Succinate in the development of overactive bladder in metabolic syndrome

Monica Velasquez Flores
Division of Experimental Surgery
McGill University, Montreal

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Abstract

The development of the overactive bladder syndrome (OAB) – a condition involving increased urinary frequency, urgency, and incontinence – remains to be properly described. Consequently, treatment of the condition falls short, begging for new directions in which to shift the research. Multiple studies have shown a relationship between OAB and the metabolic syndrome, suggesting that similar events give rise to the two conditions. The disrupted metabolism found in the metabolic syndrome is manifested throughout different cellular processes, such as in the citric acid cycle, where fluctuations in the level of intermediates have been reported. This is specifically the case for succinate, the role of which is now understood to extend beyond that of an intermediate of glucose metabolism. Along with its membrane receptor, GPR91, increased succinate levels have been implicated in multiple aspects of disease, including those of the metabolic syndrome. Thus, we predicted that the increased succinate levels reported in metabolic syndrome, are involved in the development of OAB. This study was divided into two parts:

(1) An *in vitro* cell culture study that tested the acute effects of succinate. This part was heavily focused on the response of urothelial cells to succinate, specifically those already reported in other cell types. Similar experiments were performed on urothelial cells with the knockdown of the GPR91 succinate receptor to assess its contribution to the succinate-mediated effects. These studies were complemented using inhibitors of G-

protein couple receptor pathways, giving a more complete view of the signaling of succinate in urothelial cells. These studies confirmed the presence and activity of GPR91 in urothelial cells. Through this receptor, succinate activated the PKC-MAPK pathway in a $G\alpha_q$-dependent manner and inhibited the production of cyclic AMP through $G\alpha_i$. The activation of this receptor by succinate in urothelial cells resulted in increased nitric oxide production, decreased basal PGE_2 levels and increased cytosolic calcium.

(2) An *in vivo* rat study that looked at the effects of chronic succinate exposure. We began by introducing a rat model associated with metabolic syndrome. The bladder function of this rat was compared to that of a healthy rat. In parallel, we administered succinate daily for a period of 4 weeks and compared bladder parameters in these animals to those in the vehicle group. Rats associated with metabolic syndrome showed a bladder overactivity phenotype when compared with healthy rats. The succinate administrated resulted in high levels of it in the urine, and was possibly responsible for the induction and worsening of the bladder overactivity phenotype in healthy rats and metabolic syndrome-associated rats, respectively.

These studies come together to show that increased succinate levels are capable of provoking disruption in bladder function, possibly through the activation of GPR91 expressed by urothelial cells. Given these findings, we offer a new area that, with further research, may give rise to new tools to prevent and treat OAB.

Résumé

Le syndrome de la vessie hyperactive (SVH) se caractérise par des envies urgentes d'uriner, l'incontinence et une fréquence de miction élevée. Le processus par lequel le SVH se développe n'est toujours pas élucidé. Il en résulte que les traitements efficaces font défaut, ce qui nous oblige à nous orienter vers de nouvelles voies de recherche. De nombreuses études ont établi un lien entre le SVH et le syndrome métabolique et suggèrent que des causes communes sont á l'origine des deux pathologies. Le métabolisme dysfonctionnel observé lors du syndrome métabolique se manifeste au niveau de diverses voies cellulaires, comme par exemple dans celle du cycle de Krebs, dans lequel les niveaux des intermédiaires métaboliques fluctuent de manière importante. Ceci est le cas en particulier du succinate, dont on a récemment découvert que son rôle au sein des cellules dépasse celui de simple intermédiaire métabolique. Avec son récepteur membranaire, le GPR91, le succinate s'est révélé un acteur majeur dans de nombreuses pathologies, dont le syndrome métabolique. Nous suggérons ici qu'une augmentation des niveaux circulants de succinate est impliquée dans le développement du SVH. Cette étude se divise en deux parties :

(1) Une étude *in vitro* sur des cellules en culture mettant en évidence les effets à court terme du succinate. En nous basant sur les travaux publiés sur d'autres types cellulaires, nous nous sommes focalisés sur les réactions des cellules urothéliales en culture mises en présence de succinate. Les mêmes expériences ont été réalisées après le 'knockdown' du récepteur GPR91 pour s'assurer de la spécificité des réponses. Nous avons complété ces

résultats en utilisant des inhibiteurs de la voie des récepteurs couplés aux protéines G, nous offrant ainsi une vue plus générale des voies de signalisation du récepteur du succinate. Les résultats obtenus ont confirmé la présence et l'activité du récepteur GPR91 dans les cellules urothéliales. En se liant á son récepteur, le succinate active la voie PKC-MAPK au travers des protéines $G\alpha_q$ et inhibe la production d'AMP cyclique par l'activation des protéines $G\alpha_i$. L'activation de ce récepteur par le succinate dans les cellules urothéliales entraine aussi une augmentation de la production d'oxide nitrique (NO), une diminution de la production basale de PGE_2 et une augmentation des niveaux cytosoliques de calcium.

(2) Une étude *in vivo* chez les rats qui examine les effets chroniques de l'exposition au succinate. Nous avons commencé par introduire un modèle murin associé au syndrome métabolique. L'activité de la vessie de ce modèle a été comparée à celle d'un rat normal. En parallèle, le succinate a été administré quotidiennement aux deux types de rats sur une période de 4 semaines et nous avons comparé les caractéristiques de leur vessie. Le groupe de rats associés au syndrome métabolique présentait le phénotype d'une vessie hyperactive, alors que le groupe contrôle présentait des paramètres normaux. Le succinate injecté s'est abondamment retrouvé dans l'urine et pourrait donc être responsable de l'induction et de la détérioration du phénotype hyperactive chez les rats contrôles et les rats associés au syndrome métabolique, respectivement.

Ces études combinées démontrent qu'une augmentation des concentrations de succinate peut entrainer une perturbation du fonctionnement de la vessie, possiblement par l'activation du récepteur GPR91 exprimé par les cellules urothéliales. Grâce à ces

découvertes, nous ouvrons une nouvelle aire de recherche qui pourrait déboucher sur des outils innovateurs pour le traitement du SVH.

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Preface

This work is presented in a traditional thesis format.

Chapter 4 is an *in vitro* study on urothelial and smooth muscle cells of the bladder.

Chapter 5 is an animal study (*in vivo* and *in vitro*) describing the general effects succinate on bladder function and morphology.

Author contributions are listed in the introduction of the two chapters.

Table of Contents

List of Figures and Tables

Figures

Tables

Abbreviations

ATP	Adenosine triphosphate
BOO	Bladder outlet obstruction
BP	Blood pressure
BTX-A	Botulinum-A Toxin
CAC	Citric acid cycle
cAMP	Cyclic adenosine monophosphate
Dahl/SS	Dahl Salt-Sensitive
EFS	Electrical field stimulation
ERK	Extracellular signal-regulated kinase
GPCR	G-protein coupled receptor
GPR91	G-protein receptor 91
HDL-C	High-density lipoprotein cholesterol
ICS	International Continence Society
IDF	International Diabetes Federation
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
JNK	c-JUN N-terminal kinase
MAPK	Mitogen-activated protein kinases
mRNA	Messenger RNA
NO	Nitric Oxide
OAB	Overactive bladder
OAB-q	Overactive bladder questionnaire

PBOO	Partial bladder outlet obstruction
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PTX	Pertussis toxin
SD	Sprague-Dawley
SDH	Succinate dehydrogenase
shRNA	Short hairpin RNA
VAChT	Vesicular acetylcholine transporter
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.1 Overview of Overactive Bladder

Definition

The overactive bladder syndrome (OAB), as defined by the International Continence Society (ICS), is composed of urinary urgency, increased daytime and nighttime (nocturia) frequency, with or without incontinence [1]. It is estimated that millions of people over the age of 18 experience OAB [2], with about a fifth of Canadians over the age of 35 affected [3]. OAB takes a toll on the quality of life, placing a burden on simple tasks of everyday life [4], while simultaneously placing an economic one on healthcare [5]. Often, patients who present with OAB have an underlying condition [6], adding emphasis to the burden of OAB on quality of life.

Diagnosis

OAB is a symptom complex which requires a comprehensive assessment of history and clinical examination, in order to exclude other conditions [7]. This may also be complemented with invasive procedures like cystometry which measure volume and pressures inside the bladder as it fills and empties [8]. Improving this area would involve a physiological marker for OAB, which would aid in detecting its development early on.

Treatment

Symptoms of OAB may be targeted by simple lifestyle changes such as modifying fluid intake [9]; however, it may require more than conservative management. Since the

symptoms of OAB stem from the increased contractions of bladder detrusor muscle mediated through muscarinic receptors, treatment tends to involve antimuscarinic agents [10]. The disadvantage of this therapy is that these drugs not only target the muscarinic receptors of the bladder, and can result in side effects such as dry mouth, blurry vision and constipation [10]. Complex cases of OAB may involve Botulinum-A Toxin (BTX-A) injections or surgery [11]. The β3 adrenoceptor agonist mirabegron is considered second-line therapy for OAB, activating β3 adrenoceptors of detrusor muscle leading to its relaxation [12]. This is a drug of interest because the side effects are less than those of anticholinergics [12].

Nevertheless, although these therapies mainly aim to target the symptoms of OAB, their effectiveness is not ideal and the side effects lead to patients discontinuation [13]. These findings highlight the need for a cure and/or preventative measures for OAB.

The Study of OAB

Different challenges arise when studying the development of OAB. While symptoms such as nocturia are objective, other OAB characteristics are subjective, making them more difficult to assess [14]. This is the case for the urinary urgency experienced by OAB patients. Not only does the severity of this symptom vary between patients, assessing urgency in an animal is not feasible [14]. As a result, animal models specific to OAB are not available. The next best tools are animal models displaying certain aspects of OAB. Yet, studies using these models tend to fall short in pinpointing the factors involved in the development of this syndrome [14]. New ways of looking at the problem are necessary to make available new animal models in which to study OAB.

1.2 Metabolic Syndrome

The lack of effective therapies for OAB arises from the lack of understanding of the mechanisms leading to its development. OAB may arise from a neurological cause but a good portion of it is classified as idiopathic [15]. As mentioned, patients with OAB often experience an underlying condition [6]. Many of these conditions include obesity, hypertension and type 2 diabetes [16, 17], which are pathologies that fall under the metabolic syndrome. Criteria for diagnosis of the metabolic syndrome, as set by the International Diabetes Federation (IDF), involves central obesity plus two of the following measures: elevated triglycerides, reduced high-density lipoprotein cholesterol (HDL-C), elevated fasting glucose, and elevated blood pressure [18].

As we can deduce, these criteria are associated with metabolic disturbances that will bring about changes in the body, explaining why metabolic syndrome is associated with multiple aspects of disease [19-21].

1.3 Metabolic Syndrome and Voiding Dysfunction

Numerous studies have associated metabolic syndrome and voiding dysfunction (Figure 1). Furthermore, many of these studies link metabolic syndrome and OAB [22-24]. In particular, one group reported that in their studied female population, more than 60% of women with OAB also had metabolic syndrome, as defined by the IDF criteria [25].

The association of these metabolic abnormalities and OAB suggest that factors leading to the development of metabolic syndrome, may also be involved in the development of OAB.

Explanations for the incidence of OAB in a metabolic syndrome background involve the end-organ damage associated with the different aspects of the syndrome [26]. However, a clear link has not yet been established. For this reason, a better grasp of the molecular events behind metabolic syndrome is necessary.

The criteria set by the IDF for the diagnosis of metabolic syndrome suggest a disruption in energy metabolism. Vast research is available in how the cell produces ATP. From glycolysis to oxidative phosphorylation, the enzymes and substrates involved have been clearly identified. Yet, only recently has it been understood that these metabolic players have roles that extend beyond the production of energy. The intermediates of the citric acid cycle (CAC) have been implicated in other physiological and pathophysiological cell processes.

Of most interest to us, is the intermediate succinate, as a role for it has been suggested in the context of metabolic syndrome, such as for obesity, hypertension and insulin signaling dysfunction [27].

Succinate dehydrogenase (SDH), which converts succinate to fumarate, is not only a crucial player in the CAC, but is also the second complex of the electron transport chain [28]. A good amount of studies on SDH focus on its inhibition, such as loss-of-function mutations, and are associated with the development of cancer [29].

More recently, succinate was found to be the substrate for GPR91, a G-protein coupled receptor (GPCR) [30]. A role for this receptor has been described in tissues like the kidney, liver, and adipocytes [31]. However, the role of succinate either in a metabolic way or by its action on GPR91 has not yet been clearly defined for the bladder.

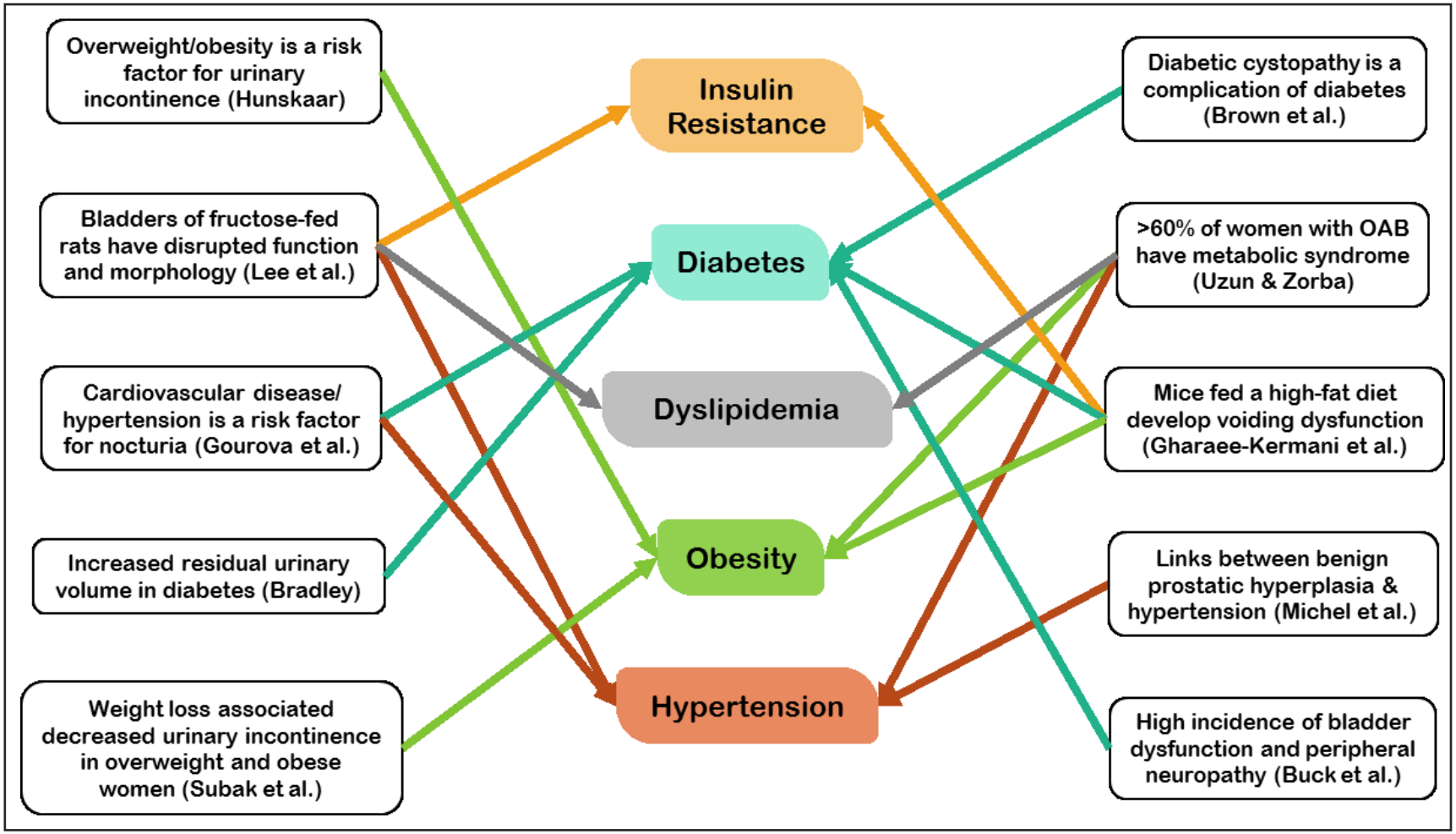


FIGURE 1. ASSOCIATIONS BETWEEN COMPONENTS OF METABOLIC SYNDROME AND VOIDING DYSFUNCTION.

