (F). * P<0.05 vs. pre-training for aged only. † P<0.10 vs. pre-training for aged only, † with line above the bars represents the tendency for a main effect for training (P<0.10).

Figure 8. **Fold change in skeletal muscle total protein in response to exercise.** Fold change (post-training relative to pre-training) in total protein in response to endurance (A) and resistance (B) training in young and aged individuals (n= 6-8, young and aged each). P≤0.05 with the line above the bars represents main effect for training.

Figure 9. **Representative western blots for Figure 3, Figure 4, Figure 7, and Figure 8.** Representative blots for young and aged individuals under basal and insulin-stimulated
conditions in response to endurance training (A) and resistance training (B). Due to limited
skeletal muscle availability the effects of endurance and resistance training were only measured
under basal conditions for PDK2 and PDK4 (C).

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A. Endurance Training

- Young
- Aged

Plasma Lactate AUC / M

Pre-Training  Post-Training

B. Resistance Training

- Young
- Aged

Plasma Lactate AUC / M

Pre-Training  Post-Training
A. **Endurance Training**

- PDH Total
- PDP1
- PDP2
- PDK2
- PKD2
- GSK3α Total
- GSK3β Total
- LDH Total
- Hexokinase II
- MPC1

Fold Change
Total Protein/Actin (AU)

B. **Strength Training**

- PDH Total
- PDP1
- PDP2
- PDK2
- PKD2
- GSK3α Total
- GSK3β Total
- LDH Total
- Hexokinase II
- MPC1

Fold Change
Total Protein/Actin (AU)