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Sex-dependent expression of brain medullary MAP and PI3 kinases in adult sheep with antenatal betamethasone exposure

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Abstract

Antenatal betamethasone (BM) therapy for women in jeopardy of premature delivery accelerates the lung development in preterm infants and reduces infant mortality rates, but may induce early programming events with chronic cardiovascular consequences. In a sheep model of fetal programming, *in utero* BM-exposed (BMX) offspring as adults exhibit elevated mean arterial pressure (MAP), decreased baroreflex sensitivity (BRS) for the control of heart rate and insulin resistance accompanied by dysregulation of the brain renin–angiotensin (Ang) system (RAS). However, the status of signaling mechanisms in the brain dorsomedial medulla (DMM) of the BMX sheep that comprise both the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) cellular pathways is unknown. Given the importance of these signaling pathways in the actions of Ang peptides as well as baroreflex function and autonomic integration, we applied both a kinase signaling array and associated individual immunoblots of the dorsomedial brain medulla from adult female and male sheep with antenatal BMX. MAPK and PI3K pathways were altered significantly in the BMX sheep in a sex-dependent manner. A protein array for kinases (PathScan® Intracellular Signaling Array Kit, Cell Signaling) and subsequent verification by immunoblot revealed that within the DMM, female BMX sheep exhibit lower expression of proteins in the PI3K pathway, while male BMX sheep show greater expression of p-MAPK pathway proteins extracellular signal regulated kinase (ERK) 1/2. We conclude that maladaptive changes in these two kinase pathways in the DMM may contribute to the chronic dysregulation of blood pressure in this model of fetal programming.

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Author contribution

D.I.D., M.C.C., H.A.S., and A.S.H. designed the study. A.S.H. performed all experiments and drafted the manuscript. A.S.H. and H.A.S. were involved in data collection. A.S.H. and B.M.W. analyzed the data with input from D.I.D., H.A.S., and M.C.C. All authors contributed to data interpretation and revision and approval of the final manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Introduction

Administration of glucocorticoids (GCs) is a widely accepted therapy for women in jeopardy of preterm labor that facilitates the development of the fetal pulmonary system and reduces infant mortality [1]. However, the consequences of antenatal GC exposure may include increased susceptibility to developing cardiovascular and metabolic diseases in adult life [2–9]. In an experimental model of fetal programming, baroreflex sensitivity (BRS) for control of heart rate is attenuated in sheep with betamethasone (BM) exposure (BMX) *in utero* in comparison with age-matched unexposed control sheep as early as 2 months of age, indicating that dysregulation of blood pressure clearly precedes the elevated blood pressure in adult BMX animals [10,11]. BMX sheep exhibit attenuated expression of the angiotensin (Ang)-converting enzyme (ACE) 2 (ACE2) and the Ang-(1–7)/Mas receptor, as well as lower Ang-(1–7) content in cerebral spinal fluid (CSF) and the dorsomedial medulla (DMM), which may reflect enhanced metabolism of the peptide by ACE and dipeptidyl peptidase 3 (DPP3), as well as the reduced conversion of Ang II into Ang-(1–7) by ACE2 [12–15].

Phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) are major intracellular signaling pathways in the dorsal medulla that contribute to the regulation of sympathetic outflow and baroreceptor regulation [16,17]. The ACE–Ang II–AT₁ receptor axis promotes ROS production, which influences a number of cellular signaling events that include increased MAPK activity, enhanced expression of pro-inflammatory cytokines, and greater sympathetic tone [17–19]. Conversely, the activation of the ACE2–Ang-(1–7)–AT₇/Mas receptor axis stimulates the PI3K signaling cascade [20,21]. PI3K activates nitric oxide synthase (NOS) and the generation of NO is a key modulator of blood pressure in the nucleus of the solitary tract (NTS) that results in a GABA-mediated tonic inhibitory effect leading to decrease in blood pressure, heart rate, and renal sympathetic nerve activity as shown in rodents [22]. Indeed, the beneficial cardiovascular effects of Ang-(1–7) in the NTS appear to be mediated by the PI3K-NOS signaling pathway [23]. Last, both PI3K and MAPK play key roles in the regulation of inflammatory cytokines and chronic inflammation that are now considered important contributors to cardiovascular disease [24–27]. The activation of MAPK may lead to transcription of pro-inflammatory cytokines, while the PI3K pathway may stimulate anti-inflammatory cytokines [27]. Therefore, we hypothesize that MAPK and PI3K pathways are differentially altered in adult sheep brain medulla by antenatal BMX *in utero*. To address this premise, we performed a protein kinase array on isolated dorsomedial medullary tissue from adult female and male BMX sheep, as well as unexposed control animals. The array data were subsequently analyzed by discrete correlate summation (DCS) to identify changes in the major signaling pathways for both female and male groups. The altered kinase pathways identified from the array were then verified by individual immunoblots utilizing antibodies based on the array.