Selected studies highlighting the associations between components of the metabolic syndrome and voiding dysfunction in humans and animal subjects [22-25, 32-37].

Chapter 2: Literature Review

Information on the action of succinate in the bladder is almost non-existent. A review of the literature available on the role of succinate in other contexts, including metabolic syndrome, will aid in providing a good foundation for hypotheses about succinate's potential action on the bladder.

2.1 Succinate and Cancer

A hallmark of cancer is the increased energy production needed to sustain proliferation of malignant cells. Oxidative phosphorylation, the cell's normal pathway for ATP production, is halted and glycolysis is revved up [38]. The hypoxic environment, which is common for tumors, allows for the stabilization of the hypoxia inducible factor 1α (HIF-1α) [39]. HIF-1α increases the expression of proteins involved in glycolysis [38]. However, cancer cells can have HIF-1α expression even without a hypoxic environment. This can be achieved by inhibition of SDH leading to an accumulation of succinate [40]. In turn, succinate leads to the inhibition of the inhibitory subunit of HIF-1α, favouring glycolysis, angiogenesis, and cell survival [39, 40].

In a bladder context, HIF-1α has also been considered, with its overexpression correlated to poor prognosis of urothelial carcinomas [41]. In terms of voiding dysfunction, HIF-1α has been proposed to play a role in the adaptation to hypoxia that occurs during partial bladder outlet obstruction (PBOO) in a rat model [42]. Upregulation of HIF-1α leads in this setting, to an angiogenic response through the upregulation of the vascular

endothelial growth factor (VEGF) and angiopoietin-1 [42]. Similar findings are available in detrusor smooth muscle cells isolated from humans, adding that while these cells maintain their viability in response to hypoxia, their proliferation is blocked thus hurting their ability to cope with the hypoxic stress, contributing to the dysfunction of the bladder [43]. Koritsiadis and colleagues add that, while the urothelium and smooth muscle may be resistant to the hypoxic insult, it is in the stromal cells where the most expression of HIF-1α occurs [44]. They hypothesize that these cells may mediate changes in the urothelium and smooth muscle through HIF-1α-induced mediators [44].

Therefore, it is suggested that increased succinate levels surrounding the bladder could lead to the activation of HIF-1α in this organ, eliciting a response that could lead to bladder dysfunction.

2.2 Succinate and Inflammation

The activation of inflammatory cells follows similar events as those described for cancer. Oxygen levels tend to be low in sites of inflammation, allowing HIF-1α to start rapid ATP production and an increase of biosynthetic capacity of immune cells [45]. As is the case for cancer, in settings of inflammation, HIF-1α activation in normal oxygen conditions has also been observed. In mice, the response of macrophages to LPS has been shown to increase succinate levels leading to the stabilization of HIF-1α and induction of interleukin-1β (IL-1β) [46].

Furthermore, the succinate receptor GPR91 has also been given a role in inflammation [47, 48]. The expression of this receptor is induced during differentiation of human

dendritic cells [47]. In addition, these cells have a chemotactic response to succinate and, through GPR91, are incited by succinate to increase cytokine production by T cells [47]. Studies like these highlight the important role of succinate and its receptor in the inflammatory response. This is further supported by the finding that mice with GPR91 knockdown have impaired immunity [47].

Increased urinary inflammatory biomarkers in OAB patients compared to controls suggest a role for inflammation in the pathogenesis of OAB [49]. The involvement of succinate in inflammation, suggest a potential role for it in this setting as well. IL-1β, a target of HIF-1α [46], has been shown to induce human detrusor smooth muscle cells (SMCs) to release cytokines and chemokines [50, 51]. As a result, Bouchelouche et al. suggest that the SMCs may be involved in the recruitment and activation of inflammatory cells to the bladder, specifically in the setting of interstitial cystitis [51].

It is obvious that, at least under conditions of stress, succinate plays a bigger role than just being part of the CAC and that its role in pathology could easily be extended to the bladder. The following findings will focus more on the role of succinate in metabolic syndrome-related abnormalities.

2.3 Succinate and the Metabolic Syndrome

As one can imagine, altered glucose levels, such as those seen in type 2 diabetes, will lead to alterations in succinate levels. Increased levels of this metabolite have been found in diabetic animal models in tissues such as the retina [52] and kidney [53].

In the retina, the activation of GPR91 by succinate leads to increases in VEGF through an ERK/COX-2/PGE_2 pathway that is associated with impairment in vasculature and retinopathy both in the context of diabetes [52] and ischemic conditions [54].

In the kidney, these increased succinate levels (due to high glucose levels) have been found to activate GPR91 leading to the increase of intracellular Ca^{2+} in endothelial cells, but not in smooth muscle cells [53]. Furthermore, increases in nitric oxide (NO) and PGE_2 production in response to high glucose and succinate were also mediated by GPR91 in these cells [53]. All in all, these effects lead to the release of renin and are suggested to aid in the development of diabetic nephropathy [53].

The actions of succinate on the increase in renin release are also implicated in increases in blood pressure, through the renin-angiotensin system, suggesting a hypertensive effect of succinate that has also been confirmed to be mediated by GPR91 [30]. Moreover, elevated levels of plasma succinate have been found in hypertensive, diabetic and insulin resistant/obese murine models [27].

It is important to mention that increases in circulating succinate levels such as those seen in murine models of hypertension and metabolic disease have not been found for humans [27]. However, this finding does not take into account succinate accumulation that is found in the organs like retina [52], kidney [53] and heart [55]. Therefore, there is the possibility that, while increased succinate levels are not reflected in the circulation, it may be accumulating in the bladder like it does in other organs, giving us reason to study this metabolite's role in bladder dysfunction.

Chapter 3: Objectives and Hypotheses

3.1 Objectives

This projects aims to determine if succinate has a role in the development of OAB in metabolic syndrome. This will be done by studying its effects at the cellular and bladder level.

3.1.1 Objective 1

Describe the signaling cascade of succinate in bladder cells and whether these effects are mediated by the GPR91 succinate receptor.

Experimental Design

Primary urothelial and smooth muscle cells will be obtained from Sprague-Dawley (SD) rats. These cells will be tested for the presence of the succinate receptor. The downstream effects of succinate on these cells will then be assessed by measuring the phosphorylation of mitogen-activated protein kinases (MAPKs) and the production of second messengers. These studies will be repeated in the presence of inhibitors of GPCR pathways, as well as in cells with the knockout of the GPR91 receptor. These experiments will clarify the role of the receptor in the response to succinate and which GPCR signaling pathways are activated when succinate binds GPR91.

3.1.2 Objective 2

Establish the bladder effects of chronic succinate administration in a rat model of bladder overactivity associated with metabolic syndrome.

Experimental Design

Intraperitoneal injections of saline or succinate will be given daily to SD rats for a period of 4 weeks. At the end of this period, their bladder function will be assessed using conscious cystometry. The bladder of these animals will then be collected and their contractility will be measured in an organ bath setting. Furthermore, the levels of GPR91 will be measured. These experiments will also be performed in Dahl salt-sensitive (Dahl/SS) rats, which display components of metabolic syndrome, to compare their bladder function to those of SD rats and to see how their bladders are affected by increased succinate levels.

3.2 Rationale & Hypotheses

3.2.1 Rationale & Hypothesis 1

Based on studies focusing on other organs with cell types like those of the bladder, succinate has been linked to:

- Activation of ERK
- Increased intracellular Ca^{2+}
- Increases in NO and PGE_2 production

These effects have been shown to be mediated, at least in part, by the GPR91 succinate receptor.

Therefore, we hypothesize that, through GPR91, succinate activates similar pathways in cells of the bladder. Describing the pathways that succinate activates in the bladder is crucial for understanding the relationship between metabolic syndrome and OAB.

3.2.2 Rationale & Hypothesis 2

Increased succinate levels, such as those seen in metabolic syndrome, are believed to mediate dysfunction of systems like the circulatory system and tissues such as the retina and kidney.

Therefore, we hypothesize that simulating increased succinate levels in rats will induce changes in the bladder leading to its dysfunction.

Chapter 4: The Effects of Succinate in Urothelial and Smooth Muscle Cells of the Bladder

This chapter includes work that has recently been published in *Cellular Signalling* under the title "Succinate, increased in metabolic syndrome, activates GPR91 receptor signaling in urothelial cells" [56]. The text in quotation marks in the Materials and Methods section is written verbatim. The figures in this chapter were specifically designed to be presented in this work.

Contribution of authors:

Along with Drs. Abubakr Mossa, Philippe Cammisotto and Lysanne Campeau, I contributed to the conception of the study and acquisition of the results presented here. The interpretation of the results in this chapter are entirely my own.

4.1 Introduction

The focus of this chapter is to have a general idea of the effects of succinate in the cells of the bladder, specifically in urothelial and SMCs cultured from SD rats. We begin by establishing the presence of the succinate receptor, GPR91. Then, guided by studies in other cell types, we look at the outcomes of GPR91 activation by succinate in urothelial and SMCs. Among these is the activation of ERK and JNK in response to succinate. Furthermore, we focus on outcomes that are more relevant to the function of the bladder, such as those involved in its relaxation and contraction.

These experiments are repeated on cells with a knockdown of the GPR91 receptor to see how much this receptor contributes to the observed effects of succinate.

Finally, the last part of this chapter, uses conditioned media to establish whether an interaction between urothelial and SMCs occurs in the presence of succinate.

4.2 Methods

Reagents and antibodies

"Puromycin, Kanamycin A, ampicillin, Probenecid, forskolin, U73122, Cyclic AMP (cAMP) and prostaglandin E2 (PGE2) ELISA kits were from Cayman (Ann Harbor, MI, USA). Antibodies were purchased from the following companies: anti-pan cytokeratin (AE1/AE3), P-JNK, JNK, alpha-actin and cytokeratin (CK17) from ThermoFisher Scientific (Waltham, MA, USA), beta-actin from ProteinTech (Rosemont, IL, USA), Phospho-ERK and total ERK from R&D Systems (Minneapolis, MN, USA), iNOS from NovusBio (Oakville, ON, Canada). Fetal bovine serum (FBS) was from Wisent (Quebec, Canada). Penicillin/Streptomycin, tryptone was from VWR International (Quebec, Canada). Proteins were measured using a BCA protein assay kit from BosterBio (Pleasanton, CA, USA). Collagenase type IV was from Worthington Biochemical Corporation (Lakewood, NJ, USA). Tryptone agar, DMEM and MCD153 media were purchased from US Biological Life Science (Salem, MA, USA). Glutamax and Keratinocyte medium supplemented with EGF and bovine pituitary extract were from Gibco (ThermoFisher Scientific, Waltham, MA, USA). ATP, d-glucose, ethanolamine, bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Oakville, ON, Canada), adenine from Biomatik (Cambridge, ON, Canada), dihydrocortisone from MP Biochemicals (Solon, OH, USA), Y27632 from Selleckchem (Houston, TX, USA), Fluo-4 from AAT Bioquest, (Sunnyvale, CA, USA), pluronic acid from Biotium (Fremont, CA, USA), DAPI from Abcam (San Francisco, CA, USA), PD98059 from MedchemExpress, (Monmouth Junction, NJ, USA), pertussis toxin from Tocris (Bristol,

UK) and yeast extract from Alfa Aesar, (Haverhill, MA, USA). UBO was a generous gift from Dr. T. Hebert (McGill University-Montreal)" [56].

Isolation and characterization of urothelial and smooth muscle cells

"Cells were isolated from female Sprague-Dawley (SD) rats aged 2–3 months. Housing and handling of the animals conformed to the Canadian Council for Animal Care (CCAC) and all protocols were approved by the Animal Ethics Committee of McGill University (Montreal, Canada). Animals were fed on standard Purina chow and had free access to water. Primary cells were isolated using a collagenase type IV technique. Briefly, rats (2–3 months) were anaesthetized using isoflurane. Blood (8–12 mL) was withdrawn by intra-cardiac puncture to sacrifice the animals. Bladder was taken and dropped in cold PBS (pH 7.4), dissected to remove adipose tissue, cut open longitudinally, spread and the urothelium was gently scrapped and incubated for 15–20 min in DMEM medium containing 100 U/mL of collagenase IV. Cells were then washed twice with DMEM supplemented with 10% fetal bovine serum (FBS) then seeded in a 24-well plate with Dulbecco's DMEM low glucose/Keratinocyte (50/50) media containing FBS (10%), Glutamax (× 1), hormones mix (insulin 5 µg/mL, dihydrocortisone 0.5 µg/mL, adenine 15 µg/mL, ethanolamine 0.1 mM), Rho Inhibitor Y27632 (10 µM) and 1% penicillin/streptomycin (100 U/mL, 100 µg/mL) in a humidified incubator in 5% CO2 atmosphere. Culture medium was replaced every 2–3 days until cell confluency. Cells were trypsinized every week and used from passage 2 to 6. Prior to use, cells were starved for 24 h in the same medium devoid of Y27632 and with Keratinocyte medium replaced by MCDB153 medium. For smooth muscle cell

culture, detrusor muscle devoid of urothelium was minced then incubated for 45 min in DMEM containing 250 U/mL of collagenase IV, with intense shaking. Muscle tissue was then placed on a strainer (40 μm) to retain non-digested materials, washed twice with DMEM/FBS and cells were seeded on a petri dish in DMEM medium supplemented with FBS (10%), Glutamax (1%), glucose (27 mM) and penicillin/streptomycin (100 U/mL, 100 μg/mL). Incubation conditions were similar to those of urothelial cells. Prior to use, cells were starved for 72 h in the medium with low glucose (5.5 mM).

Cells were grown on glass coverslips. After confluency, cells were washed twice with warm PBS, fixed in formaldehyde 4% for 30 min then washed four times with PBS. Cells were then permeabilized with Triton × 100 (0.1%), glycine (300 mM), ammonium chloride (75 mM) in PBS pH 8 for 10 min. After washing, blocking buffer (BSA 1% in PBS) was added for 30 min then incubation with the primary antibody (AE1/AE3, CK17 and alpha-actin) was carried out overnight. The following morning, secondary conjugated antibody was added, then cells were incubated with DAPI, cover slipped and examined by fluorescent microscopy" [56].

Expression of the GPR91 receptor in urothelial and SMCs

"RNA extraction was carried out using Trigent reagent (Biomatik Corporation, Canada). Purity and concentrations of RNA were measured using a Nanodrop spectrophotometer ND-1000 (ThermoFisher Sci., Wilmington, DE, USA). Primers were designed as follows: beta-actin forward 5′-GCT ACA GCT TCA CCA CCA CA 3′, and reverse 5′-ATC GTA CTC CTG CTT GCT GA-3′; GPR91 forward 5′-TTA CGC CAC TGG GAA

CTG GA-3′and reverse 5′-TTG ATG GCC TTC TGG GAA CA-3′. cDNA was synthesized using SensiFAST cDNA Synthesis Kit (Bioline, Taunton, MA, USA) and amplified by PCR using the 2 × Taq FroggaMix (FroggaBio, Toronto, ON, Canada). PCR products were assessed on a 1% agarose gel electrophoresis with ethidium bromide and photographed under UV light" [56].

Quantitative real-time PCR

"Primer sequences used were purchased from IDT (Integrated DNA Technology, Canada) and were as follows: rat Beta-actin (forward: 5′-CAC CCG CGA GTA CAA CCT TC-3′; reverse 5′-CCC ATA CCC ACC ATC ACA CC-3′) and GPR91 (forward: 5′ -ACA GCT GTC GCC CTT TTC TA-3′; reverse 5′- TCA TGC CAA CCT CTA CAC CA -3′). Reverse transcription was performed on 5 μg of total RNA using TranScript All-in-One First-Strand cDNA Synthesis Supermix for qPCR (One-step gDNA Removal) (TransGen Biotech, Montreal, Can). Quantitative real-time PCR was carried out using the TransStart Tip SYBR Green qPCR Super Mixture (TransGen Biotech, Montreal, Canada). Samples were loaded in 96 fast-PCR plate (Sarstedt, Montreal, Canada) and inserted in an Applied Bioscience 7500 Fast Real-time PCR System. Specificity of the signal was confirmed by carrying out a dissociation curve. The data were analyzed using the 2-ΔΔCT method" [56].

