Aging is Associated With Myocardial Insulin Resistance and Mitochondrial Dysfunction


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Abstract

**Background:** Aging is associated with insulin resistance often attributable to obesity and inactivity. Recent evidence suggests that skeletal muscle insulin resistance in aging is associated with mitochondrial alterations. Whether this is true of the senescent myocardium is unknown.

**Methods:** Twelve young (Y: 4 years) and 12 old (O: 11 years) dogs, matched for body mass, were instrumented with LV pressure gauges, aortic and coronary sinus catheters, and flow probes on left circumflex artery. Prior to surgery, all dogs participated in a 6-week exercise program. Dogs underwent measurements of hemodynamics and plasma substrates before and during a two-hour hyperinsulinemic-euglycemic clamp to measure whole body and myocardial glucose and NEFA uptake. Following the protocol, myocardial and skeletal samples were obtained to measure components of the insulin-signaling cascade and mitochondrial structure.

**Results:** There was no difference in plasma glucose (Y: 90±4; O: 87±4 mg/dl) but O had higher (p<0.02) non-esterified fatty acids (Y: 384±48; O: 952±97 µmol/L); and plasma insulin (Y: 39±11; O: 108±18 pmol/L). Old dogs had impaired total body glucose disposition (Y: 11.5±1; O: 8.0±0.5 mg/kg/min, p<0.05) and insulin stimulated myocardial glucose uptake (Y: 3.5±0.3; O: 1.8±0.3 mg/min/g, p<0.05). The impaired insulin action was associated with altered insulin signaling and GLUT-4 translocation. There were myocardial *mitochondrial structural* changes observed in association with decreased expression of UCP-3.

**Conclusion:** Aging is associated with both whole body and myocardial insulin resistance, independent of obesity and inactivity, but involving *altered mitochondrial structure* and impaired cellular insulin action.

**Key Words:** aging, insulin resistance, myocardium, mitochondria, uncoupling protein-3
Advanced age is associated with the development of whole body insulin resistance and increased incidence of Type 2 diabetes in humans (4,9,14,33). Aging is also frequently accompanied by obesity and inactivity that predispose to the development of insulin resistance (6,16,17,32,39). However, it is unclear what independent role aging plays in the pathogenesis of insulin resistance in the absence of obesity and inactivity. Furthermore, it is unclear as to whether these abnormalities in insulin action extend to the senescent myocardium, where continuous contractile function makes inactivity less relevant.

Recently, skeletal muscle insulin resistance in both advanced age (1,28) and in lean offspring of patients with Type 2 diabetes (10,22,29) was associated with altered mitochondrial protein expression (28) and density (10). These abnormalities were seen in association with increased intracellular lipid accumulation (22,28,29). However, it is unknown whether these same alterations are evident in the senescent myocardium, whether they are associated with altered insulin action and whether they predispose to altered LV hemodynamics in the absence of hyperglycemia or other conventional risk factors.

Accordingly, the purpose of the present study was to determine if aging alone was associated with both whole body and myocardial insulin resistance, independent of obesity and inactivity. A second goal was to determine if myocardial insulin resistance is associated with LV, systemic or coronary hemodynamic abnormalities. A third goal was to determine the cellular mechanisms responsible for myocardial insulin resistance with advanced age. Finally, we sought to determine the role of altered mitochondrial content and function in the pathogenesis of myocardial insulin resistance. We chose the model of senescent beagles as a well characterized, large animal model of aging. Specifically, senescent beagles have been utilized to investigate age
related alterations in myocardial contractility (10), coronary and ventricular remodeling (35) and response to exercise (11,12) independent of the influence of atherosclerosis.

Methods

Acclimation

Twenty-four male dogs, all of which had a beagle background and weighing 14-18 kg, were either classified as young (Y: age 3-4 years) or old (O: 10-12 years) by the vendor. The veterinary staff confirmed the ages through dental examinations. The dogs were carefully screened for all disease known to occur commonly in canines bred for research using laboratory and radiological studies. These include at least three samples for heart worms. In addition, at the time of euthanasia, a formal autopsy was performed to ascertain whether there were any clinically undetected diseases such as cancer.

