Maternal food restriction modulates cerebrovascular structure and contractility in adult rat offspring: effects of metyrapone

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Durrant LM, Khorram O, Buchholz JN, Pearce WJ. Maternal food restriction modulates cerebrovascular structure and contractility in adult rat offspring: effects of metyrapone. Am J Physiol Regul Integr Comp Physiol 306: R401–R410, 2014. First published January 29, 2014; doi:10.1152/ajpregu.00436.2013.—Although the effects of prenatal undernutrition on adult cardiovascular health have been well studied, its effects on the cerebrovascular structure and function remain unknown. We used a pair-fed rat model of 50% caloric restriction from day 11 of gestation to term, with ad libitum feeding after birth. We validated that maternal food restriction (MFR) stress is mediated by glucocorticoids by administering metyrapone, a corticosterone synthesis inhibitor, to MFR mothers at day 11 of gestation. At age 8 mo, offspring from Control, MFR, and MFR + Metyrapone groups were killed, and middle cerebral artery (MCA) segments were studied using vessel-bath myography and confocal microscopy. Co-localization of smooth muscle α-actin (SMαA) with nonmuscle (NM), SM1 and SM2 myosin heavy-chain (MHC) isoforms was used to assess smooth muscle phenotype. Our results indicate that artery stiffness and wall thickness were increased, pressure-evoked myogenic reactivity was depressed, and myofilament Ca2+ sensitivity was decreased in offspring of MFR compared with Control rats. MCA from MFR offspring exhibited a significantly greater SMαA/NM colocalization, suggesting that the smooth muscle cells had been altered toward a noncontractile phenotype. MET significantly reversed the effects of MFR on stiffness but not myogenic reactivity, lowered SMαA/NM colocalization, and increased SMαA/SM2 colocalization. Together, our data suggest that MFR alters cerebrovascular contractility via both glucocorticoid-dependent and glucocorticoid-independent mechanisms.

In the search for mechanisms mediating fetal programming secondary to maternal food restriction, an early hypothesis was that these effects were mediated by increases in maternal glucocorticoids (42, 45). Additional evidence further suggested that maternal food restriction modified expression of glucocorticoid receptors in cerebral tissues of their offspring (4, 46). Despite strong evidence for involvement of glucocorticoids in fetal responses to maternal food restriction, however, other studies have suggested parallel involvement of glucocorticoid-independent mechanisms (14), including changes in endothelial function (62), VEGF function (38), ANG II levels (52), vitamin D availability (2, 54), oxidative stress (65), and vascular miRNA (37). This diversity of evidence strongly suggests that fetal responses to maternal food restriction involve multiple interacting mechanisms that combine to produce the vascular phenotype typical of offspring from food-restricted mothers.

Interpretation of the literature relevant to maternal food restriction is complicated by the broad variety of models and tissues used in these studies, many of which exhibit markedly different responses. In the kidney, maternal food restriction leads to reductions in kidney size, decreased nephron number, abnormalities in the renin-angiotensin system, and decreased angiogenesis (38, 42, 72). Maternal food restriction also results in smaller heart sizes at birth, cardiomegaly in adult offspring, and coronary artery remodeling with fibrosis (9, 34). In the aorta, maternal food restriction can alter aortic structure leading to increased wall thickness and stiffness (38, 40). In contrast, maternal food restriction generally “spares” the fetal brain from major effects in the short term (17), but in the long term, can significantly alter brain structure (18) and function, leading to significant behavioral abnormalities (60). Aside from these general changes in the brain, the specific effects of maternal food restriction on cerebrovascular development and structure remain largely unstudied.

To facilitate studies of the effects of maternal food restriction on the developing fetal cerebral circulation, we have developed a rat model involving 50% caloric restriction during the last 10 days of gestation with normal postnatal feeding (16, 38). This model produces a milder nutritional insult than that obtained with food restriction throughout gestation and early postnatal life, thus increasing the fraction of pups surviving through adulthood (30). Because this model reduces only total caloric intake, it is also less severe than protein restriction models (5), but still produces the adult-onset hypertension and obesity characteristic of maternal undernutrition (39). With this model, we have found that maternal food restriction can reduce VEGF expression and angiogenesis, can increase matrix metalloproteinase expression, and can alter multiple microRNA...
species that influence vascular development and function (37–39).