NO and PGE_2 assays

“NO was measured using a sulphanilamide/1-(naphthyl-ethylenediamine dihydrochloride (NEDD) colorimetric method as described [19]. PGE2 was estimated in the medium by ELISA kit according to the manufacturer's protocol (Cayman: Ann Harbor, MI, USA)” [56].

Calcium mobilization

“Cells were trypsinized and seeded on Ibidi slides (Madison, WI, USA). After 48 h, cells were washed twice in warm PBS and incubated for 30 min at 37 °C in the dark with Fluo4 (5 μM final) in red phenol free-DMEM without FBS containing pluronic acid (0.1%) and probenecid (25 mM). Cells were then washed twice in PBS, let to rest for 15 min then processed immediately for confocal microscopy. Quantification of calcium induction in the obtained images were measured using ImageJ software” [56].

Western blotting

“Whole cells were lysed in RIPA buffer containing an anti-protease mix (Roche Diagnostics, Indianapolis, IN, USA) on ice. 20 to 40 μg of proteins were resolved on 8% polyacrylamide gel and then electro-transferred to PVDF membranes. Blocking in TBST milk 5% was followed by overnight incubation with primary antibodies in concentrations as recommended by manufacturers. Secondary antibodies were conjugated with HRP and bands revealed using a Luminata Crescendo HRP substrate (Millipore, Billerica, MA, USA)” [56].

GPR91 shRNA

"Scrambled shRNA and recombinant GPR91 shRNAs (construct A:CACCGTGGTGTTCGGCTACCTCTTCTGCA and construct B: CAGCAGGCAACTGTGCTATCGCTGAACAA) were designed and packaged by OrigGene (Rockville, MD,USA) [22]. The 29mer shRNA constructs for GPR91 were synthesized and inserted into retroviral untagged vectorpRS under U6 promoter for mammalian cell expression containing resistance for ampicillin. Plasmids were then transfected into competent stellar bacteria. After selection by ampicillin, single colonies were grown in liquid broth (tryptone/yeast extract) and plasmids were purified using Presto miniprep (Geneaid, Taiwan). Characterization of the plasmids was carried out on agarose gel. The retroviruses carrying scrambled and GPR91 shRNAs were produced by co-transfection of plasmids pUMVC and pMD2G in HEK293 cells. Viral supernatants were collected after 48 h, filtered through 0.45 μm filter and used on non-confluent urothelial cells. After puromycin selection, efficiency of the transfection was assessed by qPCR using specific primers" [56].

Statistical analysis

"Results are shown as median and standard error of the mean (SEM). Statistical significance was considered when P value is < 0.05. Comparisons were carried out using independent student t-test and one-way ANOVA (post hoc Tukey HSD test) as indicated in the legends. GraphPad software was used for the statistical analyses" [56].

4.3 Results

Establishing primary bladder urothelial and SMCs

One of the first steps in our study was to establish reliable primary cells lines of urothelial and SMCs of the bladder from SD rats. Figure 2 shows the immunofluorescence labeling of both cells types. Urothelial cells are characterized by the AE1/AE3 and cytokeratin 17 antibodies, while for SMCs we used the α-actin antibody.

Expression of the GPR91 receptor in urothelial and SMCs

The next step was to determine the presence of the succinate GPR91 receptor in these cells. At the time this experiment was performed, we were aware of the presence of this receptor in multiple tissues such as the kidney [53], retina [54] and heart [57]. However, its presence in the bladder had not been investigated. Recently, a study analysed the presence of this receptor in multiple murine organs, including the bladder [58]. We took a deeper look into the expression of GPR91 and not only identified its presence in the two types of bladder cells mentioned (Figure 3A), but also showed how GPR91 levels varied from each cell type. We saw that compared to SMCs, urothelial cells have higher expression of the GPR91 receptor at the mRNA level (Figure 3B). While it would be ideal to also show the expression of GPR91 at the protein level, we are limited by a lack of a specific antibody for the receptor.

Response to succinate stimulation

The following experiments focused on the response of these bladder cells to stimulation with mainly 200 μM succinate. This concentration was based on other studies which used succinate in concentrations ranging from 10 μM to 5 mM [59-61]. Preliminary experiments in our lab gave the clearest overall results when 200 μM succinate was used.

Activation of ERK and JNK by succinate

The activation of the mitogen-activated protein kinases (MAPKs) by succinate has been reported in a variety of cell types. In GPR91-activation by succinate, the phosphorylation of the extracellular signal-regulated kinase (ERK) has been linked to proliferation [62], release of renin [63], and angiogenesis through increases in VEGF [64]. Similar findings are available for the c-JUN N-terminal kinase (JNK) [65], although in the majority of these studies, ERK phosphorylation is predominant [66]. Guided by these findings, we looked at the activation of MAPKs in our bladder cells in response to succinate.

Treatment of urothelial cells with 200 μM succinate resulted in increased ERK 1/2 and JNK 1/2 phosphorylation (Figure 4A). However, the same treatment on SMCs did not yield a change in ERK 1/2 phosphorylation and while it appeared to have an increase in JNK 1/2 activation (Figure 4B), this change was not significant.

Effects NO and PGE_2 production by succinate

Considering the absence of MAPK activation by succinate in SMCs and the lower expression of the GPR91 receptor in these cells compared to urothelial cells, we chose to

focus our attention on the latter. This decision was encouraged by a study on the involvement of GPR91 in the renin angiotensin system. Toma et al. suggest that the activation of GPR91 by succinate on juxtaglomerular endothelial cells leads to the release of second messengers NO and PGE_2, which in turn, may act on the vascular smooth muscle cells [53].

The levels of NO and PGE_2 have also been reported to be controlled by the MAPK pathway [67, 68] and are relevant to our studies since these second messengers have important effects on bladder function [69, 70].

Therefore, our next step was to see if succinate also induced the release of these second messengers in our cells. As we can see in Figure 5, succinate, at both low and high concentrations, induces the production of NO by urothelial cells (Figure 5A), while decreasing the levels of PGE_2 (Figure 5B), but only at higher concentrations.

In the bladder, increased PGE_2 production is associated with pathology [71]. However, in this case we observed a decrease in the basal levels of PGE_2 production by urothelial cells, which may indicate a negative response of these cells to succinate.

We set to find out if the increased NO production was mediated by the inducible nitric oxide synthase (iNOS). The induction of this enzyme usually takes place under stressful situations such as infection or inflammatory diseases [72]. This leads to the overproduction of NO and NO-radicals that can help the body fight pathogens or exacerbate a diseased state [72]. In fact, it has been previously reported that LPS treatment of rats triggers iNOS expression in urothelial cells [73]. As expected, the expression of iNOS in our urothelial cells followed the same pattern as the production of

NO in cells treated with succinate (Figure 5C), and together, suggest an inflammatory response of urothelial cells to succinate.

Inhibition of cAMP production by succinate

Cyclic AMP (cAMP) was another second messenger we chose to consider since it has also been linked to GPR91 activation, through the $G\alpha_i$ subunit [74]. Succinate alone has no effect on the production of cAMP by urothelial cells (Figure 6A). Forskolin was used to stimulate the its production, however, pre-incubation of the cells with succinate (100 nM - 1 mM) results in an impaired cAMP production by forskolin. Interestingly, at higher succinate concentrations (10 mM), forskolin can induce the production of cAMP, suggesting that at higher concentrations, the effects of succinate on cAMP are blocked (Figure 6B).

Succinate activation of Gα proteins

These next steps focused on testing if succinate acting through GPRC-coupled pathways.

Succinate activation of $G\alpha_q$ pathway

ERK activation in our urothelial cells suggest the involvement of the $G\alpha_q$ subunit. We relied on different drugs for different steps on this pathway to show that the $G\alpha_q$ subunit is also activated in urothelial cells in response to succinate.

First, we used the PD98059 compound, which inhibits the kinase MEK, a downstream kinase of $G\alpha_q$ signaling responsible for phosphorylation of ERK [75]. As expected, the

activation of ERK by succinate was inhibited by PD98059 (Figure 7A). We also used U73122, an inhibitor of phospholipase C (PLC) [76], with which succinate was not able to induce the activation of ERK (Figure 7B). The involvement of protein kinase C (PKC) was also tested in this pathway using its inhibitor BIM-1 [77]. Incubation of urothelial cells with BIM-1 also inhibited ERK phosphorylation by succinate (Figure 7C). We completed these studies with UBO, an inhibitor of the $G\alpha_q$ protein [78]. Blocking the $G\alpha_q$ signaling prevents the phosphorylation of ERK in the presence of succinate (Figure 7D), confirming that the activation of ERK is elicited by $G\alpha_q$.

Succinate activation of $G\alpha_i$ pathway

The inhibition of cAMP production by succinate suggests the involvement of the $G\alpha_i$ subunit. For this case, we used pertussis toxin (PTX) to inhibit the $G\alpha_i$ subunit [30]. As expected, pre-incubation with PTX, prevented the inhibition by succinate of forskolin-induced cAMP production (Figure 8A). To confirm that this effect is solely $G\alpha_i$-mediated, we also performed a similar experiment with UBO and saw that blocking the $G\alpha_q$ protein did not prevent the inhibition by succinate of forskolin-induced cAMP production (Figure 8B).

GPR91 knockdown

The activation of ERK and JNK and the inhibition of cAMP production in the presence of succinate suggest the activation $G\alpha_q$ and $G\alpha_i$, respectively [79]. However, up to this point, we cannot be certain that the cellular effects of succinate were mediated through the GPR91 receptor. One of the challenges that emerged was the lack of a specific

GPR91 antagonist. Thus, to identify the involvement of this receptor in the succinate response of urothelial cells, we resorted to its knockdown using retroviral delivery of shRNA. Figure 9 shows the knockdown of GPR91 in urothelial cells using two different shRNA constructs, shRNA A and shRNA B. Both constructs caused a significant decrease in the mRNA expression of GPR91 by urothelial cells, with construct A being more effective than construct B.

GPR91 activation by succinate causes ERK and JNK phosphorylation

The succinate treatment of urothelial cells transfected with a control plasmid led to an increase in phosphorylation of ERK 1/2 and JNK 1/2 (Figure 10), as was seen in normal urothelial cells (Figure 4A). However, succinate-triggered increase in the phosphorylation of these kinases was not present in urothelial cells with the knockdown of the receptor.

GPR91 activation by succinate increases NO and decreases PGE_2 production

As expected, the incubation of urothelial cells with succinate led to an increase in NO production (Figure 11A) along with an increase in iNOS expression (Figure 11B) and decreased PGE_2 production in cells transfected with the scram plasmid (Figure 11C). These effects were not observed in GPR91-knockdown cells.

GPR91 activation by in low succinate concentration inhibits cAMP production

We previously observed that in low concentrations (200 μM) succinate inhibited the production of cAMP by forskolin, while higher concentrations (10 mM) restored cAMP levels. These findings were similar in urothelial cells transfected with the control plasmid (Figure 12). On the other hand, in cells with GPR91 knockdown (especially those with construct A), low succinate concentrations did not inhibit the production of cAMP. As expected, in the presence of higher concentrations of succinate (10 mM) did not inhibit forskolin-mediated cAMP production in cells with GPR91 knockdown.

Succinate and Cytosolic Ca^{2+}

Another reported response to succinate is the release of intracellular Ca^{2+} stores, also associated to the $G\alpha_q$ response [30]. We confirmed this response in our urothelial cells using confocal microscopy (Figure 13A). Upon stimulation with succinate, there was an increase of Ca^{2+} in the cytosol like that produced by ATP. Furthermore, this effect was absent in our GPR91-knockdown cells (Figure 13B).

In this setting, we also decided to look at SMCs. We previously alluded to the possibility of succinate having an indirect effect on SMCs through the release of a factor by urothelial cells. Incubation of SMCs with media collected from urothelial cells treated with succinate resulted in decreased Ca^{2+} release into the cytosol (Figure 13C). It should, however, be pointed out that this effect was only significant when the urothelial cells were treated with a higher succinate concentration (10 mM).

4.4 Discussion

We were successful in showing the presence of the succinate GPR91 receptor in cells of the rat bladder. In addition, we proved that this receptor is active and signals through, at least in urothelial cells, a $G\alpha_q$ and $G\alpha_i$ manner.

We have seen that activation of the GPR91 receptor by succinate leads to an increase of NO production and a decrease of PGE_2 production by urothelial cells. Furthermore, succinate hinders the production of cAMP and leads to an increase of intracellular Ca^{2+} in urothelial cells. Our last experiment allowed us to believe that while the effects of succinate may be focused on the urothelium, it may also have an indirect impact on SMC function through the release of factors not yet confirmed.

These findings highlight the importance of the urothelium as more than just a passive barrier, and puts forward a new path on which to look for therapies for bladder disorders [80]. More work is needed to investigate if succinate accumulation could occur in the bladder as it does in other organs under metabolic stress and if these levels are enough to activate the GPR91 succinate receptor. Future work should also focus on investigating if other Gα proteins are involved in the signaling of succinate in the urothelium and most importantly, investigate the effects of this signaling on the bladder.

4.5 Figures

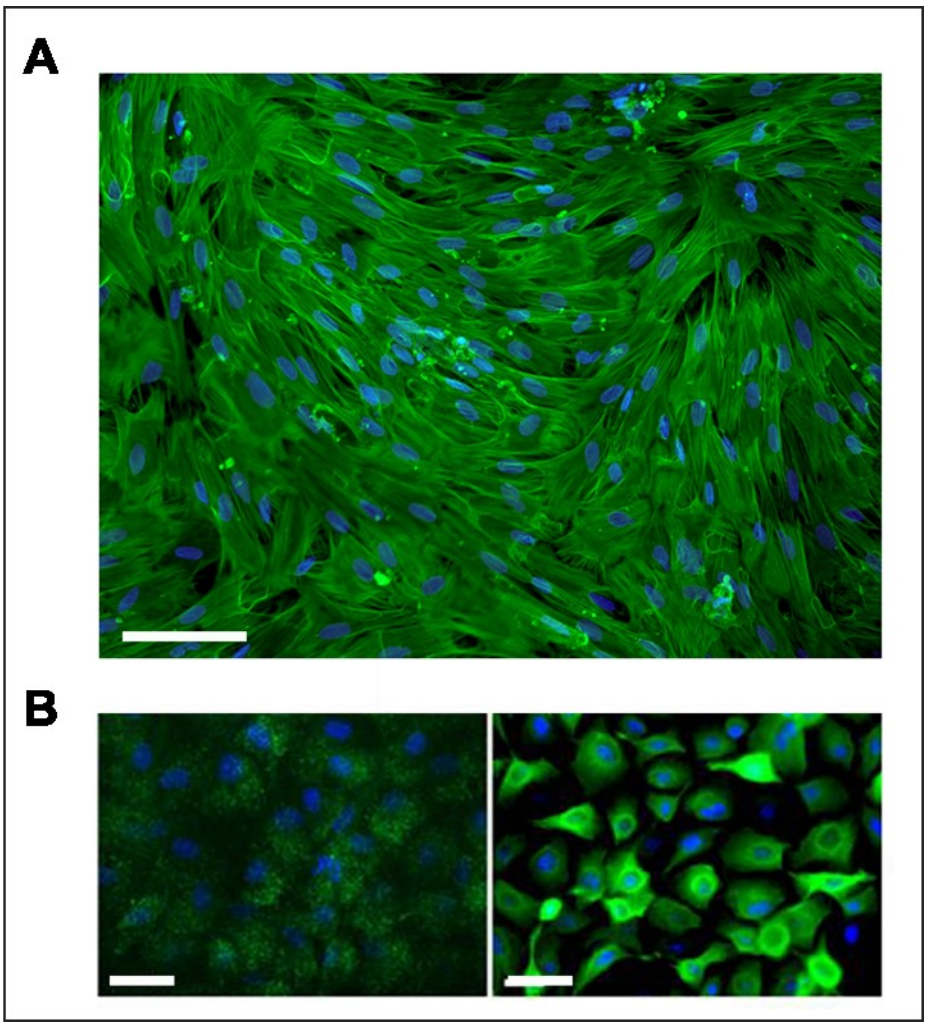


FIGURE 2. CHARACTERIZATION OF BLADDER CELLS.