The animals were acclimated to the research laboratory for 6 weeks prior to instrumentation. During the acclimation period, they were fed a standard chow once daily with a fixed carbohydrate and fat content. In addition, the dogs underwent a supervised exercise regimen on a treadmill each day for 30 minutes four times per week for six weeks prior to surgery.

Instrumentation

All dogs were instrumented as described previously from our laboratory (26,27). The dogs were allowed to recover from the surgical procedure for two weeks, during which time they were trained to lie quietly on the experimental table in a conscious, unrestrained state. Animals used in this study were maintained in accordance with the “Guide for the Care and Use of Laboratory Animal Resources” [DHHS Publication No (NIH) 86-23, Revised 1996] and the guidelines of the Institutional Animal Care and Use Committee at Allegheny General Hospital.
Experimental Protocol

Hemodynamic Measurements

Control experiments consisted of hemodynamic recordings to determine LV contractility (LV dP/dt), stroke volume (SV), cardiac output (CO), and coronary blood flow (CBF). Arterial (a) and coronary sinus (v) blood samples were obtained to calculate myocardial oxygen consumption (MvO2) as the product of the left circumflex coronary artery blood flow and the myocardial arterio-venous O2 content difference.

Metabolic Determinations

Metabolic parameters were measured at 8 am, following an overnight fast. Transmyocardial substrate balance was calculated as the difference between arterial and coronary sinus content. Basal myocardial substrate uptake was calculated as the product of myocardial substrate balance and coronary blood flow (26,27).

The measurements of plasma norepinephrine, insulin, glucagon, adiponectin, non-esterified fatty acids (NEFA), and glucose were carried out as described previously from our laboratory (26).

In all dogs, whole body and myocardial insulin sensitivity was assessed in the baseline state using the hyperinsulinemic-euglycemic clamp (26,27). In the fasting state, a primed, constant infusion of insulin (480 pmol • m^-2 • min^-1) was administered for 120 minutes to create a steady state concentration of plasma insulin (~1000 pmol/L). Arterial glucose concentrations were measured every 5 minutes and glucose was infused to maintain plasma glucose concentrations at 90 mg/dL ±10%. Myocardial glucose and NEFA balance and coronary blood flow were sampled every 15 minutes to determine myocardial glucose uptake.
Cellular Insulin Signaling

Samples of LV myocardium and gracilis (skeletal) muscle from 8 young and 8 old dogs were obtained at the time of euthanasia under basal and insulin stimulated conditions (100 U regular insulin intravenously via the right atrial catheter). These samples were obtained within 2-3 hours after the last hemodynamic measurements and clamp experiments were performed and when all parameters had returned to baseline. Briefly, under pentobarbital anesthesia, the chest was opened and the LV free wall identified. Pledgeted stay sutures were placed and a 5 gram transmural section of LV free wall was excised with hemostasis achieved by securing the sutures. Blood pressure and heart rate were monitored to assure hemodynamic stability. Simultaneously, another member of the team excised 5 grams of gracilis muscle. Insulin was administered via the right atrial catheter and after 2-4 minutes, a second sample of LV and gracilis muscle was excised prior to removing the entire heart. Samples were immediately snap frozen in liquid nitrogen and stored at -70°C. Purified sarcolemmal membranes were prepared using sucrose gradient centrifugation as described previously (34).

LV myocardium was homogenized in a buffer free of phosphatase inhibitors and subjected to electrophoretic separation by SDS-PAGE (19). Resolved proteins were transferred onto PVDF membrane (Immobilon™-P SQ, Millipore Corp., Bedford, MA) at a constant voltage (100V) for 1-2 hours at 4°C (37). Adjustments for protein loading were accomplished by normalizing bands based upon Coomassie staining of the blots (26).

