Diabetic Complications Consortium

Application Title: Targeting mitochondria to prevent obesity induced bladder dysfunction

Principal Investigator: Johanna Hannan, PhD

1. Project Accomplishments:

- Examined the time course of the development of both bladder dysfunction and changes in mitochondrial respiration in male mice fed HFD for 6 or 12 weeks
    - 6 weeks of HFD – increased bladder muscarinic contractility and did not impact mitochondrial respiration
    - 12 weeks of HFD – increased bladder muscarinic contractility and increased complex IV mediated maximal mitochondrial respiration in both detrusor and mucosal layers
- Optimized "Creatine Kinase Energetic Clamp – Respiratory Conductance" methodology in the isolated mitochondria and strips from the detrusor and urothelial layers of the bladder to assess dynamic, substrate specific mitochondrial physiology
- Extended the diet to 24 weeks and found extended HFD leads to impaired fatty acid oxidation in both the detrusor and mucosal layer in male mice
- Presented data as a poster at the Experimental Biology meeting in Orlando, FL (April 2019)
- Presented data as a moderated poster and during invited keynote at the American Urological Association Basic Science Urological Research Symposium in Chicago, IL (May 2019)

2. Specific Aims:

Specific Aim 1*:* Evaluate the contribution of reduced mitochondrial respiration in the urothelium and detrusor smooth muscle from male mice fed a short or chronic HFD to alterations in bladder function.

Results:

Short term HFD did not alter bladder mitochondrial respiration. We hypothesized that 6 weeks of HFD would lead to symptoms of detrusor overactivity with increased mitochondrial respiration and H_2O_2 emission in both the detrusor and mucosal layers of the bladder. C57BL/6N male mice were fed a low fat diet (CON) or HFD for a period of 6 weeks. Over this time, mice on HFD demonstrated weekly increases in body weight (Fig. 1A) and increased fat body mass as measured by MRI (Fig. 1B). Glucose tolerance test demonstrated a mild increase in blood glucose intolerance in HFD mice (Fig. 1C). We continue to use our novel technique to assess mitochondrial function in bladder strips while maintaining the mitochondria in their native reticular structure. Maximal mitochondrial respiration was measured in the detrusor in the presence of the following compounds added accumulatively: 1) pyruvate/malate/glutamate (PMG) to assess complex I, state IV respiration, 2) ADP to stimulate state III respiration, 3) succinate (Succ) to activate complex II mediated respiration, 4) cytochrome C (CytC) to ensure an intact mitochondrial membrane, 5) rotenone (Rot) to inhibit complex I, 6) N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) to measure complex IV respiration. Overall maximal mitochondrial respiration was not changed in the detrusor or mucosal layer of the bladder following 6 weeks of HFD (Fig 1D, 1E). Additionally, HFD did not increase bladder H_2O_2 emission (Fig. 1F). Additionally, we did notice a marked increase in overall maximal mitochondrial respiration in the mucosal layer compared to the detrusor (Fig. 2A). However, this increased respiration did not appear to be due to increased mitochondrial content per tissue weight as measured by citrate synthase concentration (Fig. 2B). Future experiments using isolated mitochondria will

assess these differences in respiration to determine if the mitochondria themselves are different and look at their density with microscopy.

Short term HFD increased muscarinic mediated bladder contraction. We also assessed bladder physiology by using void spot assays and bladder strip smooth muscle function. There was no difference in the number of voids, or the volume voids following 6 weeks of HFD (data not shown). Additionally, the bladder wet weight to body weight was not changed between groups (CON: 0.84±0.18, HFD: 0.80±0.32; $p>0.05$). All bladder smooth muscle myograph assessments were performed in bladder strips that were denuded of the mucosal layer to determine if there were changes specific to smooth muscle function. Contraction to depolarizing high potassium chloride (KCl) solution was significantly increased in HFD bladders (Fig. 3A). Similarly, HFD bladders demonstrated increased contractions to the muscarinic agonist, carbachol at high concentrations (Fig. 3B). Nerve-mediated electrical field stimulated contractions were no different between bladders from control or HFD mice (Fig. 3C). Additionally, inhibition of muscarinic receptors with atropine produced similar contractile responses between both groups (Fig. 3D). These data suggest that early HFD induced bladder dysfunction may initiate in the smooth muscle due to alterations in the contractile signaling pathways rather than changes in innervation or neurotransmitter release. At this stage these alterations are minor given that bladder function as assessed by void spot assays remained unchanged. Our future studies will also assess bladder physiology via cystometry to determine if the changes in contractile function translate to changes in bladder pressure with voiding.