Representative immunofluorescence figures of primary bladder (A) SMC and (B) urothelial cell cultures characterized using α-actin for smooth muscle cells (bar = 100 µm) and AE1/AE3 (lower left) and cytokeratin 17 (lower right) for urothelial cells (bar = 50 µm). Green: antibody; Blue: DAPI.

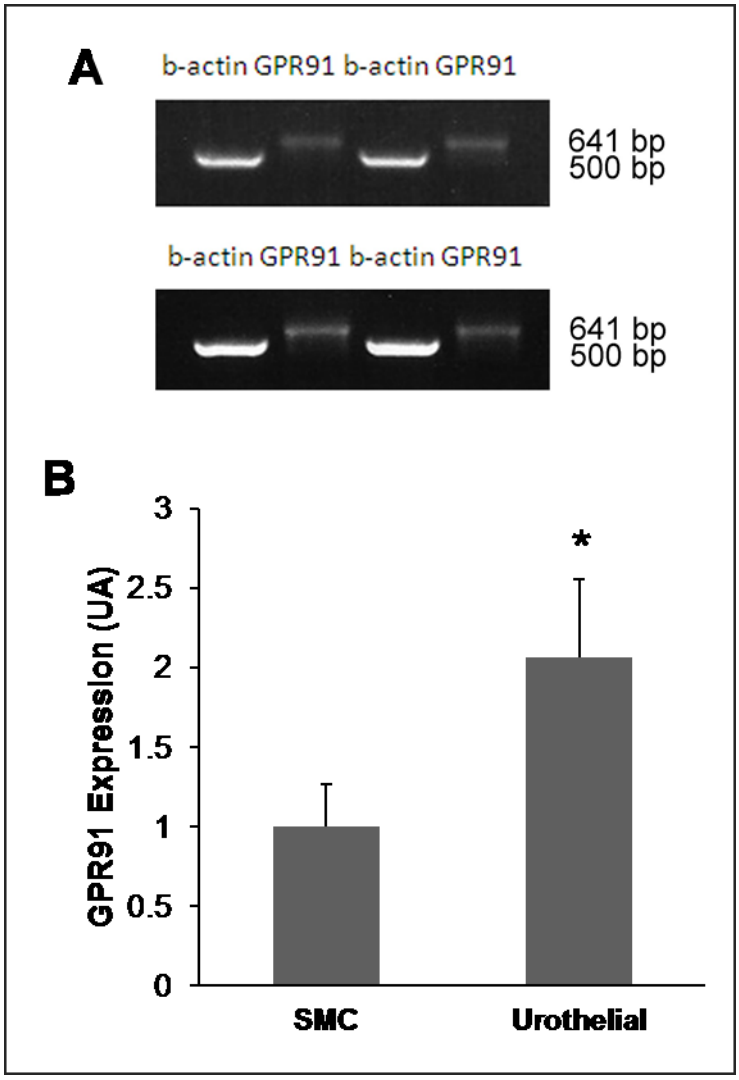


FIGURE 3. GPR91 EXPRESSION IN BLADDER CELLS.

RT-PCR of GPR91 in urothelial and smooth muscle cells. (A) RT-PCR of GPR91 on urothelial (upper gel) and smooth muscle (lower gel) cell extract; β–actin is used as size control. (B) Quantitative PCR comparing GPR91 expression between urothelial and SMCs; (n=6). $p < 0.05$; unpaired *t*-test.

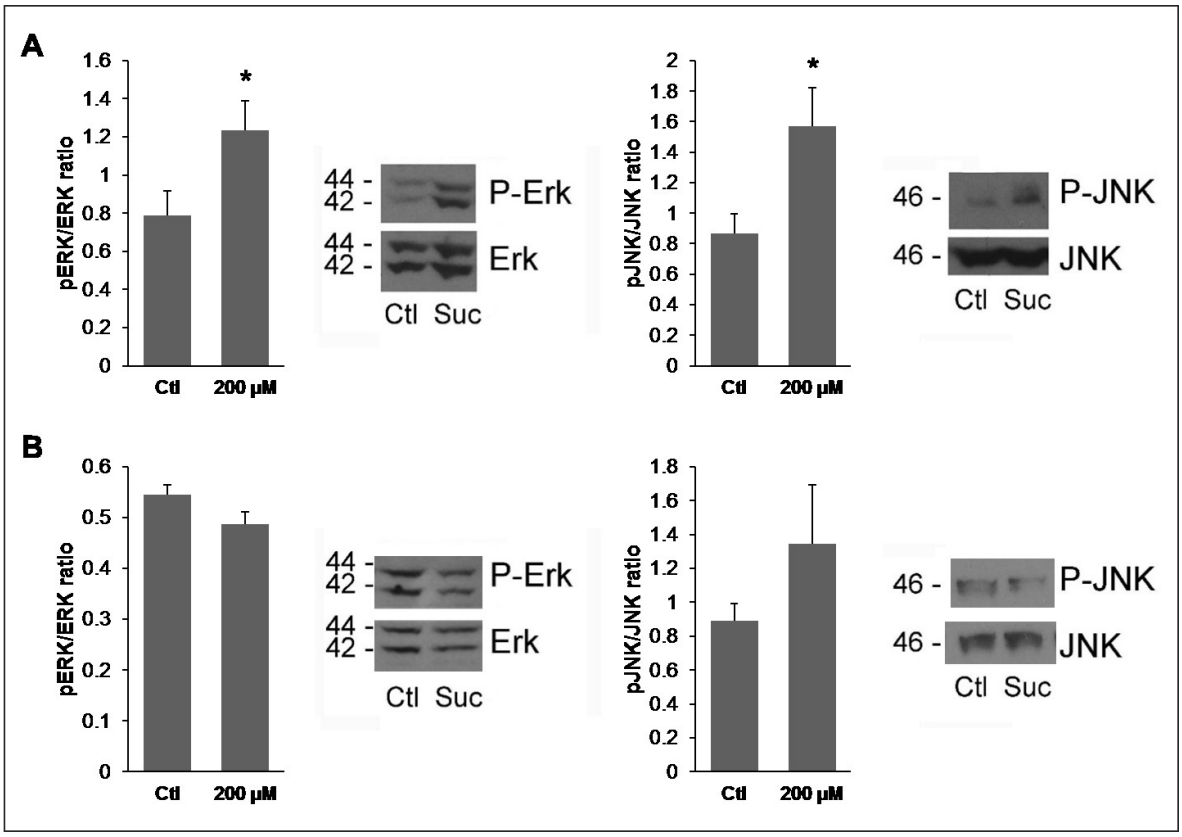


FIGURE 4. SUCCINATE ACTIVATION OF MAPK IN BLADDER CELLS.

Western blot of ERK 1/2 and JNK 1/2 in response to 2-minute incubation with 200 µM succinate in (A) urothelial and (B) SMCs. Graphs represent densitometry ratios of the phosphorylated protein over the total protein; (n=6), $p < 0.05$; unpaired *t*-test.

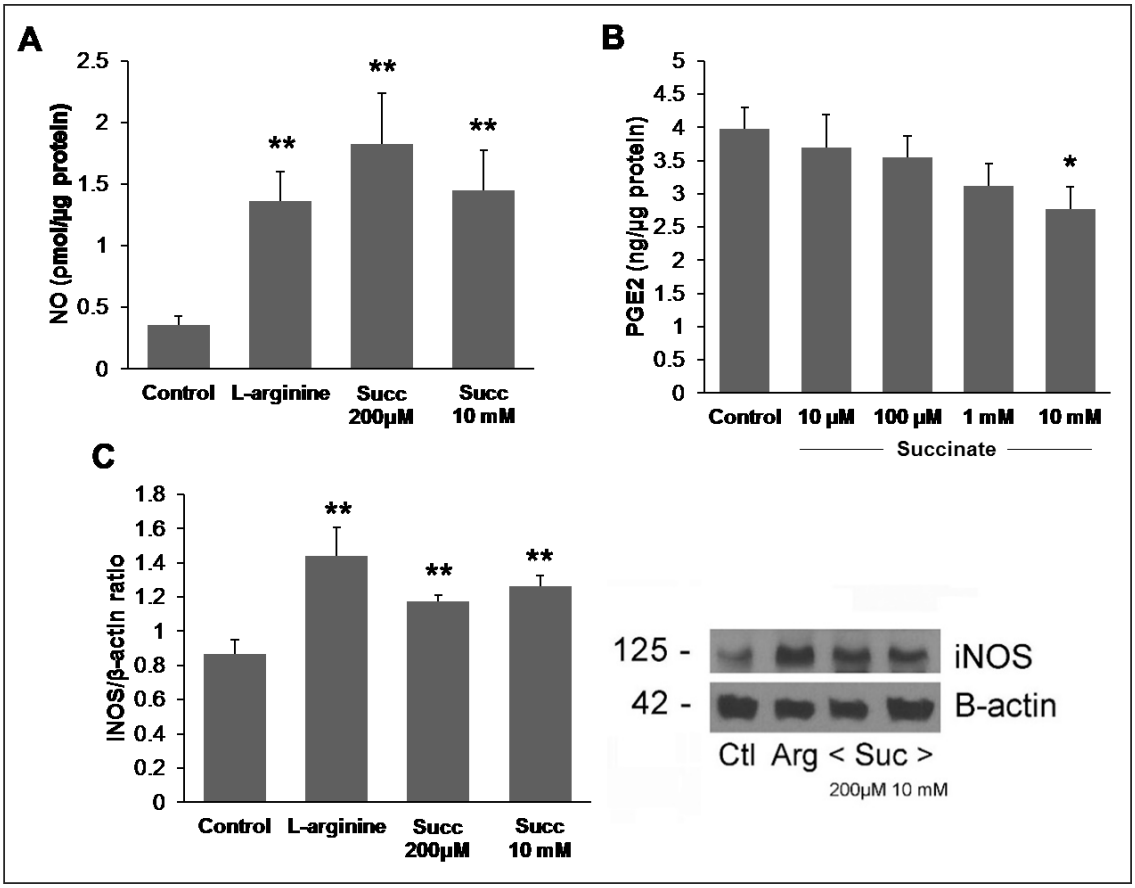


FIGURE 5. SUCCINATE REGULATION OF iNOS, NO, AND PGE_2.

Incubation of urothelial cells with different succinate concentrations for 24-hours. The secretion of (A) nitric oxide, (B) PGE2 and (C) iNOS expression were measured; (n=6). L-arginine (100 μM) is used as a positive control to induce NO production. Expression of iNOS is relative to β-actin. One-way ANOVA *P<0.05; **P<0.01 compared with control.

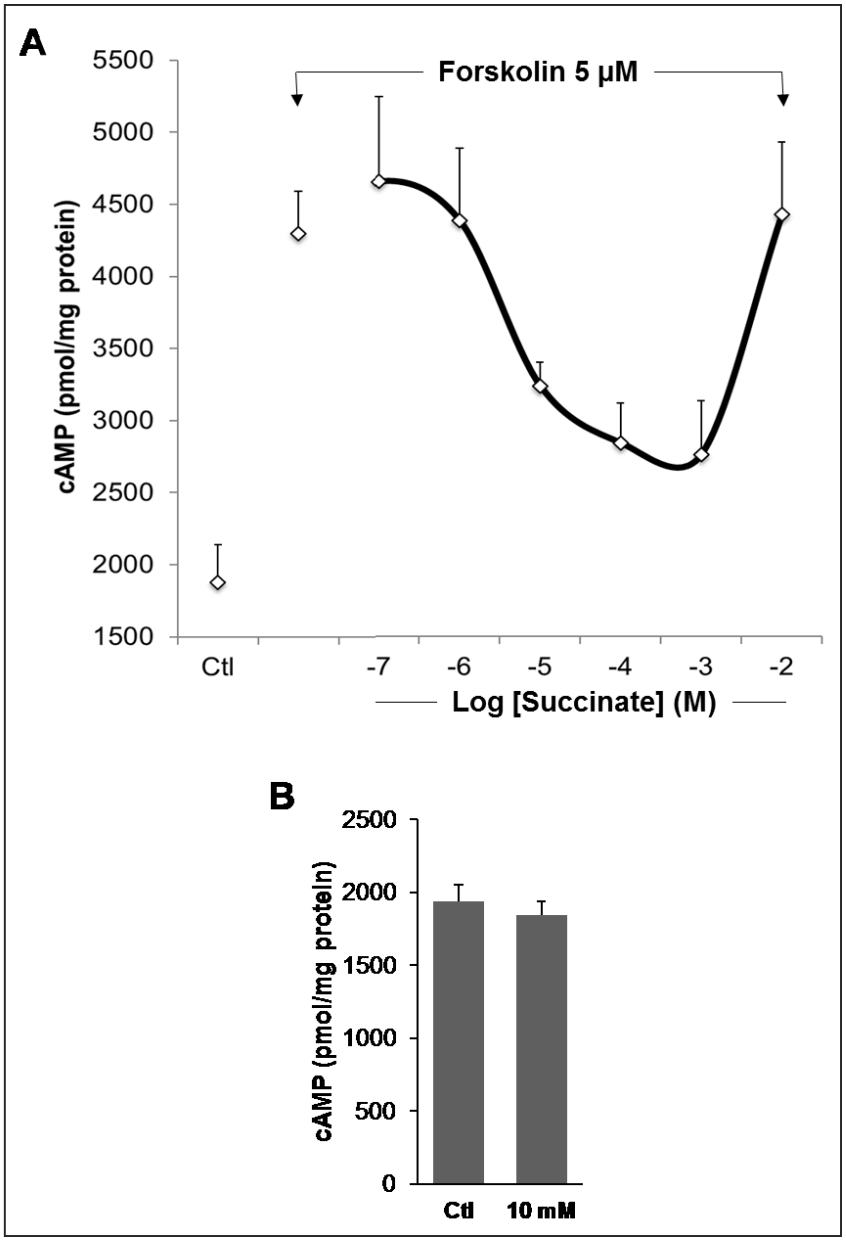


FIGURE 6. INHIBITION OF CAMP PRODUCTION BY SUCCINATE.

(A) Measurement of cAMP production in urothelial cells stimulated with forskolin 5µM and preincubated with succinate (10^{-7} to 10^{-2} M). (B) Stimulation of cAMP production in urothelial cells treated with 5µM forskolin (control) or 10 mM succinate; (n=6-7).