Polyclonal anti-Insulin Receptor-β subunit (IR-β) antibodies and normal rabbit IgG were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Goat anti-rabbit IgG-HRP conjugate, anti-phosphotyrosine (RC20)-HRPO conjugate, monoclonal mouse anti-Akt-1, polyclonal rabbit anti-phospho-Akt-1 (Ser 473), and polyclonal rabbit anti-insulin receptor substrate –1 (IRS-1), were purchased from BD Transduction Laboratories (San Diego, CA). Polyclonal anti-phosphatase and tensin homolog deleted on chromosome 10 (PTEN), polyclonal rabbit anti-GLUT-4 and GLUT-1 and Protein A/G-PLUS Agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal rabbit phospho-Akt-1 (Ser 473)
specificity was confirmed using phosphorylated and non-phosphorylated NIH/3T3 cell extracts purchased from Cell Signaling Technology, Inc. (Beverly, MA). Immunoprecipitation of IRS-1 followed by anti-phosphoserine (ser 307) western blotting was performed with 200µg of tissue lysate and pre-cleared with 0.05µg normal rabbit IgG together with 20µL of re-suspended Protein A/G PLUS-Agarose by mixing 2 hours at 4°C. The immunocomplex was captured by adding 100µL (25µL packed beads) Protein A/G-PLUS Agarose and incubating at 4°C for at least 3 hours. The supernatant was discarded and the beads were washed three times in ice-cold PBS followed by one wash with 0.5M Tris-HCl, pH 6.8. Beads were re-suspended in Laemmli buffer (19) and boiled for 5 minutes. Samples were then subjected to Western Blot analysis as described above. Data were expressed as the ratio of phospho-protein to total protein. GLUT-1 and GLUT-4 translocation under basal and insulin stimulated conditions was assessed by examining the ratio of membrane associated GLUT-4 to total (membrane and cytosolic) GLUT-4. Sarcolemmal GLUT-1 and GLUT-4 protein levels were measured on purified membranes generated using sucrose gradient centrifugation as an established method for separating sarcolemmal from intracellular membrane components.

The adenosine monophosphate kinase (AMPK) activity in the LV myocardium was determined as described previously (25). Activity was expressed as picomoles of incorporated ATP per milligram of protein per minute.

Western blots to determine protein expression of components of the signaling pathway for fatty acid uptake (polyclonal rabbit FAT/CD 36) and metabolism (polyclonal rabbit PGC-1α and PPARα, Santa Cruz) were performed on purified and cytosolic preparations respectively using methods described above.
Mitochondrial Isolation

Crude mitochondrial isolates were prepared from canine myocardium using a trypsin digestion procedure as described previously (26). The purity of the mitochondrial isolates was established by demonstrating the absence of Na+/K+ ATPase activity in mitochondrial preparations. The isolated mitochondrial samples from LV myocardium and gracilis muscle were resuspended to a final protein concentration of 0.5 μg/mL in a buffer at a final concentration of 125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromphenol blue. Membrane samples (5μg/lane for uncoupling protein-1 [UCP-3] and 2.5μg/lane for succinate dehydrogenase [SDHA] and mitochondrial cytochrome oxidase-1 [MCO1]) were subjected to electrophoretic separation by SDS-PAGE (24). Proteins resolved were transferred onto PVDF membrane (Immobilon™-P SQ, Millipore Corp., Bedford, MA) at a constant voltage (100 V) for 2 hours at 4°C (37). Nonspecific membrane protein binding sites were blocked for at least one hour with 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween-20 (TTBS) and then membranes were probed with polyclonal rabbit anti-UCP-3 antibody (1:10,000) (Alpha Diagnostics, San Antonio, TX), monoclonal mouse anti-SDHA antibody (1:100,000) or monoclonal mouse anti-MTCO1 antibody (1:100,000) (Abcam Inc., Cambridge, MA), overnight at 4°C. The blots were washed three times in TTBS, incubated 1 hour at room temperature in appropriate secondary antibody conjugated to horseradish peroxidase, and then washed as before. The immunoreactive proteins were detected by use of an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (Perkin Elmer Life Sciences, Boston, MA) and exposed to X-ray film (Hyperfilm ECL™, Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis of the bands was carried out using a Personal Densitometer SI and ImageQuant™ Software (Molecular Dynamics, Sunnyvale, CA).
Glycogen and Fat Staining:

Samples of LV myocardium were fixed in formalin (2%) and stained for glycogen content using PAS staining. Neutral lipid was identified using osmium stain. Glycogen and fat content were quantified using a Metamorph Imaging program and expressed as volume % myocardium.