Long-term HFD increased complex IV mediated bladder mitochondrial respiration.
We hypothesized that 12 weeks of chronic HFD would lead to decreased mitochondrial respiration and further increased H_2O_2 emission. Impaired mitochondrial function would contribute to the bladder shifting from a compensated state with increased contraction to a decompensated and underactive bladder phenotype. Chronic HFD lead to a greater increase in body weight (Fig. 4A) and greater accumulation of fat mass (Fig. 4B). Similar to the 6 week HFD mice, 12 weeks of HFD only mildly increased the blood glucose levels (Fig. 4C). Maximal mitochondrial respiration was unchanged in both the detrusor and mucosal layer for complex I and II mediated respiration in the presence and absence of ADP (state III and IV; Fig. 4D, 4E). In contrast, maximal respiration with the activation of complex IV was significantly increased in both the detrusor and urothelial layers. Increased respiration at complex IV often leads to increased electron leak and H_2O_2 emission, however; similar to the 6 week HFD data, chronic HFD did not increase bladder H_2O_2 emission (data not shown). These measurements were performed in small intact pieces of detrusor and mucosal layers which resulted in inconsistent data across both CON and HFD bladders. We have determined that these pieces of whole tissue are not allowing for accurate measurements of H_2O_2 emissions. We are currently performing Western blots in detrusor and mucosal tissues from both the 6 and 12 week HFD and age matched control animals to examine peroxiredoxin (PRDX) dimerization as an indicator of subcellular oxidant burden (Perkins et al., Trends Biochem Sci 2015). PRDX dimer: monomer ratio can be used a surrogate to measuring subcellular H_2O_2 as PRDXs undergo homodimerization to reduce hydroperoxides like H_2O_2. Finally, there was a decrease in mitochondrial content as measured by citrate synthase activity in the detrusor following 12 weeks of HFD (Fig. 4F).

Long-term HFD increased muscarinic mediated bladder contraction. Again, we assessed bladder physiology with behavioral void spot assays and bladder strip smooth muscle function. Similar to the 6 week HFD time point, the number of voids, or the volume voids following 12 weeks of HFD was unchanged (data not shown). However, the bladder wet weight to body weight was significantly decreased in the 12 week HFD mice (CON: 0.89±0.15, HFD: 0.63±0.07; $p<0.005$). In denuded bladder strips, the 12 week HFD data followed the same trends as the 6 week HFD data. Contractions to high KCl and increasing concentrations of carbachol were increased in HFD mouse bladders (Fig. 5A, 5B). There was no change in EFS mediated contraction in the presence or absence of atropine, indicating no change in detrusor nerve-mediated signaling with 12 weeks of HFD (Fig. 5C, 5D).

Specific Aim 2: Determine the ability of mitochondrial catalase (MCAT) overexpression to prevent HFD-induced bladder dysfunction.

Results:

Optimization of modified creatine kinase energetic clamp for dynamic mitochondrial physiology. Given that we did not see any change in H_2O_2 emission and minimal changes in bladder function with 12 weeks of HFD, we could not justify using the MCAT mice to prevent the development of bladder dysfunction. As an alternative, we tried to optimize our methods of measuring dynamic mitochondrial respiration and extended the length of the diet to 24 weeks in male mice. Using both intact detrusor and mucosal layer bladder segments and isolated mitochondria, we were able to determine mitochondrial respiration kinetics of individual components of the ETC rather than examining only maximal respiration. This approach allowed us to assess energetic capacity of the tissue while maintaining the tissue in its native state. We used a modified creatine kinase energetic clamp in the presence of different substrates. In this assay, the free energy of ATP hydrolysis (ΔG_{ATP}) can be calculated in the presence of different respiratory substrates. The following substrate conditions were tested: pyruvate/malate (Pyr/M), Glutamate, Octanoyl-L-carnitine/Malate (Oct/M), succinate/rotenone (Succ/Rot). Subsequent to the substrate additions, sequential additions of phosphocreatine (PCr: 6, 9, 15 and 21mM) was performed to gradually slow oxygen consumption rates (JO_2) back toward baseline. Plotting the calculated ΔG_{ATP} against the corresponding JO_2 reveals a linear force-flow relationship, the slope of which represents the conductance/sensitivity of the entire respiratory system under specified substrate constraints as previously described (Fisher-Wellman et al., Cell Rep 2018). Mitochondria were isolated from the mucosal layer and the detrusor. To ensure all four substrate conditions could be examined, bladders from 5 mice were pooled per representative n. Overall, the detrusor and the mucosal layers had similar preferences for pyruvate/malate, glutamate and succinate/rotenone (Fig. 6). The biggest contrast between the two tissue types was the mucosal layer's preference for octanoyl-L-carnitine/malate energized respiration (Fig. 6C). The detrusor demonstrated minimal changes in respiration following the addition of the fatty acid substrate. These data were repeated in segments of intact detrusor and mucosal layer and the same trends in respiration and substrate preference were identified (data not shown).

Chronic 24 weeks of HFD markedly increased body fat mass and increases bladder contractility. A chronic 24 week HFD lead to even greater increases in body weight (Fig. 7A) and a body composition of almost 50% fat (Fig. 7B). Blood glucose concentrations in the glucose tolerance test still remained at mildly elevated levels of glucose sensitivity (Fig. 7C). We had expected that by 24 weeks of HFD more dramatic bladder dysfunction would have developed. Although the animals have marked increases in overall adiposity, the blood glucose levels only remains mildly elevated compared to controls. Due to this mild increase, the bladder is not transitioning from a compensated contractile state to a decompensated acontractile state as seen in diabetic animals. As a result, the bladder from 24 week HFD mice remained more contractile to high KCl and carbachol stimuli (Fig. 7D, 7E) and had no changes in contractions to EFS with or without atropine (Fig. 7F, 7G).

Chronic HFD impairs bladder fatty acid energized mitochondrial respiration. Using the modified creatine kinase clamp, we found no changes in mitochondrial respiratory sensitivity in detrusor or mucosal layer mitochondria energized with the substrates pyruvate/malate, glutamate, or succinate/rotenone following chronic HFD (Fig. 8). However, both HFD bladder detrusor and mucosal layer mitochondria had decreased mitochondrial respiratory sensitivity to octanoyl-l-carnitine (Fig. 8C). In the case of HFD, there is an overload of fatty acid substrate and the bladder is unable to metabolize this substrate to convert it into fuel (ATP). The excess fats may be stored within the bladder wall or lead to excess ROS production exacerbating bladder dysfunction.