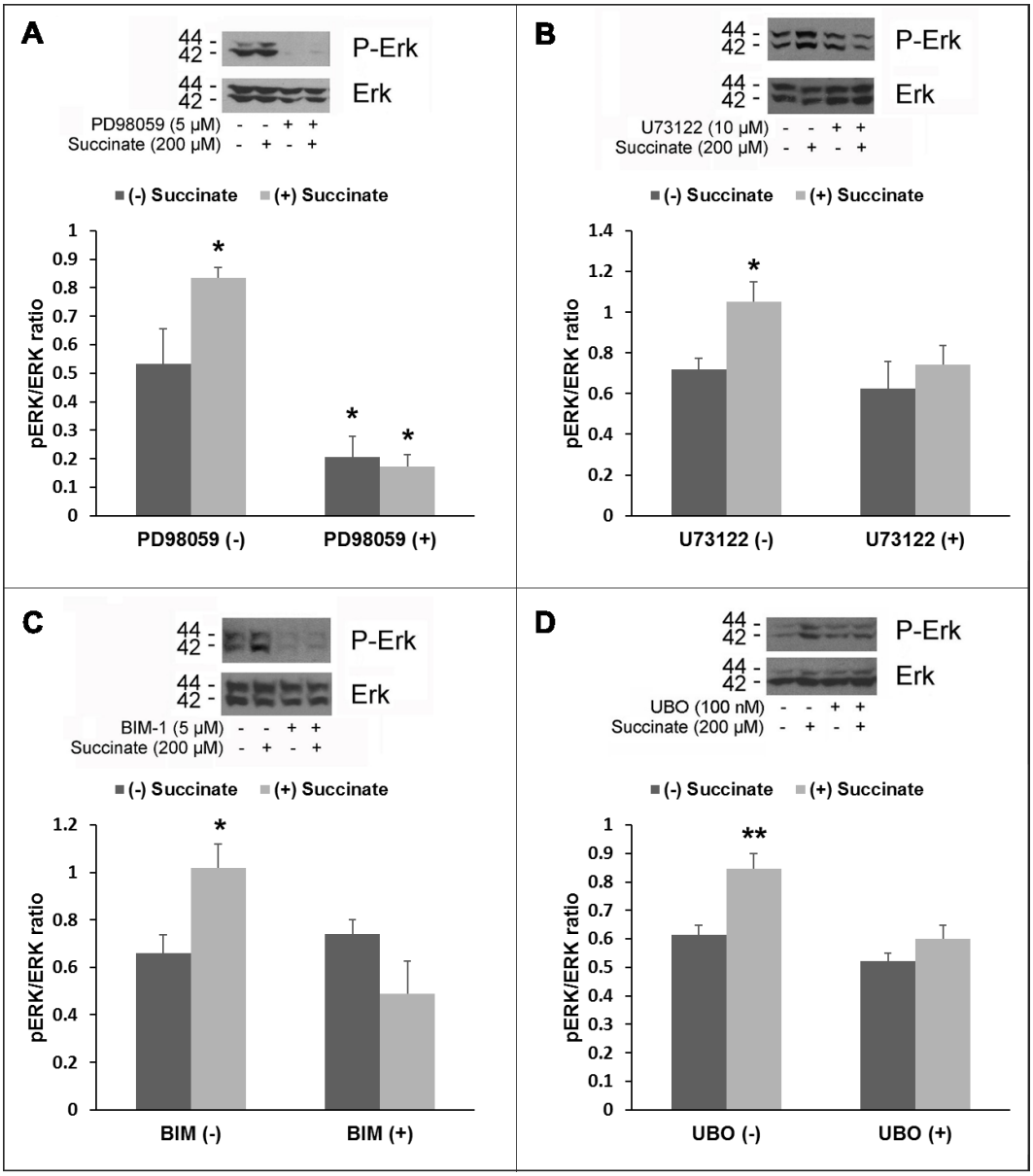


FIGURE 7. ERK ACTIVATION BY SUCCINATE IS GQ-DEPENDENT.

200 μM succinate stimulation urothelial cells with following incubation of urothelial cells with inhibitors of $G\alpha_q$ pathway. ERK phosphorylation was measured in the presence of (A) PD98059 (5 μM) MAPK inhibitor, (B) U73122 (10 μM) PLC inhibitor, (C) BIM-1 (5 μM) PKC inhibitor and (D) UBO (100 nM) $G\alpha_q$ inhibitor; (n=6). One-way ANOVA *P<0.05; **P<0.01 compared with control without succinate and inhibitor.

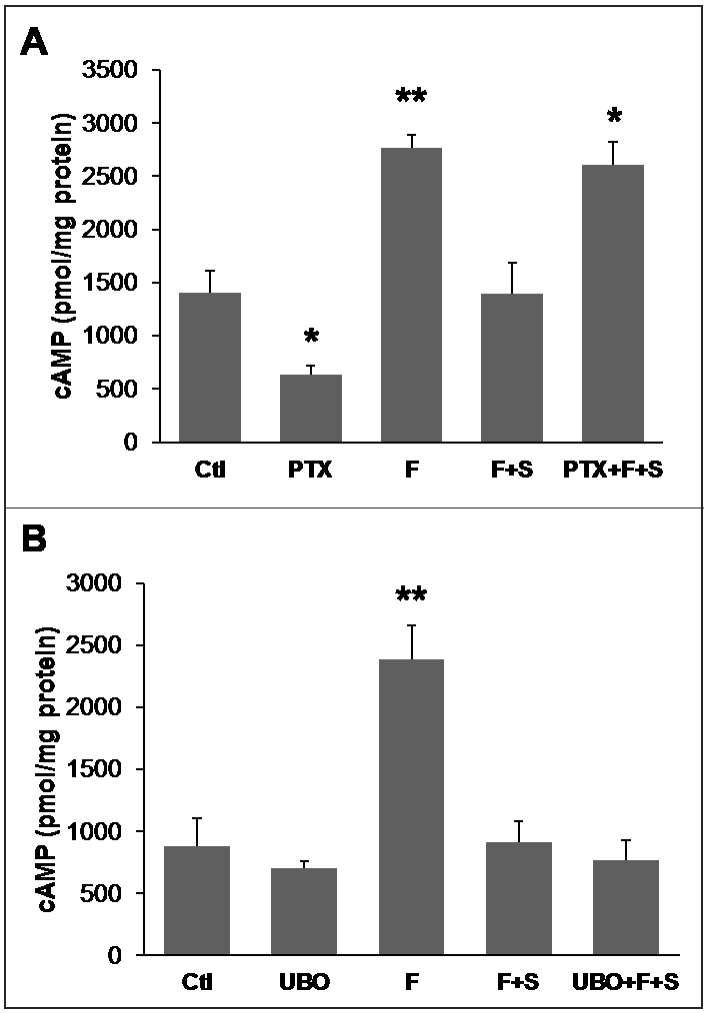


FIGURE 8. INHIBITION OF CAMP IS GI-DEPENDENT.

Production of cAMP in the presence of (A) PTX (100 ng/mL) inhibitor of $G\alpha_i$, or (B) UBO (100 nM) inhibitor of $G\alpha_q$; F: forskolin 5µM; F+S: forskolin following pre-incubation with succinate 200 µM; or pre-incubation with inhibitor followed by succinate and forskolin; (n=5-6). One-way ANOVA *P<0.05; **P<0.01 compared with control without succinate and inhibitor.

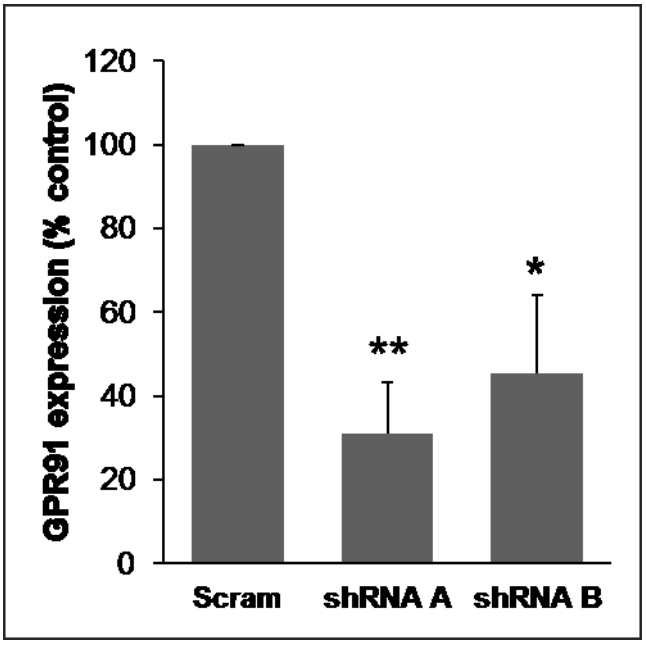


FIGURE 9. GPR91 KNOCKDOWN IN UROTHELIAL CELLS.

GPR91 expression in urothelial cells infected with viruses containing Scram, shRNA A or shRNA B constructs as measured by qPCR. (n=6). One-way ANOVA *P<0.05; **P<0.01 compared with Scram.

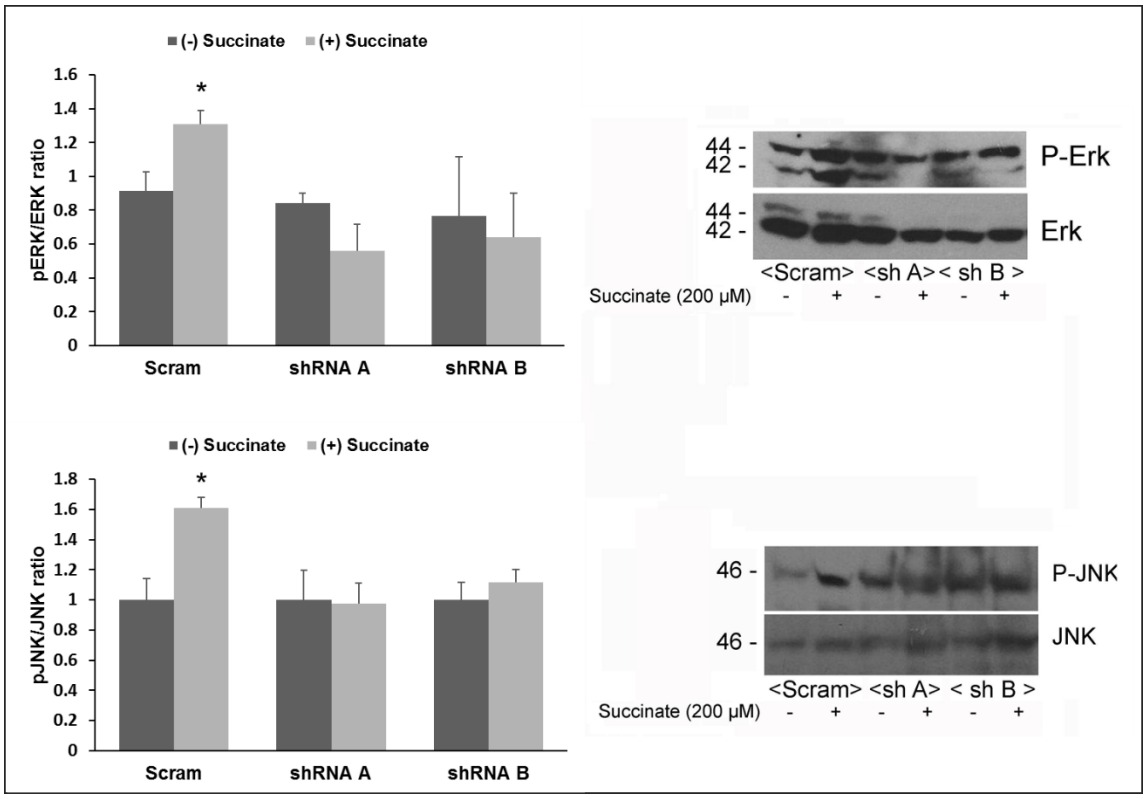


FIGURE 10. GPR91 MEDIATES MAPK ACTIVATION BY SUCCINATE.

ERK 1/2 and JNK 1/2 phosphorylation measured in the presence 200 µM succinate in urothelial cells infected with viruses containing Scram, shRNA A or shRNA B constructs. Graphs represent densitometry ratios of the phosphorylated protein over the total protein; (n=5). $p < 0.05$; unpaired *t*-test comparing cells in media with succinate *vs.* control media.

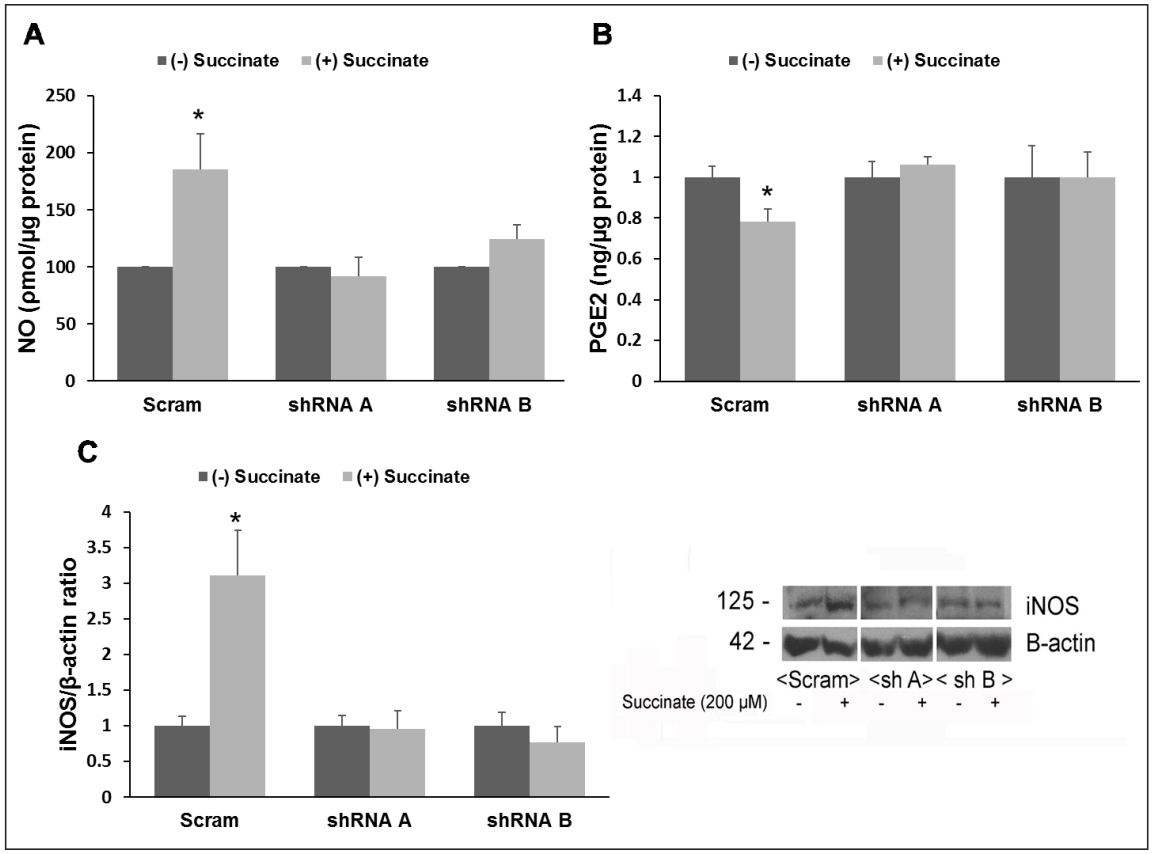


FIGURE 11. GPR91 MEDIATES SUCCINATE REGULATION OF iNOS, NO AND PGE_2.

Incubation of urothelial cells infected with viruses containing Scram, shRNA A or shRNA B constructs. The secretion of (A) nitric oxide in the presence of 200 µM, (B) PGE2 in the presence of 10 mM and (C) iNOS expression in the presence of 200 µM were measured Expression of iNOS is relative to β-actin; (n=4). p <0.05; unpaired t-test comparing cells in media with succinate *vs.* control media.