Materials and methods

Animals

Pregnant ewes were administered two intramuscular doses of 0.17 mg/kg BM acetate or saline (Control), 24 h apart at the 80th and 81st days of gestation. Offspring were delivered

full term, farm-raised, and transferred to the Wake Forest School of Medicine Animal Facility at least 4 weeks prior to necropsy. Animals were maintained on a normal diet, with access to tap water *ad libitum* and maintained on a 12:12-h light/dark cycle. Estrous cycles of all female offspring were synchronized with the insertion of a vaginal progesterone implant (Eazi-Breed CIDR, Pfizer Animal Health). Frozen brain medullae were collected by dissection along the region of the obex isolating the DMM including the NTS, the dorsal aspect of the dorsal motor nucleus of the vagus and the medial part of the gracilis nucleus during necropsy from sheep averaging at 10.5 ± 0.22 months of age whereby blood pressure is already elevated [9,11]. Brain tissue from a total of 28 animals were used in the present study. All procedures were approved by the Wake Forest School of Medicine animal care and use committee.

Intracellular signaling array

Intracellular signaling molecules were detected using the PathScan® Intracellular Signaling Array Kit Chemiluminescent Readout #7323 (Cell Signaling Technology, U.S.A.). To investigate potential regulation of central signaling pathways by antenatal BM treatment, tissue homogenates of the DMM of adult male and female BMX and control sheep were analyzed by a PathScan® cellular signaling array that assessed 18 well-characterized phosphorylated or cleaved signaling molecules. Homogenates of sheep DMM were prepared according to manufacturer's instructions and diluted to 1.0 mg/ml of protein. The array glass slides were incubated for 15 min at room temperature with the array blocking buffer (100 µl) on an orbital shaker. Seventy-five microliters of each lysate sample was then aliquoted on to each well of the antibody array-slide in duplicate ($n=4$ per group, eight total samples per slide). Slides were then incubated at 4°C on an orbital shaker overnight followed by a wash step with the array wash buffer added (100 µl) to each well for 5 min at room temperature and then decanted; the wash procedure was repeated three additional times. The detection antibody cocktail (75 µl) was then added to each of the 16 wells per slide and incubated for 60 min at room temperature. Slides were then washed three times and incubated for 30 min with HRP-linked streptavidin. For chemiluminescent detection on film, each slide was washed and treated with prepared LumiGLO® and peroxide solution. Development of the signal was accomplished using chemiluminescent film and an automated film developer. The presented relative gray densities were calculated from the average pixel densities of each particular phospho- or cleaved signal normalized to the pixel densities of the positive control spots within the chip and quantitated by ImageJ software (NIH).

Immunoblot

Frozen DMM was homogenized in PBS with protease inhibitor cocktail. Cytosolic fractions of the tissue were added to Laemmli buffer containing β-mercaptoethanol. Proteins were separated on 12% Mini-PROTEANTGX gels (Bio-Rad, U.S.A.) for 68 min at 120 Volt in Tris-glycine buffer and electrophoretically transferred on to PVDF membranes using an electrophoretic transfer cell (Bio-Rad, U.S.A.). Immunodetection was performed on blots blocked for 1 h with 5% dry milk and tris-buffered saline containing 0.1% tween-20, and then probed with primary antibodies overnight at 4°C in 5% BSA buffer. The primary antibodies were all obtained from Cell Signaling Technology to correspond to the kinase array kit (anti-total protein kinase B (PKB) (Akt) (#4691), anti-total glycogen synthase

kinase-3 β (GSK-3 β) (#12456), anti-total extracellular signal regulated kinase 1/2 (ERK 1/2) (#9102), anti-total p38 (#8690), anti-p-Akt Ser⁴⁷³ (#4060), anti-p-GSK-3 β Ser⁹ (#5558), anti-p-ERK 1/2 Thr²⁰²/Tyr²⁰⁴ (#9101), and anti-p-p38 Thr¹⁸⁰/Tyr¹⁸² (#4511)). The blots were washed three times and then incubated with anti-rabbit secondary antibody at a 1:5000 dilution (GE Healthcare Biosciences, U.S.A.). Immunodetection was accomplished using a Western Bright™ ECL immunoblotting detection kit for chemiluminescent detection (Advansta, U.S.A.). The protein bands were visually detected by MCID Elite 7.0 imaging software (Imaging Research Inc., Canada) and quantitated by ImageJ software (NIH). The results are expressed as a ratio of phosphorylated protein to total expression in relative optical density units.