In light of published evidence that maternal food restriction (MFR) alters systemic vascular characteristics in adult offspring (7), the present study explores the hypothesis that MFR also influences vascular structure and function in the cerebral circulation of adult offspring. In particular, the experimental approach examined the functional consequences of MFR for vascular stiffness and compliance in middle cerebral arteries from 8-mo-old rats whose dams underwent 50% caloric restriction during the final 10 days of gestation. Separate experiments also examined changes in cerebral artery myogenic reactivity and smooth muscle phenotype attributable to MFR. To test the corollary hypothesis that the vascular effects of MFR arise from altered glucocorticoid signaling, we compared the effects of MFR in offspring from dams treated with or without metyrapone, an inhibitor of 11-β hydroxylase, which lowers circulating levels of corticosterone. Together, these experiments offered a unique perspective of the adult-onset cerebrovascular consequences of MFR.

MATERIALS AND METHODS

General preparation. All experimental procedures were approved by the Institutional Animal Use and Care Committees at both the Harbor-University of California, Los Angeles Medical Center and Loma Linda University. First-time-pregnant Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were housed at constant temperature and humidity with a 12:12-h-light-dark cycle. At day 10 of gestation, Control group rats were provided an ad libitum diet of standard laboratory chow (Lab Diet 5001, Brentwood, MO; protein 23%, fat 4.5%, metabolizable energy 3,030 kcal/kg), while MFR and MFR + metyrapone group rats were fed a diet of the same chow restricted to 50% of the intake measured by weight in paired, Control chow-fed rats. The respective diets were given from day 10 of pregnancy to term (21 days). Metyrapone (M2696 at 0.5 mg/ml; Sigma, St. Louis, MO) was dissolved in drinking water and given from day 11 of gestation to term. The dose of metyrapone was based on previous studies showing effective blockade of maternal corticosterone synthesis (64). In light of multiple published reports that metyrapone administration during gestation had no significant effects on birth weight or blood pressure in offspring of either rats (10, 43, 12, 13), we examined changes in cerebral artery myogenic reactivity and smooth muscle phenotype attributable to MFR. To test the corollary hypothesis that the vascular effects of MFR arise from altered glucocorticoid signaling, we compared the effects of MFR in offspring from dams treated with or without metyrapone, an inhibitor of 11-β hydroxylase, which lowers circulating levels of corticosterone. Together, these experiments offered a unique perspective of the adult-onset cerebrovascular consequences of MFR.

Measurement of smooth muscle calcium concentration. Once cannulated and equilibrated at 21°C, the artery segments were loaded with Fura-2 AM (Molecular Probes, Eugene, OR) for 20 min at a concentration of 1 μM, as previously described (12, 13). After loading, the arteries were washed with PSS, and the bath temperature was increased to 37°C. Photons emitted at 510 nm were detected at a sampling rate of 3 Hz using an IonOptix photomultiplier system that automatically corrected for background fluorescence. [Ca2+]i values obtained from separate in vitro calibrations were used to convert the experimental fluorescent intensity ratios (R) to [Ca2+]i, over the physiological range by iterative fit to the Grynkiewicz equation: 

\[ [Ca^{2+}]_i = K_d[(R - R_{	ext{min}})/(R_{	ext{max}} - R)]S_I \]

Our calculations using this equation employed the following averaged values: \( S_I (20.5), R_{\text{min}} (0.3), R_{\text{max}} (6.3), \) and \( K_d (251 \text{ nM}) \).

Experimental protocols. All arteries were first exposed to PSS containing 120 mM K+ for 1 to 2 min at 60 mmHg to verify contractility. When contractile responses had stabilized, the 120 mM K+ was replaced with normal PSS and the arteries were reequilibrated until baseline diameters were reestablished (≈10 min). Next, a series of pressure steps at 20, 40, 60, 80, and 100 mmHg were applied to each artery. At each pressure, artery diameter and wall [Ca2+]i were recorded first during equilibration in normal PSS and then in PSS containing 120 mM K+. Following stabilization of the diameter and Ca2+ responses to 120 mM K+, the arteries were returned to normal PSS. For the experiments presented in this study, all artery diameters were reestablished (≈10 min). The pressure was then raised to the next level, and the measurements were repeated. Once responses were measured at 100 mmHg, the arteries were equilibrated in PSS containing zero calcium with EDTA (3 mM) to determine the maximum passive diameter for each artery. This diameter was recorded at each of the previously applied pressure steps.