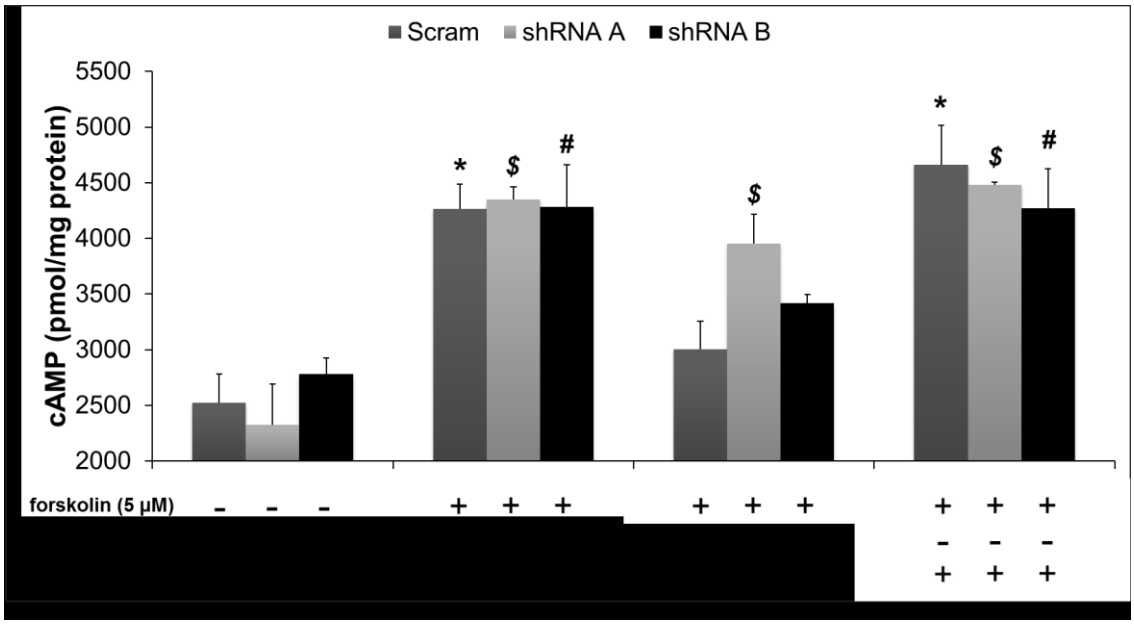
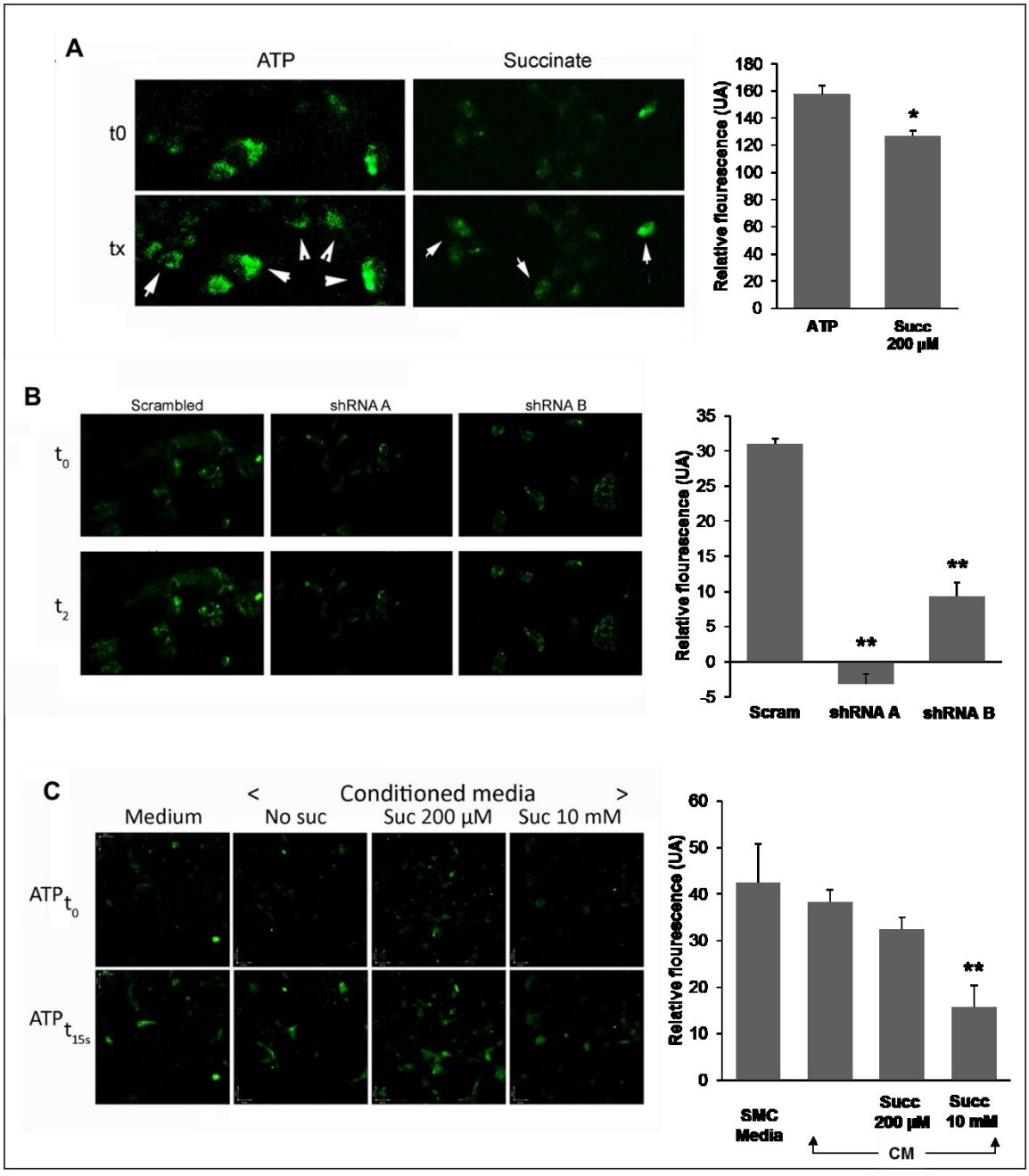


FIGURE 12. GPR91 MEDIATES CAMP INHIBITION BY SUCCINATE.

Production of cAMP in urothelial cells infected with viruses containing Scram, shRNA A or shRNA B constructs. Measurement of cAMP in the presence of forskolin 5 µM alone, or forskolin following pre-incubation with succinate (200 µM and 10 mM); (n=6). One-way ANOVA, $^{*}P<0.01$ compared to Scram control, $^{\$}P<0.01$ compared to shRNA A control, $^{\#}P<0.01$ compared to shRNA B control.


A
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Succinate
t0
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Relative flourescence (UA)
180
160
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FIGURE 13. GPR91 MEDIATES CYTOSOLIC Ca^{2+} INCREASE IN UROTHELIAL CELLS.

Intracellular Ca^{2+} in urothelial and SMCs measured by confocal microscopy on urothelial cells. (A) Stimulation of urothelial cells with 100 μM ATP or 200 μM succinate; n=7. p <0.05, unpaired *t*-test comparing succinate to ATP response. Arrows represent an increase signal. (B) 200 μM succinate stimulation of urothelial cells infected with viruses containing Scram, shRNA A or shRNA B constructs; (n=7). ***P*<0.01, one-way ANOVA compared with Scram. (C) Stimulation of SMC with 100 μM ATP following pre-incubation with SMC media or conditioned media from urothelial cells only or media from urothelial cells treated with succinate (200 μM or 10 mM) for 24 hours. ***P*<0.01, one-way ANOVA compared with cells in SMC media.

Chapter 5: Succinate worsens bladder overactivity in a rat model associated with metabolic syndrome

In this chapter, the chronic effects of succinate *in vivo* were investigated in two rat strains. The control strain is the Sprague-Dawley (SD) rat. The other strain is the Dahl Salt-Sensitive (Dahl/SS) rat, a model of hypertension associated with metabolic syndrome.

Contribution of authors:

Along with Drs. Abubakr Mossa, Philippe Cammisotto and Lysanne Campeau, I contributed to the conception of the study and acquisition of the results presented here. The interpretation of the results in this chapter are entirely my own.

5.1 Introduction

In the previous chapter, we confirmed the presence of the GPR91 receptor in cells of the bladder. Furthermore, we saw that through this receptor, succinate has the capacity to modulate the production of second messengers by urothelial cells. The findings from this study, combined with those that link succinate to metabolic syndrome and metabolic syndrome to OAB [16, 81], led us to investigate the effects of succinate in an *in vivo* setting.

The Dahl salt-sensitive (Dahl/SS) rat, developed by L. K. Dahl in 1962 through selective inbreeding [82], is a frequently used model in hypertension studies. Insulin resistance and hyperlipidemia have also been observed in this model [83, 84], making it a suitable model in which to study metabolic syndrome-associated disorders.

Here, we look beyond the hypertensive profile of Dahl/SS rats and consider the presence of bladder overactivity. In parallel, the role of succinate, implicated in metabolic syndrome, is be investigated in the development of bladder overactivity, with the intent of clarifying the link between these conditions. In this chapter, we want to demonstrate that the Dahl/SS rats display signs of bladder overactivity, when compared to healthy Sprague-Dawley (SD) rats. In addition, we want to show that chronic exposure to succinate not only worsens these signs, but it also triggers them in healthy rats.

5.2 Materials and Methods

Ethical Approval

Housing and handling of the animals conformed with the Canadian Council for Animal Care (CCAC). All protocols were approved by the Animal Ethics Committee of McGill University (Montreal, QC, Canada).

Animals

18 Male Sprague-Dawley (SD) rats were purchased from Charles River (Montreal, QC, Canada). 16 Male Dahl/SS rats (referred to as Dahl rats in this study) were bred in our animal care facility under the supervision of experienced animal care technicians. Both groups of animals had an average weight of 350 g at the beginning of the study. The rats had free access to food (standard Purina chow) and water and were kept on a 12-hour light and dark cycle. The animals were weighed weekly. Guidelines for the reduction of animals used were approved by the Animal Ethics Committee of McGill University.

Intraperitoneal injections were performed daily (morning) for 4 weeks. 10 SD rats and 8 Dahl rats received a control saline injection. The other 8 SD rats and 8 Dahl rats were treated with a succinate dose of 50 mg kg^{-1}, a concentration established in a different study [85].

Blood and Urine Analysis

At the 4–week point, the rats were fasted for 6 hours. Blood was collected from the lateral tail vein. During blood collection, fasting glucose levels were measured using the Contour next EZ blood glucose meter (Bayer, Mishawaka, IN, USA). The collected blood was centrifuged at a speed of 5000 r.p.m. for 3 minutes. Plasma samples were kept at –80 °C until ready to use. Insulin was measured using an Elisa kit (Millipore, Etobicoke, ON, Canada). Succinate levels were measured using a Megazyme enzymatic kit (Wicklow, Ireland) where plasma samples from fasted rats and 24-hour urine samples were thawed on ice, treated with perchloric acid and processed according to the manufacturer's protocol.

Blood Pressure Measurements

Tail-cuff blood pressure (BP) measurements were performed 4–6 hours after succinate injections using the MC4000 Multi Channel Blood Pressure Analysis System (Hatteras Instruments, Cary, NC, USA). Measurements were taken throughout the 4–week period to have the rats become familiar with the procedure and reduce the influence of stress on the measurements. Only measurements performed on the last week were taken into consideration for this study.

Bladder Catheter Implantation

Rats were anaesthetized under isoflurane (2%). Any pain resulting from the surgery was minimized by a single subcutaneous injection of buprenorphine slow release (1.2 mg kg^{-1}). An incision at the abdomen was made to expose the bladder. A small puncture was made in the dome of the bladder in which a polyethylene PE50, .58/.99 mm catheter (Stoelting, Wood Dale, IL, USA) was inserted. The catheter was secured to the bladder and then tunneled subcutaneously to the back of the neck and exposed through an incision. The exposed end of the catheter was sealed thermally.

Conscious Cystometry

Bladder function was assessed 3 days after catheter implantation using conscious cystometry as previously described [86]. The rats were placed in metabolic cages. The catheter was cut to unseal it and connected to a pressure transducer (Grass Technologies, West Warwick, RI, USA) and an infusion pump. A urine collector was placed below each cage and connected to a force displacement transducer. This allowed for both the intravesical pressures and micturition volumes to be recorded simultaneously. Saline was instilled into the bladder at a rate of 10 ml h^{-1} (for 1 hour). The data was analyzed using the LabScribe2 Data Recording and Analysis Software (iWorx, Dover, NH, USA).

The following parameters were analyzed: maximal pressure (highest pressure during micturition cycle), threshold pressure (pressure at initiation of voiding contraction), basal pressure (lowest pressure between voids), intermicturition pressure (average calculated pressure between voids), spontaneous activity (intermicturition pressure – basal

pressure), bladder capacity (instillation rate × average time between two voiding contractions), micturition volume (volume voided per micturition), residual volume (bladder capacity – micturition volume), and bladder compliance (bladder capacity × (threshold pressure – basal pressure)$^{-1}$).

The catheters were sealed again and the animals were placed back in their cages with food and water. The next day, the animals were sacrificed by cardiac blood withdrawal under isoflurane (3%) anesthesia, as recommended by the Animal Ethics Committee of McGill University. Their bladders were collected and kept in an ice-cold Krebs–Ringer solution containing: NaCl (119 mM), KCl (4.6 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1.2 mM), NaH_2PO_4 (1.2 mM), $NaHCO_3$ (15 mM), glucose (5 mM), pH 7.4. After weighing the bladders, the dome and trigone were removed and only the middle portion was kept. Half of the remaining sample was used for organ baths experiments and the other half kept for immunofluorescence and qPCR analysis.

Contractility studies

Each bladder was cut longitudinally to make detrusor strips that were then mounted on the 4–channel Tissue Bath System – 720MO (DMT-USA Inc., Ann Arbor, MI, USA) in 6 mL Krebs–Ringer solution at 37 °C. They were kept at 1 g tension for stabilization at the beginning, and each step was preceded by washing and resting period. The strips were then stimulated with a 60 mM solution of potassium chloride (KCl). Stimulation with 60 mM KCl was repeated to confirm consistency. The average of these two measurements was used when calculating the mean response of strips to KCl. Strips were

stimulated with increasing concentrations of carbachol (3 nM – 100 mM). Finally, strips were subjected to electrical stimulation (1, 2, 4, 8, 16 and 32 Hz) using the Grass Technologies S88 Stimulator (Grass Technologies, West Warwick, RI, USA). The strips were weighed before discarding them. All obtained values were normalized to their respective strip weights.

All organ bath data were analyzed using LabChart 7 (ADInstruments Inc., CO, USA). Carbachol responses were entered into GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) to calculate the individual E_{max} values for all detrusor strips.

Immunofluorescence

Tissues were fixed in paraformaldehyde 4% in PBS then processed for paraffin embedding. After rehydration, tissues sections were boiled in citrate buffer and washed in PBS. Tissue sections were covered with BSA 1% in PBS for 30 minutes. The sections were then incubated with previously validated primary antibodies against the pan-neural marker PGP9.5 (1:200, Cedarlane, Oakwood, ON, Canada, CL7756AP) and the cholinergic neuron marker VAChT (1:1000, Millipore, Millipore, Billerica, MA, USA, ABN100). The following morning, secondary antibody for PGP9.5 (1:250, Millipore, Millipore, Billerica, MA, USA, AP307P) and VAChT (1:250, ThermoFisher Scientific, Waltham, MA, USA, 31620) in BSA 1% was added, followed by mounting in DAPI. They were then examined under fluorescent microscopy. Images were taken by Widefield Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany). Bladder sections were taken from three different rats for each group. For quantification

purposes, random areas of mucosa and detrusor were selected avoiding blood vessels and red blood cells due to non-specific antibody binding. Quantification of fluorescence was performed by ImageJ 1.47v following a previously-used protocol [87]. The values obtained represent florescence density per area unit of the selected region of the slide, assuming that all slides were captured with uniform magnification power (400X) and illumination settings.

RNA Extraction and Quantitative PCR

Bladder RNA was isolated using TRIGent reagent/chloroform extraction (Biomatik Corporation, Cambridge, ON, Canada). RNA was dissolved in DEPC-treated water. Purity and concentrations was assessed using a Nanodrop spectrophotometer ND–1000 (ThermoFisher Scientific, Wilmington, DE, USA). Primer sequences used were obtained from IDT (Integrated DNA Technology, Canada): rat β–actin (forward: 5'–CAC CCG CGA GTA CAA CCT TC–3'; reverse 5'–CCC ATA CCC ACC ATC ACA CC–3') and GPR91 (forward: 5'–ACA GCT GTC GCC CTT TTC TA–3'; reverse 5'–TCA TGC CAA CCT CTA CAC CA–3'). Reverse transcription was carried out on 5 μg of RNA using TranScript All-in-One First-Strand cDNA Synthesis Supermix for qPCR (One-step gDNA Removal) (TransGen Biotech, Montreal, Canada). The qPCR used the TransStart Tip SYBR Green qPCR Super Mixture (TransGen Biotech, Montreal, QC, Canada). Samples were prepared in 96 fast-PCR plates (Sarstedt, Montreal, QC, Canada) and inserted in an Applied Bioscience 7500 Fast Real-time PCR System. Signal specificity was confirmed by dissociation curve. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [88].