Transmission Electron Microscopy of Myocardial and Skeletal Muscle

Tissue samples were placed in cold (4º C) 3% glutaraldehyde overnight. On the following day, the sample was post-fixed in 1% osmium tetroxide for 90 minutes, dehydrated through graded ethanol and Acetonitrile. The sample was then infiltrated with one change of 50% propylene oxide, 50% epoxy resin for 2 hours, and then pure epoxy resin for two additional hours before embedding. One-micron sections were cut and stained with methylene blue for light microscopic examination. Thin sections were cut on a Reichert Om-U2 ultra-microtome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 electron microscope at 60 kv.

Statistical analysis:

Data are expressed as the mean value ± SEM. Differences in hemodynamic and metabolic responses between the groups were determined by two-way ANOVA. Where differences were detected over time, a post hoc Student’s Newman Keuls’s test was performed to determine differences at respective time points. Differences in components of the insulin signaling cascade, mitochondrial enzymes, intramyocardial lipids, and glycogen were compared using Student T-test for unpaired data. A level of p< 0.05 was considered statistically significant.
Results

The Effects of Age on Body Mass Index and Metabolic Parameters

Table 1 illustrates the effects of age on body mass index and metabolic parameters. There was no difference in body weight, body mass index or abdominal girth between groups. Fasting plasma glucose levels were normal while plasma insulin levels and NEFA levels were significantly (p<0.01) higher in older dogs. There were no differences in plasma adiponectin or leptin levels. Notably, both plasma arterial norepinephrine and myocardial norepinephrine spillover were increased (p<0.01) in old dogs.

The Effects of Age on Heart Weight and Resting Hemodynamics

Table 2 illustrates the impact of age on myocardial mass. There was a small increase in LV+septum weight, although the difference was not significant when normalized for body weight. There were no significant differences in LV systolic or end diastolic pressures or LV dP/dt. Isovolumic relaxation was prolonged in older dogs compared to younger dogs. Older dogs had higher resting heart rates and mean arterial pressures, but comparable cardiac outputs. However, there were significant reductions in LV stroke work and myocardial external efficiency in older dogs. Older dogs had higher basal coronary blood flow and myocardial oxygen consumption. LV dimensions were not different between groups (data not shown).

The Effects of Age on Total Body and Myocardial Substrate Uptake

Figure 1 illustrates the whole body glucose disposition in young and old dogs during hyperinsulinemic euglycemic clamps. Whole body glucose disposition was reduced in older
dogs. These findings are consistent with impaired insulin mediated glucose disposition. **Figure 2** illustrates that that both basal and insulin stimulated myocardial glucose uptake were reduced in older dogs while basal myocardial NEFA uptake was increased. Hyperinsulinemia suppressed myocardial NEFA uptake and oxygen consumption to a greater extent in older dogs compared to younger dogs, yet did not reduce MVO2 to basal levels observed in the young dogs. Notably, the reduction in MVO2 during the clamp was not associated with a significant change in heart rate or blood pressure, suggesting a relative shift in metabolic preference from NEFA to glucose.

**Figure 3** illustrates the effects of aging on proximal components of myocardial insulin signaling. In cardiac muscle, there was decreased expression of insulin receptors and Akt protein abundance, while, in skeletal muscle, there was increased serine 307 phosphorylation of IRS-1, but no difference in insulin receptor or Akt protein expression. Notably, there was no difference in IRS-1 protein expression in either cardiac or skeletal (data not shown). **Figure 4** illustrates that there was diminished insulin stimulated Akt phosphorylation at serine 473 in both cardiac and skeletal muscle. This was seen in association with impaired GLUT 4 translocation in response to insulin in both the skeletal and cardiac muscle from older dogs (**Figure 4**). While there was no age related difference in basal cytosolic expression of GLUT-1 (Y: 146±23; O: 153±47 DU), aging was associated with a marked decrease in sarcolemmal expression of GLUT-1 (Y: 578±46; O: 233±72 DU, p<0.01). GLUT-1 translocation was not augmented by insulin administration.