Fluorescent immunohistochemistry. Arteries were fixed in 4% neutral buffered EM-grade formaldehyde (Electron Microscopy Sciences, 15713-S) overnight, and then dehydrated, embedded in paraffin, and cut into sections 5 μm thick. The sections were then deparaffinized, rehydrated, immersed in citrate buffer at pH 6.03, and microwaved for 5 min to facilitate antigen recovery. The sections were then incubated in 1% BSA (SC-3232, Santa Cruz Biotechnology, Santa Cruz, CA) with Triton X-100 (T-8787, Sigma-Aldrich) blocking buffer for an hour. Next, the sections were double-stained with primary antibodies reactive with smooth muscle α-actin (AS5228 @ 1:200, Sigma-Aldrich), smooth muscle 2 (SM2) myosin (AB53219 @ 1:500; Abcam, Cambridge, MA), SM1 myosin (AB681 @ 1:100; Abcam), and SM2 myosin (PRB-145P @ 1:400; Covance, Princeton, NJ), applied and incubated at 4°C overnight. The following day, the sections were washed in PBS and then equilibrated in darkness for 2 h at room temperature with two secondary antibodies labeled with Dylight-488 and Dylight-649, and then examined with our Olympus FV1000 at an optical section thickness of 1 μm, a lateral resolution of 200 nm, and a numerical aperture of 1.8.

Confocal images of coronal artery sections were analyzed using Image-Pro (v5.9; Media Cybernetics, Silver Spring, MD) to obtain...
calibrated estimates of medial wall thickness, measured from the basal elastic membrane to the adventitial-medial border. The degree of colocalization between the two markers was determined using the colocalization coefficient calculated within the FluoView software package (Olympus) because this measure was independent of absolute marker intensities. For the colocalization analysis, coronal sections were extracted from the parent images using a masking routine to eliminate background contributions to the measurement statistics. These methods have previously been described in detail (12, 13).

Chemical and reagents. All chemicals were purchased from Sigma, except pluronic acid and Fura-2 AM, which were acquired from Molecular Probes/Invitrogen (Carlsbad, CA).

Data analysis and statistics. All values are given as means ± SE, and statistical significance implies \( P < 0.05 \), unless stated otherwise. In all cases, \( n \) refers to the number of animals studied. Values of artery diameter and wall calcium were analyzed via two-way ANOVA with repeated measures using treatment (Control, MFR, and MFR + Metyrapone) and pressure as factors using SPSS (v23). Post hoc between-group comparisons were performed using the Fisher protected least significant difference analysis. Where significant between-group differences were observed, post hoc comparisons between individual means at the same pressure were performed using a Behren’s-Fisher analysis with pooled variance. Average values of medial wall thickness obtained for each section were averaged within each of the three experimental groups and were compared using a one-way ANOVA. Values for the colocalization coefficient were compared using a Behren’s-Fisher analysis with pooled variance. For analysis of myosin isoform expression, all image pixels with greater than median intensity for \( \alpha \)-actin were segregated into two groups with intensities either above or below the median intensity for each myosin isoform, respectively (SM1, SM2, and SMemb). The percentages of total pixels in each of these groups were calculated for each age group and then compared between age groups using a Behren’s-Fisher analysis with pooled variance. All data sets were normally distributed, as verified using a D’Agostino-Pearson \( K^2 \) test, and homogeneity of variance within ANOVA was verified using a Bartlett-Cochran test (71).

RESULTS

This study is based on data obtained from 15 rats: five were 8-mo-old adults from control-fed mothers; five were 8-mo old adults from food-restricted mothers; and five were 8-mo adults from food-restricted mothers treated with metyrapone. One artery segment was used from each animal for contractility studies, and the contralateral artery was taken for immunohis- tochemistry and confocal imaging. Medial wall thicknesses averaged 23.7 ± 0.4, 29.6 ± 2.1, and 30.4 ± 1.5 \( \mu \text{m} \) in Control, MFR, and MFR + Metyrapone groups, respectively (\( n = 5 \) in each group). Thickness values in the MFR and MFR + Metyrapone groups were both significantly greater than in the Control group but did not differ significantly from one another.