Statistical Analysis

All values are expressed as mean ± SEM. Statistical analyses were performed using the IBM SPSS Statistics 23. Two-way ANOVA analysis was carried out to account for both the TREATMENT (control *vs*. succinate) and STRAIN (SD *vs.* Dahl) effects on our findings. Post hoc unpaired *t*-tests were performed to observe the effect of treatment alone within each strain. $P < 0.05$ was considered significant.

5.3 Results

Physiological differences between SD and Dahl rats with and without succinate treatment

Basic physiological measurements are summarized in Figure 14. At the end of the 4-week treatment with either saline or succinate, the rats were weighed. The bladders were weighed before organ baths. A significant effect of STRAIN was observed on body weight ($F = 19.49$, $P < 0.001$) and bladder weight ($F = 6.27$, $P = 0.018$), but not on bladder-to-body weight ratio, with the Dahl rats having lower body and bladder weights than SD rats.

Tail-cuff BP measurements showed a significant STRAIN effect on systolic BP ($F = 31.57$, $P < 0.001$), which was found to be higher in Dahl rats compared to SD rats. A significant TREATMENT effect was also present in regards to systolic BP ($F = 5.95$, $P = 0.035$), where the treatment was significantly more obvious on the SD rats (t-test: $P = 0.0391$) compared to the Dahl rats (t-test: $P = 0.475$).

Neither a significant STRAIN nor a significant TREATMENT effect on fasting plasma insulin levels were found. However, a significant STRAIN effect on glucose levels was found ($F = 4.94$, $P = 0.034$), with Dahl rats having higher blood glucose levels than SD rats.

A significant STRAIN effect was found on reported fasting plasma succinate levels ($F = 26.02$, $P < 0.001$), with Dahl rats having lower succinate levels than SD rats. In the case of 24-hour urinary succinate levels, the TREATMENT effect was significant ($F = 24.94$, $P < 0.001$), with significantly higher urinary succinate levels in SD rats treated with

succinate *vs.* control (*t*-test: $P = 0.002$) and in Dahl rats treated with succinate *vs.* control (*t*-test: $P = 0.005$).

Cystometry

Cystometry (described in Table 1) provided a more detailed description of the micturition patterns of our animals. No significant STRAIN×TREATMENT interaction, nor individual effects of STRAIN or TREATMENT were found on maximal pressure, threshold pressure, basal pressure, intermicturition pressure and spontaneous activity. The STRAIN×TREATMENT interaction on bladder capacity was significant ($F = 4.58$, $P = 0.045$), with Dahl rats having smaller bladder capacities than SD rats and with succinate treatment decreasing the bladder capacity in both strains. The micturition volume was significantly lower in Dahl rats compared to SD rats ($F = 19.87$, $P < 0.001$). Residual volume was not significantly different between strains and was not significantly affected by treatment. Finally, the STRAIN effect was close on bladder compliance to significance ($F = 4.31$, $P = 0.054$) with Dahl rats having lower bladder compliance than SD rats.

Organ Baths

The response of detrusor bladder strips to KCl, carbachol and EFS are described in Figure 15. The response to 60 mM KCl stimulation was significantly affected by the STRAIN×TREATMENT interaction ($F = 8.73$, $P = 0.004$), with the detrusor of Dahl rats having a higher contractile response than those of SD rats and with the succinate treatment significantly decreasing these contractile responses. Furthermore, the

alterations of contractility elicited by succinate treatment were more effective in Dahl rats (*t*-test: $P = 0.022$) than in SD rats (*t*-test: $P = 0.367$).

The carbachol dose-response curves (3 nM – 100 μM) are summarized in Fig. 15B. The average maximal response to carbachol within each group was also significantly affected by the STRAIN×TREATMENT interaction ($F = 6.18$, $P = 0.015$), with a similar pattern as for the response to KCl (Fig. 15C). The succinate treatment resulted in significantly lower contractile responses in detrusor strips of Dahl rats treated with succinate *vs.* control (*t*-test: *P*: 0.020), as opposed to no significant difference between detrusor strips of SD rats treated with succinate *vs.* control (*t*-test: $P = 0.742$).

Response to lower frequencies of EFS (1 Hz and 2 Hz) were not significantly different between STRAIN, nor was the effect of TREATMENT significant at these frequencies (Fig. 15D-E). At 4 Hz, however, a significant TREATMENT effect starts to appear ($F = 3.99$, $P = 0.052$) where detrusor strips of rats treated with succinate have lower contractile responses compared to strips from control-treated rats (Fig. 15F). More specifically, the TREATMENT effect is close to significance in the Dahl rats (*t*-test: $P = 0.055$), but not in the SD rats (*t*-test: $P = 0.935$). The STRAIN×TREATMENT interactions become significant as the frequencies increase to 8 Hz ($F = 4.23$, $P = 0.046$), 16 Hz ($F = 5.61$, $P = 0.023$), and 32 Hz ($F = 7.89$, $P = 0.008$) (Fig. 15G-I). Detrusor strips from Dahl rats have significantly higher response to EFS than those of SD rats and the succinate treatment decreases this response significantly in Dahl rats at 8 Hz (*t*-test: $P = 0.022$), 16 Hz (*t*-test: $P = 0.015$) and 32 Hz (*t*-test: $P = 0.009$).

Expression of PGP9.5, VAChT, and GPR91

The expression of the neuronal marker PGP9.5 in bladder samples was significantly affected by TREATMENT in both the mucosa ($F = 6.52$, $P = 0.017$) (Fig. 16A) and detrusor ($F = 5.52$, $P = 0.028$) (Fig. 16B), with succinate treatment increasing PGP9.5 expression of SD rats in the mucosa (*t*-test: $P = 0.037$) and detrusor (*t*-test: $P = 0.042$) of SD rats.

Two-way ANOVA reported a significant STRAIN effect on the expression of VAChT in both the mucosa ($F = 8.82$, $P = 0.009$) (Fig. 16C) and detrusor ($F = 40.65$, $P < 0.001$) (Fig. 16D), with an overall higher expression in the Dahl rats. Succinate had an opposite effect on VACht expression in SD and Dahl rats. Bladders from succinate-treated SD rats had significantly higher expression of VAChT compared to control-treated rats (*t*-test: $P = 0.039$). Bladders from succinate-treated Dahl rats had significantly lower expression of the marker compared to control-treated rats (*t*-test: $P = 0.003$).

Finally, a significant STRAIN×TREATMENT interaction effect ($F = 1.90$, $P = 0.015$) was observed on the bladder mRNA expression of GPR91 (Fig. 17). The expression of the receptor was significantly lower in succinate-treated SD rats *vs.* control-treated SD rats (*t*-test: $P < 0.001$), but no significant changes where observed between succinate- and control-treated Dahl rats (*t*-test: $P = 0.671$).

5.4 Discussion

In this chapter, we demonstrated that in addition to a hypertensive profile, the Dahl rat displays signs of bladder overactivity when compared to SD rats. Furthermore, chronic succinate administration leads to the development of these signs in SD rats and worsens the bladder overactivity in the Dahl rat.

The Dahl rats had significantly higher blood pressure recordings than the SD rats. They also displayed significantly higher fasting glucose levels, with normal fasting insulinemia.

Cystometry provided a detailed analysis of bladder function and micturition patterns of both strains. The similar bladder-to-body weight ratios allowed valid comparisons in this setting. No difference between bladder pressures, residual volume and bladder compliance were found between strains, although this was not surprising since these parameters are more relevant in other bladder dysfunction such as bladder outlet obstruction [89, 90]. On the other hand, Dahl rats had significantly lower bladder capacities and micturition volume than SD rats, suggesting bladder overactivity in Dahl rats.

Previous bladder overactivity studies have used hypertensive rat models [91, 92], but here, for the first time, we consider bladder function in the Dahl rat. The observed differences in bladder capacity may be related to increased cholinergic activity, as suggested by the higher VAChT expression in both the mucosa and detrusor of Dahl rats compared to SD rats. Decreased bladder capacity may be a result of increased parasympathetic excitation which stimulates detrusor tone [93]. These findings are in

agreement with results on spontaneously hypertensive rats, which suggest that hyperinnervation plays a role in the bladder overactivity observed in these rats [94].

Bladder differences between SD and Dahl rats were emphasized *in vitro*. Detrusor strips of Dahl rats showed a higher contractile response to all stimuli when compared to strips from SD rats. The differences between both strains may involve the contractile signaling pathway of the smooth muscle. For instance, renovascular hypertensive rats have increased expression of bladder M3 mRNA compared to their normotensive controls, and as a result their detrusor strips have higher contractile responses to carbachol and EFS [92]. This is also supported by the higher expression of VAChT in Dahl bladders, which indicates increased cholinergic nerve activity in the bladder leading to detrusor contraction [93].

We therefore demonstrated that the Dahl rat had a bladder overactivity profile in addition to their well-known hypertensive pattern. These findings strengthen the links between hypertension and bladder overactivity and contribute a new model to study this possible causative interaction. The abnormalities in glucose metabolism, such as the increased glucose levels observed in this study and insulin resistance and hyperlipidemia reported by others [83, 84], further extends the value of the Dahl rat as a model for metabolic syndrome studies.

The lower plasma succinate concentrations observed in Dahl rats compared to SD rats is of interest as other models of metabolic syndrome have reported an increased succinate production [27]. Our finding provides an opportunity to study the isolated effects of succinate administration in a metabolic syndrome model with bladder overactivity, on voiding function and detrusor contractility. The lower plasma succinate concentrations

observed in Dahl rats may be caused by a disrupted metabolism. There may be increased flux of succinate through the citric acid cycle and/or electron transport chain [95]. In addition, the concentrations of circulating succinate in both SD and Dahl strains were unaffected by succinate treatment. This observation may be due to the time between succinate injection and blood collection, where an increase in circulating succinate might have been observed with a shorter time difference. Nevertheless, succinate treatment was shown to have a significant increase in systolic blood pressure, more predominant in SD rats, consistent with the literature [30]. The administrated succinate may therefore have as vasoactive role and, as reported by others, may accumulate in tissues [53], explaining their unaltered levels in the circulation.

The 24-hour urinary succinate was increased in succinate-treated rats, providing further explanation for the unchanged circulating succinate levels in these animals. Therefore, the administered succinate has more contact with the bladder to exert its effects. Cystometry parameters from both strains showed a trend towards bladder dysfunction in succinate-treated rats. For instance, bladder capacity was lower in Dahl succinate-treated rats *vs.* saline-treated rats, and even lower in SD succinate-treated rats *vs.* saline-treated rats. This observation is similar to the stronger effects of succinate treatment on blood pressure of SD rats compared to Dahl rats. These results are supported by the increase in peripheral nerve density, as suggested by higher levels of PGP9.5 in both bladder mucosa and detrusor of succinate-treated rats compared to saline-treated rats, with this observation being more pronounced in SD rats.

The chronic exposure to succinate in the bladder of succinate-treated rats led us to investigate changes in expression of the GPR91 succinate receptor in this organ.

Quantitative PCR results showed similar mRNA expression between SD and Dahl rats treated with saline. Receptor downregulation as a form of desensitization in response to agonist is well-described for GPCRs [96] and also occurs in succinate-GPR91 signaling [97]. This explains the decreased GPR91 mRNA observed in SD succinate-treated rats compared to saline-treated rats. Yet, administration of the same treatment to Dahl rats did not yield similar results, possibly because of the lower plasma succinate levels present in Dahl rats.

The downregulated GPR91 receptor observed in SD rats, but not in Dahl rats, could also explain why succinate treatment in only Dahl rats caused significant changes in organ bath findings. The high contractile response of detrusor strips from Dahl rats treated with saline was not present in those of Dahl rats treated with succinate. On the other hand, detrusor strips from SD rats with control and succinate treatment had comparable contractile responses, suggesting that the impact of succinate treatment was prevented by the down-regulated GPR91 expression in the succinate-treated SD rat. These findings confirm an effect of succinate on the bladder that may be mediated by the GPR91 receptor. The succinate-GPR91 signaling in the bladder may cause a decrease in cholinergic activity, as shown by lower detrusor VAChT expression in Dahl succinate-treated rats, therefore dampening the response of detrusor strips to contractile stimuli.

While we only used one dosage of succinate (50 mg/kg/day), variable dose response effect could be observed. For instance, an increase in the dose and/or duration might make the cystometry differences between saline and succinate-treated rats even more significant. We based our selected concentration on a study that investigated three

different concentrations of succinate in preliminary experiments before settling on the most effective dose [85].

As was previously described, due to the inability to assess urgency in an animal, OAB research in animals relies on other aspects of the syndrome [14]. However, these animal studies have not been able to isolate the factors involved in the development of OAB. While this study is also limited by the lack of an ideal animal model and depends on on other parameters to study bladder overactivity in the rat, it does succeed in putting forward one factor, succinate, as a potential culprit in the development of OAB.

Metabolic syndrome encompasses different metabolic abnormalities, each displaying an array of molecular characteristics that may impact the development of bladder dysfunction. As such, the Dahl rat is a model of hypertension associated with dysfunctional glucose metabolism, but they also exhibit signs of bladder overactivity, worsened in the presence of succinate.

5.5 Figures and Tables

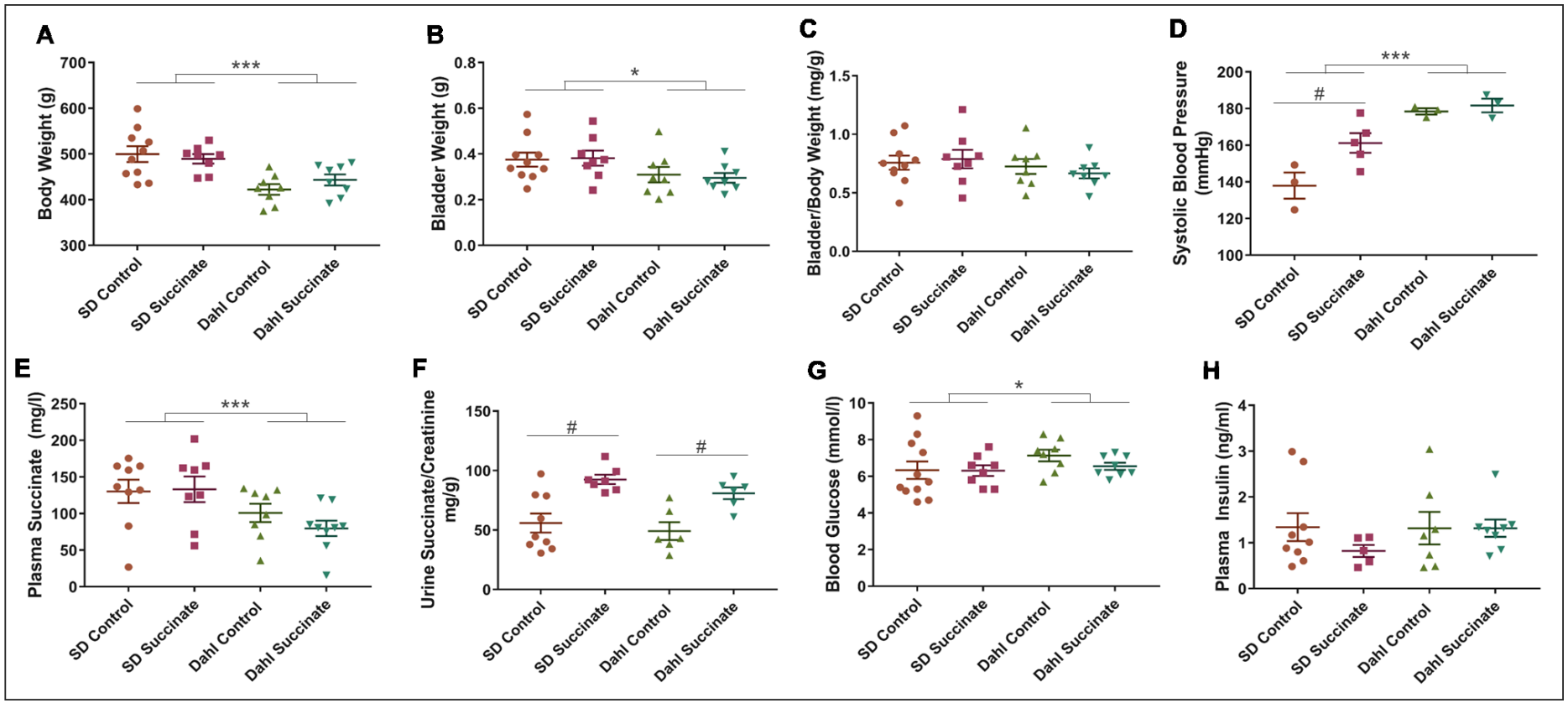

FIGURE 14. BASIC PHYSIOLOGICAL PARAMETERS.