Future studies. Lipid metabolism hinges on the availability of metabolites (acyl-carnitines and acyl-CoAs) for the fatty acids to enter the mitochondria to be utilized as a substrate. A lack of these metabolites can impair lipid metabolism and lead to changes in electron flux and creation of reactive oxygen species (ROS). In our next experiments, we will attempt to recover fatty acid metabolism in the HFD bladder smooth muscle by supplementing the tissue will additional L-carnitine and Acetyl-carnitine. We will also perform targeted metabolomics to profile Acyl-carnitine and Acyl-CoA which are essential to fatty acid metabolism This targeted metabolomic approach will provide specific mechanisms to target for future in vivo studies to prevent the development of obesity induced bladder dysfunction. We have also secured funding through an internal grant to look at the impact of HFD on female bladder physiology to compare to the male data collected with the DiaComp Pilot and Feasibility funding. We also need to consider using a HFD with a greater fat content (60% kcal fat) or a diabetic model to truly assess the role of mitochondrial to bladder dysfunction.

3. Publications and presentations:

We are currently preparing two manuscripts for publication. The first will focus on the maximal respiration data with the 6 and 12 week HFD data; the second will be establishing the phosphocreatine clamp dynamic mitochondrial respiration data in the 24 week HFD fed mice.

This funding has also allowed us to begin to look at the sex differences in bladder mitochondrial physiology with HFD by securing internal funding. An R01 application is in preparation to be submitted in February 2020.

Abstract presentations:

American Urological Association Annual Meeting, Chicago, IL, May 2019
Moderated poster presentation. Kosnik HL, Odom MR, Pak ES, Fisher-Wellman K, Hannan JL. Chronic high fat diet impairs detrusor mitochondrial fatty acid oxidation in male but not female mice. J Urol 2019;201(4s):e133-4.

Experimental Biology Annual Meeting, Orlando, FL April 2019
Poster presentation. Kosnik HL, Fisher-Wellman K, Odom MR, Pak ES, Hannan JL. Chronic high fat diet lowers male detrusor mitochondrial fatty acid oxidation while females are protected. *In press FASEB.*

Invited talks:

2019. Sex Difference in Obesity-Induced Bladder Dysfunction. *American Urological Association Annual Meeting, Chicago, IL (May 5)*