Statistics

Data are expressed as the mean $\pm$ S.E.M. Unpaired Student's *t* tests were used to determine significance between two groups for data derived from the immunoblots. The statistical analyses were performed with GraphPad Prism (GraphPad Software, U.S.A.). The criterion for statistical significance was set at $P < 0.05$. For assessment of the relationships between the 18 variables studied on the array, the entire dataset was analyzed by DCS analysis to rank the variables of the dataset with the largest correlation and mean shifts between BMX and control, as previously described [28]. In addition, the odds ratio was calculated to determine the influence of BMX between both sexes.

Results

Sex-dependent changes in MAPK and PI3K expression in BMX sheep as detected with PathScan® intracellular signaling array

Representative arrays for female and male control and BMX sheep are shown in Figure 1A,B. Relative protein expression from each array was normalized to the negative and positive control wells for quantitation and the mean data from the female and male groups are shown in Figure 1C,D, respectively ($n=4$ sheep per group). Generally, phosphorylated proteins in the PI3K pathway including phosphorylated Akt and GSK-3 β tended to be lower in the BMX females as compared with controls (Figure 1A,C), with a similar trend for proline-rich Akt substrate protein (PRAS40); the majority of the signaling proteins were unaffected by BMX. In BMX males, there were trends for greater phosphorylation of proteins in the MAPK cascade including ERK, p38, and stress-activated protein kinase/c-Jun-terminal kinase (SAPK/JNK) (Figure 1B,D). The odds ratio of 0.059, with a significant Fisher exact score of 0.035, indicated that the expression of the 18 proteins measured with the array between female and male subjects in opposite directions was significant.

DCS of protein kinase array data

DCS analysis of the quantitated data from the female and male groups generated with the kinase array is shown in Figure 2. The highest contributors to the variance in the data from BMX and control females included SAPK/JNK, Akt, PRAS40, GSK-3 β , and ribosomal protein S6 kinase (p70 S6 kinase) (Figure 2A). In the data from BMX and control males, DCS analysis revealed the major contributors to variance as MAPK/p38, signal transducer and activator of transcription 1 (STAT1), caspase-3, and GSK-3 β expression.

Validation of sex-dependent MAPK and PI3K changes by individual immunoblot analysis

Individual immunoblots for several components of the PI3K and MAPK pathways in the female and male sheep are shown in Figure 3 ($n=7-8$ sheep per group). BMX females exhibited significantly lower expression of phosphorylated GSK-3 β and Akt (Figure 3A), but no differences in either ERK 1/2 or p38 phosphorylation relative to control animals. In contrast, ERK 1/2 phosphorylation was significantly higher in the male BMX sheep with a trend for higher p-p38 ($P<0.1$) and we detected no differences in phosphorylated GSK-3 β or Akt (Figure 3B).

Discussion

Maternal treatment with the GC BM results in a consistent finding of the elevation of mean arterial pressure (MAP), as well as decreased BRS for the control of heart rate and insulin resistance in the exposed offspring of both adult female and male animals [9–11,13]. Changes in the renin–Ang system (RAS) in the DMM of adults of both sexes involve Ang II and Ang-(1–7) pathways that may contribute to the development of increased blood pressure in the BMX phenotype [13–15]. The present data reveal that BMX *in utero* influences the phosphorylation status, and thus, the activity of key signaling proteins within MAPK and PI3K signaling pathways in the DMM of adult sheep. There is abundant evidence for the importance of these pathways in the actions of Ang peptides and nitric oxide production, reactive oxygen species levels, inflammation, and insulin and leptin signaling [16,22,29]. Indeed, the patterns of change in these pathways are consistent with both the imbalance in the expression of Ang peptides in this brain area and the cardiovascular and metabolic dysfunction phenotype displayed by the BMX animals.