Physiological parameters of SD and Dahl rats after 4 weeks of saline or succinate treatment. (A) Body weight; (B) bladder weight; (C) bladder-to-body weight ratio; (D) systolic BP; (E) plasma succinate; (F) urine succinate normalized to creatinine; (G) fasting blood glucose; (H) fasting plasma insulin. Middle line represents mean with SEM. Results of two-way ANOVA (*P<0.05; ***P<0.001) and unpaired t-tests comparing succinate to control treatment within each group (#P<0.05) are summarized.

TABLE 1. SUMMARY OF CONSCIOUS CYSTOMETRY FOR SD AND DAHL RATS PERFORMED AT 4 WEEKS OF CONTROL OR SUCCINATE TREATMENT.

Parameter	Species	Control	Succinate
Maximal Pressure (cmH_2O)	SD	170 ± 32	137 ± 24
	Dahl	115 ± 39	205 ± 41
Threshold Pressure (cmH_2O)	SD	50 ± 16	49 ± 8
	Dahl	58 ± 24	62 ±19
Basal Pressure (cmH_2O)	SD	17 ± 4	14 ± 3
	Dahl	26 ± 14	14 ± 2
Intermicturition Pressure (cmH_2O)	SD	29 ± 7	25 ± 13
	Dahl	32 ± 14	22 ± 3
Spontaneous Activity (cmH_2O)	SD	12 ± 3	11 ± 3
	Dahl	6 ± 2	8 ± 2
Bladder Capacity (ml)*	SD	1.64 ± 0.21	1.26 ± 0.16
	Dahl***	0.90 ± 0.23	0.81 ± 0.11
Micturition Volume (ml)	SD	1.73 ± 0.27	1.34 ± 0.22
	Dahl***	0.86 ± 0.18	0.84 ± 0.11
Residual Volume (ml)	SD	0.07 ± 0.04	0.05 ± 0.03
	Dahl	0.08 ± 0.05	0.04 ± 0.03
Bladder Compliance ($ml\ cmH_2O^{-1}$)	SD	0.07 ± 0.01	0.05 ± 0.01
	Dahl	0.04 ± 0.01	0.02 ± 0.01

Values are expressed as mean ± SEM. Results of two-way ANOVA are summarized next to the parameter to represent a significant effect of the succinate treatment or next to the Dahl strain to represent a significant effect of the strain for that parameter (*P<0.05; ***P<0.001).

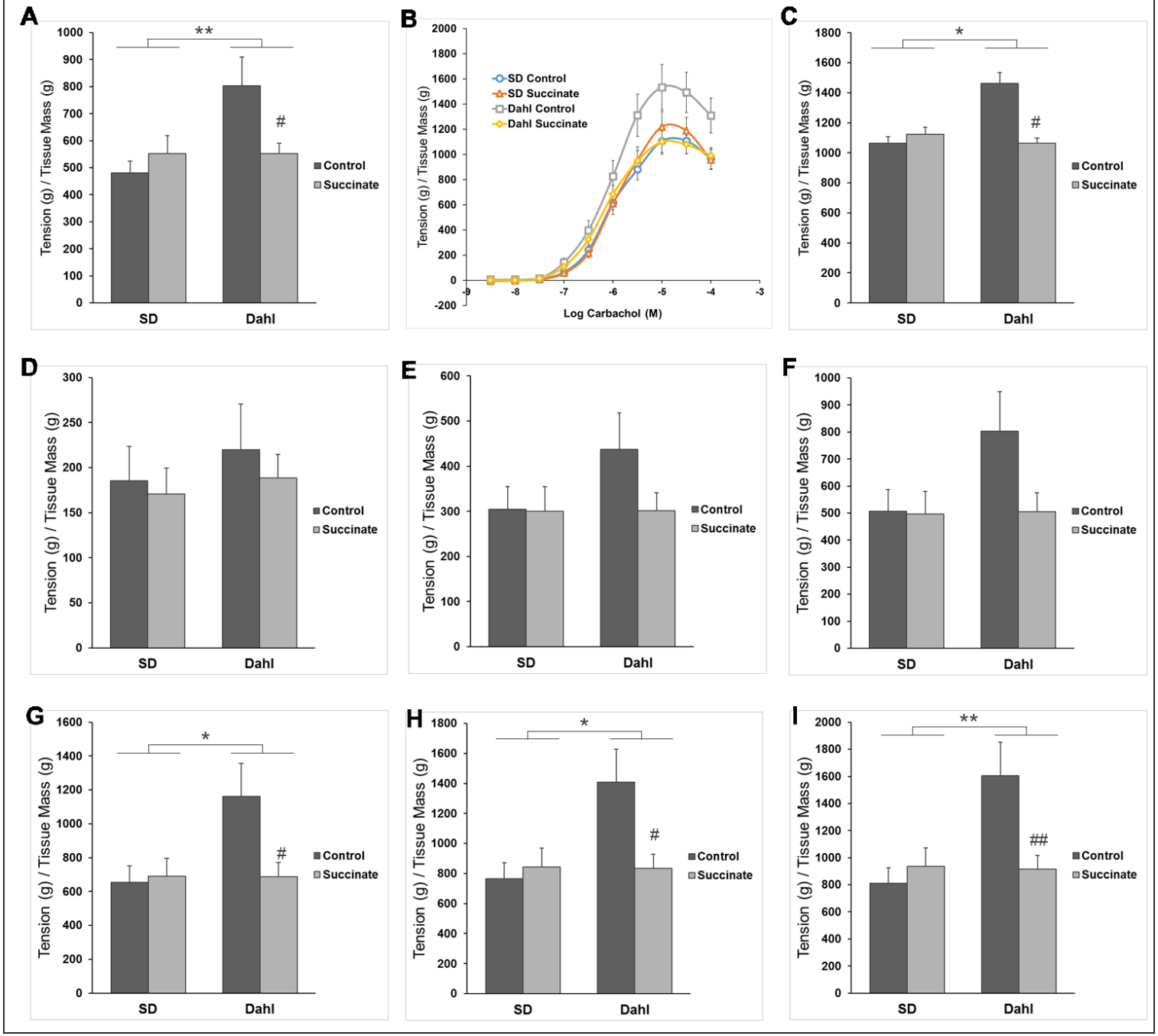
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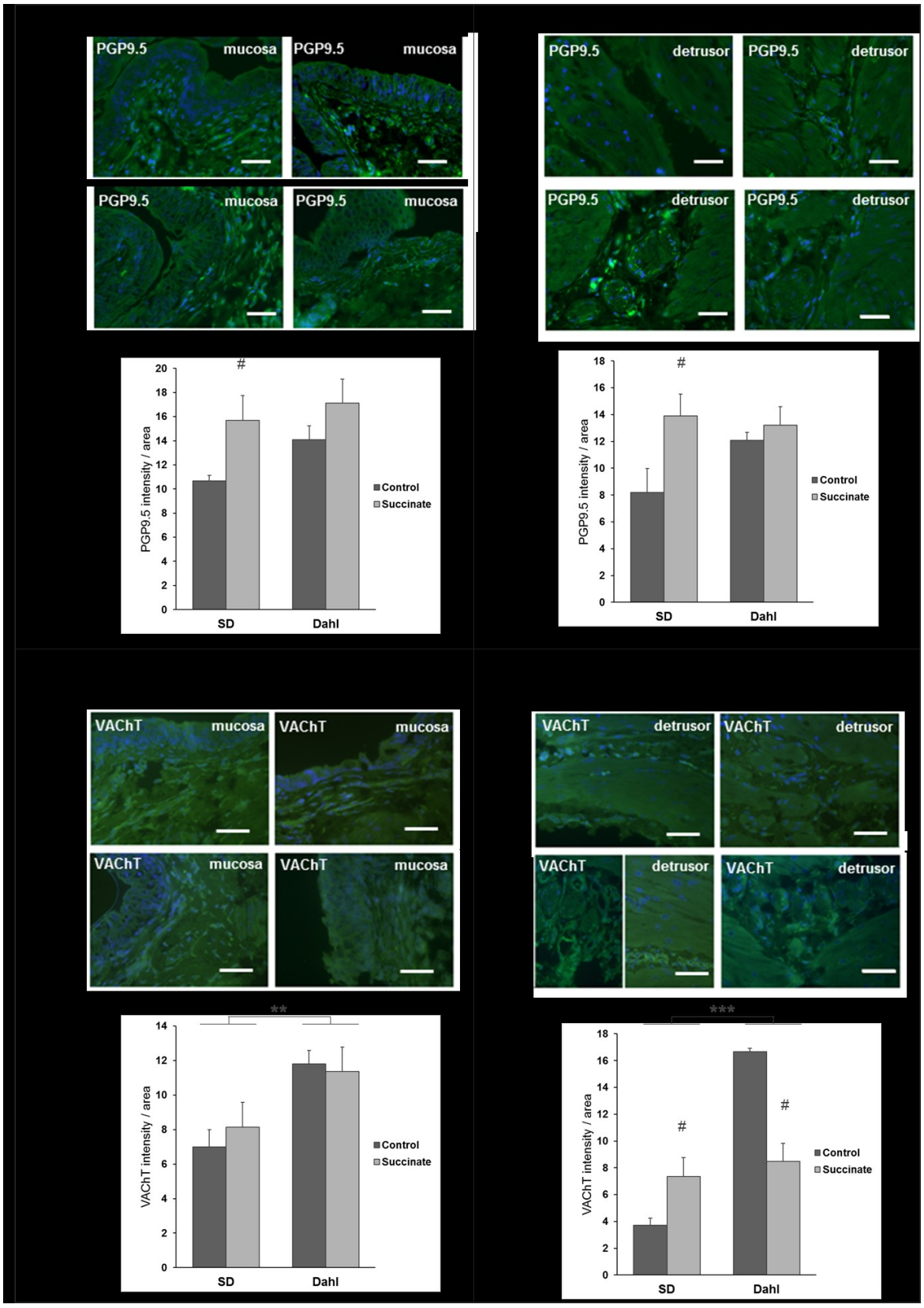
FIGURE 15. RESPONSE OF SD AND DAHL BLADDER DETRUSOR STRIPS TO CONTRACTILE STIMULI.

(A) Response to 60 mM KCl stimulation. Response to carbachol stimulation: (B) dose-response to carbachol 3 nM – 100 μM; (C) carbachol E_{max}. Response to electrical field stimulation (D) 1 Hz, (E) 2 Hz, (F) 4 Hz, (G) 8 Hz, (H) 16 Hz and (I) 32 Hz. The response is represented as tension in grams normalized to the detrusor strip weight in grams. Results of two-way ANOVA (*P<0.05; **P<0.01) and unpaired *t*-tests comparing succinate to control treatment within each group (#P<0.05; ##P<0.01) are summarized. $^{\#\#}P$<0.01).


PGP9.5
mucosa
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Succinate
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VAChT intensity / area
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Control
Succinate
SD
Dahl

FIGURE 16. BLADDER EXPRESSION OF PGP9.5 AND VACHT.

(A-D) Representative immunofluorescence images for bladder samples of each strain with control or succinate treatment with mean expression on the right. PGP9.5 expression in the (A) mucosa and (B) detrusor. VAChT expression in the (C) mucosa and (D) detrusor. Green: antibody; Blue: DAPI. 400X magnification; scale bar representative of 50 μm. Results of two-way ANOVA (***$P<0.001$) and unpaired t-tests comparing succinate to control treatment within each group (#$P<0.05$) are summarized.

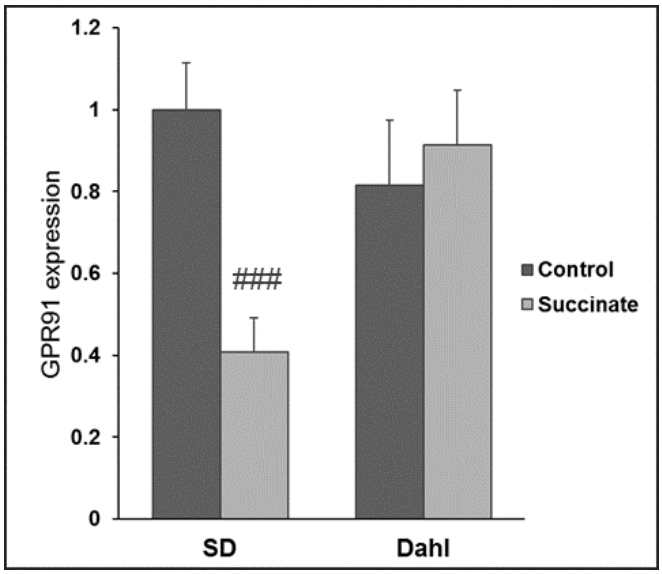

FIGURE 17. GPR91 EXPRESSION IN BLADDERS OF SD AND DAHL RATS.

Quantitative PCR of GPR91 expression in whole bladder samples relative to SD rats with control treatment. Results from unpaired t-tests comparing succinate to control treatment within each group are summarized (##P<0.001).

Chapter 6: Conclusions

The overall purpose of this master's project was to advance the understanding of OAB development. This condition, mainly considered idiopathic, does not have solid biological markers leaving much room for improvement in areas such as therapeutic interventions, preventative and diagnostic tools. Conscious of the associations of OAB with the metabolic syndrome, we put forward a promising topic – succinate and its receptor, GPR91. While this pair has been studied in other tissues affected by metabolic syndrome, their role in bladder function or dysfunction has not yet been considered until now.

The first half of this work showed that succinate, through the GPR91 receptor, prompted responses in bladder cells, specifically urothelial cells. These studies encouraged us to test the effects of succinate *in vivo*, where we confirmed that succinate, and possibly GPR91, led to detrimental effects on the bladder.

As it has been observed by other groups studying the effects of succinate in other tissues, we demonstrated that the activation of GPR91 by succinate through the $G\alpha_q$ and $G\alpha_i$ that led to changes in the release of second messengers by urothelial cells. This confirmed our first hypothesis and, in addition, hinted potential indirect effects of succinate on the smooth muscle through these second messengers.

An important part of our animal work confirmed the links made between bladder dysfunction and metabolic syndrome made in previous studies (Figure 1). Here, we showed that the Dahl rat, a model of hypertension with a disrupted glucose metabolism, showed signs of bladder overactivity when compared to the healthy SD rat. No

differences in plasma nor urinary succinate levels between the two strains were observed. However, the intraperitoneal injections of succinate resulted in increased urinary succinate levels triggering bladder overactivity in SD rats and worsening this pathology in Dahl rats, thus confirming our second hypothesis.

While it is difficult to connect the acute setting of our cell work with the chronic setting of the animal work, our studies provide enough reason to assume a pathological role for increased succinate levels on the bladder.

Combining the conclusion from this work with the knowledge of the increased succinate levels in metabolic syndrome and the links between this syndrome and OAB, we can predict the importance of succinate in this area of urology. Future studies should focus on determining if higher plasma and/or urinary succinate levels exist in OAB patients compared to patients with normal bladder function. The role of the succinate receptor in the development of OAB should also be clarified and may involve the use of GPR91 knockout mice. With this research, succinate may become useful as marker of the condition and GPR91 inhibitors may serve as new therapeutic options for OAB patients.

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