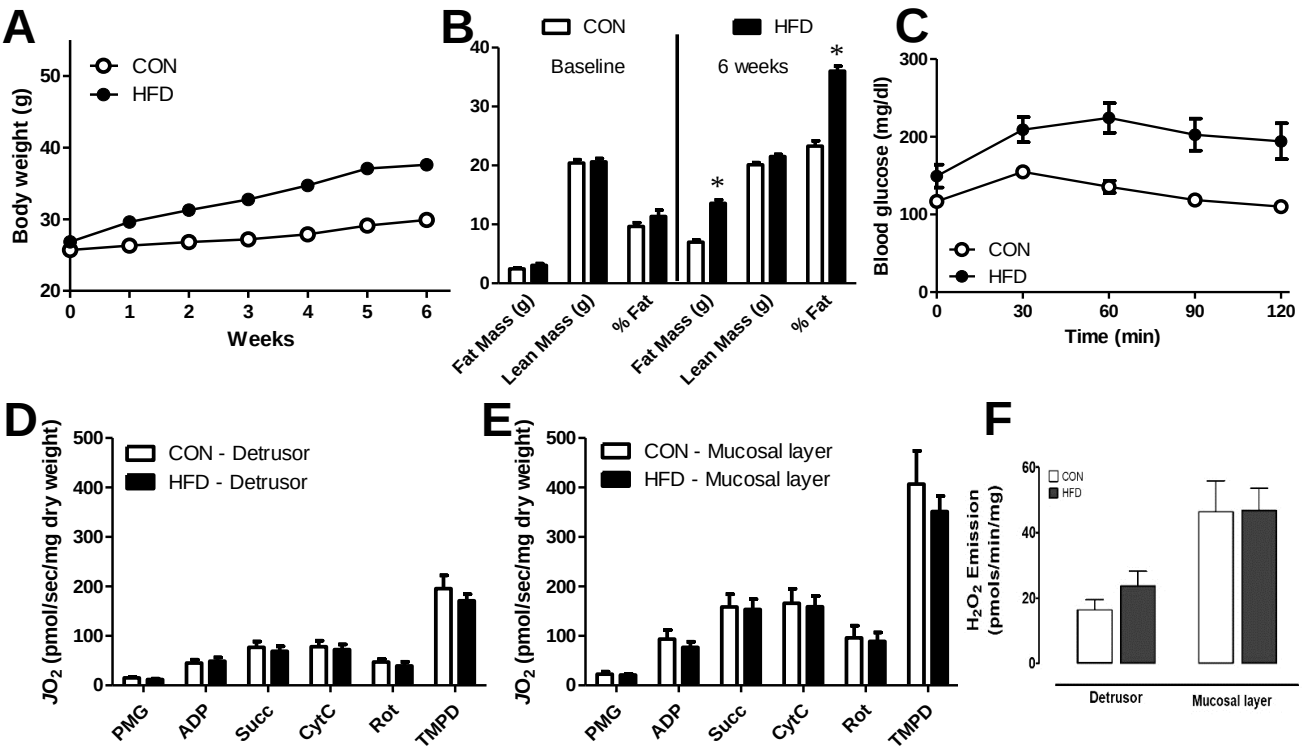

Figure 1. Short-term (6 weeks) high-fat feeding does not exacerbate mitochondrial physiology. *A*: Body weight gain during 6 weeks of HFD and CON (LFD). *B*: Body composition measurements using EchoMRI at baseline and 6 weeks for HFD and CON mice. *C*: HFD mice were slightly glucose intolerant following an IP glucose tolerance test compared to CON mice. *D*: High-resolution respirometry of detrusor smooth muscle strips was unchanged in HFD mice. *E*: High-resolution respirometry of the bladder's mucosal layer was unchanged in HFD mice. *F:* The rate of mitochondrial hydrogen peroxide emission (H2O2) was unchanged in HFD detrusor or mucosal layer. Data are mean ± SEM and were compared with one- or two-way ANOVA followed by Tukey posttest or two-tailed Student *t* test. **P* < 0.05 for HFD effect.

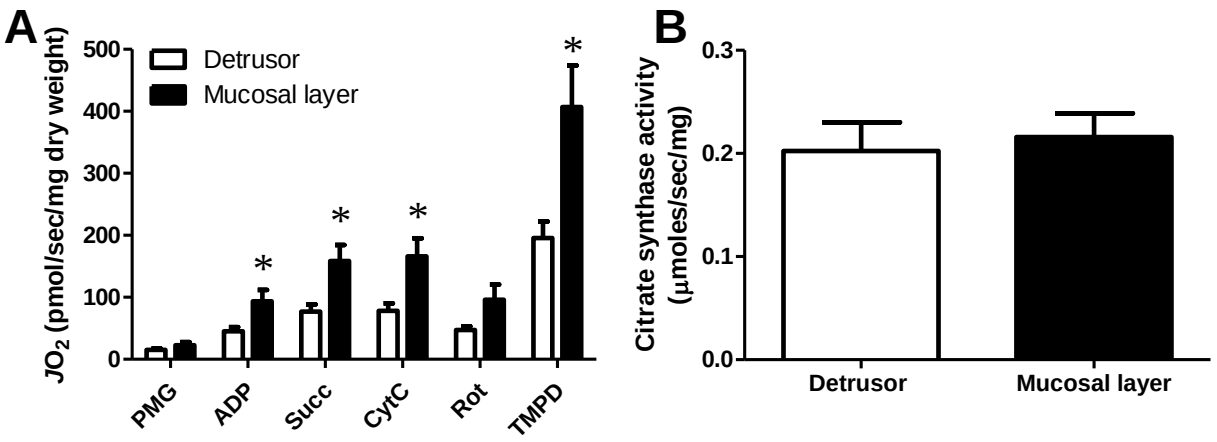

Figure 2. Increased mitochondrial respiration in the mucosal layer compared to the detrusor in control mice. *A*: High-resolution respirometry of detrusor smooth muscle strips was much lower than the mucosal layer. *B*: Citrate synthase activity was unchanged in the mucosal and detrusor layers indicating similar mitochondrial content between tissues. Data are mean ± SEM and were compared with two-way ANOVA followed by a two-tailed Student *t* test. **P* < 0.05 for mucosal layer effect.