For the current study, we applied signaling arrays coupled to DCS analysis to determine the extent of alterations in the cellular expression of various signaling pathways in the DMM of BMX adult sheep. The DCS method identified distinct profiles for the two sexes in terms of the variables that account for the majority of the variance between BMX and control groups. The analysis revealed that components of the PI3K signaling pathway are the major contributors to the variance in BMX compared with control female sheep, whereas components of the MAPK signaling pathway contributed to the variance in male BMX compared with male control sheep. The potential for quantitative differences in the pathways identified in the array were then verified in larger numbers of animals by individual immunoblots. Both phosphorylated Akt and GSK-3 β were significantly lower in the BMX females as compared with controls, while the p-MAPK proteins ERK 1/2 and p38 also tended to be lower. In contrast, p-ERK 1/2 was significantly higher and p38 tended to be higher in the BMX males, while phosphorylated Akt and GSK-3 β were unchanged. Thus, in adult male sheep, BMX appears to have a greater impact to increase protein phosphorylation of signaling molecules within the DMM, as compared with adult females where BMX appears to contribute to a loss of phosphorylation and subsequent kinase activity. We note that the status of blood pressure and baroreflex was not assessed in this group of sheep, although our previous studies have consistently shown higher blood pressure and reduced BRS in both female and male BMX sheep [11,13]. Moreover, we cannot distinguish whether an increase in blood pressure precedes changes in these signaling pathways or that altered

MAPK (males) or PI3K (females) drives changes in pressure and BRS. Additional studies are required to both block these pathways and assess changes in pressure or assess these in younger BMX sheep prior to the increase in blood pressure or reduction in BRS.

The present findings show profound sex differences in the expression and activity of signaling molecules revealing that females and males exhibit different susceptibility or vulnerability of signaling pathways to the GC exposure *in utero* and/or the subsequent disruption of the RAS despite similar cardiovascular and metabolic outcomes [9]. The observation that two distinct signaling pathways within the DMM of males compared with females were affected by GC exposure raises important questions about the validity of conclusions drawn from one sex only relative to cellular mechanisms. Consideration of these sex differences is required for effective therapeutics and based upon cellular mechanisms. Moreover, our studies certainly support the abundant evidence of sex differences in hypertension and other cardiovascular diseases.

However, despite evidence of independent actions, multiple levels of cross-talk exist between the PI3K/Akt and MAPK signaling pathways. There are inhibitory actions of Akt on ERK activity [34,35] and p38 MAPK-mediated phosphorylation of GSK-3 β occurs in brain tissue [36]. Therefore, the complexity of the interactions between these pathways may also play a role in the sex-dependent pathway profiles resulting from BMX. The activation of the MAPK and PI3K cascades are also modulated temporally by phosphatases including the MAPK phosphatases (MKPs), phosphatase and tensin homolog (PTEN), and protein phosphatase 2A (PP2A) [37,38]. Further studies on the role of phosphatases, reactive oxygen species, and nitric oxide in the DMM, along with the PI3K and MAPK pathways, in the regulation of the baroreflex and blood pressure in this model are required to discern the potential contribution of these pathways.

The local brain RAS participates in regulation of the PI3K and MAPK pathways, both of which contribute to the appropriate regulation of cardiovascular, insulin, and leptin function [31,39,40]. Our studies focussed on alterations specifically localized to the DMM nuclei involved in baroreflex control of sympathetic and vagal activity, and they support the essential role of the RAS where its dysregulation may lead to changes in neuronal function associated with changes in cardiovascular control in BMX animals. Ang-(1–7) increases dual specificity phosphatase 1 (DUSP1, also known as MKP-1) and protein tyrosine phosphatase (PTP1B), known to influence the MAPK and PI3K pathways, respectively [32,41]. Evidence also suggests that Ang-(1–7) may have a greater cardiovascular role in females [10,42–44]. The long-term impact of reductions in the ACE2–Ang-(1–7)–Mas axis on intracellular signaling within brain, however, is not established. Moreover, the diverse localization of the RAS throughout the brain suggests roles in a variety of physiological functions including neuroendocrine and cardiovascular regulation, neural differentiation, cell apoptosis, and regeneration [30]. The imbalance between Ang II and Ang-(1–7) favoring high Ang II has been implicated in several models of hypertension and ageing, and many of its deleterious cardiovascular actions are thought to occur via increased generation of reactive oxygen species through MAPK activation [31]. Likewise, the PI3K pathway is involved in maintenance of BRS and blood pressure in addition to insulin and leptin

signaling [31–33]. However, studies into these effects that incorporate both male and female subjects are limited.

