Maternal antioxidant blocks programmed cardiovascular and behavioural stress responses in adult mice

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Abstract

Intra-uterine growth restriction is an independent risk factor for adult psychiatric and cardiovascular diseases. In humans, intra-uterine growth restriction is associated with increased placental and fetal oxidative stress, as well as down-regulation of placental 11β-HSD (11β-hydroxysteroid dehydrogenase). Decreased placental 11β-HSD activity increases fetal exposure to maternal glucocorticoids, further increasing fetal oxidative stress. To explore the developmental origins of co-morbid hypertension and anxiety disorders, we increased fetal glucocorticoid exposure by administering the 11β-HSD inhibitor CBX (carbenoxolone; $12 \text{ mg} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$) during the final week of murine gestation. We hypothesized that maternal antioxidant (tempol throughout pregnancy) would block glucocorticoid-programmed anxiety, vascular dysfunction and hypertension. Anxiety-related behaviour (conditioned fear) and the haemodynamic response to stress were measured in adult mice. Maternal CBX administration significantly increased conditioned fear responses of adult females. Among the offspring of CBX-injected dams, maternal tempol markedly attenuated the behavioural and cardiovascular responses to psychological stress. Compared with offspring of undisturbed dams, male offspring of dams that received daily third trimester saline injections had increased stress-evoked pressure responses that were blocked by maternal tempol. In contrast, tempol did not block CBX-induced aortic dysfunction in female mice (measured by myography and lucigenin-enhanced chemiluminescence). We conclude that maternal stress and exaggerated fetal glucocorticoid exposure enhance sex-specific stress responses, as well as alterations in aortic reactivity. Because concurrent tempol attenuated conditioned fear and stress reactivity even among the offspring of saline-injected dams, we speculate that antenatal stressors programme offspring stress reactivity in a cycle that may be broken by antenatal antioxidant therapy.

Keywords

antioxidant; cardiovascular response; carbenoxolone; fetal programming; 11β-hydroxysteroid dehydrogenase; stress

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INTRODUCTION

Fetal glucocorticoid exposure alters brain development and programmes the development of adult hypertension [1–3]. Intriguingly, programmed hypertension is consistently demonstrated during restraint for tail cuff measurements, but not when baseline arterial pressures are recorded by unrestrained methods, including radiotelemetry [1,4–6]. Radiotelemetry studies have further shown that intra-uterine glucocorticoid exposure programmes an exaggerated hypertensive response to psychological stress, suggesting that increased stress reactivity may contribute to the tail cuff hypertension seen in other studies [5].

In addition to the programming of stress-induced hypertension, excessive fetal glucocorticoid exposure may increase the risk of developing anxiety disorders, including PTSD (post-traumatic stress disorder) [7,8]. PTSD is a mentally debilitating illness that carries an increased risk of co-morbid cardiovascular disease [9]. Although PTSD is a common disorder, most of those exposed to traumatic experiences do not develop PTSD, suggesting that PTSD susceptibility varies greatly between individuals [10]. As a prototypical example of this differential predisposition, there is a 4-fold higher incidence of PTSD in women [10]. Better knowledge of the genetic, environmental and neurobiological risk factors that contribute to PTSD susceptibility would lead to opportunities for prevention.

Intra-uterine growth restriction, an independent risk factor for adult psychiatric and cardiovascular diseases, is associated with down-regulation of placental 11β-HSD (11β-hydroxysteroid dehydrogenase) [4,11]. Down-regulation of 11β-HSD increases fetal exposure to maternal glucocorticoids [3,12] and may be an adaptive response to accelerate fetal maturation in the face of environmental adversity. To explore the developmental origins of hypertension, we previously administered the 11β-HSD inhibitor CBX (carbenoxolone) during the final week of murine gestation and demonstrated that maternal CBX increased adult blood pressures in male, but not female, offspring [13]. We further showed in a complementary maternal undernutrition model that programmed hypertension is associated with adult vascular dysfunction including exaggerated production of superoxide anion [13].

In addition to its association with exaggerated glucocorticoid exposure, intra-uterine growth restriction is associated with increased placental and fetal oxidative stress [14–16]. Although glucocorticoid excess is known to induce vascular superoxide production [17] and oxidative stress has been linked with programmed vascular dysfunction [18,19], these interactions have not been assessed from a developmental perspective. We speculated that maternal antioxidant therapy would block programmed hypertension. To further understand the mechanism contributing to programmed hypertension, we evaluated the effects of maternal tempol, a membrane-permeable SOD (superoxide dismutase) mimetic, on offspring anxiety-related behaviour and vascular reactivity. We hypothesized that maternal antioxidant therapy would attenuate the effects of maternal stress and exaggerated intra-uterine gluco-corticoid exposure on fear conditioning (a PTSD-like phenotype), stress-enhanced hypertension and aortic reactivity.

MATERIALS AND METHODS

Ethical approval

All procedures were approved by the University of Iowa Animal Care and Use Committee (protocol no. 0812259). The present investigation conforms to the *Guide for the Care and*

Use of Laboratory Animals published by the U.S. NIH (National Institutes of Health; NIH Publication No. 85–23, revised 1996).