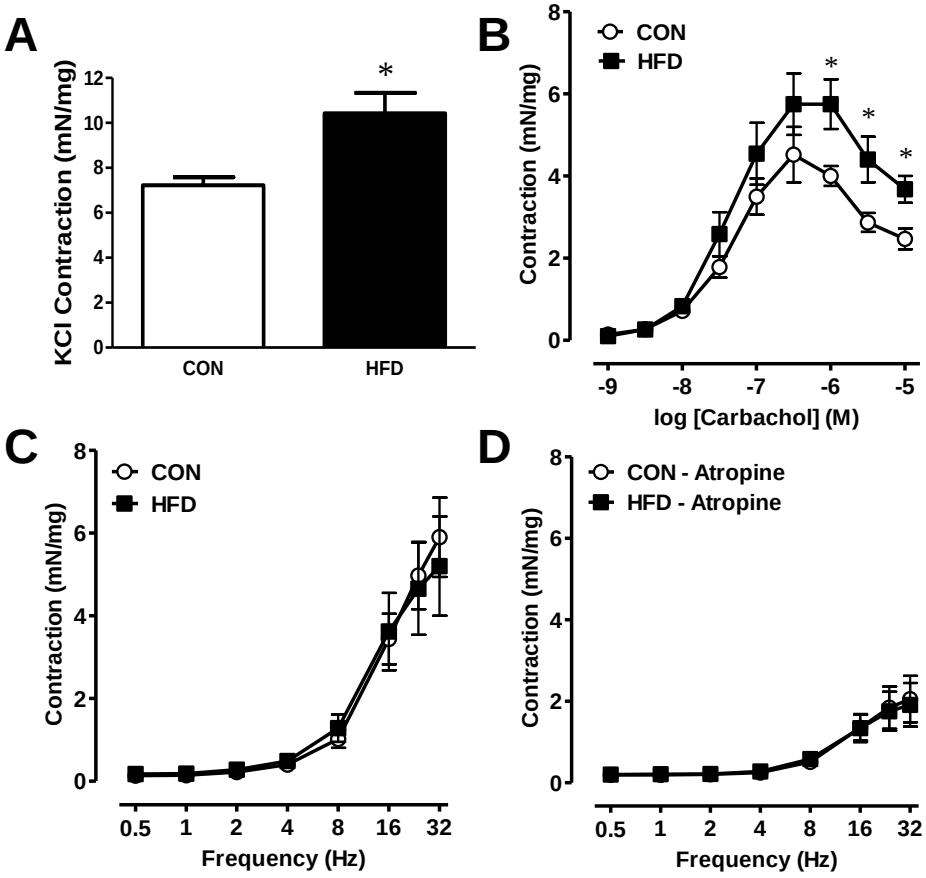

Figure 3. Short-term (6 weeks) HFD increased detrusor contractility to high potassium chloride and muscarinic agonist carbachol. *A*: High potassium chloride (KCl) contraction was increased in HFD bladders. *B*: Cholinergic contraction to the muscarinic agonist carbachol was increased in HFD bladders. *C, D:* Electrical field stimulated (EFS) contractile responses were unchanged in HFD detrusor in the presence or absence of atropine. All contractile force data are normalized to tissue strip weight. Data are mean ± SEM and were compared with a student *t* test or a two-way ANOVA followed by a two-tailed Student *t* test. **P* < 0.05 for HFD effect.

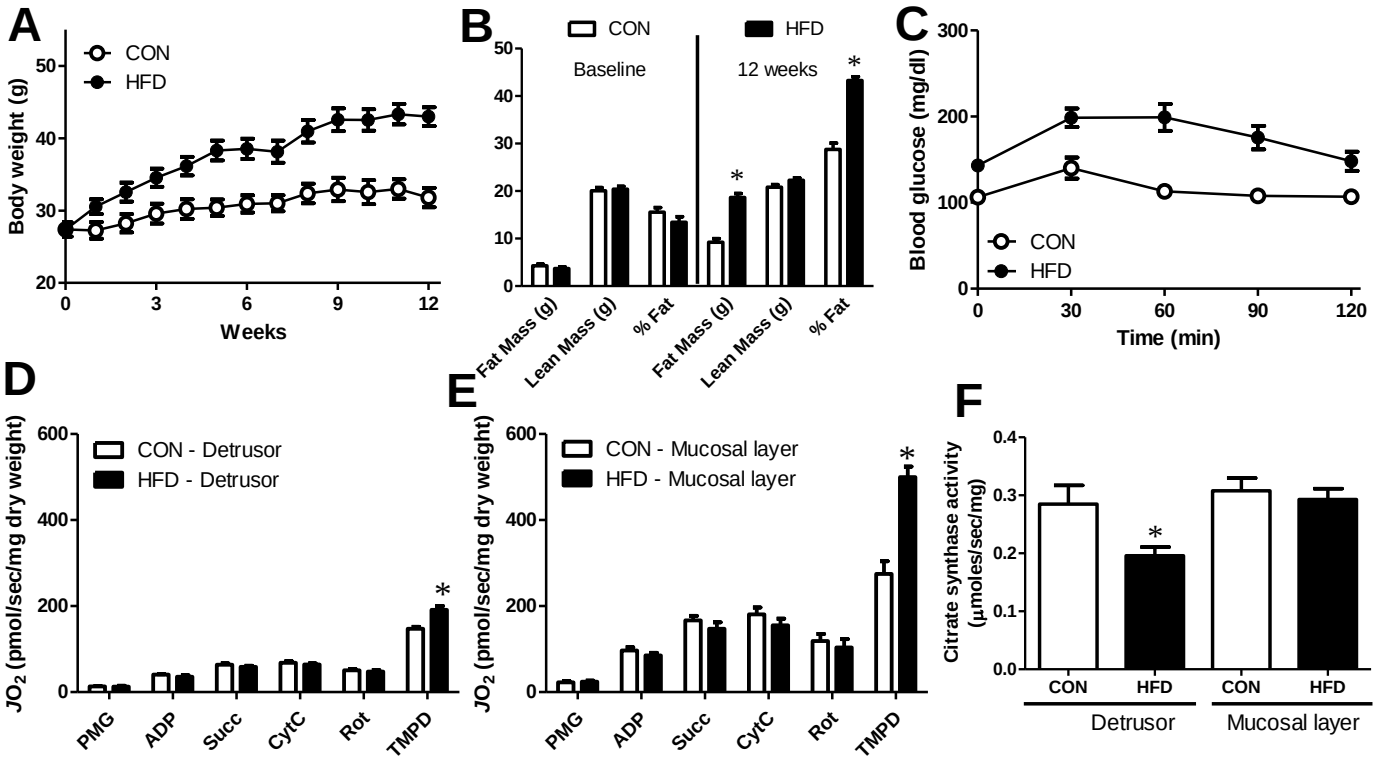

Figure 4. Long-term (12 weeks) high-fat feeding increases complex IV driven mitochondrial respiration. *A*: Body weight gain during 12 weeks of HFD and CON (LFD). *B*: Body composition measurements using EchoMRI at baseline and 12 weeks for HFD and CON mice. *C*: HFD mice were slightly glucose intolerant following an IP glucose tolerance test compared to CON mice. *D*: High-resolution respirometry of detrusor smooth muscle strips was increased with TMPD activation of complex IV in HFD mice. *E*: High-resolution respirometry of the bladder's mucosal layer was increased with TMPD activation of complex IV in HFD mice. *F:* Citrate synthase activity was decreased in the detrusor layer of bladders from HFD mice indicating decreased mitochondrial content. Data are mean ± SEM and were compared with one- or two-way ANOVA followed by Tukey posttest or two-tailed Student *t* test. **P* < 0.05 for HFD effect.