Effects of changes in pressure on maximum passive diameter in control, MFR, and MFR ± metyrapone MCAs. In Control arteries treated with EGTA to eliminate active tone, average diameters increased progressively from 220 ± 13 \( \mu \text{m} \) at 20 mmHg to 296 ± 10 \( \mu \text{m} \) at 100 mmHg (Fig. 1, top). The relation between pressure and diameter was shifted upward in the MFR group, in which diameters averaged 243 ± 7 \( \mu \text{m} \) at 20 mmHg to 299 ± 11 \( \mu \text{m} \) at 100 mmHg. The MFR values were significantly greater than observed in Controls at both 20 and 40 mmHg. Treatment with metyrapone yielded a pressure-diameter relation that was not significantly different than that in Controls at any pressure; diameters averaged 228 ± 5 \( \mu \text{m} \) at 20 mmHg and 299 ± 5 \( \mu \text{m} \) at 100 mmHg. At 20 mmHg, the diameter in the Metyrapone group was significantly less than that in the MFR group.

Calculations of incremental compliance from the slopes of the pressure-diameter relations revealed that in Control group arteries, compliance decreased progressively from 1.29 ± 0.25 \( \mu \text{m/mm Hg} \) at 30 mmHg to 0.66 ± 0.02 \( \mu \text{m/mm Hg} \) at 90 mmHg (Fig. 1, bottom). The relation between compliance and pressure was significantly shifted downward in the MFR group such that compliance averaged 0.92 ± 0.12 \( \mu \text{m/mm Hg} \) at 30 mmHg to 0.55 ± 0.0 \( \mu \text{m/mm Hg} \) at 90 mmHg. Treatment with Metyrapone yielded a pressure-compliance relation that was not significantly different than that in Controls at any pressure. In the Metyrapone group, compliance values averaged 1.22 ± 0.20 at 30 mmHg and 0.56 ± 0.09 at 90 mmHg.

Effects of changes in pressure on myogenic contractility in control, MFR and MFR ± metyrapone MCAs. Stretch-induced active (myogenic) contractions were calculated at each transmural pressure, as differences in artery diameter measured in PSS and EGTA solutions. In Control arteries, these \( \Delta D \) values...
increased from 36 ± 10 μm at 20 mmHg to a peak value of 
75 ± 7 μm at 60 mmHg. At higher pressures, the ΔD values 
decreased to 55 ± 7 μm at 100 mmHg (Fig. 2, top). The ΔD 
values in arteries from the MFR group were generally less 
than observed in the Control group, and these differences 
were significant at 40 and 60 mmHg. In the MFR + Metyrapone 
group, the ΔD values were significantly less than observed in 
the Control group at 60 mmHg but were greater than observed 
in Controls at 100 mmHg.

Stretch-induced changes in cytosolic calcium concentration 
were calculated at each transmural pressure as differences 
in concentration measured in PSS and EGTA solutions. In Con-
trol arteries, these Δ[Ca²⁺] values increased from 96 ± 3 nM 
at 20 mmHg to a peak value of 141 ± 7 nM at 80 mmHg (Fig. 
2, middle). The Δ[Ca²⁺] values in arteries from the MFR group 
were not significantly different than observed in the Control 
group at any pressure. In contrast, the Δ[Ca²⁺] values observed in 
the MFR + Metyrapone group were significantly greater 
than observed in Control arteries at all pressure and were 
significantly greater than observed in MFR arteries at 20, 60, 
80, and 100 mmHg.

Myofilament calcium sensitivity was estimated as the ratio 
between the stretch-induced ΔD response and the Δ[Ca²⁺] 
response at each pressure. The values of this ratio were 
significantly less in the MFR group than in the Control group at all 
pressures except 100 mmHg (Fig. 2, bottom). Values of this 
ratio in the MFR + Metyrapone group were also significantly 
less than in the Control group, and at all pressures.

Effects of changes in pressure on potassium-induced con-
tractility in control, MFR and MFR ± metyrapone MCAs.

Potassium-induced active contractions were calculated at each 
transmural pressure as differences in artery diameter measured 
in high potassium (120 mM) and PSS solutions. In Control 
arteries, these ΔD values did not vary with pressure and ranged 
from a minimum of 36 ± 7 μm at 100 mmHg to a maximum 
of 62 ± 9 μm at 40 mmHg (Fig. 3, top). Potassium-induced 
ΔD values in arteries from the MFR group were significantly 
greater than Control at 40 mmHg but were significantly less 
than Control at 80 mmHg. The ΔD values in the MFR + 
Metyrapone group were significantly greater than in the 
Control group only at 100 mmHg but were significantly greater 
than in the MFR + Metyrapone arteries at both 80 and 100 
mmHg.