Animal model

Adult C57Bl/6J mice (The Jackson Laboratory) were bred until discovery of a maternal plug. From embryonic day 12 to 19, dams were undisturbed or injected subcutaneously with saline (0.9% NaCl) or CBX (12.5 mg $\cdot$ kg^{-1} of body weight $\cdot$ day^{-1}; Sigma). Dams received standard diet and either standard drinking water or drinking water supplemented with the antioxidant tempol (1 mmol/l; Sigma) throughout pregnancy (from detection of copulatory plug to delivery). Pups were neither cross-fostered nor culled until weaning on day 21 of life.

Fear conditioning

At 4 months, mice were placed in a fear conditioning chamber (MED Associates). After 3 min, they were presented with a tone (80 dB, 20 s) that co-terminated with an electric foot-shock (0.5 mA, 1 s) for a total of five times at 1 min intervals [20]. The following day, context conditioning was tested by returning the mice to the original learning chamber in the absence of the electrical stimulus or auditory cue. The amount of time spent in a characteristic 'freezing' posture was recorded for 5 min. On the third day, cue conditioning was tested by placing the mice in a novel context and measuring the time spent 'freezing' during a 3 min re-presentation of the learned auditory cue (tone).

Telemetry recordings

At 6 months, radiotelemetry catheters (PA-C10; Data Sciences International) were implanted in the left carotid artery, as previously described [13]. After a 7 day recovery period, arterial pressures, heart rate and relative locomotor activity were sampled for 10 s every 5 min for 60 h (encompassing three dark cycles and two light cycles). At the end of the following light cycle, arterial waveforms were recorded at 2000 Hz during a 5 min phase of inactivity. Immediately after these baseline recordings, mice were transferred to a metabolic cage (Hatteras Instruments) to evoke a centrally mediated stress response [21,22]. The effects of psychological stress on blood pressure, heart rate and locomotor activity were assessed at the plateau phase of the stress response (40–60 min after initiation). For both control and CBX females, baseline haemodynamics (but not stress responses) have been previously reported for six mice [13]. The previously published baseline results are included to avoid biased interpretation of the primary end point (haemodynamic response to stress as a percentage of baseline). Mice weaned and maintained on a hypercaloric diet [45 kcal/g (where 1 kcal≈4.184 kJ), 41% of energy as fat; TD-06171, Harlan Teklad] contributed to baseline recordings (six female saline, six female CBX) and stress-evoked recordings (three male saline, three male CBX, six female saline, six female carbenolone). With our early results showing haemodynamic phenotypes in male but not female mice, follow-up studies investigated sex-specific effects of tempol on male haemodynamics and female aortic physiology.

Aortic reactivity

Female offspring never instrumented for blood pressure recording were killed at 6 months of age. The thoracic aorta was harvested and aortic segments were mounted on fixed pins within 5 ml myographs (Danish Myotechnology) [13]. Following equilibration with 4.5 mN resting tension, arteries were maximally constricted with 10^{-5} mol/l prostaglandin F$_{2a}$ (chosen over noradrenaline based on preliminary investigations showing it elicited more consistent preconstriction that was free of cyclic oscillations). After washing, vessels were preconstricted with consecutive additions of 10^{-6} mol/l prostaglandin F$_{2a}$ until the vessel

achieved 50% of its maximal constriction. When stable constriction was achieved, increasing concentrations of acetylcholine (10^{-9}–10^{-5} mol/l) or sodium nitroprusside (10^{-9}–$10^{-5.5}$ mol/l) were added at fixed intervals. To isolate the effects of exogenous nitric oxide, the nitric oxide synthase inhibitor l-NNA (N^G-nitro-l-arginine) was added to the myographs prior to the addition of sodium nitroprusside. Some arteries were pre-incubated in PEG [poly(ethylene glycol)] conjugates of SOD (50 units/ml) and catalase (250 units/ml) throughout the experiment. All compounds were acquired from Sigma.

Chemiluminescence

The aorta arch was harvested from the same non-instrumented female mice utilized for myography. Basal superoxide anion production was measured by lucigenin (25 μmol/l)-enhanced chemiluminescence, as previously described [13]. NADPH oxidase-dependent superoxide production was measured as the diphenyleneiodonium (10^{-4} mol/l)-inhibitable chemiluminescence measured after the addition of the enzyme substrate NADPH (10^{-4} mol/l). To further explore the relative importance of prenatal CBX and postnatal high-fat diet in the establishment of aortic superoxide production, additional mice were evaluated while receiving either the standard or high-fat diets.

Data analysis

All values are presented as means ± S.E.M. Results were compared by two-way ANOVA factoring for maternal injections and/or tempol administration. Aortic concentration–response curves were assessed by two-way repeated measures ANOVA. Post-hoc analysis (Holm–Sidak method) was performed if statistically significant differences were detected ($P < 0.05$). When a significant interaction was present between the programming stimulus (injections) and the intervention (tempol), Student's unpaired t tests were utilized to evaluate for independent effects. In all cases, the *a priori* hypothesis that maternal CBX programmes adult phenotypes was assessed by one-way ANOVA. All analyses were performed using SigmaStat 3.0 (SPSS).