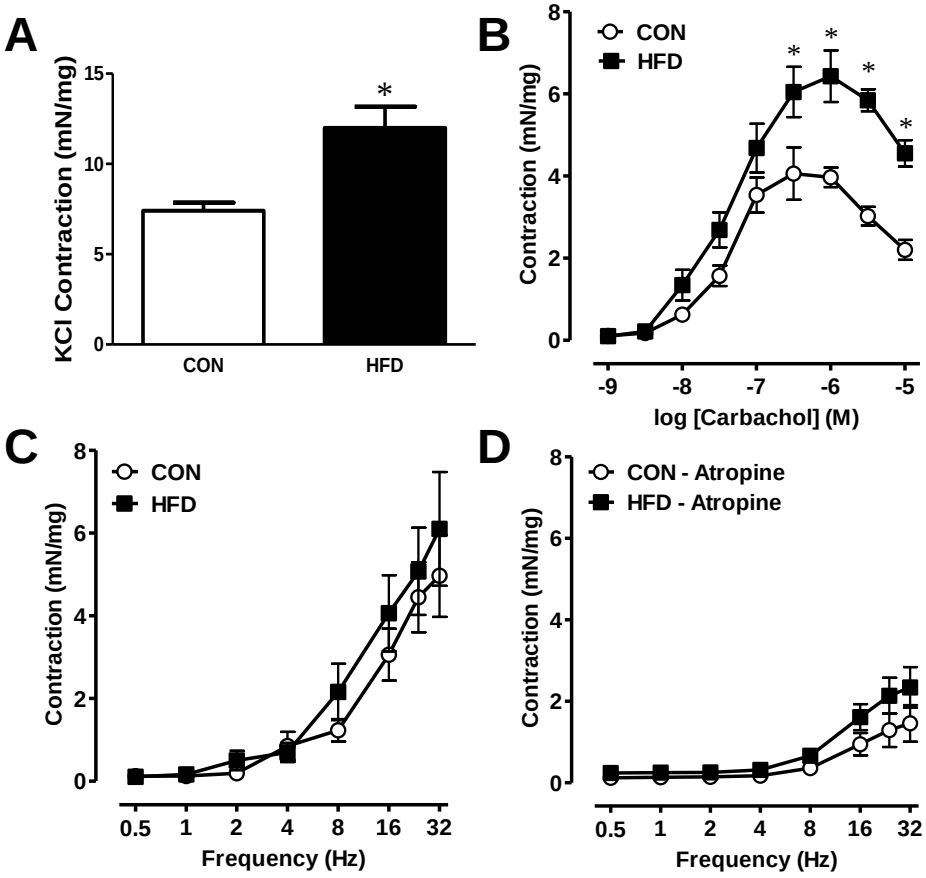

Figure 5. Long-term (12 weeks) HFD increased detrusor contractility to high potassium chloride and muscarinic agonist carbachol. *A*: Contraction to high potassium chloride (KCl) was increased in HFD bladders. *B*: Cholinergic contraction to the muscarinic agonist carbachol was increased in HFD bladders. *C, D:* Electrical field stimulated (EFS) contractile responses were unchanged in HFD detrusor in the presence or absence of atropine. All contractile force data are normalized to tissue strip weight. Data are mean ± SEM and were compared with a student *t* test or a two-way ANOVA followed by a two-tailed Student *t* test. **P* < 0.05 for HFD effect.