Potassium-induced changes in cytosolic calcium concentra-
tion were calculated at each transmural pressure as differences 
in concentration measured in high potassium and PSS solu-
tions. In Control arteries, these Δ[Ca²⁺] values ranged from a 
maximum of 211 ± 11 nM at 20 mmHg to a minimum of 
162 ± 18 nM at 80 mmHg (Fig. 3, middle). At all pressures, 
Δ[Ca²⁺] values were significantly greater in the MFR group 
than in the Control group and ranged from 302 ± 30 nM at 20 
mmHg to 230 ± 31 nM at 80 mmHg. In the MFR + 
Metyrapone group, Δ[Ca²⁺] values were also significantly 
greater than in the Control group at all pressures but did not 
differ significantly from values in the MFR group.

Potassium-induced changes in myofilament calcium sensi-
tivity were estimated as the ratio between the K⁺-induced ΔD 
response and the Δ[Ca²⁺] response at each pressure. The 
values of this ratio were significantly less in the MFR group 
than in the Control group at 20 and 80 mmHg (Fig. 3, bottom). 
Values of this ratio in the MFR + Metyrapone group were
significantly less than in the Control group at 20, 40, and 80 mmHg but were significantly greater than in the Control group at 100 mmHg. Values of this ratio in the MFR + Metyrapone group were also significantly less than in the MFR group at 20 and 40 mmHg.

Effects of maternal food restriction and metyrapone on contractile protein colocalization and smooth muscle in MCAs. To assess the effects of MFR with or without metyrapone on smooth muscle phenotype, concentric rings of rat middle cerebral arteries were immunostained to detect colocalization of smooth muscle α-actin with the nonmuscle, SM1, or SM2 isoforms of myosin heavy chain (Fig. 4). Colocalization of smooth muscle α-actin with the nonmuscle isoform was significantly greater in the MFR group than in either the Control group or the MFR + metyrapone group (Fig. 5). Colocalization of smooth muscle α-actin with the SM1 isoform was also significantly greater in the MFR group than in the Control group, but the SM1 colocalization value in the MFR + Metyrapone did not differ significantly from either the Control or MFR groups. For colocalization of smooth muscle α-actin with the SM2 isoform, the values in the Control and MFR groups were similar, but the value in the MFR + metyrapone group was significantly greater than in either the Control or MFR groups.

DISCUSSION

The present study explores the hypothesis that glucocorticoids contribute to the long-term effects of MFR on structure-function relations in the cerebral circulation of adult offspring. In middle cerebral arteries, MFR reduced incremental compliance, and this effect was largely prevented by treatment with metyrapone, a corticosterone synthesis inhibitor. In contrast, MFR increased medial thickness, and this effect was resistant to treatment with metyrapone. Myogenic reactivity was attenuated by MFR, largely through inhibition of myofilament calcium sensitivity, and this inhibition was resistant to metyrapone. MFR had mixed effects on potassium-induced contractions that included enhancement of the calcium response to potassium together with inhibition of myofilament calcium sensitivity. The effects of MFR on potassium-induced tone were minimally altered by metyrapone. Colocalization of nonmuscle myosin heavy chain (NM-MHC) with smooth muscle α-actin, an index of the proportion of functionally immature smooth muscle in the artery, was enhanced by MFR, and this effect was reduced by metyrapone. In contrast, colocalization of SM2 myosin heavy chain with smooth muscle α-actin, an index of the proportion of fully differentiated contractile smooth muscle, was unaffected by MFR but was enhanced by metyrapone. Together, these results suggest that MFR selectively alters multiple aspects of structure-function relations in cerebral arteries and that only some of these long-term effects involve the actions of glucocorticoids.

The mechanisms mediating the vascular effects of MFR remain uncertain, particularly in relation to the role of glucocorticoids. Given the capacity of MFR to increase maternal glucocorticoids (45) and the ability of glucocorticoids to increase arterial stiffness (35), our first series of experiments examined whether MFR could increase cerebral artery stiffness and thereby reduce passive compliance. These measurements revealed that MFR reduced passive compliance and, more