RESULTS

Fear conditioning

Maternal CBX or tempol had no significant effect on litter size, birth weight or sex distribution (results not shown). To test the effects of maternal CBX on offspring responses to psychological stress, we measured fear-related freezing behaviour during a 3-day fear-conditioning protocol. On the first day of testing, mice were presented with a series of tones that co-terminated with mild foot-shocks. Maternal CBX did not significantly alter the baseline responses to either the tone or shock (results not shown). Upon re-presentation of the conditioned stimuli, CBX-exposed female mice had increased context-based fear (Figure 1, main effect of CBX: $F_{1,17} = 6.7$, $P = 0.02$) and a trend towards increased cue-based fear (Figure 2, main effect of CBX: $F_{1,17} = 3.28$, $P = 0.09$). Overall, the offspring of tempol-treated dams had significantly decreased contextually conditioned freezing (Figure 1, main effect of tempol: $F_{1,59} = 7.3$, $P = 0.009$) and auditory-cue-conditioned freezing (Figure 2, main effect of tempol: $F_{1,59} = 9.3$, $P = 0.003$). In both cases, Holm–Sidak post hoc testing isolated the effect of tempol to CBX offspring ($P = 0.02$ for CBX–tempol compared with CBX–water), with no significant effects on control offspring ($P = 0.16$ for effect of tempol on context conditioned fear and $P = 0.06$ for effect of tempol on cue-conditioned fear).

Radiotelemetry

We next explored the effects of fetal glucocorticoid exposure and antioxidant intervention on adult cardiovascular responses to psychological stress. Maternal saline or CBX injections

did not independently alter baseline blood pressure, heart rate or locomotor activity (Figure 3). For males, baseline locomotor activity was influenced by a significant interaction between the injections and antioxidant therapy ($F_{2,31} = 4.1$, $P = 0.03$). By planned contrast testing (Student's t test), maternal tempol increased the activity of CBX-exposed mice ($P = 0.03$ for CBX–tempol compared with CBX–water).

When first placed in a metabolic cage to elicit psychological stress, CBX-exposed male offspring had exaggerated pressure responses (Figure 4C, $P = 0.008$ compared with undisturbed) and tachycardic responses (Figure 4D, $P = 0.013$ compared with undisturbed). Similar trends were seen in the male offspring of saline-injected dams, but these did not approach statistical significance ($P = 0.12$ and $P = 0.09$ respectively). Overall, tempol decreased the peak heart rate response to placement in the metabolic cage (Figure 4D, main effect of tempol: $F_{1,38} = 8.1$, $P = 0.007$). Holm–Sidak post hoc testing identified significant effects of tempol on the male offspring of saline-injected dams ($P = 0.02$) and CBX-injected dams ($P = 0.003$), but not the male offspring of undisturbed dams ($P = 0.83$).

At 30–40 min after placement in the metabolic cage, blood pressures and heart rates plateau (Figures 4A and 4B). During this plateau phase, male offspring blood pressure was influenced by maternal injection status, as well as an interaction between injection status and tempol administration (Figure 4E, main effect of injections: $F_{2,38} = 4.1$, $P = 0.03$; interaction: $F_{2,38} = 4.0$, $P = 0.03$). On *post-hoc* testing, male offspring blood pressures were increased by maternal saline and CBX injections ($P = 0.04$ and $P = 0.003$ compared with undisturbed offspring), and maternal tempol decreased the blood pressures of the male offspring of saline-injected and CBX-injected mice ($P = 0.04$ for saline–tempol compared with saline–water; $P = 0.01$ for CBX–tempol compared with CBX–water). Similarly, maternal saline or CBX injection increased male offspring heart rates during placement in the metabolic cage (Figure 4F, $P = 0.11$ and $P = 0.009$ compared with undisturbed offspring), but tempol did not have a significant effect on stress tachycardia (overall effect tempol: $F_{1,38} = 2.3$, $P = 0.14$; interaction between injections and tempol: $F_{2,38} = 2.5$, $P = 0.10$). Compared with the female offspring of saline-injected dams, female offspring of CBX-injected dams did not have any significant alteration in baseline or stress-evoked haemodynamics (Figures 3 and 4 respectively). There were no differences in the arterial pressures or heart rates of mice weaned to the regular diet (4 kcal/g, 6% of energy as fat, 7013; Harlan Teklad) or the hypercaloric diet (e.g. female mean blood pressures: 113 ± 2 mmHg compared with 112 ± 2 mmHg; male stress-evoked heart rate plateau: $137 \pm 5\%$ compared with $137 \pm 10\%$ baseline).

Aortic reactivity

Because CBX-exposed female mice are normotensive, they provided an opportunity to test the effects of maternal CBX and tempol on aortic vasomotor function. We tested the hypotheses that (i) maternal CBX programmes primary (blood pressure-independent) alterations in vascular function and (ii) concurrent tempol blocks the programming of aortic reactivity. Prostaglandin F_{2a} induced preconstriction did not differ between the groups (results not shown). Endothelium-dependent vasodilation to acetylcholine was significantly decreased in aorta from CBX offspring, independent of maternal tempol administration (Figure 5A, main effect of CBX: $F_{1,203} = 7.7$, $P = 0.01$; main effect of tempol: $F_{1,203} = 0.19$, $P = 0.66$). After pre-incubation in the antioxidants PEG–SOD and PEG–catalase, the effect of maternal CBX on aortic reactivity was no longer significant (Figure 5B, $F_{1,203} = 2.0$, $P = 0.17$). Maternal CBX or tempol did not significantly alter aortic responses to the endothelium-independent vasodilator sodium nitroprusside (Figures 5C and 5D).