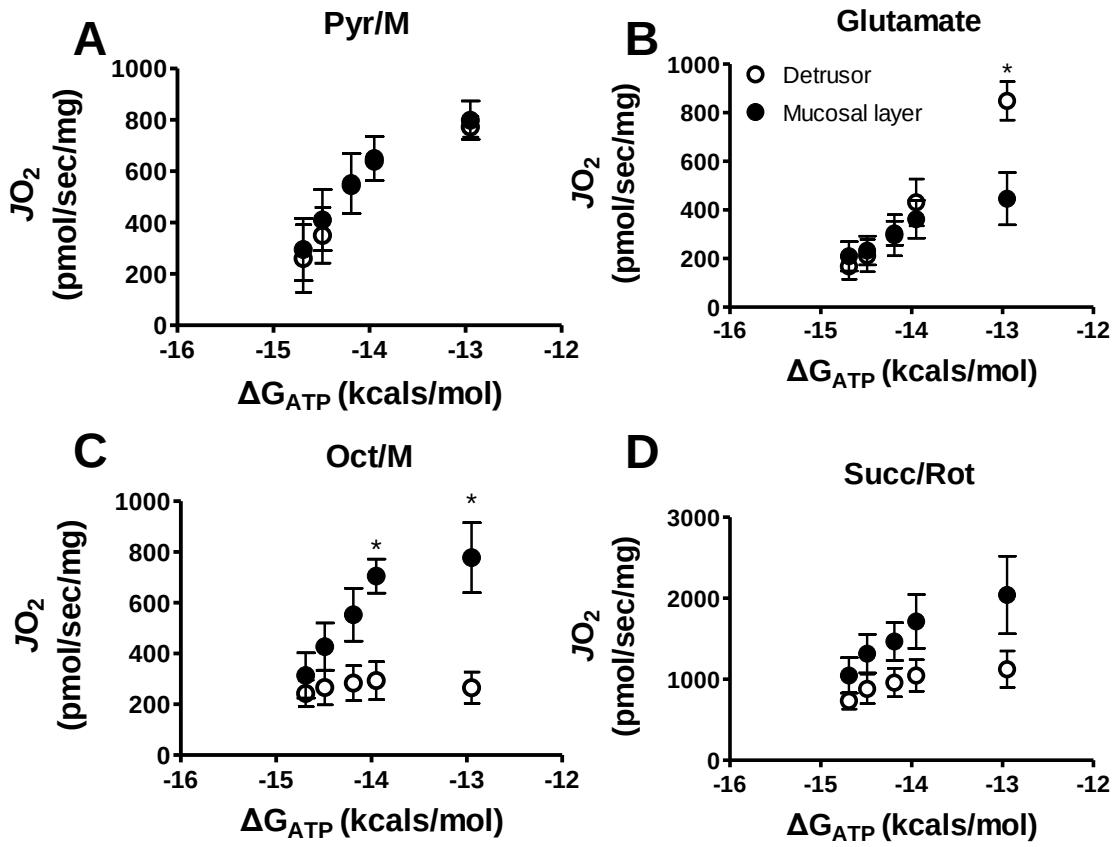

Figure 6. Respiratory oxygen consumption with different substrates between mitochondria isolated from the detrusor and mucosal layers of the bladder. *A-D*: Relationship between mitochondrial oxygen consumption (JO_2) and ATP free energy (ΔG_{ATP}) in bladder detrusor or mucosal mitochondria energized with either pyruvate/malate (Pyr/M), glutamate, Octanoyl-L-Carnitine/Malate (Oct/M) or succinate/rotenone (Succ/Rot). Data are mean ± SEM and were compared with a student *t* test or a two-way ANOVA followed by a two-tailed Student *t* test. **P* < 0.05 for mucosal layer effect.