Substantial evidence suggests that events during fetal development have profound impact on health and development into adulthood [2–9]. Understanding the consequences of events that occur within this time period in a model of gestational development with similarity to the human setting is particularly important. Many animal models exist for the study of pregnancy outcomes, yet are incapable of truly replicating the maternal and fetal placental interactions. The sheep model of fetal programming is recognized as particularly relevant in investigating human pregnancy compared with rodents. This model of fetal programming resulting from maternal BMX during peak fetal nephrogenesis considers a specific environmental event that alters expression of the brain and renal RAS (shifting away from Ang-(1–7)) producing cardiometabolic dysfunction in the adult offspring [45]. Further studies are warranted to determine the impact of the RAS on the dysregulation of PI3K and MAPK protein expression, particularly the impact of altered signaling on downstream inflammatory or oxidative stress processes within this brain area to establish the contribution of these pathways to the cardiovascular and metabolic phenotype observed in the adult sheep. Understanding the mechanisms underlying changes in cardiovascular outcomes in an animal model may be beneficial for more directed therapeutic management of young adults exposed to antenatal steroids. Differences in multiple signaling molecules between males and females in the present study reveal potential differential responsiveness and consequences to certain therapeutics and may shed light on the sex-specific pathophysiology of observed with GC exposure [46–48].

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Abbreviations

ACE	angiotensin-converting enzyme
Akt	protein kinase B (PKB)
Ang	angiotensin
Ang-(1–7)	angiotensin-(1–7)
AT₁	angiotensin II type 1 receptor
BM	betamethasone
BMX	BM exposure
BRS	baroreflex sensitivity
DCS	discrete correlate summation

DMM	dorsomedial medulla
ERK	extracellular signal regulated kinase
GABA	gamma aminobutyric acid
GC	glucocorticoid
GSK-3β	glycogen synthase kinase 3 β
HRP	horseseradish peroxidase
MAPK	mitogen activated protein kinase
Mas	angiotensin-(1–7) mas receptor
NO	nitric oxide
NOS	nitric oxide synthase
PI3K	phosphoinositide 3-kinase
PRAS40	proline-rich Akt substrate
RAS	renin–angiotensin system
ROS	reactive oxygen species
SAPK/JNK	stress-activated protein kinase/c-jun-terminal kinase

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Clinical perspectives

- The status of signaling mechanisms in the brain DMM of the BMX sheep with low GC exposure *in utero* that comprise both the MAPK and PI3K cellular pathways are unknown.
- We report that sex-dependent differences in the MAPK and PI3K pathways in a brain center involved in autonomic regulation. Importantly, the data reveal alterations in signaling in the brain of a clinically relevant non-rodent model of fetal programming in response to an *in utero* environmental challenge.
- In addressing the specific molecular consequences of GC-induced fetal programming associated with lower Ang-(1–7) tone in central cardiovascular centers, the study reveals potential targetted therapeutic approaches for cardiovascular dysfunction that may be relevant to similar *in utero* GC exposure in humans.

Figure 1. Differential patterns of signaling expression in DMM of BMX adult female and male sheep as detected using a signaling array

(A) Representative images of PathScan® intracellular array indicate altered patterns of protein expression within PI3K pathway between control and BMX female sheep and (B) altered patterns of protein expression within MAPK/PI3K pathway between control and BMX male sheep. Relative densities of the signaling array data normalized to the positive controls on each slide (0 to 1 gray scale) for female sheep (C), and in male sheep (D). Mean $\pm$ S.E.M. ($n=4$ in all groups).

Figure 2. DSC analysis of the signaling array data showing different profiles in female and male sheep

Correlate summation of the variables that account for the majority of the variance between BMX and control groups in female (A) and male (B) sheep. Signaling molecules are sorted from left to right according to the DCS score; higher DCS score indicates greater contribution to the variance between control and BMX.

Figure 3. Immunoblots of dorsomedial medullary tissue for MAPK and PI3K components from BMX and control sheep

Representative immunoblots of DMM tissues and corresponding quantitative analysis of the relative protein expression from control (–) and BMX (+) female (A) and male (B) sheep. Values presented are phosphorylated to total protein for each variable. Mean $\pm$ S.E.M. ($n=7-8$ sheep in each group); * $P<0.05$.