These results suggested that programmed endothelial dysfunction may be mediated by a persistent increase in intravascular oxidative stress. By lucigenin-enhanced

chemiluminescence, baseline aortic superoxide production was low and not influenced by maternal CBX or tempol administration (initial 7 min of Figure 6A). NADPH stimulated a dramatic increase in aortic superoxide production (subsequent 7 min of Figure 6A) that was significantly greater than from aorta of CBX offspring (Figure 6B, main effect of CBX: $F_{1,39} = 8.4$, $P = 0.006$, Holm–Sidak: $P = 0.04$ within water and $P = 0.051$ within tempol). This programmed increase in aortic superoxide production was independent of maternal tempol ($P = 0.37$ for the main effect of tempol and $P = 0.84$ for an interaction between tempol and CBX). To better understand the relative importance of CBX in the programming of aortic superoxide production, both male and female mice were evaluated after having been weaned to either a standard or hypercaloric diet (Figure 7). Once again there was an overall effect of CBX ($P = 0.03$), with subgroup analysis showing a significant increase in female mice ($P = 0.04$), but not in male mice ($P = 0.40$). This effect was independent of postnatal diet ($P = 0.12$ for regular diet compared with high-fat diet). In summary, there is converging evidence from myography and chemiluminescence showing maternal CBX programmes alterations in aortic physiology that are not blocked by maternal tempol administration.

DISCUSSION

Human studies and animal models have shown that diverse maternal environmental stressors alter adult offspring responses to psychological stress, often in a sex-specific fashion [3–5,23–25]. Accentuated maternal stress responses may ultimately underlie a trans-generational susceptibility to psychological stress [26,27]. Although placental 11β-HSD inactivates maternal glucocorticoids and protects the developing fetus from excessive steroid exposure, 11β-HSD activity is down-regulated during maternal undernutrition or fetal growth restriction [4,28–30]. To model this process, we induced 11β-HSD inactivation through administration of CBX during the final week of murine gestation.

A novel finding of the present study is that maternal CBX exposure increases conditioned fear responses in female offspring. This sex-specific programming is consistent with a higher incidence of PTSD in women [10]. Context-conditioned fear was most influenced, suggesting that maternal CBX administration may affect adult hippocampal more than amygdala function. Notably, exaggerated glucocorticoid exposure during critical developmental windows has been shown to reduce hippocampal neurogenesis and reduce adult hippocampal neuronal numbers [31,32]. In addition to cognitive effects, reduced hippocampal volumes are a key marker of increased PTSD susceptibility [33]. Further studies are necessary to further explore hippocampal structural and functional correlates in the context of antenatal stressors and antioxidant interventions. As suggested by the findings in related models, epigenetic alterations of hippocampal glucocorticoid receptors may contribute to the persistence of these phenotypes. Further studies will be necessary to delineate the ontogeny of the observed changes.

The striking effects of maternal antioxidant therapy on offspring behavioural and cardiovascular responses to psychological stress have not been previously described. In addition to negating the programming of conditioned fear by CBX, maternal tempol attenuated stress-evoked haemodynamic responses. This raises the possibility that prenatal stress and exaggerated transplacental glucocorticoid exposure may be sufficient to programme behavioural alterations in the offspring and that these may be mediated by oxidative stress. This speculation is supported by results showing that prenatal stress enhances fetal corticosterone exposure [23] and selectively increases hippocampal oxidative stress in female offspring [34]. Although further studies are necessary to localize the protective effects of maternal tempol administration within the placenta or fetus, a complementary maternal protein restriction model has demonstrated that direct tempol

administration to prehypertensive 3-week-old rats blocks the development of programmed hypertension [35].

In contrast with the female predisposition to PTSD, diverse animal models have demonstrated a sexually dimorphic male predisposition towards programmed hypertension [36–39]. Although the causes of this sexual dimorphism have not been fully elucidated, previous studies have demonstrated that the protection of females from programmed hypertension is lost with ovariectomy or aging [37–39]. Our results support this gender-specific programming of stress-evoked hypertension and extend our mechanistic understanding in linking prenatal oxidative stress with the programming of multiple psychological-stress-induced phenotypes, including fear and hypertension. Despite the lack of effect of postnatal diet on adult haemodynamics or aortic superoxide production, the inclusion of mice weaned to a hypercaloric diet may have confounded the results while simultaneously enhancing the study's generalizability. It is noteworthy that CBX increased aortic superoxide production independent of the fat content of the postnatal diet.

A potential role of persistently enhanced superoxide production in programmed cardiovascular disease was assessed by lucigenin-enhanced chemiluminescence as well as aortic reactivity in the presence of catalase and SOD. Although high concentrations of lucigenin are known to induce artifactual superoxide levels through redox cycling, we specifically chose a concentration (25 μM) below the threshold described for redox cycling in cellular systems (50 μM). The use of 25 μM lucigenin in these studies may have led to higher superoxide detection than would be seen with lower concentrations, but recent studies have shown a linear relationship between superoxide detection and lucigenin concentrations ranging from 5 to 250 μM [40]. In female offspring, combined prenatal and acute antioxidant exposure normalized the impairment in endothelium-dependent vasodilatation induced by maternal CBX, although neither treatment alone had a significant effect. These results compliment a growing body of literature demonstrating impaired endothelial function and increased vascular superoxide production after maternal undernutrition or fetal growth restriction [41–44].