In cardiac muscle, the altered Akt phosphorylation was associated with increased expression of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) in older dogs (Y: 86±12; O: 161±16 DU, p<0.01) while this was not evident in skeletal muscle (Y: 21±7; O: 28±5 DU). There was no difference in AMP kinase activity between young and old dogs in
either cardiac (Y: 3.24±0.30; O: 3.11±0.49 pmol ATP/mg/min) or skeletal muscle (Y: 2.12±0.11; O: 2.01±0.32 pmol ATP/mg/min). There was no increase in PGC-1α expression between groups in either cardiac (Y: 252±23; O: 264±6 DU) or skeletal muscle (Y: 96±10; O: 86±17 DU). There was no difference in PPARα expression in cardiac (Y: 425±25; O: 475±116 DU) or skeletal (Y: 125±25; O: 75±116 DU) muscle. However, FAT/CD36 expression in myocardial sarcolemmal membranes was significantly reduced in older dogs (Y: 1,312±123; O: 387±12 DU, p<0.05), while there was no difference in skeletal muscle (Y: 212±92; O: 187±54 DU).

Figure 5 illustrates the effects of aging on myocardial mitochondrial structure and expression of mitochondrial proteins. There were no differences in the semi-quantitative assessment of mitochondrial content in cardiac or skeletal muscle. The senescent myocardium was associated with increased vacuolization of mitochondria. These structural abnormalities were seen in association with decreased expression of uncoupling protein-3 (UCP-3) in the senescent myocardium. In addition, cytochrome oxidase-1 (MTCO-1) and succinate dehydrogenase (SDH) were also diminished. These mitochondrial abnormalities were seen in association with increases in intramyocardial lipid deposition and decreased glycogen content (Figure 6) in the senescent myocardium compared to younger dogs. In contrast, there was no difference in semi-quantitative analysis of mitochondrial density or UCP-3, MTCO-1 or SDH expression between young and old dogs in skeletal muscle samples (data not shown).

Discussion

In the present study, we determined that the senescent myocardium is associated with marked insulin resistance in the absence of obesity, physical inactivity, or cardiac contractile dysfunction. These abnormalities are seen in association with increases in plasma NEFA,
increased myocardial NEFA uptake, and increases in myocardial oxygen consumption. While both whole body and myocardial insulin resistance were observed in older dogs, there were distinct cellular abnormalities in insulin signaling in cardiac versus skeletal muscle. Finally, myocardial insulin resistance is associated with altered mitochondrial structure seen in conjunction with decreased UCP-3 expression.

While total body insulin resistance has been documented frequently in advanced age (4,9,14,33), the extent and mechanisms associated with myocardial insulin resistance in aging are unknown. We observed that myocardial insulin resistance was associated with impaired insulin induced Akt-1 phosphorylation and GLUT-4 translocation, but did not involve increased serine phosphorylation of IRS-1 as was observed in skeletal muscle in our study and has been reported previously (18,40). In contrast, we observed decreased insulin receptor and Akt expression in sarcolemmal membranes in senescent myocardium. Prior studies (36) have demonstrated a reduction in insulin receptor density in senescent myocardium, but have not examined altered Akt expression. Importantly, we observed increased PTEN expression in senescent myocardium, but not skeletal muscle. Prior studies have identified PTEN as a putative mediator of insulin resistance in skeletal muscle (21,38), but not in myocardium. We have observed previously increased PTEN activity in the hearts of young dogs with dilated cardiomyopathy (26) suggesting that the mechanism of cellular insulin resistance may vary with the target tissue examined (cardiac versus skeletal muscle).