Fig. 3. Effects of MFR and metyrapone on K+-induced pressure-diameter relations. In arteries from the MFR group (top), stretch-induced (myogenic) changes in diameter (ΔD) were significantly greater than Control at 40 mmHg but were significantly less than Control at 80 mmHg (P < 0.05). In the MFR + Metyrapone arteries, ΔD values were significantly greater than in the Control group at 100 mmHg (P < 0.05) and were greater than in the MFR + Metyrapone at both 80 and 100 mmHg (P < 0.05). Potassium-induced changes in cytosolic calcium (middle) were significantly greater in both the MFR and MFR + Metyrapone groups (P < 0.05) than in the Control group at all pressures. Values of Δ[Ca2+]i in the MFR and MFR + Metyrapone groups did not differ significantly from one another at any pressure. Estimates of myofilament calcium sensitivity (bottom, ΔD/Δ[Ca2+]i) were significantly less in the MFR group than in the Control group at 20 and 80 mmHg (P < 0.05). Values of this ratio in the MFR + Metyrapone group were significantly less than in the Control group at 20, 40, and 80 mmHg but were significantly greater than in the Control group at 100 mmHg. Values of this ratio in the MFR + Metyrapone group were also significantly less than in the MFR group at 20 and 40 mmHg.

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importantly, that treatment with metyrapone significantly attenuated this effect (Fig. 1); however, MFR also increased medial thickness \( \approx 25\% \), and this effect persisted in the MFR + Metyrapone group, suggesting its independence of glucocorticoids, consistent with the well-established antiproliferative acute effects of glucocorticoids in vascular smooth muscle (21, 58). Given the key roles of elastin and collagen as determinants of arterial stiffness (63, 67), together with the known effects of glucocorticoids on stiffness (35), these findings suggest that MFR reduces cerebrovascular compliance through changes in matrix protein abundance and organization, mediated by increased activity of corticosterone during gestation (36) on the glucocorticoid receptor (6, 44) and possibly also on the mineralocorticoid receptor (48). These results are also consistent with possible long-term glucocorticoid-dependent effects of MFR on matrix metalloproteinase activities or tissue inhibitors of metalloproteinases, as suggested by other studies (22, 28, 39). Although the precise mechanisms mediating these diverse effects remain unclear, the results suggest that MFR can have long-term, “programming” effects via glucocorticoid-independent effects on medial wall thickness and glucocorticoid-dependent effects on cerebrovascular compliance and stiffness.

Compared to their effects on vascular structure and compliance, the long-term effects of glucocorticoids on vascular function are more poorly understood, due largely to their highly artery- and tissue-specific effects (23, 26, 70). To evaluate whether MFR might produce long-term influences on cerebrovascular contractility through a glucocorticoid-dependent pathway, our second series of experiments assayed pressure-dependent myogenic contractility in cerebral arteries isolated from adult offspring of food-restricted dams. In these experiments, MFR depressed myogenic contractility (Fig. 2, top) but had very little effect on the relation between transmural pressure and cytosolic calcium concentration (Fig. 2, middle). Because contractile tone is the product of cytosolic calcium concentration and myofilament calcium sensitivity (61), the observed pattern suggests that the long-term effects of MFR on myogenic contractility were attributable largely to decreased myofilament calcium sensitivity. Consistent with this interpretation, MFR decreased the ratio between artery diameter and cytosolic calcium (Fig. 2, bottom), an index of myofilament calcium sensitivity (12). Regarding the role of glucocorticoids in these “programming” effects of MFR, Metyrapone had very little influence on MFR-induced depression of myogenic contractility (Fig. 2, top), but interfered significantly with regulation of cytosolic calcium (Fig. 2, middle). This latter finding suggests that glucocorticoids are essential for calcium homeostasis in cerebral arteries, at least in adult offspring of food-restricted dams. Because the effects of MFR and metyrapone during gestation were observed 8 mo after birth, it seems probable that the observed changes in calcium regulation are attributable to a programming effect on the expression patterns for calcium-regulating proteins. From this perspective, our data raise the possibility that gestational food restriction in the absence of glucocorticoids causes epigenetic control.
Changes in gene expression for calcium-regulating proteins and that glucocorticoids protect against such effects. Although glucocorticoids can produce long-term depression of contractility in cerebral arteries (19) and, over the short term, can inhibit myofilament sensitivity in airway smooth muscle (24) and uterine arteries (69), metyrapone had very little long-term effect on myofilament calcium sensitivity in cerebral arteries of MFR offspring (Fig. 2, bottom). Altogether, these findings suggest that MFR attenuates cerebrovascular myogenic contractility through long-term depression of myofilament calcium sensitivity via mechanisms largely independent of glucocorticoids.