Consistent with our present results, the endothelial dysfunction seen in femoral arteries isolated from neonatal dexamethasone-exposed rats is not altered by concurrent antioxidant therapy [45]. Our results support these studies showing that programming stimuli elicit blood-pressure-independent endothelial dysfunction that may be mediated by an imbalance between reactive oxygen and reactive nitrogen species. The role of RAS (renin–angiotensin system) activation in this pathway is an area of ongoing investigation. The lack of blood pressure phenotype in CBX females suggests that these vascular alterations are a primary consequence of maternal CBX administration, although they may not play a causative role in the programming of hypertension. Alternatively, the aortic myography may not model the resistance vasculature, and our results do not exclude a role for alterations in resistance arteries in programmed hypertension.

The use of maternal CBX administration to increase fetal exposure to maternal glucocorticoids replicates the decrease in 11β-HSD seen during maternal undernutrition [4,28,29]. It is important to note that, in addition to inhibiting placental 11β-HSD, maternal CBX blocks related dehydrogenases and disrupts gap junctions at high concentrations [46]. Each of these alterations may influence maternal, placental or fetal physiology. To overcome these limitations, others have induced programmed phenotypes by administering synthetic glucocorticoids that are not 11β-HSD substrates [1,5,7]. It is reassuring that exogenous glucocorticoid administration, endogenous glucocorticoid exposure (through 11β-HSD inhibition) and natural intra-uterine growth restriction induce similar adult phenotypes [1–5].

Our results demonstrate that the intra-uterine environment and antenatal interventions influence phenotypic responses to postnatal psychological stress. We have identified novel programmed phenotypes that support an important role of altered neuroregulation in the establishment of adult disease susceptibility. We speculate that intra-uterine glucocorticoid exposure alters central blood pressure and behavioural regulation, and intra-uterine oxidative stress plays a key role in the establishment of adult cardiovascular and behavioural responses to stress. Moreover, these results suggest that antioxidant administration, targeted to pregnancies complicated by maternal stress or fetal growth restriction, could help prevent the emergence of psychiatric disease-and stress-evoked hypertension. Because antioxidant exposure may have both beneficial and detrimental effects based on the end point assessed and the type or severity of prenatal stress, further studies (including biomarkers of oxidative stress) are needed to better understand the role of maternal, placental and fetal oxidative stress in the programming of adult physiology.

Acknowledgments

Abbreviations

CBX	carbenoxolone
11β-HSD	11β-hydroxysteroid dehydrogenase
PEG	poly(ethylene glycol)
PTSD	post-traumatic stress disorder
SOD	superoxide dismutase

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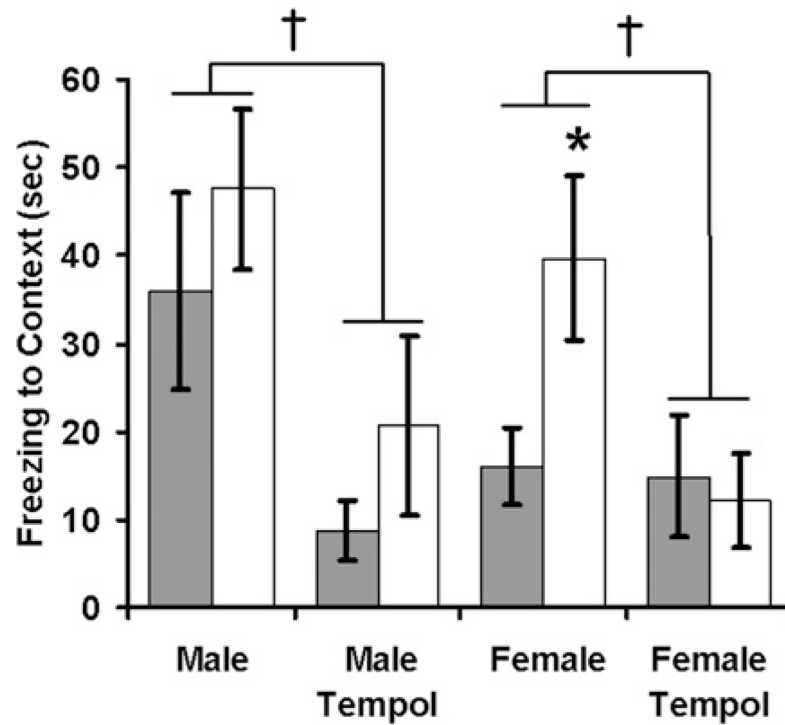
Figure 1. Maternal antioxidant therapy attenuates conditioned fear and blocks the programming effects of CBX