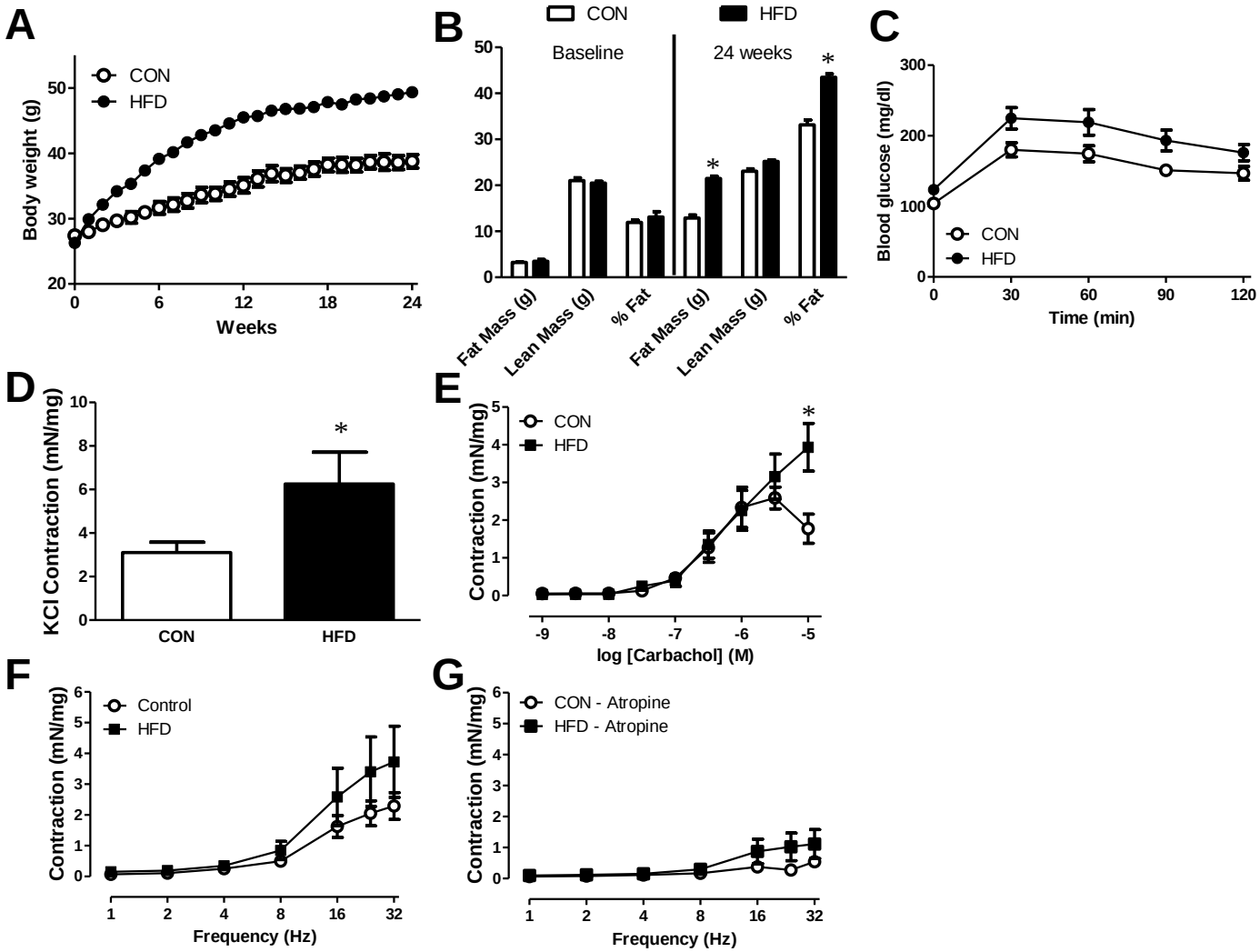

Figure 7. Chronic (24 weeks) high-fat feeding increases bladder contractility. *A*: Body weight gain during 24 weeks of HFD and CON (LFD). *B*: Body composition measurements using EchoMRI at baseline and 24 weeks for HFD and CON mice. *C*: HFD mice were slightly glucose intolerant following an IP glucose tolerance test compared to CON mice. *D*: Contraction to high potassium chloride (KCl) was increased in HFD bladders. *E*: Cholinergic contraction to the muscarinic agonist carbachol was increased in HFD bladders. F,G*:* Electrical field stimulated (EFS) contractile responses were unchanged in HFD detrusor in the presence or absence of atropine. All contractile force data are normalized to tissue strip weight. Data are mean ± SEM and were compared with one- or two-way ANOVA followed by Tukey posttest or two-tailed Student *t* test. *$P < 0.05$ for HFD effect.

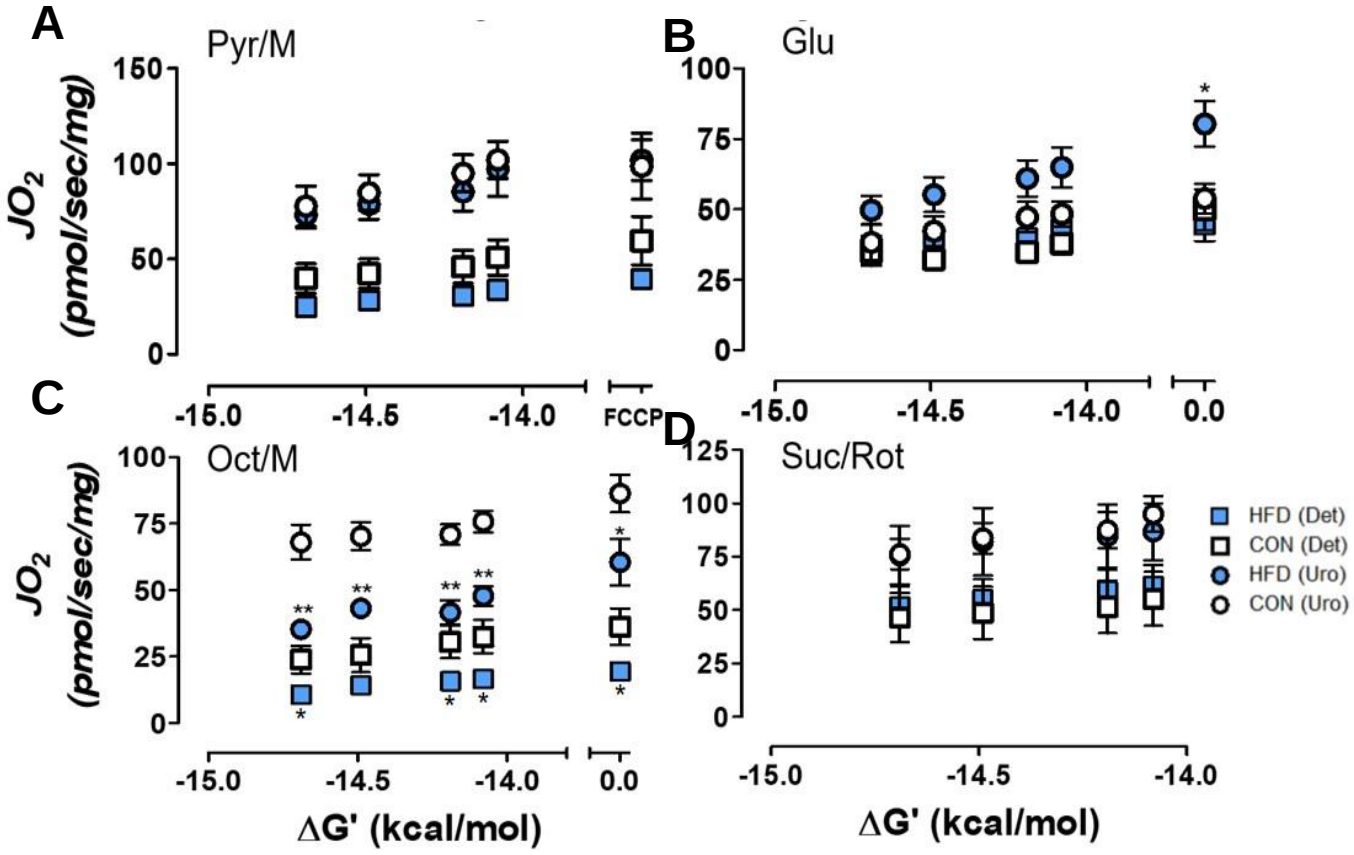

Figure 8. Respiratory oxygen consumption energized with fatty acid Octanoyl-L-Carnitine is reduced in both detrusor and mucosal layers from chronic (24 weeks) HFD mice. *A-D*: Relationship between mitochondrial oxygen consumption (JO_2) and ATP free energy (ΔG_{ATP}) in bladder detrusor or mucosal layer intact tissue energized with either pyruvate/malate (Pyr/M), glutamate, Octanoyl-L-Carnitine/Malate (Oct/M) or succinate/rotenone (Succ/Rot). Data are mean ± SEM and were compared with a student *t* test or a two-way ANOVA followed by a two-tailed Student *t* test. **P* < 0.05 for HFD effect.