Altered myocardial insulin mediated glucose uptake and impaired cellular insulin action were observed in association with increased basal NEFA uptake and myocardial oxygen consumption. These findings were seen in association with increased intramyocardial lipid accumulation and altered mitochondrial structure in the myocardium of senescent dogs. Notably,
the increase in NEFA uptake and intramyocardial accumulation in the senescent myocardium occurred in the setting of reduced FAT/CD36, but unaltered levels of PPARα and PGC-1α. The mechanism responsible for increased NEFA uptake under circumstances of reduced FAT/CD36 remains to be determined. Prior studies have demonstrated reduced mitochondrial content in skeletal muscle of lean offspring of Type 2 diabetes (10,22,29) and as well as mitochondrial dysfunction in skeletal muscle of elderly subjects with insulin resistance (1,28) in association with intracellular lipid accumulation. Recent evidence suggests that these findings are associated with reduced transcription factors such as PGC-1α (8,20) or other cellular signaling pathways implicated in mitochondrial biogenesis, such as AMP kinase (31,41). We did not observe differences in AMP kinase activity or PGC-1α expression in the myocardium between young and old in the present study, suggesting that differences in mitochondrial biogenesis did not account for the observed differences in mitochondrial structure or protein expression.

**Altered mitochondrial structure and function** has been identified frequently as a trigger to cellular aging (3,13,15) as well as insulin resistance (10,22,28,29). Reduced uncoupling protein expression has been identified as a mediator of these events (15). We observe reduced UCP-3 content in mitochondria of older hearts in association with intramyocardial lipid accumulation. Recent evidence has suggested that these uncoupling proteins may be involved not only in mitigating the intra-mitochondrial accumulation of oxygen free radicals (15) through dissipation of the proton gradient across the inner mitochondrial membrane, but also in reverse transport of excess fatty acyl-CoA anion and peroxide from mitochondria (15,29). Although uncoupling proteins and mitochondrial dysfunction have been implicated in cellular aging (3), ours is the first evidence to implicate altered UCP-3 expression in myocardial insulin resistance in advanced age.
Oxidative stress has been shown to lead to lipid peroxidation of mitochondrial membrane phospholipids, resulting in vacuolization of the mitochondria similar to that seen in our study (3). Although we cannot establish a direct cause and effect relationship, the altered mitochondrial structure by electron microscopy decreased UCP-3, MTCO-1, and SDH expression, coupled with the accumulation of intramyocardial fat recapitulate a pathophysiologic framework that has been established for insulin resistance in skeletal muscle (10,22,29), but extends the mechanism to the pathogenesis of myocardial insulin resistance. Taken together, these data suggest that normal aging is associated with both whole body and myocardial insulin resistance in the absence of significant obesity, physical inactivity, or contractile dysfunction. Notably, the cellular mechanisms of insulin resistance vary between skeletal muscle and myocardium as reflected in the distinct roles played by the increased expression of \( p\text{IRS-1 ser 307} \) and PTEN respectively. In addition, there appear to be differences in the respective tissues in the nature and extent of mitochondrial structural changes with aging. Both mechanisms appear to be linked to increased intracellular lipid accumulation (25,26).

Despite the magnitude of whole body and myocardial insulin resistance and the nature and extent of mitochondrial structural alterations observed in these old dogs, the consequences to myocardial structure and function are modest at this stage. We observed modest, but significant increases in resting heart rates and blood pressures and modest increases in LV mass, but not LV/body weight ratios seen in association with increased plasma norepinephrine and cardiac norepinephrine spillover. There is an extensive literature linking chronic hyperinsulinemia associated with whole body insulin resistance to increased sympathetic nervous system activation (5,7,30), leading to hypertension. In this regard, there was significant impairment in isovolumic relaxation time, LV stroke work, and external mechanical efficiency. The impairment
in cardiac external mechanical efficiency is interesting in light of excessive myocardial fatty acid uptake in older dogs under basal fasting conditions. The suppression of myocardial NEFA uptake and the associated reduction in MVO₂ observed during the hyperinsulinemic-euglycemic clamp in older dogs resulted in a significant improvement in external mechanical efficiency. The extent to which these age-related myocardial metabolic abnormalities predispose to altered systemic hemodynamics and LV function or predispose to increased risk consequences from superimposed cardiac injury remains to be determined.