One day after training mice to associate a specific context with an aversive stimulus, they were returned to the conditioned context. Fearful behaviour (time spent freezing) was measured in adult offspring of dams that received either saline (solid bars) or CBX (open bars) during pregnancy. $n = 13$ male saline, $n = 10$ male CBX, $n = 6$ male saline–tempol, $n = 6$ male CBX–tempol, $n = 14$ female saline, $n = 5$ female CBX, $n = 5$ female saline–tempol, $n = 4$ female CBX–tempol. $*P < 0.05$ CBX compared with saline; $†P < 0.01$ tempol compared with no tempol.
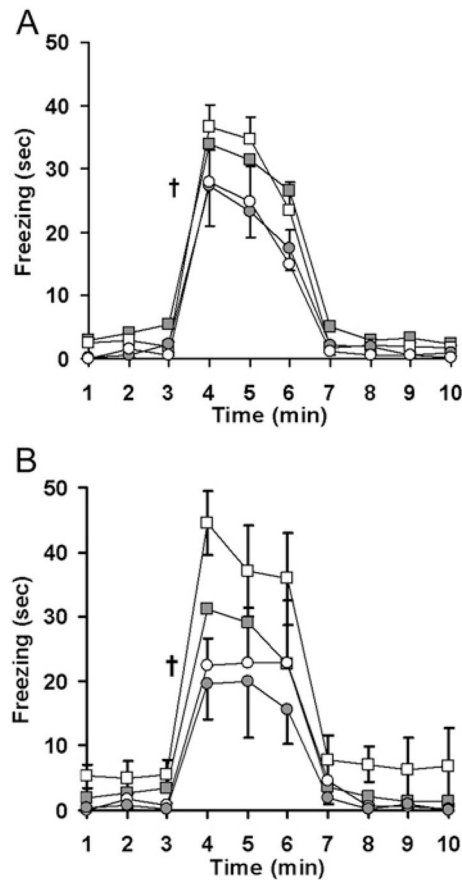
Figure 2. Maternal antioxidant therapy attenuates conditioned fear in mice programmed by exaggerated fetal glucocorticoid exposure

Mice trained to associate an auditory cue with an aversive stimulus were placed in a masked context for 3 min prior to a 3 min exposure to the conditioned cue. Fearful behaviour (freezing) was measured in adult male (A) and female (B) offspring of dams that received either saline (solid symbols) or CBX (open symbols) along with either water (squares) or the antioxidant tempol (circles) during pregnancy. $n = 13$ male saline, $n = 10$ male CBX, $n = 6$ male saline–tempol, $n = 6$ male CBX–tempol, $n = 14$ female saline, $n = 5$ female CBX, $n = 5$ female saline–tempol, $n = 4$ female CBX–tempol; †$P < 0.01$ tempol compared with no tempol.
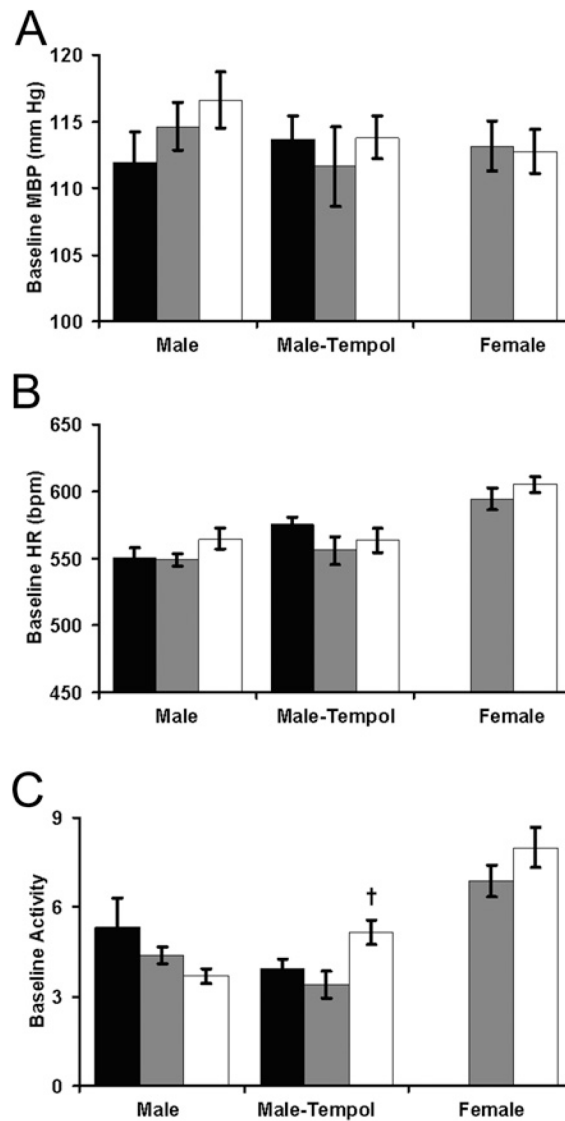
Figure 3. Antenatal saline, CBX and tempol do not significantly alter baseline haemodynamics
Mean blood pressure (MBP, A), heart rate (HR, B) and relative locomotor activity (C) were assessed in adult offspring of dams that were either undisturbed during pregnancy (black bars) or received daily third trimester injections of saline (grey bars) or CBX (open bars). These baseline recordings were obtained every 5 min for 60 h. $n = 6$ male undisturbed, $n = 6$ male saline, $n = 6$ male CBX, $n = 5$ male undisturbed–tempol, $n = 6$ male saline–tempol, $n = 8$ male CBX–tempol, $n = 12$ female saline ($n = 6$ weaned to a hypercaloric diet), $n = 12$ female CBX ($n = 6$ weaned to a hypercaloric diet); †$P < 0.05$ CBX–tempol compared with CBX–water.
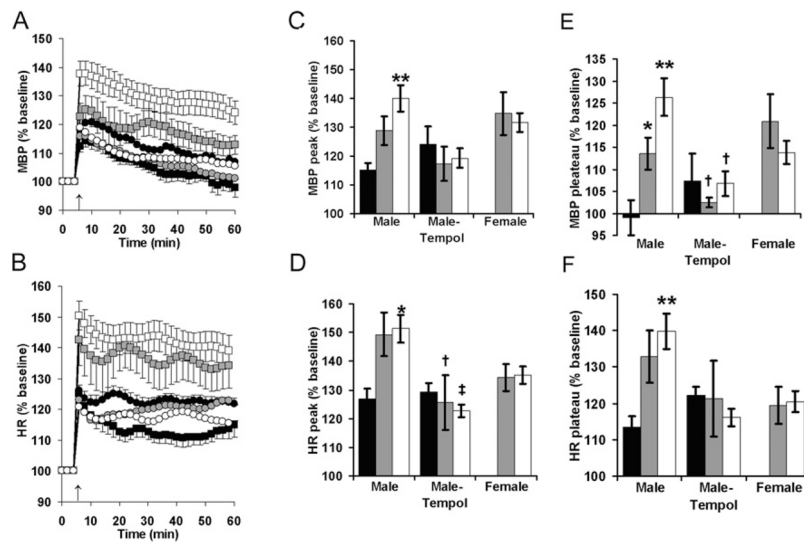
Figure 4. Maternal antioxidant attenuates programmed cardiovascular responses to psychological stress