Another important characteristic of vascular function is maximum contractile capacity, which can be elucidated by exposure to high concentrations of potassium that depolarize the smooth muscle membrane, enable the influx of extracellular calcium, and maximally stimulate the contractile apparatus in a receptor-independent manner (33). In our experiments, MFR produced long-term effects on contractile responses to potassium depolarization in a pressure-dependent manner (Fig. 3) that were quite distinct from the pattern observed for myogenic contractility (Fig. 2). Compared with Control responses, MFR responses to potassium depolarization were elevated at 40 mmHg, but depressed at 80 mmHg, suggesting that the fundamental stress-strain relationship in these arteries underwent long-term alteration by MFR. These results at lower transmural pressures (20–60 mmHg) generally agreed with the findings of Ozaki et al. (57), who reported that MFR enhanced potassium-induced contractility of isolated femoral arteries. In turn, the inconsistencies between the present results at higher pressures and the Ozaki findings could be due to differences in artery type (femoral vs. cerebral), method of contractile measurement (pressurized myography vs. wire-mount isometric myography), or food restriction model (70% MFR for 0–18 days gestation vs. 50% MFR for 11–21 days gestation).

Unlike the Ozaki study, however, the present experiments examined the sustained effects of MFR on calcium regulation and revealed that MFR increased cytosolic calcium concentrations at all pressures, suggesting that the long-term effects of MFR on overall contractility were not dependent primarily upon pressure-dependent changes in calcium handling (Fig. 3, middle). Instead, the data suggest that MFR increased either release or influx of calcium or, alternatively, decreased extrusion or sequestration of calcium in these arteries (47). The contractile effects of the elevated calcium levels in the arteries from the MFR offspring were partially offset by parallel depression of myofilament calcium sensitivity between 20 and 80 mmHg (Fig. 3, bottom). The ability of MFR to modulate myofilament sensitivity further suggests that MFR either decreased the ratio between myosin light-chain kinase and myosin light-chain phosphatase activities (thick filament regulation), and/or depressed the ability of activated myosin heavy chain to generate force through cross-bridge interactions with actin (thin filament regulation) (55). Altogether, these results suggest that MFR can exert long-term effects on either calcium regulation through possible alterations in the function of key proteins such as L-channels, plasma membrane calcium ATPase, or sarcoplasmic and endoplasmic reticulum calcium ATPase, or alternatively may modulate abundances and activities of proteins governing myofilament calcium sensitivity such as myosin light-chain kinase, myosin light-chain phosphatase, CPI-17, Rho-kinase, caldesmon, and HSP27 (3, 11).

One of the possible mechanisms through which MFR could modulate multiple key vascular proteins is through activation of glucocorticoid receptors (19, 42, 51). Consistent with a role for glucocorticoids in MFR-induced changes in contractility, metyrapone blocked the effects of MFR on potassium contractions at low (40 mmHg) and high (80 mmHg) pressures (Fig. 3, top). Despite these effects of metyrapone on potassium-induced contractility, metyrapone had no significant effect on cytosolic calcium, suggesting that glucocorticoids are not involved in the programmed effects of MFR on calcium handling during potassium contractions (Fig. 3, middle). This result agrees with previous findings in cardiac myocytes that L-channel density is increased, and that SERCA pump abundance is decreased by short-term exposure to glucocorticoids (15), leading to increased cytosolic calcium concentrations. In contrast, metyrapone further depressed myofilament calcium sensitivity at low pressures (20 and 40 mmHg) but increased it at high pressure (100 mmHg) in potassium-contracted arteries, suggesting that glucocorticoids probably contribute little to the long-term effects of MFR on calcium sensitivity (Fig. 3, bottom). In combination, these results emphasize that MFR influences calcium handling and calcium sensitivity very differently in stretch-induced and potassium-induced contractions through mechanisms that are largely independent of the programming effects of glucocorticoids.

Another general mechanism through which MFR could alter cerebrovascular contractility is by modulation of the phenotype of smooth muscle (56) in cerebral arteries. A transition from a contractile to a noncontractile smooth muscle phenotype can dramatically alter calcium handling, contractility, and structure (29, 68), and such dedifferentiation can be identified through changes in patterns of contractile protein expression and colocalization (1, 12, 31). From this perspective, MFR produced...
long-term increases in the fraction of smooth muscle α-actin colocalized with NM-MHC (Fig. 4), suggesting an increase in the fraction of noncontractile smooth muscle (20, 56). This result suggests that MFR could promote either inward migration of progenitor smooth muscle cells, or dedifferentiation and/or proliferation of preexisting contractile smooth muscle (59). Arguing against dedifferentiation, which would be expected to decrease the number of contractile smooth muscle cells, MFR had no effect on the colocalization of SM2 MHC with smooth muscle α-actin, suggesting that the proportion of contractile smooth muscle cells was unchanged (Fig. 5). In addition, the colocalization of SM1 MHC with smooth muscle α-actin, which is an indicator of partially differentiated contractile smooth muscle (20), was increased by MFR. Together, these findings suggest that MFR produced long-term increases in the total fractions of smooth muscle cells in both the contractile and noncontractile phenotypes, which could be explained only by proliferation of existing smooth muscle, or inward migration and/or differentiation of smooth muscle progenitors in the artery wall (56).