This is the first study to demonstrate the magnitude and the cellular basis of insulin resistance in myocardium from a relevant large animal model of cardiovascular aging (10,11,12,35). There are relatively few studies that have examined these metabolic derangements in senescent beagles or have looked at associated hemodynamic alterations with aging in this species. Prior studies in non-human primates have demonstrated alterations in insulin receptor autophosphorylation in obese rhesus macaques and impaired insulin sensitivity in non-diabetic aged rhesus macaques. Notably, we chose to control for the effects of obesity and activity level in our study to focus on the effects of aging per se. Similarly, we chose to study a model that does not develop coronary atherosclerosis in order not to confound the effects of aging per se. Nonetheless, there are several questions that remain to be elucidated. **We did not measure lactate uptake during the hyperinsulinemic clamps nor did we measure glucose or NEFA oxidation. We did not examine the effects of superimposed stress on the age related responses. Such important inquiry will remain the subject of future investigations.**

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**Disclosures section**

There are no disclosures.

**References**


**Figure 1**: Hyperinsulinemic-euglycemic clamp in young (n=12) versus older (n=12) dogs. Panel A: Matched levels of hyperinsulinemia Panel B: Reduced glucose infusion in older dogs consistent with total body insulin resistance. Panel C: The suppression of NEFA by hyperinsulinemia. *p<0.05 young versus old at respective time points.*

**Figure 2**: Myocardial substrate uptake during hyperinsulinemic euglycemic clamp. Panel A: Insulin mediated myocardial glucose uptake. Panel B: Insulin mediated suppression of myocardial NEFA uptake. Panel C: Myocardial oxygen consumption during insulin stimulated glucose uptake. *p<0.05 young (n=8) versus old (n=8) at the respective time points.*

**Figure 3**: Basal expression of proximal components of the insulin signaling cascade in cardiac and skeletal muscle from young (n=8) and old (n=8) dogs. Panel A: IR-β= insulin receptor measured with an antibody directed against the beta subunit. Panel B: The phosphorylation of insulin receptor substrate-1 (IRS-1) at serine 307 inhibiting the action of the docking protein. Panel C: Protein expression of Akt.

**Figure 4**: Basal and insulin stimulated phosphorylation of Akt at serine 473 (Panel A) and GLUT 4 translocation (Panel B) in both cardiac and skeletal muscle from young (n=8) and old (n=8) dogs.

**Figure 5**: Transmission electron micrographs of myocardium from young (Panel A, n=7) and old dogs (Panel B, n=8)). There was no difference in myocardial mitochondrial density, but
decreases in mitochondrial protein expression including cytochrome oxidase-1 (MCOI), succinate dehydrogenase (SDH), and uncoupling protein-3 (UCP-3).

**Figure 6:** Representative illustration of myocardial accumulation of glycogen (A) and neutral lipid (B) in young (n=11) and old (n=10) myocardium and quantitative assessments, * p<0.05 young vs old.
Table 1

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<td>Age (years)</td>
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<td>32 ± 3*</td>
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Figure 1

Hyperinsulinemic Euglycemic Clamp

Plasma Insulin (pmol/L)

Time (minutes)

Glucose Infusion Rate (mg/kg/min)

Time (minutes)

Plasma NEFA (umol/L)

Time (minutes)
Effects of Age on Myocardial Substrate Utilization

Myocardial Glucose Uptake

Myocardial NEFA Uptake

Myocardial O₂ Consumption

* indicates statistical significance.

Copyright Information
The Effects of Aging on Insulin Signaling

IR-β  p-IRS-1 ser 307  AKT protein

Young  Old

Figure 3
Figure 4

The Effects of Aging on Insulin Signaling

p-AKT ser 473

GLUT 4 Translocation

60 kDa

Copyright Information
Figure 5

**Young**

**Old**

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**Copyright Information**
Figure 6

Glycogen and Fat Content in Young and Old Myocardium

Young

Old

[Bar charts showing the comparison of glycogen and fat content between young and old myocardium.]

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