Mean blood pressure (MBP) (A, C and E) and heart rate (HR) (B, D and F) were recorded 5 min before and 60 min after placement in a metabolic cage for adult offspring of dams that were either undisturbed during pregnancy (black) or received daily third trimester injections of saline (grey) or CBX (white). In (A and B) only male results are presented, with offspring of dams receiving standard drinking water denoted by squares and offspring of dams receiving tempol during pregnancy denoted by circles. $n = 5$ male undisturbed, $n = 9$ male saline ($n = 3$ weaned to a hypercaloric diet), $n = 13$ male CBX ($n = 3$ weaned to a hypercaloric diet), $n = 5$ male undisturbed–tempol, $n = 6$ male saline–tempol, $n = 6$ male CBX–tempol, $n = 12$ female saline ($n = 6$ weaned to a hypercaloric diet), $n = 11$ female CBX ($n = 6$ weaned to a hypercaloric diet); $*P < 0.05$ or $**P < 0.01$ compared with undisturbed; $\dagger P < 0.05$ or $\ddagger P < 0.01$ compared with no tempol.
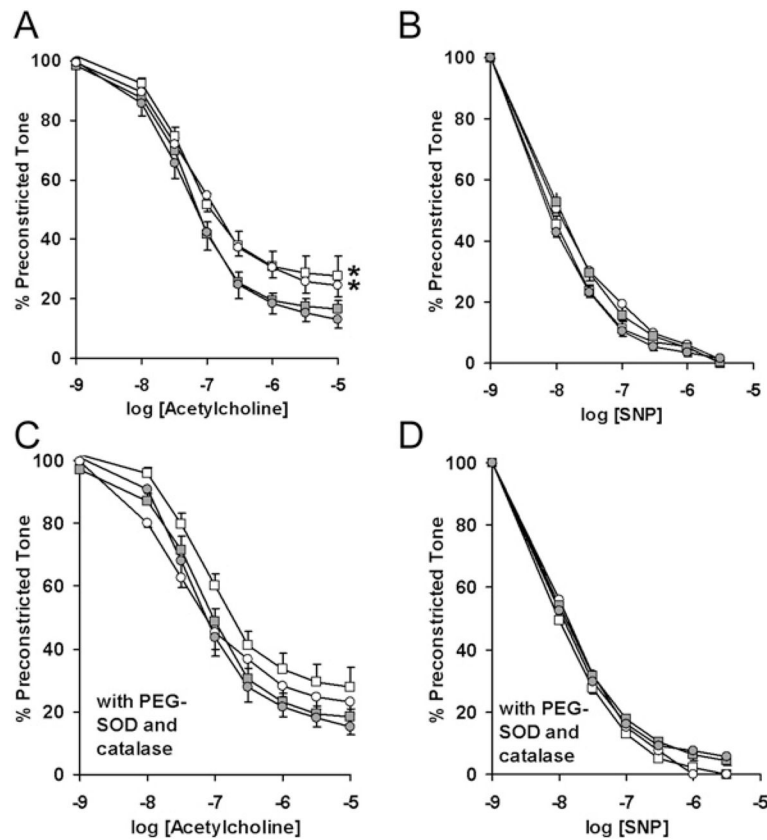
Figure 5. Maternal antioxidant does not correct glucocorticoid-programmed endothelial dysfunction
Aortic dilation to acetylcholine (A) or sodium nitroprusside (SNP) (B) was evaluated for female offspring of dams that received injections of either saline or CBX in addition to treatment with either tempol or water. Additional aortic rings were incubated in a combination of PEG-conjugated catalase and SOD prior to the addition of the vasodilators (C and D). Responses were compared by repeated measures ANOVA after the addition of cumulative concentrations of the vasodilator. $n = 11$ saline (■), $n = 7$ CBX (□), $n = 8$ saline–tempol (●), $n = 6$ CBX–tempol (○). $*P < 0.05$ CBX compared with the control.
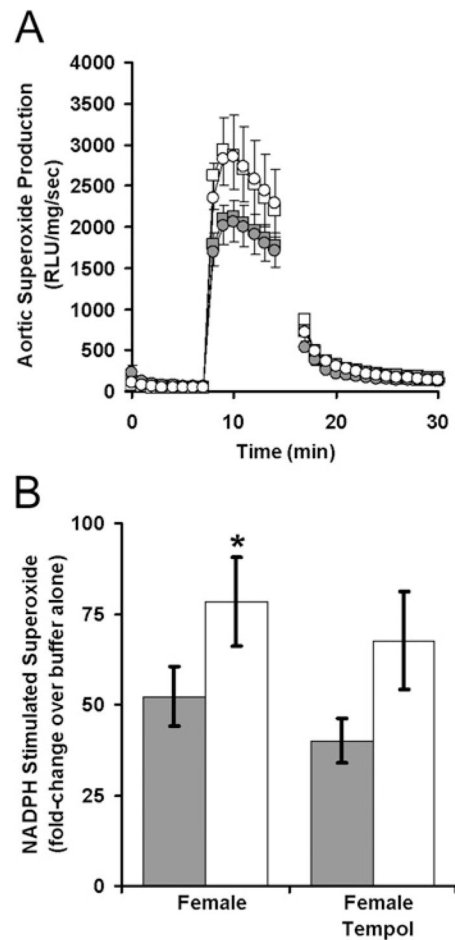
Figure 6. Maternal antioxidant therapy does not correct glucocorticoid-programmed aortic superoxide production