With respect to the involvement of glucocorticoids in the long-term effects of MFR on smooth muscle phenotype, treatment with metyrapone significantly decreased colocalization of NM-MHC with smooth muscle α-actin (Fig. 5). This finding suggests that long-term effects of glucocorticoids contributed to the MFR-induced increases in colocalization of NM-MHC with smooth muscle α-actin, which again could indicate inward migration of progenitor smooth muscle cells, dedifferentiation, or smooth muscle proliferation (59). Whereas metyrapone had minimal effects on SM1 colocalization, it significantly enhanced colocalization of SM2 MHC with smooth muscle α-actin, which supports the view that glucocorticoids promote long-term contractile dedifferentiation in response to MFR. This interpretation is in contrast with previous reports that antenatal dexamethasone treatment can accelerate short-term vascular differentiation (27), and emphasizes that short-term and long-term effects of glucocorticoids are not always equivalent. On the other hand, the present results agree with previous reports that glucocorticoids promote proliferation in vascular smooth muscle cells (66), and thereby could contribute to the long-term effects of MFR on smooth muscle phenotype. These changes in smooth muscle phenotype, in turn, could help explain the complex effects of MFR on calcium handling, myofilament calcium sensitivity, and overall contractility.

Perspectives and Significance

Overall, the present experiments support the hypothesis that maternal food restriction during the latter half of gestation fundamentally alters long-term structure-function relationships in the middle cerebral arteries of adult offspring, as revealed by pressure myography and confocal microscopy. MFR significantly decreased compliance in offspring cerebral arteries, and this effect was partially rescued by treatment with the corticosterone synthesis inhibitor metyrapone, suggesting that this effect of MFR was mediated by increased glucocorticoid exposure during gestation. While MFR increased vessel thickness, thickness remained increased following treatment with metyrapone, suggesting an additional glucocorticoid-independent mechanism. MFR also caused long-term depression of myogenic reactivity due largely to attenuation of myofilament calcium sensitivity through mechanisms that were resistant to metyrapone, and thus appeared independent of glucocorticoids. Similarly, MFR programmed potassium-induced contractions but in a manner distinct from its effects on myogenic contractility; potassium contractions were enhanced at low pressure, and depressed at high pressure, by MFR. During potassium contractions, MFR increased cytosolic calcium at all pressures through mechanisms resistant to metyrapone, but MFR generally depressed myofilament calcium sensitivity through mechanisms that were enhanced by metyrapone at low pressures, but antagonized at high pressures. In relation to smooth muscle phenotype, MFR gave rise to enhanced proportions of cells in a noncontractile phenotype without diminishing the proportion of fully differentiated contractile cells, suggesting either a long-term shift toward an increased proliferation or inward migration of smooth muscle progenitors. Metyrapone generally antagonized the effects of MFR on smooth muscle phenotype, suggesting that glucocorticoids contribute to changes in smooth muscle phenotype in both the long term, as well as the short term. Owing to the strong relationships among the smooth muscle phenotype, contractility, and structure, the sustained influences of MFR on smooth muscle phenotype help explain both the glucocorticoid-dependent and -independent general effects of MFR programming on cerebrovascular structure and function. These findings tempt speculation that MFR might increase vulnerability to stroke and other cerebrovascular diseases, in parallel with its ability to increase the risk of cardiac disease in adult offspring of food-restricted mothers (7, 8).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: L.M.D. performed experiments; L.M.D. and W.J.P. analyzed data; L.M.D., O.K., J.N.B., and W.J.P. interpreted results of experiments; L.M.D. and W.J.P. drafted manuscript; L.M.D., O.K., J.N.B., and W.J.P. edited and revised manuscript; L.M.D., O.K., J.N.B., and W.J.P. approved final version of manuscript; O.K. and W.J.P. conception and design of research; W.J.P. prepared figures.

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