Superoxide production was measured by lucigenin-enhanced chemiluminescence among aorta obtained from female offspring of dams that received CBX and/or tempol during pregnancy. After the measurement of relative light unit (RLU) production during 7 min incubation in a buffer, superoxide was measured for 7 min after the addition of NADPH and for another 14 min after the addition of diphenyleneiodonium (A). NADPH-stimulated superoxide production was compared in the offspring of dams that received saline or CBX during pregnancy (B). $n = 13$ saline (■), $n = 7$ CBX (□), $n = 11$ saline–tempol (●), $n = 11$ CBX–tempol (○). *$P < 0.05$ CBX compared with the control.
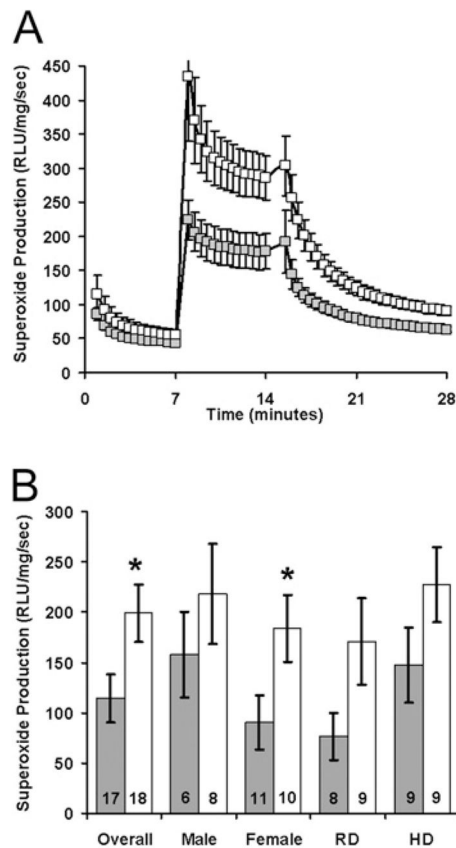
Figure 7. Antenatal CBX consistently increases aortic superoxide production among adult female offspring
Superoxide production was measured by lucigenin-enhanced chemiluminescence among aorta obtained from adult offspring of dams that received either saline (■) or CBX (□) during pregnancy. These mice were weaned to either regular diet (RD) or hypercaloric diet (HD) until tissue collection. Relative light unit (RLU) production was measured for 7 min with only lucigenin and NADPH, then for 7 min with the aortic segment added, and finally for 14 min after the addition of diphenyleneiodonium (A). NADPH-augmented, diphenyleneiodonium-inhibitable superoxide production was determined by subtracting superoxide production at 28 min from that at 14 min (B). The numbers at the base of the bars represent aggregate numbers. $n = 6$ female saline–RD, $n = 4$ female CBX–RD, $n = 5$ female saline–HD, $n = 6$ female CBX–HD, $n = 2$ male saline–RD, $n = 5$ male CBX–RD, $n = 4$ male saline–HD and $n = 3$ male CBX–HD; *$P < 0.05$ CBX compared with the control.